1. INTRODUCTION

1.1. Background

The research of a chemopreventive agent for colorectal cancer had been widely conducted for decades, regarding its third position of the most diagnosed cancer in the world. Among cases, 70% was a sporadic case where the patient did not have any family history and genetic mutation on colorectal cancer, therefore it was proposed that diet and lifestyle might be the factors that trigger cancer formation (De Almeida *et al*., 2019). Diet, in specific, was the focus in most of the research as the major factor in triggering colorectal cancer (Wong *et al*., 2019). This was related to the role of the colon as one part of the intestinal tract where all the fermentation occurs with the help of gut microbiota.

Among dietary compounds, anthocyanin has been reported to depend on gut fermentation in order to be absorbed (Ozdal *et al.*, 2016). The breakdown of anthocyanin will result in the release of aglycone and sugar, from which sugar was an energy source for microbiota. Anthocyanin aglycone was also reported with its antibacterial activity toward pathogenic bacteria, thus the theory of anthocyanin consumption could affect gut microbiota composition was presented (Zhang, *et al*., 2016). Other than that, this compound was known with high antioxidant activity and had been reported for other functional properties, such as inhibition to cancer cell proliferation (Zhang *et al.*, 2005).

Jaboticaba (*Myrciaria jaboticaba*), originated from Brazil, is known for its high anthocyanin content that mainly present in its dark-coloured peel. With anthocyanins ranging from 58.1 - 315 mg, the highest concentration was actually on par with blueberry anthocyanin content (Rufino *et al*., 2010). Dominated by delphinidin- and cyanidin-3-*O*-glucoside, Jaboticaba fruits were reported with strong antioxidant activity compared to other species of *Myrtaceae* fruits. An *in vivo* study reported plasma antioxidant level was increased 1.7 times in the animal model after consumption of freeze-dried Jaboticaba peel (2% d.w., Leite *et al*., 2011). Jaboticaba fruits was able to stimulate the enzyme antioxidant system in obese rats (Lenquiste *et al*., 2015). Leite-Legatti *et al*., (2012) reported a promising result on that Jaboticaba obtained cytotoxic effect toward different cancer cell lines, without the exception of colorectal cancer cell line, HCT116 and HT29, from which active compounds of Jaboticaba exerted a moderate cytotoxic effect.

However, there is no study of animal model related to the effect of Jaboticaba fruit on the development of colorectal cancer. In terms of chemoprevention and gut health, the relationship between the intake of Jaboticaba and its anthocyanins to the gut flora and microbial metabolites is also unknown. Therefore, this study aimed to investigate the correlation between Jaboticaba extract, intestinal flora composition, microbial metabolites, and chemopreventive effects of colorectal cancer.

1.2. Literature Review

1.2.1. Colorectal Cancer (CRC)

Colorectal cancer ranks third among the most diagnosed cancers in the world, and ranks second in the number of cancer deaths worldwide (Wong *et al*., 2019). In 2018, the World Health Organization recorded 1,849,518 new cases and 880,792 related deaths (WHO, www.who.int). Among them, half of these cases occur in Asia (Wong *et al*., 2019). Most cases are sporadic cases (70%), which are caused by environmental factors such as diet (high fat, low fiber, high red meat intake) and alcohol consumption; the other 30% are hereditary (De Almeida *et al*., 2019).

In regard to the type of case (sporadic and hereditary), the occurrence of colorectal cancer started from a mutation in cancer- or proliferation-related genes, for example, the *APC Wnt* signaling pathway (Cancer Genome Atlas Network, 2012). The mutation can be triggered by either an original mutation in the gene (hereditary case) or by high exposure to radical species due to environmental factor (sporadic case). These mutations in the gene will alter protein expression, which will further lead to uncontrolled cell proliferation or cancer (Medic, Tramer, & Passamonti, 2019). In this dissertation, the following sections will briefly explain two possible causes of mutation.

1.2.2. Colorectal cancer and Microbiota

There are about 10^{14} bacterial cells in the human gut, which can regularly maintain the body's homeostasis (Whitman, Coleman, & Wiebe, 1998). This microbiota community was usually referred as gut microbiota. Several factors may trigger changes in the composition of intestinal flora, such as changes in fiber intake or the occurrence of gastrointestinal diseases. The composition of intestinal flora in patients with Crohn's disease and ulcerative colitis is different from that in normal patients (Kaur *et al*., 2011).

Approximately 90% of colon cancer biopsy of CRC patients are found with high concentration of bacteria, mainly *Escherichia coli* and other *coli*-like bacteria (Swidsinski *et al*., 1998).When reared under germ-free condition, the genetic-altered mice (model for colorectal cancer study) was found with lower tumor count when housed in a germ-free condition, indicating a significant role of gut microbiota in the development of colorectal cancer (Zhu *et al*., 2014).

CRC patients' guts habit pathogenic bacteria that usually exist in low number-to-none in the gut of healthy subject (Mangifesta *et al*., 2018). In addition, high population of pathogen bacteria was only observed on cancer/tumor site while the normal cell site in the same subject was unaffected. Furthermore, cancer usually appears starting in the mucosal tissue, from which it has high exposure to microbiota normally. All these facts lead to the hypothesis that a specific microbial community as an infectious agent can promote the incidence of cancer. Hausen (2009) further confirmed this hypothesis. He reported that human papillomavirus and *Helicobacter pylori* are the main causes of cervical cancer and gastric cancer, respectively. About 20% of cancers are actually related to the microbiome. Several bacteria, such as Enterotoxin-producing *Bacteroides fragilis* (ETBF) (Huycke, Abrams, & Moore, 2002) and *Escherichia coli* strain with *pks* genomic island can induce DNA damage on cell (Cuevas-Ramos *et al*., 2010). Silva-García, Valdez-Alarcón, & Baizabal-Aguirre (2014) proposed another mechanism of that bacteria could activate *Wnt* signaling pathway which triggers uncontrolled proliferation.

