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In Vitro Growth-Inhibitory Effect of Stimulant Beverages Against Intestinal Bacteria Associated with Colorectal Cancer

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Declaration

I hereby declare that I have done this thesis entitled *In Vitro* Growth-Inhibitory Effect of Stimulant Beverages Against Intestinal Bacteria Associated with Colorectal Cancer independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

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Kateřina Čerňanská

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Abstract

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. Its development depends on individual attributes such as age, healthy diet, habits, and lifestyle. Recently, there is rising evidence that specific bacteria play a significant role in the colorectal tumour microenvironment. Although currently available treatments such as chemotherapy, surgery, radiotherapy, and faecal microbiota transplantation are quite effective, further research in the area of prevention of the disease is still needed. Since stimulant beverages are largely consumed worldwide (coffee and tea consumption of all non-alcoholic beverages was 4.38% in 2022) and their antibacterial effects are already proven by some studies, we decided to evaluate the *in vitro* effects of these beverages on the growth of representatives of bacterial pathogens associated with CRC risk. In this thesis 6 representatives of stimulant beverages, namely *Aspalathus linearis*, *Camellia sinensis var. sinensis*, *C. sinensis var. asamica*, *Coffea arabica*, *C. canephora* and *Ilex paraguariensis* were tested against 6 pathogenic bacteria by broth microdilution method. As a result, all samples of stimulant beverages produced no inhibitory activity (MIC > 512 μg/ml) against *Bacteroides fragilis*, *Clostridium septicum*, *Escherichia coli*, *Fusobacterium necrophorum, Peptostreptococcus anae*robius and *Streptococcus bovis*. For this reason, we decided to use bacterial growth kinetics but only *E. coli* and *S. bovis* were used for this method. From the growth lag phase duration measurement, it was found that the concentration 512 μg/ml of *I. paraguariensis* significantly inhibited the growth of *E. coli* ATCC 25922. On the other hand, *A. linearis*, *C. arabica* and *C. canephora* showed significant supportive growth effect of *E. coli* at concentrations 64, 128 and 256 μg/ml respectively. In general, the effects of stimulant beverages were much more significant on *E. coli* growth than on *S. bovis.* These results represent experimental background for future studies focused on monitoring of the effect of foods and beverages on growth of bacteria associated with CRC.

Key words: antibacterial, colorectal cancer, extracts, stimulant beverages

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ATCC – American Type Culture Collection

BHI – Brain Heart Infusion

CCM – Czech Collection of Microorganisms

CLSI – Clinical and Laboratory Standards Institute

CRC – colorectal cancer

DMSO – dimethyl sulfoxide

EC – epicatechin

ECG – epicatechin gallate

EGC – epigallocatechin

EGCG – epigallocatechin gallate

FMT – faecal microbiota transplantation

LAB – lactic acid bacteria

MHB – Mueller-Hinton broth

MIC – minimal inhibitory concentration

WCB – Wilkins-Chalgren broth

1. Introduction

Colorectal cancer is the third leading cancer in the world in terms of incidence and mortality (Qiao et al. 2021). Most cases of CRC are detected in Western countries and its incidence is increasing each year. The risk of developing CRC is related to personal characteristics or habits such as age, past chronic illness, and lifestyle (Mármol et al. 2017). The assessment of the human microbiota may be crucial in this regard, as disruption of the normal gut bacterial community is an important aspect of CRC development (Tarashi et al. 2019). Several studies have identified bacterial species whose presence may increase the risk of CRC, including prominent examples such as *Fusobacterium nucleatum*, enterotoxigenic *Bacteroides fragilis* and *E. coli* (Dougherty & Jobin 2023). Considering all these aspects, the number of research and studies on alternative treatments for CRC is starting to increase. In particular, many studies are focusing on prevention of this disease and the effect of a healthy diet on its development. Non-alcoholic beverages are also part of a healthy diet, some of which have stimulant effects, such as coffee (*Coffea arabica*, *C. canephora*) black and green tea (*Camellia sinensis var. sinensis*, *Camellia sinensis var. assamica*) or maté (*Ilex paraguariensis*). There are other studies that have examined the effects of stimulant beverages and their impact on this disease. Some of these studies show both positive and negative effects for the development of this illness. However, there is not yet a clearly established result as to whether stimulant beverages increase or decrease the risk of developing this cancer.

