Investigating Microbial Metabolism of Garcinia mangostana by the Human Gut Bacterium Clostridium sporogenes

Anna Liu
University of Connecticut, anna.liu@uconn.edu

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Investigating Microbial Metabolism of *Garcinia mangostana* by the Human Gut Bacterium *Clostridium sporogenes*

Anna Liu

B.S., University of Connecticut, 2021

A Thesis Submitted in Partial Fulfillment of the Requirements to Graduate as an Honors Scholar for the Doctor of Pharmacy Degree at the University of Connecticut 2023

Honors Thesis Advisor: Marcy J. Balunas, Ph.D.

Honors Academic Advisor: Brian Aneskievich, Ph.D.
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Acknowledgements

I would first like to thank my principal investigator, Dr. Marcy J. Balunas of the School of Pharmacy at University of Connecticut. Without her assistance, involvement, and support in every step of this process, this experiment and thesis would’ve never been accomplished. Thank you so much for your guidance and mentorship throughout the years. I’ve learned and grown tremendously throughout my time in the Balunas Lab to be a more well-rounded thinker, scientist, and student. Thank you for the opportunity to experience science and create science!

I’m extremely grateful to my fellow lab members; Mariam Zedan, Bethlehem Abebe, Sara Puckett, Kojo Acquah, Robert Samples, Zachary Lane, and Shekar Sunderesh for the friendly lab environment and support on my project whenever I needed it. In addition, I would also like to acknowledge Mariam Zedan and Shekar Sunderesh for their comments and revisions in writing this thesis. Thank you to Akshay Narula and Dr. Na Li for their help in using the anaerobic chamber, and Dr. Jen Liddle from the UConn Proteomics and Metabolomics Facility for her assistance in LC-MS and data analysis. A special thank you to Dr. Brian Aneskievich for guidance and support throughout my academic career.

Finally, I’d like to thank the Dr. Karl A. Nieforth Pharmacy Student Research Award and School of Pharmacy PharmD Honors Program Research Scholarship for funding of this project.
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Abstract

The human gut microbiome is a burgeoning field of research due to recent recognition of its profound impact on the human hosts’ homeostasis, physiology and metabolism, and disease. Botanical natural products have been utilized for disease prevention and medical treatment since ancient times. Increased consumption of botanical natural products for their health benefits have led researchers to investigate interactions between the gut microbiome and botanical natural products. *Garcinia mangostana*, also known as mangosteen, is a fruit from Southeast Asia that is known for its health benefits such as anti-inflammatory, antihypertensive, and anticancer properties. Its benefits have been linked to bioactive xanthones found in the pericarp of the fruit, with α-mangostin being one of the abundant xanthones. Despite health benefits from α-mangostin, low bioavailability has been reported, likely resulting from first pass metabolism. Due to the low bioavailability of α-mangostin, further research is needed to determine how α-mangostin exerts its effects. The human gut microbiota plays a significant role in the interaction with orally ingested compounds, including those from mangosteen. There is a lack of understanding regarding how mangosteen compounds are metabolized by the human gut microbiome. This project aims to investigate the metabolism and biotransformation of mangosteen metabolites by *Clostridium sporogenes*, a member of a prominent phyla of gut bacteria. Metabolism of α-mangostin, mangosteen extract, and risperidone by *C. sporogenes* were confirmed through incubation in an anerobic environment. Results from this experiment can be used to guide future exploration into specific mechanisms of metabolism of gut bacteria.
Introduction

Human Microbiome

The human gut microbiome is a burgeoning field of research due to recent recognition of its profound impact on the human host's homeostasis, physiology, and metabolism, and disease (Thursby et al., 2017). The impact of the microbiome has long been overlooked and has been coined the “forgotten organ” and the “second genome” (Spanogiannopoulos et al., 2016). With more recent studies being published, the contributions of the gut microbiota on human health have become clearer; yet continued progress is needed to understand these complex interactions.

The microbiome impacts human hosts in a wide range of physiologic functions. These include protection against pathogens, regulation of immunity, and collection of energy from dietary intake (Thursby et al., 2017). Home to over a trillion microorganisms, the human gut microbiome includes bacteria, microscopic fungi, archaea, viruses, and protozoa (Thursby et al., 2017). These microorganisms are generally in a mutualistic relationship with the human host and are tightly linked to environmental and social factors which can change microbial composition. Variations in individual gut microbiomes are common and can be due to factors such as genetics, diet, health status, geography, and age (O’Sullivan et al., 2015).

