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Analysis of *Staphylococcus aureus* and *Escherichia coli* inhibition from varying concentrations of Vitamin C

Victoria Suslovitch, Madeleine Kenton, and Eric C. Freundt[1]
Department of Biology, University of Tampa, Tampa, FL 33606, [1]Faculty Advisor

ABSTRACT

The effects of ascorbic acid (vitamin C) on the gram positive bacteria *Staphylococcus aureus* (*S. aureus*) and the gram negative bacteria *Escherichia coli* (*E. coli*) were studied using a serial dilution of concentrated vitamin C applied in a Kirby-Bauer disk diffusion assay. It was observed that higher concentrations of ascorbic acid are more effective in killing bacteria. It was also observed that the gram positive bacteria, S. aureus, is more susceptible than the gram negative bacteria, E. coli. Lastly, it was shown that a saturation of vitamin C at high concentrations was not achieved within the experimental parameters, as no plateau effect was observed. The possibility of ascorbic acid as an antimicrobial agent is worthy of future research due to its safety, versatility, and availability.

1 INTRODUCTION

Ascorbate (Figure 1), more commonly known as vitamin C, is a substance essential to the human body for the growth and repair of tissues, the synthesis of collagen, and the proper function of the immune system (Crandon et all, 1940). It is pertinent that humans obtain a satisfactory amount of ascorbic acid through their diet in order to stay healthy. If one is deprived of this vitamin, he or she will be at an immunological disadvantage and may even develop a condition called scurvy (Crandon et all, 1940). This extreme example of ascorbic acid depravation is characterized by fatigue, the formation of wounds, and the bleeding of mucous membranes, all due to decreased generation of collagen (Crandon et all, 1940). Furthermore, ascorbic acid aids in the growth and maturation of white blood cells, consequently strengthening the immune system (National Research Council, 2000). Thus, it is evident that ascorbic acid is a relevant nutrient with regard to human health. It is recommended by the Institute of Medicine that men obtain a minimum of 90 mg of per day and women obtain 75 mg per day of vitamin C (National Research Council, 2000). As ascorbic acid is water soluble, excess amounts will be passed out in the urine and confer no detrimental effects physiologically (National Research Council, 2000).

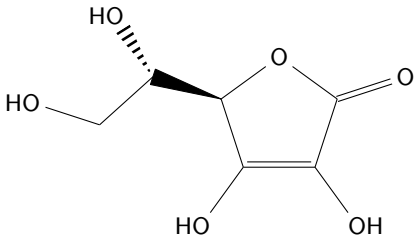

Fig. 1: Molecular structure of ascorbic acid.

In addition to its nutritional value, it is thought that ascorbic acid may act as an antimicrobial agent. Previous research shows that it has an inhibitory effect on bacteria in the stomach, such as *Helicobacter pylori*, the newly identified causative agent of ulcers (Jarosz et al., 1998). Also, studies have shown that ascorbic acid has the ability to kill strains of *Mycobacterium tuberculosis* that are resistant to most other antibiotic drugs (Vilcheze et al., 2013). Similar studies were demonstrated in which fruit juices were used as antimicrobials; hypotheses in these studies suggest ascorbic acid as an antimicrobial property (Opara et al., 2009). These scientists further argue that vitamin C has the potential to be even more efficient than antibiotics, as it supports the immune system directly in addition to its antimicrobial effects.

However promising, most of the existing research on ascorbic acid in the scope of microbiology focuses primarily on gut flora and there are still many unanswered questions. For example, it is unknown at which concentrations ascorbic acid best inhibits bacterial growth; this was investigated in the following study. It was hypothesized that higher concentrations of ascorbic acid are more antimicrobial, but may plateau above a certain concentration. This was predicted because logically more antimicrobial agent will confer more inhibition, but after a certain point the growth environment may be saturated with the ascorbic acid. Furthermore, it was hypothesized that the gram positive bacteria, *S. aureus*, is more susceptible than the gram negative bacteria, *E. coli*. This hypothesis is based on the knowledge that the larger layer of peptidoglycan in the gram negative bacterial cell wall aids in the protection of the cell from intrusion of foreign chemicals (Beveridge, 1999). Additionally, many gram negative bacteria have efflux pumps that can rid the internal cell environment of unwanted substances (Nikaido, 1996). These hypotheses were tested using the Kirby-Bauer disk diffusion assay. The treatment with the largest average zone of inhibition was supported as the more effective antimicrobial agent for the respective *S. aureus* and *E. coli* trials. This study is pertinent to microbiology, human health, and other areas of concern as it provides a possible alternative to treatment with traditional antibiotics. As bacteria acquire resistance to overused antibiotics, human medicine must evolve as well. With the knowledge that ascorbic acid can inhibit bacteria such as *Mycobacterium tuberculosis*, it will be interesting for researchers to determine its effects on other pathogens as well. Quantification of effective ascorbic acid concentrations is important to ensure efficiency.