2. Colorectal cancer (CRC)

The gastrointestinal tract is the passageway of the digestive system that leads from the mouth to the rectum, and it contains all the major organs of the digestive system such as the esophagus, stomach and intestines (Montgomery et al. 1999). Among various diseases affecting gastrointestinal health, several types of cancer can arise in the colon and rectum (Shao et al. 2022). CRC is the third most frequent type of cancer and the fourth most frequent cause of cancer associated death. Most cases of CRC are reported in Western countries and its incidence is increasing each year. The risk of developing CRC is associated with personal attributes or habits such as age, history of chronic disease and lifestyle (Mármol et al. 2017).

2.1. Epidemiology

CRC is the most frequent malignant disease of the gastrointestinal tract, the third most common cancer and the second most deadly cancer which caused 600000, 881000 and 910000 million deaths in 2015, 2018 and 2020, respectively (Ferlay et al. 2015; Rawla et al. 2019; "Colorectal Cancer Awareness Month 2021 – IARC" 2020). There is good scientific evidence that the digestive organs account for more cancers than any other system (Boland et al. n.d.). China, United States and Japan have the highest estimated number of new cases (Xi & Xu 2021), where 4.4% of men and 4.1% of women will be diagnosed with CRC in their lifetime. It is the third and the second most common type of cancer for men (10% of all cancers) and women (9.2% of all cancers) worldwide (Ferlay et al. 2015). In developing regions, the lack of an effective and safe therapy and the prevention for CRC as well is a global health concern, especially for children under the age of five years.

The Czech Republic is one of the countries with the highest incidence of cancer in the world (Sung et al. 2021). According to (Ferlay et al. 2020), around 7,700 patients are newly diagnosed with this cancer annually in the Czech Republic and approximately 3,400 patients die from it. However, over the last 10 years, there has been a very significant decline in CRC: 3.2% in men and 2.8% in women, mainly due to screening programmes introduced in the Czech Republic (Pehalova et al. 2021) According to The International Agency for Research on Cancer CRC will increase by 56% between 2020

and 2040, to more than 3 million new cases per year mainly in countries with high human development index.

2.2. Causes

The human gut microbiome includes microorganisms, such as eukaryotes, archaea, protozoa, viruses, and predominantly bacteria (both commensal and pathogenic), together with their common genetic material, microorganisms have a significant impact on both local and systemic immune responses of gut system (Azimi et al. 2022). They also play an important role in the synthesis of amino acids, enzymes, vitamins, absorption of minerals and nutrients, and production of short-chain fatty acids (Lloyd-Price et al. 2016). Colonization of these commensal microorganisms in the gastrointestinal tract after birth is essential for the maturation of the immune system, especially for the development of the intestinal mucosa, which influences host tolerance mechanisms to distinguish between commensal bacteria and pathogenic bacteria (Thaiss et al. 2016)

Metabolites derived from commensal bacteria regulate the process of CRC development by influencing various factors such as immune cell-mediated tumour killing, tumour cell growth and survival (Huaman et al. 2018). In colon cancer, disruption of the colonic surface barrier leads to infiltration of commensal organisms and their metabolites, which subsequently activate tumour myeloid cells and induce local inflammation. In this process, pathobiontic and pathogenic bacteria invade normal colorectal tissues and promote inflammation and tumorigenesis. Bacteria that carry genotoxic markers promote the accumulation of genetic changes in intestinal epithelial cells and initiate the development of cancer (Azimi et al. 2022).

It was discovered that some bacteria, such as enterotoxigenic *Bacteroides fragilis*, secrete specific toxins, causing expansive inflammation, hyperplasia, colitis and multiple intestinal neoplasia, which subsequently leads to the development of CRC as well (Wu et al. 2009). Also, some strains of *E. coli*, can cause DNA damage that eventually progresses to CRC and inflammatory bowel disease by secreting a genotoxin called colibactin (Arthur et al. 2012; Kostic et al. 2013).

The risk of developing CRC is closely related to poor dietary habits, obesity, diabetes, smoking, intestinal inflammation, polyps, genetic factors, and aging. Ninety

percent of patients who are diagnosed with such disease, are over 50 years of age in average. However, CRC is more aggressive in patients who are diagnosed at a younger age (Granados-Romero et al. 2017; Ponz de Leon & Roncucci 2000). Gut dysbiosis is another one of the factors associated with an increased risk of developing intestinal cancer.

2.3. Management

The gastrointestinal tract is the interface with our diet. The key information is that manipulation of diet, gut microbiota and the gastrointestinal environment are important factors in the prevention of colon and gastrointestinal cancers (Boland et al. 2000).