The composition of gut microbial species is important in maintaining homeostasis (Wu et al., 2012). A disrupted microbiome, due to a variety of factors, can lead to homeostatic imbalance and disease. Mice studies in this area have shown the impact of diet on the microbiome. Mice fed with anthocyanins and other phenolic acid metabolites over an 8-week period resulted in a reduced body weight gain and improved glucose metabolism compared to mice with an antibiotic-disrupted microbiome (Esposito et al., 2015). Anthocyanins are water-soluble pigments found in many plants, including flowers, fruits, and roots. Anthocyanins have been shown in animal models, human clinical trials, and cell culture studies to have antioxidative, antidiabetic, anti-inflammatory
capabilities and many other health effects (Khoo et al., 2017). Animal and human model studies of the microbiome have indicated strong research potential in the link between human gut metabolism and disease.

Botanical Natural Products

Botanical natural products have been utilized for disease prevention and medical treatments since ancient times (Schmidt et al., 2007). Approximately one-fourth of all drugs prescribed today originate from plant species. When botanical substances reach the gut, several interactions may occur. For example, botanicals can interact with other consumed botanicals, or botanicals can interact with the gut bacteria to biotransform into novel secondary metabolites. These botanical secondary metabolites or their biotransformation products may be bioactive, producing various effects in the body (Schmidt et al., 2007). Investigation of these metabolites might result in new therapeutics. Examples of important metabolites from botanical supplements include α-mangostin from mangosteen (Balunas et al., 2008, Cui et al., 2010, Gutierrez-Orozco et al., 2013, Li et al., 2013, Pedraza-Chaverri et al., 2008), resveratrol from red wine (Stervbo et al., 2007), ECGC from green tea (Singh et al., 2011) and many more. There has been an ongoing increase in consumption of these over-the-counter botanical supplements due to their perceived influence on health (Clarke et al., 2015). National estimates of the use of complementary health approaches in adults across timepoints of 2002, 2007, and 2012 indicated that dietary supplements remained the most popular complementary health approach used by adults in the United States (Clarke et al., 2015). One such botanical supplement of focus is *Garcinia mangostana*, also known as mangosteen.
Garcinia mangostana

One of the most widely used botanical supplements is *Garcinia mangostana* (Im et al., 2017). *G. mangostana* is a tropical tree found predominantly in Southeast Asia, known for its health benefits such as anti-inflammatory, antihypertensive, anticancer, and microbial health promotion (Balunas et al., 2008, Gutierrez-Orozco et al., 2013). These benefits have been linked to bioactive xanthones found in the pericarp of the fruit, with α-mangostin being one of the prominent xanthones. α-Mangostin, a major constituent of mangosteen, is a prenylated xanthone shown to provide anti-cancer activities, especially in the lung and breast, as well as anti-inflammatory properties (Pedraza-Chaverri et al., 2008, Cui et al 2010). However, an area of intense research focus for α-mangostin and other natural products is their low bioavailability in the human body.

Through bioavailability studies of α-mangostin, low bioavailability has been reported, often resulting from first pass metabolism (Li et al., 2013). First pass metabolism refers to the metabolism of any orally ingested molecule before entering the systemic circulation (Lin et al., 1999). The bioavailability of a compound, whether botanical or pharmaceutical, is essential because the higher the bioavailability of the substance, the more of the compound reaches target cells and has an effect. In contrast, if a compound has low bioavailability, it may signify more intensive first pass metabolism and less of the compound reaching the target cell. After the compound is orally ingested, it travels down the digestive tract and will encounter the microflora present in the small intestine, the large intestine, and the colon (Clarke et al., 2019). The compound can undergo reactions due to enzymes produced by gut microbiota, including hydrolytic reactions, carbon-carbon cleavages, ring-fusion, deconjugation, reduction and glucuronidation (Le Chatelier et al., 2013, Aura et al., 2008). After gut metabolism, the compound will travel to the liver through the hepatic portal vein and be further metabolized by liver enzymes. Then, the compound will be
released into the systemic circulation. For a metabolite to exert its effect on human physiology and disease, an adequate amount needs to reach systemic circulation. Due to the low bioavailability of \( \alpha \)-mangostin (Li et al., 2013), further research is necessary to determine how \( \alpha \)-mangostin exerts its effects on the host.

**Research Aims**

The human gut microbiota plays a significant role in interaction with orally ingested compounds, including those from mangosteen. Despite being extensively studied, there is still a lack of understanding about how mangosteen compounds are metabolized in the body, especially by the microorganisms present in the human gut. These interactions can potentially result in alteration of molecular structure, modification of mangosteen metabolites half-lives, bioavailability, and biological effects. However, gut bacterial metabolites and their biotransformation of botanical compounds may undermine the effects of mangosteen metabolites on human physiology. While the link between the human microbiome and health has become an area of intense research interest, the interaction between microbiota and botanical supplements, such as mangosteen, is still underexplored.