Furthermore, lower population of beneficial bacteria in the gut of colorectal cancer patient will lower short chain fatty acid (SCFA) production (Den Besten *et al*., 2013). Acting as an energy source, SCFA is an important metabolite for maintaining the health of colon cells. The ATP content of mouse colon cells in C57BL / 6 sterile mice was 56% lower than that of normal mice, and colonization by conventional microbial colonies increased the ATP level (Donohoe *et al*., 2011).

1.2.3. Colorectal Cancer and Radical Species

High activity of radicals may induce mutation in animal body, due to high exposure to radical or incapability of endogenous defense system to neutralize the radicals (McCord, 2000). The main defense system of the human body is our antioxidant enzymes

which consist of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Mates, Perez-Gomez, & De Castro, 1999)

SOD is known as major enzyme in the antioxidant system as it is the first line of defense toward radical species. SOD acts first on the most reactive species, the superoxide anion, followed by catalase which neutralizes superoxide anion into hydrogen peroxide, compound with less reactivity than superoxide anion (Figure 1). To be noted, catalase exists in all parts of the cell except for mitochondria (Ighodaro & Akinloye, 2018). Therefore, hydrogen peroxide presents in mitochondria will be neutralized by GPx. GPx uses glutathione (GSH) to reduce the hydrogen peroxide (the reaction can be seen in Figure 1.3.). Glutathione is an important antioxidant that can act either in a direct or indirect way to neutralize radical species. Glutathione directly scavenges free radicals and oxidizes itself to glutathione disulfide (GSSG) or it takes part in the GPx reaction mentioned above (indirect scavenging) (Wu *et al*., 2004). Three enzymes work together to protect cells from oxidative stress (Mates *et al*., 1999).

(1) $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

(2) $2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2$

(3) $ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$

Figure 1. Enzyme Reaction of Superoxide Dismutase (1), Catalase (2), and Glutathione Peroxidase (3). (Mates *et al*., 1999).

If the system is unable to neutralize large amounts of free radicals, oxidative stress may occur. A particular danger in oxidative stress is that it triggers the production of inflammatory cytokines and radical species. High concentration of radical species will cause the oxidation of DNA, RNA and cell walls lipid which could trigger cell mutation. Furthermore, ROS can induce DNA damage by triggering strand break base modification and addition of double bond to pyrimidine base (Lobo *et al.*, 2010). This will further cause hypersensitivity, inflammation and even an autoimmune condition (Mates et al., 1999).

Many diseases related to high oxidative stress or DNA mutation caused by oxidative stress, especially cancer (Lobo *et al*., 2010). Patients with inflammatory bowel disease was found 1.5 – 2 times more prone to colorectal cancer while teenager with ulcerative colitis which cover whole colon lining have >15% risk to colorectal cancer. Research reported a linear association between ulcerative colitis and colorectal cancer with much steeper slope for patient with severe colitis (Beaugerie & Itzkowitz, 2015) which indicates a close relationship between inflammation and cancer development.

1.2.4. Anthocyanin as Chemopreventive Agent

Carcinogenesis on colon cells had been reported to be reduced by chemopreventive agents extracted from dietary constituent (Corpet & Taché, 2002). Anthocyanin was among them and had been widely researched for past decades. This natural compound, founded mainly in fruit with dark color (for vegetable, only found in high concentration on eggplant and red cabbage), purple corn, and cereal, is famous for its antioxidant properties due to its chemical structure (phenolic and a hydroxyl group). The activity of anthocyanin as a chemopreventive agent has also been reported, starting from the pure anthocyanin compound to food-high-in-anthocyanin. Regarding to its consumption, the daily intake can vary depending on the diet (±1-100 milligrams). These compounds have not been found in processed food such as bread or canned food, despite their claim to be high in anthocyanin, therefore, limiting the anthocyanin consumption. Unto today, there is no recommended daily intake for anthocyanin (Martin *et al*., 2017).

Many *in vitro* studies found that anthocyanin's anti-cancer activities include a variety of activity, such as its antioxidant effect, anti-cell proliferation, apoptosis induction, and other activity. Wang & Stoner (2008) conduct a special review on these. The inhibitory effect of anthocyanin on colorectal cancer have be reported by many *in vivo* studies using different rat/mice model. Feeding anthocyanin extracts in bilberries (0.3% of the diet) reduced tumors by 30%, while pure anthocyanin 3-glucoside compounds at the same concentration reduced tumors by 45% in APC^{Min} mice (Cooke *et al*., 2006). In AOM-induced F344 rats, 10% of lyophilized extract of black raspberry was able to decrease 71% of the total tumor (Harris *et al*., 2001) while the anthocyanin-rich

extract of bilberry, chokeberry, and grape were able to reduce 70%; 59%; 27% of ACF (abberant crypt foci) formation, respectively (Lala *et al*., 2006).

Anthocyanin was able to protect the DNA from free radical-induced oxidative damage (Ghosh *et al.,* 2006). However, the bio-activities of each extract from antioxidant sources may be differ. In studies using bilberry, chokeberry and grape, it was found that certain extracts might have protective effects on oxidative damage to DNA, while others did not. For example, certain extracts were more effective in reducing the number of medium to large ACFs, while small size ACFs were not significantly affected (Lala *et al*., 2006). In studies of anthocyanin-rich sausages, it had been reported that anthocyanins could significantly inhibit the formation of polyps in the colon without affecting the total number of tumors (Fernández *et al*., 2018). These results indicate that anthocyanin may be more effective as a protective agent against colorectal cancer, so its activity against ACF and polyps is higher than that of tumor (Fernández *et al*, 2018). Therefore, the need to investigate each anthocyanin extract from a different type of dietary source is important to determine each mechanism of chemopreventive agent (Medic *et al*., 2019).