The two main factors in preventing CRC are normal gut flora and dietary fibre. Butyrate plays an important role in this protection. One of the first observed effects of butyrate on the degree of DNA methylation is probably related to altered gene expression, the consequences of which are as yet unknown, particularly in the context of colon cancer. However, butyrate may also directly increase cell proliferation in normal cells and suppress proliferation in transformed cells. Induction of enzymes by butyrate or microflora and increased prebiotic activity may be an important mechanism of protection against CRC. (Singh et al. 2014; Feng et al. 2018; Hague et al. 1995; (Marchetti et al. 1997)

Treatment options depend on the stage of the disease, the condition of the patient and on the molecular composition of the tumour (Stintzing 2014). The staging is based on the size of the primary tumour, lymph node involvement and the presence of distant metastases (O'Connell et al. 2004). Depending on the pathological characteristics of the tumour, there are different treatment modalities for CRC. Laparoscopic surgery is usually performed for early stages of the primary disease, open surgical resection of the tumour for cases with metastases and adjuvant radiotherapy for unresectable cases. Other treatments for CRC are neoadjuvant and palliative chemotherapy (Hurwitz et al. 2004; Heemskerk-Gerritsen et al. 2015). In 1989 the clinical and histological improvement after faecal microbiota transplantation (FMT) in ulcerative colitis was reported for the recipient after administration of donor microbiota via faecal enema for the first time (Bennet & Brinkman 1989). Following this report, the use of FMT has been described in over 70

cases with varying results (Colman & Rubin 2014). FMT has provided physicians with the most effective method available to replace the pathogenic microbiome ecosystem with a healthy microbiome ecosystem (Ianiro et al. 2018). Currently, the experimental evidence for the efficacy of FMT is mainly focused on the treatment of *Clostridium difficile* infection, while its use in other gastrointestinal diseases, especially CRC, is largely unexplored (Fong et al. 2020). FMT is the most direct method, supported by the highest level of evidence of efficacy in non-cancer diseases. Moreover, a favourable microbiome has the potential to overcome resistance to immunotherapy and alleviate immune-related adverse effects. To this end, clinical trials are underway to evaluate the potential of FMT and microbiota-enhanced approaches to immunotherapy in CRC (Park et al. 2020).

2.4. Bacteria associated with CRC

Up to 500 species of bacteria can be present in the human colon. According to several reports, five genera represent most viable forms of anaerobic bacteria: *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Peptostreptococus* and *Fusobacterium*. A variety of facultative and aerobic organisms are also present in the colon. Overall, it is estimated that bacteria make up 35-50% of the volume of the contents of the human colon (Salminen et al. 1995). There is rising evidence that bacteria are a significant part of the tumour microenvironment. It may contribute to CRC metastasis by signalling through metabolites, promoting epithelial-mesenchymal transition, creating an immunosuppressive microenvironment, and disrupting the gut-vascular barrier (Patel et al. 2022). According to various studies bacteria species associated with this disease were recorded in patients suffering from CRC, namely *Bacteroides fragilis*, *Clostridium septicum*, *C. perfringens*, *Coprobacillus*, *Escherichia coli*, *Eubacterium eligens*, *E. rectale Faecalibacterium preusnitzii*, *Fusobacterium necrophorum*, *F. nucleatum*, *Helicobacter pylori*, *Lachnospiraceae bacterium*, *Peptostreptococcus anaerobius*, *Prevotella copri*, *Ruminococcus obeum*, *Streptococcus bovis* (Ayele et al. 2020; Keenan & Frizelle 2020; Marchesi et al. 2011). *E. coli* and *S. bovis* represent very good laboratory models of bacteria for testing due to their good cultivation.

E. coli is a Gram-negative rod-shaped facultatively anaerobic bacterium. It is an important component of the normal gut microbiota of humans and other mammals and is

also widely used as a host for recombinant DNA cloning technology. Several different strains of *E. coli* cause various intestinal and extraintestinal diseases through virulence factors that affect a wide range of cellular processes (Kaper et al. 2004; Pakbin et al. 2021). Most strains present in the gastrointestinal tract do not cause any disease, however some of them are significantly involved in incidental gastroenteritis. *E. coli* tumorigenic mechanisms can be classified as inflammation that leads to tumour formation and virulence factors that modulate host cells, which later on can cause the development of CRC (Leung et al. 2015). The disease-producing *E. coli* have specific characteristics that are typical of their pathogenicity. The main types of pathogenic *E. coli* are as follows: enteropathogenic (adhering with unknown enterotoxins), enterotoxigenic (adhering to the small intestine, producing enterotoxins) and enteroinvasive (invading the colonic mucosa) (Kühn et al. 1986).