A key obstacle to studies in this area is the complexity of the systems. It is very challenging to study the *in vivo* effects given the diversity of gut microbes coupled with that of dietary components. For example, recent studies have revealed links between the microbiome to cancer biology, neuroscience, and metabolic diseases but robust links have yet to be determined (Roopchand et al., 2015, Kostic et al., 2013, Foster and Neufeld et al., 2013). Future studies should fill in the gap of the biochemical processes within the gut microbiota and the potential links to bioactivity of molecules. To better understand the role of dietary supplement and microbiome
interactions, these transformations and metabolic process must be examined in controlled settings so that more translational studies can be done.

To investigate the metabolism and biotransformation of mangosteen metabolites by gut bacteria, a literature search was performed to determine the optimal method to carry out these experiments. Zimmermann and colleagues assessed the metabolism capacity of 76 bacterial species/strains which represent the major phyla of the human gut microbiome. The bacterial species were incubated with 271 pharmaceutical drugs from a selection of diverse clinical indications and physiochemical properties (Zimmermann et al., 2019). To narrow down to one gut bacterium for this study, overall bacterial metabolism was assessed.

*Clostridium sporogenes*, a member of the most prevalent bacterial phyla found in the human gut and with extensive metabolizing capability, was chosen to examine microbial interactions with mangosteen, a popular botanical supplement. Prior investigation of gut microbiota found potential positive impacts on human health from *C. sporogenes* generating antioxidant and neuroprotective indole acetic acid from dietary tryptophan (Gao et al., 2018). In addition, *C. sporogenes* was found to metabolize >20% of 30 drugs, >50% of 20 drugs and >80% of 15 drugs (Zimmerman et al., 2019). *C. sporogenes* was one of the few bacterial species that metabolized risperidone almost completely, making it a promising choice for metabolic studies (Zimmerman et al., 2019). The present study will determine the effects of *C. sporogenes* on mangosteen to provide guidance for subsequent isolation and identification of metabolites from mangosteen resulting from interactions with *C. sporogenes*. Understanding these metabolite transformations will allow us to further explore the links between botanical health benefits and microbial transformation.
Methods

Growth Media and Agar Plates for Culturing of *C. sporogenes*

Culture media components were purchased from Fisher Scientific, Sigma Aldrich, and BD. Degradation assays included *C. sporogenes* (purchased from ATCC 15579), α-mangostin, and risperidone (purchased from Fisher Scientific), and mangosteen extract [prepared from mangosteen provided by Nature’s Sunshine Products (Spanish Fork, UT)].

Gut microbiota media (GMM) was used for degradation assays and incubation. All ingredients were purchased from Fisher Scientific unless noted otherwise. Media was prepared following the protocol in Zimmermann et al., 2019. Based on the protocol, the following media components were needed: tryptone, yeast extract (purchased from BD), d-glucose (purchased from BD), L-cysteine (purchased from BD), cellobiose, maltose, fructose, meat extract (purchased from BD), MgSO₄·7H₂O, NaHCO₃, NaCl, FeSO₄ (purchased from Sigma Aldrich), Millipore water, acetic acid, isovaleric acid, propionic acid, butyric acid, KH₂PO₄, CaCl₂, histidine hematin solution, tween 80, ATCC vitamin mix (purchased from ATCC), ATCC trace mineral mix (purchased from ATCC), menadione, and resazurin (purchased from Sigma Aldrich). Tryptone peptone, yeast extract, D-glucose, L-cysteine, cellobiose, maltose, fructose, meat extract, MgSO₄·7H₂O, NaHCO₃, NaCl, FeSO₄, and Millipore water were combined in an Erlenmeyer flask. Acetic acid, isovaleric acid, propionic acid, and butyric acid were added to the Erlenmeyer flask. KH₂PO₄ and CaCl₂ were in a powdered form that needed to be in solution before addition to the Erlenmeyer flask, with pH adjustment to 7.2 for KH₂PO₄. The solution was then autoclaved at 121°C to ensure sterility. After autoclaving, the solution was cooled to an appropriate temperature (~47 °C), after which ATCC vitamin mix and ATCC trace mineral mix were added. The histidine hematin solution, tween 80, menadione, and resazurin were prepared and filter sterilized via Millex sterile syringe filters with PVDF membrane and pore size of 0.22 μm (purchased from Sigma Aldrich). These components
were added to the solution to complete the gut microbiota media (GMM). The media was immediately placed in anaerobic conditions.

