Interaction of Cocoa Powder with Intestinal Microbiota

Martha M. Escoto Sabillon
Louisiana State University and Agricultural and Mechanical College

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INTERACTION OF COCOA POWDER WITH INTESTINAL MICROBIOTA

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Nutrition and Food Sciences

by

Martha Marina Escoto Sabillón
B.S., Zamorano Pan-American Agricultural School, Honduras, 2014
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Cocoa is the fully fermented and dried seed of the cacao tree (Theobroma cacao L.) which has prebiotic properties, due to their high concentration of polyphenols. Therefore, the ingestion of cocoa could cause changes in the proportions of the intestinal microbiota that can influence the intestinal immune response. The objective of this study was to determine the effect of alkalization process of the cocoa bean in the diversity of the gut microbiota. The samples were “lavado” unprocessed cocoa powder, “natural” unprocessed cocoa powder, “D-11-S” as alkalized cocoa powder, “D-11-B” heavily alkalized cocoa powder, and raw cocoa “shells” and a control of fecal matter. The cocoa powders are rich in polyphenols and anthocyanins that are pH sensitive exhibiting different colors as their structure changes. Analyses of pH and color correlated to microbial diversity can help understanding for which forms of polyphenols and anthocyanins will be more active. To analyze the samples a digestion was conducted by simulating the human digestion system in vitro, with five samples and one control (fecal samples without cocoa). Microbial diversity and composition were analyzed with Illumina HiSeq with methods via bTEFAP® DNA analysis. Segments of the bacterial genome were amplified with the 515F and 806R primers specific for the universal Eubacterial 16S rRNA gene. Final operational taxonomic units (OTUs) were taxonomically classified using BLASTn against a database derived from GreenGenes/RDP/NCBI. Monte Carlo simulation was performed to detect features with significant differences. *Firmicutes, Proteobacteria,* and *Bacteroidetes* were the most predominant phyla in samples comprising >96% of all sequences (p<0.05). Overall, alkalization process did
affect the diversity of the gut microbiota, but the effect was not consistent for only alkalinized cocoa. The reduction of *Firmicutes* and *Bacteriodetes* (F:B) by natural cocoa and D-11-B affected the diversity of the gut microbiota promoting a normal stable variety of OTUs. These data suggest that cocoa powder consumption aids in the prevalence of a beneficial microbiota in the human gut.
CHAPTER 1. INTRODUCTION

Cocoa beans originate as seeds in fruit pods of the *Theobroma cacao* tree. Raw cocoa has an astringent unpleasant taste and for this to be consumed it has to be fermented, dried, and then roasted to obtain the characteristic “cocoa” taste and flavor. The fermentation process takes place in the pulp, where microbiological activity in the pulp leads to the initiation of various biochemical processes important for taste and flavor, which takes place inside the beans (Watson *et al.*, 2013). The use of cocoa and cocoa-derived products has continued through the earliest centuries, and several studies have attributed it to beneficial and curative effects (Ackar *et al.*, 2013).

Cocoa powder is an important ingredient for plenty of foods, beverages and confectioneries. It is used in significant volumes in the manufacture of syrups and coatings, but also in non-confectionary food usages like baking, beverages, flavorings, and icing. Out of the total of U.S. imports in cocoa bean-related ingredients, 37% corresponds to cocoa powder (USDA, 2001). For 2004 in the United States there was more than 130 million pounds of cocoa powder consumed. Statistics of total importations by North America for the year 2010 were around 505,000 tons of cocoa powder, with 84% of the tons belonging to the United States (Sarris, 2003).

Recent studies suggest the consumption of cocoa powder in regard to the potential health benefits may be related to it being a prebiotic. Non-digestible nutritional food ingredients such as dietary fiber that are fermented and known as a prebiotic increase the beneficial bacterial loads in the colon (Ramachandran & Baojun, 2015). The carbohydrates in cocoa powder are generally dietary fiber, which form complexes with polyphenols that are fermented by microorganisms in the human colon providing health beneficial compounds to the host. These polyphenols in cacao are flavonoids, substances that have the ability to remove free radicals, chelate metals and other
pro-oxidative compounds. The constant consumption of a food rich in flavanols has the potential to support gut health by the ability to exert a nutritional pressure that favors some types of bacteria over others (Tremaroli & Backhed, 2012).

The major steps contributing to the development of these benefits and the characteristics of color and flavor in the cocoa and chocolate are the fermentation, roasting, alkalization, and an agitation process called conching. Subsequently, after the fermentation in the cacao seeds, the seeds are dehulled and roasted at 100-150°C. The roasted cocoa beans are generally transformed from a solid phase to a suspension called cocoa liquor, which contains cocoa butter and nonfat fines (Kamphuis, 2009). Afterward, the liquor is mechanically pressed to extract the cocoa butter and creating a solid cake, which is ground into small particles and this is known as cocoa powder. Commonly, cocoa powder holds a residual of cocoa butter from about 10-12%. The highest percentages of the chocolate flavors and polyphenol antioxidants reside in the cocoa powder (Miller et al., 2008).

Products from cacao such as the chocolate liquor and the cocoa powder can be modified by a process called “Dutching” or alkalization. In this process, the liquor or cocoa are generally washed with a sodium or potassium carbonate solution which darkens the cocoa ingredients. Likewise, it might change the flavor by reducing bitterness and increasing the dispersibility of cocoa powder (Beckett, 2009; World Cocoa Foundation, 2012). The alkali-treated powders are mostly used in the non-confectionery manufacture. For the production of chocolate confectionery, there are some large brands of dark chocolate that used the alkalized powder and liquor as sub-ingredients (Wollgast and Anklam, 2000).

The main issue for the Dutching process is that phytonutrients, polyphenols, and flavanols, may be reduced, modified or destroyed at an alkaline pH. In 2000, a U.S. patent showed that only
19% of the total amount of procyanidins remain in the alkali-treated cocoa powder (Kelly, 2000). In a study done by Gu and collaborators (2006), they compared three commercially prepared untreated cocoa powders to two commercially prepared alkalized cocoa powders demonstrating that the alkalized cocoa powders had 78% fewer flavanols. Last but not least, a study simulating the alkaline conditions of the colon, stated that catechin, epicatechin, or procyanidin dimers were degraded at a pH of 7.4 after 24 hours, and by 100% after 4 hours at a pH of 9.0 (Zhu et al., 2002). Despite this information that alkali cocoa powder destroys flavanols, very few data exist on the fermentation of alkali cocoa powder and the effect on the microbiome.

The objective of this study was to determine the effect of varying degrees of alkali processing on the diversity of the gut microbiota and the fermentation of cocoa by human colonic microbiota. This thesis is divided into five chapters, where the first is a brief introduction and justification of this study. The second and third chapters comprise a literature review and explain materials and methods, respectively. The fourth chapter describes the results and discussion. A summary and conclusions are contained in the fifth chapter. After the references section, the appendices include additional materials used in this study. The vita of the author is also provided.
CHAPTER 2. LITERATURE REVIEW

2.1 Cocoa

Cocoa traces have been found in Mesoamerica and date back to 2000 B.C. The use of cocoa and cocoa-derived products has continued back through the earliest centuries, and several studies have attributed its beneficial and curative effects (Ackar et al., 2013). Cocoa beans originate as seeds in fruit pods of the *Theobroma cacao* or “Fruit of the Gods” tree. The beans were used as a commercial trading currency by the Mayas, Olmecs, Toltec, and Aztec people of Mexico and Central America as observed in Columbus’s voyages (ICE Futures U.S., 2007). Cocoa tree farms are in tropical environments within 15-20° of latitude from the equator. Cocoa is a sensitive and delicate crop, with proper care, at the fifth-year cocoa trees begin to yield pods at high production levels (Baptista, 2009).

Farmers begin to harvest the ripe pods using long-handed steel tools. Pods are collected and split with a sturdy stick removing all the beans inside. Each pod of cacao contains approximately 30 to 45 beans embedded in a mucilaginous pulp. This pulp is rich in glucose, fructose, and sucrose. Raw cocoa has an astringent unpleasant taste and for this reason, to be consumed, it needs to be fermented, dried, and roasted to obtain the characteristic “cocoa” taste and flavor. To make a pound of chocolate 400 beans are approximately required (World Cocoa Foundation, 2012; Watson et. al 2013).

Cocoa fresh seeds are packed and typically fermented in boxes or heaped in piles covered with mats or banana leaves. The fermentation process takes place in the pulp that surrounds the beans. The pulp heats up for three to seven days, where microbiological activity leads to the initiation of various biochemical processes important for taste and flavor, which takes place inside
the beans. After, the beans are dried in the sun or by solar dryers for several days they are transported to processing facilities (Miller et al., 2008; World Cocoa Foundation, 2012).

2.1.1 Processing Cocoa Powder

For further processing cocoa beans must be cleaned and inspected to remove non-cocoa particles; and this removal is done by sieving, magnets, or combined with cleaning steps. After this, the cleaned beans are deshelled. Proper removal of the shell is a prerequisite of a good quality product since it does not contribute to the flavor of the final product. Ideally, the shell should separate perfectly leaving the nib, which is the inside of the cocoa bean, almost intact. In some cases, the shell around the bean cannot be removed easily and companies usually separate it by a swing-hammer type of breaker. After breaking, the shells and nib are divided by winnowing and unbroken beans are returned for reprocessing.

Roasting matures the flavor in the beans from the prior processes, fermentation, and drying. The beans are roasted at temperatures ranging between 100-150°C (230-284°F). Once the beans were roasted, the nib is ground into a suspension. The heat generated during this process causes the cocoa butter in the nib to melt, changing phase to “cocoa liquor”. The cocoa liquor is poured into hydraulic presses that separate liquor into cocoa butter and cocoa cakes. The solid cocoa cake is then ground into the common product known as cocoa powder. Cocoa powder normally holds a residual of 10 to 12% of cocoa butter, but including mainly nonfat cocoa solids. The cocoa powder maintains the common chocolate flavor and the polyphenol antioxidants are present.

The alkalization step process, also known as Dutching, introduces specific flavors and is predominantly used for the production of cocoa powder. In this process, the nibs are normally treated with an alkaline solution such as sodium or potassium carbonate and water. Depending upon the cocoa beans, the percentage of alkaline solution, time, and temperature it will result in a
change in flavor, color, and pH. The pH of the non-alkalized cocoa cake can also be increased by adding dry alkali, resulting in a higher pH powder after pulverizing but, produce less effects in color (Beckett, 2009; World Cocoa Foundation, 2012). Natural cocoa powders have an extractable pH of 5.3-5.8. Alkalized cocoa powders are grouped into lightly treated (pH 6.50-7.20), medium-treated (pH 7.21-7.60), and heavily treated (pH 7.61 and higher) (Miller et al., 2008).

