Gut Health Benefits of Natural and Alkali-Processed Cocoa (*Theobroma cacao*) with and without Inulin

Lauren A. Essenmacher

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of
Master of Science in Life Science
In
Food Science and Technology

Monica Ponder, Chair
Andrew Neilson
Joshua Lambert
Cristina Fernandez-Fraguas
Sean O'Keefe
Amanda Stewart

May 7, 2020
Blacksburg, Virginia

Keywords: flavanols, bioactivity, cocoa, *in vitro* fecal fermentation, *in vitro* digestion, procyanidins, alkalization, alkali processing, gastrointestinal barrier function, intestinal inflammation

Gut Health Benefits of Natural and Alkali-Processed Cocoa (*Theobroma cacao*) with and without Inulin

Lauren A. Essenmacher

ABSTRACT

Chronic conditions such as obesity, inflammatory bowel disease (IBD), and colitis are associated with gastrointestinal (GI) inflammation and compromised GI barrier integrity. Cocoa may be a potential dietary strategy to mitigate gut-related conditions and been shown to elicit anti-inflammatory, antioxidant, and prebiotic effects. Alkali treatment of cocoa was once thought to reduce its bioactivity, but new evidence suggests it may enhance cocoa's health properties, through the formation of new, potentially bioactive high molecular weight compounds. Inulin, a fructose-containing plant polymer, exerts prebiotic effects and has also been investigated in the mitigation of IBD. This study aims to 1) investigate effects of alkali processing on gut health related bioactivity and phytochemical composition of cocoa and 2) evaluate potential additive benefits of combining cocoa and inulin.

Polyphenolic and flavanol compounds in natural cocoa, alkalized cocoa, and inulin powders were characterized using Folin-Ciocalteu (total polyphenols) and 4-dimethylaminocinnamaldehyde (total flavanols) assays, thiolysis , and HILIC UPLC-MS/MS. Treatments of cocoa and inulin were made in 1:2 cocoa:inulin and 1:4 cocoa:inulin mixtures for both natural and alkalized cocoas. Cocoa mixtures, in addition to both cocoa powders and inulin alone, were subjected to an *in-vitro* digestion to generate material for an *in-vitro* fecal fermentation. Samples collected from the fermentation at 0, 6, 12, and 24 hours were analyzed via HPLC-MS for microbial metabolites, applied to HT-29 colon cancer cells to assess anti-inflammatory activity, and applied to a florescence assay measuring PLA_2 inhibitory activity.

The alkalized cocoa powder was found to have a significantly lower concentration of total polyphenols and total flavanols, as well as a lower mDP, suggesting that alkalization may affect larger procyanidins more than smaller flavanol compounds. Inulin enhanced the inhibition of the PLA2 enzyme and enhanced the IL-8 anti-inflammatory properties of cocoa, although the trends were weak. Overall, we did not see any clear, significant effects of alkalization or the addition of inulin to cocoa's colonic metabolite formation or its gut bioactivity *in vitro*. However, we have demonstrated that colonic fermentation of cocoa may have a negative effect on its bioactivity *in vitro*. Future research should further explore flavanol DP and bioactivity, fiber's interaction with polyphenols, colonic metabolism of cocoa, and cocoa's gut health effects *in vivo*.

Gut Health Benefits of Natural and Alkali-Processed Cocoa (*Theobroma cacao*) with and without Inulin

Lauren A. Essenmacher

GENERAL AUDIENCE ABSTRACT

Gut conditions like obesity-associated inflammation and inflammatory bowel disease are highly prevalent, debilitating, and currently have no cure. Cocoa has been investigated as a possible dietary strategy for the mitigation and prevention of chronic inflammatory gut conditions due to its anti-inflammatory and enzyme inhibiting properties. Most attribute these effects of cocoa to its abundance of compounds called polyphenols. It is widely thought that the ability of cocoa to promote health is lost when cocoa beans are processed, because of the loss of polyphenols. Alkalization, or "Dutching", is an optional step in cocoa processing that some manufacturers perform to enhance flavor and color formation. Dutching cocoa can promote the polymerization of many smaller, flavanol, protein, and other compounds into larger, indigestible compounds. These indigestible compounds will not be absorbed in the small intestine and may be broken down in the large intestine by colonic bacteria, forming new metabolites. We obtained cocoa powders, one natural (not alkalized) and one alkalized and compared them in terms of content of polyphenols, bioactivities, and anti-inflammatory abilities. Additionally, we added a known prebiotic, inulin, to our cocoa formulations to determine if there are additive benefits of cocoa and inulin together. Ultimately, we found that alkalized cocoa contained lower concentrations of all polyphenolic compounds, even the larger compounds. Inulin enhanced the inhibition of digestive enzymes and the anti-inflammatory properties of cocoa, though not significantly. Inulin also reduced the pH (i.e. increased the acidity) of a simulated gut environment, which may be beneficial. Alkalization did not significantly affect cocoa's enzyme inhibitory activity or anti-inflammatory activity. Overall, the addition of inulin to cocoa does not seem to be effective in increasing cocoa's ability to treat and prevent gut diseases, but more information is needed.

Acknowledgments

I would not have been able to complete this project without the support of many faculty and staff members as well as other graduate students at Virginia Tech and at the Plants for Human Health Institute.

I would like to first thank my advisors, Dr. Andrew Neilson and Dr. Monica Ponder. Dr. Neilson took me on as his student before moving institutions, but made sure to keep my degree on track and give me all the help I needed, even when I broke my foot and again when a global pandemic hit. Dr. Ponder was willing to take over as my committee chair in Spring 2020, gave me valuable mentorship, and taught me many microbiology, lab, and life skills. I am grateful for everything you both have taught me, inside and outside the classroom.

Thank you to the rest of my committee, Dr. Sean O'keefe, Dr. Cristina Fernandez-Fragus, and Dr. Amanda Stewart for their valuable feedback, support, and encouragement throughout my time. I would especially like to thank Dr. Joshua Lambert for graciously hosting me in his lab at Penn State for a week and helping me with cell culture experiments.

Thank you to my cohort in the department of Food Science and Technology at Virginia Tech, graduate students at the Plants for Human Health Institute, and Neilson lab mates throughout my time, especially Kat Racine, for her endless advice, friendship, and help at the bench.

Finally, I would like to thank my friends and family. My mom and dad for always supporting and believing in me and my brother, Brian, for always being a source of comic relief when I needed it. Last but not least, my golden girl, Lucy, for being my constant companion through the uncertainties of graduate school.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

IBD, inflammatory bowel disease, GI; gastrointestinal, UC; ulcerative colitis, DP; degree of polymerization; HMW; high molecular weight, SCFA; short chained fatty acids, UPLC/MS; ultra high performance liquid chromatography/mass spectrometry, MLCK; myosin light chained kinase, TEER; transepithelial resistance, ZO; zona occludins, ZO-1; zona occludin-1, LPS; lipopolysaccharides, PPO; polyphenol oxidase, HMF; hydroxymethylfurfural, MRP; Maillard reaction product, HDAC; histone deacetylase; TNF-α; Tumor Necrosis Factor alpha, IL-8; Interleukin 8, iNOS; inducible nitric oxide synthase, COX2; cyclo-oxygenase-2, GSE; grape seed extract, AOM; azoxymethane, CTFR; cystic fibrosis transmembrane conductance regulator, GAE; gallic acid equivalents, DMAC; 4-dimethylaminocinnamaldehyde, mDP; mean degree of polymerization, TQD; triple quadrupole, MRM; multi-reaction monitoring, CID; collision-induced dissociation, ESI; electrospray ionization, PLA2; phospholipid enzyme 2, FITC-D; fluorescein isothiocynate-dextran, DSS; dextran sodium sulfate, PCB2; procyanidin B2

Attributions

Several colleagues contributed to Chapters 3-4 of this thesis. Below is a brief explanation of their contributions:

Andrew P. Neilson, PhD, a current faculty member at the Plants for Human Health Institute in the Department of Food, Bioprocessing, and Nutrition Sciences at North Carolina State University assisted with the study design, data analysis, and completion of the manuscript.

Monica A. Ponder, PhD, a current faculty member in the Department of Food Science and Technology at Virginia Tech assisted in study design, compilation, and completion of the manuscript.

Kathryn C. Racine, MS, a current PhD student at the Plants for Human Health Institute in the Department of Food, Bioprocessing, and Nutrition Sciences at North Carolina State University assisted with cocoa and inulin extraction, Folin and DMAC assays, HILIC analysis, and data processing and interpretation.

Laura E. Griffin, PhD, a formal post-doctoral scholar at the Plants for Human Health Institute of North Carolina State University assisted with sample preparation and conduction of thiolysis, HILIC, and Folin assays, and data interpretation.

Joshua D. Lambert, PhD, a current faculty member in the Department of Food Science at Pennsylvania State University contributed to the study design, assisted with cell culturing of HT-29 cells, data analysis and interpretation, and completion of the manuscript.

Sydney A. Corbin, a current research assistant at the Plants for Human Health Institute of North Carolina State University assisted with culturing Caco-2 TC-7 cells, performing the FIT-C dextran assay, data collection, and extraction of fermentation metabolites.

Candace M. Nunn, a current research assistant at the Plants for Human Health Institute of North Carolina State University assisted with the extraction of fermentation metabolites.

Kim M. Waterman, a current food microbiology lab manager in the Department of Food Science and Technology at Virginia Tech, assisted with freeze drying the fermentation products.

Talia N. Seymore, a current undergraduate student in the Department of Toxicology at Pennsylvania State University assisted with the preparation of fermentation sample solutions in DMSO and the sample preparation and conduction of the IL-8 assay

CHAPTER 1. INTRODUCTION AND JUSTIFICATION

Inflammatory Bowel Disease (IBD), Celiac disease, and colitis are chronic conditions associated with gastrointestinal (GI) inflammation and compromised GI barrier integrity, but their exact mechanisms remain unknown [1,2]. Additionally, chronic low-grade inflammation of the gut is seen in obesity and the metabolic syndrome [3]. These gut conditions have no permanent drug cure, increase the risk of developing colorectal cancer, and contribute to a decrease in overall heath and quality of life [1,2]. Recently, cocoa (*Theobroma cocoa*) has been explored as a possible treatment for gut-related conditions. Bioactive compounds in cocoa, known as polyphenols, have shown to help mitigate some causative agents of poor gut health like inflammation and barrier function [4–6].

The polyphenols, specifically flavan-3-ols, present in cocoa are made up of repeating monomeric compounds catechin and epicatechin which polymerize to form procyanidins with varying degrees of polymerization (DP)[7]. Flavan-3-ols have been shown to differ in bioactivity based upon their DP and location of the target tissue [7]. Understanding the bioactivity of these compounds is important for predicting their efficacy of eliciting a physiological response *in vivo*. Smaller flavanols of cocoa like catechin and epicatechin are absorbed quickly by the small intestine and display protective activity in the cardiovascular system [6,8]. Larger DP flavanols however, have shown to be more bioactive in gut tissue [4,7].

Traditional cocoa processing is widely thought to have a negative impact on cocoa's health properties of as it results in the loss of polyphenols [9–11]. However, alkali processing or "dutching" cocoa can promote the polymerization of smaller flavanol, protein, and other compounds which creates many new, higher DP flavanols, and other high molecular weight (HMW) compounds [9,12]. When ingested, these compounds will not be absorbed readily and will reach bacteria in the colon and be formed into various metabolites which could potentially be bioactive. Furthermore, alkali treatment reduces cocoa into smaller, more soluble particles, which could have increased fermentability to gut microbes.

In addition to cocoa, prebiotics, or substrates that are selectively utilized by the host microorganisms, conferring a health benefit, have been shown to improve gut health and reduce symptoms of IBD.[13]. Inulin, a fructo-polymer and prebiotic, influences gut health by promoting the growth of beneficial bacteria and the creation of short chained fatty acids (SCFAs) [14]. Combining both inulin and cocoa could provide a full spectrum of benefits for gut health.

Our overall objectives for this experiment are to determine the impact of alkali processing on the health-related bioactivity and phytochemical composition of cocoa and to evaluate the potential additive benefit of combining alkali processed cocoa and inulin. This experiment is a pilot study, in which a single, low, dose was used for each assay in order to identify which treatments might warrant further investigation in future studies.

Our central hypotheses are:

1. alkali-treatment of cocoa may improve gut health-related functionality and this effect may be due to the formation of novel bioactive cocoa compounds and improvement in the fermentability of cocoa
2. the combination of alkali-treated cocoa and inulin will have enhanced gut-health-related activity compared to either treatment alone

In order to achieve our overall objectives, the following specific aims are proposed:

1. To determine the impact of alkalization, the addition of inulin, or their combination on the gut-health related activities of cocoa powder in vitro, we will apply our treatments to human colon cancer cell lines to determine their abilities to mitigate inflammation in the gut as determined by measuring IL-8 cytokine expression. We will also apply our treatments to a florescence assay determining PLA_2 enzyme activity.

Due to the compounds formed during alkali processing and the prebiotic effects of inulin, we predict that alkalized cocoa powders with inulin added 4x inulin followed by 2x inulin) will result in the largest reductions in inflammation-related cytokine release and enzyme activity. We predict the natural powder with added inulin (4x inulin followed by 2x inulin) will present the next largest reductions, followed by the alkalized powder alone, then the natural powder alone.

2. Using UPLC/MS analyses, we prosed to identify compounds in these cocoa powders that serve as effective predictors of bioactivity in cell culture and thus *in vivo*.

We predict the alkalized powder will have larger polyphenolic compounds compared to the natural powder which will correspond to improved bioactivity in the gut

CHAPTER 2. REVIEW OF THE LITERATURE

2.1 GUT HEALTH

The gastrointestinal (GI) tract is a long canal from mouth to anus comprised of the oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, and anal canal. It primarily functions to ingest, digest, absorb, and excrete waste from foods, with the help of accessory organs like the liver, gallbladder, and pancreas. Four layers, the outer serosa, muscularis externa, submucosa, and inner mucosa, form the wall of the intestines. The mucosal layer is further divided into 3 sections: the outermost muscularis mucosa, a wall of smooth muscle, the lamina propria, a layer of blood and lymph tissue, and the epithelial layer, a single layer of specialized cells. The epithelial cells function mainly to secret a protective layer of mucus, which keeps tissues moist, protects the host from pathogens and foreign substances, and absorbs and transport nutrients. The proper functioning of the mucosal layer plays a key role in maintaining gut homeostasis, barrier function, and the symbiotic relationship between the gut microbiome and the host [15,16].

Increasing evidence suggests the importance of gut health and its influence on other bodily systems and overall health. The term "gut health" can be defined as a set of criteria that must be maintained to possess a healthy upper and lower gastrointestinal (GI) tract [16]. These criteria include effective digestion and absorption of food, absence of GI illness, normal and stable intestinal microbiota, effective immune strength, and an overall state of wellbeing/quality of life. These criteria for gut health are further described in the table below, adapted from Bischoff 2011 [16].

The two entities most responsible for determining gut health are the GI barrier and the gut microbiome, which interact considerably. The intestinal epithelial cells form a barrier segregating the gut microbiota from immune cells. Additionally, intestinal epithelial cells react to gut microbes and their metabolites to produce mediators/signals to induce responses in lymphoid (immune) tissues. If either of these systems are compromised, intestinal inflammatory states ensue.

Gut Barrier Function

The intestinal mucosal barrier is a complex structure comprised of both physical and chemical components separating the internal bodily environment from the luminal contents.

Table 2.1 Gut Health adapted from Bischoff 2011 [16].

5 Major Criteria for a healthy GI system	Specific Signs of GI Health
Effective Digestion and Absorption of food	• Normal nutritional status and effective absorption of food • Regular bowel movement, normal transit time with no abdominal pain • Normal stool consistency and rare nausea, vomiting, diarrhea, constipation, and bloating
Absence of GI illness	• No acid peptic disease, gastroesophageal reflux disease, or other gastric inflammatory disease • No enzyme deficiencies or carbohydrate intolerances • No IBD, celiac disease, or other inflammatory state • No colorectal or other GI cancer
Normal/Stable Intestinal Microbiota	• No bacterial overgrowth • Normal composition and vitality of the gut microbiome • No GI infections or antibiotic-associated diarrhea
Effective Immune Status	• Effective GI barrier function, normal mucus production and no enhanced bacterial translocation • Normal numbers and normal activity of immune cells • Immune tolerance and no allergy or mucosal hypersensitivity
Status of well-being	• Normal Quality of life • Balanced serotonin production and normal functioning of the enteric nervous system

This intestinal mucosal barrier represents the largest surface for interactions between bodily cells and their external surroundings and therefore must maintain a delicate balance of protecting the host from invasion of foreign microorganisms and antigens while allowing essential fluids and nutrients to be absorbed [17].

The main component of the physical mucosal barrier is the intestinal epithelium, a single layer of specialized subtypes of cells: Enterocytes, goblet cells, Paneth cells, and enteroendocrine cells, in addition to immunity cells like lymphocytes and dendritic cells. Three types of junctions, tight junctions, adherence junctions, and desmosomes, join the cells of the epithelium and regulate paracellular permeability. The other physical components of the barrier include the

vascular endothelium and the mucus layer. In addition to the physical barrier, digestive secretions, immune molecules, cells products like cytokines, inflammatory mediators, and antimicrobial peptides comprise the chemical portion of the barrier [17–19].

Bischoff *et al*. defines intestinal permeability as "a functional feature of the intestinal barrier at given sites, measurable by analyzing flux rate" [17]. They further describe normal intestinal permeability as "a stable permeability found in healthy individuals with no signs of intoxication, inflammation, or impaired intestinal functions" and impaired intestinal permeability as "a disturbed permeability being non-transiently changes compared to the normal permeability leading to a loss of intestinal homeostasis, functional impairments, and disease" [17]. Factors that can alter intestinal permeability include modifications of the gut microbiota, changes in the mucus layer, epithelial damage, alcohol, stress, diet, among many others. Direct epithelial cell damage via cytotoxic agents or mucosal irritants result in a marked loss of barrier function. Loss of intestinal barrier function can occur abruptly or gradually, leading to chronic inflammatory diseases, although the mechanisms are not yet precisely understood [18] .

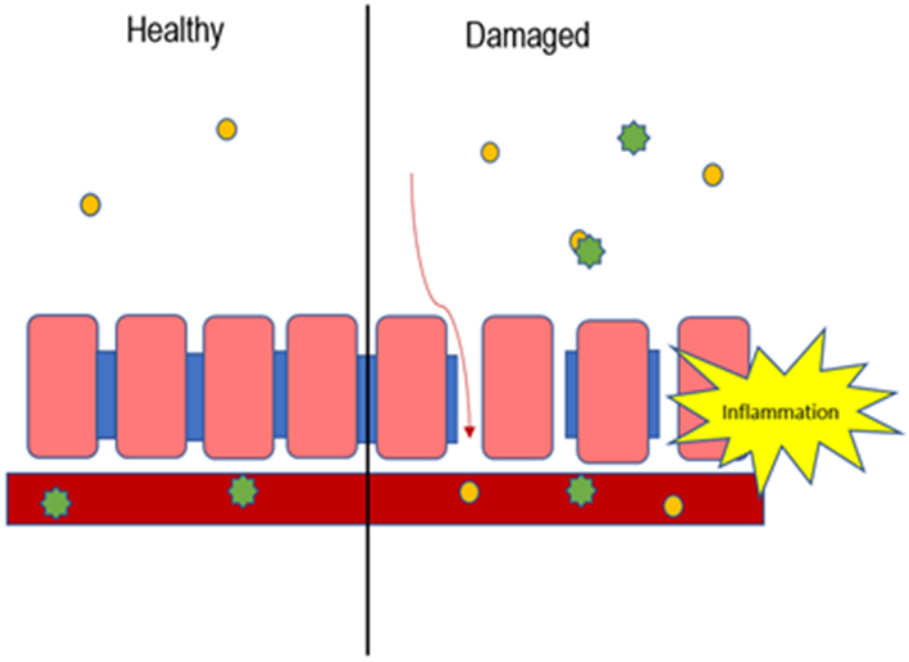


Figure 2.1.1 Gut Barrier: Healthy and Damaged. The left panel shows a healthy, well-functioning gut barrier that separates the luminal contents from the bodily environment. The right panel shows a damaged and improperly functioning gut barrier, where luminal and bodily contents are allowed to mix, inciting inflammation.

Tight junctions are complex multiprotein structures with transmembrane, extracellular, and cytoplasmic domains that adhere adjacent cells and separate the apical and basolateral domains of the membrane. Tight junctions, along with regulatory molecules, such as kinases, are primarily responsible for maintaining the integrity of the mucosal barrier by limiting solute flux along the paracellular pathway [18,20]. The structure of tight junctions is shown below (**Figure 2.1.2**)

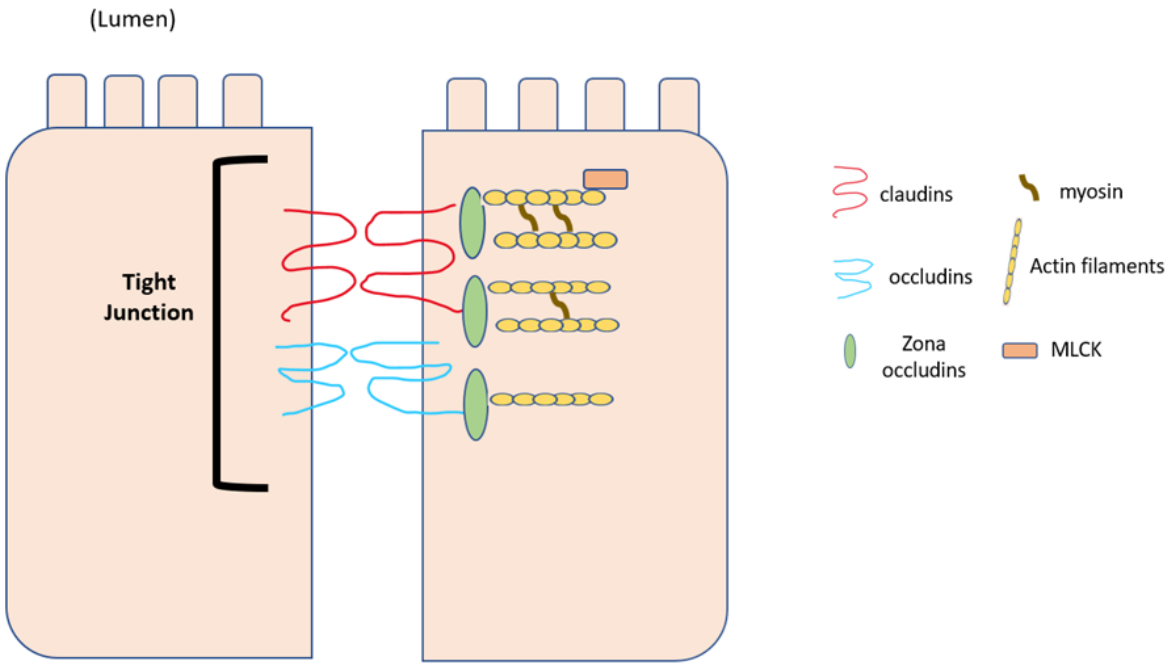


Figure 2.1.2 Simple Tight Junction Structure. Two adjacent intestinal epithelial cells connected via the transmembrane proteins claudins and occludins, the scaffolding proteins zona occludins, the cell cytoskeleton formed of proteins actin and myosin, and the regulatory myosin light chain kinase (MLCK) protein.

There are two major routes of paracellular flux, the pore and leak pathways. The pore pathway is high-capacity and selective in terms of size and charge. Claudin channels, discussed below, are the anatomical sight of flux via the pore pathway. On the other hand, the leak pathway is low-capacity, selective only in terms of size, and allows paracellular transport of large solutes like proteins and bacterial LPS [18,19]. Both pathways can be affected by disease states which can contribute to overall barrier dysfunction.

The most important of transmembrane proteins in tight junctions are members of the claudin family, which are expressed differently by different types of epithelial cells types. Some

function to create paracellular claudin channels to support solute flow and others seal the intercellular space to enhance the barrier. For example, claudin-2 forms pores for smaller charged solutes and water and are associated with reductions in transepithelial resistance (TEER), a measure of barrier function [20]. Meanwhile, claudin-4 expression increases TEER and it is considered a barrier-enhancing claudin [20].

Other tight junction proteins include occludins, which can regulate flux pathways by their ability to modulate the junction strand structure and claudin channel function [19,20]. Proteins of this family interact directly with periplasmic scaffolding proteins such as members of the zonula occludins (ZO) protein family. ZO-1 contributes to the regulation of the leak pathway and other data suggest that in addition to other ZO proteins, it contributes to cortical actin organization [17,19,20]. Another important protein is myosin light chain kinase (MLCK), which when active, drives actomyosin contraction and increases tight junction permeability [19]. These major tight junction proteins and their functions are summarized in the table below (**Table 1.2).**

When epithelial cell damage occurs, a third pathway of paracellular flux, an unrestricted

Table 2.2 Major Tight Junction Proteins

Protein	**Major Functions**
Claudins	transmembrane proteins, regulate paracellular ion selectivity; some are thought to create paracellular "claudin channels" (the pore pathway), where others are thought to seal the intercellular space
Occludins	transmembrane proteins, interact directly with claudins and actin; modulate strand architecture and claudin channel function
Zonula occludins	Peripheral membrane (scaffolding) proteins, interact with transmembrane proteins and actin filaments; required for tight junction assembly and maintenance; crucial to regulation of the leak pathway
MLCK	Regulates actin and myosin contractions affecting barrier function

pathway, can open to allow the passage of large proteins, viruses, and bacteria. Barrier loss can be detected as increased flux across any of the aforementioned pathways. When the intestinal barrier is dysfunctional, it can expose luminal content to the rest of the body, which can lead to inappropriate immune responses characteristic of chronic inflammatory conditions [15,18].

Human Gut Microbiome

The human intestine is colonized by ~100 trillion bacteria, termed the gut microbiome, with functions ranging from roles in metabolism to immune defense to behavior [21,22]. Development of the gut microbiota begins in utero and continue to change with age until a relatively stable adult-like intestinal microbiome is reached around age 3. Its composition varies greatly among individuals and is affected by many factors including mode of infant delivery and feeding, the aging process, diet composition, geography, medications, stress, and host immune system [23].

Despite the variation among individuals' gut microbiomes, metagenomic analysis has identified several major bacteria phyla present: Bacteroidetes, Firmicutes and Actinobacteria, are in the most abundant concentrations, and Verrucomicroba, Proteobacteria, Fusobacteria, and Cyanobacteria are present in lower concentrations [22,23]. The relative proportion and species membership of these phyla may vary dramatically from person to person, even among healthy, disease-free individuals. Some bacteria species are considered beneficial while others are considered pathogenic. Several studies have shown some members of the genera, *Enterococcus*, and *Klebsiella* are involved in intestinal inflammation and some strains possess pathogenic properties. In contrast, species of *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* are often associated with promotion of intestinal barrier integrity and the reduction of inflammation [15]

Characteristics associated with a healthy microbiome include microbial diversity, relative abundance of certain microbes, microbial gene richness, and resilience of the microbial populations [24]. Some evidence suggests that a lean phenotype has more *Bacteriodetes* than *Firmicutes.* Additionally, most studies in genetically obese mice, diet induced obese mice, and obese humans have shown increase in abundance of *Firmicutes*, although some studies have shown the opposite [22]. Identifying traits of healthy microbiomes has been of great interest to researchers lately, as it is becoming more evident just how many physiological functions are affected by this community.

The gut microbiome has several functions in metabolism, one of which is breaking down otherwise indigestible dietary fibers into short chained fatty acids (SCFAs) [15,17,21,22]. SCFAs like butyrate, propionate, and acetate assist in regulating immune responses and metabolism and are important for normal function of the gut, maintenance of the epithelial barrier, and promoting anti-inflammatory effects [21,25]. Colonic microorganisms are involved in the synthesis of micronutrients such as vitamin K, B12, biotin, folic acid, and pantothenates as well as the absorption of calcium, magnesium, and iron. The gut microbiota can also convert amino acids into a variety of signaling molecules and antimicrobial peptides [21].

In addition to metabolism, the microbiome has a complicated bi-directional relationship with the innate and adaptive immune system [23]. Studies, mostly in animals, have implicated that the gut microbiome is involved in the structural development and function of gut-associated lymphoid tissues, T cells, and B cells [22]. In addition, the microbiome influences the regulation of secretion of both pro-inflammatory and anti-inflammatory cytokines by macrophages, dendritic cells, and neutrophils in the epithelial layer. In an ill-functioning microbiome, these antigenic factors may be inappropriately generated and trigger persistent inflammation in the intestinal mucosa, like what is seen in Inflammatory Bowel Disease [21–23].

Gut Inflammation

As previously mentioned, dysfunction of the gut mucosal barrier and microbiome dysbiosis can initiate various physiological responses leading to gut inflammation. One of the most prevalent of these inflammatory states is Inflammatory Bowel Disease (IBD), which had 6.8 million cases globally in 2017, a large increase from the 3.7 million global cases reported in 1990 [26].

Inflammatory Bowel Disease encompasses 2 conditions, Ulcerative Colitis and Crohn's disease, both characterized by chronic inflammation causing damage to the GI tract. Symptoms of both include abdominal pain, rectal bleeding or bloody stools, weight loss, fatigue, and persistent diarrhea [2,18]. Ulcerative Colitis is described as damaged areas of the GI tract with inflammation only present in the inner most layer of the colon lining, the mucosa, while Crohn's disease is described as inflammation in multiple layers of the GI tract with damaged areas occurring discontinuously in patches. The exact etiology of these conditions are not completely understood, but both Ulcerative Colitis and Crohn's disease are associated with claudin-2

upregulation, MLCK activation, and occludins downregulation, suggesting intestinal permeability plays a key role in the pathology of IBD [27].

A low-grade, systematic, chronic inflammation in the gut is seen in individuals with the metabolic syndrome, especially among the aging population. A condition known as metabolic endotoxemia occurs when a component of bacterial cell walls, bacterial lipopolysaccharides (LPS), accumulate at high concentrations in the body [15,25]. LPS is present in low concentrations in tissues among healthy individuals, but reach high concentrations in those with obesity. When bacterial cells are lysed, LPS is released into the gut, and if mucosal barrier function is poor, LPS can enter circulation via paracellular transport. Once in the circulation at high enough concentrations, LPS can infiltrate tissues (like liver and adipose tissue) and lead to expression of genes encoding for proinflammatory factors [19]. In skeletal muscle, high concentrations of LPS stimulate cytokine expression, inducing inflammation and muscle atrophy gene expression, ultimately leading to the wasting away of lean tissue [28].

It has been shown that chronic inflammation in the gut, like that of IBD and endotoxemia, promotes the initiation of colorectal cancer by inducing carcinogenic mutagenesis [15,25]. This process is exacerbated by ineffective surface barrier function, which allows commensal bacteria to become a driving factor of tumor-promoting inflammation [24].

Because of their influences on gut inflammation and associated diseases, gut barrier function and the gut microbiome have been explored as potential targets for disease prevention and therapy. Current treatments for gut related conditions are accompanied by adverse side effects and complications. Alternative intervention strategies are being developed, looking to naturally abundant bioactive compounds to assist in the mitigation of disease. One product that has received considerable attention recently is cocoa, due to its potential anti-inflammatory, among other, health effects.