Tryptic soy agar (TSA) supplemented with 5% sheep blood was prepared by following the protocol from ATCC. Based on the protocol, the following media components were needed: tryptic soy broth (purchased from BD), defibrinated sheep blood (purchased from Thermo Fisher Scientific), agar (purchased from BD), and Millipore water. Tryptic soy broth, agar, and Millipore water were measured and combined to be autoclaved at 121 °C. After the sterilized media was cooled to ~47 °C, room temperature defibrinated sheep blood was added. The media was mixed and dispensed in agar plates. After the agar had solidified, these were placed in anaerobic condition.

Anaerobic Chamber

All culturing and anaerobic work was performed in a Coy Vinyl Anaerobic Chamber (Figure 1). Frozen glycerol stocks of *C. sporogenes* were plated on trypticase soy agar supplemented with 5% sheep blood and incubated at 37 °C for 24 hours.

A saturated silver nitrate (AgNO₃) solution was used in the chamber to act as a hydrogen sulfide (H₂S) absorbent. Anaerobic bacteria are known to produce H₂S which can be detrimental to chamber health and maintenance (Pridmore et al., 1998). A saturated solution of AgNO₃ was prepared with 25 mL of micelle water (H₂O) and 100 g of solid AgNO₃. Using a foil-wrapped Erlenmeyer flask to prevent exposure to light, 1 mL of H₂O was added at a time followed by sonicication until the solid was completely dissolved. This solution was placed inside the chamber for the entirety of the experiment.
Figure 1. **Anaerobic Chamber.** A photo of the anaerobic chamber used for these experiments. A & B are the outer and inner lock of the airlock of the chamber, respectively. They function to keep the anaerobic air separate from aerobic air. C displays the gas regulator of the nitrogen and anaerobic gas mix which is kept at 20 psi. D shows the vinyl gloves where hands are inserted to work inside the chamber. E is a catalyst and fan box. The catalyst reacts with oxygen (O\textsubscript{2}) and hydrogen (H\textsubscript{2}) to make water, and the fan box circulates air in the chamber around. F is the humidifier to absorb water from the catalyst to maintain anaerobic conditions.
Culturing of *C. sporogenes* in Anaerobic Chamber

Since *C. sporogenes* arrived as freeze-dried pellet, it was necessary to rehydrate the strain in the anaerobic chamber following ATCC protocols. Briefly, to rehydrate *C. sporogenes*, the vial was brought into the anaerobic chamber. Using a tube filled with 3 mL of TS broth supplemented with 5% sheep blood, 0.5 mL was used to rehydrate 1/3 of the pellet. The suspension (0.5 mL) was transferred back to the tube and mixed well. This suspension was used to plate on either TS agar supplemented with 5% sheep blood, or TS broth supplemented with 5% sheep blood to determine retrieval of *C. sporogenes*. Because of the freeze-dried nature of the strain, 96 hours were necessary for full retrieval of the strain as indicated by growth on TS agar supplemented with 5% sheep blood or turbidity of TS broth supplemented with 5% sheep blood (ATCC).

*C. sporogenes* cultured on TS agar supplemented with 5% sheep blood was used for degradation assays, including dose response and time series, and for cryopreservation.

Figure 2. **Growth of *C. sporogenes***. Photos of the growth of *C. sporogenes* in the anaerobic chamber after 48 hours at 37 °C.
Degradation Assay: Determining metabolism by *C. sporogenes* based on incubation time and dose of compound

**Dose Response and Time Series**

All experiments were conducted in biological triplicate. Five individual bacterial colonies were selected and inoculated in GMM and allowed to grow for 24 hours. Three stocks were prepared, including GMM containing α-mangostin, risperidone, or mangosteen extract, respectively, to a concentration of 20 mM (or 20 mg/mL for mangosteen extract). These stocks were then combined with the previously inoculated GMM, followed by dilution with additional GMM to obtain 5 mL total volume for final concentrations of 2 μM (or 2 μg/mL), 50 μM (or 50 μg/mL), or 100 μM (or 100 μg/mL) of α-mangostin, risperidone, or mangosteen extract, respectively, in anaerobic culture tubes. The different concentrations of each compound/extract were used to test dose response. For the time series, each culture vial was incubated with *C. sporogenes* and compound at baseline (0 hours), 12 hours, 24 hours, and 50 hours until collected by removing from the anaerobic chamber and stored in -80 °C until extraction.

Extraction of the samples included the addition of an internal standard mix (as described below in the *Extraction Protocol*), as well as MeOH and ACN, following published protocol as described below (Zimmermann et al., 2019). The sample was then centrifuged to collect the supernatant. An internal standard mix is commonly used in mass spectrometry to correct for sample variation during extraction and to compensate for variability in signal intensity due to ion suppression (Jeanne Dit Fouque et al., 2018). It is also important to note that compounds added as internal standard mix should be chemically similar to compound being analyzed but not expected to be naturally present in the sample.

