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Oral Dosages of the NSAID Aspirin Decreased the Growth Rate of Species Found in the Human Gut Microbiome Including Akkermansia muciniphila, Bacteroides fragilis, Clostridium sordellii, and Clostridium difficile

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Cover Page Footnote

We thank Professor Charles B. Coughlin for his dedication to guiding us through the process of producing this article. Professor Coughlin spent the time, money, and energy to provide us with useful skills in biology that will be utilized for many years to come.

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Abstract

Over past few decades, new insight has been revealed in the scientific community about the importance of the human gut microbiome relating to general health. It is known that imbalances in the species that reside in the human gut can cause organism-wide problems in humans. When prescribing or injecting oral medications, the thought of the downstream effects on the gut microbiome are not always considered. By exposing known healthy members of the gut; *Akkermansia muciniphila*, *Bacteroides fragilis, Clostridium sordellii*, and *Clostridium difficile* to the Aspirin, this study attempted to provide insight into the effects of the drug on bacterial growth. While these species only account for a small percentage of the total biodiversity of the gut microbiome, they are some of the most thoroughly studied and well known. *A. muciniphila* is known to occur in higher concentrations in healthy, low body mass index individuals which suggests that aspirin alternatives may be beneficial in some clinical cases. To accomplish the goal of this study, time courses were designed to analyze if different dosages of Aspirin inhibited the growth curve of each species when compared to growth curves of the same species in drug-free media. Aspirin was found to have a dose-dependent effect in growth rate of *A. muciniphila*, *B. fragilis, C. sordellii*, and *C. difficile* resulting in a significant decrease in the exponential growth phase of all four species. This suggested that aspirin inhibited cell culture growth in a dose-dependent manner. Aspirin's toxic affect to these important commensal species of the human gut should be considered by practitioners prior to prescription.

Introduction

The human gut microbiome is a new area of study within human health. It is known that the different species and their concentrations can directly affect the health of the human commensal partner (Althani et al., 2016). An imbalance of the gut microbiome has been related to dozens of symptoms and diseases (Althani et al,. 2016). Additionally, the gut microbiota performs multiple important functions that can benefit the human body. These important functions include immunity, metabolism, homeostasis, and acquisition of nutrients (Lazar et al., 2018). It is thought that some medications can be harmful to the metabolism or growth of the bacterial species within the gut (Feng et al., 2010). Unfortunately, many individuals consume over-the-counter (OTC) drugs such as aspirin without considering what the consequences might be for the balance of their gut microbiome or the downstream impacts on their health associated with these changes. By exposing important members of the gut flora; *Akkermansia muciniphila, Bacteroides fragilis,*

Clostridium sordellii, and *Clostridium difficile* to aspirin, this study attempted to provide insight into the effects of the drug on bacterial growth.

Aspirin, acetylsalicylic acid, is a non-steroidal anti-inflammatory drug (NSAID) that is the most used medication worldwide for pain management and can be taken daily by those dealing with cardiovascular diseases to reduce the risk of heart attack (Chi et al., 2021). Aspirin works by targeting cyclooxygenase-2 (COX-2) to inhibit prostaglandin synthesis, and salicylates like aspirin have been shown to reduce biofilm formation in the human microbiota (Damman, 2013). Additionally, the drug has been documented to destroy intestinal epithelium in vivo (Upreti et al., 2010). Due to this evidence of the destructive effects of aspirin, it is suspected that the consumption of this drug may be altering the natural growth of bacteria within the stomach. It is suspected that decreased dehydrogenase and esterase activity within the stomach epithelium generated by aspirin exposure may also decrease the metabolic activity of the bacterial species within the stomach, thereby altering natural growth (Upreti et al., 2010).