2.2 THEOBROMA CACOA CULTIVATION AND PROCESSING

Origin and Cultivation

Cocoa beans are the seeds harvested from cocoa pods, the fruit of the *Theobroma cacao* tree. Raw beans undergo processing to obtain chocolate liquor, cocoa powder, and cocoa butter, which are used as ingredients in chocolate and other products around the world [29]. The

Theobroma cocao plant, generally known to have originated from South America, was considered divine in origin and consumed for the first time by the Maya and Aztecs over 4,000 years ago [30].

Cocoa plants thrive in environments with temperatures ranging from 65-95°F, 40-100 inches of rain each year, and high humidity. Regions between 10-20° north and 10-20° south of the equator, especially Africa, Asia, and Latin America, have optimal climates and are the primary growing regions for cocoa. Cocoa trees are typically grown with other food crops such as banana or coconut trees for protection of the plant against strong sun and winds and to provide shade needed during the plant's development [31].

The beginning of cocoa bean development is marked by the emergence of a bud of the cocoa flower through the bark of the tree. Once the flower is matured and fertilized, cocoa beans develop inside a pod for about 5-6 months until they are harvested. Each pod contains 20-50 cocoa beans within a sweet, white, mucilaginous pulp [31]. Harvesting the pods from trees can be done with tools or by hand, after which the pods may be allowed to sit for a few days before opening, depending on the practices in that growing region. This practice, known as "pod storage", improves initiation of fermentation and gives better quality beans [29,31].

Raw cocoa beans have an astringent, bitter taste and must undergo post-harvest processing to produce their characteristic flavor and color. After being harvested and potentially stored, pods are broken open to extract the beans, which exposes the beans and pulp to microbial activity, initiating the process of fermentation [31].

Fermentation

During fermentation, cocoa beans are packed into piles or boxes, covered with banana leaves, and allowed to ferment for 4-7 days. The fermentation process allows the beans to develop color, flavor, and aroma and aids in the removal of the bulk of the pulp which helps in drying. The sugary pulp, containing a mix of sucrose, glucose, and fructose, is used as the fermentation substrate [29,31].

Towards the end of fermentation, as temperature and oxygen levels increase, the heat and acids formed penetrate through the bean testa. As the cellular components within the seed freely mix, enzymatic reactions are stimulated, killing the bean. Bean death initiates a variety of reactions that create many flavor and aromatic compounds. When the dead bean is exposed to

oxygen, polyphenol oxidase (PPO) oxidizes epicatechin, which causes the cotyledon (nib) to turn brown, signaling the end of fermentation [31,32].

Drying

Drying of cocoa beans can be done using solar, open air, or hot air over dryer methods. It is usually performed within a 5-7 day period where both enzymatic and nonenzymatic reactions continue to take place. This process lowers the moisture content of the beans to ~5-8%, to help avoid degradation of the beans by microorganisms. It also allows the acids in the cocoa to evaporate off and helps to produce a low acid, high cocoa flavored product. Major polyphenol oxidizing reactions are catalyzed by PPO, forming new flavor compounds and giving the beans brown color [33]. Once the 5-8% moisture content is achieved, almost all reactions stop and the drying phase is completed. The beans then leave the farm for further processing in a commercial setting [29].

Roasting

Roasting is primarily performed to promote the formation of roasted and sweet compounds characteristic of cocoa and the removal of any remaining undesired low boiling point compounds [34,35]. This process is typically carried out between 95-170°C, for 5-120 min, depending on the manufacturer and the desired final product [35]. During roasting, flavor precursors that were created during fermentation (amino acids and sugars) undergo a non-enzymatic browning known as the Maillard reaction and Strecker degradation. In addition, the shells/husks of the cocoa beans are loosened, the moisture content of the beans decreases to about 2%, and the number of microorganisms present in the beans is further reduced [31,35].

Winnowing

Following roasting, the bean shells or husks are removed and separated typically by the kibbling and winnowing processes. Kibbling involves crushing of the roasted beans, which breaks apart the shells from the nibs. The shells and nibs are then fed into a winnowing cabinet that blows a stream of air, separating the lighter particles (shells) from the heavier particles (nibs). If the shells are not removed, the trace amounts of pulp left in the shells will create off flavors in the final product [29].

Grinding and Pressing

Once isolated, the cocoa nibs are ground into a paste using a mill. The heat generated in this process causes the cocoa butter in the nib to melt and form cocoa liquor [29]. A hydraulic press is then used to remove the cocoa butter from this liquor which is collected for further processing. Once the cocoa butter is pressed off, it leaves what is known as a pressed cake. This dry pressed cake will be finely ground to make cocoa powder which is sold and used in commercial and home settings [29,32].

Alkalization

Alkalization or "Dutching/ Dutch processing" of cocoa is an optional step in cocoa processing that involves treating cocoa nibs, liquor, or powder with an alkali solution [11,36]. This process was originally developed in the 18th century by Coenraad Johannes, a chocolatier who wanted to enhance the solubility of his cocoa product [29]. Dutch processing is used in the commercial cocoa industry to neutralize cocoa beans' natural acidity, promote development of various darker colors in the final powder product, promote reactions between reducible sugars and proteins within the beans, and to increase the final powder's dispersibility. Cocoa that has been processed with alkali at the nib, liquor, or cake stages are referred to as "alkalized cocoa", "cocoa processed with alkali" or "Dutch process cocoa", while cocoa that has not been processed with alkali is referred to as natural cocoa [36].

The most prevalent method of alkalization used in the US and Europe is nib alkalization, due to its coloring and flavoring effects the other methods do not produce [36]. Depending on the desired method, the cocoa nib, liquor, or cake will be heated in a closed mixing vessel with warm alkali solution for generally 10-30 min [11,36]. Although strength and composition of alkali solutions vary among manufacturers, the most commonly used solutions are NaOH and K_2CO_2. The excess moisture introduced during this process is removed via heat and/or drying, or via roasting if it is alkalized prior. The resulting cocoa has a higher pH and a darker color than natural cocoa [9,12,36].

Several factors contribute to the degree of change observed in cocoa following dutch processing. These include the strength of the alkali solution used, the alkali selected, the moisture level during the reaction, the length of the reaction, the reaction temperature, and the

drying temperature and duration [10,37]. Longer alkalization times, higher temperatures, and more basic pH conditions favor more intense color formation due to sugar degradation, the Maillard reaction, and anthocyanin polymerization [12,37]. Controlling these parameters can allow control of the final properties of the cocoa product like color, flavor, and composition.

2.3 THEOBROMA CACAO RAW COMPOSITION

Polyphenols

Polyphenols are secondary metabolites of plants comprised of multiple phenol structures that exhibit many diverse biological functions consistent with disease prevention such as antioxidant activity, regulation of xenobiotic metabolizing enzymes, and anti-inflammatory activity, among others [38,39]. Due to their health-promoting properties, polyphenols have been of great interest in nutrition and health research, especially among those aiming to combat chronic diseases associated with oxidative stress like cancer and cardiovascular disease. Cocoa is considered an abundant source of polyphenols as both raw and processed beans contain significantly more polyphenols than coffee, black and green tea, and red wine [33].

The major polyphenolic compounds in cocoa are flavonoids, which are a broad class of phytochemicals that can be further divided into anthocyanidins, flavonols, flavones, flavanones, and flavanols (flavan-3-ols). Flavan-3-ols occur in monomeric form as catechin and/or epicatechin and in oligomeric and polymeric forms as compounds known as proanthocyanidinss (also called condensed tannins). Proanthocyanidins comprised exclusively of catechin and epicatechin moieties are known as procyanidins, which play a key role to the health properties of cocoa [33].

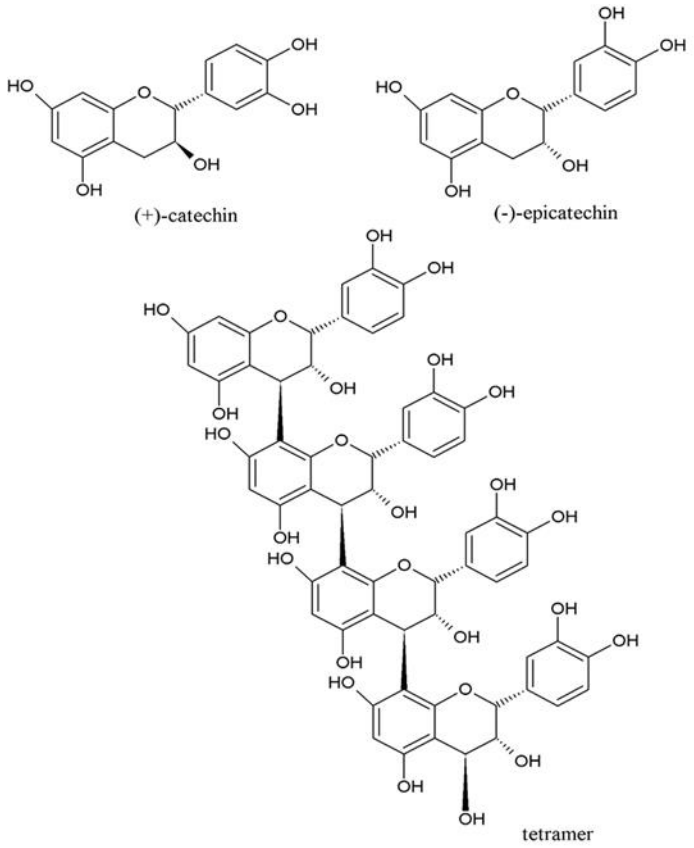


Figure 2.3.1. Representative Flavanols of Cocoa: (−)-epicatechin, (+)-catechin, and a procyanidin tetramer

Raw cocoa beans contain about 18-20% polyphenols by dry weight, the majority of which are made up of flavanols and anthocyanins. The most prominent monomeric flavanols are (−)-epicatechin and (+)-catechin which together make up 35% of the total phenolic compounds in beans [33]. It is important to note, during the roasting process, (+)-catechin becomes primarily epimerized to (−)-catechin, making cocoa one of the few foods that contains an appreciable amount of (−)-catechin [33]. Dimers, trimers, and high oligomers of flavanols make up most of the phenolic content of raw cocoa beans (58%) and are mainly represented by procyanidins B1, B2, B5, C1, and D. Anthocyanins, mainly cyanidin-3-O-glactoside and cyanidin-3-O-arabinoside, contribute 4% of the phenolic content of raw cocoa. The remaining phenolic content is comprised of small quantities of flavonols, flavones, flavanones, benzoic acid derivatives, and hydroxycinnamic acids [33,35].

Lipids

About half of the weight of cocoa beans is attributed to fat, typically referred to as "cocoa butter" or "cocoa fat". The lipid profile is majority triglycerides (95%) with the remaining 5% represented by mono and di glycerides, glycolipids, sterols and phospholipids [31].

Fiber

Definitions of dietary fiber (DF) differ among groups who define it. For the purposes of this review, dietary fiber is defined as substances that are not digested in the upper GI tract and reach the colon intact where they may be subjected to transformation via the gut microbiota [40].

Constituents of dietary fiber (DF) include plant carbohydrate polymers like lignin, celluloses, hemicelluloses, pectic substances, gums, and resistant starch. These carbohydrate polymers may exist as large biopolymers associated with lignin that are cross linked with phenolic compounds and other non-carbohydrate components (e.g. polyphenols, waxes, saponins, phytates, resistant protein) [41].

The majority of cocoa's fiber content is contained in the bran, which is almost completely lost throughout processing [42]. The high polyphenol content of cocoa is important to note, as some dietary polyphenols are constituents of DF [43]. As cocoa undergoes processing, its DF content is seen to increase, which is thought to be due to its high polyphenolic content. Once in powder form, cocoa does still have an appreciable amount of fiber (specifically lignin), making up about 5-10% of its composition, depending on the processing it undergoes [44].

Carbohydrates

Starch is the primary carbohydrate in cocoa, found in the nibs. Soluble carbohydrates include stachyose, raffinose, and sucrose, glucose, and fructose [31]. Sucrose makes up ~90% of sugars in unfermented beans, but this changes during the fermentation process where it is broken down into its constituents, glucose and fructose.

Proteins

Proteins are classified into groups based on solubility and electrophoretic mobility. Two major protein groups in cocoa are albumin (52%) and globulins (43%) [31,35]. Free amino acids present are mostly leucine, proline, and lysine [31].

Minerals, Methylxanthines, and Organic Acids

Cocoa beans contain magnesium, copper, potassium, and calcium, all of which are associated with health benefits [42]**.** Small amounts of caffeine and theobromine are the major xanthines in cocoa. These compounds interact with adenosine receptors and have notable health promoting and psychological effects. Additionally, cocoa contains organic acids, mainly citric acid, which is important in processing reactions [31,42].

2.4 PROCESSING EFFECTS ON COMPOSITION

The raw composition of cocoa beans changes drastically throughout processing as new flavor and aroma compounds characteristic of cocoa/chocolate form. Most notable is the overall decrease in polyphenols that removes some of the bitterness and astringency found in raw beans, which is desirable for chocolate manufacturers. These changes begin in the pod storage phase, where small amounts of (−)-epicatechin and (+)-catechin are reduced [32]. Throughout fermentation, many important flavor and aroma precursors are formed, as the degradation of polyphenolic compounds continues.

Fermentation

The formation of free amino acids and peptides via proteolytic enzyme reactions is triggered at the beginning of fermentation. The amine moieties of these molecules will participate in the Maillard reactions during roasting, which will determine what final flavor and aroma compounds are formed [32]. Additionally, invertase hydrolyzes sucrose into glucose and fructose. Fructose and glucose, as well as other reducing sugars mannitol, and inositol, also important for later reactions during roasting [31,32].

Polyphenol oxidase (PPO) is the main enzyme responsible for oxidizing (−)-epicatechin and (+)-catechin. When oxidized, these compounds will polymerize with other phenolic compounds or amino acids to form high molecular weight tannins [35]. Higher molecular weight tannins and have lower digestibility in the human body than the monomers, epicatechin and catechin. Anthocyanins are hydrolyzed to anthocyanidins and sugars (arabinose and galactose) and will also eventually form into condensed tannins. The procyanidin content of the beans is also drastically reduced. By the end of fermentation, total polyphenol content is reduced by

approximately 10-50% and soluble polyphenol content is reduced to about 20% of the original concentration [33,38].

Drying

During drying, oxidation and polymerization reactions of phenolic compounds continue. Proteins and sugars present in beans may begin to participate in non-enzymatic browning reactions to form pyrazines. Minor changes in fatty acid composition and decreases in phospholipid levels may also occur [32,35].

Roasting

Polyphenolic compounds continue to be affected during roasting, where significant decreases in remaining (−)-epicatechin, (+)-catechin, and procyanidin content occurs. However, some of this loss may not be due to degradation, as roasting of cocoa beans causes epimerization in flavanol monomers, like the aforementioned (+)-catechin epimerizing (−)-catechin. Epimerization also occurs among flavanol dimers, trimers, and possibly in procyanidins. High temperatures, especially when combined with alkaline conditions, accelerate these reactions [32,33].

The most drastic composition changes during roasting occur through non-enzymatic browning via the Maillard reaction. The Maillard reaction involves the interaction between the carbonyl groups of reducing sugars and the amino groups of peptides, amino acids, and proteins under high temperatures. It has been established that the decrease in reducing sugars (glucose and fructose) and amino acid concentration (especially of leucine, alanine, phenylalanine, and tyrosine) that occur upon roasting of cocoa beans is due to the Maillard reaction taking place [42,45]. In the first step of the Maillard reaction, the amino group reacts with the carbonyl group to form glycosylamines, which then isomerize into intermediate amadori compounds. Acidic conditions favor the formation of hydroxymethylfurfural (HMF), while alkaline conditions favor the formation of fission products and reductones. These intermediate compounds eventually undergo condensation and polymerization reactions to form melanoidins and other Maillard reaction products [45].

Although great losses of polyphenol compounds occur during roasting, antioxidant capacity of cocoa is partially restored by the development of Maillard reaction products (MRPs),

particularly melanoidins, which have been explored for their antioxidant, antimicrobial, and tumor growth inhibiting properties [42,46]. The exact structures of melanoidins remain ambiguous, but what is known is they are large, nitrogen containing, polymers.

Throughout roasting, total dietary fiber content is not affected, but a redistribution of polymers and a sharp increase in Klason lignin occur. Klason lignin is described as "a fraction of plant material which remains insoluble after treatment with 12 M H_2SO_4 for 3 hr at room temperature and for another 2 hr in 1M H_2SO_4 at 100°C" [34]. It has been shown cocoa beans before roasting have 4.0-4.3% Klason lignin content, but after roasting this increased to 8.6-9.2% [34]. In many plants, this insoluble material known as "Klason Lignin" is represented by lignin exclusively, however, tannin/protein complexes and MRPs formed during roasting are also largely insoluble after the H_2SO_4 treatment. It is likely that the increase in Klason lignin seen in cocoa beans with roasting is due to the associations promoted between polysaccharides, proteins, polyphenols, and MRPs, leading to the formation of insoluble tannin/protein complexes that are detected in the Klason lignin measurement. Lignin is included in the classic definition of dietary fiber, while tannin/protein complexes and MRPs are not, which may affect estimations of dietary fiber in these materials [34].

Normally, MRPs are studied and quantified independently of dietary fiber, but it has been shown in a bread model that MRPs formed during heating contributed to the increase in insoluble dietary fiber that was quantified from wheat flour to bread [43]. Condensed tannins and resistant proteins have been found in dietary fiber residues in many foods. In grape pomace extracts, it has been shown that condensed tannins and resistant proteins are not hydrolyzed by digestive enzymes. These compounds remained and were quantified in insoluble dietary fiber residues as well as Klason lignin residues [47,48]. These interactions are similar to reactions that have been characterized further in coffee beans. In coffee beans, which undergo a similar roasting process to cocoa beans, dietary fiber becomes "maillardized" under roasting [49]. The MRP formed during coffee bean roasting has been shown to include phenolic compounds like chlorogenic acid and caffeic acid. These complexes formed via associations of MRP to polysaccharides, polyphenols, and lignin are referred to by some as "maillardized dietary fiber" [43]. Additionally, "antioxidant dietary fiber" has been used to refer to antioxidant compounds associated with the indigestible fraction of foods [43]. Cocoa, which has a similar polyphenolic

profile to grape pomace, is likely to also participate in these kinds of linkages during roasting that may affect the "fiber-like" properties of cocoa.

It is clear from these data how quantification of fiber and MRP can be complicated by their interactions. Complex DF-protein-MRP-polyphenol molecular structures exist in these thermal processing systems, like cocoa bean roasting, and their effects should be further studied.

Alkalization

Cocoa is naturally acidic and has a pH range of ~5-5.6. Alkalization neutralizes the slight acidity of the cocoa to the more neutral pH 7-8 range, influencing the flavor. Alkali processing of cocoa powder also results in a darker color, increased solubility, and reduced bitterness. Increasing pressure, alkali concentration, or reaction time during processing all result in a darker cocoa powder [11,37,50].

Alkalization is a further step of the Maillard reaction and promotes interactions between polyphenols and some earlier MRPs. During alkalization, some volatile compounds are reduced, but some are also produced which is thought to be a part of the Strecker degradation of amino acids [9]. Strecker degradation involves the oxidative deamination and decarboxylation of alpha-amino acids in the presence of alpha-dicarbonyl compounds (which are formed during the Milliard reactions during roasting). The continuation of the Maillard reaction with the modified pH also promotes further associations between polysaccharides, proteins, polyphenols, and MRPs.

During alkalization, polyphenols (anthocyanins, procyanidins, and catechins) are transformed into quinones which undergo polymerization to form high molecular weight (HMW) insoluble brown compounds (melanoidins) via protein linking [10]. Hydrophobic amino acids are degraded dramatically by alkalization (more so than total amino acids) suggesting polyphenols binding to hydrophobic amino acids. Pyrazine levels are shown to decrease with alkalization, which is attributed to the hydrogen bonding of polyphenols to the amide carbonyl groups. Quinones that are formed during fermentation and roasting via oxidation of epicatechin and catechin can also participate in complexing with amino acids [9,12].

It is well established that a loss of total polyphenols occurs with the alkalization process, but the observed decreases vary greatly, from 20.4% to 60.5% [10]. For monomers, 63.5% reduction in epicatechin and 43.1% reduction in catechin has been reported during Dutch

processing. It is thought that epimerization of epicatechin to catechin also occurs during this processing because high temperatures (especially when combined with alkaline conditions) accelerate the epimerization of the flavanols monomers [9]. Proanthocyanidin content of cocoa decreases on average about 67.1% in alkalized powders compared to natural powders. These great losses seen could be due to either 1) monomerization during alkalization or 2) transformation into quinones and further condensing afterwards to higher degree of polymerization [9].

2.5 INULIN

Inulin is a naturally occurring plant storage carbohydrate found in leeks, onions, wheat, asparagus, garlic, Jerusalem artichoke, and chicory root. Inulin has a wide range of commercial food applications due to its solubility, gelling properties, and stability. When mixed with water, inulin forms an acid and heat stable particle gel network that creates a white, creamy structure easily incorporated into foods. Its stability and versatility make inulin popular as a sugar and fat replacer and stabilizer in the food industry [51,52].

Inulin belongs a class of fructose containing polymers known as fructans. Its basic chemical structure consists of a glucose moiety linked to a chain of fructose moieties via α(1,2) bonds. It can be represented as GFn, where G symbolizes the glucose moiety, F symbolizes the fructose moiety, and n is the number of fructose units linked to each other by β-(1,2) linkages (**Figure 2.5.1)** [51].

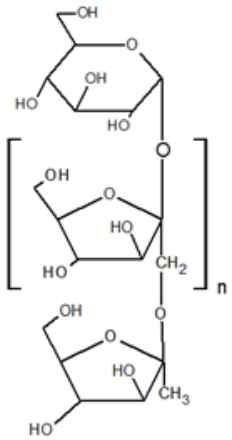


Figure 2.5.1 Basic Molecular Structure of Inulin

The degree of polymerization (DP) of inulin describes the number of fructose units in each polymer. Inulin is most commonly sourced from the fleshy taproot of Chicory (*Cichorium intybus*). The DP of native chicory inulin typically ranges from 2-60, meaning it contains both oligosaccharides (DP 2-10) and polysaccharides (DP greater than 10). Due to its ß-(1,2) bonds, inulin cannot be digested by the upper GI tract and it reaches the large intestine mostly intact. There, it is fermented by colonic bacteria. This fermentation produces short chained fatty acids, increases fecal biomass, influences gut microbiota, and decreases colonic pH. Due to these properties, inulin is considered both a soluble fiber and a prebiotic, both of which have shown to be beneficial for gut health [51,52].

2.6 PREBIOTICS AND GUT HEALTH

Prebiotics

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [40]. These substrates are mostly complex carbohydrates and plant polysaccharides that are indigestible by dietary enzymes and instead become metabolized by microbes in the lower gut into fermentation products. The main products produced are short chained fatty acids (SCFAs), acetate, propionate, and butyrate, and gasses like H_2 and CO_2.

In addition to modulating gut microbiological communities and altering toxin metabolism, the positive gut-health benefits associated with prebiotics are attributed to their formation of short chained fatty acids (SCFAs), or fatty acids with fewer than 6 carbons [13]. SCFAs influence GI epithelial cell integrity, glucose homeostasis, lipid metabolism, appetite regulation, and immune function [13,53]. Butyrate, in particular, is considered very important for the maintenance of colonic health, as it acts as a direct source of energy for colonocytes. Several in vitro, ex vivo, and animal studies have exhibited positive effects of SCFAs for reducing colonic inflammation, preventing carcinogenesis, and promoting anastomotic healing of the gut [53]. These benefits are attributed to their ability to interact with signaling pathways within the gut lumen. For example, SCFAs interact with G-protein coupled receptors in immune cells of colon, which promote the expression of specific chemokines in the colonic epithelium and affect insulin sensitivity in adipocytes and peripheral organs [15]. Additionally, SCFAs inhibit histone

deacetylase (HDAC) which represses NF-kB, which affects the production of inflammatory markers like IL-2 and IL-10 [53].

As aforementioned, inulin is considered prebiotic and has shown to exhibit effects in managing IBD. Formation of SCFAs induced by inulin metabolism has shown to enhance growth of lactobacilli and/or bifidobacteria, both associated with decreased mucosal lesions and diminished mucosal inflammation [51]. In a rat model of human ulcerative colitis, daily administration of inulin was shown to increase lactic acid bacteria counts and decrease the pH in the colon. Additionally, these rats showed decreases in mucosal inflammation and histological damage scores compared to controls [51].

In vitro models have shown colonic bacteria to ferment cocoa powder and form SCFAs [54], suggesting the potential prebiotic-like and fiber-like properties of cocoa products. Complex, indigestible compounds formed during processing like melanoidins, Klason lignin, and condensed tannins, may actually have a positive influence on cocoa's bioactivity, which is contradictory to the central dogma of bioactivity loss during processing due to losses of total polyphenols.

2.7 COCOA AND GUT HEALTH

Metabolism of Polyphenolic Compounds

The bioavailability and absorption of flavanol compounds are largely influenced by their degree of polymerization (DP) and the composition of the food matrix. When ingested, flavanol compounds are released from their food matrix before being solubilized by the gut lumen. Once solubilized, these stable compounds are then transported by the intestinal epithelial cells across their unstirred water layer, where they are absorbed and metabolized [39]. Monomeric flavan-3-ols (+)-catechin and (−)-epicatechin have the highest rates of absorption in the small intestine (~20%), while procyanidin dimers and trimers show limited absorption rates and reach the lower gut intact, where they have local functions [38]. Procyanidin compounds larger than DP 4 cannot be absorbed in the small intestine. These larger compounds, along with unabsorbed monomeric, dimeric, and trimeric flavan-3-ols pass through the GI tract unaltered and reach the colon where they are subject to degradation via microbial metabolism into verolactones, phenolic acids, and benzoic acids [38,39]. The poor bioavailability of these compounds in the upper GI tract make

them ideal for targeting the gut microbiome and gut epithelium, as they will reach their major point of exposure, the colon, intact. Some polyphenols remain bound to macromolecules despite this microbial metabolism and these compounds are thought to act as "fiber" and elicit a prebiotic effect [55]. For these HMW compounds that become further metabolized by the colonic bacteria, it has been recently considered that the health benefits of these compounds are not due to the native compounds found in foods, but to their metabolites that are created and come into contact with and are absorbed by the gut lumen. For this reason, much investigation is now directed towards characterizing these microbial metabolites.

Cocoa and IBD

The effects of cocoa on IBD and intestinal inflammation have been demonstrated mostly in cell culture and animal studies. Cocoa procyanidins have shown to inhibit tumor necrosis factor alpha (TNF-α), a prominent pro-inflammatory immune marker, and cell oxidant increase in Caco-2 cells [5]. In the same cell line, it has shown to reduce inflammatory markers like Interleukin-8 (IL-8), cyclooxygenase-2 (COX-2), and nitric oxide synthase (iNOS) expression that is induced by TNF-α production. High molecular weight, polymeric procyanidins were shown in a cell model to be more effective than oligomeric and monomeric fractions in reducing IL-8 expression corresponding to gut inflammation [7]. These data suggest that larger flavanol compounds may be the most bioactive in gut tissue. In a mouse model of UC, oral administration of a polyphenol rich extract significantly reduced the severity of the colon inflammation and decreased crypt damage and leukocyte infiltration in the intestinal mucosa [56]. In a similar mouse model, a cocoa enriched diet was shown to downregulate serum TNF-α, colon iNOS activity, and decrease colon cell infiltration [5]. The compounds in cocoa have also shown to have an effect on barrier function and the gut microbiome, which are discussed in more detail in the sections below. The lack of research and promising preliminary data for cocoa and its effects in mitigating IBD warrant further investigation of these compounds and their activity in the gut.

Cocoa and the Gut Microbiome

It has been shown *in vitro* that cocoa is fermented by colonic bacteria and results in the formation of SCFAs [54]. These effects have also been studied *in vivo* in both animals and humans. Rodents on a 6-week cocoa treatment (100 g cocoa/ kg chow) compared to a reference

group showed decreases in *Bacteroides*, *Staphylococcus* and *Clostridium* genera, which some members are associated with disease. After 4 weeks of treatment diet with a cocoa powder, pigs experienced growth of Bifidobacteriaceae and Lactobacillaceae compared to control [57]. In a double blind, randomized, crossover study in human subjects, it was demonstrated that consuming a high-cocoa flavanol drink (494 mg cocoa flavanols/day) for 4 weeks daily significantly increased the populations of *Bifidobacterium* and *Lactobacillus* (genera generally associated with a healthy gut) in feces compared to patients consuming a low cocoa flavanol group (23 mg cocoa flavanols/day) diet. The high-cocoa flavanol group also had a decrease in Clostridia counts compared to the low flavanol group [58].

Cocoa Barrier Function/Leaky Gut

Flavanol consumption is correlated with increased tight junction protein expression and improvements in gut permeability. It was found that administrating 100 mg/kg/day grape seed extract [(GSE) (as 0.1% GSE in drinking water)] increase occludin expression in the proximal colons of healthy rats vs. a control group. Has also been found that GSE in standard chow diet (250 mg/kg/day) increased ZO-1 and occluding expression and decreased intestinal permeability in the small intestine of healthy rats [4,59]. In high fat fed mice, it was shown that long term supplementation of 8% unsweetened natural cocoa powder for 18 weeks improved gut barrier function, as measured by increased plasma levels of glucagon-like peptide-2 [60]

Cocoa procyanidin dimers and trimers shown to protect Caco-2 cells from loss of biolayer integrity induced by oxidants. In this model, the hexameric procyanidin fraction interacted with the interface without affecting barrier integrity and inhibited deoxycholate-induced cytotoxicity and partially prevented the generation of oxidants [61]. Additionally, cocoa extracts and cocoa procyanidin fractions shown to significantly inhibited dextran sodium sulfate-induced loss of barrier function, with the polymer rich fraction possessing the greatest protective activity [7]. Finally, the co-administration of a grape seed procyanidin extract was shown to inhibit losses of barrier integrity in rats fed an obesity inducing diet [62].

Cocoa and Colon Cancer

Cocoa flavanols have been investigated for the prevention and treatment of certain cancers as they have the capacity to interact with multiple carcinogenic pathways involved in

inflammation, proliferation, and apoptosis of initiated malignant cells. In a genetic model of multiple intestinal neoplasia, adding 1% and 0.1% catechin to the diet was able to diminish the formation of intestinal tumors by 75% and 71%, respectively [5]. Animal studies have shown that the polyphenols in cocoa may prevent and/or slow down the initiation-progression of different types of cancers like prostate, liver, and colon cancers. A model of male Wistar rats with azoxymethane (AOM)-induced aberrant crypt foci, pre-cancer lesions in the colon, has been utilized to study the chemo-preventive ability of a cocoa rich diet. A 12% cocoa diet in these rats for 8 weeks led to a downregulation of proinflammatory cytokines COX-2 and iNOS, inhibiting NF-kB signaling, ultimately leading to suppressed AOM- intestinal induced inflammation. The same rat model has been reported to reduce cell proliferation and leads to a lower number of early, pre-neoplastic lesions in the colon when fed a diet supplemented with dark chocolate [61]. Although no studies have been performed in humans to determine the effects of cocoa on colon cancer, the plethora of promising cell culture and in vivo evidence warrants further investigation.