Samples were labeled alphabetically (A) for α-mangostin with *C. sporogenes*, (B) for risperidone with *C. sporogenes*, (C) for mangosteen extract with *C. sporogenes*, (D) for α-
mangostin without C. sporogenes, (E) for risperidone without C. sporogenes, and (F) for mangosteen extract without C. sporogenes. The number following each letter indicated concentration of corresponding compound/extract added with number 1 indicating samples with 2 μM of the corresponding compound/extract, 2 indicating samples with 50 μM of the corresponding compound/extract, and 3 indicating samples with 100 μM of the corresponding compound/extract. Following the numerical value, a lowercase letter “a” indicates the first of the three biological replicates, a lowercase “b” indicates the second biological replicate, and a lowercase “c” indicates the third replicate.

Extraction Protocol

Extraction followed published protocols (Zimmermann et al., 2019). Briefly, a sample of 100 μL of incubation culture was transferred to a centrifuge tube. An internal standard mix of sulfamethoxazole, caffeine, ipriflavone and yohimbine was added to the sample to reach a final concentration of 80 nM of each. To obtain 80 nM, each compound was added to the same 100 μL of Millipore water. The molecular weights of sulfamethoxazole, caffeine, ipriflavone and yohimbine are 253.28 g/mol, 194.19 g/mol, 280.32 g/mol, and 390.91 g/mol, respectively. Using the molecular weight of each substance and a volume of 100 μL, a 10 mM solution would result from the addition of 0.25 mg of sulfamethoxazole, 0.19 mg of caffeine, 0.28 mg of ipriflavone, and 0.39 mg of yohimbine. To further dilute the solution, 1 μL of the 10 mM solution was added to 25 mL of Millipore water for a concentration of 400 nM. Lastly, 25 μL of the 400 nM solution was added to incubation culture for a final concentration of 80 nM. Following the addition of the internal standard mix, cold (-20 °C) acetonitrile and methanol were added in a 1:1 ratio to the sample. The mixture was allowed to incubate for at least an hour at -20 °C. Following incubation,
the mixture was centrifuged at 3200 relative centrifugal force (rcf) for 15 minutes. The supernatant was collected for further analysis.

**Figure 3. Overview of Extraction Protocol.** First, ACN and MeOH were put in the freezer. Bacterial samples were removed from -80 °C, and internal standards, and cold ACN and MeOH were added to each sample. Samples were then allowed to incubate in freezer for at least an hour. These were then centrifuged to obtain the supernatant (created with BioRender.com).
Inoculation Procedures

Following Zimmermann et al. (2019), risperidone was chosen to be the positive control due to its known metabolism by \textit{C. sporogenes} (Zimmermann et al., 2019). Mangosteen extract in addition to isolated \(\alpha\)-mangostin, an abundant xanthone found in mangosteen, were chosen to be the two experimental treatments. The negative control contains the culture media with and without \textit{C. sporogenes} incubation. Risperidone, \(\alpha\)-mangostin, and mangosteen extract were all solubilized in 0.5\% dimethyl sulfoxide (DMSO) to be added to the culture media.

Day 1: Experimental Setup & Media Preparation

The anaerobic chamber was set up to contain and maintain anaerobic conditions necessary for \textit{C. sporogenes} growth. Silver nitrate solution was prepared and set aside, away from direct light, prior to being placed in the chamber. Trypticase-soy agar (TSA) supplemented with 5\% sheep blood and gut microbiota media (GMM) was prepared and cooled before moving into the chamber. All supplies necessary for the experiment were gathered and sterilized with \(\text{LpH}\).

Day 2-4: Bacterial Culture

Supplies, culture media, plates and silver nitrate solution were moved into the anaerobic chamber. From frozen glycerol stocks, \textit{C. sporogenes} was plated on TS agar with 5\% sheep blood and allowed to incubate for 48 hours at 37 °C.

Day 5: Inoculation to Liquid Media

Five individual bacterial colonies of \textit{C. sporogenes} were inoculated into 30 mL of pre-reduced GMM and incubated at for 24 hours. Outside the chamber, the botanical stock solutions, \(\alpha\)-mangostin and mangosteen extract, and risperidone were prepared as described above and moved to the chamber to be pre-reduced.
Day 5-7: Incubation

In brief, bacterial cultures were added to pre-reduced GMM containing either risperidone, α-mangostin, or mangosteen extract. Samples were incubated anaerobically at 37 °C for 0, 12, 24, and 50 hours before collection and storage at -80 °C for further processing.

Figure 4. Overview of Experimental Procedure. C. sporogenes was plated on TS agar for 48 hours in 37 °C and 5 individual colonies were selected and inoculated in GMM. Bacteria colonies were allowed to grow in GMM for 24 hours in 37 °C and then diluted in GMM media with
botanical stock. This was then incubated 0, 12, 24, or 50 hours, before being collected (created with BioRender.com).

