The Effects of Saccharin on Gram-Negative and Gram-Positive Bacteria

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Abstract

A common misbelief is that non-nutritive sweeteners (NNS) are healthier than natural sugars, only because of their lower caloric content. Individuals are unaware of the consequences that high amounts of NNS pose to the bacteria in the gut microbiome. In the current research, the viability of two common bacterial phyla found in the gut microbiome, Proteobacteria and Firmicutes, was analyzed after being introduced to varying ratios of saccharin, a common NNS. A gram staining technique was adapted and a microplate assay method was utilized to quantify the difference in ratios between gram-negative Proteobacteria, *E. coli*, and gram-positive Firmicute, *S. aureus*. Saccharin posed an inhibitory effect on both *E. coli* and *S. aureus*, while *E. coli* responded with a linear dose-dependent trend and *S. aureus* illustrated a modest response to increasing ratios of saccharin. This further suggests that excess NNS has a negative impact on the gut microbiome by altering the natural bacterial ratios. If individuals are aware of the consequences caused by varying dosages of NNS, diets can be altered in order to increase overall health and lower the likelihood of becoming obese, in turn lower obesity rates in America.

Introduction

Approximately one-third of all Americans (160 million individuals) are either obese or overweight [1]. Research has fallen short in discovering the prevention of obesity. While the disease is partially genetic, recent studies suggest the ratio of species in the gut microbiome may be an underlying cause of obesity. A common misbelief is that non-nutritive sweeteners (NNS) are healthier than natural sugars, only because of their lower caloric content [2]. Many individuals are unaware of the consequences that high amounts of NNS pose to the bacteria in the gut microbiome. If individuals are aware of the consequences caused by varying dosages of NNS, diets can be altered in order to increase overall health and lower the likelihood of becoming obese, in turn lower obesity rates in America.

More than 98% of the gut microbiome consists of bacteria that either belong to the Firmicutes or Bacteroidete*s* phyla [2]. Studies have shown that the ratio of Firmicutes to Bacteroidetes (F:B) is negatively correlated with body mass index [3]. As an individual's body mass index increases, their abundance of Firmicutes increases while their abundance of Bacteroidetes decreases [3]. Firmicutes are gram-positive, largely facultative anaerobes, while Bacteroidetes are gram-negative, obligate anaerobes. The difference in gram type is due to the thickness of the peptidoglycan cell wall that surrounds the bacterium. According to a phylogenetic tree, gram-negative Bacteroidetes and Proteobacteria are closely related, with a recent common ancestor [4]. This common lineage allowed for the substitution of the obligate anaerobic Bacteroidetes with the facultative aerobe Proteobacteria without deviating from the focus of the gut microbiome, as these represent logical experimental equivalencies.

Escherichia is a gram-negative Proteobacteria, while *Staphylococcus* is a gram-positive Firmicute [5]. *Escherichia coli* and *Staphylococcus aureus* were compared to determine if, and what, the relationship is between gram-negative and gram-positive bacteria in the gut microbiome. The scope of current research was to determine if excess NNS would produce the same inhibitory effects as compared to the F:B when looking at the gram-type rather than at phyla. Understanding how altering the amount of each abundant bacteria in the gut will help individuals to comprehend how diets that are high in NNS affect the biodiversity of the gut microbiome.

Background studies are insufficient, not because of the lack of information, but because of the wide diversity within the gut microbiome. Some studies have focused on the gut microbiome community as a whole, but not on individual or isolated species. One specific study found that sucralose, a common NNS, altered the F:B ratio in the gut microbiome of mice [6]. Saccharin was used in the current study, as it is another common NNS in the daily diet. While this research has widened the understanding of the gut microbiome, previous research has yet to demonstrate the effect of NNS of bacteria from different phyla or gram types.

Our research aims to study how various ratios of NNS, such as saccharin, selectively affect the individual members of a gut community, with varying genetic and biochemical properties. If gram-negative *E. coli* and gram-positive *S. aureus* react comparably to the F:B ratio in the presence of saccharin, then *E. coli* viability will be inversely proportional, while *S. aureus* viability will be directly proportional to the ratio of saccharin. Viability was quantified via fluorescence using a microplate assay. This new approach of studying the gut microbiome helps

in further understanding the effects that common artificial sweeteners have on both gram-positive and gram-negative bacteria, and ultimately obesity.