Melanoidins that are formed during roasting have demonstrated the ability to suppress tumor growth in vitro- due to S and G2/M-phase cessation [42]. Melanoidins (mostly of HMW) also have some antioxidant activity. Melanoidins extracted from heated potato fiber has shown inhibit the proliferation of several types of cancer cells and that it decreased cancer cell motility, produced remarkable morphological changes, induced apoptotic cell death, and reversed the stimulatory effect of insulin-like growth factor [42].

Cocoa and Diarrhea

In cell cultures of human colon epithelium, cocoa flavanols were shown to target intestinal cystic fibrosis transmembrane conductance regulator (CTFR) Cl- channel, the main Cl- channel in the enterocytes of the intestine. Increases in cellular cAMP and cGMP activate these channels, which, once activated, initiate diarrhea. Cocoa flavanols are shown in T84 colon epithelial cells to serve as mild inhibitors of cAMP-stimulated Cl- secretion in the intestine. Other oligomeric flavanols from tree bark have also demonstrated to inhibit CFTR-mediated currents and improve diarrheal symptoms in patients with AIDS [63].

Cocoa and obesity related inflammation/endotoxemia

Cocoa has shown to inhibit many digestive enzymes, such as α-amylase and α-glucosidase, which affects carbohydrate digestion, inhibiting the glucose transporters and promoting an incretin response [4]. An 18-week cocoa treatment in male mice was shown to be effective in reducing plasma endotoxin levels by 40.8% compared to a high fat mouse group. Other animal studies have demonstrated cocoa's ability to decrease total triglyceride levels, total cholesterol levels, and serum glucose levels in diabetic rats fed diets supplemented with cocoa extract. Additionally, polyphenol rich cocoa extracts have shown to dose-dependently inhibit activity of phospholipase A_2 *in vitro* [64]. Cocoa may also help reduce systemic toxin exposure via improvement of the gut barrier and gut microbiome [4].

Conclusion

While many fascinating discoveries have been made in regard to cocoa, inulin, and gut disease, many key gaps in knowledge remain. How cocoa reduces inflammation, influences lipid and carbohydrate metabolism, modifies the microbiota, affects the intestinal barrier, among its many other activities is poorly understood. It is also unknown exactly what chemical properties of cocoa correlate to bioactivity, what compounds are bioactive in what tissues, and what their mechanisms of action are. Are native flavanols responsible for cocoa's bioactivities, or are their microbial metabolites? Additionally, how does DP affect flavanol bioavailability? Further research is needed looking into the relationship between cocoa processing, mDP, and bioactivity in the gut.

More information should also be gathered regarding how exactly polyphenols and fiber interact with tissue and with each other within the gut. Some hypothesize that some fiber can entrap polyphenols the gut lumen, further reducing their bioavailability in the small intestine and increasing its availability for bacterial metabolism, while others suggest the supply of fermentable fiber actually alters bacterial metabolism away from the polyphenolic compounds, similar to what is seen in protein metabolism [65]. Understanding these interactions can help us better characterize the metabolism of both substances and predict their effects *in vivo*.

Finally, the pathology of IBD and related conditions must be more completely understood in order to develop a well-targeted and effective treatment. The potential for cocoa to play a role in the mitigation of disease is clear. Further investigation is needed to determine the mechanisms of action of cocoa, the disease, and other unknowns.

CHAPTER 3. EXPERIMENT

3.1 MATERIALS AND METHODS

3.1.1 Chemical Characterization

In order to identify and quantify individual compounds in each treatment, chemical characterizations were performed on extracts of the natural cocoa, alkalized cocoa, and inulin powders.

Cocoa Powder, Chemicals, and Standards

Two commercial cocoa powders, one natural and one alkalized, were provided from The Hershey Company (Hershey, Pennsylvania, USA). The powders were processed on 8/30/2018 and 9/5/2018 using beans sourced from Ivory Coast and stored at -20°C prior to the experiment. Beans were roasted at very hot temperatures, a reported 500°F (~260°C). Alkalization was performed by the manufacturer after roasting, grinding, and pressing, on the pressed cake (i.e., when cocoa was in powder form). Inulin from chicory root was purchased from Acros Organics (New Jersey, USA) (Lot #A0390044 and #A0396361) and stored at room temperature (~25°C) in a desiccator. The inulin from both lots were mixed thoroughly to ensure a homogeneous distribution of the two powders to account for any possible differences in DP or composition.

Extraction

Hexane extraction was performed for each treatment for de-fatting, followed by a polyphenol extraction using a 70:28:2 acetone:water:glacial acetic acid mixture as reported in Racine et al [66]. About 15 g of Cocoa powder (or inulin) was mixed with ~150 mL hexane in a 1000mL beaker and sonicated for 10 min at ~22˚C. The mixture was then centrifuged for 5 minutes at 5000 rcf (x g). The supernatant was discarded, and the remaining solids were transferred back into the beaker. After repeating the fat extraction process 2 times, the solids were transferred to a drying plate and allowed to dry at room temperature under a fume hood for 2 hours. The defatted powder was then removed and placed in a beaker with 150 mL of extraction solution (70:28:2 acetone: water: glacial acetic acid). The mixture was sonicated for 10 min at ~22°C and then centrifuged for 5 min at 5000 xg. The supernatant was collected, and

the extraction was repeated at least three times, until the supernatant was essentially colorless. The collected supernatants from each repetition were combined and transferred into a round bottom flask and placed under a rotary evaporator at 40°C until all the acetone was evaporated. The remaining extract was collected and stored at -80°C until it was freeze dried for about 72 hrs. Weight of cocoa/inulin extracts were recorded, yield calculated, and stored at -80°C until ready for analysis.

Extraction Yield: (g extract/g powder) x 100% =

The calculated % yields were used when determining concentrations total polyphenols and total flavanols.

Folin-Ciocalteu Assay

To determine total polyphenol content of the cocoa and inulin samples, the Folin-Ciocalteu colorimetric method was used as described by Dorenkott et al [67]. Each treatment was diluted to a final concentration of 0.2 mg/mL or 2.0 mg/ml using 40% reagent alcohol. Approximately 100 µL of each sample was mixed with 900 µL distilled deionized water and 2.5 mL 0.2N Foli-Ciocalteu reagent (Sigma, St Louis, MO). This was followed by the addition of 2 mL sodium carbonate (7.5% w/v) and thorough vortexing of each sample. The samples were then incubated for 2 hours at room temperature (~25°C). Their absorbances were measured at 765 nm and compared to standard curves of (+)-catechin hydrate and gallic acid. The total polyphenol concentration is expressed in mg catechin (+C) equivalents/g extract and mg Gallic Acid Equivalents (GAE)/g extract. These concentrations will be used to determine if trends between polyphenol content and gut protective effects exist.

4-dimethylaminocinnamaldehyde (DMAC) Assay

In order to assess total flavanol amounts in each cocoa powder sample, 4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay was performed as described Dorenkott et al [67]. DMAC solution was prepared by adding 3.0 mL stock HCl to 27 mL EtOH and cooling the solution at 4°C for 15 min, followed by the addition of 0.03 g DMAC. Each sample was diluted with EtOH to 0.1 mg/mL or 1.0 mg/mL. Procyanidin B2 was diluted with 1:1

EtOH:water to prepare the standard curve. The final concentrations used for the curve were 1, 10, 50, and 100 ppm. Approximately 50 µL of either EtOH blank solution, procyanidin B2 standard solution, or diluted sample was added to each individual well. Approximately 250 µL DMAC solution was immediately pipetted into each well and the absorbance was read at 640nm.

Thiolysis

Thioloysis was used to determine the mean degree of polymerization (mDP) of polymers within the cocoa and inulin extracts and be performed according to the procedure by Dorenkott *et al* [7]. Cocoa and inulin extracts were diluted with methanol to a concentration of 0.5 mg/mL or 5mg/mL. Approximately 50 µL of methanol diluted extract solutions were added to a microcentrifuge tube along with 50 µL HCl regent (3.30% HCl in water) and 100 µL benzyl mercaptan reagent (5% benzyl mercaptan in methanol). Unthiolyzed controls were prepared with 50 µL diluted cocoa extracts and 150 µL methanol. The tubes were placed in a 90°C water bath for 5 min and then cooled on ice for 5 min to stop the reaction. To prepare for LC/MS analysis, 100 µL of each sample was combined with 900 µL of 95:5 0.1% formic acid in water (phase A) : 0.1% formic acid in ACN (phase B) solution in an HPLC vial.

The samples were analyzed on a Waters Acquity H-class separations module with an Acquity UPLC HSS T3 column (2.1 mmx 100 mm, 1.8 µm particle size) at 40°C. Binary gradient elution was performed using 0.1% formic acid in water (Phase A) and 0.1% formic acid in acetonitrile (Phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution is as followed: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7-10.5 min). (–)-electrospray ionization (ESI) together with tandem mass spectrometry (MS/MS) was used to analyze UPLC effluent on a Waters Acquity triple quadrupole (TQD) MS. (–) mode electrospray ionization (ESI) was performed with capillary, cone, and extractor voltages of –4.24 kV, 30.0 V, and 3.0 V respectively. Source temperature was 150 °C and desolvation temperature was 400°C. Cone gas was at a flow rate of 75 L/h and desolvation gas as a rate of 900 L/h. Argon (0.25 mL/min) was used as the collision gas in MS/MS. Multi-reaction monitoring (MRM) with a mass span of 0.2 Da was performed on parent ions and collision-induced dissociation (CID) on daughter ions. Inter channel delays and inter scan time was 1.0 s each. Additional calculations were done to account for native monomers and will reported as DP of total flavanols. mDP of oligomers and polymers and mDP of total flavanols were calculated as follows:

$$\boldsymbol{mDP\ (O+P) = \frac{net\ number\ of\ monomers + net\ number\ of\ thiolytic\ derivatives}{net\ number\ of\ monomers}}$$

$$\boldsymbol{mDP\ (total\ flavanols) = \frac{total\ monomers + net\ number\ of\ thiolytic\ derivatives}{total\ monomers}}$$

The mDP and mDP total flavanols were used to determine if correlations exist between certain DP cocoa flavanols and gut barrier and anti-inflammatory activities. We also determined the differences between the DP of alkalized cocoa powder vs natural cocoa powder.

HILIC UPLC-MS/MS (Hydrophilic interaction liquid chromatography)

In order to separate and quantify high DP procyanidins, an aqueous normal phase (Hydrophillic interaction liquid chromatography, HILIC) UPLC method with post column ESI adjuvant infusion was used as described by Racine et al [68]. A Waters Acquity H-class separation module equipped with an Acquity Torus DIOL column (2.1 mm × 100 mm, 1.7 µL, 45°C) and Torus DIOL VanGuard Pre-column (2.1 mm × 5 mm, 1.7µL) was used in the analysis. Using 2% acetic acid in acetonitrile (Phase A) and 3% water and 2% acetic acid in methanol (Phase B), binary gradient solution was performed. The solvent flow rate was set to 0.8 mL/min and the linear gradient elution was carried out using the following parameters: 100% A (0 min), 55% A (5.7 min), 5% A (6.0 min), 100% A (6.7-9.0 min). The UPLC-eluent was analyzed using (-)- mode ESI coupled to tandem mass spectrometry (MS/MS) on a Waters Acquity triple quadrupole (TQD) MS. To enhance the ionization of the HMW compounds, ammonium formate (0.04 M in water, 5 microLmin) was added to the eluent flow stream port-column. Ionization settings were: (-) mode, capillary voltage: -4.5 kV, cone voltage: 60.0 V, extractor voltage: 1.0 V, source temperature: 150°C, and desolvation temperature: 500°C. N2 was used for cone gas at a flow rate of 50 L/h and desolvation gas at a flow rate of 1000 L/h. Ar was used as a collision has with a 0.1 mL/min flow rate for the MS/MS analysis. Parent ions and signature daughter ions followed by collision-induced dissociation (CID) were subjected to multi-reaction monitoring (MRM) with a mass span of 0.2 Da and 1.0 sec of inter-channel delays and inter-scan times. A calibration curve was prepared for DP 1-9. The curve was analyzed with dilutions ranging from 6.93×10^{-7} – 0.091 mg/mL. MassLynx software was used to collect data.

This HILIC method was employed for improved ionization and detection of procyanidins. It has been shown that procyanidins with different DP have different bioactivities, so using a method that can more distinctly separate these compounds, like this HILIC method, is desirable for identifying activities of specific DP PCs.

3.1.2 *In vitro* experiments

Treatment Formulation

Cocoa powders were either alkalized or natural. Inulin was either omitted, added in a 1:2 cocoa:inulin (w/w) ratio, or added in a 1:4 cocoa:inulin ratio. A total of 6 treatment combinations were formulated as shown below (**Table 3.1.2.1**), in addition to controls.

Table 3.1.2.1 Treatment Formulation Scheme

		Alkalization (Dutching)	
		—	+
Inulin (Cocoa:Inulin)	**None**	natural cocoa powder	alkalized cocoa powder
	1:2	natural cocoa powder + 2x inulin	alkalized cocoa powder + 2x inulin
	1:4	natural cocoa powder + 4x inulin	alkalized cocoa powder + 4x inulin

The cocoa/inulin mixtures were made immediately prior to the digestions performed according to the formulations above (**Table 3.1.2.1)**. For each mixture, the designated masses of cocoa powder and inulin were added to a plastic bag. The bag was sealed and the contents inside were mixed thoroughly by shaking. The masses used for the in vitro digestions and subsequent fecal fermentation and cell culture assays are shown in the table below (**Table 3.1.2.2)**. A control or "blank" digestion was performed along with the treatment digestions which did not include cocoa powder or inulin. Additionally, one digestion was performed with inulin and no cocoa powder.

Table 3.1.2.2 Specific Treatment Formulations for in-vitro digestion (dry weight, n=1)

Treatment	Mass of Cocoa (g)	Mass of Inulin (g)	Total Mass (g)
Natural	3	0	3
Alkalized	3	0	3
Natural 1:2	3	6	9
Natural 1:4	3	12	15
Alkalized 1:2	3	6	9
Alkalized 1:4	3	12	15
Inulin only	0	9	9
Control Digesta	0	0	0

Through the total masses differ among samples, the same mass (g) of cocoa powder was used for each treatment, only amounts of inulin added were adjusted. The difference in total mass among treatments was considered when calculating the amount of digesta to be added to each fermentation at an equal concentration.

In vitro Digestion

Three Stage *in vitro* digestion was performed to simulate the digestion of cocoa powder/mix through the small intestine to form a cocoa digesta product similar to that which would be used *in vivo* for fermentation by colonic bacteria (simulated later by *in vitro* fecal fermentation). This method was employed by Racine et al [66] and has been validated as a model for polyphenol bioacessability. Oral phase base solution was prepared the week of analysis by combination of water (1L) with potassium chloride (1.792 g), sodium phosphate (1.776 g), sodium sulfate (1.140 g), sodium chloride (0.596 g), and sodium bicarbonate (3.388 g). The oral phase began by adding 54 mL of oral phase base solution to each reaction bottle, along with urea (0.4 mg/mL base solution), uric acid (0.03 mg/mL base solution), α-amylase (10.6mg/mL base solution), and mucin (0.05 mg/mL base solution). The volume of oral phase used for each formulation was calculated to the volume needed for the median of the masses (9g) at the concentration of 6 mL/g digestion substrate. Cocoa powder, inulin, and mixtures were added to each of the 500 mL reaction bottles at masses according to the table above (**Table 3.1.2.2**).

Though the masses differ among treatments, yields were considered when dosing subsequent experiments using the products from digestion and the final volumes of the digestions were adjusted to be the same for each treatment. The bottles were blanketed with nitrogen gas, capped and sealed, then placed in a 37°C water bath and shaken at 85 oscillations per minute (opm) for 10 minutes, completing the oral phase.

The gastric phase began by removing the reaction vessels from the water bath and immediately placing them on ice. Saline was added until a volume of 300 mL was reached and the pH of the solution was adjusted to 2.5±0.1 using 1.0 M HCl. Carefully to avoiding foaming, 20 mL of 40 mg/mL pepsin in 0.1M HCl solution was added, followed by the addition of saline solution to bring the total volume to 400 mL. The reaction vessels were blanketed with nitrogen gas, capped and sealed, then placed in a shaking water bath (37°C, 85 opm) for 1 hour, completing the gastric phase.

The intestinal phase began with the removal of the reaction bottles from the water bath, which were immediately placed on ice. The pH of the solution was adjusted to 6.0± 0.1 using 1M NaOH. Approximately 20 mL 10mg/mL Pancreatin/ 5mg/mL Lipase was added, followed by approximately 30 mL of 40 mg/mL Bile extract solution (all in 100 mM $NaHCO_3$). After ensuring a pH of 6.0 ± 0.1 using 1N NaOH, the mixtures were brought to a total volume of 500 mL with saline. The vessel was blanketed with nitrogen gas, capped, and placed in a shaking water bath (37°C, 85 opm) for 2 hours. The contents were then frozen at -80°C and freeze dried.

In Vitro Fecal Fermentation

Microbial metabolites of flavanols should be considered as compounds that could contribute to health effects as they are more bioavailable and extensively produced upon the digestion of flavanols. Furthermore, fermentation may modify other components of cocoa, such as fiber, and the added inulin into more bioactive forms. To be able to investigate these metabolites, we subjected the flavanols to metabolism of lower gut microbiota via an *in vitro* fecal fermentation based on methods published by Mills *et al* and Alqurashi *et al* [69,70]. The inocula used for the fermentations was human fecal microbiome transfer material obtained from OpenBiome (Somerville, Massachusetts, USA) [Fecal Microbiota Preparation for Research (FMP-R)]. FMP-R packs contain 25 1ml aliquots of prepared stool material sourced from a rigorously screened healthy stool donor, and are designed to support non-clinical science,

including humanized animal models. Fecal material was pooled from 2 donors to reduce the effects of a single donor. OpenBiome is a nonprofit biorepository that provides therapeutic Fecal Microbiota Transplantation (FMT) material for physicians, as well as FMP-R material for clinical and non-clinical researchers. FMP-R is obtained from rigorously screened donors, for which the health histories, clinical data, pathogen screen results and 16S rRNA sequences are available. Samples are collected under IRB approval from a pool of donors. Material is processed in a sterile 12.5% glycerol and 0.9% saline buffer at 2.5 ml of buffer per gram of stool. This buffer acts as a cryo-protectant for long-term storage. Each sample is passed through a 330 µm filter to remove large particulate matter and facilitate liquid handling for gavage and molecular analysis.

Fermentation media was produced by adding peptone water powder (2 g), yeast extract (2g), NaCl (0.1g), K_2HPO_4 (0.04 g), KH_2PO_4 (0.04 g), $MgSO_4 \cdot 7H20$ (0.01 g), $CaCl_2 \cdot 6H2O$ (0.01 g), $NaHCO_3$ (2 g), tween 80® (2 mL), hemin (0.05 g), Vitamin K1 (10 µL), bile salts (0.5 g), resazurin (1 mg), and L-cysteine (0.5 g/L) to 1 L of DDI water. The fermentation media, along with freshly made PBS, were sparged overnight with 100% N_2 gas until the resazurin indicator turned clear, indicating the solutions were anaerobic.

Fermentations were performed in duplicate in a 4-glove anaerobic chamber (855-ACB, Plas-Labs, Lansing, MI) maintained at 37°C with an atmosphere of 85% N_2, 10% CO_2 and 5% H_2. The atmosphere was maintained at <200 ppm O_2 by purging with the mixed gas as well as a palladium catalyst designed to neutralize residual O_2. The atmosphere composition was monitored using an anaerobic monitor from Coy Labs (Grass Lake, MI). All materials were autoclaved or otherwise sterilized before introduction into the chamber. Fermentations were performed in duplicate with each treatment in 50 mL conical tubes. Human fecal homogenates were thawed for 5-10 min inside the chamber and then used to create a 10% fecal slurry using PBS. To create a 1:50 fecal homogenate, fermentation media (41.16 mL) was combined with 10% fecal slurry (0.84 mL) inside each 50 mL conical tube. Dried digesta powder was added to each tube at a 1/5 concentration (0.2X concentration) to provide a physiologically relevant dose (5x diluted from small intestine to large intestine). To determine the mass of digesta needed, we used the yields (**Table 4.2**) from digestion to calculate a digesta mass that corresponded to 8.4 mL, as 8.4 mL is ~1/5 the total volume of each fermentation (42 mL). Additionally, a "background control" fermentation was performed with fecal inoculation and media, but no

digesta added, to account for anything that may be in the fecal matrix or media. A "nonmicro control" was performed with fermentation media, natural digesta powder, but instead of fecal inoculation, PBS was added. The nonmciro control accounts for any chemical degradation that may have occurred that is unrelated to the microbiome. The formulations for all treatments are shown in the table below (**Table 3.1.2.3**).

Table 3.1.2.3 In Vitro Fecal Fermentation Formulations

Treatment	Fecal slurry (mL)	Media (mL)	Compound/Digesta (g)
Background control	0.84	41.16	None
Non-micro negative control	None- 0.84 PBS	41.16	0.131
Natural	0.84	41.16	0.131
Alkalized	0.84	41.16	0.120
Natural 1:2	0.84	41.16	0.239
Alkalized 1:2	0.84	41.16	0.140
Natural 1:4	0.84	41.16	0.271
Alkalized 1:4	0.84	41.16	0.257
Inulin only	0.84	41.16	0.127
Blank digesta	0.84	41.16	0.086

The caps were left slightly ajar as the samples were allowed to ferment for 24h. At hours 0, 6, 12, and 24, the tubes were inverted to mix and 10 mL samples were taken from each replicate. The pH of each sample was measured and recorded followed by the addition of 1 mL of 1.0M HCL. Finally, samples were frozen at -80°C, and freeze dried.

Fermentation Metabolite Analysis

Following fecal fermentation, samples were analyzed for microbial metabolites. Freeze-dried fermentation samples were added to a test tube (15 mg) with 80% MeOH in 2% formic acid (2.5 mL), vortexed 20-30 seconds, sonicated 20 min, and centrifuged for 4 min (3700 xg). The supernatants were collected. The vortex, sonication, centrifugation process was then repeated twice, but instead using 98% MeOH in 2% FA (2.5 mL). The supernatants from all three steps were combined in the same tube and vacuum dried. The vacuum-dried samples were then resolubilized by adding 0.1% FA in H_2O (2 mL), and vertexing 20-30 seconds, then sonicating for 2 min. The samples were then filtered (0.45 µm) into a 96 well plate, blanketed in N_2 gas, and kept at -80°C until UPLC analysis.

UPLC separations were performed according to Goodrich et al [71], with modifications, on a Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm particle size). The column temperature was set to 40 ◦C, and the samples were maintained at 10°C. The binary mobile phase system was comprised of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in ACN (phase B). The system flow rate was 0.55 mL/min. Elution was performed based on the following linear gradient: 95% A at 0 min held until 0.5 min, 65% A at 6.5 min, 20% A at 7.5 min held until 8.75 min, 95% A at 8.85 min held until 10.0 min. An injection volume of 50μL was used for samples and standards. MS/MS analysis of colon a Waters Acquity TQD (triple quadrupole) mass spectrometer effluent was performed by (−)-electrospray ionization (ESI) equipped with a Z-spray electrospray interface. The ESI capillary were 150°C and 400°C, respectively.

Table 3.1.2.4 Multi-reaction-monitoring (MRM) of Native Flavanols and selected Fecal Microbial Metabolites

Compounds	**t_R[a] (min)**	**MW (g mol^{-1})**	**[M – H]$^-$ (m/z)[b]**	**Daughter Ion (m/z)**
Catechin	3.18	290.028	289.03	245.06
epicatechin	3.79	290.092	289.09	245.06
Procyanidin B2	4.03	578.136	577.14	425.10
3-hydroxybenzoic acid	3.60	137.874	136.874	92.963
4-hydroxybenzoic acid	2.90	137.882	136.882	92.966
3,4-dihydroxybenzoic acid	2.10	153.948	152.95	108.99
phenylacetic acid	5.40	135.942	134.942	90.956
3-hydroxyphenylacetic acid	3.65	151.948	150.948	107.020
4-hydroxyphenylacetic acid	3.30	151.948	150.948	107.064
3,4-dihydroxyphenylacetic acid	2.50	167.968	166.968	122.973
4-hydroxymandelic acid	0.88	167.958	166.958	122.968
caffeic acid	3.60	179.968	178.968	134.977
vanillic acid	3.60	167.962	166.962	152.002
3-(3-hydroxyphenyl)propionic acid	4.50	165.958	164.958	105.982
3-(4-hydroxyphenyl)propionic acid	4.14	165.952	164.952	121.014
3-(3,4-dihydroxyphenyl) propionic acid	3.30	181.968	180.968	108.984
5-(3’-dihydroxyphenyl)-γ-valerolactone	5.22	192.071	191.071	147.090
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	3.94	208.066	207.066	163.010
5-(3’,4’-dihydroxyphenyl)valeric acid	5.16	210.082	209.082	191.090
5-(3’-hydroxyphenyl)valeric acid; 5-(4’-dihydroxyphenyl)valeric acid; 5-hydroxy-5-phenylvaleric acid[b]	5.20	194.087	193.087	175.050
5-phenylvaleric acid	7.97	178.032	177.032	159.086

[a] *retention time*

[b] *collectively referred to as “hydroxyphenyl valeric acids” in text; isomers cannot be distinguished by retention time or MRM*

The desolvation gas and cone voltage was −4.25 kV, and the source and desolvation temperatures gasses were N2 at flow rates of 900 L/h and 75 L/h, respectively. The MS/MS collision gas was argon. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points/peak with an average peak width of 6 s. The autodwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s for each transition. The TQD was operated in quantitation mode, with the mass resolution of the first and second quadrupoles set at 1.0 and 0.75, respectively. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μL/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min.

Phospholipid Enzyme (PLA2) Inhibitory Activity

In order to determine the inhibitory effects of cocoa/inulin on phospholipase A2, a florescence assay kit was used (EnzChek Phospholipase A2 Assay Kit Cat. No. E10217).

Table 3.1.2.5 DMSO stock solutions equivalent to 10mg/mL.

Treatment	0 h	6 h	12 h	24 h
Blank Digesta*	10*	14	25	17
Background control**	10	10	10	13
Nonmicro control***	17	18	12	11
Natural	22	18	14	16
Alkalized	22	22	14	17
Natural 1:2	22	21	12	16
Alkalized 1:2	24	15	15	15
Natural 1:4	27	22	22	12
Alkalized 1:4	30	21	18	12
Inulin	20	20	14	10

**The yield of the blank digetsa at time 0 was used as a baseline for normalization. The blank digesta fermentation used the digesta performed with no cocoa powder or inulin. **The background control represents the fermentation performed with the fecal inoculation and the fermentation media, but no digesta added. ***The nonmicro control represents that fermentation performed with natural powder digesta, fermentation media, and PBS in place of the fecal inoculation.*

Cocoa/Inulin fermentation products from time points 0, 6, 12, and 24 hours were diluted to 10 mg/mL (normalized to their yields) in DMSO. Normalization was calculated by dividing the yields of each treatment at each fermentation time point (**Table 4.3**) by the yield of the blank digetsa (32.05 g) at time 0 hours. That ratio was then multiplied by 10 (mg/mL) to obtain the concentration of the normalized solutions equivalent to 10 mg/mL, shown above (**Table 3.1.2.5**).

PLA2 buffer (70 µL) was dispensed into each well followed by diluted enzyme solution (10 µL). Cocoa diluted fermentation products (10 µL) and diluted PLA2 substrate were also added to each well (10 µL) and the plate was incubated at 10 min at room temperature. Fluoresence was determined at λex =485 nm and λem =538 nm using a Fluoroskan flouresence detector. The inhibition of each sample was calculated by using the control ("blank") digesta at t=0 hours as 100% enzyme activity, as this control accounted for all the material in both the three-stage digestion and the fecal fermentation.

$$\%\ \boldsymbol{enzyme\ acitivty} = \frac{\boldsymbol{fluorescence}}{\boldsymbol{fluoresence\ blank\ digesta\ 0h}}\boldsymbol{x\ 100}\%$$

3.1.4 Cell Culture Experiments with Fermentation Products

In order to examine the effects of fermentation metabolites on gut health, cell culture experiments were performed according to Bitzer et al [7] with modifications as described below.

Cell Culture Conditions

Caco-2 TC7 and HT-29 human colon cancer cells were maintained in sub-confluence in Dulbecco's modification of Eagle's medium or McCoy's 5A medium. The media were supplemented with 10% fetal bovine serum, 100IU/mL penicillin and 100 micrograms/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Trypsinization was used to subculture the cells.

Inflammation (IL-8 secretion following TNF-a stimulation)

HT-29 cells were allowed to reach 80% confluence in 24 well plates. Lyophilized cocoa fermentation products were prepared in DMSO at and added to each well at 100 µg/mL final concentrations (n=3) using the normalized solutions created in DMSO above (10 mg/mL) (**Table 3.1.2.5**). Cells were pretreated with fermentation products for 36 hours before being stimulated

with 5 ng/mL tumor necrosis factor (TNF)-α for 6 hours. Interleukin (IL)-8 levels in the medium were determined by enzyme-linked immunosorbent assay [IL-8 ELISA: R&D Systems (Cat# D8000C)].

Barrier function (FITC flux following DSS damage)

In order to examine the ability of cocoa fractions to mitigate colon permeability in vitro, the apical to basolateral flux of fluorescein isothiocynate-dextran (FITC-D) across differentiated Caco-2 cell monolayers was measured. The caco-2 cells were seeded in corning 0.4 μM polycarbonate trans well inserts and allowed to confluence and differentiate for 27 days. All monolayers used has a transepithelial electric resistance (TEER) of 200-300 Ω cm^2. The treatments, in DMSO solutions normalized to 10 mg/mL (**Table 3.1.2.5**), were added to the differentiated monolayers (100 μg/mL) 2 hours before the addition of 2% dextran sodium sulfate (DSS) to the media to induce a loss of the cells' epithelial membrane integrity. The cells were incubated for an additional 36 hours. FITC-labeled dextran was added to the apical compartment (concentration 1 mg/mL) and the cells were further incubated for 6 hours. Every 30 min, basolateral media was be removed (50μL) and florescence determined using a plate reader (wavelength 493 nm, wavelength 517 nm). The rates of increase in basolateral fluorescence were determined and normalized to the values derived from monolayers that were not treated with DSS. The results from this assay were not included in the analyses of this project. The results from our preliminary trials are presented in the appendix.

3.2 STATISTICS AND DATA ANALYSIS

Analyses were performed using GraphPad Prism 8 (GraphPad, La Jolla, CA, USA). One-way ANOVA, two-way ANOVA, and mixed effects analyses with Tukey's post hoc test were used to determine significance (see specific figure captions). All values are expressed as mean ± SEM.