**Liquid Chromatography – Mass Spectrometry**

Using the supernatant from the incubated samples, samples were prepared for LC-MS using a 1:1 ratio of supernatant:water. Samples were run in single injections followed by two additional injections of the same samples on separate dates. These were run on a Waters Synapt G2-Si mass spectrometer (MS) attached to a Waters Acquity ultra-high-performance (UPLC) liquid chromatography system at the UConn Proteomics and Metabolomics Facility. The acquisition method used was an 11-minute fast data-independent-acquisition method with positive polarity, resolution mode with photodiode array detector (PDA). Each sample (10 μL) was injected and separated over a Waters Acquity UPLC HSS T3 column (2.1 x 150 mm, 1.8 μm) and heated to 45 °C. A mobile phase of H2O with 0.1% formic acid, referred as A, and acetonitrile with 0.1% formic acid, referred as B, was used. The gradient elution of the LC-MS run began with 5% B and 95% A for 0.5 minute, increased to 60% B and 40% A over 3.5 minutes, increased to 98% B and 2% A over 4 minutes, and remained for another 1.2 minutes before ramping down to 5% B and 95% A for 1.8 minutes. Samples were eluted directly into a Waters Synapt G2-Si mass spectrometer and ionized by ESI with a 2kV capillary voltage and 40V sampling cone voltage. MS1 data was collected in continuum mode over a scan range of 150-2000 m/z, with a scan time of 0.2 sec. MS² data was collected over the same scan range of 150-2000 m/z, and fragmented using a fixed collision energy of 30V. Real-time lock-mass correction was achieved by concomitant infusion of leucine enkephalin using the measurement of the singly charged peptide ion over a 3-scan average.
MS Data Processing

LC-MS data was analyzed using Waters MassLynx™ software. For each sample, the total ion chromatogram (TIC), extracted ion chromatogram (EIC) and area under the curve (AUC) was analyzed and compared.

TICs of each sample were analyzed to match the retention time of the pure compounds. Mass searches of 411.1807 m/z were used for α-mangostin and mangosteen extract while the mass search of 411.2196 m/z was used for risperidone, both to obtain the EIC for each sample. The AUC was calculated from the EIC using the integrate function in Waters MassLynx™ Software. Parameters used in the integrate function included a peak-to-peak (PtP) baseline noise, and minutes for peak width, smoothing parameters, ApexTrack peak detection parameters, and response threshold which were all automatically determined for each peak.
Results & Discussion

Analysis

For the analysis, the samples with 2 μM at 12 hours were analyzed for comparison of the positive control (risperidone) to previous literature (Zimmermann et al., 2019). All other samples were stored in -80 °C until future analysis. In analyzing the AUC mean value for metabolism of cultures with C. sporogenes and cultures without C. sporogenes, there was found to be a 33% decrease in risperidone detected, indicating metabolism by C. sporogenes (Figure 5). The mean AUC calculated from risperidone samples without C. sporogenes was 86,023.2 μAU*minute with a standard error of the mean (SEM) of 11,789.9 μAU*minute. The mean AUC calculated from risperidone samples with C. sporogenes was 57,410.6 μAU*minute +/- 19,243.4 μAU*minute. There was a 66% decrease in α-mangostin, indicating metabolism by C. sporogenes. The mean AUC calculated from α-mangostin samples without C. sporogenes was 14,594.5 μAU*minute +/- 4,717.4 μAU*minute. The mean AUC calculated from α-mangostin samples with C. sporogenes was 5,042.2 μAU*minute +/- 1,173.0 μAU*minute. In samples testing metabolism of mangosteen extract, there was a 42% decrease in α-mangostin from mangosteen extract detected, indicating metabolism by C. sporogenes. The mean AUC calculated for α-mangostin from mangosteen extract samples without C. sporogenes was 1,904.9 μAU*minute +/- 941.7 μAU*minute. The mean AUC calculated for α-mangostin from mangosteen extract samples with C. sporogenes was 1,111.7 μAU*minute +/- 338.2 μAU*minute.

A two-tailed T-test using an α-value of 0.05, was conducted to determine the p-value for metabolism of risperidone, α-mangostin, and mangosteen extract with and without C. sporogenes present. Risperidone and α-mangostin values were statistically significantly different with p-values of 0.0216 and 0.0018, respectively. Mangosteen extract values were not statistically significant with a p-value of 0.0943.
Figure 5. Metabolizing potential of *C. sporogenes*. AUC mean data from risperidone, α-mangostin, and mangosteen extract with and without *C. sporogenes*. Error bars depict standard error of the mean (SEM). Additional plots, TICs, EICs, and AUCs can be found in supplemental information.