1.2.5. Jaboticaba Fruit (*Myrciaria cauliflora)*

1.2.5.1. Jaboticaba fruit introduction

Jaboticaba (*Myrciaria cauliflora*) is one of the species from Myrtaceae family. The other species from Myrtaceae family called *Myrciaria jaboticaba* is a similar species to *M. cauliflora*. *M. cauliflora* tree is small in size with a height of 3 – 6 meters with smooth gray bark and long leaf (2 – 6 meter long). In comparison, *M. jaboticaba* is bigger (3 - 6 meter tall, which makes them known as 'Great Jaboticaba') and have much shorter leaves (2 – 4 cm long) (Leite-Legatti *et al*., 2012). Jaboticaba trees will produce fruit once every year, sometimes twice a year. Major harvest can be collected in late March and April. Both of them produce edible fruit called Jaboticaba with sweet and acid taste (pH range of 3.3 to 4.1) on the white pulp part covered with dark-colored skin (Inada *et al*., 2015).

Figure 2. Picture of Jaboticaba (Wu, Long, & Kennelly, 2013)

1.2.5.2. Nutrition and physiological component

Jaboticaba contains a high amount of phenolic compounds, especially 556.3 g GAE (Gallic acid equivalent)/kg in peel (Wu *et al*., 2012). Jaboticaba is a good source of minerals such as potassium (13.2 mg/100 g), phosphorus (9.2 – 34.6 g/100 g), calcium (6.3-7.6 mg/100 g) and iron (0.49-0.87 mg/100 g). Other than that, vitamins such as vitamin B1, B2, and C are also found in the fruit. A complete nutritional composition is listed in Table 1 (Wu *et al*., 2013).

Table 1. Nutritional Composition of per 100-gram Jaboticaba Fruits

Caloric and nutritional composition	Values
Calories	45.7 – 51.7 units
Water	87.1 g
Protein	0.11 – 0.32 g
Fat	<0.01 g
Carbohydrate	12.58 g
Ash	0.2 g
Calcium	6.3 – 7.6 mg
Phosphorus	9.2 – 34.6 mg
Iron	0.49 – 0.87 mg
Potassium	13.2 mg
Vitamin B1	0.04 mg
Vitamin B2	0.09 mg
Niacin	1.3 mg
Fiber	0.08 mg

Table 1. (continued)

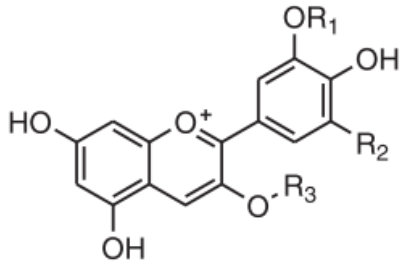
Caloric and nutritional composition	Values
Riboflavin	0.02 mg
Tryptophan	1.0 mg
Lysine	7.0 mg
Ascorbic acid	17.7 – 238 mg
Total anthocyanin	58.1 – 315 mg
Total phenolics	460.9 mg
Total carotenoids	0.32 mg

(Wu *et al*., 2013).

Bioactive compounds are a major constituent that has been focused during research for their functional properties. The bioactive compounds in Jaboticaba include anthocyanins, phenolics, and carotenoids with a content of 58.1 ~ 315 mg; 460.9 mg and 0.32 mg respectively (refer to Table 1). In addition, Jaboticaba fruit also contains flavonoids, gallic acid tannin, ellagitannin and other phenolic compounds, making it a fruit containing multiple biologically active compounds (Wu *et al*., 2013).

Numerous research that had started from 1972 until now had reported several types of anthocyanin on Jaboticaba, including (1) peonidin and peonidin-3-*O*-glucoside; (2) cyanidin-3-*O*-glucoside; (3) delphinidin-3-*O*-glucoside; (4) pyranocyanin B (was written by the order they are identified). The structure of this four anthocyanin can be seen in Figure 1. (Wu *et al*., 2013). Among variety of anthocyanin in Jaboticaba fruit, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside are the major anthocyanins (Wu *et al*., 2013).

Figure 3. Chemical Structure of Jaboticaba's Major Anthocyanin



Peonidin (**1**) $R_1 = CH_3$; $R_2 = R_3 = H$
Peonidin-3-*O*-glucoside (**2**) $R_1 = CH_3$; $R_2 = H$; $R_3 = Glc$
Delphinidin-3-*O*-glucoside (**3**) $R_1 = H$; $R_2 = OH$; $R_3 = Glc$
Cyanidin-3-*O*-glucoside (**4**) $R_1 = H$; $R_2 = H$; $R_3 = Glc$

Cyanidin-3-*O*-glucoside was the most abundant bioactive compounds in Jaboticaba fruit, followed by gallic and ellagic acid (Table 2, Inada *et al*., 2015) A 100 gram of dry Jaboticaba contains about 433 mg cyanidin-3-*O*-glucoside and 81 mg delphinidin-3-*O*-glucoside. The peel contains high amount of anthocyanins, making the peel dark color, and the anthocyanins in the peel are 4.5 ~7.8 times higher than that in whole fruits. In addition, other types of flavonoid, tannin, and depsides were found as secondary bioactive on this fruit (Wu *et al*., 2013).

Table 2. Bioactive Compounds of Jaboticaba and Its Fractions (mg/100 g dwb)

Compound	Whole fruit	Pulp	Peel	Seed
Cyanidin-3-*O*-glucoside	280±18	0.4±0.0	1,261±18	58±4
Delphinidin-3-*O*-glucoside	48±1	ND	269±11	11.8±0.4
Gallic acid	7.9±0.1	3.2±0.0	21±1	30±1
Rutin	77±2	7.1±0.6	247±5	241±12
Myricetin	0.4±0.0	0.2±0.0	4.3±0.2	2.0±0.1
Quecertin	0.4±0.0	ND	3.5±0.0	0.4±0.0
m-coumaric acid	0.3±0.0	0.6±0.0	0.2±0.0	0.6±0.1
Myricitrin	3.5±0.1	0.7±0.0	20±0	1.8±0.0
Ellagic acid	34±1	5.3±0.3	178±5	83.8

(Inada *et al*., 2015).