CHAPTER 4. RESULTS

4.1 CHEMICAL CHARACTERIZATIONS OF COCOA POWDERS AND INULIN

Chemical characterization was performed on each of the two cocoa powders and inulin to identify and quantify polyphenolic and flavonoid compounds. These values will allow us to correlate *in vitro* activity in the gut with the presence of certain compounds.

4.1.1 Total Polyphenols

The natural cocoa powder had about 4 times total polyphenol content (98.67 ± 1.5 mg Gallic acid equivalent /gram cocoa powder) vs alkalized powder (24.44± 0.34 mg gallic acid equivalent) ($p<.0001$) (Figure 4.1.1) Inulin showed a slight, but negligible response to this assay.

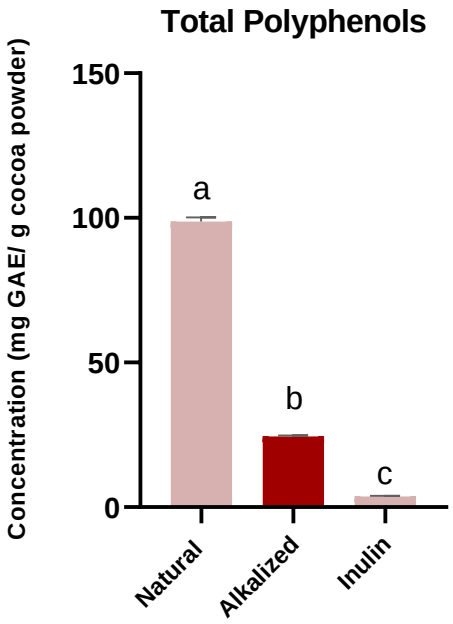


Figure 4.1.1 Total polyphenols in each cocoa powder (natural and alkalized) extract and inulin extract were determined via the Folin-Ciocalteu colorimetric assay. All values (n=3) are expressed as mg gallic acid equivalents/ g (cocoa or inulin) powder and reported as mean ± SEM. Significance between treatments was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

4.1.2 Total Flavanols

The Natural cocoa powder contained significantly more total flavanols (8.09 ± 0.38 mg PCB2 equiv) compared to the alkalized cocoa powder extract (2.33 ± 0.076 mg PCB2 equiv)

(p<0.0001). This is similar to the pattern seen above in the Folin assay measuring total polyphenols, with the natural powder containing about 3.5-fold the amount of flavanols as the alkalized powder. The slight difference in these ratios can be explained by the specificity of the assays. The Folin assay detects all polyphenols and some other reducing agents, including those that are not flavanols, while the DMAC assay only detects flavanols.

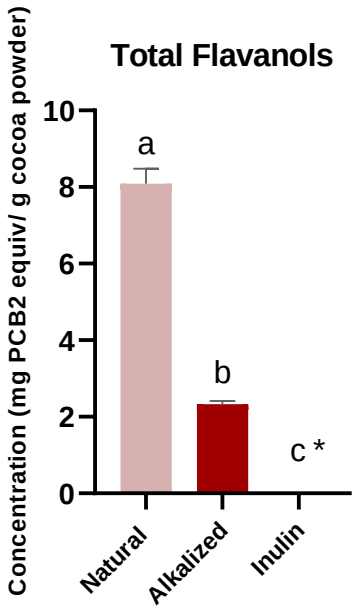


*Figure 4.1.2. Total Flavanols in each cocoa powder (natural and alkalized) extract and inulin extract were determined in triplicate (n=3) via the 4-dimethylaminocinnamaldehyde (DMAC) colorimetric assay. All values are expressed as mg gallic acid equivalents/ g (cocoa or inulin) powder and reported as mean ± SEM. Significance between treatments was determined by one-way ANOVA and Tukey's HSD post-hoc test (p<0.05). Different letters above treatments indicate statistically different values. *Inulin did not elicit a detectable response for this assay.*

4.1.3 Mean Degree of Polymerization

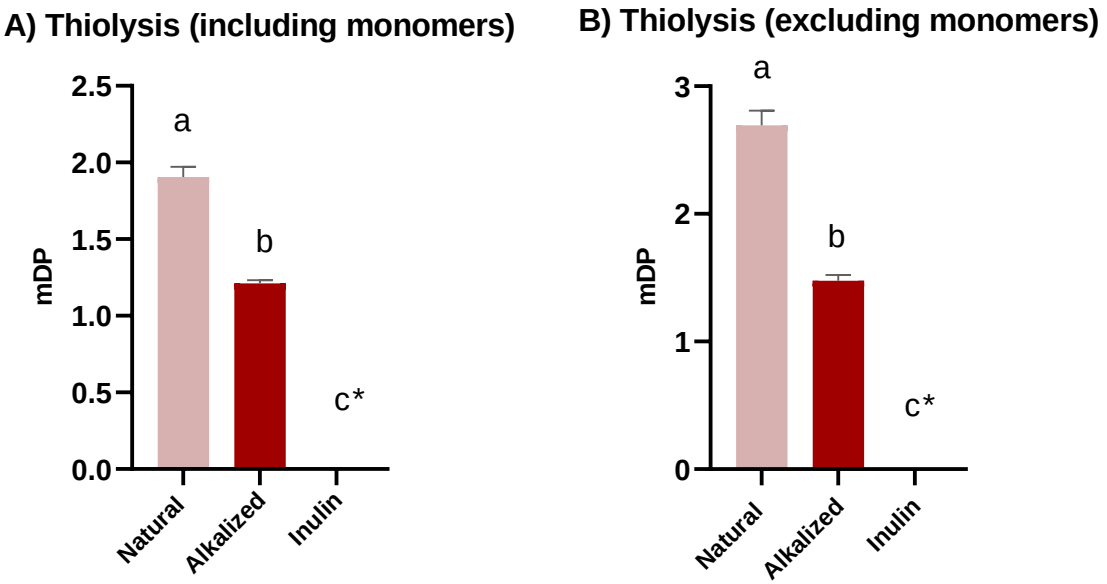


*Figure 4.1.3 Mean Flavanol Degree of Polymerization (DP) in each cocoa powder (natural and alklized) and inulin extract determined by thiolysis. A) represents the calculated flavanol DP including native monomers and B) represents the same values excluding native monomers from the calculation. All values (n=5) reported as mean ± SEM. Significance between treatments was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values. *Inulin elicited no response.*

Figure 4.1.3 A shows the results of thiolysis including native monomeric compounds in the calculation, the natural cocoa extract had an mDP of about 2, while the alkalized extract had an mDP just above 1 (**Figure 4.1.3A**). This value represents the mDP of total flavanols in these samples. Figure 4.1.3 B displays mDP excluding native monomeric flavanols from the calculation. The natural powder showed an mDP of about 3 while the alkalized powder showed an mDP or about 1.5 (**Figure 4.1.3B**). These values represent the mDP of the oligomeric and polymeric flavanol compounds in these samples. When calculated with only the net monomers (excluding native monomers), mDP is larger for both powders, as the native monomers aren't influencing the average. Inulin elicited no response for this assay.

4.1.4 Analysis of Procyanidins

The natural cocoa powder contained significantly more flavanols of every size (DP 1-6) than both the alkalized powder and the inulin, which elicited no response **(Figure 4.1.4**). The natural powder was the richest in dimers and hexamers, with an appreciable amount of monomers, trimers, tetramers, and pentamers also present. The alkalized powder displayed low

levels of all compounds, but monomers, dimers, and hexamers were the most abundant. No procyanidin compounds were detected in the inulin extract (**Figure 4.1.4 E**)

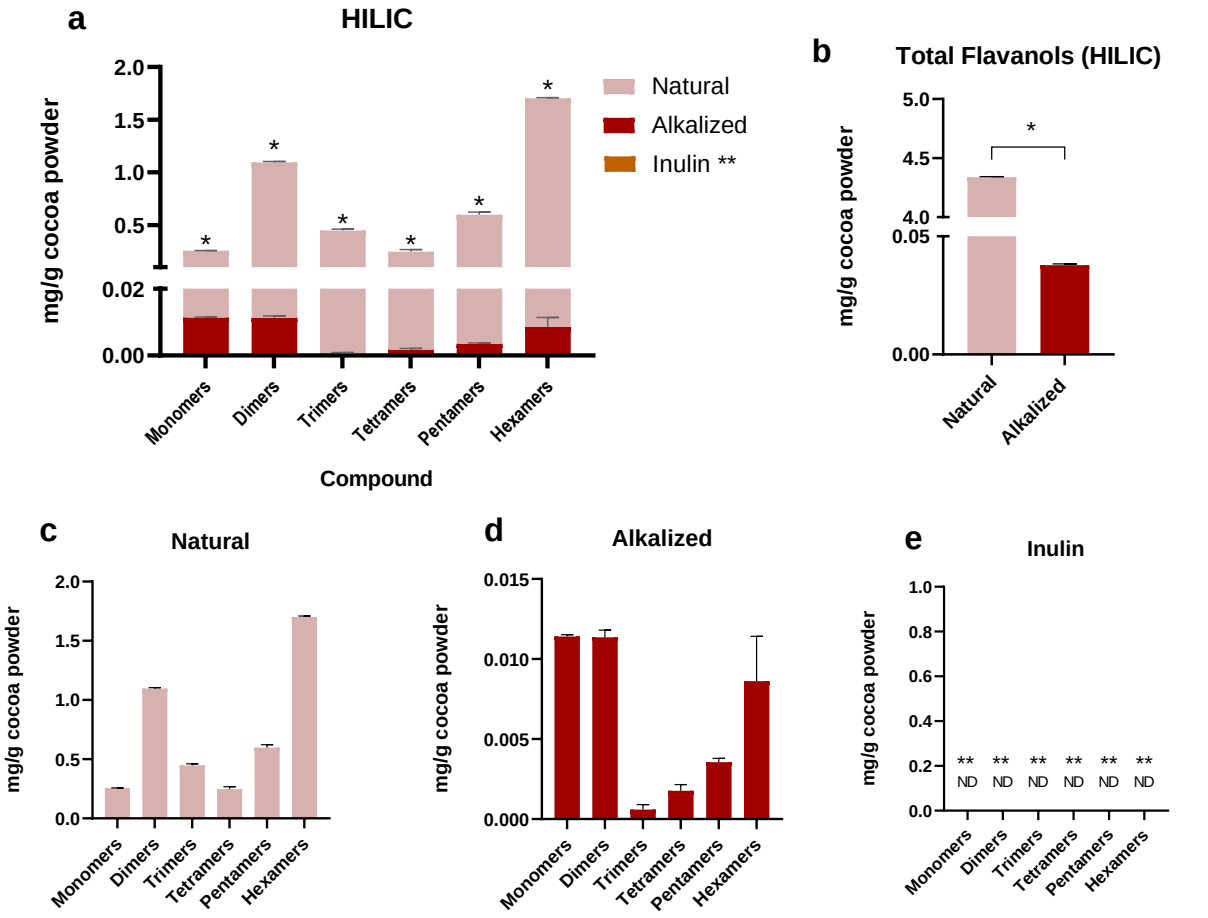


*Figure 4.1.4 (A-E) Levels of procyanidin compounds in powders were measured up to DP=6 via HPLC-MS/MS analysis. All values (n=3) reported as mean ± SEM. Significance between treatments was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). * above treatments indicate statistically different values between the natural and alkalized extracts. **ND above treatments indicates no detection.*

The total flavanol value obtained from this assay is lower than that obtained from the DMAC assay, likely for 2 reasons. The first is the difference in sensitivities of each assay. The second is using this HILIC method we only measured up to DP=6, so there could be some larger compounds unaccounted for (**Figure 4.1.4 C**).

4.2 *IN VITRO* DIGESTION

Three stage *in vitro* digestion was performed to simulate human digestion of cocoa/inulin from the mouth through the small intestine. The product formed was freeze dried, then used as the substrate in the *in vitro* fecal fermentation in order to represent a physiologically relevant sample of partially digested cocoa/inulin similar to what would be fermented by colonic bacteria *in vivo*. The yields from the dried digesta are shown below (**Table 4.2**)

Table 4.2 Total digesta yields from three-stage invitro digestion (dry weight, n=1)

Treatment	Total Mass digested* (g)	Total Mass post-digestion (g)
Natural	3.0	7.2
Alkalized	3.0	7.1
Natural 1:2	9.0	13.4
Natural 1:4	15.0	16.4
Alkalized 1:2	9.0	8.54
Alkalized 1:4	15.0	15.6
Inulin only	9.0	8.20
Control Digesta	0.0	4.58

mass includes mass of "food" (cocoa and inulin mixtures) only; it does not include mass of digestion reagents added

4.3 IN VITRO FECAL FERMENTATION

In vitro fecal fermentation was performed to generate microbial metabolites similar to what would be produced during the colonic digestion of cocoa/inulin. The yields from each of the 10 mL samples taken at each time point (0, 6, 12, 24 hours) for each treatment are shown below (**Table 4.3**)

4.3.1 Fecal Fermentation pH changes

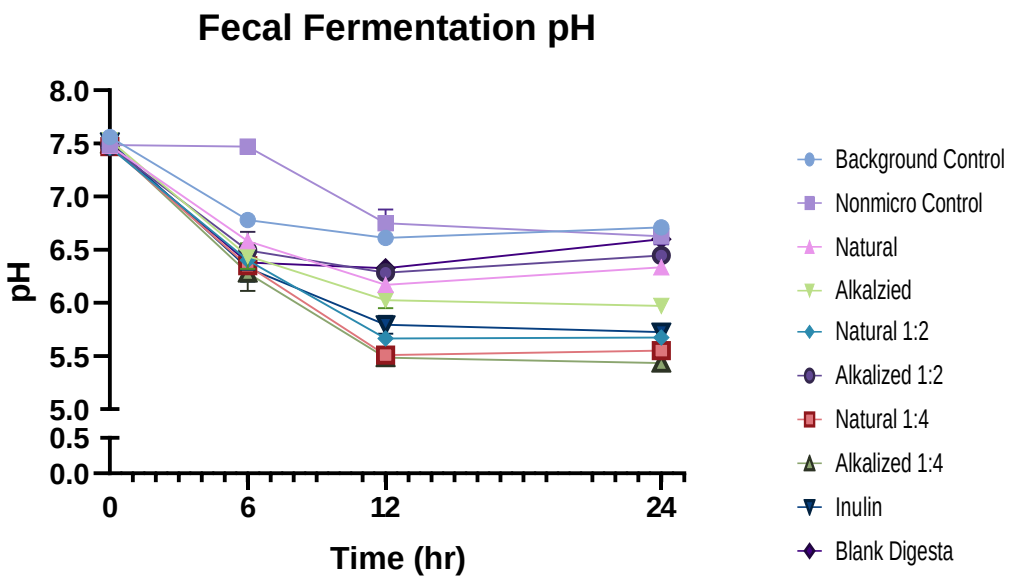
The pH changes that occurred during the fermentation are shown in the figure below **(Figure 4.3.1.1).** All treatments showed an overall decrease in pH over the 24-hour fermentation, as expected. Nonmicro control had the highest pH overall, which may relate to a lack of SCFAs being produced due to a lack of microorganisms in the fermentation. All samples containing

inulin had an overall lower pH than samples without inulin, except the Alkalized 1:2. Inulin is a known prebiotic, so this lower pH may be explained by the formation of more SCFAs in these samples. However, we did not measure SCFA production, so this cannot be confirmed. Changes in pH seem to be more dependent upon inulin concentration. Below, individual pH changes are shown and discussed in more detail in **Figure 4.3.1.2**.

Table 4.3 In vitro Fecal Fermentation Yields (dry weight). Each value represents the mean (n=2) of the dried mass of a 10 mL sample taken from fecal fermentation at time points 0, 6, 12, and 24 hours.

Treatment	0 h Yield (mg)	6 h Yield (mg)	12 h Yield (mg)	24 h Yield (mg)
Blank Digesta*	32.05 *	45.62	81.38	55.50
Background control**	28.40	27.42	25.13	41.43
Nonmicro control***	55.05	58.15	38.31	35.05
Natural	71.34	60.81	44.93	51.21
Alkalized	72.13	73.09	45.79	54.16
Natural 1:2	71.23	68.19	39.95	50.41
Alkalized 1:2	78.05	48.39	47.58	49.94
Natural 1:4	85.56	73.02	73.815	36.85
Alkalized 1:4	94.63	67.44	56.03	36.85
Inulin	62.97	63.38	43.69	32.71

**The yield of the blank digetsa at time 0 was used as a baseline for normalization. The blank digesta fermentation used the digesta performed with no cocoa powder or inulin. **The background control represents the fermentation performed with the fecal inoculation and the fermentation media, but with no digesta added. ***The nonmicro control represents that fermentation performed with natural powder digesta, fermentation media, and PBS in place of the fecal inoculation.*



4.3.1.1 In vitro fecal fermentation pH changes for each cocoa and inulin formuation (Table 3.1.2) were measured using a benchtop pH meter at time points 0, 6, 12, and 24 hours. All values (n=2) reported as mean ± SEM. Significance between and within treatments was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$).

The pH of the natural powder fermentation's pH changed significantly at each time point, 0, 6, 12, and 24 hours (**Figure 4.3.2**) compared to the alkalized powder, alkalized 1:2, and background control fermentation pHs, which decreased significantly from 0h to 6h, but then did not significantly change from 6-12h or 12-24h. The natural 1:2, natural 1:4, alkalized 1:4, and inulin fermentation pHs dropped significantly from times 0-6 h and 6-12h, but then did not change significantly from 12-24 hours. The blank digesta pH significantly dropped from time 0-6h, remained stable for 6-12h, then significantly increased from time 12-24h. Interestingly, the non-micro control pH did not significantly decrease from time 0-6 hours, unlike every other treatment and control, and also did not significantly change between time points 12 and 24 hours.

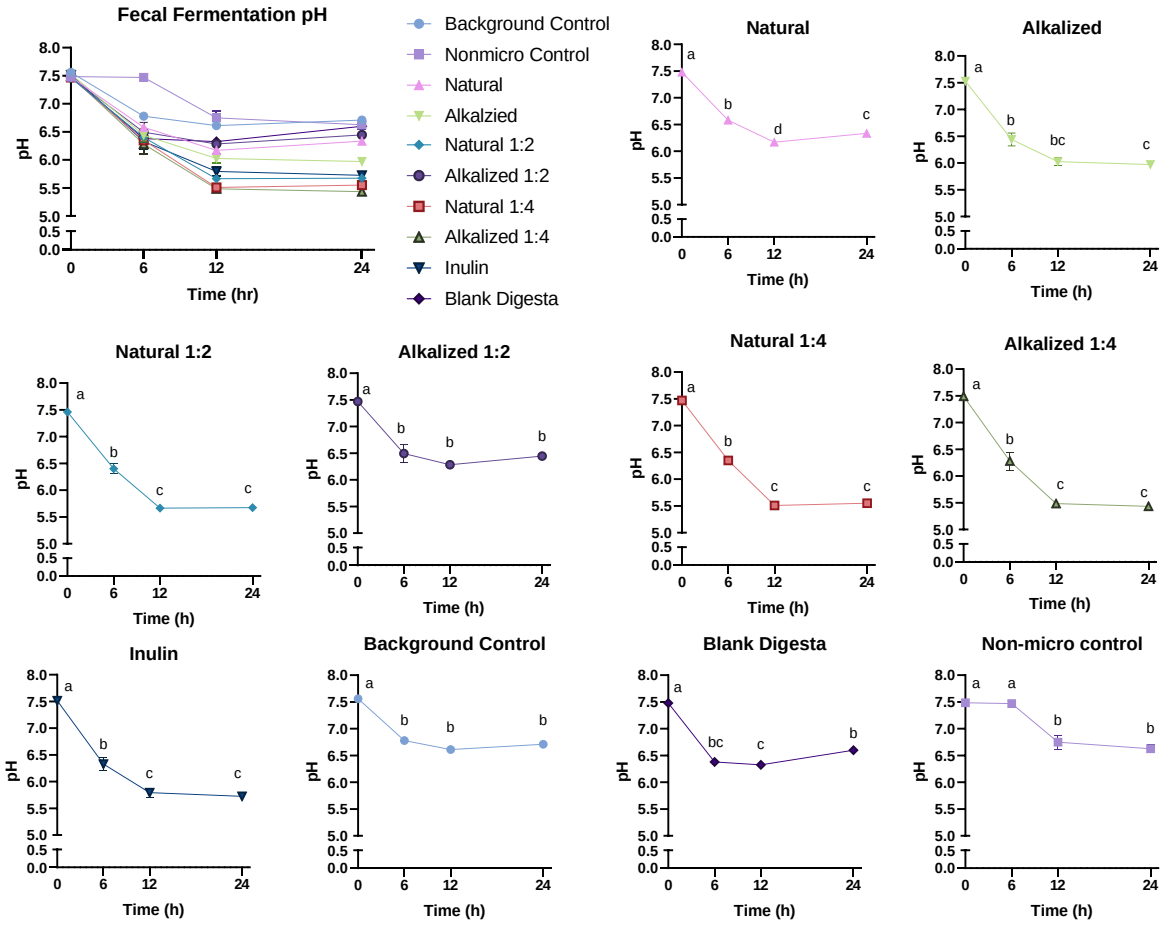


Figure 4.3.1.2. Fecal Fermentation Individual pH changes for each cocoa and inulin formulation: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin only, Blank Digesta (product of the blank three stage digestion used for fermentation), a Non-micro Control (no fecal inoculation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above times indicate statistically different values

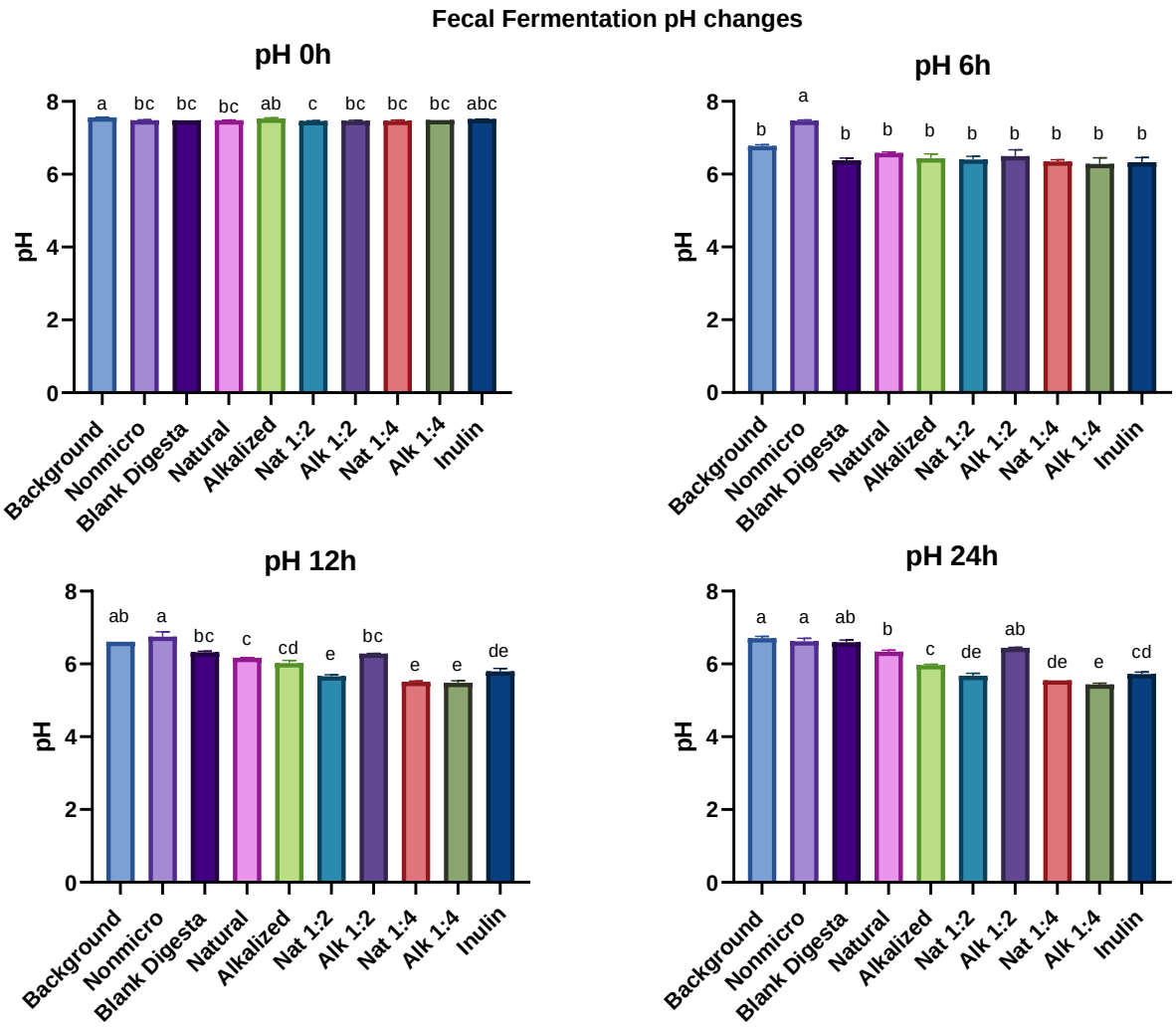


Figure 4.3.1.3. pH between all treatments at time points 0, 6, 12, 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin only, Blank Digesta (product of the blank three stage digestion used for fermentation), a Non-micro Control (no fecal inoculation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between time points 0, 6, 12, and 24 hours of each treatment was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above times indicate statistically different values.

Throughout the fermentations, different treatments displayed different changes in pH. Although all the fermentations began within a small pH window (7.46-7.56), there were still differences seen at the start. At time 0 hours, the background control has the highest pH at 7.56, which is significantly different than all treatments other than Alkalized and Inulin. The Natural 1:2 fermentation, with a pH of 7.46, is significantly lower than the pH of the background control

and Alkalized fermentations (**Figure 4.3.1.3**). At time 6 hours, the pH of the nonmicro control was 7.47, significantly higher than the pH of every other treatment. The nonmicro control fermentation was the also only treatment that did not show a significant difference in pH between times 0 and 6 hours (**Figure 4.3.1.2)**. At time 12 hours, the nonmicro, background, and blank digesta fermentations displayed the highest pH readings (6.75-6.325), followed by the Alkalized 1:2, Natural, and Alkalized, and Inulin fermentations (6.285-6.025). The Natural 1:2, Natural 1:4, and Alkalized 1:4 fermentations all displayed lower pH measurements than all other treatments, except inulin, at this time point, with measurements ranging from 5.66-5.485 (**Figure 4.3.1.3**). At 24 hours, the trends were similar to those seen at 12 hours: The background control, nonmicro control, blank digesta, and Alkalized 1:2 have the highest pH measurements (6.71-6.445), followed by the Natural fermentation (6.335) and the Alkalized fermentation (5.97). Inulin, Natural 1:2, Natural 1:4, and Alkalized 1:4 fermentations were on the lower end of the record pHs ranging from 5.725-5.435 **(Figure 4.3.1.3).**

The nonmicro control and background control remain among the 2 highest pH measurements from 6-24 hours. All fermentations containing inulin (except Alkalized 1:2) saw the lowest pH measurements from 12-24 hours, likely due to the formation of SCFAs, as inulin is a known prebiotic and creates these when fermented by colonic bacteria [51].

4.3.2 In vitro Fecal Fermentation Metabolites

To investigate the effects of alkali processing and the addition of inulin to cocoa during colonic fermentation, fecal fermentation metabolites were identified and quantified at times 0, 6, 12, and 24 hours for each treatment used in the *in vitro* fecal fermentation. General trends of each metabolite investigated for each treatment at all time points are discussed below.

Catechin

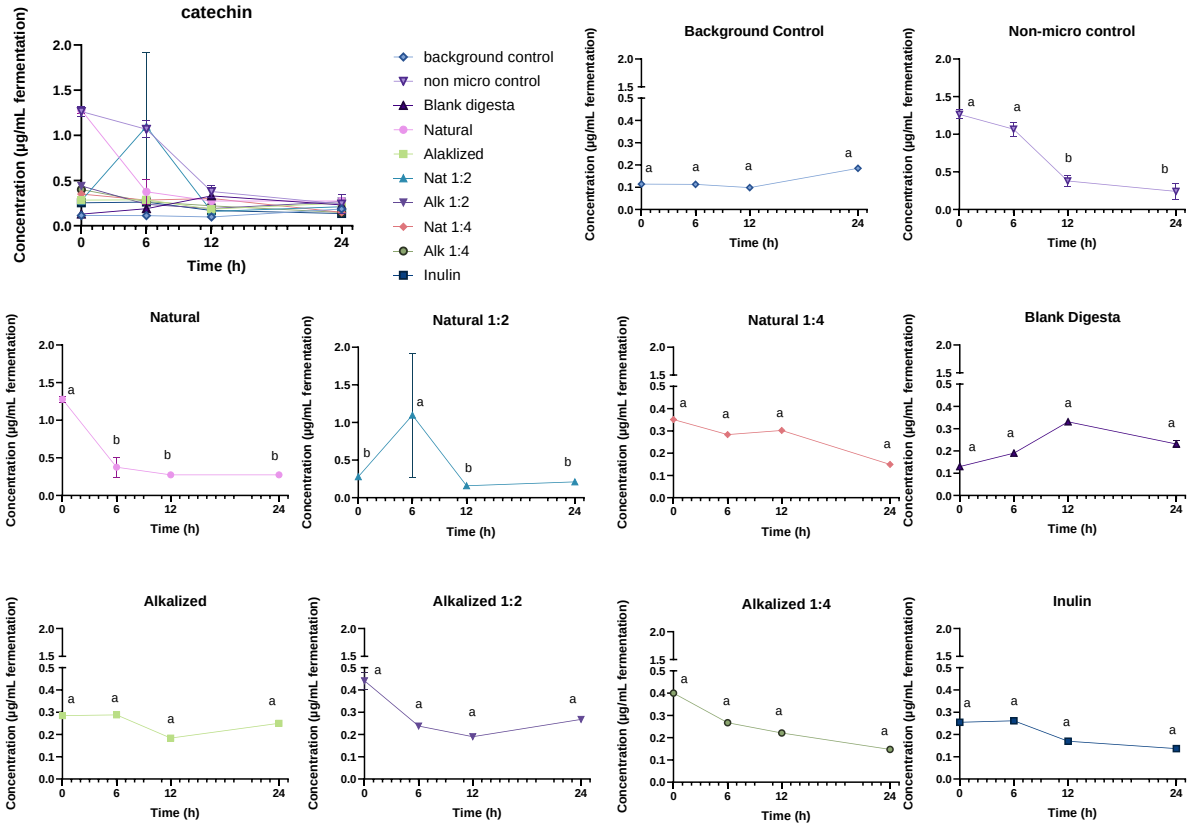


Figure 4.3.2.1. (±)-Catechin quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

Catechin was detected in appreciable amounts at time 0 hours in the non-micro control, and natural fermentations. The catechin level in the nonmicro fermentation stayed fairly level until 12 hours, where the levels finally dropped off, likely due to other (non-microbial) degradation of the compound. The natural fermentation, however, saw an immediate decrease of catechin levels at time 6 hours, suggesting the fecal bacteria was involved in the breakdown of this compound, especially towards the beginning of fermentation. The natural 1:2 cocoa saw a small spike in catechin level at time 6 hours, which perhaps could be explained by bound polyphenols being released at a later time in colonic digestion, although the error for these

samples was large. It is interesting that the natural and nonmicro control started out at about the same level of catechin, but the natural 1:2 and natural 1:4 both started out about the same but at a lower concentration. This could indicate some effect of the inulin in these samples on the concentration of catechin detected. The background control, blank digesta, inulin, and alkalized samples all showed slight responses, which is explained by the lack of cocoa in the inulin and controls, and the alkalization process degrading catechin in those samples (**Figure 4.3.2.1**).