Materials and Methods

Bacterial Strains and Glycerol Stocks

Escherichia coli (Strain: K-12) was purchased from Carolina (Item# 155068). *Staphylococcus aureus* subsp. *aureus* Rosenbach (Strain ID: NCTC-8532) was purchased from ATCC (Catalog Number: 12600). Overnight bacterial cultures of *E. coli* and *S. aureus* were grown in Tryptic Soy Broth (TSB) to be used for the creation of glycerol stocks. A 50% glycerol solution was made by diluting 100% glycerol in DI H_2O. Overnight bacterial growth of *E. coli* was combined with the 50% glycerol solution in a 1:1 ratio and the solution was mixed. The glycerol stock was divided into 1mL microfuge tubes and stored at -80C. The same method was used to create *S. aureus* glycerol stocks, which were also stored at -80C.

Creating Reagent Stocks and Working Solutions

Creation of 10 mL Tris-HCl Stock Solution

The Tris-HCl solution was used to dilute the stains to working concentrations in a neutral buffer. 0.5mL 1M Tris-HCl pH 8 was diluted in 49.5mL DI H_2O [7]. The pH was confirmed at 7.4 on pH paper. Solution was stored at room temperature.

Preparation of SYTO 13 Working Solution

SYTO 13 was used to quantify *E. coli*, a gram-negative bacteria. 5mM solution in DMSO (provided by manufacturer) was diluted in 10mM Tris-HCl in a 1:10 ratio to create a 0.5mM working solution [7]. The working solution was divided into multiple light-resistant 1mL microfuge tubes and was stored at -80C.

Preparation of HI Working Solution

HI was used to stain *S. aureus*, a gram-positive bacteria. 5mg of HI was dissolved in 1mL DMSO to create a stock solution of 5mg/mL. The stock solution was diluted in 10mM Tris-HCl in a 1:50 ratio to create a 0.1mg/mL working solution [7]. The working solution was divided into multiple light-resistant 1mL microfuge tubes and was stored at -80C.

Aerobic Broth

In order for *E. coli* and *S. aureus* to grow, an aerobic media was created. Per 150mL DI H_2O, 4.50g TSB and 0.600mL 0.25% Resazurin were dissolved in solution. Resazurin is an O_2 indicator and was included in order to observe the O_2 content of the media during bacterial growth. For each experimental trial, 3 replicates of broth were created with the above values of TSB and Resazurin. Saccharin (Catalog Number: 240931) was purchased from Sigma Aldrich. Varying concentrations of saccharin were added to the tubes according to Table 1. Ratios of saccharin were calculated using the amount of saccharin found in 1 packet of NNS. Dosages were designated as no NNS with 0.00g/mL, a low dosage with 0.08g/mL (equivalent to 1 packet of NNS), and a high dosage with 0.22g/mL (equivalent to 3 packets of NNS). The same broth was used for the tubes with the same ratio of saccharin. 12mL of each broth was added to 15mL capped tubes labeled 1-18, which were autoclaved at the lowest setting and allowed time to cool completely.

Tube	Saccharin	Bacteria

1-3	0.00g/mL	*E. coli*
4-6	0.08g/mL	*E. coli*
7-9	0.22g/mL	*E. coli*
10-12	0.00g/mL	*S. aureus*
13-15	0.08g/mL	*S. aureus*
16-18	0.22g/mL	*S. aureus*

Table 1. Saccharin dosages for *E. coli* and *S. aureus*

Inoculation of *E. coli* and *S. aureus* for Individual Growth

Bacteria needed to be added to the tubes of autoclaved broth. A 1mL tube of thawed *E. coli* in glycerol stock was centrifuged at 30,000 RPM x 15 minutes. The concentrated bacterial pellet was resuspended in 750uL no NNS TSB. Three flames were lit on the benchtop to aid in prevention of excess oxygen contamination. 50uL of bacterial suspension was added to each labeled tube of autoclaved broth. This was completed for *S. aureus*, using the same no NNS TSB and inoculation technique. Tubes were then allowed to incubate at 37C for 48 hours.