Even though the identified anthocyanin of Jaboticaba is lower in variety than other dark-colored fruits, the total anthocyanin of Jaboticaba is almost comparable to blueberry. 100 grams of Jaboticaba fruits contain 58.1 - 315 mg of anthocyanins while the total phenolic content was 460.9 mg (Rufino *et al*., 2010). Yuan *et al*. (2011) reported total anthocyanin and total phenolics in blueberry were 200-350 mg and 350-490 mg, respectively

1.2.5.3. Functional properties of Jaboticaba

Traditionally, this fruit has been used in Brazil to treat many diseases, such as diarrhea and asthma. Many research conducted antioxidant, antibacterial and anticancer properties of Jaboticaba fruit, among them, the antioxidant activity of Jaboticaba was the most studied. The methanol/water (70:30, v/v) extract of Jaboticaba obtained 62 ± 6 mmol Trolox eq. kg^{-1} (Abe, Lajolo, & Genovese, 2012). The IC_{50} of Jaboticaba methanol extract was 19.4 ± 0.28 mg mL^{-1} (Reynertson *et al.*, 2008). A Jaboticaba whole fruit contain

31.63 ± 0.1 g GAE kg^{-1} of dry weight (Reynertson *et al*., 2006). Compared with other *Myrtaceae* fruits, Jaboticaba has the highest antioxidant activity (Wu *et al*., 2013). Furthermore, Dai *et al.* (2009) reported that Jaboticaba contained higher GAE than various blueberry species. *In vivo* animal model showed that oral treatment of freeze-dried Jaboticaba peel (1% and 2%) was able to increase the antioxidant level of blood plasma (1.7x by TEAC assay and 1.3x by ORAC assay) (Leite *et al*., 2011). In addition, intake of Jaboticaba peel could stimulate the enzyme antioxidant system and decrease the oxidative stress in obese-rats (Wu *et al*., 2013).

Jaboticaba has a cytotoxic effect on cancer cell. Jaboticabin and 2-*O*-(3,4 dihydroxy-benzoyl)-2,4,6-trihy-droxy-phenylacetic acid extracted from Jaboticaba, were classified as having moderate cytotoxicity toward HCT116 and HT29 (Reynertson *et al*., 2006). Using HCT116 and SW480 cell lines as tested subjects, delphinidin-3-*O*-glucoside also showed cytotoxicity effect, while cyanidin-3-*O*-glucoside showed low cytotoxicity effect (Reynertson *et al*., 2006). In addition to colorectal cancer, both polar and non-polar extract of Jaboticaba peel also exhibited cytotoxic effects on 11 other tumor cell lines. Polar extracts had the highest activity on K-562 cell line, leukemia cancer cells, while non-polar extracts have the highest activity on PC-3 cell line, prostate cancer cells (Leite-Legatti *et al*., 2012).

1.2.6. Modulation of anthocyanin on intestinal microbiota

The functional properties of anthocyanin are not limited to their antioxidant effect. Many studies had documented the ability of anthocyanin to stimulate the growth of intestinal flora. In addition to anthocyanins, other biologically active compounds such as phenolic acid and flavonoids have the same effect (Ozdal *et al*., 2016). Most of these bioactive compounds interact with intestinal flora due to the low absorption rate of bioactive compounds in the small intestine and 90-95% of the bioactive compounds entering the large intestine for further absorption (Faria *et al.*, 2014). The above also applies to the bioactive compounds of Jaboticaba and anthocyanins from other fruits (Quatrin *et al*., 2020). In the large intestine, polyphenols are converted into their metabolites (aglycones) by the intestinal microbiota, which are easily absorbed by large intestine cells, therefore increasing their bioavailability (Keppler & Humpf, 2005). In this

regard, polyphenols can stimulate the growth of intestinal microbiota (Cardona *et al.*, 2013).

The mechanism of anthocyanin metabolism in bacteria is related to the hydrolysis reaction of microbial α, L-rhamnosidase and β, D-glucosidase, which cleaves the sugar moiety of the anthocyanin structure and converts it to aglycone. Since the aglycon was unstable in the pH environment of the large intestine, the aglycon usually independently converted into a quinoid bases and then further degraded to another form (Keppler & Humpf, 2005). Protocatechuic acid was reported to be the main degradation product of anthocyanins in the large intestine because 73% of the cyanidin-O-glucoside (Jaboticaba anthocyanins) was converted to protocatechuic acid (Vitaglione *et al.*, 2007). In this regards, *in vivo* studies had also reported the metabolism of anthocyanins in the large intestine. *In vivo* study in rats and humans had shown that most anthocyanins (delphinidin 3-*O*-glucoside, etc.) were absorbed in the large intestine. Then the unabsorbed part was excreted in the form of glycosylation. After ingesting food for 4 hours, 85% of blueberry anthocyanins have been found in the colon (Kahle *et al*., 2006) and 69% of them disappeared (Aura *et al*., 2005). According to Hassimotto, Genovese, & Lajolo (2008), the anthocyanins in mulberry red can be completely degraded within a few hours after ingestion by intestinal flora.