Epicatechin

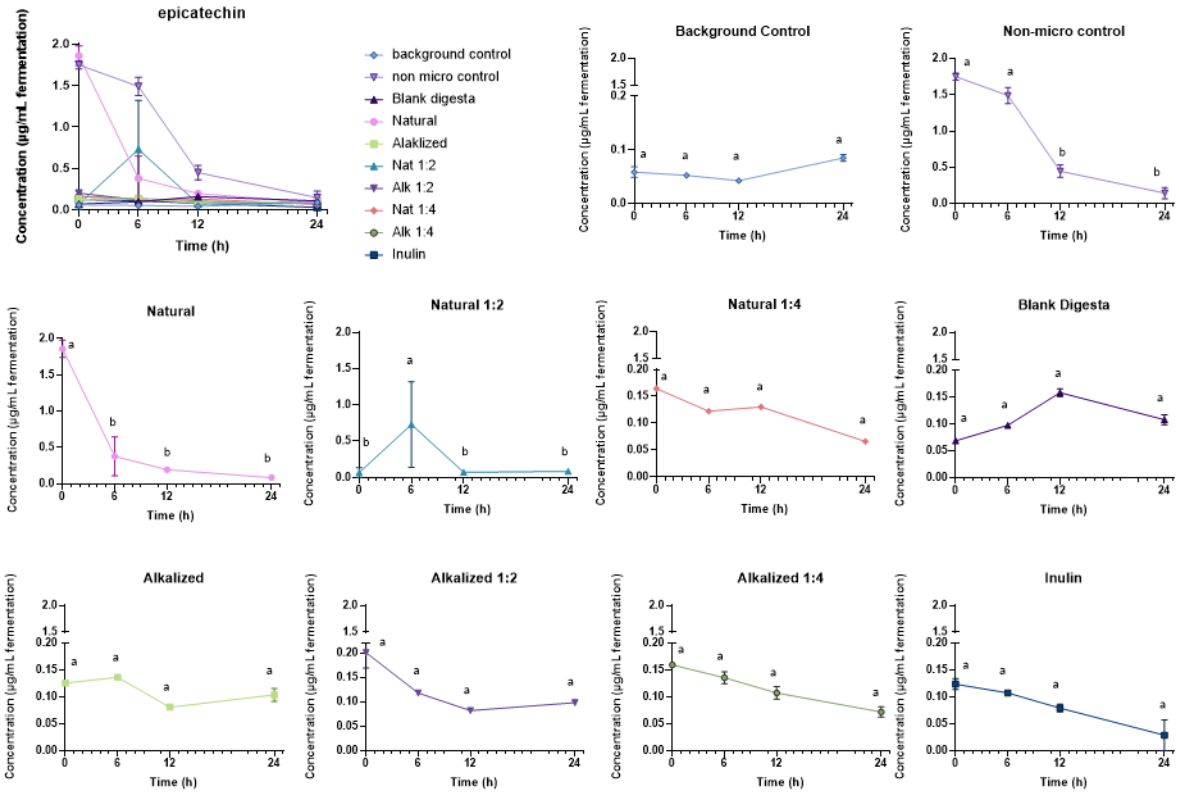


Figure 4.3.2.2. (±)-epicatechin quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

Epicatechin presented the same trends as catechin, with natural and non-micro having the highest amounts at time 0, non-micro staying consistent at time 6 hours, and dropping off at 12 hours and natural dropping off at 6 hours. The natural 1:2 treatment again showed a spike in epicatechin levels at time 6 hours, which could potentially be due to binding, although again, these values had a large standard error (**Figure 4.3.2.2**).

Procyanidin B2

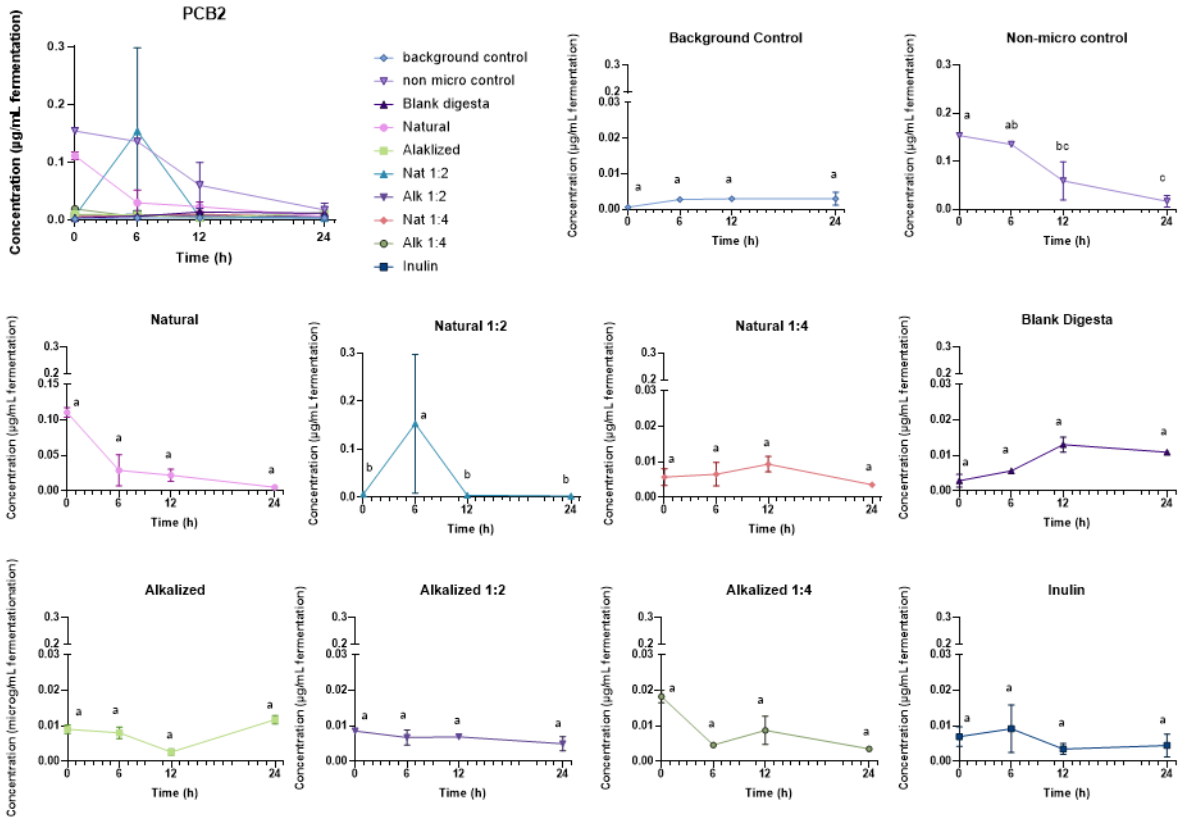


Figure 4.3.2.3. Procyanidin B2 quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

The non-micro control, natural, and natural 1:2 treatments elicited responses similar to catechin and epicatechin, likely because they are all compounds native to raw cocoa. Again, the nonmicro control dropped off more slowly than the natural treatment, likely due to a lack of fecal microorganisms and therefore less microbial catabolism of PCB2. The natural 1:2 saw a spike at time 6 hours, but presented a very high error rate (**Figure 4.3.2.3**). Additionally, the natural, natural 1:2, natural 1:4, and nonmicro control do not start fermentation with consistent levels of PCB2, even though they were all dosed with the same amount of natural powder.

3-hydroxybenzoic acid

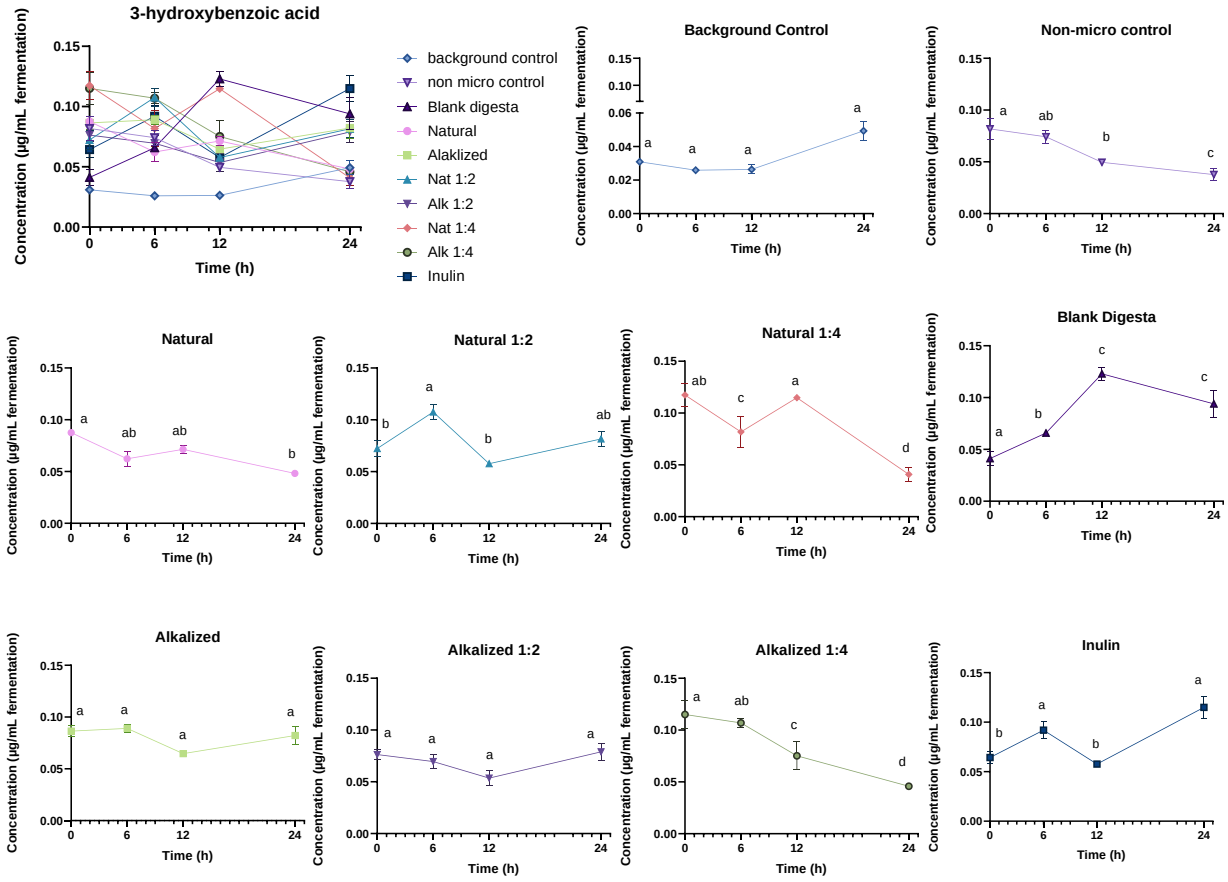


Figure 4.3.2.4 3-hydroxybenzoic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was

determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

3-hydroxybenzoic acid was detected at the highest level in the blank digesta at time 12 hours. The blank digesta at levels almost as high as the levels in all of these treatments, so any 3-hydroxybenzoic acid detected could just be due to things present in the background digestion reagents (**Figure 4.3.2.4**). Alaklized 1:4 and Natural 1:4 both saw significant drops in concentration at time 24 hours, Background control, alkalized, and alkalized 1:2 showed no significant changes in concentration throughout fermentation. Natural 1:2 again showed a spike in concentration at 6 hours.

4-hydroxybenzoic acid

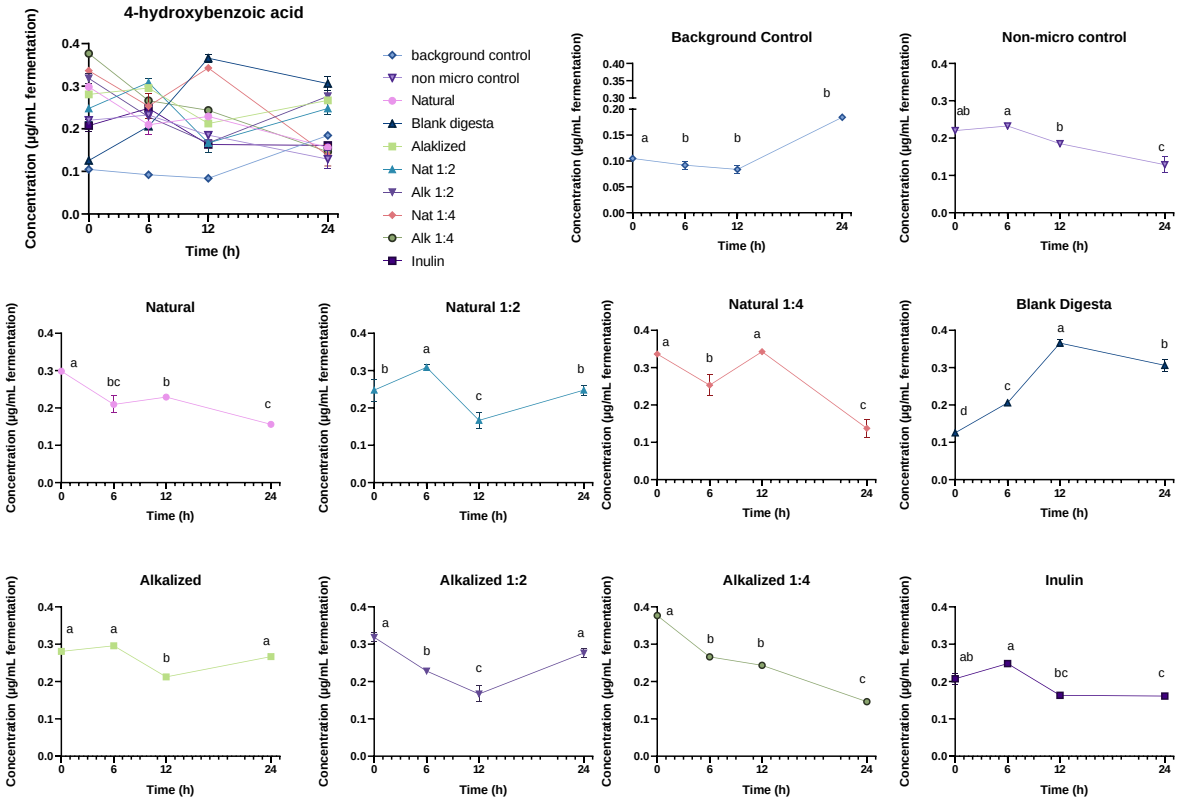


Figure 4.3.2.5 4-hydroxybenzoic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a

Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

4-hydroxybenzoic acid was detected in each treatment in similar trends to 3-hydroxybenzoic acid, as they have very similar molecular structures. (**Figure 4.3.2.5**). The Natural 1:2, Alkalized, and alkalized 1:2 all saw significant drops in concentration at time 12h, then increased in concentration at 24 hours. Meanwhile, the blank digesta saw a significant increase at time 6 and 12 hours, and a decrease at 24 hours. All treatments had fairly low levels of this metabolite.

3,4-dihydroxybenzoic acid

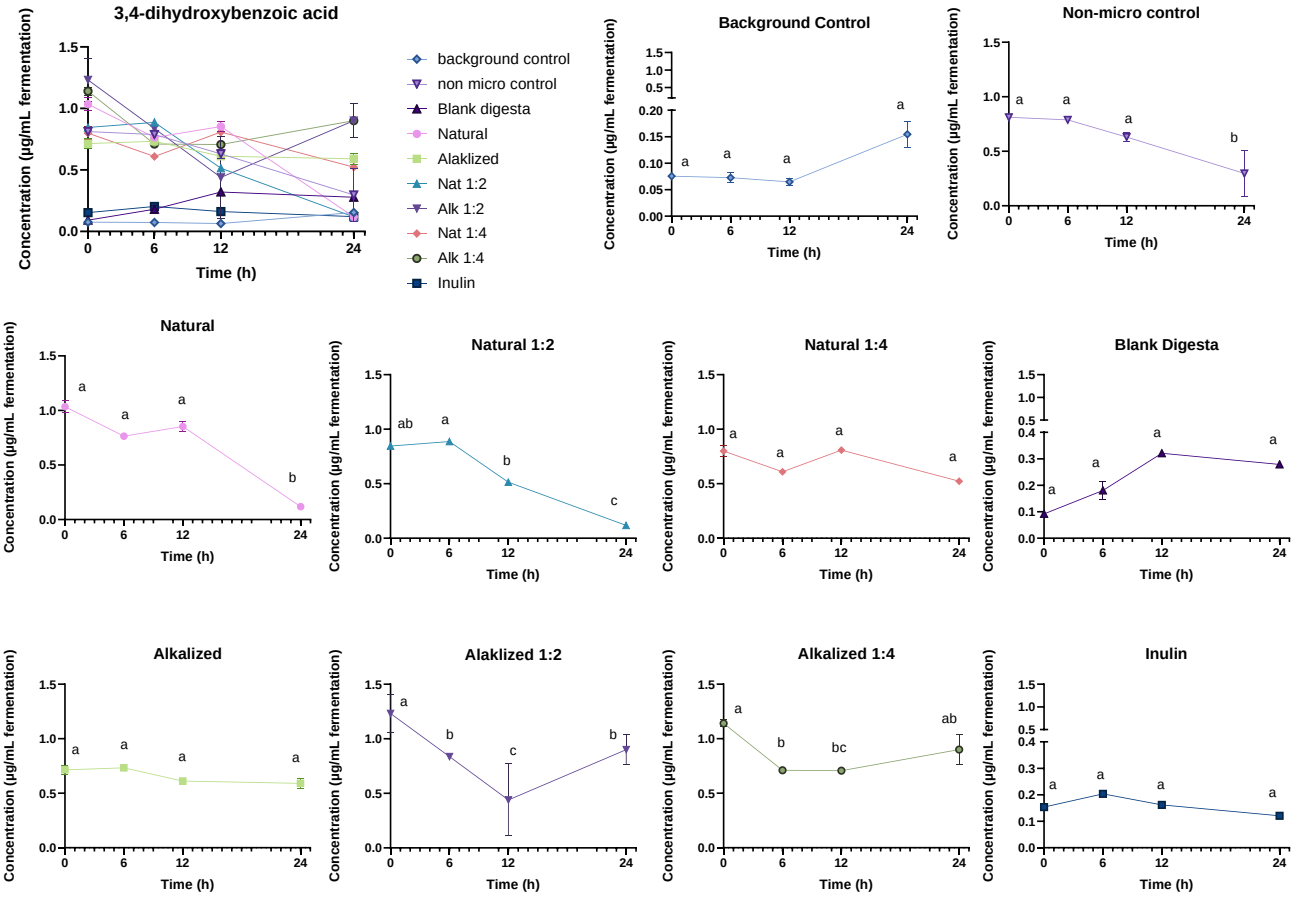


Figure 4.3.2.6. 3,4-dihydroxybenzoic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and

inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

The alkalized 1:2 and alkalized 1:4 samples had the highest concentrations of 3,4-dihydroxybenzoic acid at time 0, then significantly decreased in concentration until 24 hours, where it increased again. The natural, non-micro control, and natural 1:2 treatments all displayed a general decrease throughout fermentation. Inulin, background control, alkalized, and blank digesta all showed no significant changes in concentration of this compounds from times 0-24 hours (**Figure 4.3.2.6**).

Phenylacetic acid

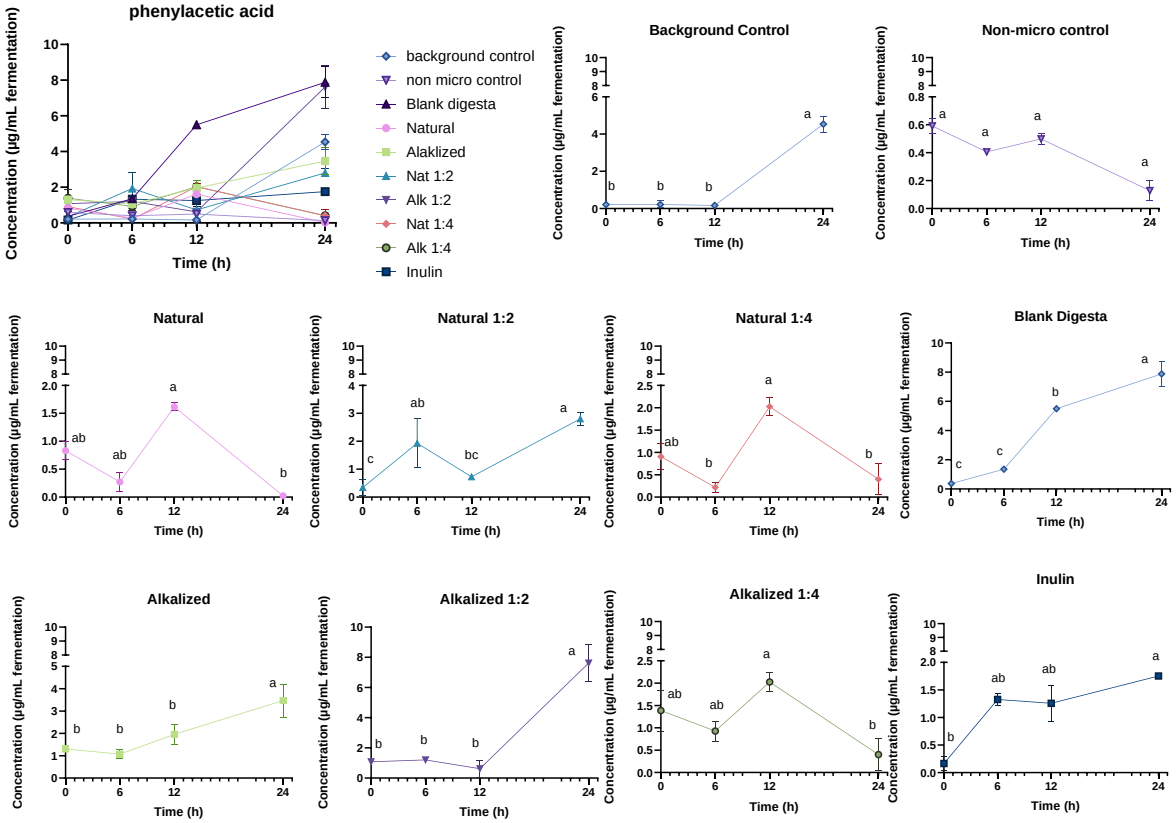


Figure 4.3.2.7. Phenylacetic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin

formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

The blank digesta and alkalized 1:4 samples detected the greatest concentrations of phenylacetic acid at time 24 hours. Both of these samples displayed a fairly low amount of phenylacetic acid at the beginning of fermentation and showed increases later on, at 12 hours for the blank digesta and not until 24 for the alkalized 1:2. The natural powder, natural 1:4 and narual 1:2 powders had the same trends of having the highest concentrations at 12 hours and dropping off at 24 hours. The inulin and background control had the highest amounts at 24 hours. The nonmicro control saw a decrease over time, although the changes were insignificant (**Table 4.3.2.7**). Interestingly, the background control saw a large spike in phenylacetic acid concentration at 24 hours, suggesting that a portion of this metabolite at that time may have been present in the fecal slurry.

3-hydroxyphenylacetic acid

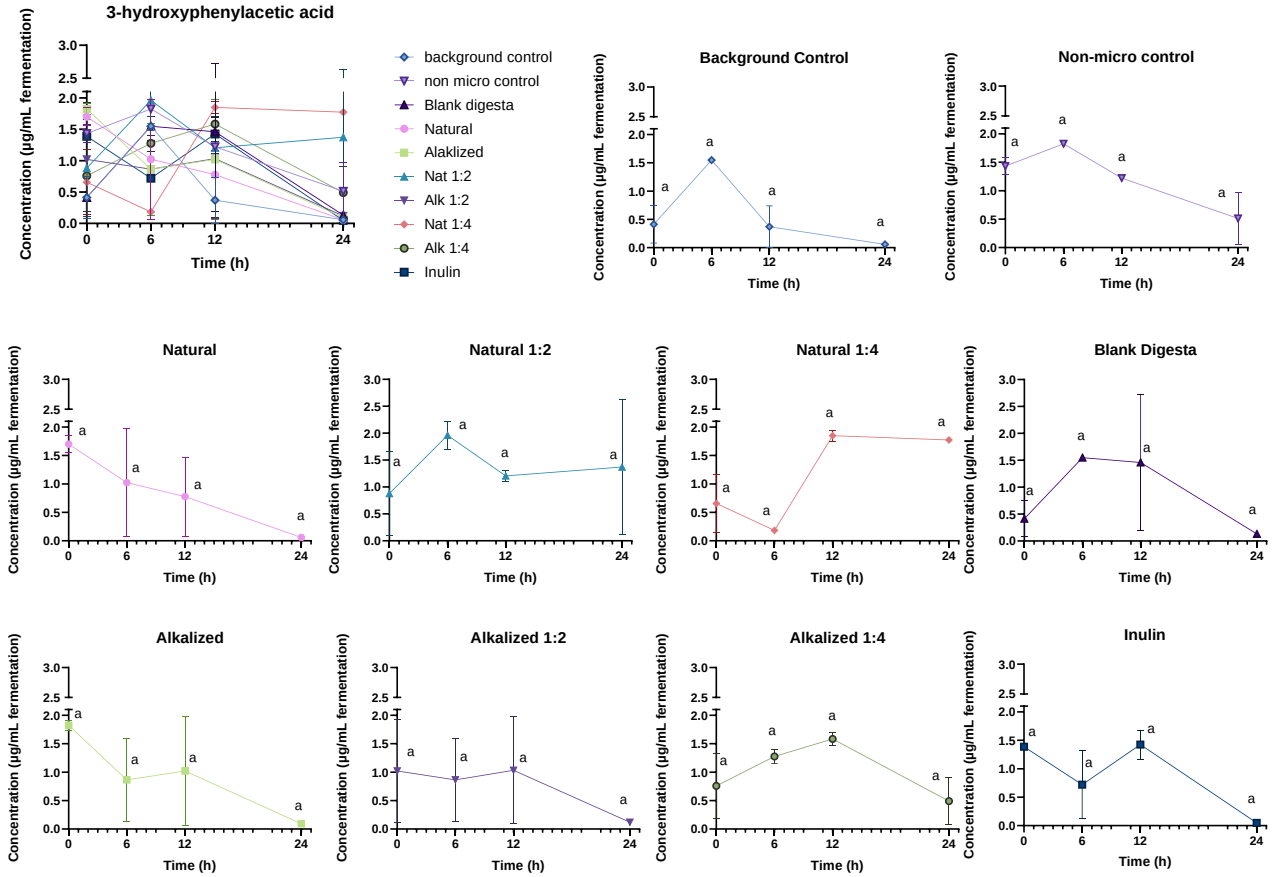


Figure 4.3.2.8. 3-hydroxyphenylacetic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

3-hydroxyphenylacetic acid showed no significant changes over time during fermentation for any treatment, and were detected at similar concentrations in each treatment (**Figure 4.3.2.8**).

4-hydroxyphenylacetic acid

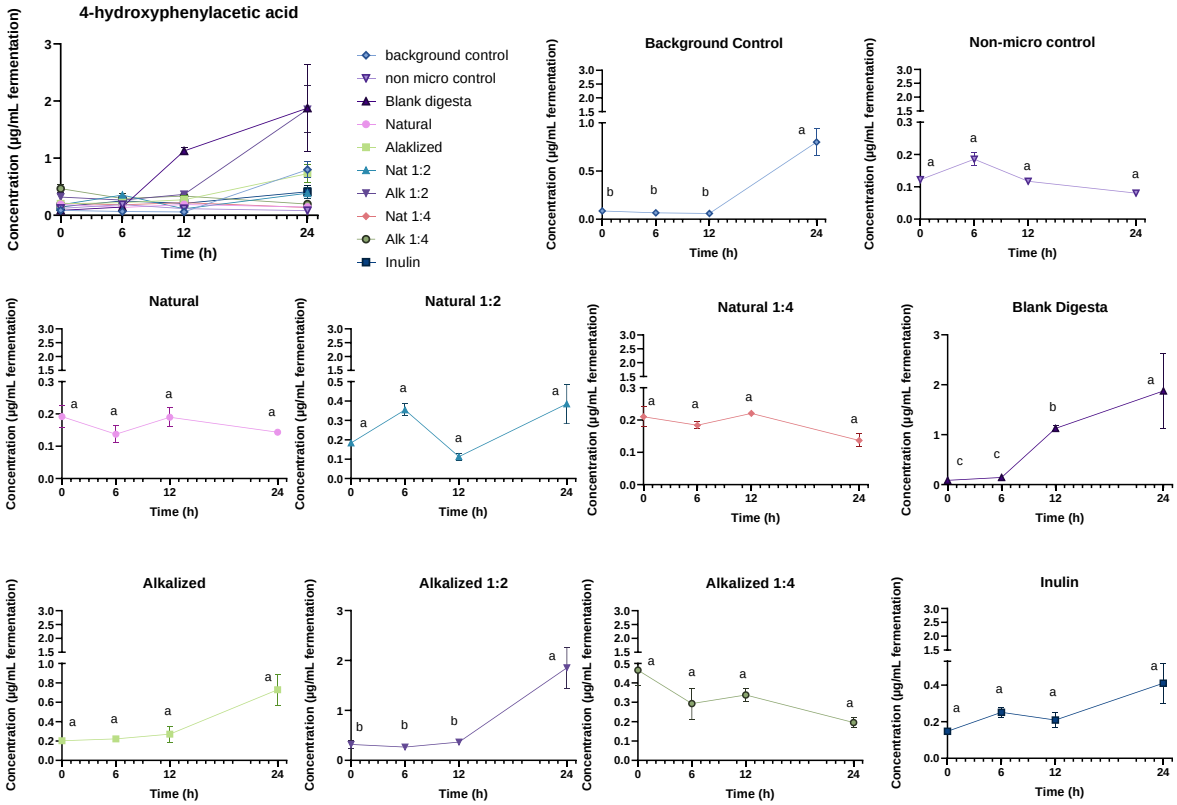


Figure 4.3.2.9. 4-hydroxyphenylacetic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

4-hydroxyphenylacetic acid was detected at the highest amounts in the background control, blank digetsa, and alkalized 1:2 samples at time 24 hours. The blank digesta showed a significant increase in concentration at times 12 and 24 hours, while the alkalized 1:2 and background control only saw significant increases at time 24 hours. All other treatments showed no significant changes in concentration of 4-hydroxyphenylacetic acid over the 24 hour fermentation (**Figure 4.3.2.9**).