Fluorescent Protocol for Quantification of Bacterial Viability

Incubated tubes were centrifuged at 3,000 g x 15 minutes to isolate a bacterial pellet. The supernatant was decanted and the pellet was resuspended in 1mL DI H_2O. Solution was transferred to a 1mL microfuge tube, labeled and placed in the refrigerator. 2uL of SYTO 13 working solution and 5uL of HI working solution were added to each microfuge tube. Mixed bacterial suspensions with stains were incubated in a dark cabinet for 15 minutes at room temperature to allow stains to bind to the bacteria. Each tube was centrifuged again at 10,000 g x 15 minutes. Pellets were decanted and resuspended in lmL PBS broth. This double centrifugation technique removed the unbound stain from the suspension, thus reducing background in the microplate assay. 200uL of each suspension was added to a light-sensitive 96 well-plate and 2 wells were filled with PBS broth as a negative control. The 96 well-plate was placed in a FilterMax F3 Multi-Mode Microplate Reader and SoftMax Pro software was used to measure intensities of fluorescence. For the green channel, excitation was set at 485 nm and emission was set at 535 nm, while for the red channel, excitation was set at 518 nm and emission was set at 595 nm [7].

Statistical Methods and Analysis

The microplate assay reader produced output intensity values of fluorescence for each of the experimental replicates. These values were normalized to the no NNS dosage and were analyzed using the statistical functions in Microsoft Excel. A two-tailed T-test was run between each experimental dosage to determine the statistical significance of the data. Results were considered significant at a p value < 0.05. The graphing functions in Microsoft Excel were used to generate graphical representations of bacterial viability via intensity of fluorescence and percent inhibition. Standard error of the mean (SEM), N=2 error bars are included in graphical representations, along with asterisk markers which represent significance of the p values between doses of NNS. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

Results

Viability of *E. coli* is inversely proportional to saccharin ratio

E. coli is a gram-negative Proteobacteria closely related to Bacteroides. One study found that saccharin and other NNS have a bacteriostatic effect on *E. coli* viability [5]. However, it is unknown if this effect was due to the phyla of *E. coli* or the gram-type. To further investigate the effect of saccharin, we treated *E. coli* in an isolated culture with increasing concentrations of saccharin to test the hypothesis that gram-negative bacteria will decrease in the presence of saccharin. Viability was measured via a double staining method of SYTO 13 and HI. While the HI showed some fluorescent signal, this was predominantly due to bleed over into the excitation wavelength (Supplemental Fig.2). A two-tailed T-test was run to determine if the difference in fluorescence was significant or not. Analysis of the green, SYTO 13-stained channel demonstrated an overall decrease in the viability of *E. coli* with an increasing ratio of saccharin after 48 hours of incubation. According to Fig.1, a significant decrease in the intensity of fluorescence of *E. coli* was detected between each experimental group. This decrease was proportional to the ratio of saccharin added to the TSB broth. There was a significant decrease of 19.9% in viability between no NNS and a low dosage with a p value of 0.0071. A significant decrease of 35.7% was also demonstrated between no NNS and a high dosage with a p value of 0.0002. Thus, the viability of gram-negative *E. coli* decreased in a dose-dependent manner when cultured with saccharin, which supported our hypothesis.