S. bovis is a primary inhabitant of the gastrointestinal tract of humans and animals. It is a catalase-negative and oxidase-negative, immobile, non-sporulating, Gram-positive lactic acid bacterium that grows in pairs or cocci chains (Schlegel et al. 2003). *S. bovis* is one of the main causes of bacterial endocarditis and is involved in colon cancer in humans, probably due to a chronic inflammatory reaction at the site of intestinal colonisation (Herrera et al. 2009). According to the study of Deng et al. (2020) the presence of *S. bovis* is higher in patients with progressive CRC than in healthy individuals. By analysing the relationship between the amount of *S. bovis* and clinicopathological characteristics, it was found that *S. bovis* is more likely to be present in patients with larger tumour size. These data suggest that *S. bovis* is associated with advanced CRC and may promote the development of this disease (Deng et al. 2020).

3. Diet and CRC

Many factors, including diet, environment, genetics, and immunity, influence the occurrence and development of CRC. Among them, dietary factors have been shown to greatly affect the progression of this disease (Zheng et al. 2022).

3.1. Dietary factors

Many articles have studied the role of dietary factors in the outcome of CRC and the results are still inconclusive. However, increasing evidence showed that dietary patterns influence the risk of CRC and affect its treatment. People who suffer from this ailment or are at higher risk of this disease are advised to eat a plant-based diet rich in fruits, vegetables, and whole grains with adequate fibre intake and to avoid high amounts of processed and red meat, and highly refined cereals (Zheng et al. 2022). In the review of Hou et al. (2013) it was found that intake of garlic, vitamin B6 and magnesium, an active lifestyle, maintaining a healthy weight, and avoiding hormone replacement therapy in women can significantly protect against the development of CRC. Alcohol consumption, smoking, and a diet high in fat or sugar can increase the risk of this disease (Van Meer et al. 2013). To give an example, one study reported that men who ate beef, pork, or lamb more than five times a week had a threefold higher risk of developing CRC compared with men who ate less than one meal a month that included these meats (Tuan & Chen 2016).

Dietary factors influencing CRC also include stimulant beverages, which have been found in some studies to both promote and reduce CRC development. However, there are still more studies that confirm the positive effect of these beverages on this disease than studies that have found a negative effect. For example, some experiments have shown that coffee increases the risk of CRC in the non-smoking population, whereas the opposite has been observed in the cigarette-smoking population (Slattery et al. 1999). Similarly, according to Kim et al. (2019) it was found that high green tea consumption was associated with a reduced risk of CRC, with or without healthy lifestyle factors. Mild green tea consumption, however, increased the risk of CRC in regular smokers and drinkers, and in a group with a highly inflammatory diet. In conclusion of this study,

increased green tea consumption could help reduce CRC risk in those with unhealthy lifestyles.

3.2. Dietary components

There are seven main components contained in food that are essential for healthy microflora: carbohydrates, proteins, fats, fibre, vitamins, minerals, and water. These components may have a probiotic and prebiotic effect. However, according to Borugian et al. (2002) increased carbohydrate consumption is associated with an increased risk of CRC in both men and women. Study of Liao et al. (2019) shows that replacing animal protein, especially red meat, with plant protein is associated with a lower risk of CRC. Previous studies have shown that the risk of CRC is higher in groups of high intakes of fats than in those of low intake. In addition, studies of CRC risk and its association with different types of fat have revealed a higher risk of CRC in individuals with high intakes of both saturated fat and cholesterol (Nkondjock et al. 2003; Tayyem et al. 2015). With regard to dietary fibre, the results of animal carcinogenesis studies are variable, but insoluble fibre sources, including wheat bran, appear to be more protective than soluble fibre, and some fibres appear to enhance carcinogenesis (Harris & Ferguson 1993). In the case of vitamins, several mechanisms have been proposed for the role of vitamin B9 in preventing carcinogenesis through molecular mechanisms such as DNA synthesis, repair and methylation (Lamprecht & Lipkin 2003). Vitamin B6 may influence colorectal carcinogenesis through its role in DNA synthesis and methylation (Selhub 2002). In addition, animal models have shown that supplemental vitamin B6 inhibits cell proliferation and reduces the number of tumours in the colon (Komatsu et al. 2002). At the same time, a study of Martínez et al. (1996) suggested an inverse association between total vitamin D intake and the risk of this disease. As for minerals, a review by Swaminath et al. (2019) summarised research on the effect of minerals on CRC. The review concluded that multiple minerals including calcium, magnesium, manganese, zinc, selenium, potassium, iodine, iron, copper, phosphorus, and sodium are likely to influence CRC risk, however individually they may be moderately associated with the risk of this disease. Nevertheless, there is currently no study that compares all seven dietary components in relation to the risk or prevention of CRC.