During the cleavage of the sugar moiety, the sugar component is naturally released, and the bacteria themselves can use it as a source of nutrients. Through research, it was found that anthocyanins from pomegranate could stimulate the growth of *Bifidobacterium* spp. and *Lactobacillus* spp (Bialonska *et al*., 2010) Jucara pulp, a fruit very similar to Jaboticaba (including bioactive components) can stimulate the growth of *Bifidobacterium in vitro* (Guergoletto *et al.*, 2016). A study using Jaboticaba peels reported that it has no significant effect on stimulating the above bacteria, but it can inhibit the growth of *Enterobacteria* (pathogenic bacteria) (Quatrin *et al*., 2020). Other studies using blackberry anthocyanins had also reported the stimulation of the growth of butyrate-producing bacteria and the inhibition of *Campylobacter*, *Desulfovibrio* spp. and other pathogenic bacteria species (Chen *et al*., 2018). These results indicate that anthocyanins can be used as prebiotic compounds (Sun *et al.*, 2018).

The activity of anthocyanins in stimulating the growth of beneficial bacteria or inhibiting pathogenic bacteria can lead to a reduction in the intestinal inflammation. In addition, studies have documented an increase in the production of short-chain fatty acids (Chen *et al*., 2018).

1.2.7. Short Chain Fatty Acid (SCFA)

SCFA is a group of saturated aliphatic organic acids with variation in the carbon numbers, depending on the type. The ratio of acetic acid, propionic acid and butyric acid produced by fermentation of undigested carbohydrates (resistant starch, plant cell wall polysaccharides) is 60:20:20 (Cummings, 1981). These three acids were known as the major SCFA with concentration ranging from 70-140 mM in the proximal and 20-70 mM in the distal colon (Topping & Clifton, 2001). Colon cells cover 95% of total SCFA, leaving the remaining 5% excreted in the feces. In an adult, approximately 400-600 mmol of SCFA are produced per day and 5-15 mmol is excreted in the feces per day (Bergman, 1990).

The decrease in SCFA concentration as it was absorbed throughout colon from proximal end to distal end leads to a decrease in acidity due to SCFA acidic nature. Related to this, it is expected that high concentrations of SCFA can prevent bacterial growth which have certain sensitivity to pH. Exposure to 0.5 – 0.7 mol/L of propionic acid at pH 5 was reported to kill 90% of population of *Escherichia coli* K12 and *Salmonella* spp. (Cherrington *et al*., 1991).

Roseburia spp. and *Faecalibacterium prausnitzii* of *Firmicutes* (butyrate producer) can optimally grow at pH 5.5 in comparison to pH 6.5. *Roseburia* population reduce significantly at pH 6.5 (Walker *et al.*, 2005). As the pH increase to 6.5, these butyrate-producing bacteria decrease significantly, being replaced by acetic acid- and propionic acid-producing bacteria such as *Bacteroides*. At pH 5.5, the growth of *Bacteroides* in YCFA anaerobic media was inhibited (Duncan *et al.*, 2009). Using 16s rRNA, the level of *Bacteroides* increase from 20 to 75% by shifting the pH from 5.5 to 6.5 (Walker *et al*., 2005). Therefore, differences in bacterial communities in different parts of human intestine was due to the differences in pH (Den Besten *et al*., 2013).

The microbiome responsible for SCFA production varies with each fatty acid. Among three of the major acid, acetic acid can be produced by several type of bacteria while

propionic and butyric acid had a quite specific pathway of production (Morrison & Preston, 2016). Propionic acid and butyric acid are produced by only a few bacteria, for example, *Ruminococcus bromii* (Ze *et al.*, 2012) and *Faecalibacterium prausnitzii* (Louis *et al.*, 2010). *Bifidobacterium, Lactobacillus,* and *Ruminococcus* were also mentioned as acetic acid producer (Louis, Hold, & Flint, 2014). From the analysis of phylum class, *Bacteroidetes* contribute to the production of acetic acid and propionic acid, and *Firmicutes* phylum produce butyrate. Among gut microbiota, *Bacteroidetes* was known as the primary fermenters (Den Besten *et al*., 2013). Specific substrates are also needed, where deoxy sugars is a preference for propionic acid (Reichardt et al., 2014) and starch is a preference for butyric acid (Ze *et al.*, 2012).

The metabolization of SCFA after absorption also differ in regard to SCFA type. Butyrate will be used as major energy source (70 – 90%) for colon epithelium cell (Hijova & Chmelarova, 2007). Any residual butyrate would be transported to the liver as a substrate for gluconeogenesis reaction. Majority of propionate and 50-70% of acetate also serve as substrates for the reaction as well. Additionally, any acetate residual from liver would be used by the muscle cell for energy generation by oxidizing acetic acid (Hijova & Chmelarova, 2007).

Shortly mentioned above, SCFA holds an important role in maintaining the ideal pH value. In the colon, the increase in the pH value is majorly caused by ammonia. Ammonia is produced from the degradation of urea, protein, and amino acid by intestinal microbiota. In the case of limited dietary fiber, the intestinal microbiota will use urea, protein, and amino acid as their substrate, resulting in an increase in the colonic pH. High pH value had been associated to an increased risk of colorectal cancer because it can stimulate secondary bile formation and long chain fatty acid (LCFA) ionization, which irritate colonic epithelium cell and is a major factor in the development of colorectal cancer (Lupton & Newmark, 1990). Some studies even consider the secondary bile acid to be colorectal cancer promotor/carcinogen and high exposure to secondary bile acid can cause oxidative stress and DNA damage (Ajouz et al., 2014). In addition, in patients with adenoma colorectal cancer, they express high concentration of 7-α-dehydrolase, an enzyme that produces secondary bile acid (Lupton & Newmark, 1990).

SCFA, especially butyric acid, is the main energy source for healthy colon cells while triggering apoptosis of cancer cells. Tested on 3 different adenoma colorectal cancer line,

butyric acid showed highest activity in triggering apoptosis (Hague *et al.*, 1995). Butyric acid was also able to differentiate between normal colonocyte and cancer cell as exposure of 10 mmol/L butyric acid for 150 minutes to colonic mucosa showed no DNA fragmentation. In reverse, incubating colonic mucosa with no butyric acid in 150 minutes trigger apoptosis on those cell (Busche *et al.*, 1996).