In 2008, Miller et al. discussed that a main concern of the Dutch process was that the components of the cocoa, such as polyphenols and flavanols may be modified or destroyed at alkaline pH. A study conducted by Gu and collaborators compared commercial natural cocoa powder to alkalized cocoa powder and found the processed powder had 78% fewer flavanols (Gu et al., 2006). Finally, a study simulating an in vitro digestion revealed that the cocoa components are pH-dependent. A comparison of pH 5.0 versus pH 9.0, showed that the stability of all four compounds, epicatechin-(4â-8)-epicatechin (Dimer B2) and epicatechin-(4â-6)-epicatechin (Dimer B5) was greater at lower pH than the stability at higher pH (Zhu et al., 2002).

2.1.2 Cocoa Powder Composition

Cocoa powder is high in palmitic and stearic saturated fats, but is low in cholesterol and sodium. Also, the powder is a source of protein, potassium, and zinc, a very good source of dietary fiber, iron, magnesium, phosphorus, copper, and manganese (Ilanep, 2005). Usually cocoa powder ended up containing 10-12% of residual cocoa butter is considered as being a “nonfat” cocoa solid. The nonfat cocoa solids refer to the brown particulate material of the seed. The major steps contributing to the development of these benefits and the characteristic of color and flavor in the cocoa and chocolate are the fermentation, roasting, alkalization, and conching. Subsequently, after the roasting, alkalization of cocoa beans is generally applied to develop organoleptic and
technological qualities of cocoa powder (Kamphuis, 2009). The process of alkalization is done by washing the cocoa with potassium carbonate solution that neutralized or changed cocoa acidity, making changes in the pH (Wollgast & Anklam, 2000).

2.1.3 Benefits of Cocoa

Recent research has shown that cocoa and chocolate consumption is associated with several health benefits such as prevention of cancer, cardiovascular and neurodegenerative diseases, diabetes, obesity, and slowing of aging - attributed to the content of polyphenols (Miller et al., 2008; Martín and Ramos, 2016). Cocoa is a rich source of polyphenols compounds, especially flavanols. Procyanidins, oligomers of flavanols, like catechin and epicatechin, constitute the majority of the cacao bean and is one of the more concentrated sources. Epicatechin is the most abundant monomeric flavanol in cocoa, representing 35% of the total phenolic content (Wollgast and Anklam, 2000).

The constant consumption of a food rich in flavanols has the potential to support gut health by the ability to exert a nutritional pressure that favors some bacteria growth. The carbohydrates in cocoa are generally dietary fiber that form complexes with polyphenols. These complexes may be fermented by microorganisms in the human colon providing health benefits to the host (Tremaroli & Backhed, 2012). However, the remaining number of polyphenols in the cocoa powder will directly depend on the methods used in the cocoa beans processing.

2.1.4 Consumption and Income in the USA

North America is considered the world's second largest cocoa consuming area and is likely to grow by 3.6 percent per annum and reach 703,000 tons (Sarris, 2003). Out of the total from U.S. imports of cocoa bean-related products including the chocolate liquor, cocoa butter, and chocolate paste, 37% represents cocoa powder (USDA, 2001). In 2004 in the United States of America
people consumed more than 130 million pounds of cocoa powder (Miller et al., 2008). FAO projected consumption for 2010 in the U.S. was on an average of 634,000 tons. North American total importations for 2010 statistics were around 505,000 tons, including 424,000 tons to the Unites States, decreasing their growth rate of importations by -0.2% (Sarris, 2003).

According to a World Cocoa Foundation (WCF) publication in 2014 consumers have thousands of different ways to taste chocolate, estimating a consumption of more than 3 million tons of cocoa beans annually. The demand for post-processing cocoa powder and cake measured in nominal dollar value of imports is $781,154. (World Cocoa Foundation, 2012).

2.2 Gastro-Intestinal Tract

The human gastrointestinal (GI) tract is a complex biological system. The GI tract consists of oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, rectum, and anus, with the combination of bacteria, archaea, yeast, filamentous fungi and protozoans constitute the digestive system (Rajilić-Stojanović et al., 2007; Maukonen and Saarela, 2015). The human gastrointestinal tract is bacteria-free before birth. During birth, infants have direct contact with mother’s skin, vagina, feces, and the environment, developing a faster bacteria proliferation in the infant’s gastrointestinal system than the caesarean babies (Morelli, 2008). However, in adults, the pooled microbial population in the human body exceeds $10^{14}$ microbial cells, which is greater than the number of human cells.

The adult human GI tract is constituted by all three domains of life: bacteria, archaea, and eukarya. Bacteria living in the human gut achieve the highest cell densities recorded for any ecosystem. In all 98% of the species that live in the gastrointestinal tract belong basically to few bacterial phyla; Firmicutes, Actinobacteria, Proteobacteria, and Bacteriodetes. Despite the fact that
the bacterial phyla community in the GI tract is diverse, only eight of the 55 known bacterial phyla have been identified, and of these, 5 are infrequent (Gerritsen et al., 2011; Bäckhed et al., 2016).

The different types of bacterial populations in the GI tract will depend on the environmental conditions (temperature, pH, redox potential, water activity (Aw), salinity, light, and atmospheric composition) of the different anatomical sites (Table 1.). Each microorganism plays a role in the digestion and absorption of intestinal contents during the passage throughout the GI tract. The human oral cavity has the second largest population of bacteria (approximately $10^{10}$/ml; more than 500 bacterial species); being the major inhabitant of the most microbial divisions that is colonized by Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria, and Cyanobacteria (Maukonen and Saarela, 2015) (Figure 1).

In the stomach, the bacterial population reduces due to the high acidity and digestive enzymes. The stomach becomes a barrier restricting access of bacteria from the outside environment to the rest of the GI tract. The bacteria that get to the stomach cavity are attached to gastric epithelial or are present in mucus. The most predominant bacteria in the stomach are gram positive and the micro-organism gram-negative aerobic, *Helicobacter pylori* (Tlaskalová-Hogenová et al., 2004).

The small intestine is divided into three parts. The duodenum, mostly known as the first and shortest part of the small intestine, is the part of the GI tract where most of the host enzymatic digestion of food occurs. The microbial density increases along the gastrointestinal tract; in the stomach and duodenum there is an increase from $10^{1}$-$10^{4}$/ml, respectively. The remaining two parts of the small intestine, jejunum and ileum have a microbial density from $10^{5}$-$10^{9}$/ml. The conditions in the ileum are more favorable for microbial growth, for example, the pH is less acidic.
and bile acids are reabsorbed. Therefore, ileum microbial density can be higher \((10^9/ml)\) compared with the duodenum. The small intestine contains a relatively higher concentration of bacteria than the stomach, it involves mainly Firmicutes \((Lactobacilli, Bacilli,\) and Gram-positive Cocci\), but some Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria \((\text{Tlaskalová-Hogenová et al., 2004; Booijink et al., 2007).})

The large intestine is one-fifth of the whole length of the gastro-intestinal tract. Also known as the large bowel, the large intestine has a microbial cell density from \(10^{10}-10^{12}/ml\) in the colon and feces, and most of the bacterial populations are either anaerobic or facultatively anaerobic. The predominant phyla are Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria \((\text{Tlaskalová-Hogenová et al., 2004; Booijink et al., 2007).})

**Table 1.** Human GI Tract Segments with dimensions and the corresponding microbial density. \((\text{Tlaskalová-Hogenová et al., 2004; Rajilić-Stojanović et al., 2007).})

<table>
<thead>
<tr>
<th>Gastrointestinal Segment</th>
<th>Length (cm)</th>
<th>Density of microbiota cells/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>12</td>
<td>(10^4) - (10^7)</td>
</tr>
<tr>
<td>Duodenum</td>
<td>25</td>
<td>(10^7) - (10^9)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>160</td>
<td>(10^5) - (10^7)</td>
</tr>
<tr>
<td>Ileum</td>
<td>215</td>
<td>(10^7) - (10^9)</td>
</tr>
<tr>
<td>Cecum</td>
<td>6</td>
<td>(10^{10}) - (10^{11})</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Transverse colon</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Descending colon</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Sigmoid Colon</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>18</td>
<td>(10^{10}) - (10^{11})</td>
</tr>
</tbody>
</table>

Physiologically, humans do not have in their gastrointestinal tract the enzymes capable of metabolizing dietary fiber. When non-digestive carbohydrate (dietary fiber) get to the large intestine, they reach an anaerobic metabolic system with an environment comprised of bacteria
which contain enzymes that ferment fermentable fiber and the products are often bioavailable to the host (Louis et al., 2007). The large bowel is a fermentation vessel that harbors the majority of our gut microorganisms. These microbiota generate by-products that can be utilized by the host and improve the host’s health with the production of energy, nutrients, and the protection against diseases (Bäckhed et al., 2016).

**Figure 1.** Compositional differences in the microbiome by anatomical site (Cho and Blaser, 2012). The presence (+) or absence (-) of H. pylori, can lead to permanent and marked perturbations in the community composition.
2.3 Digestive System

The digestive system is the most multipart and complex biological system in animals and in all of life. The four main regions of the digestive system are the oral cavity, stomach, small intestine and large intestine. The digestion process begins in the mouth. During the oral phase of digestion, a mechanical breakdown of food structure begins. At the same time, food will mix with saliva, \( \alpha \) amylase hydrolysis will then occur, and finally, bolus formation occurs prior to swallowing (Woolnough et al., 2008).

The second segment of digestion is the gastric phase, which takes place in the stomach. It is within the confines of the stomach acids confined where the hydrolysis of protein and fat occurs. Protein digestion is initiated by pepsin and hydrochloric acid resulting in peptide formation; half portion of the protein leave the stomach in smaller peptides. Carbohydrate digestion began by salivary amylase stops as the amylase is denatured, and lipid digestion occurs with gastric lipase.

The third phase of the digestive system is the ileal phase, the food in the small intestine will be hydrolyzed. Protein and peptide passing from the stomach are further hydrolyzed by pancreatic enzymes such as trypsin, chymotrypsin, elastase, and the aminopeptidases, and carboxypeptidase. Dissolved peptidases will finally contribute to be hydrolyzed prior to absorption of free amino acids and smaller peptides. The absorption of amino acids will occur principally in the proximal jejunum (Grimble and Silk, 1989). The hydrolysis of carbohydrates is produced by pancreatic amylase in the lumen of the small intestine. The carbohydrates will be digested to maltose, dextrins, and limit dextrins. Dextrins are degraded to maltose by amylase, isomaltase degrades limit dextrins to glucose as it can digest alpha 1-4 and alpha 1-6 glycosidic bonds, and
arylase degrades maltose to glucose. Lactase digests lactose to the monosaccharides glucose and galactose, and sucrase digests sucrose to the monosaccharides glucose and fructose (Boisen & Eggum, 1991).