3-3,4 dihydroxyphenylacetic acid

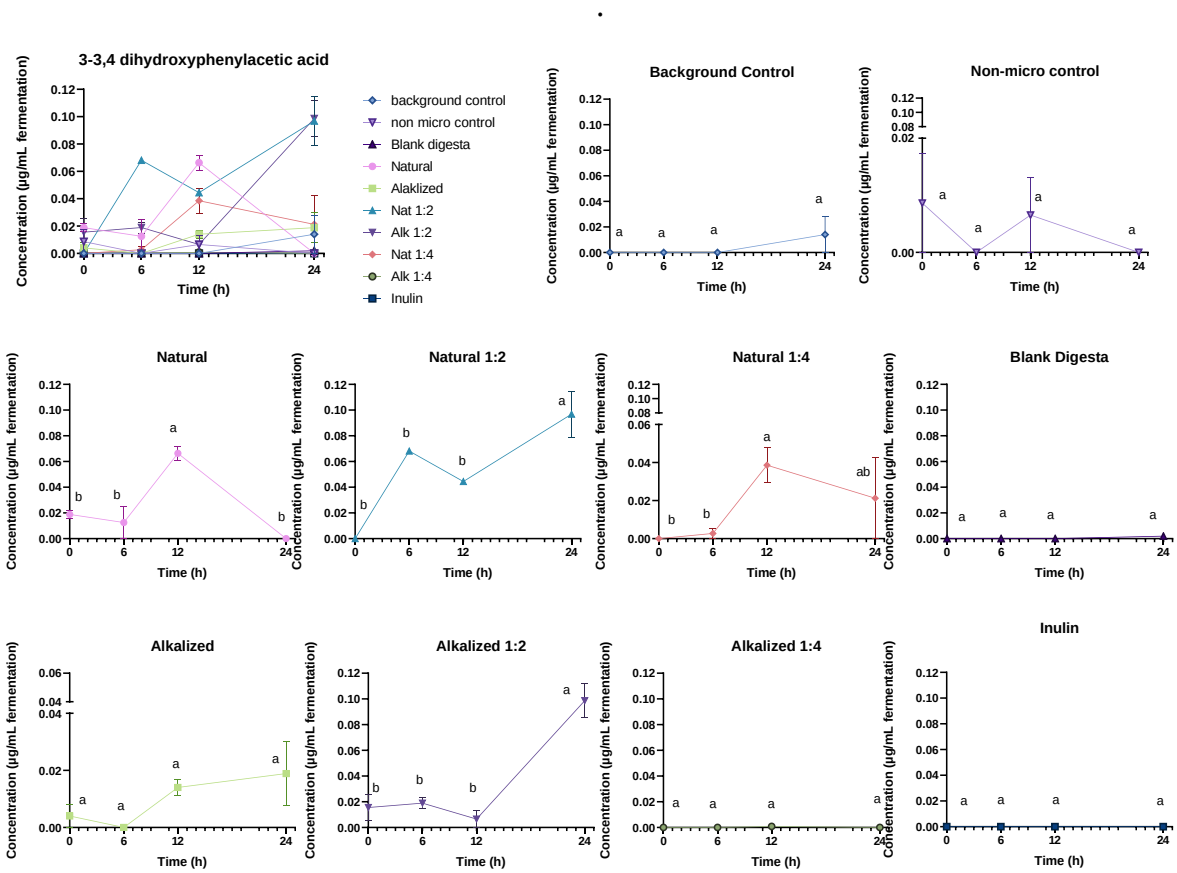


Figure 4.3.2.10. 3-3,4 dihydroxyphenylacetic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

3-3,4 dihydroxyphenylacetic acid was detected in the highest concentrations at time 24 hours in the natural 1:2 samples and the alkalized 1:2 samples. The natural and natural 1:4 powders displayed significant increases of this compound at time 12 hours. No other treatments displayed significant differences in the concentration of 3-3,4 dihydroxyphenylacetic acid throughout the fermentation (**Figure 4.3.2.10**).

4-hydroxymendelic acid

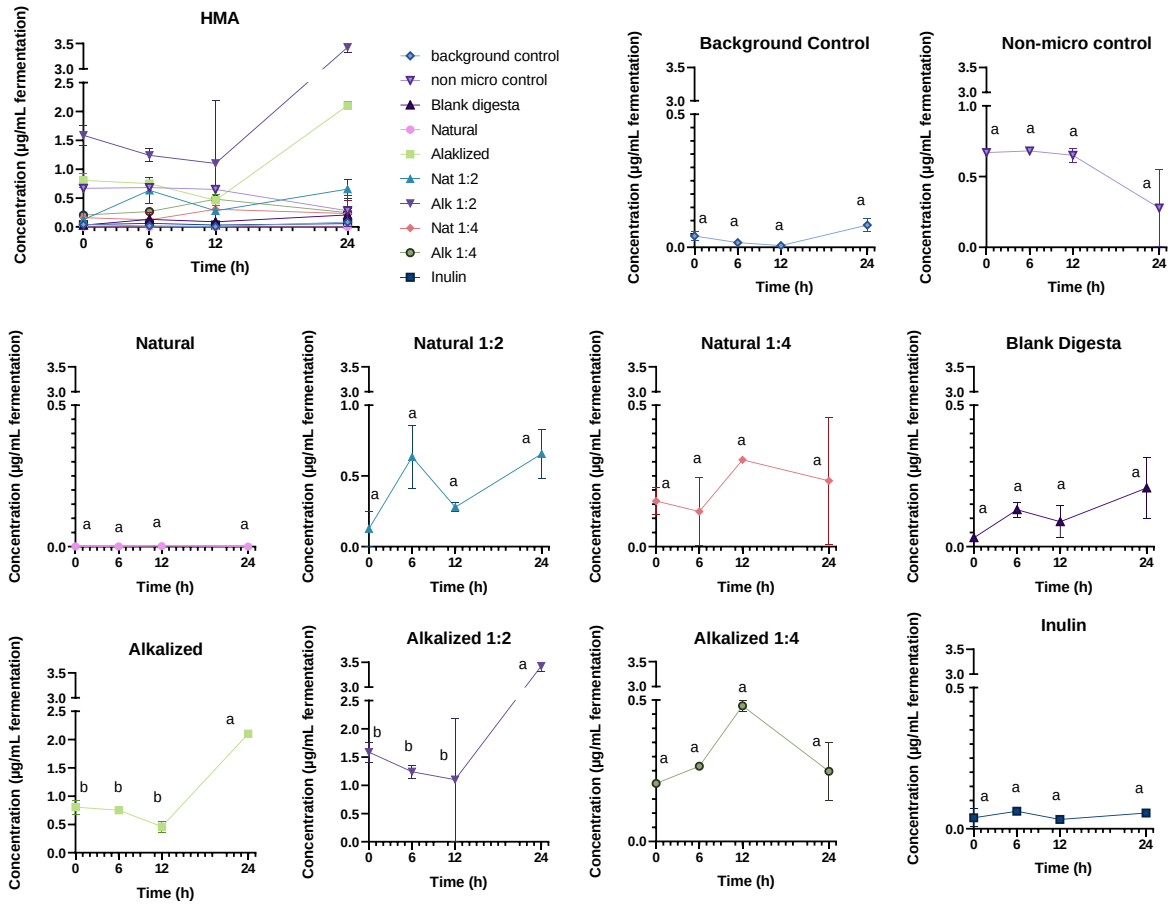


Figure 4.3.2.11. HMA quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

4-hydroxymendelic acid was detected in the highest concentrations in the alkalized 1:2 sample at time 24 hours, followed by the alkalized sample at 24 hours. Both of these samples shown a significant increase in the concentration of HMA at time 24 hours. No other treatments displayed significant changes or trends over time (Figure **4.3.2.11**).

Caffeic acid

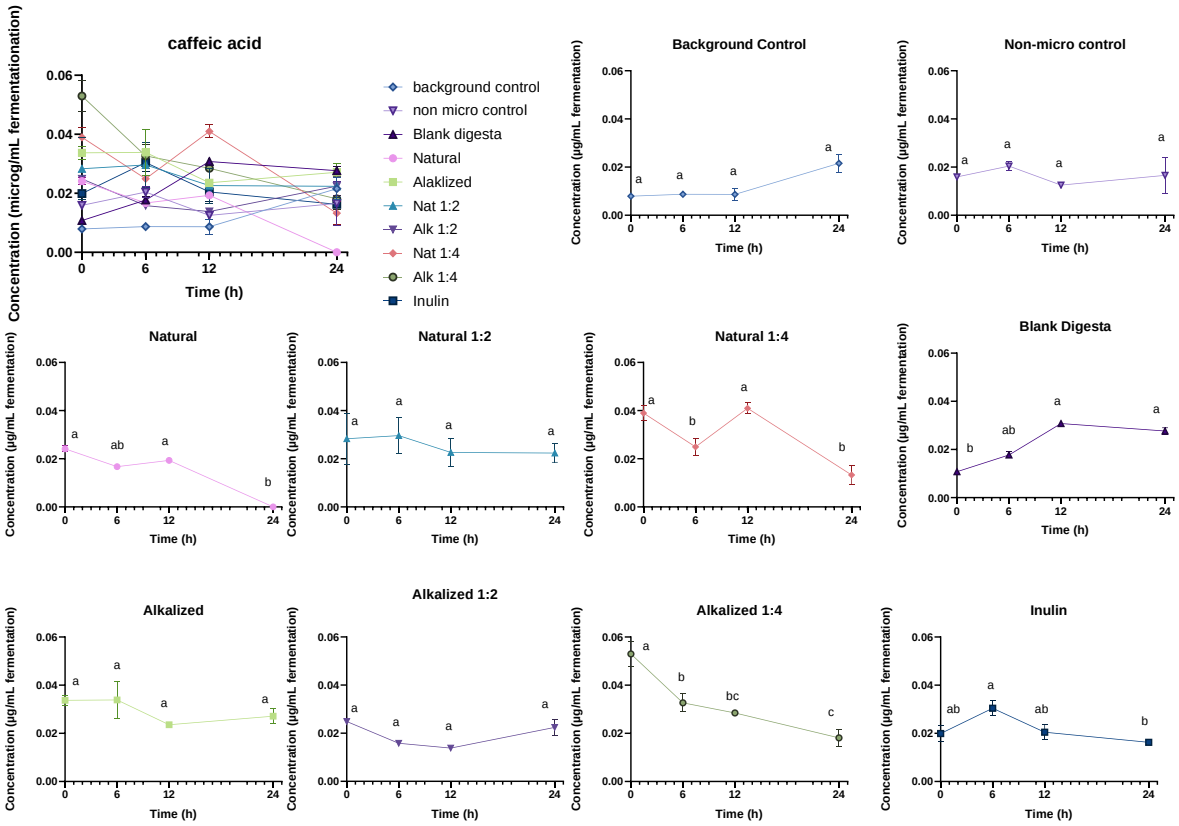


Figure 4.3.2.12. Caffeic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

The concentration of caffeic acid detected was the highest in the alkalized 1:4 treatment at time 0 hours, followed by the natural 1:2 and alkalized samples at 0 hours. The concentration in the alkalized sample stayed fairly consistent throughout fermentation, while other treatments generally decreased in caffeic acid concentration over time (**Figure 4.3.2.12**).

Vanillic acid

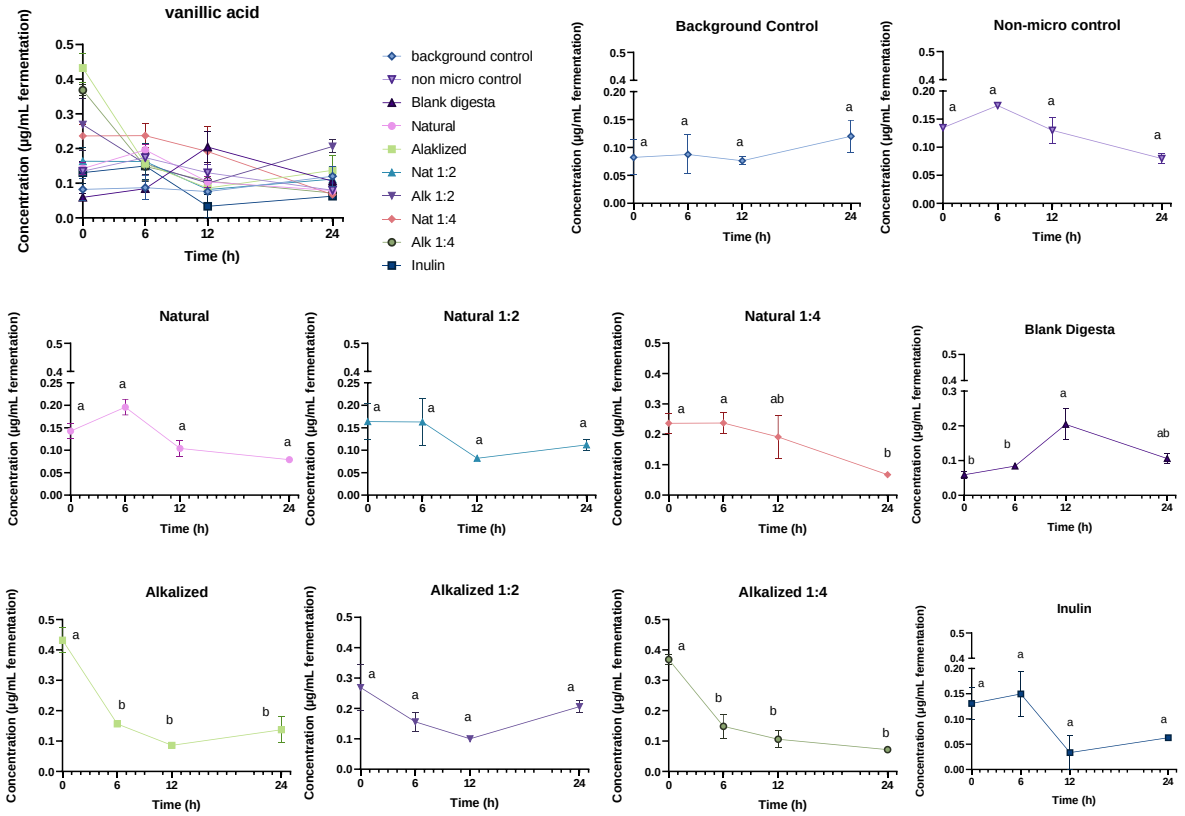


Figure 4.3.2.13. Vanillic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

Vanillic acid was detected in the highest amounts in the alkalized, alkalized 1:4, and alkalized 1:2 samples, respectively, at time 0 hours. The concentrations in these treatments then decreased at 6 hours, and leveled off for the remainder of fermentation (**Figure 4.3.2.13**). The natural 1:4 treatment showed an overall significant decrease over the 24 hour time period, and the blank digesta displayed a significant increase in vanillic acid concentration at time 12 hours. No other significant changes or trends were detected among treatments for vanillic acid.

3-(3-hydroxyphenyl)propionic acid

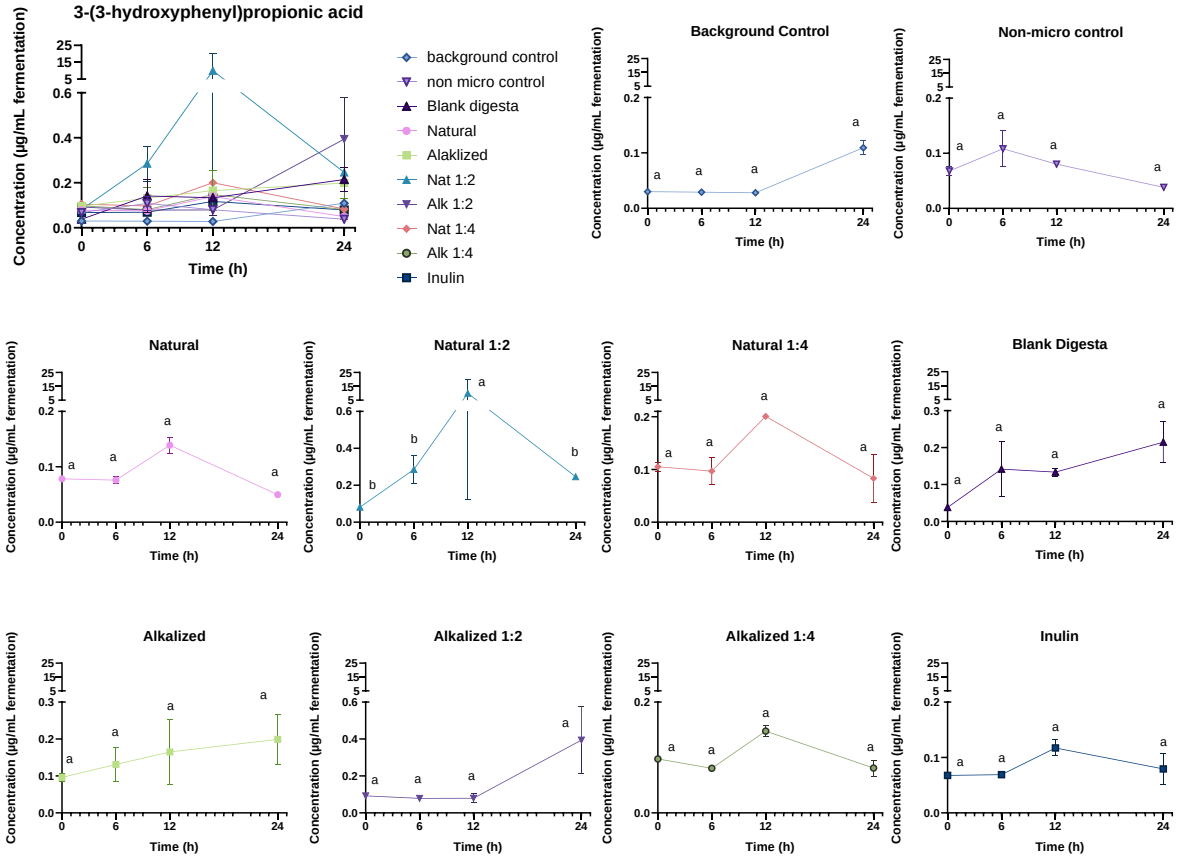


Figure 4.3.2.14. 3-(3-hydroxyphenyl)propionic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

3-(3-hydroxyphenyl)propionic acid displayed significant changes only within the natural 1:2 treatment, which had a significantly large spike in concentration at time 12 hours. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.14)**.

3-(4-hydroxyphenyl)-propionic acid

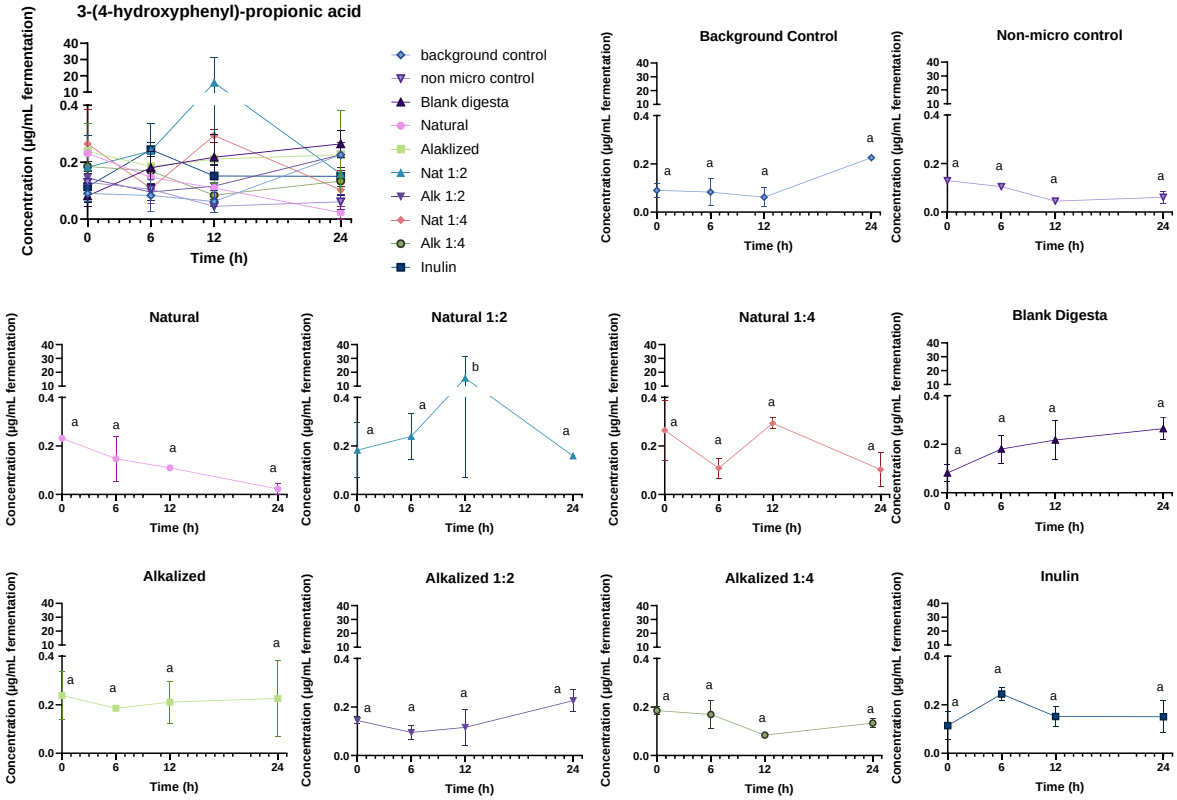


Figure 4.3.2.15. 3-(4-hydroxyphenyl)-propionic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

3-(4-hydroxyphenyl)propionic acid displayed similar trends to 3-(3-hydroxyphenyl)propionic acid. Significant changes occurred only within the natural 1:2 treatment, which had a significantly large spike in concentration at time 12 hours. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.15**).

3-(3,4-dihydroxyphenyl) propionic acid

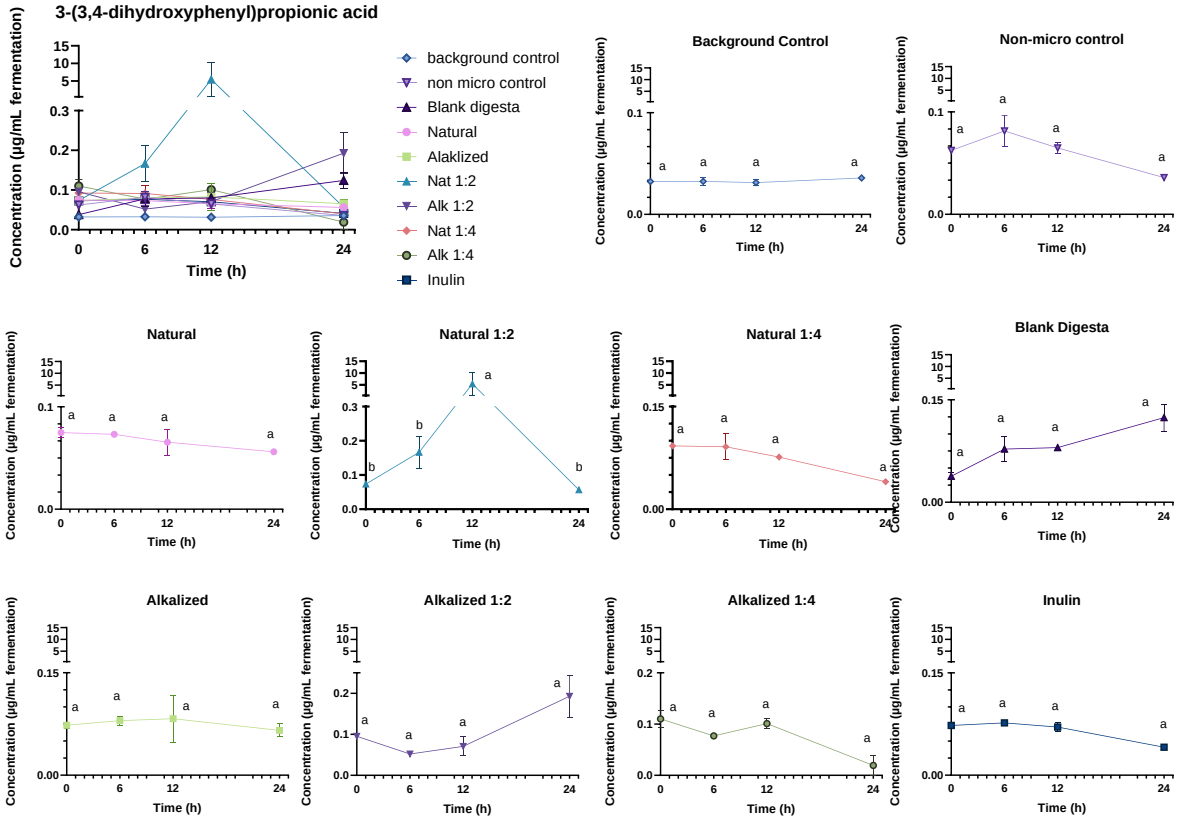


Figure 4.3.2.16. 3-(3,4-dihydroxyphenyl) propionic acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

3-(3,4-dihydroxyphenyl)propionic acid had a similar trend to the other propionic acids. The only significant change was the significantly large increase in concentration detexted at time 12 hours in the Natural 1:2 treatment. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.16**).

5-(3-dihydroxyphenyl)-γ-verolactone

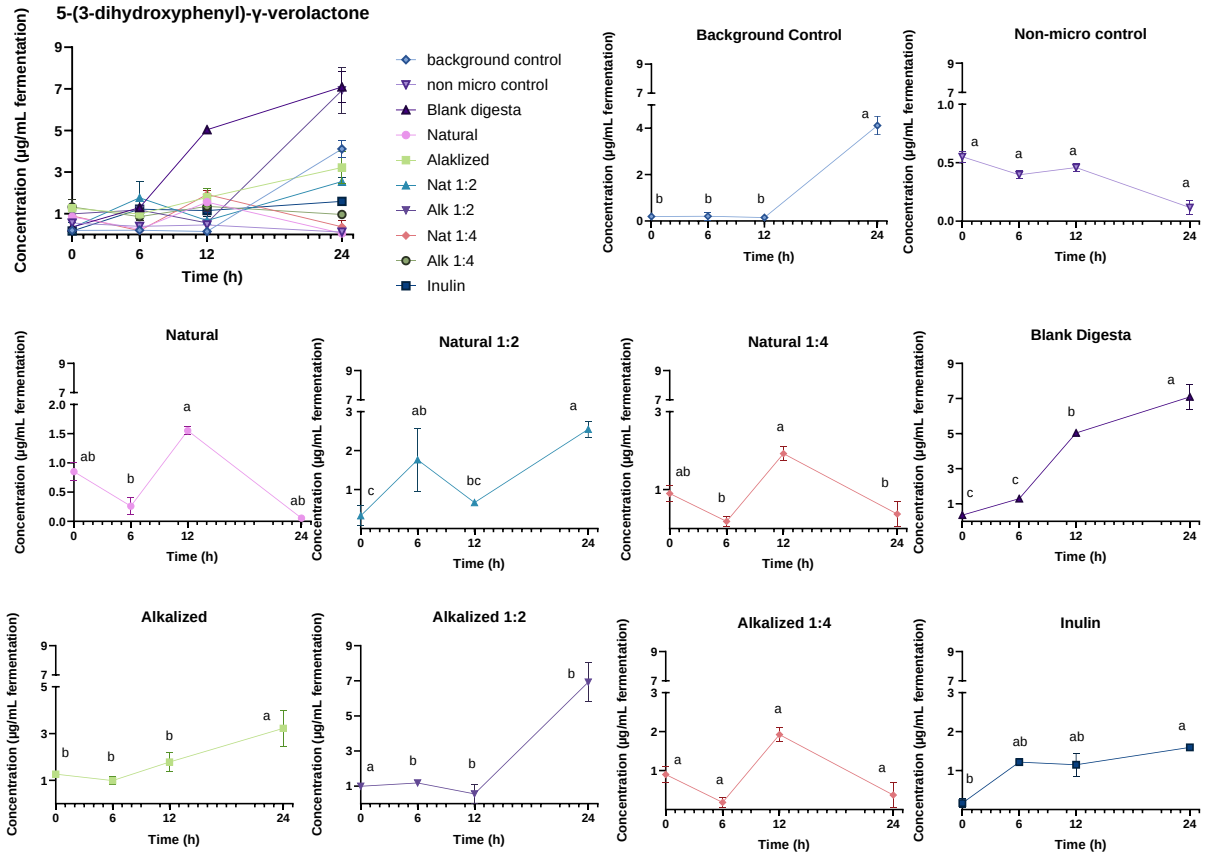


Figure 4.3.2.17 5-(3-dihydroxyphenyl)-γ-verolactone quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

5-(3-dihydroxyphenyl)-γ-verolactone was detected in the highest concentrations at time 24 hours in the alaklzied 1:2 and blank digesta samples. The natural and natural 1:4 samples displayed significant increases in concentration at time 12 hours, while the alkalized and background control treatments shown a significant increase in concentration at 24 hours. The natural 1:2 treatment was the only one to show a significant increase in concentration at 6 hours (**Figure 4.3.2.17).**

5-(3,4-dihydroxyphenyl)-γ-valerolactone

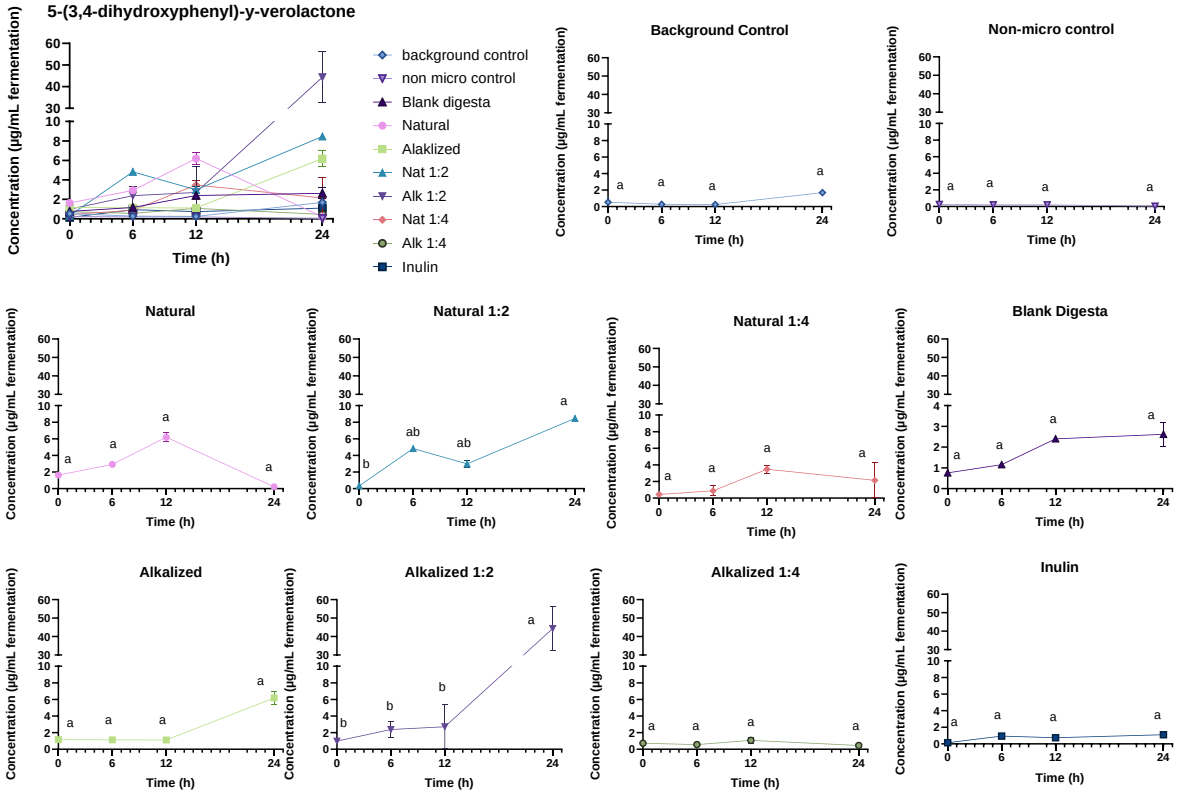


Figure 4.3.2.18. 5-(3,4-dihydroxyphenyl)-γ- verolactone quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

5-(3,4-dihydroxyphenyl)-γ-verolactone was detected in the highest concentration in the alkalized 1:2 sample at time 24 hours, a significant increase from the amount detected in that treatment at all other times. The natural 1:2 sample showed a significant increase in concentration from 0-24 hours. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.18**).