Excluding *Clostridium difficile,* the bacterial species chosen were those that are known to reside in the gut of healthy humans (Althani et al., 2016). Four species were tested: *Akkermansia muciniphila, Bacteroides fragilis, Clostridium sordellii,* and *Clostridium difficile*. The first of the three species has recently been discovered to be directly related to healthy BMI in humans (Geerlings et al,. 2018). Across many studies, it was found that low BMI individuals had higher concentrations of *A. muciniphila* and high BMI individuals had low concentrations of the species (Geerlings et al,. 2018; Iwaza et al., 2022). It is also known that high BMI individuals are at a higher risk for cardiovascular disease (CVD) (Davidson et al., 2022). Since aspirin (81 mg) is commonly prescribed as a CVD preventative drug, the exposure of aspirin to *A. muciniphila* was tested. B. fragilis is a Gram-negative and commensal bacterium that inhabits the lower GI tract of most mammals. *Bacteroides fragilis* is very abundant in healthy people, helps to direct the host immune system, and can protect its host from dangerous *Clostridium difficile* associated infections (Huang et al., 2011). *C. difficile* is a pathogenic, anaerobic, and Gram-positive species of spore-forming bacteria that is one of the leading causes of antibiotic-associated diarrhea and can cause colitis from disruption of the normal healthy bacteria in the colon. *B. fragilis* can inhibit *C. difficile* colonization through modulation of the gut microbiota, thus relieving pathogenic colitis (Deng et al., 2018). If the growth of *B. fragilis* is inhibited, it can lead to the gut being more susceptible to infections by pathogenic species. Because *B. fragilis* concentration regulates *C. difficile* concentration in the Gut, both species were exposed to aspirin to identify if the drug exposure attenuated the growth of one, or both species.

The last species investigated was *C. sordellii.* Although this species has been identified to reside in the gut of healthy individuals, irregular populations sizes can cause diseases such as toxic shock syndrome (Fischer et al., 2005). Understanding the effect of aspirin exposure on *C. sordellii* may provide insight into the pathology of symptoms experienced by aspirin users.

This study aimed to provide insight on the potential toxic effects of common medication on the important species of the human gut. The hypothesis was that aspirin will inhibit the growth of *A. muciniphila, B. fragilis, C. difficile,* and *C. sordellii* in the simulated gut with a dose-dependent relationship. Careful consideration of the conditions of the human stomach was utilized to design a study representing the drug-bacteria interaction. To simulate the conditions of the human gut, the Hungate method was used to culture the bacteria anaerobically. The cultures

were incubated at 37° C on a continuous agitation shaker to simulate human body temperature and the contraction of the smooth muscles of the stomach. The growth of the bacterial cultures was detected by optical density using spectrophotometry. If aspirin inhibits the growth of the bacteria, there will be less change in optical density relative to the uninoculated control.

Methods

Replicating Gastric Conditions

Because these experiments tested the effects of orally administered medications on the microbiome of the stomach, the conditions of the human stomach were required to be simulated. Because the full volume of the human stomach was not used, the experiment was scaled down appropriately. Three conditions of the human stomach were simulated including the microaerobic environment, temperature, and "churning" action generated by smooth muscle contractions known as peristalsis.

The average, empty human stomach volume is about 0.9 L (Radcliffe, 1998). Assuming the oral medications are taken with a small glass of water (0.1 L), the average stomach volume was determined to be 1 L. To make the synthetic stomach more manageable, the 1 L volume was scaled down to 10 ml. This reduced volume was easily tested utilizing 10 ml Hungate tubes. To replicate the microaerobic environment, the atmospheric gas within each Hungate tube was replaced by N_2 gas using the Hungate method (Hungate & Macy, 1973). A laboratory incubator at 37° C was utilized to replicate core body temperature. To reproduce the constant mixing of gastric fluid due to contractions of the smooth muscle lining of the stomach, also known as peristaltic movement, the tubes were placed on a gently agitating shaker within the incubator.

Culture Technique

The bacterial strains utilized were *Clostridium difficile* (ATCC© 43593-FZTM), *Clostridium sordellii* (ATCC® 9715™), *Bacteroides fragilis* (ATCC© 25285TM), and *Akkermansia muciniphila* (ATCC® BAA-835™). Due to the metabolic specificity of bacterial species, four different media mixes were used. Modified Reinforced Clostridial (MRC) media (ATCC® 2107) was used in the experiment involving *C. sordellii* and De Man, Rogosa and Sharpe (MRS) broth was utilized to culture *B. fragilis*. Tryptic Soy Broth (TSB) was used to culture *C. difficile* and, *A. muciniphila* was cultured in brain/heart infusion (BHI) broth supplemented with 1% porcine mucin (Type III, Sigma-Aldrich M1778-10G).