Lipid hydrolysis is catalyzed in the small intestine by three enzymes such as pancreatic lipase, carboxylic ester hydrolase, phospholipase, and a co-enzyme called colipase. Pancreatic lipase splits triacylglycerols into monoacylglycerols and fatty acids; the carboxylic ester hydrolase breaks down carboxylic esters, and phospholipase hydrolyzes fatty acids in the 2-position of glycerol-phospholipid. The presence of bile salts improved the activity of pancreatic lipase and it increased the absorption of long-chain fatty acids and monoacylglycerols. The amino acids, peptides, monosaccharides, and digested fatty acids are absorbed mainly in the jejunum (Boisen & Eggum, 1991). The small intestine percentage of fat digestion and absorption is 98%, the remaining 2% of undigested fat, proteins, and carbohydrates are transferred to the large intestine (Saunders & Sillery, 1988).

The internal microbiota of the large intestine is directly involved with the fermentation process of proteins and carbohydrates. In the large intestine is recovered from 10-20 % of nitrogen from protein, which is attributed to the microflora (Gerritsen et al., 2011). Protein (Mucin) and dietary carbohydrates (fermentable fiber, oligosaccharides, and Inulin) are further broken down by bacterial enzymes. Since undigested fatty acids cannot be fermented by bacterial enzymes during the passage through the large intestine, lipids are marginally influenced. The by-products of the dietary fiber and protein fermentation are short-chain fatty acids (SCFA) and a variety of gases. Humans absorb the SCFA and they have a variety of roles in the body and butyrate is the major energy source for colonocytes (Boisen & Eggum, 1991).
2.3.1 Fermentation in the Digestive System

By means of digestive enzymes, complex food components are able to be absorbed and used throughout the body. Many dietary fiber because of their structure are not digested in the upper GI tract. However, dietary fiber are fermented in the large intestine, also called the colon, containing an exceedingly complex society of microflora. The fermentation of these carbohydrates depend on the microbes available, the non-digested substrate present, the enzymes produced and most important the anaerobic conditions (Duncan et al., 2002; Valeur and Berstad, 2010).

The fermentation process of dietary fiber (non-digested carbohydrate), SCFAs and gasses such as carbon dioxide, hydrogen, and methane, are produced in the colon where there is an anaerobic condition (Cherbut, 2002). Carbohydrate fermentation in the colon is known for maintaining a host homeostasis, it exerts an influence on host physiology through nutritional, regulatory, and immune-modulatory properties. The end products of this fermentation are the SCFAs acting as signals for the regulation of virulence genes in enteric pathogens. Additionally, SCFAs prevent the growth of pathogens by decreasing the luminal pH and stimulate intestinal motility (Lin et al., 2014).

The major short-chain fatty acid (SCFA) that are formed by microbial fermentation are acetate, propionate, and butyrate; the acetate is metabolized by the muscle, butyrate by the colonic epithelium, and propionate by the liver. SCFA can promote large intestine functions, which include modulation of colonic motility, promotion of visceral blood flow, providing an additional amount of energy, and prevention of the overgrowth of potential pathogens in the lumen. In some studies, typical ratios in feces of SCFA are around 3:1:1 acetate-propionate-butyrate (Cummings and Macfarlane, 1997; Duncan et al., 2002; Sato et al., 2008).
Acetate is the major SCFA produced by the colonic microflora. Acetate is not metabolized in the colon because it is rapidly absorbed after its production and transported to the liver. Residual acetate is used further by the colonic microbiota and converted into butyrate. The major benefits of acetate are that it acts as an energy substrate for muscles, is essential for cholesterol synthesis in the body, and also has been shown that it suppress harmful bacteria (Cummings and Macfarlane, 1997; Hijova and Chmelarova 2007).

Propionate is the primary precursor for gluconeogenesis and may inhibit lipogenesis and protein synthesis (Louis et al., 2007). Propionate inhibits the synthesis of fatty acids in the liver and is also involved in the control of hepatic cholesterol synthesis. It helps to lower plasma cholesterol concentrations by inhibiting hepatic cholesterol-genesis through colonic fermentation. In recent research the production of propionate is associated to obesity and could be considered an important factor that contributes to gain weight (Schwiertz et al., 2009).

Butyrate is the major energy source for epithelial cells of colonic mucosa and stimulates cells proliferation, particularly the distal colon (Cummings, 1981; Sato et al., 2008). It was reported that butyrate gives protection against cancer and ulcerative colitis by blocking the absorption of carcinogenic substances and making the colon less susceptible to DNA damage (Kushkevych, 2014). In addition, the absorption of calcium (Ca) increased because butyrate helps in the maintenance of a healthy epithelium (Gibson and Mccartney, 1993; Asp et al., 1996). Butyrate has the ability to maintain a normal colonocyte phenotype, a characteristic that can contribute to reducing the risk of colorectal cancer (Encarnação et al., 2015).
2.3.2 Bacteria related with Fermentation

During the fermentation process of dietary fiber, the bacteria will attach to starch molecules especially *Bacteroides thetaiotaomicron, Bifidobacterium longum* and some *Lactobacillus* spp. (Bird *et al.*, 2000; Louis *et al.*, 2007; Xu *et al.*, 2017). However, *Bacteroides* spp, *Lactobacillus* spp. and *Bifidobacterium* spp. do not produce butyrate as a final product from dietary fiber. The end fermentation products for *Bacteroides* spp. are acetate, propionate, and succinate. Lactate and acetate are produced by *Bifidobacterium* spp. and *Lactobacillus* spp. when fermenting dietary fiber. Therefore, these bacterial species stick to the surface of starch molecules and ferment dietary fiber into intermediate products that are converted by other species to butyrate (Duncan *et al.*, 2004; Louis *et al.*, 2007).

The genera *Coprococcus* spp. and *Roseburia* spp. and the species *Faecalibacterium prausnitzii* produce butyrate from acetate (Duncan *et al.*, 2002). However, butyrate was also an end product of fermentation produced by lactate utilizing microorganisms of human feces (Duncan *et al.*, 2004). Also, the species *Eubacterium limosum* has the ability to convert lactate into acetate and butyrate, but it has to be with the presence of *Bifidobacterium longum*. Studies have shown that the majority of butyrate-producing isolates are related to the *Clostridium coccoides-Eubacterium rectale* group (Sato *et al.*, 2008).

2.3.2.1 *Bifidobacterium* spp.

*Bifidobacterium* spp. are classified under the phylum Actinobacteria. This genus is a gram-positive, nonmotile, strictly anaerobic branched rod that contributes to the lactate formation in the human colon. *Bifidobacterium* genus is the third most abundant in the human intestine after the genera *Bacteroides* and *Eubacterium*, making up 6% of total fecal bacteria (Matsuki *et al.*, 2004; Sato *et al.*, 2008). *Bifidobacterium* spp. can be used as a probiotic since it improves the digestion,
absorption, and helps the immune system. The use of *Bifidobacterium* spp. helps to lower the side-effects of antibiotic therapy. Also, it brings protection against enteric pathogens, putrefactive substances, and is involved in the reduction of cholesterol levels and anti-tumoral activity (Leahy *et al.*, 2005).

**2.3.2.2 Lactobacillus spp.**

The genus *Lactobacillus* spp. belongs to the phylum Firmicutes. *Lactobacillus* is a gram-positive rod-shaped non-spore forming bacteria belonging to lactic acid bacteria (LAB) category. The major acid during fermentation of sugars is lactic acid; it also produces acetic, succinic, and formic acids in lower amounts. *Lactobacillus* strains can grow under aerobic conditions, as well as anaerobic conditions in the colon (Reid, 1999). This genus helps to reduce the permeability in the intestinal mucosal preventing pathogen adhesion by producing biosurfactants. For the strain of *Lactobacillus fermentum* there is evidence of reduced adhesion and competitive exclusion of pathogenic *Escherichia coli*, *Listeria monocytogenes*, *Shigella sonnei*, and *Salmonella typhimurium* in *in-vitro* and *in-vivo* studies (Brown *et al.*, 1997)

**2.3.2.3 Bacteroides spp.**

The *Bacteroides* spp. genus is part of the Bacteroidetes phyla. *Bacteroides* genera are strictly anaerobic Gram-negative, and a dominant bacillus bacterial group in the human GI tract. *Bacteroides* spp. could break down a wide variety of indigestible dietary carbohydrates by producing acetate and succinate as the main metabolic products. This organism starts its fermentation by attaching to starch molecules. *Bacteroides* grows quickly after the introduction of prebiotics such as a dietary fiber (Wang and Gibson, 1993; Brown *et al.*, 1997). The
*Firmicutes/Bacteroidetes* ratio increases with age that is from birth to adulthood and is further altered with advanced age. The highest diversity of genus *Bacteroides* could be seen in an elderly population compared to younger populations (Mariat *et al.*, 2009).

### 2.3.2.4 *Roseburia* spp.

*Roseburia* spp. is classified under the phylum Firmicutes. This genus is gram-positive, and are strictly anaerobic bacteria that inhabit the human colon. *Roseburia* spp. produce butyric acid from acetate. When the presence of *Roseburia* spp. in the human colon is increased it will be associated with weight loss and reduced glucose intolerance (Wiele *et al.*, 2011).

### 2.4 Probiotic and Prebiotic

Probiotic is a term defined as single or mixed cultures of live microorganisms that when consumed live in adequate numbers confer a health benefit to the host improving the original microflora balance (Fuller, 1989; Stanton *et al.*, 2001). These live organisms are present in dairy products, salami, sauerkraut, fermented cereals, and other plant-based food. They can be protected in their way through the gastric system and stimulated in the colon. Products that are labeled as probiotics have to be consistent with certain parameters such as there should be food products in addition to microorganisms; the concentration of microbes should be sufficient to cause a health effect; and they have to be generally recognized as safe status (GRAS) (Cani & Delzenne, 2007).