5-(3,4-dihydroxyphenyl)-valeric acid

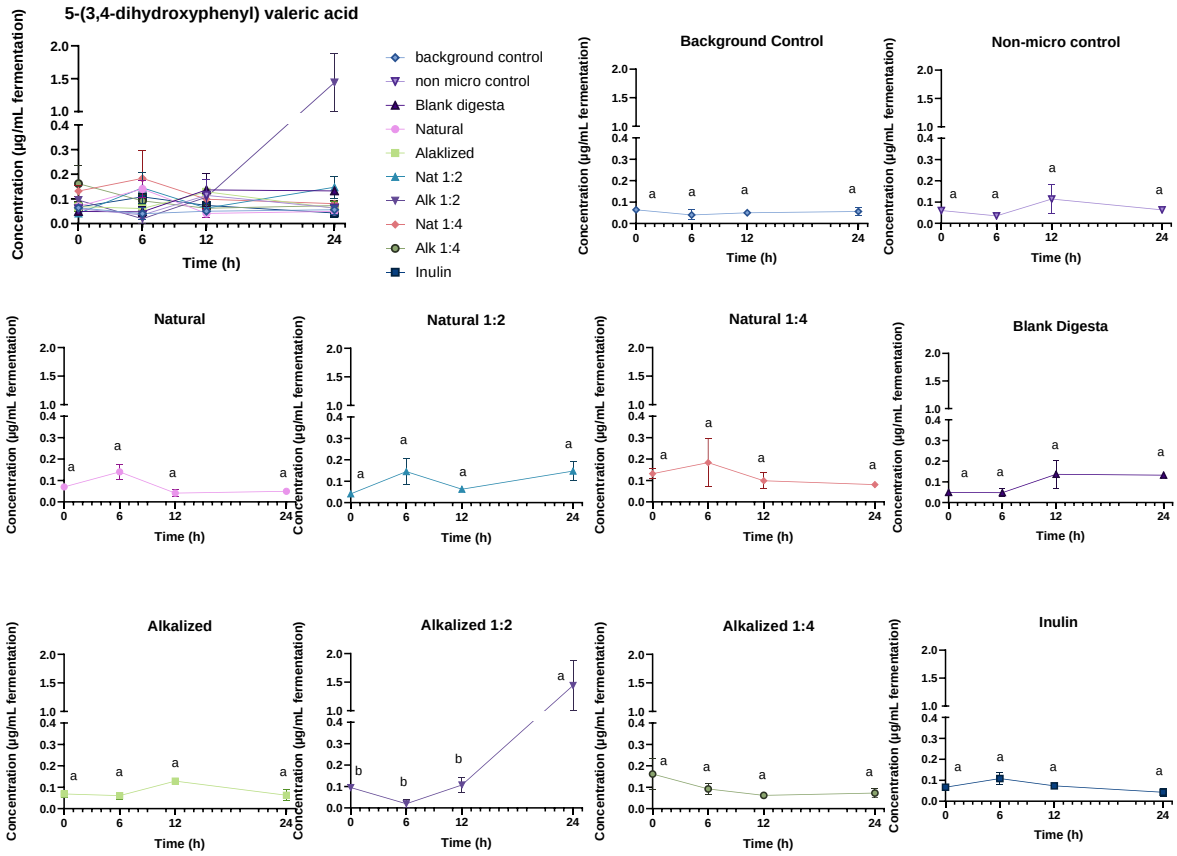


Figure 4.3.2.19. 5-(3,4-dihydroxyphenyl)-valeric acid quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

5-(3,4-dihydroxyphenyl)-valeric acid was seen in the highest concentration in the alkalized 1:2 sample at 24 hours, a significant increase in concentrations seen in this treatment at time 0-12 hours. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.19**).

Hydroxyphenylvaleric acids

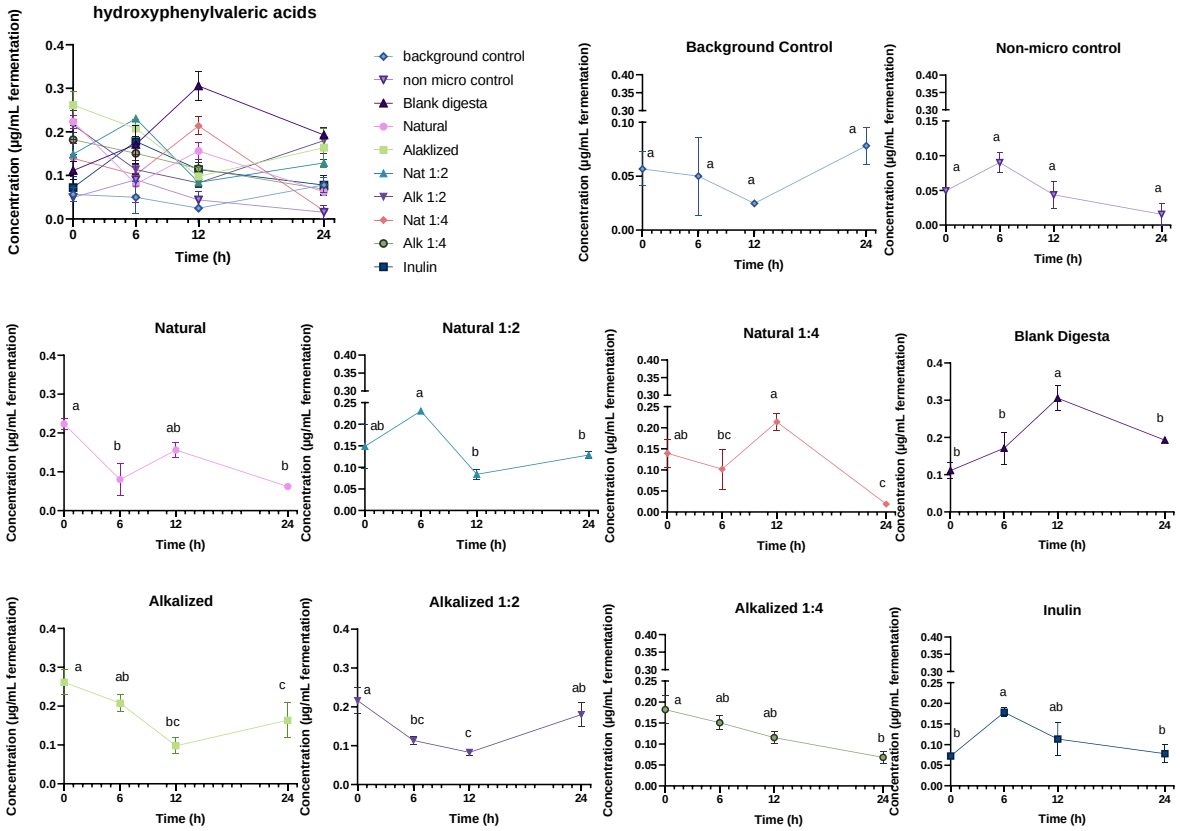


Figure 4.3.2.20. Hydroxyphenyl valeric acids quantified (µg/mL fermentation) via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin, Blank Digesta (product of the blank three stage digestion used as treatment for fermentation), a Non-micro Control (no fecal inoculation added to fermentation), and a Background Control (no treatment, only media and fecal inoculation added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values.

Hydroxyphenylvaleric acids were detected modestly throughout fermentation, but interestingly showed the highest concentrations in the blank digesta treatment at time 12 hours. Concentrations tended to decrease at later times (12 hours, 24 hours) of the fermentation, although these trends were variable among treatments (**Figure 4.3.2.20)**.

5-phenylvaleric acid

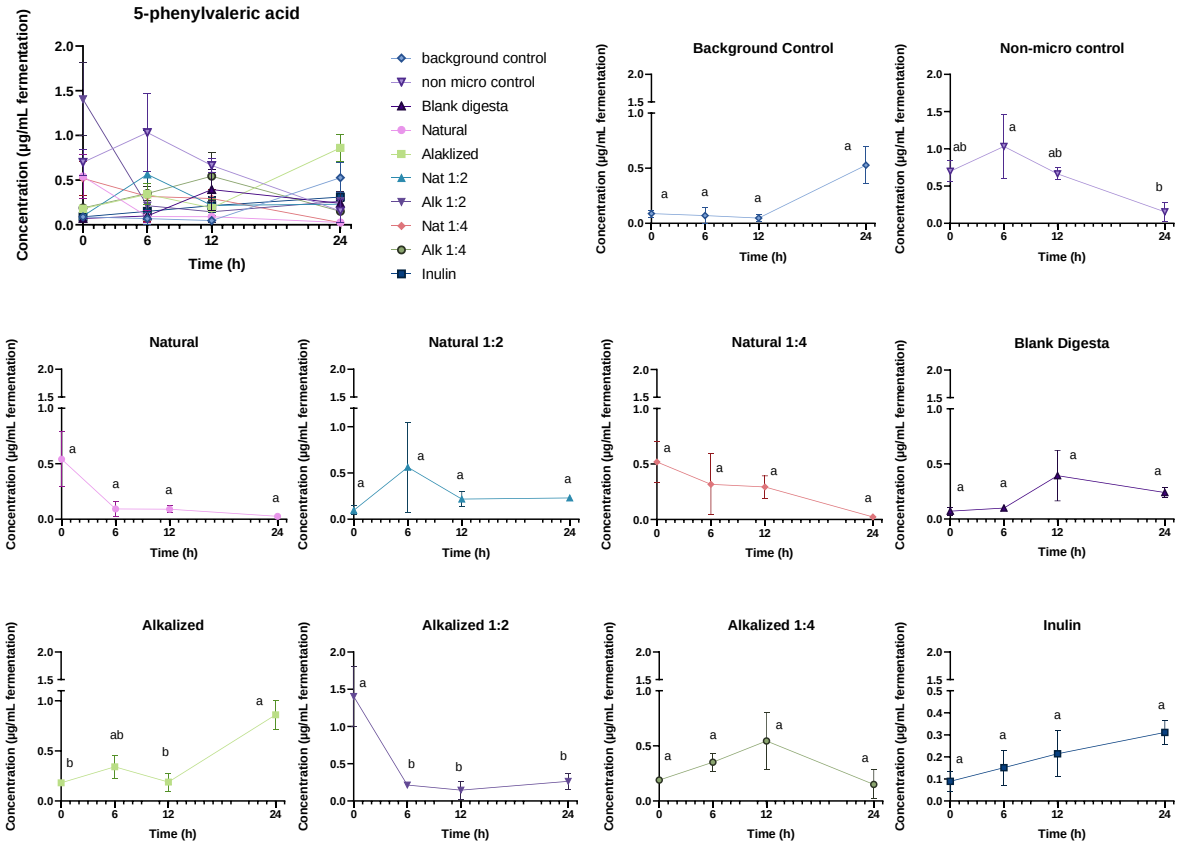


Figure 4.3.2.21. hydroxyphenyl valeric acids quantified via HPLC analysis in each of the fecal fermentation samples taken at time points 0, 6, 12, and 24 hours. Cocoa and inulin formulations shown: Natural, Alkalized, Natural 1:2, Natural 1:4, Alkalized 1:2, Alkalized 1:4, Inulin only, blank digesta (product of the blank three stage digestion used for fermentation), a non-micro control (no fecal inoculation), and a background control (no treatment added to fermentation). All values (n=2) reported as mean ± SEM. Significance between times for each treatment was determined by two-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above treatments indicate statistically different values

5-phenylvaleric acid was found in the highest concentrations in the alkalized 1:2 sample at 0 hours. The alkalized sample saw a significant increase 0 and 12 to 24 hours, while the nonmicro control saw a spike in concentration at time 6 hours, significantly higher than the concentration seen at 24 hours. No other significant changes or trends were detected among other treatments at times 0-24 hours (**Figure 4.3.2.21**).

A few general trends were observed within the fecal fermentation metabolites. One of the most abundant metabolites at the beginning of the fecal fermentation was 3,4-dihydroxybenzoic acid, which was present in all of the natural and all of the alkalized samples, including the

nonmicro control, around 1.0 µg/mL of fermentation. The levels of this compound either remined level or dropped off towards the 24 hour endpoint of the fermentation. Hydroxybenzoic acids tend to be a later metabolite of the colonic digestion of flavanols, so concentrations of these compounds seen at the beginning of fermentation are likely due to their presence in the media or fecal inoculation, as it was detected in both the nonmicro and background controls.

Native flavanols like catechin, epicatechin, and PCB2 were present in the highest levels at the beginning of fermentation, although the absolute values of these metabolites were still fairly low. This is likely explained by the degradation of some of these native compounds throughout the previously performed 3-stage in vitro digestion. Additionally, in the nonmicro controls for all of these native compounds maintained concentration at time 6 hours only in the nonmicro control, suggesting the decreases in concentrations seen at least from time 0-6 hours is likely due to the metabolism of colonic microorganisms. All three native compounds also saw a sharp increase in the natural 1:2 sample at time 6 hours, although the error rates were high. This increase seen could be due to the release of some bound forms of these flavanols or another unexplored mechanism.

Interestingly, the propionic acid metabolites, 3-(4-hydroxyphenyl)-propionic acid, 3-(3,4-hydroxyphenyl)-propionic acid, and 3-(3-hydroxyphenyl)-propionic acid, all saw a spike in concentration at 12 hours for the natural 1:2 samples. No other treatments at any other times had an appreciable amount of these metabolites.

Phenylacetic acid was also one of the most abundant metabolites, but this tended to be later in the fermentation. In the alkalized and natural 1:2 samples, there was a large spike in concentration of phenylacetic acid at 24 hours, while the natural and alkalized 1:4 samples saw a spike at 12 hours and then a drop at 24 hours. Most interesting to note is the highest concentrations of phenylacetic acid was detected in the blank digesta as well as the alkalized 1:2 sample at 24 hours. Another acetic acid metabolite, 4-hydroxyphenylacetic acid, was also seen to be most abundant at 24 hours again in the alkalized 1:2 and blank digesta samples.

The alkalized 1:2 fermentation at 24 hours was shown to be very abundant in 5-(3-dihydroxyphenyl)-γ-verolactone abundant, 5-(3-dihydroxyphenyl)-γ-verolactone, and 5-(3,4-dihydroxyphenyl) valeric acid. These metabolites all tended to increase in concentration as fermentation went on.

The effects of alkalizing and/or adding inulin to cocoa on its microbial metabolism has not been previously investigated to our best knowledge. Future endeavors should include more replications and higher doses to determine what influence these factors may have.

4.4 GUT ACTIVITY ASSAYS

In order to investigate cocoa's bioactivity in the lower gut, several *in vitro* assays were performed assessing enzyme inhibition and inflammatory activity.

4.4.1 PLA_2 Enzyme Inhibition

The PLA_2 assay measures the activity of the enzyme phospholipase A_2, an enzyme involved in phospholipase digestion and cell signaling associated with inflammatory response, namely eicosanoids. High levels of PLA_2 are present in those with obesity and the metabolic syndrome and have long been associated with increase in intestinal inflammation and atherosclerosis [72]. Inhibition of this enzyme has been investigated as an intervention in inflammatory related disease like IBD [73]. Lower activities of this enzyme activity detected in this assay indicate an inhibitory effect of that treatment.

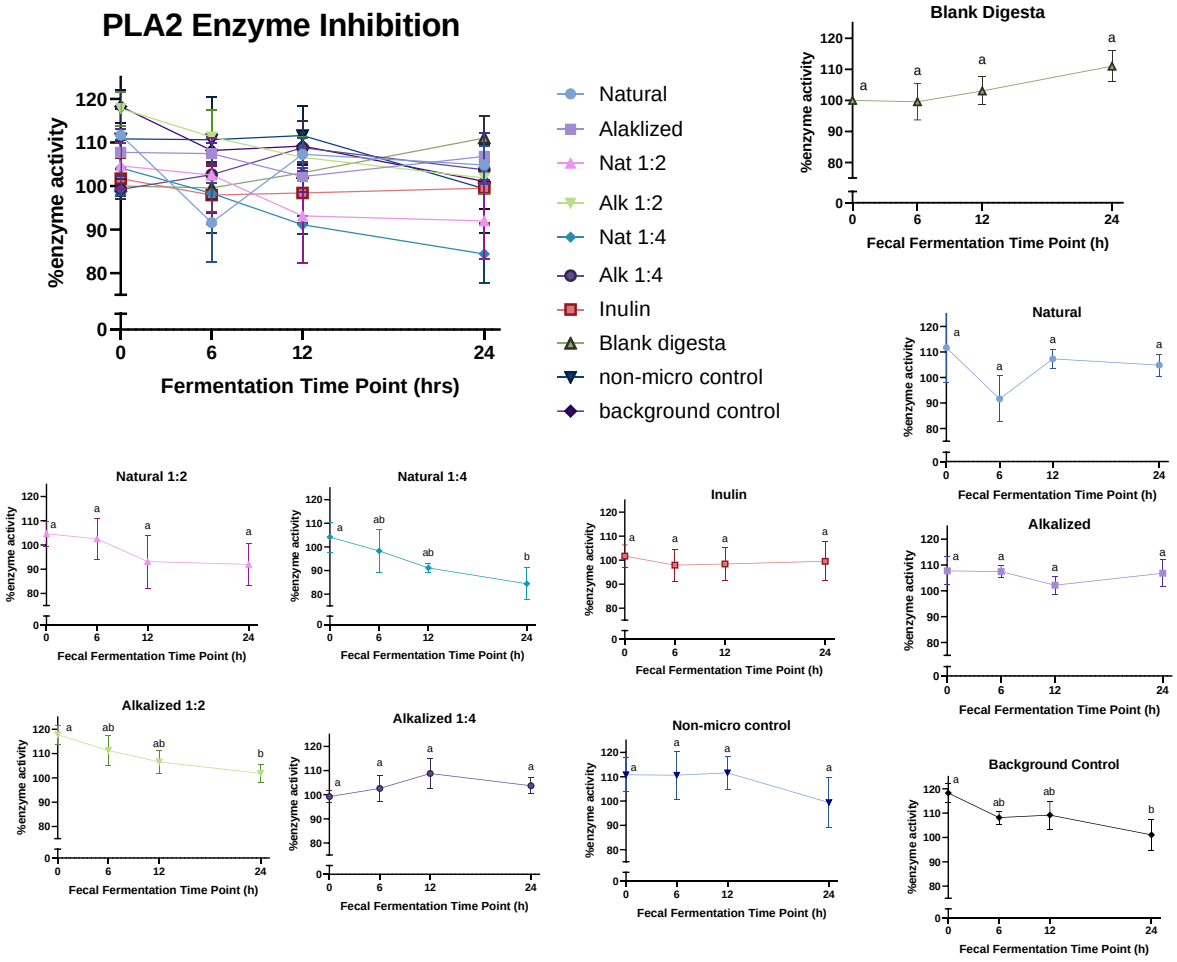


Figure 4.4.1.1 PLA2 % Enzyme Activity determined by ELISA of all treatments sampled from times 0, 6, 12, and 24 hr of fecal fermentation. The % enzyme activities of PLA2 are expressed as mean ± SEM (n=3). Values were normalized to the blank digesta at time 0 (100%). Significance between times was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$). Different letters above times indicate statistically different values.

In general, later fermentation mixtures seem to be better inhibitors of this enzyme, but the blank digesta shows the opposite trend over the 24 hours. This suggests that our treatments may have had even more of an effect than what is displayed because they were combating this trend from the background digestion reagents. It is important to note that pancreatin and lipase were added during the *in vitro* digestion of the samples and caused some of the samples to elicit >100% enzyme activity at some time points.

Natural powder was more inhibitory than alkalized in most cases. Alkalized, natural 1:2, alkalized 1:4, inulin, blank digesta, and nonmicro control did not have any significant

differences. Alkalized1:2, natural 1:4, and background control had significant differences only between 0h and 24 h samples.

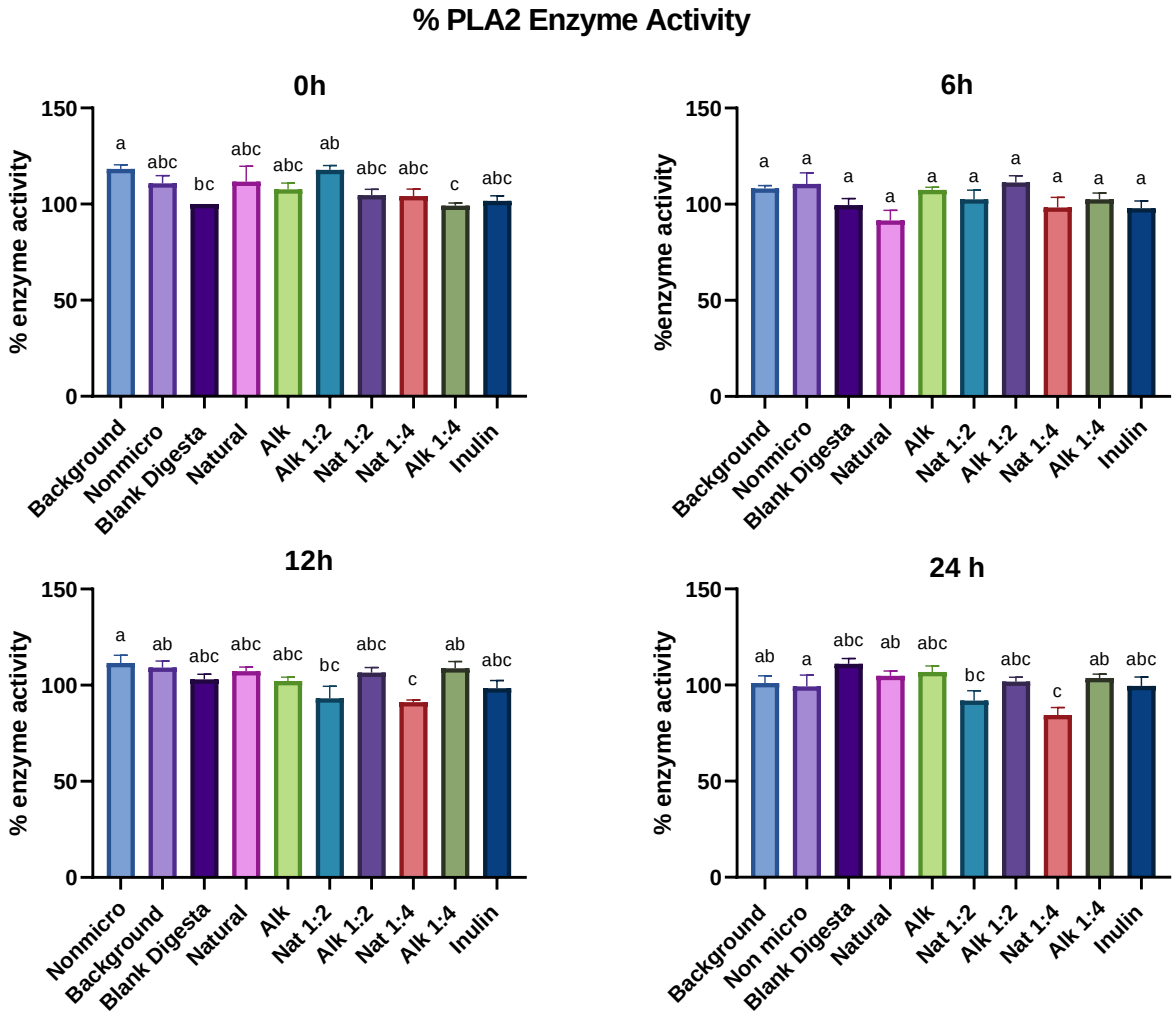


Figure 4.4.1.2 PLA2 % Enzyme Activity determined by ELISA of all treatments sampled from times 0, 6, 12, and 24 hr of fecal fermentation. The % enzyme activities of PLA2 are expressed as mean ± SEM (n=3). Values were normalized to the blank digesta at time 0 (100%). Significance between treatments at times 0, 6, 12, and 24 hours was determined by one-way ANOVA and Tukey's HSD post-hoc test ($p<0.05$).

PLA2 enzyme activity was over 100% for all treatments at 0 hours, except Alkalized 1:4 which had a mean of 99.2% enzyme activity. None of the treatments were significantly different from each other in influencing PLA2 enzyme activity at 6 hours. At 12 and 24 hours, the inulin,

Natural 1:2, and Natural 1:4 treatments displayed the lowest enzyme activities, and therefore the greatest enzyme inhibition.

4.4.2 Gut Inflammation (IL-8 Cytokine Expression)

IL-8 is a cytokine produced by many different types of cells that lead to an inflammatory response and has been reported to promote tumor growth in colon cancer cells [74]. Patients with IBD present elevated levels of IL-8 in their colonic mucosa [75]. IL-8 expression in HT-29 colon cancer cells after stimulation with TNF-α following pre-treatment with cocoa and inulin fermentation products for 36 hours were measured to determine the gut anti-inflammatory effects of cocoa, inulin, and cocoa/inulin mixes. Lower levels of IL-8 expression indicate less inflammation, and potential anti-inflammatory effects of the treatment.

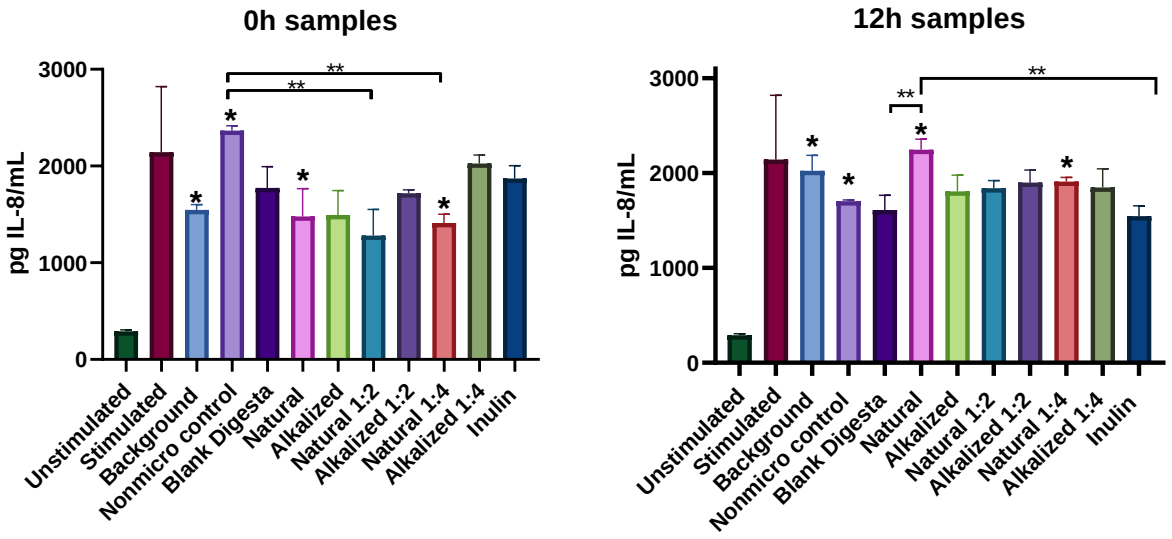


Figure 4.4.2 IL-8 measured via ELISA after pretreating HT-29 cells for 36 hours with samples taken from fermentation at times 0 and 12 hours for all treatments, followed by s hour treatment with TNF-α. All values reported in mean ± SEM (n=3) and were normalized to the unstimulated well. Significance between treatments at times 0 and 12 hours, were determined by a 1-way ANOVA and Tukey's post-hoc test ($p<0.10$) Significance between times 0 and 12 hours for each treatment were determined by individual T-tests ($p<0.10$) above bars indicate statistically different values between (0 vs. 12 h) times within treatment.**over bars represents significant difference between treatments within that time point.*

Interestingly, the background control, containing only the media and fecal inoculation, showed significantly increased IL-8 levels from 0-12 hours, suggesting that things present in the media or fecal slurry could be affecting the results from this assay. Non micro control,

containing only the fermentation media and natural powder digesta, had the highest IL-8 levels at 0 hours of all treatments, which decreased significantly at 12 hours. This suggest that chemical degradation of these compounds (as opposed to microbial degradation) may actually improve the anti-inflammatory effects of cocoa.

The natural, natural 1:2, and natural 1:4 treated cells all saw increases in IL-8 levels from 0-12 hours, suggesting that cocoa and cocoa/inulin mixes are better inhibitors of inflammation at 0 hours colonic fermentation vs 12 hours fermentation. These data suggest that colonic fermentation does not increase the bioactivity of cocoa / cocoa and inulin in vitro over time, but rather it appears to worsen it.

Within the 0 hour fermentation samples, the natural, and natural 1:4 treatments had significantly lower levels of IL-8 compared to the nonmicro control. The natural 1:2 also followed this trend, although the change was not statistically significant. Within the 12 hour fermentation samples, the blank digesta and inulin samples had significantly lower levels of IL-8 than the natural sample (**Figure 4.4.2**).

4.5 DISCUSSION

Overall effect of Alkalization

Alkalization of cocoa powder has major impacts on its phytochemical composition. In this study, the total polyphenol content of the alkalized powder was about of 24.7% of the total polyphenol content in the natural powder. Additionally, we demonstrated that the alkalized powder had about 28.75% of the total flavanols. These results were expected, as it has been well established that alkali processing of cocoa results in lower (up to 90%) total flavanol and total polyphenol concentrations [11,12].