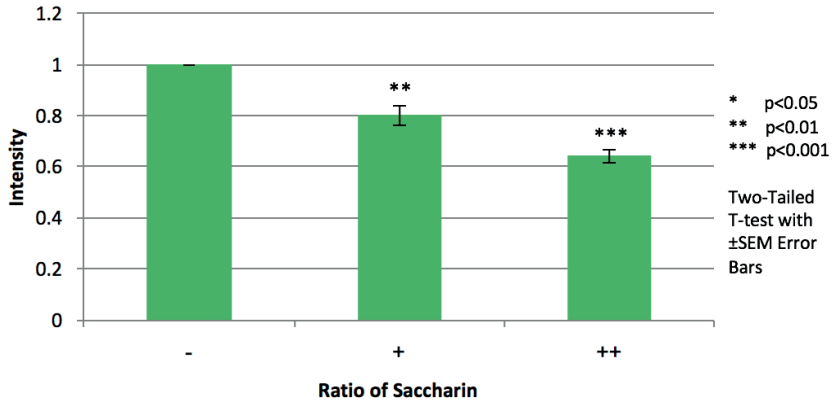

Fig.1 SYTO 13 Fluorescence of *E. coli* in the Presence of Saccharin. Data was normalized to *E. coli* with no NNS (-). Low dosage (+) is equivalent to one packet of NNS, while high dosage (++) is equivalent to three packets of NNS. Error bars represent ± Standard Error of the Mean (SEM), N=2. A two-tailed T-test was run to analyze the fluorescence of *E. coli* using α=0.05. A linear, dose-dependent decrease in *E. coli* was observed.

Viability of *S. aureus* decreases with an increasing ratio of saccharin

S. aureus is a gram-positive Firmicute, which is highly concentrated in the gut microbiome. However, it is unknown what the effect of excess NNS in an otherwise nutrient rich broth might be on this class of microbe. Therefore, we treated *S. aureus* in an isolated culture with increasing concentrations of saccharin to test the hypothesis that gram-positive bacteria will increase in the presence of saccharin. While *S. aureus* was also stained with SYTO 13, the viability of gram-positive bacteria is better represented in the red channel, as HI binds exclusively to gram-positive bacteria and the effects of SYTO 13 are reportedly quenched (Supplementary Fig.1). A two-tailed T-test was run to determine if the difference in fluorescence was significant or not.

Analysis of the red, HI-stained channel demonstrated an overall decrease in the viability of *S. aureus* with an increasing ratio of saccharin after 48 hours of incubation. As seen in Fig.2, there was a significant decrease of 21.3% in the intensity of fluorescence between no NNS and a low dosage which was noted with a p value of 0.0293. A significant decrease of 24.8% was also demonstrated between no NNS and a high dosage with a p value of 0.0254. Surprisingly, an insignificant decrease of 3.43% in intensity was noted between a low dosage and a high dosage. As there was a decrease in the viability of *S. aureus* with the addition of saccharin, our hypothesis was rejected for the gram-positive bacteria.

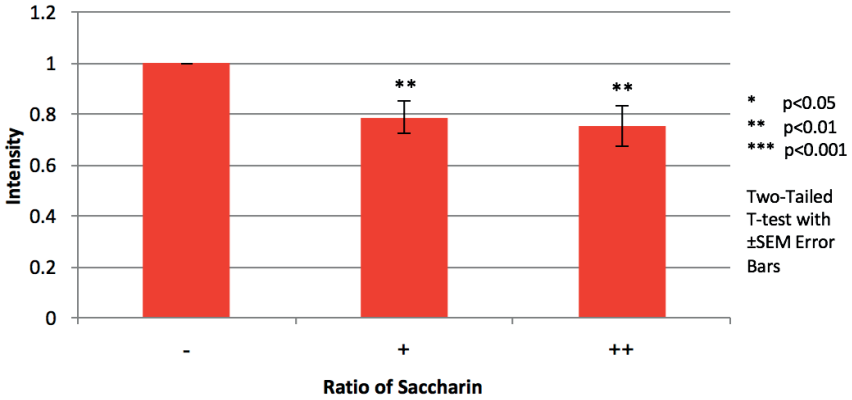

Fig.2 HI Fluorescence of *S. aureus* in the presence of Saccharin. Data was normalized to *S. aureus* no NNS (-). Low dosage (+) is equivalent to one packet of NNS, while high dosage (++) is equivalent to three packets of NNS. Error bars represent ± Standard Error of the Mean (SEM), N=2. A two-tailed T-test was run to analyze the fluorescence of *S. aureus* using α=0.05. A nonlinear, modest response was observed in *E. coli*.