The selectivity of butyric acid was due to gene mutation in cancer cells. It was observed that when colon cells mutate into cancer cells, they will shift from aerobic respiration to anaerobic glycolysis (De Morgan, 1871). Normally, colon cells will rely on aerobic respiration to meet its energy need, but due to mutation on the *Mpc1* gene (a gene that encodes mitochondrial pyruvate carrier 1), the mitochondria uptake rate of pyruvate decreases thus cause the system to conduct anaerobic glycolysis (Schell *et al*., 2014). During the anaerobic glycolysis, the biosynthesis of lipid is limited that leads to the accumulation of butyric acid in the cytoplasm. High concentration of butyric acid will then increase the sensitivity of colon cancer cells and trigger the apoptotic mechanism (McNabney & Henagan, 2017).

In addition, SCFA also regulates the intestinal barrier by regulating tight junction proteins by activating 5' adenosine monophosphate (AMP) kinase (Beek *et al.*, 2017; Wang *et al.*, 2012). Maintaining the integrity of the intestinal barrier is important because it prevents harmful bacteria or their metabolites from transferring to the blood or other organs. Bacterial translocation triggers inflammatory cytokines and causes diseases such as insulin resistance (Cani *et al*., 2008).

1.2.8. Possible mechanism of anthocyanin on colorectal cancer

In the research, anthocyanin is reported to induce apoptosis, tight junction modulation, suppress cell proliferation, decreasing oxidative stress and inhibiting tumor progression. The anthocyanins used in the research range from pure compounds (such as anthocyanins and delphinidin) to the use of anthocyanin-rich extracts (such as grapes and black currant). Therefore, the anthocyanin concentration in those studies also varied from 0.01 µM to 240 µM. (Medic *et al*., 2019).

Cyanidin 3-O-glucoside and delphinidin 3-O-glucoside, the most reported anthocyanin in food products, show inhibitory activity against EGFR tyrosine kinase, which is related to cancer cell survival and apoptosis (Cooke *et al*., 2006). The activation

of apoptotic pathway is related to the activation of caspase, which will stimulate the expression of pro-apoptotic proteins (such as Bax) and inhibit the expression of anti-apoptotic proteins (such as Bcl-2) (Gross, McDonnell, & Korsmeyer, 1999). Other study supported this by reporting delphinidin ability to stimulate Bax and inhibit Bcl-2 protein that leads to the arrest of HCT 116 cells after 48 hours incubation (Yun *et al.*, 2009).

Comparison between pure anthocyanin compounds and anthocyanin aglycone reveal higher activity of the aglycone in cytotoxicity. Tested on Caco-2 cells, cyanidin chloride (CY), the aglycon of cyanidin 3-O-glucoside could reduce ROS at a low concentration (25 µmol / L) compared to anthocyanin 3-O-glucoside which requires medium to high concentration (25-200 µmol/L) (Renis *et al*., 2008).

Anthocyanin had also been reported for its selective cytotoxicity toward cancer cells by increasing the rate of ROS (radical oxygen species) accumulation in cancer cells, while the normal cell was left unaffected. Anthocyanin-rich extract (ARE) of bilberry and blackcurrant was reported to stimulate apoptosis by caspase-3 activation and inhibit proliferation by triggering ROS accumulation in Caco-2 cell (colon cancer cell). However, no accumulation of ROS occurs on normal fibroblast cell (NIH/3T3) indicating selective cytotoxicity due to ROS level (Anwar *et al*., 2016). Compared with normal cells, cancer cells have higher ROS levels, so it also has higher oxidase activity, which can overcome ROS levels and therefore the cell was able to survive (Medic *et al*., 2019).

1.2.9. Key tips for research on anthocyanin anti-intestinal cancer

1.2.9.1. Anthocyanin Sample Extraction

Anthocyanins are mainly present inside the plant matrix and have various structures, so the source of the matrix and its interaction with other compounds need to be considered (Martin *et al*., 2017). Light, temperature, pH, sugar, oxygen, and another factor also needs to be taken into account (Cavalcanti, Santos, & Meireles, 2011). Regarding their structure, anthocyanins are polar compounds, contributed by their aromatic rings with polar substituents (—OH, —C═O, or —OCH3). This also applies to flavonoid glycosides, which are usually polar compounds (Bueno *et al*., 2012). Concerning this, cold solvent extraction with either water or polar organic solvents (methanol, alcohol, ethanol, etc.) is the most common method for extracting anthocyanin (Shi *et al*., 2005).

To optimize the extraction, pretreatment is usually carried out, the most common is to dry or powdered the plant material (air-drying, freeze-drying, grinding, filtration, etc.), and under mild condition (Martin *et al*., 2017). For now, freezing or lyophilization is used as a pretreatment method because low temperature can protect biologically active compounds (Tura & Robards, 2002). After pretreatment, samples can be directly extracted by the conventional solvent extraction or other novel extraction methods, such as pressurized fluid extraction (SFE), countercurrent chromatographic methods, and other new methods (Martin *et al*., 2017).

1.2.9.2. In Vivo study to explore the anti-tumor effect

In vivo study is a study that is done in the living organism as the word itself is the Latin for 'the living' (Woods & Foster, 1964). For research related to colorectal cancer, several experimental models can be used to investigate various topics of colorectal cancer. Experimental models were divided into two groups, induced and transgenic animal models. APC (+/-) mice were one of the transgenic animal models, which were mutated by homologous recombination in codons that responsible for the expression of the APC tumor suppressor gene (Heyer *et al.*, 1999). In the induced-model, 1,2 dimethylhydrazine (DMH) and azoxymethane (AOM) are commonly used for induction. This model has similar characteristics to human sporadic colorectal cancer with a similar response to several treatments (Perše & Cerar, 2011).