The main probiotic microorganisms are in the genera of *Lactobacillus*, *Bifidobacterium*, and certain strains of *Enterococcus* and *Saccharomyces* spp. Some of the species include *Lactobacillus plantarum*, *Lactobacillus delbrücki*, *Lactobacillus bulgaricus*, *Bifidobacterium (B.) breve*, *B. longum*, *B. bifidum*, and *Streptococcus thermophilus*. The gram-negative *Escherichia coli* strain Nissle 1917, various lactic acid producing *Lactobacillus* strains, and a number of other bifidobacterial species and strains represent the main microorganisms classified as probiotic.
agents. Additionally, the butyrate producer, *Roseburia* genus, and the mucin-degrading bacterium, *Akkermansia muciniphila*, has also been reported as probiotics. Some of the beneficial effects of the probiotics are that they have been shown to have: enhanced the immune response, and increased the ability to digest food and alleviate many common digestive disorders such as constipation, diarrhea, and irritable bowel syndrome (IBS) (Lin *et al.*, 2014).

A non-digestible food ingredient, mostly oligosaccharides, that is beneficial to the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon is what we know as prebiotic; a term related to dietary fiber (Gibson & Roberfroid 1995). Fiber can be classified into three main categories which are dietary, functional, and total fiber. Dietary fiber includes non-digestible carbohydrates and lignin that are intrinsic and intact in plants. Functional fiber includes isolated, non-digestible carbohydrates that have beneficial physiological effects in humans; and total fiber is a combination of both (Cani *et al.*, 2004; Wang *et al.*, 2004; Falony *et al.*, 2006; Topping & Clifton, 2001).

Dietary fiber which increase the beneficial bacterial loads in the large intestine are known as prebiotic. Cocoa is considered as a prebiotic (Ramachandran *et al* 2015). The carbohydrates in cocoa powder are generally dietary fiber, which form complexes with polyphenols that are fermented by microorganisms in the human colon providing health benefit(s) to the host. These polyphenols in cacao are flavonoids, substances that have the ability of remove free radicals, chelate metals and others pro-oxidative compound. The constant consumption of a food rich in flavanol has the potential to support gut health by the ability to exert a nutritional pressure that favors some bacteria (Tremaroli & Backhed, 2012).
Synbiotics are defined as probiotic bacteria plus complex carbohydrates as prebiotics. When use combination of live probiotics with specific prebiotics as a symbiotic, that combination will help for the survivability of probiotics. Prebiotics provides a specific substrate which required for probiotic bacterial growth; from this combination, the host is getting benefits from both, probiotics and prebiotics (de Vrese et al., 2001; Schrezenmeir & de Vrese, 2001).

2.5 *In vitro vs. In vivo Digestion*

Developing *in vitro* digestibility techniques, it is logical to simulate one step incubations with pepsin or other proteases such as trypsin, papain, rennin, or more steps occurring at the *in vivo* conditions. *In vivo* determinations are time-consuming and costly, therefore much determination has been dedicated to the development of *in vitro* procedures. The *in vitro* technique will vary between experiment to experiment, making a design on which specific enzymes are necessary. The enzymes selected will be either to obtain the highest digestibility values or the measurement of the initial rate of hydrolysis. In both techniques, *in vivo* or *in vitro*, the enzymes used should have specificities similar to those present in the human digestive tract. In general, there are some key requirements for the development of *in vitro* digestibility assays: matching *in vivo* enzymes in presence, sequence, enzyme:substrate ratios standardizing enzyme activities and specificities; controlling co-enzymes and co-factors, pH and temperature; separating digested from undigested material while considering the inhibition of end products of digestion; and agreeing for the effects of sample size, particle size and particle size distribution (Boisen & Eggum, 1991).

Other methods have simulated gastric and intestinal digestion using a 2-stage *in vitro* digestion. The 2-stage digestion begins involving a pepsin-hydrogen chloride mixture neutralization and then digested with pancreatin, trypsin or intestinal fluid. The pancreatin is
suggested for solubilizing the potential digestible nutrients. However, amylase activity in pancreatin can become limited for starch degradation, while fat needs an addition of bile salts. The degradation of dietary fiber can be done only by the presence of microbial enzymes. Therefore, it can be simulated by rumen fluid, caecal fluid, fecal extract, or an appropriate enzyme complex of microbial carbohydrases (Ferguson et al., 1990).

Overall, there has been good agreement between these in vitro results and in vivo rat true fecal nitrogen digestibility. In vitro digestion technique has the capacity to give useful information of an in vivo amino acid and protein digestibility for humans. An in vitro method to measure the extent of digestion of protein must be precise, rapid, cheap, simple, vigorous, adjustable and relevant to the processes of digestion, absorption, and metabolism.

The complex in vitro methods including the computer controlled models of the digestive tract are more precise at mimicking the processes of digestion and absorption. Although, it might be too expensive and time consuming, but are useful tools for expanding the understanding of digestion processes. Despite the complexity of digestion, all these methods require an independent validation with in vivo data from the target species or an adequate animal model (Butts et al., 2012).

**2.6 Gut microbiota and human microbiome**

Gut microbiota is also known formerly as the “the normal flora”, which are the microbial organisms that constitute the microbiome. The flora composition in a community can vary considerably between environmental sites, among host niches and between health and diseases (Cho and Blaser, 2012). The microbiota in the gut can contribute to the development of healthy
environments within the intestinal tract by suppressing colonization of pathogenic organisms; this is called the state of “normobiosis”. Normobiosis is fundamental for normal gut homeostasis and ideal development of the host (Lin et al., 2014).

Microbiota composition differences exist across body sites and each human lifespan (Cho and Blaser, 2012). Before birth, the human gut is essentially sterile, but immediately after birth, it becomes a natural reservoir for an ample number of microorganism’s species (Forsythe et al., 2010; Petersen & Round, 2014). Lactobacilli is the pioneer microbial community in a baby’s gastrointestinal tract corresponding to the concentration of lactic acid bacteria (LAB) producing in the mother’s vagina and milk. As a result for the presence of this bacteria is a well prepare gastrointestinal tract for subsequent microbial successions until microbial maturity is reached (Palmer et al., 2007).

This “symbiotic ecosystem” is complex but with the largest conglomeration of microorganisms in the lumen and outside mucus layer of the colon (Gareau et al., 2016). Our gut microbiota aids to increase the thickness of the villi in the bowel wall and exhibits fast epithelial cell turnover (Leser and Mollbak, 2009). It contributes to the host nutrition by enhancing the efficacy of energy intake from ingested food, synthesis of essential vitamins, and the fermentation of dietary fiber and dietary proteins. It also affects a broad range of physiological properties of the human host, controlling the energy balance, pH, the development of the human immune system, stimulating the gut motility, and protection against pathogens. When is an imbalance of the intestinal microbiota there can incline individuals to a variety of diseases such as inflammatory bowel diseases (IBD), allergies, and obesity (Urokawa et al., 2007; Delzenne et al., 2011).
Microbiomes naturally consist of environmental or biological niches holding complex communities of microbes. The totality genes information and the milieu of resident microbes living and interacting within and on humans are referred to as the human microbiome (Cho and Blaser, 2012; Johnson and Versalovic, 2012). In adults, the microbial community in the human body exceeds 100 trillion microbial cells, which are about 10 times more than the total of the human cells. The microbiome can also be known as a “superorganism” because it is made up of the human cells and the complex ecosystem of the microbes. Hence the human genes are naturally mixed with trillions of microbes which colonized in our bodies. Of course, microbes do not colonize the gut only, but they are present on everybody surface that comes into contact with the environment. The microbiome is impacted by and impacts three potential causal determinants: the host, the agent, and the environment. Therefore, the composition of the microbiome differs by every human anatomical site (Bäckhed et al., 2005; Urokawa et al., 2007; Johnson and Versalovic, 2012; Hanson and Weinstock, 2016)(Figure 1).

The isolation of human genes from the microbe genes is impossible. Likewise, microbiome will be out of reach of in-depth scientific inquiry by culture-based method because of the short range of different taxonomy through this method. However, recent technological approaches such as DNA sequencing and computational methods (“next-generation” sequencing) have been used to analyze bacterial communities using the 16 rRNA gene for phylogenetic analysis. Metagenome is the term applied to the complex interactions of the human genome with the microbial genome. Using these methods will give a deeper understanding of the commensal residents, beneficial bacteria, and the contribution to human health. (Turnbaugh et al., 2006; Hattori and Taylor, 2009; Hanson and Weinstock, 2016).
Human-associated microbial communities likely play a significant role in host nutrition, development of immunity, and protection from diverse pathogens. The human body contains plenty different sites colonized by bacteria communities during newborn and childhood development and throughout the lifetime of each individual (Johnson and Versalovic, 2012). In adults, the gastrointestinal (GI) tract harbors a vast majority of microbiota achieving the highest cell densities recorded for any ecosystem. Even though the bacterial community in the GI tract is extremely diverse; the majority of 98% species live in gastrointestinal tract belongs to the few bacterial divisions or phyla. Those bacterial phyla are namely *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%), and *Actinobacteria* (3%). The divisions that dominate are Bacteroides (*Bacteroides* genera) and Firmicutes (*Clostridium* and *Eubacterium* genera), each contains around 30% of the microbes in feces and the mucus covering the intestinal epithelium (Bäckhed *et al.*, 2005; Hattori & Taylor, 2009) (Table 2.).

The remaining species belong to minor taxonomic features that are secondary to the majority. As described in Bäckhed and coworkers 2005, the adult human GI contains other than bacteria the two remaining domains of life, archaea, and eukarya. The major archaeal communities found and studied in the human gut are *Methanobrevibacter smithii, Methanosphaera stadtmanae,* and *Methanobrevibacter ruminantium.* The main eukaryote fungi in the human intestine are *Candida, Aspergillus* and *Penicillium.* Also, in fecal human samples have been identified more than 1200 viral genotypes. (Rajilic-Stojanovic *et al.*, 2007; Hattori & Taylor, 2009).
Table 2. Description of predominant bacterial phyla in the human body (Johnson and Versalovic, 2012).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Characteristics</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>Bacilli; Clostridia</td>
<td>Gram-positive; diverse in their morphology (rod, coccoid, spiral), physiology (anaerobic, aerobic); include commensal and beneficial bacteria</td>
<td>Lactobacillus; Ruminococcus; Clostridium; Staphylococcus; Enterococcus; Faecalibacterium</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Bacteroidetes</td>
<td>Gram-negative; composed of 3 large classes widely distributed in the environment, including soil, seawater, and guts of animals</td>
<td>Bacteroides; Prevotella</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Gammaproteobacteria; Betaproteobacteria</td>
<td>Gram-negative; include a wide variety of pathogens</td>
<td>Escherichia; Pseudomonas</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
<td>Gram-positive; diverse morphology; major antibiotic producers in the pharmaceutical industry</td>
<td>Bifidobacterium; Streptomyces; Nocardia</td>
</tr>
</tbody>
</table>

2.7 Analyzing the Gastro-Intestinal(GI) Microbiota

Purely culturable bacteria are necessary for comprehensive characterization of their biological and genetic natures. Culture-based methods are not sufficient for sampling communities of hundreds of different taxa present at a range of abundances in the microbiome. Only 10% to 40% of complex bacterial communities cultured through the selective growth media and special growth conditions (Hanson and Weinstock, 2016). However, a solution was created in 2007 when the National Institutes of Health supported “the Human Microbiome Project (HMP)” with the purpose to collect and integrate the composition and evolution of the human microbiome. Also, HMP displays aspects that will impact or affect the microbiome, and whether the human microbiome will be directly correlated toward particular diseases (Hattori & Taylor, 2009; Johnson and Versalovic, 2012).
The arrival of new molecular methods and technologies has been beneficial for the detection of uncultured microbes and may enable more microorganisms in the future. The culture-independent known methods are DNA pyrosequencing, microarrays (Phlytochip), next generation DNA sequencers such as 454-FLX (Roche), SOLiD (Applied Biosystems), PacBio, and Genome Analyzer (Illumina). DNA sequencing technologies and computational methods had been used to identify prokaryotic taxonomy in complete environmental samples such as the microbiome by working with the 16S ribosomal RNA gene (16S rRNA). The phylogenetic data analyses are required for a deeper understanding of the commensal residents, beneficial microorganisms, and their role in human health (Quail et al., 2008; Hattori & Taylor, 2009; Johnson and Versalovic, 2012).