2 MATERIALS AND METHODS

Pharmaceutical vitamin C pills ($500\,\mathrm{mg}$ / each pill) were obtained. Two pills ($1000\,\mathrm{mg}$) were dissolved using a vortex mixer into $10\,\mathrm{mL}$ of sterile deionized water, creating a solution with a concentration of $100\,\mathrm{mg}$ vitamin C / $1\,\mathrm{mL}$. Besides ascorbic acid, the pill also contained cellulose gel, hydroxypropyl cellulose,

Table 1. Zones of Growth Inhibition (mm) in Decreasing Concentrations (mg/mL) of Vitamin C for *S. aureus* and *E. coli*.

Vitamin C Concentrations (mg/mL)	Zones of Inhibition (mm)							
	Staphylococcus aureus				*Escherichia coli*			
	Trial 1	Trial 2	Trial 3	**Average**	Trial 1	Trial 2	Trial 3	**Average**
Stock	15	14	15	**14.67**	11	12	11	**11.33**
10^{-1}	14	13	12	**12.33**	10	11	11	**10.67**
10^{-2}	10	11	11	**10.67**	11	12	9	**10.67**
10^{-3}	10	10	10	**10.00**	10	10	9	**9.66**
10^{-4}	11	9	9	**9.67**	9	8	11	**9.33**
10^{-5}	11	8	9	**9.33**	10	10	8	**9.33**
10^{-6}	8	9	9	**8.67**	10	9	9	**9.33**
10^{-7}	9	8	8	**8.33**	8	9	9	**8.66**
10^{-8}	7	7	7	**7.00**	9	9	7	**8.33**
Control	0	0	0	**0.00**	0	0	0	**0.00**

croscarmellose sodium, stearic acid, magnesium stearate, and silicon dioxide. As ascorbic acid was listed the only active ingredient and no ingredient ratios were given, it was assumed that the other components would make only negligible impacts on the experiment. A serial dilution of this stock solution was performed in 1:10 dilutions until all concentrations through 10^{-8} of the original solution were achieved. This was achieved by mixing 100 µL of the starting solution of vitamin C with 900 µL of sterile deionized water. The resulting solution was then used in the next dilution, and in this way, 100 µL of each consecutive solution was mixed with 900 µL of sterile deionized water.

A disk diffusion assay was used to test the susceptibility of each bacterial species. Twenty Mueller-Hinton agar plates were inoculated with a broth culture using micropipettes. The spread plate technique was then employed in attempt to achieve a lawn of bacteria on these plates. Ten plates were infected with *S. aureus* and ten were infected with *E. coli* using sterile cotton swabs. *S. aureus* and *E. coli* were chosen because they represent one gram-positive and one gram negative species respectively, they are easily grown on Mueller-Hinton agar plates, and the generation times required to achieve a lawn of growth were within reasonable time constraints. Furthermore, both *S. aureus* and *E. coli* are naturally associated with the human body; *S. aureus* is found on skin, hair, and nails, and within the nasopharangeal cavity and *E. coli* is found in the intestine (Hentges, 1993). Thus, human flora that is found both in and on the body and is representative of both gram reactions are represented by these two species. Sixty blank paper disks were soaked; six disks were soaked in 1 mL of the solution at each specific concentration: three disks for each plate of *S. aureus* and three for each plate of *E. coli*. The three disks that were soaked in the same solution were placed equidistantly on each Mueller-Hinton agar plate using sterile tweezers. Controls were established by using three paper disks that were not soaked in any solution for both *S. aureus* and *E. coli*. The plates were incubated at 37 °C for 24 hours to form the lawn of bacteria. When removed from the incubator, the diameters of each zone of inhibition around the paper disks were measured using rulers and recorded in millimeters.

3 RESULTS

The zones of inhibition recorded for each disk were compiled and the average zone of inhibition was calculated at each concentration for both S. aureus and *E. coli*. The data are displayed in Table 1. In general, *S. aureus* was inhibited more than *E. coli* at higher concentrations of vitamin C. In the stock solution, *S. aureus* displayed zones that were 2–4 mm larger than those displayed by *E. coli*. The 10^{-1} concentration also portrayed *S. aureus* as being inhibited more than *E. coli*, since the zones were 1–4 mm larger than those observed on the E.coli plates. At 10^{-2} however, the two gram-reaction bacterias displayed similar zones of inhibition and this trend continued as the concentration of vitamin C decreased. These trends were also observed when the average zones of inhibition were compiled into a chart, as can be viewed in Figure 2.