The individual oral medications tested were dosed appropriately for a 68 kg (150 lb.) human and scaled down to the 10 mL volume of media within the Hungate tubes. The normal adult dose of aspirin for analgesic use is 325 mg. A variety of dosages were utilized in each test, varying from 1/16 of a normal dose to 100 times a normal dose. The different dosages were used to possibly account for a dose dependent effect on the growth rates of the species. For all four experiments, each tube was loaded with the appropriate media for the species, and every tube excluding the positive and negative control were loaded with a dosage of medication. Three replicate tubes of each dosage were utilized. The Hungate method was then used to remove the atmospheric air from each tube and create the microaerobic environment simulating the stomach (Hungate & Macy, 1973). This method utilizes microfiltered compressed nitrogen to remove and replace the atmospheric air in each test tube. The tubes were then autoclaved, and ready for inoculation.

To begin each test, insulin syringes were used to transfer 0.1 mL of bacterial stock inoculated ~24 hours prior to the study into each tube using sterile technique. The positive control tube was

inoculated, and the negative control tube was not. The negative control tube served as the baseline for optical density measurements. Because the positive control tubes contained no drug dosage, the growth measured within the tubes was considered "unimpeded growth" and provides a comparison point for the aspirin-dosed tubes. After all appropriate tubes were inoculated, the tube's absorbance at 620 nm was measured with a spectrophotometer immediately and at fixed time intervals thereafter. A Model 340 Sequoia-Turner spectrophotometer was used in all experiments except the *C. difficile*, which used a Thermo Spectronic Educator Digital Spectrophotometer. After measuring the absorbance, the tubes were incubated. The time between measurements and the total length of the study varied depending on the previously determined growth rate and pattern of each species. Once the data were collected, statistical analyses of the growth differences in maximal culture density were completed using unpaired, one-tailed t-tests (p = 0.05) between the slopes of the exponential phases of the different dosages of aspirin for each individual species.

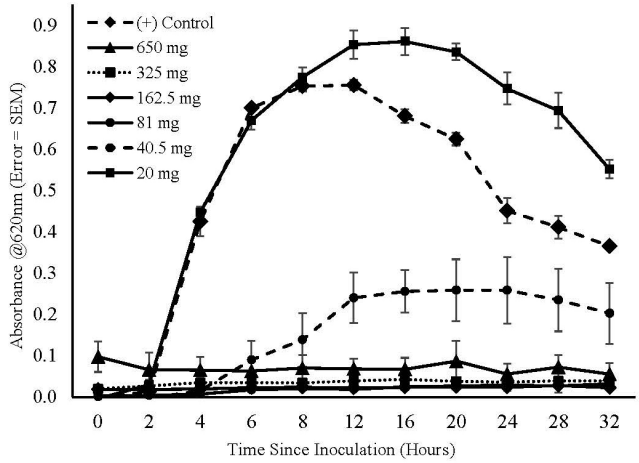
Results

Akkermansia Muciniphila Growth Was Inhibited by Aspirin

The growth rate of *Akkermansia muciniphila*, was determined by dividing the change in absorbance for each dosage from hour 2 to hour 12 by 10, the length of the growth phase. The rate of the exponential phase of growth demonstrated that the growth rate of the 40.5 mg, 81 mg, 162.5 mg, 325 mg, and 650 mg aspirin dosage values were significantly less than the positive control, while the 20 mg and positive control were statistically indistinguishable. Significance was determined by completing an unpaired, one-tailed t-test (p = 0.05) between the rate for each amount and that of the positive control (Figure 1). Additionally, the growth rate of the 40.5 mg, 81 mg, 162.5 mg, 325 mg, and 650 mg dosage values were significantly less than the 20 mg dosage (Figure 1). This was determined using the same t-test performed between the rates of the 40.5 mg, 81 mg, 162.5 mg, 325 mg, and 650 mg dosages, against the 20 mg dose.

Figure 1

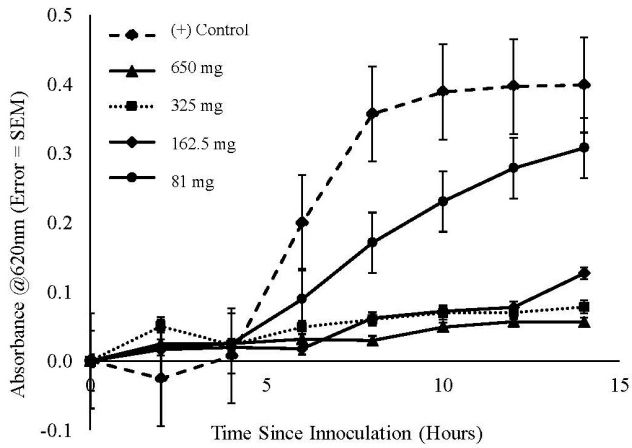
Change in Absorbance at 620 nm Light Over 28 Hours of A. muciniphila Cultures Exposed to Various Dosages of Aspirin



Note. Time "0" represents the time that the sterile medium was inoculated, and the test tube's optical density was determined immediately. The aspirin dosage values are represented as a multiplier of the dosing appropriate for a 68 kg adult human. The error bars represent the standard error of the mean. Three test tube replicates were averaged to make each data point in these profiles.