The metabolism of *C. sporogenes* in risperidone samples (33% reduction) indicates the study design was successful since risperidone was used as a positive control in the experiment because of its known metabolism by *C. sporogenes* (Zimmermann et al., 2019). The greatest metabolism was shown in samples incubated with α-mangostin (66% reduction) suggesting that *C. sporogenes*’ metabolism may include a functional group present in α-mangostin. In samples with mangosteen extract, there was a 42% reduction in α-mangostin for samples incubated with *C. sporogenes*. Compared to pure α-mangostin (66% reduction), mangosteen extract, which contains α-mangostin as well as other xanthone constituents, had a reduced metabolism of α-mangostin. A preliminary hypothesis to explain this phenomenon could be that *C. sporogenes* metabolized a suite of xanthone constituents, reducing its ability to metabolize α-mangostin. Another possibility
includes possible antibacterial properties of other mangosteen constituents, reducing *C. sporogenes* ability to metabolize α-mangostin.

**Risperidone**

Risperidone was first analyzed via LC-MS, resulting in a prominent peak at an elution time of 3.9 minutes, confirmed using TICs for pure risperidone with elution time of 3.9 minutes (Figure 6). After analyzing the TICs for risperidone samples, a mass search of 411.2196 *m/z* with 20 parts-per-million (ppm) window resulted in the EICs in Figure 7, confirming the peak at 3.9 minutes to be risperidone. The EICs of risperidone were integrated to give the AUC for risperidone in the control and treated samples (Figure 8). The AUC values of risperidone samples without *C. sporogenes* were used to compare to samples with *C. sporogenes* (Table 1).
Figure 6. **Total Ion Chromatograms (TICs) for Risperidone-treated Samples.** The total ion chromatogram for risperidone-treated samples including samples of risperidone without *C. sporogenes* (A) and samples of risperidone with *C. sporogenes* (B).
Figure 7. **Extracted Ion Chromatograms (EICs) for Risperidone-treated Samples.** The extracted ion chromatogram for risperidone-treated samples including samples of risperidone without *C. sporogenes* (A), and samples of risperidone with *C. sporogenes* (B).
Figure 8. **Area Under the Curve Chromatograms (AUCs) for Risperidone-treated Samples.**

The area under the curve chromatograms for risperidone-treated samples including samples of risperidone without *C. sporogenes* (A), and samples of risperidone with *C. sporogenes* (B). The values above each selected peak indicate the AUC value.
Table 1. **Area Under the Curve (AUC) Table for Risperidone-Treated Samples.** AUC values were obtained for each risperidone-treated sample with or without *C. sporogenes*. Samples were injected three times to obtain technical replicates (named “_r2” or “_r1”).

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<thead>
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<th>Risperidone</th>
<th>With (W) or Without (WO) C. sporogenes</th>
<th>Area Under the Curve (μAU*minute)</th>
<th>Mean (μAU*minute)</th>
<th>Standard Deviation</th>
<th>Standard Error of the Mean (μAU*minute)</th>
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% metabolized by *C. sporogenes* 33.3

**α-mangostin**

Pure α-mangostin was first analyzed via LC-MS to observe a prominent peak at an elution time of 8.45 minutes. TICs for pure α-mangostin were confirmed with the elution time of 8.45 minutes (Figure 9). After analyzing the TICs for pure α-mangostin, a mass search of 411.1807 m/z with 20 ppm window resulted in the EICs in Figure 10, which were further confirmed to be α-mangostin. The EICs of α-mangostin were integrated to give the AUC of α-mangostin in the control and treated samples (Figure 11). The AUC values of α-mangostin-treated samples without *C. sporogenes* were used to compare to samples with *C. sporogenes* (Table 2).
Figure 9. **Total Ion Chromatograms (TICs) α-Mangostin-treated Samples.** The total ion chromatogram for α-mangostin-treated samples including samples of α-mangostin without *C. sporogenes* (A), and samples of α-mangostin with *C. sporogenes* (B).
Figure 10. Extracted Ion Chromatograms (EICs) for α-Mangostin-treated Samples. The extracted ion chromatogram for α-mangostin-treated samples including samples of α-mangostin without *C. sporogenes* (A), and samples of α-mangostin with *C. sporogenes* (B).
Figure 11. Area Under the Curve Chromatograms (AUCs) for α-Mangostin-treated Samples.

The area under the curve chromatogram for α-mangostin-treated samples including samples of α-mangostin without *C. sporogenes* (A), and samples of α-mangostin with *C. sporogenes* (B). The values above each selected peak indicate the AUC value.
Table 2. **Area Under the Curve (AUC) Table for α-mangostin-treated Samples.** AUC values were obtained for each α-mangostin-treated sample with or without *C. sporogenes*. Samples were injected three times to obtain technical replicates (named “_r2” or “_r1”).