***E. coli* shows a linear, dose-dependent response to saccharin and *S. aureus* shows a nonlinear, modest response to saccharin**

As both bacteria were negatively inhibited in the presence of saccharin, the overall hypothesis that *E. coli* viability would be inversely proportional and *S. aureus* viability would be directly proportional to the ratio of saccharin was rejected. When graphing the percent inhibition of increasing saccharin ratios on *E. coli* and *S. aureus*, both demonstrate a similar, linear inhibition from no NNS to a low dosage. In Fig.3, this is seen as an inhibition of approximately 20% from no NNS to the equivalent of one packet of NNS. A divergence of inhibition is observed from a low dosage to a high dosage. *E. coli* responds to an increasing ratio of saccharin in a linear, dose-dependent fashion. *S. aureus*, on the other hand, shows a modest response to an initial dose of saccharin. Unlike *E. coli*, saccharin appears to have a maximal inhibition of 20-30% in *S. aureus*, as increasing doses do not affect its viability after an initial low dose.

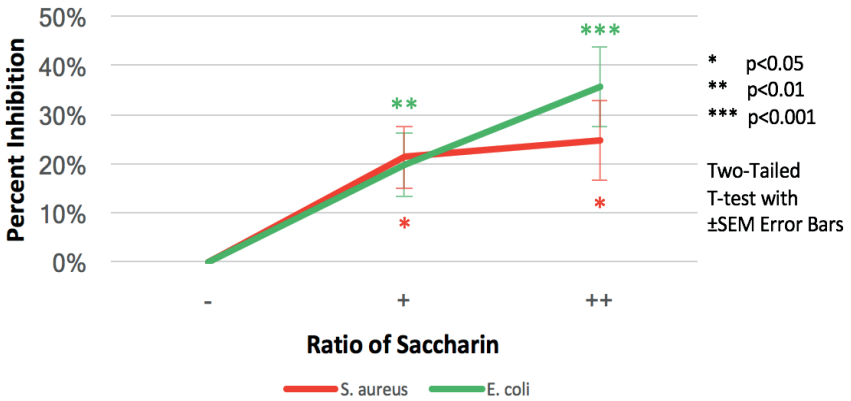

Fig.3 Percent Inhibition of *E. coli* and *S. aureus* in the Presence of Saccharin. *E. coli* shows a linear response to an increasing ratio of saccharin, whereas *S. aureus'* response appears to level off at a ratio greater than a low dosage.

Discussion

Previous research has demonstrated the direct correlation between the viability of gut microbiota and the overall health of an individual. In this study, we examined the effect of increasing saccharin ratios on the viability of *E. coli* and *S. aureus*. Viability of bacteria was quantified using fluorescent output from a microplate assay, which utilized SYTO 13 to quantify gram-negative *E. coli* and HI to quantify gram-positive *S. aureus*. We found that an increase in saccharin decreased the viability of both *E. coli* and *S. aureus* when compared to the negative control group of no NNS. However, the magnitude of that effect varied, as *E. coli* demonstrated a dose-dependent effect and *S. aureus* was not impacted by increasing dosages after the initial dose of saccharin.

Several studies have addressed the effect of NNS on gram-negative and gram-positive bacteria. A majority of the gut microbiome consists of bacteria that either belong to the gram-positive Firmicutes or gram-negative Bacteroidetes phyla [2]. Studies have shown that the ratio of Firmicutes to Bacteroidetes (F:B) is negatively correlated with body mass index [3]. Due to methodological limitations in the laboratory, the obligate anaerobic Bacteroidetes were replaced with facultative aerobes Proteobacteria, namely *E. coli*. According to a phylogenetic tree, gram-negative Bacteroidetes and Proteobacteria are closely related, with a recent common ancestor. Further, both Bacteroidetes and Proteobacteria are equally represented and important in the composition and health of the gut microbiome. Thus, Proteobacteria was a logical model organism from which to launch current studies. Previous research has yet to demonstrate the effect of NNS on the co-culture ratio of bacteria from different phyla or gram types. This study makes strides towards identifying how NNS may selectively affect bacteria with different genetic and biochemical properties.