1.2.9.3. DMH (mechanism, CRC induction)

DMH can induce colorectal cancer by activating in the liver and then a veriety reactions occur to produce methyldiazonium ion (Fiala & Stathopoulos, 1984) (Figure 4). Methyldiazonium ion (MAM) is known to be a strong carcinogenic metabolite and can translocate to colon via bile acid or blood circulation (Perše & Cerar, 2011). Matsumoto & Higa (1966) reported that MAM can cause DNA methylation on various sites and lead to apoptosis and cell proliferation of healthy colon.

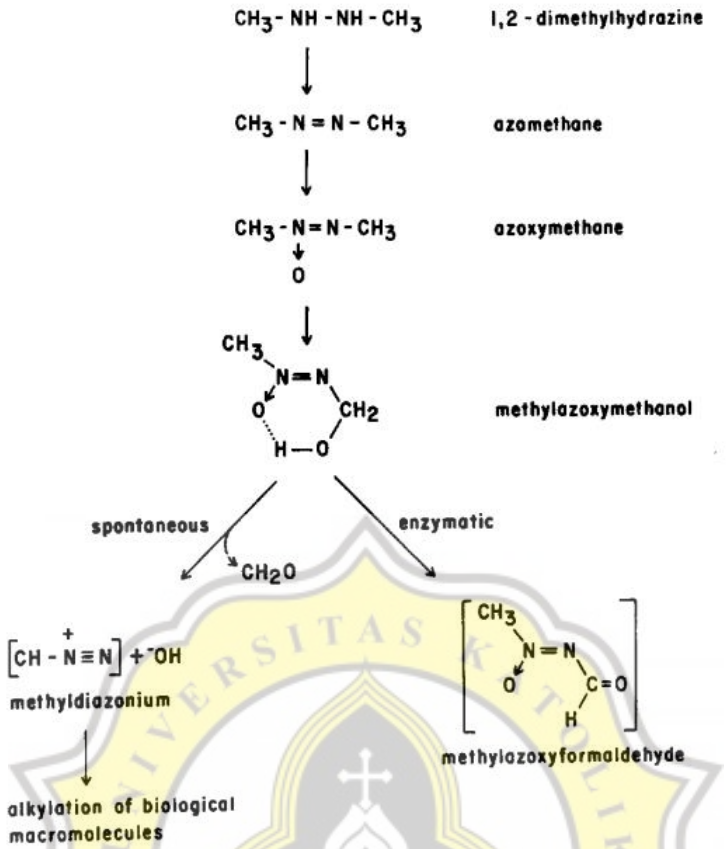


Figure 4. Pathway of 1,2-DMH Conversion to Carcinogenic (Fiala & Stathopoulos, 1984).

Commonly, six weeks of male Wistar or Sprague-Dawley rats are used to induce colorectal cancer. The induction itself is usually done by injecting DMH subcutaneously (15-25 mg/kg) twice a week (Perse & Cerar, 2005). Formation of ACF can be observed starting on the second week after injection, appeared originally as single crypt. ACF formation will increased dependent with time as well as the crypt number (Bird & Good, 2000). On the other hand, tumor can be observed starting on 30th weeks (Rodrigues *et al.*, 2002).

ACF is a lesion found on colon cells and may develop into an adenoma, thus making the lesion one of the biomarkers for colorectal cancer (Bird & Good, 2000). The lesions can be seen by histological sections of colonic cell mucosa or stained with methylene blue on the entire colonic colon surface and then magnified with microscope. However, the latter one can only provide visualization and identification of lesions, but cannot give appropriate histological results (Bird, 1987).

Compared with normal crypts, ACF's epithelial cell lining is larger and thicker. Shape of ACF can be rounded or elongated and the shape of a luminal opening is elliptical while a normal mucosa is circular (Orlando *et al*., 2008). The difference between ACF and normal foci shows in Figure 5 (dos Reis *et al*., 2019; Wargovich *et al*., 2010). ACF can be divided into hyperplastic and dysplastic cells. Hyperplastic ACF is common in sporadic colorectal cancer, while dysplastic ACF is more common in patients with familial adenomatous polyposis (FAP). To differentiate, dysplastic ACF was larger and contain more apoptotic bodies compared to hyperplastic ACF. Its structure is similar to adenomatous polyps, so dysplastic ACF is more likely to develop into adenoma (Orlando *et al*., 2008).

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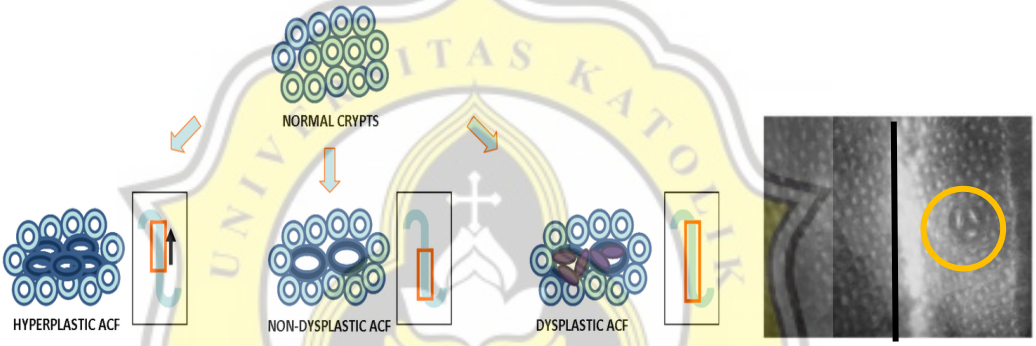


(a) normal crypt (b) an ACF

Figure 5. Identification of Aberrant Crypt Foci

(dos Reis *et al*., 2019; Wargovich *et al*., 2010)