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<th>α-mangostin</th>
<th>With (W) or Without (WO) <em>C. sporogenes</em></th>
<th>Area Under the Curve (μAU*minute)</th>
<th>Mean (μAU*minute)</th>
<th>Standard Deviation</th>
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</table>

% metabolized by *C. sporogenes* 65.5

**Mangosteen Extract**

The mangosteen extract was first analyzed via LC-MS to observe a prominent peak of α-mangostin constituent in mangosteen extract at an elution time of 8.45 minutes. TICs for mangosteen extract confirmed α-mangostin in mangosteen extract with the elution time of 8.45 minutes (Figure 12). After analyzing the TICs for mangosteen extract-treated samples, a mass search of 411.1807 m/z with 20 ppm resulted in the EICs seen in Figure 13. EICs further confirmed peak to be α-mangostin within the mangosteen extract. The EICs of α-mangostin within mangosteen extract were integrated to give the AUC of α-mangostin in mangosteen extract (Figure 14). The AUC values of mangosteen extract samples without *C. sporogenes* were used to compare
to samples with *C. sporogenes*. The AUC values for each sample were calculated, resulting in the mean AUC for samples with or without *C. sporogenes* (Table 3).

Figure 12. **Total Ion Chromatograms (TICs) of Mangosteen Extract-treated Samples.** The total ion chromatogram for mangosteen extract-treated samples without *C. sporogenes* (A) and with *C. sporogenes* (B).
Figure 13. **Extracted Ion Chromatograms (EICs) for Mangosteen Extract-treated Samples.**

The extracted ion chromatogram for mangosteen extract-treated samples without *C. sporogenes* (A) and with *C. sporogenes* (B).
Figure 14. **Area Under the Curve Chromatograms (AUCs) for Mangosteen Extract-treated Samples.** The area under the curve chromatograms of mangosteen extract-treated samples without *C. sporogenes* (A) and with *C. sporogenes* (B). The values above each selected peak indicate the AUC value.
Table 3. **Area Under the Curve (AUC) Table for Mangosteen Extract-treated Samples.** AUC values were obtained for each mangosteen extract-treated samples with or without *C. sporogenes*. Samples were injected three times to obtain technical replicates (named “_r2” or “_r1”).

<table>
<thead>
<tr>
<th>Mangosteen Extract</th>
<th>With (W) or Without (WO) C. sporogenes</th>
<th>Area Under the Curve (μAU*minute)</th>
<th>Mean (μAU*minute)</th>
<th>Standard Deviation</th>
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**% metabolized by C. sporogenes** 4.6
Conclusion

Using LC-MS to detect and analyze abundance of α-mangostin or risperidone in each sample demonstrated metabolism of each compound by *C. sporogenes*. There was the greatest percentage of metabolism shown in samples with α-mangostin (66%) although less so in samples testing for α-mangostin in mangosteen extract (41.6%), and risperidone (33%). Despite testing for the same compound (α-mangostin) in samples with only α-mangostin as compared to those with mangosteen extract demonstrated decreased metabolism observed from comparison of 66% to 42%. This suggests a possible mechanism in which *C. sporogenes* may be inhibited, requiring more time to metabolize, or possible interactions with other xanthone constituents in mangosteen extract. Further studies are necessary to determine the metabolism and possible resulting bioactivity of other xanthone constituents in mangosteen extracts following *C. sporogenes* metabolism.

In addition, the results and analyses presented in this thesis were from only a small portion of the incubated samples. Other samples yet to be tested include variable concentrations of α-mangostin, risperidone and mangosteen extract including 50 μM and 100 μM treatments at multiple timepoints including 0, 12 hour, 24 hour, and 50 hour, as well as the 12 μM treatment at 0 hour, 24 hour, and 50 hour. Further analysis and comparison of these samples may result in additional insight in the metabolic potential of *C. sporogenes* as it relates to time (time series) or concentration (dose response). In addition, degradation products of risperidone or α-mangostin in response to metabolism by *C. sporogenes* may be assessed to determine possible changes in bioactivity or bioavailability.

Future directions in the research of gut microbiota metabolism include exploration of enzymes of different gut bacteria that contribute to metabolism of these molecules and more
botanical supplements such as ECGC in green tea and resveratrol in red wine. The identification of metabolized constituents in botanical supplements may lead to bioactivity studies such as anti-inflammatory and anti-proliferative assays to further establish the link of gut microbiota metabolism to bioactivity of natural products. There is a continued need to understand the role of gut microbiome and to establish in vitro to in vivo relevance prompting further research in this field.
References


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