Figure 2

Change in Absorbance at 620 nm Light Over 14 Hours of B. fragilis Cultures Exposed to Various Dosages of Aspirin



Note. Time "0" represents the time that the sterile medium was inoculated, and the test tube's optical density was determined immediately. The aspirin dosage values are represented as a multiplier of the dosing appropriate for a 68 kg adult human. The error bars represent the standard error of the mean. Three test tube replicates were averaged to make each data point in these profiles.

***Bacteroides fragilis* Growth Was Inhibited by Aspirin**

The data showed inhibition of growth for all aspirin concentrations except for the positive control and the 81 mg (Figure 2). The negative control showed some measurements are likely due to the turbidity of the media. By visual inspection, the slopes of the doses below the 81 mg were similar and did not appear to have exponential growth. A slope analysis was performed to compare the slopes of the positive control and 81 mg. There was no significant difference observed. A slope analysis performed to compare the slopes of the 81 mg and 162.5 mg showed a significant difference in the slopes ($P < 0.002$).

***Clostridium sordellii* Growth Was Inhibited by Aspirin**

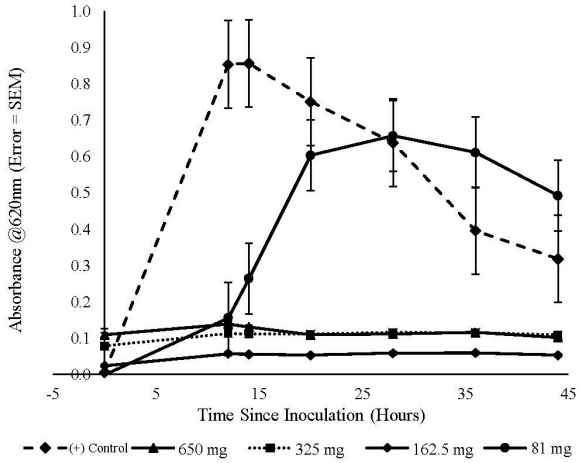
The data showed inhibition of growth for all aspirin concentrations except for the positive control and the 81 mg dose (Figure 3). By visual inspection, the slopes of the doses below the 81 mg dose were similar and did not appear to have exponential growth. A slope analysis performed to compare the slopes of the positive control and 81 mg dose showed a significant difference in the slopes ($P < 0.04$). It is likely that the turbidity of the media and the aspirin in some test tubes were more optically dense due to the aspirin dosage differences. A slope analysis was performed to compare the slopes of the 81 mg dose and 162.5 mg dose. There was no significant difference observed. Some data points have no observable SEM bars due to small SEM values of the measurements.

***Clostridium difficile* Growth Was Inhibited by Aspirin**

The data showed inhibition of growth for all aspirin concentrations except for the positive control and the 81 mg dose (Figure 4). By visual inspection, the slopes of the doses below the 81 mg dose were similar and did not appear to have exponential growth. A slope analysis performed to compare the slopes of the positive control and 81 mg dose showed no significant difference in the slopes. A slope analysis was performed to compare the slopes of the 81 mg dose and 162.5 mg. There was no significant difference observed.

Figure 3

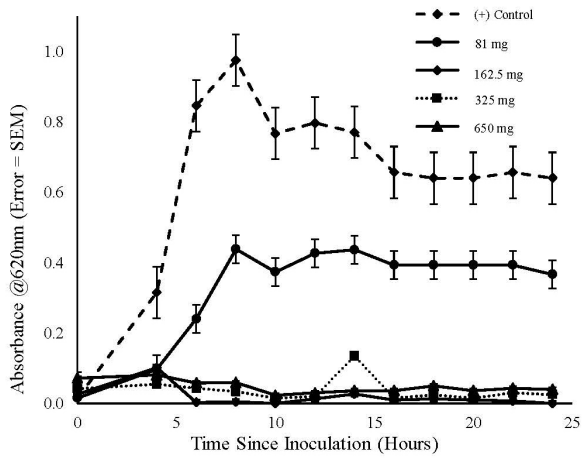
Change in Absorbance at 620 nm Light Over 45 Hours of C. sordellii Cultures Exposed to Various Dosages of Aspirin