Both methods, the culture-based, and 16-based have limitations for functional analysis. It is with metagenomics, which made possible to comprehensively explore the biological nature of complex communities (Weng et al., 2006). For sequencing the 16S rRNA gene it is important to make several considerations selecting the platform that will be used. The most important attention is to have sequence quality, also known as reads. The second consideration is the number of reads that each can obtain per run and dollar. Finally, the last consideration for 16S rRNA studies is the length of the study, as long the sequences are the fastest way to assign the operational taxonomic unit (OTU) (Kozich et al., 2013).

2.7.1 Next-Generation Sequencing Method: Illumina

Next generation sequencing (NGS) is innovating the molecular biology studies throughout a wide and rapidly growing range of applications. Due to the NGS improvements, microbial ecology has shifted its research from observational analyses to experimental characterization of the taxonomy in communities through the practice of complex experimental designs. This change
has been taken since the next-generation sequencing cost had become inexpensive and the availability of bioinformatic tools have increased. The Illumina sequencing technology has been under constant improvements, concerning instrumentation, processing software, and the sequencing chemistry, to accomplish the development of more data and longer reads (Minoche et al., 2011).

Illumina works using a chip-based bridge amplification procedure followed by a sequencing by synthesis utilizing reversible terminator dye nucleotides. Illumina depends on reagents and two platforms that are distinguished by their capacity of cycles, HiSeq2000 and MiSeq, which obtain 300 and 500 cycles respectively. The HiSeq2000 generates more than 50 Gbps per day using 100-nucleotide reads (i.e., during 10.8 days run produces 1.6 billion pairs of reads). By contrast, the MiSeq is a one-day run experiment and produces 1.5 Gbps per day using paired 150-nt reads (i.e., 5 million pairs of reads) (Caporaso et al., 2012). The platforms need the same reagents since the only difference is the number of cycles, so logistically is more difficult to fill up a 500 cycle than 300. Reagents for HiSeq2000 (300 cycles) are $500 per lane more expensive than MiSeq. HiSeq2000 platform is the standard method for shotgun metagenomic sequencing because it generates depth reads. However, MiSeq has better potential for 16S rRNA gene sequence studies because it produces longer reads for a low cost (Kozich et al., 2013).
CHAPTER 3. MATERIALS AND METHODS

3.1 Samples collection

3.1.1 Cocoa Powder Samples

Four cocoa powders and one hull sample were received from the Hershey’s Company®. The difference in the cocoa powder samples were chosen to evaluate the impact effect of alkalization process in the diversity of the gut microbiota. The samples were “lavado” unfermented unprocessed cocoa powder, “natural” unprocessed cocoa powder, “D-11-S” as alkalized cocoa powder, “D-11-B” heavily alkalized cocoa powder, and raw cocoa “shells” and a control of fecal matter (Table 3.). The difference in the cocoa powder samples were purposely chosen to evaluate the impact of these processing steps on the gut microbiota. All samples were analyzed for color and pH.

Table 3. Description of the cocoa powder samples from the Hershey’s Company®.

<table>
<thead>
<tr>
<th>Product Code/Sample ID</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lavado(^a)</td>
<td>Unprocessed cocoa powder (no alkalized)</td>
</tr>
<tr>
<td>Natural(^b)</td>
<td>Unprocessed cocoa powder (no alkalized)</td>
</tr>
<tr>
<td>D-11-S(^c)</td>
<td>Dutch (alkalized)</td>
</tr>
<tr>
<td>D-11-B(^d)</td>
<td>Dutch (alkalized)</td>
</tr>
<tr>
<td>Shells</td>
<td>Grinded hulls</td>
</tr>
</tbody>
</table>

\(^a\) “Un-dutch” or no alkalized cocoa powder, unfermented beans with roasting. \(^b\) Unsweetened that had most of its fat removed before being ground into powder. \(^c\) Dutch-process cocoa. \(^d\) Cocoa heavier Dutch than usual.

3.1.2 Fecal Samples

The human fecal samples were collected from 7 different human subject donors. Donors were free of antibiotics for at least three weeks prior to the collection of fecal samples. Each donor kept records of his/her diet for five days; their diets were based on dairy products, bread, chicken, vegetables, fruits, and beef.
The fecal sample of each contributor was collected on the fifth day of their diets. Samples were immediately stored in an ultra-low temperature freezer (-80°C) until it was used.

3.2 Color Analyses

The samples’ color measurement was determined per treatment as triplicates to characterize each sample. The color was measured using a chroma meter LABSCAN XE (Hunterlab, VA) fitted with a pulsed xenon lamp and an aperture diameter of 13 mm. Each sample was placed into a sample tray and fitted into the aperture to record the surface color. CIELAB color scales were used and reported as $L^*$, $a^*$ and $b^*$ values. $L^*$ values describe the lightness (100) to darkness (0) measurements on the axis. The $a^*$ values assess the red-green hues, corresponding positive values to redness and negative to greenness. The $b^*$ axis refers to yellow-blue hues, with positive values referring to yellowness and negative representing blueness (Sharma 2003).

3.3 pH measurement

The pH was measured using a Mettler Toledo pH meter. The pH is an important parameter in fermentation assessment because it can reflect microbiota growth. Also, the pH parameter is associated with the alkalization process of cocoa samples.

3.4 Cocoa sample pre-digestion

A protocol mimicking the human digestion and absorption process in the stomach and small intestine was established (Goita, 2013). Cocoa substrates were pretreated in the model *in-vitro* digestive system to simulate the products that would reach the colon microbiota for fermentation. The enzymatic digestion involved pepsin digestion followed by pancreatin, to remove the digestible, non-fibrous contents of the substrate. Pepsin is an enzyme used to degrade the protein into mostly water-soluble peptides; and the pancreatin (a mixture of amylase, lipase, and protease) degrades protein, peptides, starches, and lipids. The protease works to hydrolyze
proteins into oligopeptides; amylase hydrolyzes starches into oligosaccharides and the 
disaccharide maltose, and lipase hydrolyzes triglycerides into fatty acids, monoacylglycerols, and 
glycerol. The digestion process was conducted as followed:

Sixty (60) g of freeze dried cocoa sample was added to 200 mL distilled water. After, 
hydrochloric acid (HCl) was added to bring the solution to a pH of 2. The mixture was incubated 
for 10 minutes in a shaking water bath to bring the temperature to 37°C. Next, 0.5 g (3500 U/mg) 
of pepsin was added to the mixture and incubated while shaking for another 3 hours. Then the 
sample pH was brought to 7.5 with a sodium hydroxide (NaOH) solution and incubated for 10 
minutes to bring back the temperature to 37°C before adding 4.0 g of pancreatin (SIGMA-
ALDRICH) and 1 g of bile salts (sodium cholate and sodium deoxycholate mixture from SIGMA-
ALDRICH). The solution was mixed thoroughly for 10 minutes and then incubated at 37°C for an 
extra 2 hours. The mixture was microwaved for 3-5 minutes for the enzymes denaturation and then 
filtered to obtain the solids. Those remaining solids from the mixture were washed with ethanol 
and filtered again and freeze dried overnight. The dried substrate was a powder that was kept 
frozen in a -80°C freezer until utilized for the fermentation step.

3.5 Cocoa sample fermentation

Throughout the fermentation process, an anaerobic buffer solution was used and it was 
prepared according to the following steps. The anaerobic solution was created with two solutions, 
A and B, at a 9:1 ratio. The solutions were prepared as mentioned in the following steps:
Solution A was used as a redox (oxidation-reduction) indicator. To prepare solution A several 
components were added, 11.76 g of sodium bicarbonate (NaHCO₃), 11.1 ml of hemin (0.78 
mmol/L of water), 1.1 ml of menadione (0.36 mmol/L of water) and 1.1 ml of resazurin (3.98 
mmol/L of water) to 1 liter of distilled water. The solution was autoclaved for 15 minutes at 121°C.
Solution B had a composition of 0.48 mmol of sodium chloride (NaCl), 0.02 mmol of dipotassium phosphate (K₂HPO₄), and 0.63 mmol L-Cysteine-HCl, in a liter of autoclaved distilled water.

The *in-vitro* bacterial inoculum was prepared using 50 g of fecal sample mixed with 200 mL of the anaerobic buffer. Then the mixture was filtered with either cheesecloth or a filter whirl pack bag. The solution was incubated at 37°C under anaerobic conditions for an hour. Sterile glass bottles contained 175mL of the anaerobic solution (solution A and B) and 5 g of the fermentation substrate. Then, an aliquot of 25mL of the incubated fecal bacteria solution was added to each vessel and flushed with an anaerobic gas composition (10% CO₂, 80% N₂ and 10% H₂) for 10 minutes. Subsequently, samples were incubated at 37°C while stirring. Finally, processed samples were collected in 15 mL falcon tubes at 0, 6, 12, and 24 hours, and stored at -80°C until usage. The *in-vitro* fermentation in a digestive model system was done in duplicates for each separate experiment.