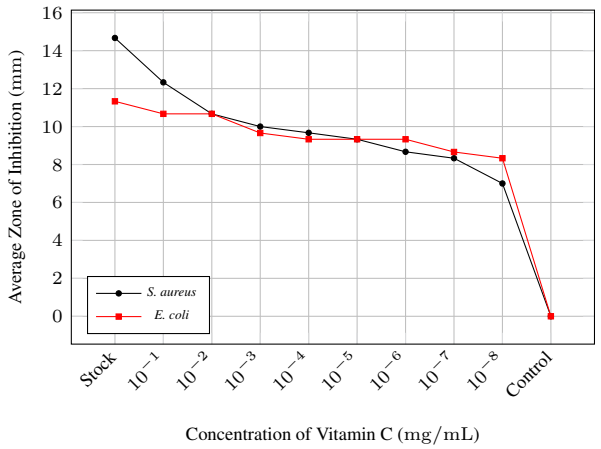

Fig. 2: Zones of Growth Inhibition (mm) in decreasing Concentrations (mg/mL) of Vitamin C for *S. aureus* and *E.coli*.

4 DISCUSSION

The hypothesis that higher concentrations of ascorbic acid are more antimicrobial was supported by the data collected. The stock concentration caused the largest zones of inhibition in both *S. aureus* and *E. coli*. Furthermore, with each consecutive dilution, the zones of inhibition decreased for both bacterial species as well. No zones of inhibition were observed for the control plates, confirming ascorbic acid as the antimicrobial factor.

The hypothesis that antimicrobial activity would plateau above a certain concentration was not supported by the data collected. No saturation was observed as the inhibition correlated directly with concentration. A larger range of concentrations would need to be observed in future studies in order to discover if and at what concentration saturation is reached.

The hypothesis that the gram positive bacteria, *S. aureus*, is more susceptible than the gram negative bacteria, *E. coli*, was supported by the data collected. For the stock solution, the average zone of inhibition was $3.34\,\mathrm{mm}$ larger for *S. aureus* than that for *E. coli*. Similarly, for the 10^{-1} solution, the average zone of inhibition was $1.66\,\mathrm{mm}$ larger for *S. aureus* as well. For all of the concentrations lower than 10^{-1}, the average zones of inhibition were relatively similar between *S. aureus* and *E. coli*. The outliers in this overall trend occurred at the 10^{-6} and 10^{-8} dilutions, in which the average zones of inhibition for *E. coli* were larger than those for *S. aureus*. This unexpected pattern could be due to the ascorbic acid being so dilute that the variable response between gram positive and gram negative bacteria is less distinguished.

In addition to its relative support of the hypotheses, these data are in agreement with the previous research regarding ascorbic acid and microbiology. As shown by and Jarosz et al. and Vilcheze et al., vitamin C inhibits the human pathogens *Helicobacter pylori* and *Mycobacterium tuberculosis* (3,4). This newly developing understanding of ascorbic acid could lead to novel uses of vitamin C as an antibiotic or preventative health measure. This is especially pertinent because a decreasing number of antibiotics are effective in treating infection as populations of resistant bacteria are rapidly increasing as they are subjected to antibiotic selection pressures (Liu, 1999). Thus, novel techniques for mitigating bacterial growth are especially valuable to modern medicine. However, this experiment provides a model for conditions that may commonly occur within even healthy human bodies, as *S. aureus* and *E. coli* are a part of the human bodys normal flora (Hentges, 1993). While demonstrating that vitamin C can be used as an antibiotic against pathogenic bacteria, it must also be taken into account that it could have adverse effects towards benign, beneficial, or symbiotic bacteria on or within the human body as well (Reid, 2001). The task of killing harmful bacteria while keeping beneficial normal flora alive and thriving is a difficult but important task.

If this experiment is replicated, it would be interesting to significantly increase the range of concentrations tested and observe whether the same results occurred. It would also be interesting to see whether or not a plateau would be reached as concentration increases or decreases. Improvements to the experiment could be made by testing additional types of bacteria or by including more disks per petri dish, in order to increase the sample size of the data. Additional attention could also be allocated concerning the amount of solution added to each disk, because the disks were not wiped off after coating; it is possible that they contained differing amounts of solution when applied to the plates.

In the future, it would be feasible to examine the effects of vitamin C obtained from natural sources. The antimicrobial effects observed could be compared to those of vitamin C pills, in order to further understand which source is most beneficial. It could also be beneficial to observe if high doses of vitamin C could have any effect on human flora. Obtaining pure ascorbic acid and testing its efficiency compared to that of the pharmaceutical tablets could also improve this experiment. Due to the fact that most people tend to consume vitamin C through many typical food products, it is possible that taking an additional vitamin C supplement could affect their microbiota, despite the fact that the extra acid is passed out. Overall, the possibility of ascorbic acid as an antimicrobial agent is worthy of future research due to its antimicrobial properties, safety, versatility, and availability.

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