Note. Time "0" represents the time that the sterile medium was inoculated, and the test tube's optical density was determined immediately. The aspirin dosage values are represented as a multiplier of the dosing appropriate for a 68 kg adult human. The error bars represent the standard error of the mean. Three test tube replicates were averaged to make each data point in these profiles.

Figure 4

Change in Absorbance at 620 nm Light Over 24 Hours of C. difficile Cultures Exposed to Various Dosages of Aspirin



Note. Time "0" represents the time that the sterile medium was inoculated, and the test tube's optical density was determined immediately. The aspirin dosage values are represented as a multiplier of the dosing appropriate for a 68 kg adult human. The error bars represent the standard error of the mean. Three test tube replicates were averaged to make each data point in these profiles.

Discussion

The commensal and pathogenic species in the gut microbiota that were tested in the present study were *Akkermansia muciniphila, Bacteroides fragilis, Clostridium difficile,* and *Clostridium sordellii.* These species of bacteria have coevolved with humans in a symbiotic relationship where ingested food and drugs can influence the gut microbiota's function (Althani et al., 2016). Aspirin, or acetylsalicylic acid, is a non-steroidal anti-inflammatory drug (NSAID) that has an inhibitory effect on most commensal and pathogenic species of bacteria in the cardiovascular system (Upreti et al., 2010). The hypothesis was that aspirin will inhibit the growth of *A. muciniphila, B. fragilis, C. difficile,* and *C. sordellii* in the gut microbiota in a dose-dependent relationship and that there would be a differential impact of aspirin on the growth patterns of each species of bacteria.

The experiment involving *A. muciniphila* demonstrated a dose-dependent decrease in the slope of the growth rate. The positive control and 20 mg dose were statistically the same, indicating that 20 mg of oral aspirin dose does not affect the normal growth rate of the species. Simultaneously, the growth rate of the 40.5 mg dosing was significantly less than both the positive control and 20 mg dose, and significantly steeper than all other doses. This result may indicate that the decrease in growth rate of *A. muciniphila* is dependent on the dose of aspirin taken in vivo. All doses higher than 40.5 mg had a growth rate of ~0, indicating no change in species number. Both the normal human dose for analgesic effect (325 mg) and cardiovascular disease prevention (81 mg) fall within this complete growth inhibition range for *A. muciniphila.*

The reason for this dose dependent change of growth rate is not currently fully understood. Aspirin is known to irreversibly inhibit platelet cyclooxygenase-1 (COX-1), causing downstream pathway changes that alter the function of platelets (Floyd & Ferro, 2013). Assuming the drug could enter the cell, *A. muciniphila* may have a COX-1 pathway directly linked to metabolism. The inhibition of this hypothetical metabolic pathway may result in a dose dependent decreased growth rate. A previous study by demonstrated that aspirin dose-dependently decreased the growth rate of *E. coli, Pseudomonas sp., Lactobacillus sp.*, and *Staphylococcus sp* (Upreti et al., 2010). The same study also indicated that aspirin was dose-dependently toxic to intestinal epithelium. The researchers attributed this effect to the inhibition of dehydrogenase and esterase activity within the epithelium (Upreti et al., 2010). If *A. muciniphila* utilizes either of these metabolic enzymes, this may account for the decrease in growth rate seen in the current study. The 20 mg aspirin dosing may be slightly reducing the metabolic rate of the species, thereby reducing the rate in which mucin is consumed (Geerlings et al., 2018). Because the bacteria are metabolizing the mucin slower, and each tube contained the same concentration of mucin, the plateau and death phases were elongated when compared to the control (Kosciow & Deppenmeier, 2020). It is worth noting that pH change is also a possible explanation for the effect, but whether it is a metabolic effect or chemical effect does not change the fact of the observed impact on culture growth inhibition.