Research has already identified that the gut microbiome is influenced by many factors, including, but not limited to, genetics and diet. The study at hand focused on the dietary influence, in particular how the addition of NNS into the diet will affect the viability of gram-negative and gram-positive bacteria. A previous study found that the presence of NNS, specifically saccharin, had a bacteriostatic effect on *E. coli* [6]. The results of our study support previous studies, as an overall decrease of 35.7% in the viability of *E. coli* was demonstrated with the addition of

saccharin. The current study saw a significant decrease in the viability of both *E. coli* and *S. aureus* in the presence of an increasing ratio of saccharin. According to Fig.1, *E. coli* appears to be directly dependent on the amount of saccharin present. A significant, linear decrease in intensity of fluorescence in *E. coli* was observed between both no NNS to a low dosage and no NNS to a high dosage. In Fig.2, there was also a significant decrease of over 20% in the viability of *S. aureus*. This inhibition appears to have a greater dependence on the presence or absence of saccharin, rather than on the amount of saccharin added, as the difference in inhibition between no NNS to low dosage and no NNS to high dosage was insignificant. The expected results based on phyla were not reproduced when focusing on gram type.

According to the inhibition trends seen in Fig.3, it can be assumed that if we were to further increase the saccharin ratio, these trends would continue. We would expect *E. coli* to continue in a linear, dose-dependent fashion, while a further increase in the saccharin ratio past a low dosage will have little to no effect on the viability of *S. aureus*. An increasing ratio of saccharin has a greater inhibitory effect on *E. coli*, as there is a linear, dose-dependent rate of inhibition. In regards to *S. aureus*, it appears as if saccharin is unable to have an inhibitory effect greater than 30%. This suggests that increasing from 2 to 3 packets of NNS will have a greater effect on the viability of *E. coli* than it will on *S. aureus*.

While the mechanism which causes the difference in inhibitory trends is currently unknown, a noteworthy difference between gram-positive and gram-negative bacteria is the thickness of the peptidoglycan cell wall. Further testing is warranted with other Firmicutes and gram-positive bacteria from other phyla to determine if the thickness of the peptidoglycan cell wall has an effect on inhibition. If these studies are inconclusive, the difference in inhibition could be due to Firmicutes having evolved different metabolic functions as compared to those of other phyla.

SYTO 13 stains all bacteria, both gram-negative and gram-positive. Therefore, *E. coli* and *S. aureus* were both represented in the green channel. HI stains and quenches the green fluorescence of SYTO 13 only in gram-positive bacteria, such as *S. aureus*. As seen in the Supplemental, an overlapping in the excitation and emission wavelengths in the green and red channels of the microplate assay resulted in fluorescence of both *E. coli* and *S. aureus* in both channels. Due to this overlap and quenching, analyzing the green channel in Fig.1 is more representative of the viability of gram-negative *E. coli*, while analyzing the red channel in Fig. 2 is more representative of the viability of the gram-positive *S. aureus*. As shown in Supplemental Fig.1 and Fig.2, the spectral overlap has some effect on the quantification of *S. aureus*, as it is not entirely quenched. However, *E. coli* is relatively unchanged. One way to overcome this would be to refine the excitation and emission wavelengths of the channels, as well as reduce the amount of background in the assay.

In this study, the viability of bacteria was only observed in individual cultures. The gut microbiome consists of hundreds of species of bacteria, all of which interact in an overall anaerobic environment. This study did not take into consideration how these interactions would affect the viability of *E. coli* and *S. aureus*, in addition to differing ratios of saccharin. If time and funding permits, a future study would look at the viability of *E. coli* and *S. aureus* in a co-culture, as opposed to individual cultures. The future study would also take into consideration the anaerobic nature of the gut microbiome. An additional future study could be performed that

looks at the effects of gram-negative and gram-positive bacteria of different phyla, to determine how this variation would affect a bacterial ratio. This would hopefully provide a more representative conclusion as to how bacteria interact in an intact gut microbiome.

The strengths of the current research are its significance and novelty. There is a current controversy over diets varying in NNS concentration and the impact they have on the gut microbiome. In addition, research has yet to identify the effect of NNS on gram-negative and gram-positive bacteria. Another strength is that an optimal growth range was identified for each bacteria. This ensured that the bacteria reached its exponential growth phase before saccharin was added.