### 3.6 Nucleic Acid Extraction and sequencing

#### 3.6.1 DNA Extraction

Using the PowerSoil® DNA Isolation Kit (MoBio), DNA was extracted from 250 µl of stool added to the power beads tube with lysis buffer to ensure bacterial cell lysis. Purified genomic DNA was subjected to fragmentation, ligation to sequencing adapters for identification of individuals samples, and purification. After the amplification and denaturation steps, libraries were pooled and sequenced. The quantity of DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Purified DNA was stored for future analysis at -20°C.
3.6.2 Sequencing

The bacterial genomic DNA was amplified with the specific primers for the universal Eubacterial 16S rRNA gene 515F (5´-GTGCCAGCMGCCGCGGTAA-3´) and 806R (5´-GGACTACHVGGGTWTCTAAT-3´). These primers result in amplicons that are produced from hypovariable and hypervariable regions of the gene and were used to assess the microbial ecology of all samples using Illumina HiSeq methods by bTEFAP® DNA analysis service. All single-step 30 cycle PCRs were performed using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). On the first cycles of denaturation the settings used were 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds. In the annealing process, a condition of 53°C for 40 seconds and 72°C for 1 minute were established. Finally, the elongation step was performed at 72°C for 5 minutes. All amplicon products from samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were pooled and sequenced utilizing the Illumina HiSeq 2500 chemistry following manufacturer’s recommendations.

3.6.3 Bioinformatics

Raw Illumina sequencing reads obtained from the sequencer were denoised using a proprietary pipeline from MR. DNA software (MR DNA, Shallowater, TX). The resulting Illumina reads were filtered according to barcodes to identify different samples. Preliminary quality control steps included the removal of sequences shorter than <200bp, and all reads containing ambiguous base calls and sequences with homopolymer runs longer than 6 nucleotides. Chimera sequences arising from the PCR amplification were detected and excluded from the denoised sequences.
3.6.4 Phylogenetic analysis

Operational taxonomic units (OTUs) were generated and defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from GreenGenes/RDP/NCBI. The RDP runs were quality-controlled, aligned for annotation of Bacterial and Archaeal 16S rRNA sequences. For the metagenomics visualization, a web browser known as Krona was set. Krona is a new visualization tool that allows intuitive exploration of relative abundances and confidences within the complex hierarchies (Ondov et al., 2011).

3.7 Data Analysis

3.7.1 Color and pH analysis

Data was statistically analyzed using SAS software version 9.4 (SAS Institute Inc., Cary, NC). Mean values of triplicate analyses were reported with their standard deviations. To determine differences between treatments and within treatments during the fermentation time, Analysis of Variance (ANOVA) and Tukey’s studentized range test was used at a significance level of $P \leq 0.05$.

3.7.2 Alpha and beta diversity statistical analysis

The OTUs that reached a 97% nucleotide similarity level were used for the alpha diversity (Shannon-Wiener) and rarefaction curve analysis using Qiime. Alpha diversity calculates how many bacterial species are within each given sample, and is expressed by the number of species found in the ecosystem. A heat map was created on the basis of the relative abundance of OTUs using “R” (The “R” Project for Statistical Computing). Phylogenetic beta diversity was performed using OTUs for each sample using the Qiime program. This analysis creates an individual
phylogenetic tree for each sample. Afterward, the tree is evaluated statistically for each sample and subsequently, a coordinate analysis is applied to visualize ten separate jackknife iterative comparisons.

The beta diversity specifically allows the comparisons of the bacteria community as a whole, in consideration of the variety of things in each sample and how these unknown sequences are phylogenetically related. The principal coordinate analysis (PCoA) was conducted according to the weighted UniFrac distance matrix, calculating the relationships between organisms phylogenetically. Weighted UniFrac is the dominant method in the field for pairwise distance measurements. This method requires higher presence of different OTUs rather than just the mere presence as in unweighted. Also, it is considered a powerful method for analyzing the complex data sets, with many samples and extensive metadata, created in microbial ecology (Lozupone et al., 2010; Golob et al., 2017). PCoA and UniFrac were used to examine the correlation between hours of fermentation and treatments.

The statistical analysis was done using a variety of computer packages including XLstat, NCSS 2007, and NCSS 2010. Monte Carlo simulation was performed to detect features with significant differences from the samples remaining. All tests considered had to represent statistical significance of p<0.05.
CHAPTER 4. RESULTS AND DISCUSSION

4.1 Color analysis

The texture, flavor and color of cocoa powder is dependent on the type of processing it goes through. The “Dutch” process, also known as alkalinization, affects these characteristics. Alkali treatment involves adding agents, NaOH or K₂CO₃, dissolved in water directly to the powder allowing the mixture to react. This process neutralizes the normal cocoa acidity, raises the pH, darkens the cocoa, reduces the acid and astringent taste of cocoa improving its flavor (Li et al., 2012). Representative samples of natural cocoa, along with a series of dark brown/black and reddish alkali-processed were used in this study.

Polyphenols in cocoa powder are associated with health benefits and also are involved in the production of the brown cocoa color and the astringent taste (Li et al., 2014). The cocoa samples that have not been alkalinized, natural and lavado, have a pale to light brown color. The D-11-S and D-11-B showed a range going from red/brown to dark brown/black (Table 4). Although all alkalinized cocoa powders can vary in color from reddish brown to darker brown, and this characteristic is dependent of the alkalinization process. As Li and collaborators (2012) explained, this process improves the flavor and appearance of a wide range of products and therefore is an important procedure for chocolate and food manufacturers.

Table 4. Characteristics of the cocoa samples in color and pH before the digestion and then fermentation.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Color Description⁷</th>
<th>Color L*</th>
<th>Color a*</th>
<th>Color b*</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-11-B</td>
<td>Dark brown/black</td>
<td>23.73±0.41ᵃ</td>
<td>8.32±0.06ᵈ</td>
<td>10.76±0.14ᵈ</td>
<td>7.65±0.01ᵃ</td>
</tr>
<tr>
<td>D-11-S</td>
<td>Red/Brown</td>
<td>40.63±0.37ᵇ</td>
<td>18.81±0.01ᵃ</td>
<td>26.70±0.02ᵃ</td>
<td>6.87±0.03ᵇ</td>
</tr>
<tr>
<td>Natural</td>
<td>Light brown</td>
<td>50.23±0.58ᶜ</td>
<td>15.64±0.04ᵇ</td>
<td>28.17±0.17ᵃ</td>
<td>5.19±0.04ᵈ</td>
</tr>
<tr>
<td>Lavado</td>
<td>Pale brown</td>
<td>52.95±1.74ᵈ</td>
<td>12.09±0.38ᶜ</td>
<td>20.02±0.84ᵇ</td>
<td>5.41±0.06ᶜ</td>
</tr>
<tr>
<td>Shell</td>
<td>Dark and light brown</td>
<td>35.74±1.68ᵉ</td>
<td>11.41±1.13ᶜ</td>
<td>14.91±1.97ᶜ</td>
<td>5.17±0.04ᵈ</td>
</tr>
</tbody>
</table>

The means and standard error with the same letters are not significantly different (P<0.05). ⁷Color description refers to a visual observation.ᵃ–ᵈ means with a different letter along each column represents significantly difference between treatments.
The colors of the cocoa samples were characterized by a chroma meter LABSCAN XE using $L^*$, $a^*$ and $b^*$ values (Table 4). The lavado and natural cocoa powder had the highest $L^*$ values being significantly different. While the powders that have gone further with the Dutch process showed a significant decrease in $L^*$ values. Due to the fact that D-11-B is a sample that has been heavily alkalized it has the lowest $L^*$ value. The $a^*$ and $b^*$ values were significantly different almost in every sample, only lavado and shells were the same on $a^*$; and for $b^*$ D-11-S and natural reported similarities.

The $a^*$ scale reveals that un-alkalized samples and shells have values between 11 to 15, but the heavily alkalized D-11-B have the lowest $a^*$ value; indicating a significant reduction of the red component. As the color increases to brown/black a progressive reduction in polyphenol content was evident. During alkalization the anthocyanins, procyanidins, and catechins, are changed into quinones, which form high molecule weight insoluble brown compound. Hence, the darker the cocoa powder is, less astringent, and a basic pH but a decreased in the polyphenol content will be notice (Li et al., 2014).

4.2 Gut microbiota

A total of 3,799,034 sequences were parsed and 3,309,403 were clustered. After a high-quality sequence curation, the Bacteria and Archaea domain were utilized for the final microbial analyses with a total of 3,308,382 sequences identified; the remaining 1,021 sequences belonged to the domain of Eukaryota, Metazoa, and Viridiplantae domains. For the final microbial analyses, 3,301,825 sequences were identified within the bacteria domain, representing 99.7% of high-quality reads (Table 5).
At 0 hours, the sample that showed the highest microbial diversity was D-11-S, followed by D-11-B and shells. For the 6 and 12 hours, besides the control, samples that had more sequence counts were D-11-B and lavado. At 24 hours, the order of maximum readings were D-11-S, shells, and lavado.

**Table 5.** Comparison of sequence amount estimation of the 16S rRNA gene libraries at 97% similarity from the Illumina sequencing analysis before trimming and quality filtering.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0 hours</th>
<th>6 hours</th>
<th>12 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>263714</td>
<td>39286</td>
<td>38290</td>
<td>223730</td>
</tr>
<tr>
<td>D-11-B</td>
<td>278152</td>
<td>34950</td>
<td>30598</td>
<td>217703</td>
</tr>
<tr>
<td>D-11-S</td>
<td>282044</td>
<td>29876</td>
<td>22853</td>
<td>244252</td>
</tr>
<tr>
<td>Natural</td>
<td>252275</td>
<td>33955</td>
<td>25765</td>
<td>231307</td>
</tr>
<tr>
<td>Lavado</td>
<td>177988</td>
<td>32914</td>
<td>31804</td>
<td>239968</td>
</tr>
<tr>
<td>Shells</td>
<td>273241</td>
<td>28328</td>
<td>29686</td>
<td>240724</td>
</tr>
</tbody>
</table>

After cleaning, quality filtering and dereplication, the input sequences were ordered according to their abundances considering that the high abundance reads are more likely correct and convenient to use as cluster seeds. Based on the results tested by Mr. DNA lab, the operational taxonomic units (OTU) analysis were defined after the removal of singleton sequences, clustering at 3% (97% similarity).

For alpha and beta diversity analysis, samples were rarefied to 25,000 sequences and bootstrapped at 20,000 sequences. The Shannon-Wiener Index curve plot reaches a plateau at approximately 5,000 sequences indicating that sequencing depth was sufficient to capture the full scope of microbial diversity making the data reliable. The Shannon-Wiener Index (Figure 2), is a measured to define the alpha-diversity, both evenness and number of distinct organisms within samples, within an environment.
Figure 2. Ecological representation of microbial communities: alpha diversity. (A) Shannon-Wiener curve, alpha diversity captures the richness of a sample and the evenness of the organisms’ abundance distribution. (B) Rarefaction curve, the plot of the number of species as a function of the number of samples. (A) and (B) were calculated based upon 97% similarity.