Alkalization had a large impact on flavanol mDP, demonstrated by the thiolysis assay, where the mDP both including and excluding monomers was significantly lowered in the alkalized powder compared to the natural powder. The HILIC assay, assessing procyanidins, also showed a significantly lower level of flavanols of every size for the alkalized powder. This data suggests that alkalization of cocoa has a greater effect in decreasing concentrations of procyanidins vs monomeric cocoa flavanol compounds. It has been reported in a US patent that one alkalized cocoa powder had no detectable flavanols >DP5 and had significantly less overall

procyanidins and total polyphenols compared to other natural powders, although the origins and other processing parameters were not specified [11]. Miller *et al* analyzed 20 different commercial cocoa powders obtained from 5 different manufacturers, showed a trend of decreasing total flavanol concentration with increasing degree of alkalization (increased pH). However, in that analysis, it was found that flavanols of every size (DP 1-10) decrease in concentration proportionately to the level of processing (i.e. more processing, less polyphenols) [11]. This is contradictory to our findings that suggest the dutching process may have a larger impact on procyanidin levels compared to smaller flavanols. It is important to note that polyphenol composition also greatly affected by growing conditions, cultivar, and other processing parameters, adding an additional challenge to the comparison of this and all other cocoa characterization data.

Alkalization did not seem to have a large impact on the pH changes during fecal fermentation. It has been shown before in an *in vitro* fecal fermentation of both alkalized and natural cocoas, the pH of the alkalized cocoas did not decrease to the same degree as the pH of natural cocoas throughout the 24 hours [54]. In our fermentation, the pH changes of the alkalized powder were not significantly lower or higher than the pH changes seen in the natural powder.

Alkalized treatments did not have a significantly different PLA_2 inhibitory activity compared to natural powder treatments. These results contrast with what has been previously reported about alkalized cocoa having a lower inhibitory activity compared to natural cocoa for the PLA_2 enzyme [64]. The same study also demonstrated that larger cocoa procyanidins have more inhibitory activity compared to smaller flavanols. Our analysis of the procyanidins and mDP in our cocoa powders showed that our alkalized powder not only had lower total flavanols but to an even greater degree, lower procyanidin concentration compared to the natural powder. With lower levels of large procyanidins (and all flavanols in general), we would have expected PLA2 enzyme activity to be worsened in the alkalized powder, but this was not seen in the present study.

Finally, alkalization had no effect on the IL-8 assay at 12 hours, implying that it does not have an overall effect on the anti-inflammatory effects of cocoa. It has been shown before that larger cocoa procyanidins are the most effective at reducing IL-8 concentrations in vitro compared to smaller procyanidins, so we would’ve expected our natural powder to be a better inhibitor of cytokine expression [7].

Overall effect of adding inulin

Inulin showed no or a negligible response in the Folin, DMAC, and thiolysis assays, measuring total polyphenols, total flavanols, and degree of polymerization, respectively. These results were expected, as inulin does not contain any polyphenolic compounds.

Inulin seemed to have an impact on fecal fermentation pH, as all treatments containing inulin (except the alkalized 1:2 treatment) had a larger drop in pH over 24 hours compared to the samples without inulin. This drop is likely explained by the formation of SCFAs, as inulin is a known prebiotic and creates these when fermented by colonic bacteria [51]. This drop in pH could have implications regarding the stability of flavanol compounds, as it has been demonstrated that flavan-3-ols are generally less stable at alkaline pHs. The lower pH may also have positive implications for gut health. Lowering the pH of the gut lumen has shown to elicit a change in microbiota composition and can help prevent overgrowth of pH-sensitive pathogenic bacteria like *Enterobacteriaceae* and *Clostridia* [76].

For the PLA2 assay, at 12 and 24 hours, the inulin, natural 1:2, and natural 1:4 treatments displayed the lowest enzyme activities, and therefore the greatest enzyme inhibition, suggesting that inulin and the combination of natural cocoa and inulin may play a role in the inhibition of this enzyme. This inhibition is likely not due only to the drop in pH, as the alkalized 1:4 sample, which had the lowest overall pH at 24 hour fermentation, did not have as much of an inhibitory effect on PLA2 as the natural powders with inulin or inulin alone.

Inulin treated fermentation samples did not significantly influence the IL-8 response from TNF-α stimulated HT-29 colon cancer cells. At 0 hours, most cocoa treatments containing inulin are lower than the treatments alone and at 12 hours, and the inulin only treatment displays the lowest levels of IL-8. These results are somewhat surprising, as other prebiotics have shown to decrease pro-inflammatory cytokines, including IL-8, in both *in vivo* and *in vitro* models. Additionally, inulin specifically has shown to decrease the concentration of pro-inflammatory cytokines *in vivo* due to interactions with G-protein coupled receptors [77]. In rats, inulin dietary intervention repressed the synthesis of tissue IL-8 following an acute induction of colitis [78]. We would have expected inulin to significantly lower IL-8 levels in every treatment that contains it, but this is not what was observed in the present experiment. The interpretation of our IL-8

data are complicated by the fact that the nonmicro control significantly decreased IL-8 levels from 0-12 hours while the background control significantly increased IL-8 expression.

Effects of Colonic Fermentation

Interpreting the effects of colonic fermentation on the bioactivity of cocoa is complicated by the fact that gut microbiomes vary among individuals. Although we used a pool fecal sample, which eliminates some of the issue of heterogenicity among individuals, it does not eliminate it completely. In the IL-8 assay, 0h samples of every treatment seem to be better inhibitors of inflammation (or of IL-8 expression) vs. 12-hour samples, particularly for those containing natural cocoa. These data would suggest that native cocoa or earlier metabolites are superior for affecting gut tissue, which then suggests upper regions of the gut are more protected (small intestine, ascending colon) than the lower gut. Conversely, later samples (12 and 24 hours) seem to be better inhibitors of the PLA_2 enzyme compared to earlier samples (0 and 12 hours), but these effects were not significant for many of the treatments. It is still unknown for these endpoints whether protecting native flavanol compounds from microbial digestion may be more advantageous for bioactivity throughout the colon, rather than promoting fermentation and the production of microbial metabolites.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Overall, our data suggests that neither alkalization nor the addition of inulin to cocoa have universal effects on cocoa's bioactivity in the gut, although differences were observed in specific assays. This study was novel in several ways. First, the majority of studies investigating flavanol bioactivity utilize pure extracts as treatments, while our study attempted to create a more physiologically relevant product of *in vivo* digestion and colonic fermentation using *in vitro* simulations. Additionally, no other studies have explored the addition of inulin to cocoa. The use of these digestion and fermentation products in biological assays is not common, and in the future, experiments should be optimized for the use of these products rather than extracts to reduce "noise" seen in data.

Although the novelty of this project is well-demonstrated, it was not without limitations. This was a pilot study, in which only 1 fermentation trial was performed, and 1 dose was used for the PLA2 enzyme inhibition and IL-8 cell culture assays. These were fairly low doses based off of pure cocoa extract used in previous experiments. Future work should reduce the study to fewer treatments and use higher or multiple different doses, while still employing physiologically relevant levels. Additionally, while *in vitro* models of digestion and fermentation allow tight control of experimental parameters, they do not account for all of the variables present in true, real-world human digestion. The digestion model we chose to use models the bioaccessability of polyphenols, but there are other *in vitro* models we could have employed. In the future, it may be beneficial to optimize this model to digest the specific macronutrients in cocoa, which could affect our outcomes. *In vivo* models would produce valuable information on this topic and can account for things like nutrient and water absorption. Changes in the microbiome, SCFA production, tight-junction protein expression, endotoxemia, intestinal wall damage, and barrier function should all be assessed.

The specific compounds and mechanisms by which cocoa reduces inflammation in the gut and other tissues, affects the intestinal barrier, and inhibits digestive enzymes should also be investigated. Are cocoa flavanols responsible for bioactivity in the gut, or are its prebiotic effects and SCFA formation what is providing these health effects? Additionally, more work is needed to determine pathology of IBD and related conditions in order to develop a well-targeted and effective treatment. While this current work does not clearly indicate an added benefit of adding

inulin to cocoa, above and beyond lowering the pH of the colon, it certainly shows promising evidence for each substance's individual ability to influence enzyme activity and inflammation *in vitro* and that their combination does affect bioactivity, but it is still unclear exactly how and why [79,80]. The potential for cocoa and inulin to play roles in the mitigation of disease is clear. Further investigation is needed to determine the mechanisms of action of cocoa, relationship between size and bioactivity, interactions with macromolecules like fiber, and other unknowns.

References

[1] Nishida A, Inoue R, Inatomi O, Bamba S, Naito Y, Andoh A. Gut microbiota in the pathogenesis of inflammatory bowel disease. Clin J Gastroenterol 2018;11:1–10. https://doi.org/10.1007/s12328-017-0813-5.

[2] Zhang YZ, Li YY. Inflammatory bowel disease: Pathogenesis. World J Gastroenterol 2014;20:91–9. https://doi.org/10.3748/wjg.v20.i1.91.

[3] Saltiel AR, Olefsky JM, Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease Find the latest version : Inflammatory mechanisms linking obesity and metabolic disease. J Clin Invest 2017;127:1–4. https://doi.org/10.1172/JCI92035.systems.

[4] Strat KM, Rowley Iv TJ, Smithson AT, Tessem JS, Hulver MW, Liu D, et al. REVIEWS: CURRENT TOPICS Mechanisms by which cocoa flavanols improve metabolic syndrome and related disorders ☆. J Nutr Biochem 2016;35:1–21. https://doi.org/10.1016/j.jnutbio.2015.12.008.

[5] Goya L, Martín M, Sarriá B, Ramos S, Mateos R, Bravo L, et al. Effect of Cocoa and Its Flavonoids on Biomarkers of Inflammation: Studies of Cell Culture, Animals and Humans. Nutrients 2016;8:212. https://doi.org/10.3390/nu8040212.

[6] Magrone T, Russo MA, Jirillo E. Cocoa and dark chocolate polyphenols: From biology to clinical applications. Front Immunol 2017;8:677. https://doi.org/10.3389/fimmu.2017.00677.

[7] Bitzer ZT, Glisan SL, Dorenkott MR, Goodrich KM, Ye L, O'Keefe SF, et al. Cocoa procyanidins with different degrees of polymerization possess distinct activities in models of colonic inflammation. J Nutr Biochem 2015;26:827–31. https://doi.org/10.1016/j.jnutbio.2015.02.007.

[8] Ferri C, Desideri G, Ferri L, Proietti I, Di Agostino S, Martella L, et al. Cocoa, Blood Pressure, and Cardiovascular Health. J Agric Food Chem 2015;63:9901–9. https://doi.org/10.1021/acs.jafc.5b01064.

[9] Li Y, Zhu S, Feng Y, Xu F, Ma J, Zhong F. Influence of alkalization treatment on the color quality and the total phenolic and anthocyanin contents in cocoa powder. Food Sci Biotechnol 2014;23:59–63. https://doi.org/10.1007/s10068-014-0008-5.

[10] Li Y, Feng Y, Zhu S, Luo C, Ma J, Zhong F. The effect of alkalization on the bioactive

and flavor related components in commercial cocoa powder. J Food Compos Anal 2011;25:17–23. https://doi.org/10.1016/j.jfca.2011.04.010.

[11] Miller KB, Hurst WJ, Payne MJ, Stuart DA, Apgar J, Sweigart DS, et al. Impact of Alkalization on the Antioxidant and Flavanol Content of Commercial Cocoa Powders. J Agric Food Chem 2008;56:8527–33. https://doi.org/10.1021/jf801670p.

[12] Li Y, Feng Y, Zhu S, Luo C, Ma J, Zhong F. The effect of alkalization on the bioactive and flavor related components in commercial cocoa powder. J Food Compos Anal 2011;25:17–23. https://doi.org/10.1016/j.jfca.2011.04.010.

[13] Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes 2017;8:172–84. https://doi.org/10.1080/19490976.2017.1290756.

[14] Shoaib M, Shehzad A, Omar M, Rakha A, Raza H, Rizwan Sharif H, et al. Inulin: Properties, health benefits and food applications. Carbohydr Polym 2016;147:444–54. https://doi.org/10.1016/j.carbpol.2016.04.020.

[15] Boulangé CL, Neves AL, Chilloux J, Nicholson JK, Dumas ME. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. Genome Med 2016;8:1–12. https://doi.org/10.1186/s13073-016-0303-2.

[16] Bischoff SC. “Gut health”: a new objective in medicine? 2011. https://doi.org/10.1186/1741-7015-9-24.

[17] Bischoff SC, Barbara G, Buurman W, Ockhuizen T, Schulzke J-D, Serino M, et al. Intestinal permeability--a new target for disease prevention and therapy. BMC Gastroenterol 2014;14:189. https://doi.org/10.1186/s12876-014-0189-7.

[18] Michielan A, Incà RD. Intestinal Permeability in Inflammatory Bowel Disease : Pathogenesis , Clinical Evaluation , and Therapy of Leaky Gut 2015;2015. https://doi.org/10.1155/2015/628157.

[19] Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 2009;9:799–809. https://doi.org/10.1038/nri2653.

[20] France MM, Turner JR. The mucosal barrier at a glance. J Cell Sci 2017;130:307–14. https://doi.org/10.1242/jcs.193482.

[21] Hakansson A, Molin G. Gut Microbiota and Inflammation. Nutrients 2011;3:637–82. https://doi.org/10.3390/nu3060637.

[22] Yadav M, Verma MK, Chauhan NS. A review of metabolic potential of human gut

microbiome in human nutrition. Arch Microbiol 2018;200:203–17. https://doi.org/10.1007/s00203-017-1459-x.

[23] D'Argenio V, Salvatore F. The role of the gut microbiome in the healthy adult status. Clin Chim Acta 2015;451:97–102. https://doi.org/10.1016/j.cca.2015.01.003.

[24] Chen J, Pitmon E, Wang K. Microbiome, inflammation and colorectal cancer. Semin Immunol 2017;32:43–53. https://doi.org/10.1016/j.smim.2017.09.006.

[25] Saad MJA, Santos A, Prada PO. Linking Gut Microbiota and Inflammation to Obesity and Insulin Resistance. Physiology 2016;31:283–93. https://doi.org/10.1152/physiol.00041.2015.

[26] Alatab S, Sepanlou SG, Ikuta K, Vahedi H, Bisignano C, Safiri S, et al. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Gastroenterol Hepatol 2020;5:17–30. https://doi.org/10.1016/S2468-1253(19)30333-4.

[27] Radavelli BL, Zanella PB, Silva AS, Alba VD, Radavelli BL, Zanella PB, et al. Diet , microbiota and in fl ammatory bowel disease : review 2018. https://doi.org/10.1108/NFS-07-2017-0156.

[28] Frost RA, Nystrom GJ, Lang CH. Lipopolysaccharide regulates proinflammatory cytokine expression in mouse myoblasts and skeletal muscle. Am J Physiol - Regul Integr Comp Physiol 2002;283. https://doi.org/10.1152/ajpregu.00039.2002.

[29] Shavez Beg M, Ahmad S, Jan K, Bashir K. Status, supply chain and processing of cocoa - A review 2017. https://doi.org/10.1016/j.tifs.2017.06.007.

[30] Afoakwa EO. Chocolate science and technology. n.d.

[31] Afoakwa EO. Cocoa production and processing technology. Cocoa Prod Process Technol 2016.

[32] Kongor JE, Hinneh M, de Walle D Van, Afoakwa EO, Boeckx P, Dewettinck K. Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile — A review. Food Res Int 2016;82:44–52. https://doi.org/10.1016/J.FOODRES.2016.01.012.

[33] Oracz J, Zyzelewicz D, Nebesny EWA. The Content of Polyphenolic Compounds in Cocoa Beans (Theobroma cacao L .), Depending on Variety , Growing Region , and Processing Operations : A Review The Content of Polyphenolic Compounds in Cocoa

Beans (Theobroma cacao L .), Depending on Variety , G 2015;8398. https://doi.org/10.1080/10408398.2012.686934.

[34] Redgwell RJ, Trovato V, Curti D. Cocoa bean carbohydrates: roasting-induced changes and polymer interactions. n.d.

[35] Giacometti J, Jolić SM, Josić D. Cocoa Processing and Impact on Composition. Process Impact Act Components Food 2015:605–12. https://doi.org/10.1016/B978-0-12-404699-3.00073-1.

[36] Dyer B. Alkalized Cocoa Powders. n.d.

[37] Kostic M. Cocoa Alkalization, n.d.

[38] Oracz J, Nebesny E, Zyzelewicz D, Budryn G, Luzak B. Bioavailability and metabolism of selected cocoa bioactive compounds: A comprehensive review. Crit Rev Food Sci Nutr 2019:1–39. https://doi.org/10.1080/10408398.2019.1619160.

[39] Neilson AP, Ferruzzi MG. Influence of Formulation and Processing on Absorption and Metabolism of Flavan-3-Ols from Tea and Cocoa. Annu Rev Food Sci Technol Is Annu Rev Food Sci Technol 2011;2:125–51. https://doi.org/10.1146/annurev-food-022510-133725.

[40] Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol 2017;14:491–502. https://doi.org/10.1038/nrgastro.2017.75.

[41] Elleuch M, Bedigian D, Roiseux O, Besbes S, Blecker C, Attia H. Dietary fibre and fibre-rich by-products of food processing: characterisation, technological functionality and commercial applications: a review 2010. https://doi.org/10.1016/j.foodchem.2010.06.077.

[42] Katz DL, Doughty K, Ali A. COMPREHENSIVE INVITED REVIEW Cocoa and Chocolate in Human Health and Disease n.d. https://doi.org/10.1089/ars.2010.3697.

[43] Pérez-Jiménez J, Díaz-Rubio ME, Mesías M, Morales FJ, Saura-Calixto F. Evidence for the formation of maillardized insoluble dietary fiber in bread: A specific kind of dietary fiber in thermally processed food. FRIN 2014;55:391–6. https://doi.org/10.1016/j.foodres.2013.11.031.

[44] Natural Unsweetened Cocoa Powder 8 oz Cans | HERSHEY'S n.d. https://www.hersheys.com/en_us/products/hersheys-natural-unsweetened-cocoa-8-oz-

cans.html (accessed April 27, 2020).

[45] Aprotosoaie AC, Luca SV, Miron A. Flavor Chemistry of Cocoa and Cocoa Products-An Overview. Compr Rev Food Sci Food Saf 2016;15:73–91. https://doi.org/10.1111/1541-4337.12180.

[46] Langner E, Rzeski W. Biological Properties of Melanoidins: A Review. Int J Food Prop 2014;17:344–53. https://doi.org/10.1080/10942912.2011.631253.

[47] Saura-Calixto F. Dietary Fiber as a Carrier of Dietary Antioxidants: An Essential Physiological Function. J Agric Food Chem 2011;59:43. https://doi.org/10.1021/jf1036596.

[48] Jung HG, Fahey GC. Nutritional Implications of Phenolic Monomers and Lignin: a Review. J Anim Sci 1983;57:206–19. https://doi.org/10.2527/jas1983.571206x.

[49] Silván JM, Morales FJ, Saura-Calixto F. Conceptual Study on Maillardized Dietary Fiber in Coffee. J Agric Food Chem 2010;58:12244–9. https://doi.org/10.1021/jf102489u.

[50] Mesías M, Delgado-Andrade C. Melanoidins as a potential functional food ingredient. Curr Opin Food Sci 2017;14:37–42. https://doi.org/10.1016/j.cofs.2017.01.007.

[51] Akram W, Garud N, Joshi R. Role of inulin as prebiotics on inflammatory bowel disease. Drug Discov Ther 2019;13:1–8. https://doi.org/10.5582/ddt.2019.01000.

[52] Kalyani Nair al K, Kharb S, Thompkinson DK, Kalyani Nair K. Inulin Dietary Fiber with Functional and Health Attributes-A Review. Food Rev Int 2010;26:0–0. https://doi.org/10.1080/87559121003590664.

[53] van der Beek CM, Dejong CHC, Troost FJ, Masclee AAM, Lenaerts K. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. Nutr Rev 2017;75:286–305. https://doi.org/10.1093/nutrit/nuw067.

[54] Li Z. Interaction of Cocoa Powder with Intestinal Microbiota INTERACTION OF COCOA POWDER WITH INTESTIAL MICROBIOTA. n.d.

[55] Fogliano V, Corollaro ML, Vitaglione P, Napolitano A. In vitro bioaccessibility and gut biotransformation of polyphenols present in the water-insoluble cocoa fraction 2011:44–55. https://doi.org/10.1002/mnfr.201000360.

[56] Andújar I, Recio MC, Giner RM, Cienfuegos-Jovellanos E, Laghi S, Muguerza B, et al. Inhibition of Ulcerative Colitis in Mice after Oral Administration of a Polyphenol-Enriched Cocoa Extract Is Mediated by the Inhibition of STAT1 and STAT3

Phosphorylation in Colon Cells. J Agric Food Chem 2011;59:6474–83. https://doi.org/10.1021/jf2008925.

[57] Solano-Aguilar GI, Lakshman S, Jang S, Beshah E, Xie Y, Sikaroodi M, et al. The Effect of Feeding Cocoa Powder and Lactobacillus rhamnosus LGG on the Composition and Function of Pig Intestinal Microbiome. Curr Dev Nutr 2018:1–11. https://doi.org/10.1093/cdn/nzy011.

[58] Tzounis X, Rodriguez-Mateos A, Vulevic J, Gibson GR, Kwik-Uribe C, Spencer JP. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study 1-3 n.d. https://doi.org/10.3945/ajcn.110.000075.

[59] Goodrich KM, Fundaro G, Griffin LE, Grant A, Hulver MW, Ponder MA, et al. Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin: A secondary analysis of healthy Wistar Furth rats. Nutr Res 2012;32:787–94. https://doi.org/10.1016/j.nutres.2012.09.004.

[60] Gu Y, Yu S, Park JY, Harvatine K, Lambert JD. Dietary cocoa reduces metabolic endotoxemia and adipose tissue inflammation in high-fat fed mice☆ 2014. https://doi.org/10.1016/j.jnutbio.2013.12.004.

[61] Martín M, Goya L, Ramos S, Martín MA, Goya L, Ramos S. Preventive Effects of Cocoa and Cocoa Antioxidants in Colon Cancer. Diseases 2016;4:6. https://doi.org/10.3390/diseases4010006.

[62] Gil-Cardoso K, Ginés I, Pinent M, Ardévol A, Blay M, Terra X. The co-administration of proanthocyanidins and an obesogenic diet prevents the increase in intestinal permeability and metabolic endotoxemia derived to the diet. J Nutr Biochem 2018;62:35–42. https://doi.org/10.1016/j.jnutbio.2018.07.012.

[63] Schuier M, Sies H, Illek B, Fischer H. Cocoa-Related Flavonoids Inhibit CFTR-Mediated Chloride Transport across T84 Human Colon Epithelia. J Nutr 2005;135:2320–5. https://doi.org/10.1093/jn/135.10.2320.

[64] Gu Y, Hurst WJ, Stuart DA, Lambert JD. Inhibition of Key Digestive Enzymes by Cocoa Extracts and Procyanidins. J Agric Food Chem 2011;59:5305–11. https://doi.org/10.1021/jf200180n.

[65] Edwards CA, Havlik J, Cong W, Mullen W, Preston T, Morrison DJ, et al. Polyphenols

and health: Interactions between fibre, plant polyphenols and the gut microbiota. Nutr Bull 2017;42:356–60. https://doi.org/10.1111/nbu.12296.

[66] Racine KC, Wiersema BD, Griffin LE, Essenmacher LA, Lee AH, Hopfer H, et al. Flavanol polymerization is a superior predictor of α-glucosidase inhibitory activity compared to flavanol or total polyphenol concentrations in cocoas prepared by variations in controlled fermentation and roasting of the same raw cocoa beans. Antioxidants 2019;8. https://doi.org/10.3390/antiox8120635.

[67] Dorenkott MR, Griffin LE, Goodrich KM, Thompson-Witrick KA, Fundaro G, Ye L, et al. Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. J Agric Food Chem 2014;62:2216–27. https://doi.org/10.1021/jf500333y.

[68] Racine KC, Lee AH, Stewart AC, Blakeslee KW, Neilson AP. Development of a rapid ultra performance hydrophilic interaction liquid chromatography tandem mass spectrometry method for procyanidins with enhanced ionization efficiency. J Chromatogr A 2019;1594:54–64. https://doi.org/10.1016/j.chroma.2019.02.007.

[69] Mills CE, Tzounis X, Mottram DS, Gibson GR, Spencer JPE. In vitro colonic metabolism of coffee and chlorogenic acid results in selective changes in human faecal microbiota growth 2019:1220–7. https://doi.org/10.1017/S0007114514003948.

[70] Alqurashi RM, Alarifi SN, Walton GE, Costabile AF, Rowland IR, Commane DM. In vitro approaches to assess the effects of açai (Euterpe oleracea) digestion on polyphenol availability and the subsequent impact on the faecal microbiota n.d. https://doi.org/10.1016/j.foodchem.2017.04.164.

[71] Goodrich KM, Neilson AP. Simultaneous UPLC-MS/MS analysis of native catechins and procyanidins and their microbial metabolites in intestinal contents and tissues of male Wistar Furth inbred rats. J Chromatogr B Anal Technol Biomed Life Sci 2014;958:63–74. https://doi.org/10.1016/j.jchromb.2014.03.011.

[72] Sato H, Taketomi Y, Murakami M. Metabolic regulation by secreted phospholipase A 2 2016. https://doi.org/10.1186/s41232-016-0012-7.

[73] Krimsky M, Ligumsky M, Aptekar L, Schwob O, Goshen G, Gruzman A, et al. Articles in PresS. Am J Physiol Gastrointest Liver Physiol 2003.

https://doi.org/10.1152/ajpgi.00463.2002.

[74] Itoh Y, Joh T, Tanida S, Sasaki M, Kataoka H, Itoh K, et al. IL-8 promotes cell proliferation and migration through metalloproteinase- cleavage proHB-EGF in human colon carcinoma cells. Cytokine 2005;29:275–82. https://doi.org/10.1016/j.cyto.2004.11.005.

[75] Daig R, Andus T, Aschenbrenner E, Falk W, Schölmerich J, Gross V. Increased interleukin 8 expression in the colon mucosa of patients with inflammatory bowel disease. Gut 1996;38:216–22. https://doi.org/10.1136/gut.38.2.216.

[76] Den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 2013;54:2325–40. https://doi.org/10.1194/jlr.R036012.

[77] Shokryazdan P, Faseleh Jahromi M, Navidshad B, Liang JB. Effects of prebiotics on immune system and cytokine expression. Med Microbiol Immunol 2017;206:1–9. https://doi.org/10.1007/s00430-016-0481-y.

[78] Use Of The Prebiotic Inulin In The Prevention Of Adverse Signs Of Acute Colitis in: Acta Veterinaria Volume 65 Issue 3 (2015) n.d. https://content.sciendo.com/view/journals/acve/65/3/article-p339.xml (accessed April 25, 2020).

[79] Petry N, Egli I, Chassard C, Lacroix C, Hurrell R. Inulin modifies the bifidobacteria population, fecal lactate concentration, and fecal pH but does not influence iron absorption in women with low iron status. Am J Clin Nutr 2012;96:325–31. https://doi.org/10.3945/ajcn.112.035717.

[80] Welters CFM, Heineman E, Thunnissen FBJM, Van den Bogaard AEJM, Soeters PB, Baeten CGMI. Effect of dietary inulin supplementation on inflammation of pouch mucosa in patients with an ileal pouch-anal anastomosis. Dis Colon Rectum 2002;45:621–7. https://doi.org/10.1007/s10350-004-6257-2.

APPENDIX

The FIT-C dextran assay was performed with Caco-2 TC-7 colon cancer cells as described in the methods section, but this experiment was preliminary and is still in the trouble shooting phase. Results from 2 trails are shown in the figures below.

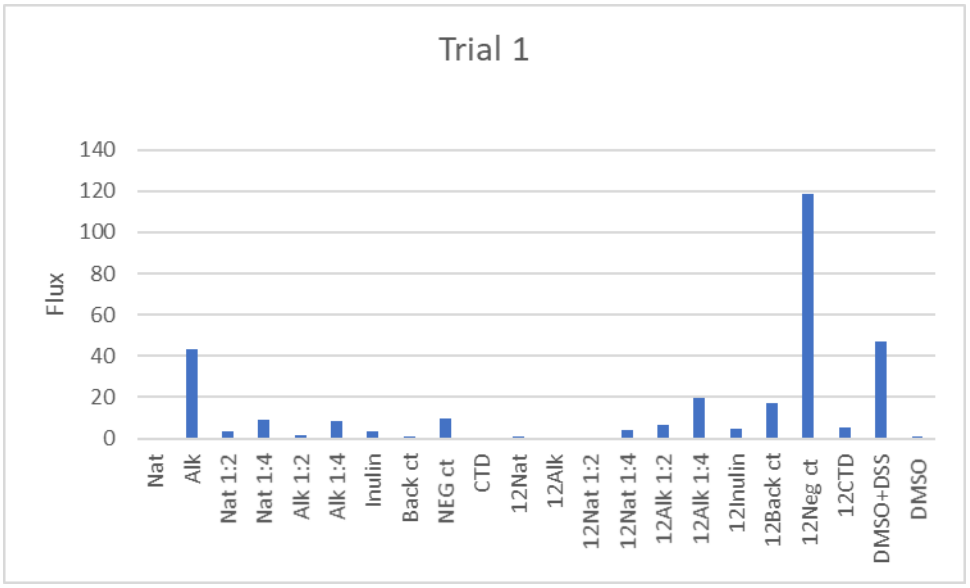


Figure A1: FIT-C dextran flux in Caco-2 cells Trial 1

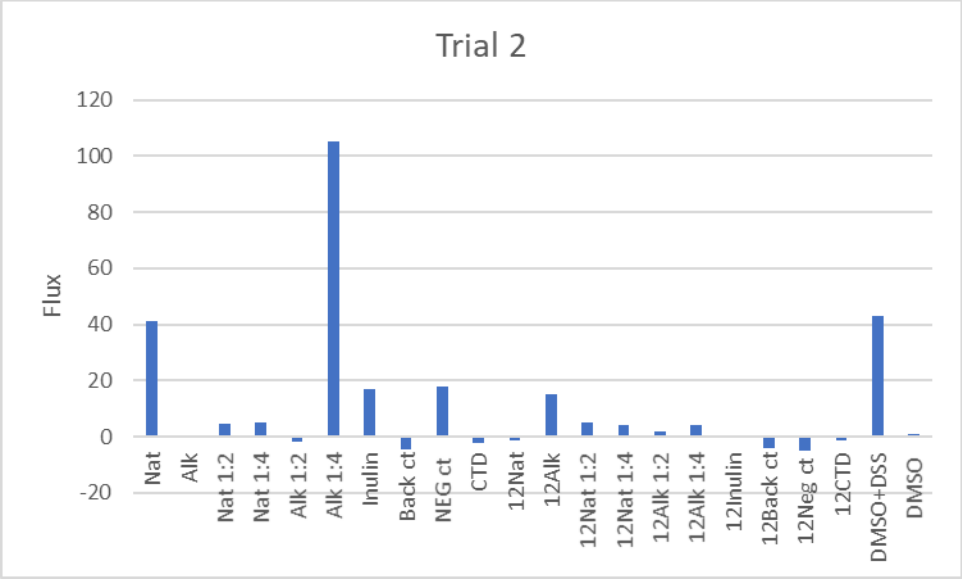


Figure A2: FIT-C dextran flux in Caco-2 cells Trial 2