Conclusion

In the current study, we explored the effect of an increasing ratio of saccharin on the viability of gram-negative *E. coli* and gram-positive *S. aureus*. Seeing that both *E. coli* and *S. aureus* decrease in the presence of saccharin, a high NNS diet has a negative impact on the overall health of one's gut microbiome. While this study only looked at the bacteria in individual cultures, additional studies are needed with co-cultures in order to further explore the relationship between gram-negative and gram-positive bacteria in the gut microbiome.

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Supplemental

SYTO 13 stains both gram-negative and gram-positive bacteria. Thus, both *E. coli* and *S. aureus* were stained by SYTO 13. Fluorescence of *E. coli* stained by SYTO 13 is shown in Fig.1. Fig.4 shows the intensity of fluorescence of the SYTO 13-stained *S. aureus* read in the green channel of the microplate assay. This figure shows that an increasing ratio of saccharin had no impact on the intensity of fluorescence of *S. aureus*. Given that HI stains only gram-positive bacteria and quenches the effects of SYTO 13, *S. aureus* fluorescence is best represented in the red channel, as seen in Fig.2.

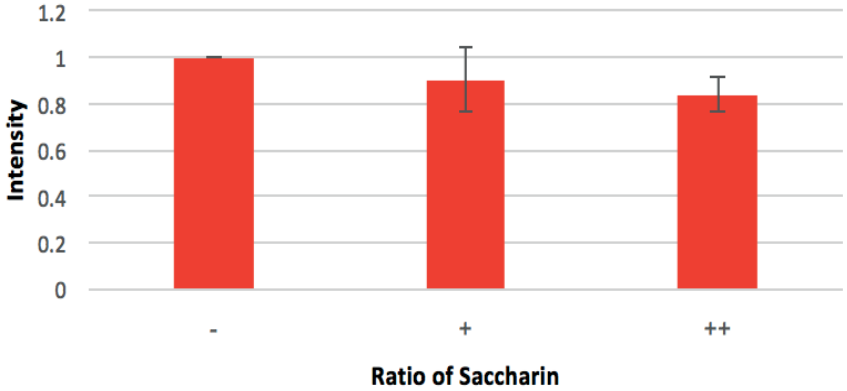

Fig.4 SYTO 13 Fluorescence of *S. aureus* in the Presence of Saccharin. SYTO 13-stained *S. aureus* did not demonstrate a meaningful decrease in the presence of saccharin.

A slight increase in the intensity of fluorescence of *E. coli* can be seen in Fig.5. This was deemed unremarkable and can be attributed to the variability of the assay. HI only stains gram-positive bacteria, in this case *S. aureus*. *E. coli* fluoresced as there was an overlap in the excitation and emission wavelengths between the green and red channels of the microplate assay. *E. coli* intensity is better represented in Fig.1.

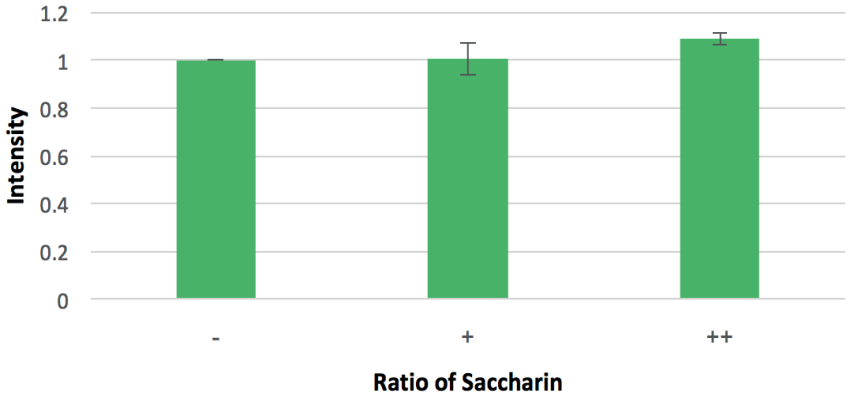

Fig.5 HI Fluorescence of *E. coli* in the Presence of Saccharin. A minimal increase in the intensity of *E. coli* was observed.