1.2.9.4. Sprague Dawley (SD) Rat

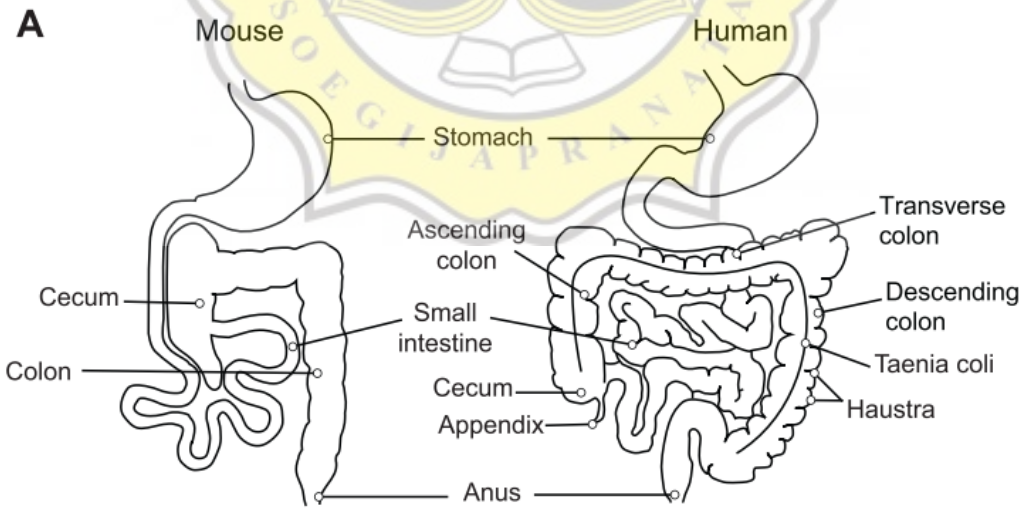
Rats are one of the animals commonly used in *in vivo* studies, from which it considers the similarities in the genetic information as well as the anatomy to the target organism, human in the majority (Nguyen *et al.*, 2015). SD-rat in specific is generated by breeding Wistar rats with a hybrid of laboratory-wild-rats in 1920 by Robert S. Dawley. These rats were then improved by the laboratory in terms of their longevity, microbial status and any other parameters into the Sprague Dawley rat that are used today. Onto today, SD-rat is widely used as an animal model for cancer, diabetes, cardiovascular disease, and obesity as well (Brower *et al*., 2015).

This breed is a breed of albino rats and is known for its calmness, so it is easier to handle than other breeds. The average body weight is 450 -520 grams for males and 250-300 grams for females. In experimental rats, their life span is short to moderate, ranging

from 2.5 to 3.5 years. They have similar physiques as Wistar rats, but the ratio of tail to body length is longer (Cavigelli, Michael, & Ragan, 2013).

1.2.9.5. Association of Rat to Human in Gut Microbiota Experiment

As mentioned earlier, the similarity between rats and humans is very important for using rats as animal models. In the study of intestinal flora, the composition of the organs is similar, so the model is widely used in research. However, Nguyen *et al*. (2015) pointed out several differences. First, the microbial fermentation in rats occurs in the cecum rather than the colon. The cecum ratio of rats is larger than that of humans, and the ratio of the entire gastrointestinal tract is also larger than that of humans. In opposite, the human cecum is relatively small, has an anatomical structure similar to the colon, and does not have a specific function. Secondly, because the main fermentation occurs mainly on the cecum, rat's colon composed of thin muscularis mucosa, therefore, a smooth colon surface. Compared with the human colon, rat's colon has no clear division, while the human colon is divided into three parts, namely ascending, lateral and descending. Besides, transverse folds exist only in rat's cecum and proximal colon, but in all parts of the human colon (Figure 6). (Nguyen *et al*., 2015).



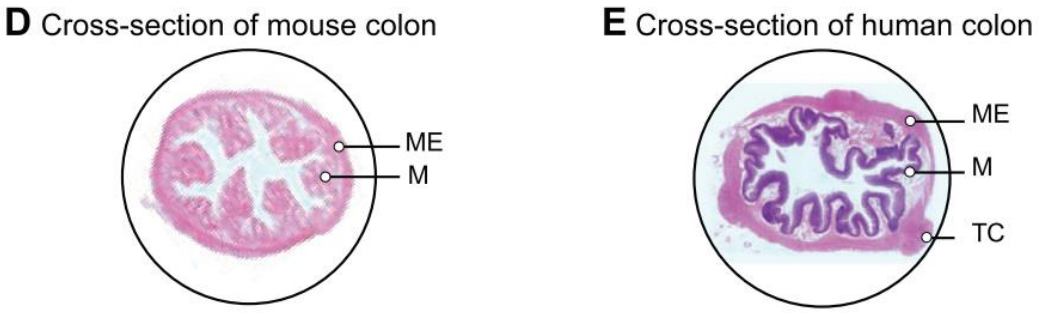


Figure 6. The difference between rat and human gastrointestinal tract. M: mucosa; ME: muscularis externa; TC: taenia coli (Nguyen *et al*., 2015).

The last point, the goblet cells are well distributed in the human colon, distributed equally from cecum to rectum. In rats, these cells are only abundant in the proximal colon and gradually decrease from the distal end to the rectum. Goblet cell is the cell that responsible for mucin production, while Paneth cells' role is the secretion of antimicrobial compounds. These cells are present in the cecum and proximal colon of humans, but only in the cecum of rats. Study reports that antibacterial compounds are also different between rats and humans (Nguyen *et al*., 2015).

1.3. Objectives

To explore the chemopreventive potential of Jaboticaba extract on DMH-induced colorectal cancer in Sprague-Dawley rats, by evaluating the stimulatory effect of Jaboticaba extract on the intestinal microflora and its short-chain fatty acids.