Fourteen phyla were found based in all the extractions of all the samples. Three phyla (Firmicutes, Proteobacteria, and Bacteroidetes) were the most predominant in cocoa fermented with fecal samples and comprised >96% of all the sequences. The Firmicutes are composed of gram-positive bacteria, Proteobacteria and Bacteroidetes are gram-negative bacteria.
The highest proportion (p<0.05) of Firmicutes was present in sample D-11-B within the non-fermented cocoa (0 hours) compared to the fermented D-11-B cocoa (24 hours). After fermentation, the presence of Firmicutes in D-11-S, lavado, and cocoa shells were reduced by 60%, 55%, and 55%, respectively. Before fermentation Proteobacteria had a low percentage of relative abundance in all samples. After 24 hours, the abundance of this phylum increased in lavado and D-11-S by 57% and 54%, respectively. Conversely, Bacteroidetes relative abundance without fermentation (0 hours) presented an average between 16% and 23%. Cocoa shells and D-11-S samples had an increased percentage of Bacteroidetes of 22% and 8%, respectively after fermentation (Table 6).

Table 6. Comparison of relative abundance of phylum level between the control and samples during the fermentation time.

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Phylum (%)</th>
<th>Time</th>
<th>Control</th>
<th>Phylum per Sample (%)</th>
<th>D-11-B</th>
<th>D-11-S</th>
<th>Natural</th>
<th>Lavado</th>
<th>Shells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Firmicutes</td>
<td>51</td>
<td>0h</td>
<td>78</td>
<td>77</td>
<td>76</td>
<td>72</td>
<td>70</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6h</td>
<td>80</td>
<td>68</td>
<td>46</td>
<td>61</td>
<td>53</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12h</td>
<td>62</td>
<td>60</td>
<td>40</td>
<td>55</td>
<td>57</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>26</td>
<td>33</td>
<td>16</td>
<td>36</td>
<td>15</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>29</td>
<td>0h</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6h</td>
<td>8</td>
<td>20</td>
<td>40</td>
<td>21</td>
<td>33</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12h</td>
<td>32</td>
<td>30</td>
<td>49</td>
<td>30</td>
<td>29</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>70</td>
<td>54</td>
<td>56</td>
<td>56</td>
<td>60</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>18</td>
<td>0h</td>
<td>16</td>
<td>17</td>
<td>19</td>
<td>21</td>
<td>23</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6h</td>
<td>5</td>
<td>8</td>
<td>13</td>
<td>14</td>
<td>12</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12h</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>12</td>
<td>11</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>2</td>
<td>11</td>
<td>27</td>
<td>6</td>
<td>24</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>2</td>
<td>0h</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6h</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12h</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24h</td>
<td>0.8</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

These results were in accordance with the study done by Camps-Bossacoma and other (2017), where they tested the effect of a cocoa-enriched diet (40.18 mg/g of catechin) on gut microbiota in rats. In this study, cocoa diet influenced the bacterial pattern by decreasing the
percentages of Firmicutes phylum and increasing Bacteroidetes, Proteobacteria, and Actinobacteria as the result of this cocoa product. Camps-Bossacoma and collaborators associate their outcome with the cocoa composition, which is rich in polyphenols (flavonoids), also carbohydrates, proteins, lipids, fiber, and minerals. It has been reported that polyphenols have diverse prebiotic effects and antimicrobial activities, and excessive amounts of polyphenols may inhibit the growth of beneficial microorganisms (Chodak, 2012).

Another possible mechanism is the content of polyphenols on the gut microbiota composition. Processing cocoa through the alkalization process had shown that components such as polyphenols are lost or reduced (Li et al., 2013). Many studies have been conducted to investigate the ratio between the most abundant phylum in the GI tract, Firmicutes and Bacteroidetes. The F:B ratio has been associated with obesity or weight loss (Cho and Blaser, 2012; Sanz et al., 2013).

In the study by Duda-Chodak and collaborators (2015) they determined that higher levels of F:B were found in obese subjects than those maintaining a natural weight. This indicates that it may be possible to incorporate D-11-B or natural cocoa into a diet for a possible decrease in the ratio of F:B in the gut microbiota to maintain a natural weight. Comparatively the increase in the presence of Bacteroidetes from the D-11-S, lavado, and shells could lessen weight gain when incorporated into a diet. Methylxanthines induce acid and pepsin secretions in the GI tract. A synergistic interaction between methylxanthines and flavonoids is suggested by Dulloo (2011) and may be involved in the effects on body weight. The interaction of cocoa polyphenols and dietary fiber repress the growth of certain bacteria modifying microbiota composition and immune response. However, the sole presence of cocoa polyphenols does not have a direct effect on body weight (Massot-Cladera et al., 2015).
The enhancement of the growth of beneficial bacteria produced by cocoa diets could be partially attributed by the fecal pH. The acidification can be a consequence of the prebiotic effect of fiber, promoting the growth of SCFA-producing bacteria, which are capable of surviving in this environment. These conditions suppress as well the growth of Proteobacteria. In other studies, they compared the ratio of Firmicutes, Bacteroidetes, and Proteobacteria, associating to food allergies, obesity, and inflammatory bowel disease (IBD) (Tremaroli & Backhed, 2012; Ling et al., 2014).

**Figure 3.** Estimated predominant genera present in samples.
The height of each band of color is proportional to the relative abundance of each sequence type. Color boxes represent the phylum that genera belong: purple (Proteobacteria), red (Bacteroidetes), gray (Actinobacteria), blue (Verrucomicrobia); genera that do not have a box belongs to Firmicutes.

Sequence reads from the fermented cocoa samples could be classified into 165 genera. Of the total number of genera identified in the samples analyzed, 32 genera were predominant. The most predominant genera were *Bacteroides* (14%), *Escherichia* (11%), *Eubacterium* (9%), *Faecalibacterium* (8%), *Shigella* (6%), *Clostridium* (6%), *Comamonas* (6%).
Ruminococcus (5%), Subdoligranulum (4%), Enterobacter (3%), Blautia (3%), Bifidobacterium (2%), Roseburia (2%), Oscillospira (2%), and Parabacteroides (2%). Among the shared predominant genera in all the treatments, Bacteroides and Parabacteroides belonged to the phylum Bacteroidetes. Escherichia, Comamonas, Enterobacter, and Shigella are members of Proteobacteria phylum. Correspondingly, for Actinobacteria it would be Bifidobacterium and the remaining nine genera belonged to Firmicutes. (Figure 3).

In the control group (fecal sample without cocoa), fifteen genera were predominantly detected. The genera that increased in percentages through fermentation time belonged to the Proteobacteria phylum. Specifically, the microbiota of the control after fermentation was characterized by a depletion of the two phyla of bacteria, Firmicutes and Bacteriodetes. A disruption of homeostasis, by environmental or host factors, such as a low-fiber diet can cause dysbiosis with a growth of Proteobacteria in the gut (Shin et al., 2015). Proteobacteria increasing ratios through fermentation can be due to substrate limitations. The most prevalent growing genus through time was the Comamonas. The species detected was Comamonas kerstersii that is considered non-pathogenic, and a non-fermenting Proteobacteria (Opota et al., 2013). The species of Comamonas related to human diseases is Comamonas testosteroni, but they are rarely clinically significant as all Comamonas (Arda et al., 2013).

With regard to the D-11-B group, heavily alkalized cocoa, ten genera were identified. Of the ten, four genera reside in the Proteobacteria phylum including Escherichia, Shigella, Enterobacter, and Comamonas. Escherichia and Shigella had a significantly increase at the 6 hours fermentation period. The point of growth for Enterobacter and Comamonas was at 12 and 24 hours, respectively. Bacteroides and Parabacteroides stayed consistent through the 24 hours of fermentation while Firmicutes decreased.
The fermentation of the D-11-S cocoa sample group, alkalized treatment, had seven genera that were predominant. Some changes in the microbiota composition appeared due to limited substrate for the samples and the change in pH (Massot-Cladera et al., 2015). It is worth noting that the genus *Bacteroides* percentage increased at the 24 hours, as well as *Parabacteroides*. Also, some bacteria like *Bifidobacterium* colonized the mucus layer and start growing. Further five bacterial genera from the Firmicutes phylum almost disappeared during the cocoa fermentation after 24 hours.

Observing the results on the natural cocoa samples, *Bacteroides* genus decrease in percentages during 24-hour fermentation, in contrast with the rest of the samples. The principal genera representing Firmicutes such as *Eubacterium*, *Faecalibacterium*, *Clostridium*, and *Ruminococcus* remained constant over time, showing a minimal decrease at the 24 hours of fermentation. *Escherichia*, *Shigella*, and *Enterobacter* genera from Proteobacteria, at the 0 hours percentages was minimal having a growth at the 24 hours.

In regard with lavado and shells, these two samples had similar results. Proteobacteria represented genera increase and Firmicutes decrease through time. It is worth observing that the quantity of *Bacteroides* and *Parabacteroides* had an increase in percentages at 24 hours. Mirpuri and collaborators (2014) describe that Firmicutes or lack of Bacteroidetes expansion is due to competition or inhibition by Proteobacteria, however, the precise mechanism is unknown. As previously stated, Proteobacteria is abundant in the gut microbiota; Camps-Bossacoma and others (2017) stated that the more relative abundance of Proteobacteria, the higher are the IgA levels.

To provide a visual overview combined with the previous analysis, we utilize a dual hierarchal dendrogram to display the data for the predominant genera with clustering related to the different groups. The heatmap is compared with the same proportion of bacterial diversity. The
analysis revealed four major groups of microbial communities. The most diverse communities were those at the end of the fermentation, and this was reflected in the Shannon diversity (Figure 3). Based on the clustering in Figure 4, it showed a significant difference between the 0 hours and 24 hours collection samples. Upon closer analysis, it also displayed similarities in the microbial community of the populations treated with shells and D-11-S at hours 6 and 12 closely resemble the microbial composition of the natural or D-11-B populations at hour 24.

Genera that were in the samples but almost absent or disappear during the fermentation were Butyrivibrio, Akkermansia, Lysinbacillus, Acetobacter, Bacillus, Klebsiella, Methanobrevibacter, Lachnoclostridium, Dialister, Preludibacter, and Lactobacillus. Genera growing through fermentation were Enterococcus, Enterobacter, Shigella, Escherichia belonging to Proteobacteria; Bacteroides and Parabacteroides belonging to Bacteroidetes.