The experiments utilizing *B. fragilis, C. difficile*, and *C. sordellii* had similar outcomes to the experiment with *A. muciniphila*. The results on the growth curves showed that all concentrations tested, except the 81 mg dose, inhibited growth of *B. fragilis, C. difficile,* and *C. sordellii.*

Overall, we conclude that aspirin has a bacteriostatic growth attenuation effect on the commensal and pathogenic gastrointestinal species, *Bacteroides fragilis, Clostridium sordelii,* and *Clostridium difficile* similar to the effect seen

on *A. muciniphila*. Another study tested a similar hypothesis where aspirin and other salicylates may inhibit the growth of pro-inflammatory bacteria in a dose-dependent matter but used a different methodology in which healthy volunteers received either a standard dose of aspirin or a placebo and provided stool samples for six weeks that were analyzed to determine the microbial composition. It was found that aspirin influenced multiple taxa in the gut microbiota including Bacteroides (Prizment et al., 2020). This differs from the present study which was performed in vitro. The present study allowed more experimental control and the ability to distinguish the interactions between just the bacterial species being studied and the aspirin without any extra factors such as diet affecting the results and the community of the microbiome. The slope analysis performed in the present study showed no significant difference in the slopes between each species but there was a differential effect observed between the different aspirin concentrations within each species of bacteria. It is unclear whether *B. fragilis, C. difficile,* and *C. sordellii* in the gut microbiota might be differentially affected when aspirin is ingested. If they are differentially affected, one species could potentially dominate in the gut and lead to disease such as gastric distress as seen in *C. difficile* "blooms."

Despite the experimental optimization, the present study had some constraints. One limitation with the paradigm was that large dosages of aspirin oversaturated the media and distorted optical density readings, preventing the use of higher doses. Also, comparisons of the growth rates of each species could not be done simultaneously. Ideally, the tests would have been done using an actual human gut. The study was performed in vitro which allowed more experimental control but did not have the same environment as an actual human gut.

Conclusion

Supported by the work of Upreti and Pant (2010), the decrease of growth rate and intensity seen in *A. muciniphila* with aspirin exposure provides a strong case of evidence that aspirin is toxic to common gut bacteria. An interesting dilemma appears when the relationship of *A. muciniphila* concentrations and healthy BMI are factored in. Many past studies have indicated that individuals with a healthy BMI have a much higher proportion of *A. muciniphila* in their gut than those with a high, unhealthy BMI (Iwaza et al., 2022). Simultaneously, individuals with a high BMI are at a higher risk for cardiovascular disease, and therefore are more likely to take a daily 81 mg aspirin as a CVD preventative (Davidson et al., 2022). The present study found that an 81 mg (1/4x normal dose) of aspirin completely inhibited the growth of *A. muciniphila* (Figure 1). This may indicate that aspirin usage is making obesity worse by preventing the growth of *A. muciniphila.* Unfortunately, this is only speculative as the problem of obesity has hundreds of factors, but these data may provide insight for future studies. Ideally, an in vivo metagenomic analysis of the gut of high BMI patients before and after the usage of daily aspirin would be performed. If compared to BMI change, a study of this type would help isolate the aspirin related reduction of *A. muciniphila* as an obesity factor. If found to be true, CVD prevention drug alternatives may be implemented in obese patients.

Future studies could involve testing the effects of aspirin on more commensal and pathogenic species in the gut microbiota and testing the effects of other medications such as Aleve or Tylenol on the gut microbiota. Other methods that could be performed include following through with standard plate counts to quantify bacterial growth, testing the pH before and after each time course is run, and performing

molecular testing on individual species of bacteria within the time courses.

This study provided insight into the often-forgotten importance of the human gut microbiota. Care providers often overlook the complicated commensal system of gastric microscopic organisms when prescribing medications and building a care plan for each patient. The present study demonstrated that this oversight may be resulting in a tradeoff of health benefits in patients suffering from cardiovascular disease. It was demonstrated that the low dosages of aspirin typically prescribed to patients who are suffering or at risk of CVD are toxic to *A. muciniphila*. Many past studies have demonstrated that this species is positively correlated with healthy, low BMI individuals. This being said, patients at risk for CVD typically have a higher BMI and other health problems. Aspirin ingestion in these patients may be reconsidered by practitioners to avoid further diminishing the population of the healthy species *A. muciniphila.* The same principle should be followed for *Bacteroides fragilis*, a species whose growth rate is also diminished by aspirin exposure. *B. fragilis* presence in the gut prevents the overgrowth of *C. difficile*, a species known to cause potentially lethal infections when overgrown in the gastrointestinal system.

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