By the abundance of Bacteroides and Parabacteroides, and the presence of Bifidobacterium, D-11-B and natural cocoa at 6 hours of fermentation array a healthy pattern of the diversity in the gut microbiota. This healthy pattern is attributed to the SCFA, acetate, propionate, and succinate, produced by the fermentation of the Bacteroides as previously stated by Duncan and collaborators (2004), and Louis and collaborators (2007). The presence of Bifidobacterium, a probiotic, improves the digestion absorption in the immune system while enhancing Eubacterium ability to convert lactate into acetate and butyrate (Leahy et al. 2005; Sato et al. 2008).

It is well known that non-digestible complex carbohydrates are metabolized by the human microbiota to oligosaccharides and then fermented to SCFA (Massot-Cladera et al., 2015). After the fermentation of cocoa powder and fiber, both reach the colon intact. There the commensal bacteria have the opportunity to metabolize and influence the intestinal environment and the
immune system. As a genus of importance in this study was *Bacteroides*, Bird and collaborator (2000) state in their investigation that *Bacteroides* by itself does not produce butyrate as the final product.

**Figure 4.** Dual hierarchal dendrogram evaluation of the taxonomic classification data. Sample clustered on the X-axis is labeled based upon the treatment. Samples with more similar microbial populations are mathematically clustered closer together. The genera (consortium) are used for clustering. Thus, the samples with a more similar consortium of genera cluster closer together with the length of connecting lines (top of heatmap) related to the similarity, shorter lines between two samples indicate closely matched microbial consortium. The heatmap represents the relative percentages of each genus. The predominant genera are represented along the right Y-axis. The legend for the heatmap is provided in the upper left corner.
Therefore, Louis and collaborators (2007) mentioned that *Bacteroides* stick to the surface of starch molecules and ferment dietary fiber into intermediate products and with the collaboration of other species they can produce butyrate. A sample that has high percentages of these genera was cocoa shells, and it was also the sample that presents the lowest pH, affirming the investigation of Walker and others (2005) that butyrate production is done in anaerobe acidify environment.

Making this relation, butyrate is related as the major source of energy for epithelial cells of colonic mucosa and stimulates cells proliferation (Cummings, 1981). Also, gives protection against cancer and ulcerative colitis by blocking the absorption of carcinogenic substances and making the colon less susceptible to DNA damage (Kushkevych, 2014). In addition, the absorption of calcium (Ca) increased through the butyrate helping in the maintenance of a healthy epithelium (Gibson and Mccartney, 1993; Asp et al., 1996). The growth of *Bacteroides* and *Parabacteroides* after the 24 hours of fermentation is commonly related to positive benefits in health.

For beta diversity analysis, a principal coordinate analysis plot was generated based upon the weighted UniFrac distance matrix. The coefficient shows the monotonicity between the true distance between communities and the times. Figure 5 displays the weighted UniFrac plot of the collection time (Fig.5A.) and to complement the weighted UniFrac plot of the treatment (Fig. 5B.). After examining each UniFrac plot, it appears there is a strong correlation between phylogenetic assemblage and collection time. Additionally, there appears to be phylogenetic assemblage among each treatment group collected at both 6 hours and 12 hours that is significantly different from the remaining collection times; with exception of the natural and D-11-B samples collected at 6, 12, and 24 hours. The shells demonstrated the highest percent variation at the 24 hours.
4.3 pH

The pH in cacao is closely related to the proportion of fatty acids and the fermentation process. As what appeared in (Table 7) the control was significantly different from all the samples within the hours (P<0.05). At the starting time (0 hours) pH in the *in vitro* fermentation, there was a statistical difference between D-11-S and shells. The major decrease in pH during fermentation was reflected after 6 hours when all the treatments show a significant reduction over time. In the pH shells had a consistent tendency of having the more acidic values, followed by lavado and natural that were statistically similar. The alkalinized treatments, D-11-B and D-11-S, regardless of the strength of the process showed a statistically equal pH over time. Of all the treatments, the control had the more basic pH. These results of pH reduction can be attributed to the presence of microorganisms in the samples fermenting carbohydrates.
Table 7. The pH change of cocoa samples when fermented in an in-vitro digestive model system.

<table>
<thead>
<tr>
<th>Samples</th>
<th>0 Hour</th>
<th>6 Hour</th>
<th>12 Hour</th>
<th>24 Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.85±0.14aA</td>
<td>7.83±0.08abA</td>
<td>7.70±0.02bcA</td>
<td>7.59±0.07cA</td>
</tr>
<tr>
<td>D-11-B</td>
<td>7.61±0.06aBC</td>
<td>7.35±0.04bbB</td>
<td>7.24±0.02cbB</td>
<td>7.11±0.04dB</td>
</tr>
<tr>
<td>D-11-S</td>
<td>7.63±0.14abB</td>
<td>7.34±0.03bbB</td>
<td>7.23±0.02bcB</td>
<td>7.20±0.03dB</td>
</tr>
<tr>
<td>Natural cocoa</td>
<td>7.56±0.11aBC</td>
<td>7.09±0.02bcC</td>
<td>7.02±0.03bcC</td>
<td>6.95±0.05cC</td>
</tr>
<tr>
<td>Lavado</td>
<td>7.60±0.02aBC</td>
<td>6.96±0.09bCD</td>
<td>7.00±0.04bcC</td>
<td>6.93±0.08bcC</td>
</tr>
<tr>
<td>Shell</td>
<td>7.44±0.06aC</td>
<td>6.92±0.13bD</td>
<td>6.90±0.09bD</td>
<td>6.69±0.14cD</td>
</tr>
</tbody>
</table>

The means and standard error with the same letters are not significantly different (P<0.05). a-d means with a different letter along each row are significantly different in each treatment through time. A-D means with a different letter along each column represent significantly difference within hours between treatments.

Maekivuokko and collaborators (2007) associated pH and fermentation process with the production of short chain fatty acids, influencing the human microbiome. These results remark that production of short-chain fatty acids in cocoa powder as a substrate. Koziółek and others (2005) demonstrate that SCFA and pH had an inverse relationship if SCFA increases pH reduction will be noticeable. Similarly, Walker and others (2005) found that in anaerobic fermentation, increased butyrate production resulted in a lower pH (5.5 to 6.5) and increased acetate produced a higher pH. Considering that the shell sample has the lowest pH, reasonably it will have a diverse microbiota, and for further experiment have a greater butyrate concentration. Likewise, the pH fluctuations and SCFAs production indicate that carbohydrates in cocoa can be used as prebiotic by human microbiota.
CHAPTER 5. SUMMARY AND CONCLUSIONS

In this study, we determine the effect of the alkalization process of the cocoa bean in the diversity of the gut microbiota throughout an in-vitro fermentation. Analyses performed for color and pH were directly related to the presence of polyphenols in the cocoa samples.

Concerning color and pH study, polyphenols in cocoa powder are associated with health benefits and also are involved in the production of the brown cocoa color and the astringent taste. These characteristics are directly affected during alkalization. The anthocyanins, procyanidins, and catechins are changed into quinones, which form high molecular weight insoluble brown compound (Li et al., 2014). Sample D-11-B was the darkest cocoa with a basic pH. The processed samples, D-11-S, natural, and shells showed dark color but lighter than D-11-B. Making lavado the lightest cocoa powder because it is unfermented and unprocessed. Thus, the darker cocoa powder is, the less astringent it will taste, but it will decrease polyphenol content.

Overall the alkalization process did affect the diversity of the gut microbiota, but the effect was not consistent for only alkalinized cocoa. The reduction of Firmicutes and Bacteriodetes (F:B) by the non-alkalinized natural cocoa and the alkalinized D-11-B affect the diversity of the gut microbiota and promote a natural weight. The percentage of Bacteriodetes increase in the alkalinized D-11-S, the non-alkalinized natural cocoa, and the shells attributing to a reduce weight gain when incorporated into a diet. Increases in the growth of the gut microbiota from all samples of cocoa were present.

The analysis of pH correlated to microbial diversity and helped us understand what form of cocoa was more active during the fermentation, alkalized or non-alkalized. The major decrease in pH during fermentation was reflected after 6 hours. The non-alkalized shells had the largest drop in pH over the 24 hours. Based on the microbial diversity of Bacteroides and
Parabacteroides, and the presence of Bifidobacterium in samples D-11-B and natural cocoa at 6 hours of fermentation array a healthy pattern in the gut microbiota. This healthy pattern is attributed to the fermentation products as acetate and butyrate by Bacteroides, Parabacteroides, Bifidobacterium and Eubacterium.

In conclusion, cocoa powder can be used as a prebiotic and its consumption may aid in the prevalence of a beneficial microbiota in the human gut. Of the samples presented both D-11-B and natural cocoa presented microbial diversity in the gut microbiota. They both had reduced ratios of Firmicutes to Bacteriodetes that are associated with weight management. The color profile of the D-11-B is a dark/brown, which had been through alkalization to darken the powder and remove bitterness. Natural cocoa has a light brown color and has not been processed. The pH of the alkalized D-11-B is basic as compared with the acidic pH of the natural cocoa. These color and pH variation between the two samples could allow manufacturers to utilize microbial diverse cocoa and have the option of color and pH dependent upon its future use. Also, cocoa shell has a good profile for the consumption and develop a diversity microbiota.
REFERENCES


Kushkevych, I. V. (2014). Etiological Role of Sulfate-Reducing Bacteria in the Development of Inflammatory Bowel Diseases and Ulcerative Colitis, 2, 63–73.


Appendix A. Cocoa samples appearance.
Appendix B. The pH change of cocoa samples when fermented in an in-vitro digestive model system.
Appendix C. Complete metagenome – phylotype tree of all samples during the entire experiment.
Appendix D. Complete metagenome – phylotype tree of control sample at 0 and 24 hours.
Appendix E. Complete metagenome – phylotype tree of D-11-B sample at 0 and 24 hours.
Appendix F. Complete metagenome – phylotype tree of D-11-S sample at 0 and 24 hours.
Appendix G. Complete metagenome – phylotype tree of the natural sample at 0 and 24 hours.
Appendix H. Complete metagenome – phylotype tree of lavado sample at 0 and 24 hours.
Appendix I. Complete metagenome – phylotype tree of shells sample at 0 and 24 hours.
VITA

Martha Marina Escoto Sabillón was born and raised in Tegucigalpa, Honduras. She attended her elementary and high school in the present city. In 2010, she enrolled as an undergraduate student at Zamorano Pan-American Agricultural School and she later performed a senior internship at Louisiana State University in Louisiana, USA. She obtained her Bachelor of Sciences in Food Science and Technology in December 2014. After completion, she became a graduate student in the School of Nutrition and Food Sciences in January 2015 at Louisiana State University and expects to graduate in December 2017.