These aforementioned components are also commonly found in some stimulant drinks, which contain many other substances that can influence the development of CRC as well. Most stimulant beverages contain the same or similar substances. The most well-known of these stimulants is coffee, which is why studies examining the effects of coffee in relation to cancer are prevalent. Coffee contains various anticarcinogenic substances including polyphenols, diterpenes and melanoidins that may be beneficial in improving CRC survival by alleviating systemic disturbances caused by metabolic reprogramming of the cancer or by promoting an anticarcinogenic microenvironment that slows tumour progression (Alicandro et al. 2017). Caffeic acid has the ability to inhibit DNA methylation in cancer cells and is associated with inactivation of apoptosis, stress and inflammatory responses, and regulation of cell cycles involved in the tumour development process (Dong et al. 2011). Further study examined the relationship between coffee consumption after diagnosis and survival in CRC and found that higher coffee consumption was associated with a reduced risk of recurrence and death in patients with stage III. However, whether these findings can be generalized to patients with less advanced stages of CRC remains unclear (Guercio et al. 2015). Another important compound present in coffee are acrylamide, furan, and furfural derivatives, which are thought to have adverse health effects. These substances are formed in a very complex reaction network that occurs during coffee roasting (Gökmen 2016). The most important of these is acrylamide, which is formed by the breakdown of free asparagine in the presence of sugars (Yaylayan et al. 2003; Mottram et al. 2002; (Stadler et al. 2002). According to the International Agency for Research on Cancer acrylamide classifies as a probable human carcinogen in Group 2A (International Agency for Research on Cancer (IARC) 1994).

In the study of Dik et al. (2014) it was examined whether coffee or tea consumption was associated with an altered risk of CRC. The main interest was whether genetic variations in two enzymes involved in caffeine metabolism (CYP1A2 and NAT2) could affect this risk. The results of this study suggest that neither consumption patterns nor genetic differences in caffeine metabolism appear to have a significant effect on CRC risk. Another potentially beneficial stimulant is yerba maté (*Ilex paraguariensis*). It is known to be rich in a variety of phytochemicals such as polyphenols (phenolic acids and flavonoids), alkaloids such as methylxanthines (caffeine, theobromine, theophylline), and terpenes such as carotenoids and saponins (Heck & De Mejia 2007; Barroso et al. 2019).

Phenolic compounds are the group of bioactive substances most associated with the development of a favourable gut microbiota and the maintenance of human health (Shi et al. 2021). The microbiota metabolises the phenolic compounds contained in yerba mate and releases hydroxycinnamic acids in the colon. These phenolic metabolites are responsible for regulating the microbiota population, inhibiting pathogenic bacteria, and stimulating beneficial bacteria (Dueñas et al. 2015; Ozdal et al. 2016; Krga & Milenkovic 2019).

3.3. Effect of diet on gut microbiota

Gut microbiome has an important role in human body defence system. The imbalance between gut microbiome and immune system may contribute to diseases (Van Praet et al. 2014). Consuming a diet high in fat and sugar alters the composition of a healthy microbiota, leading to an imbalance of the microbial population in the gut, a phenomenon known as 'gut dysbiosis'. In addition, long-term consumption of a high-fat diet is associated with a decline in cognitive function (Proctor et al. 2017).

Thousands of microorganisms are an integral part of the gut microbiota and contribute to the normal functioning of this environment. Probiotics and prebiotics also add to the maintenance of normal intestinal microflora. Probiotics are viable microorganisms that (when ingested) have a beneficial effect in the prevention and treatment of specific pathological conditions. Prebiotics, on the other hand, are non-digestible food components that benefit the health of the host by selectively stimulating the growth and/or activity of 1 or a limited number of bacteria in the colon (Chow 2002). They increase the concentration of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria*, and reduce the levels of pathogenic microorganisms. This potential of probiotics and prebiotics has the ability to inhibit the development and progression of neoplasia through mechanisms such as reduction of intestinal inflammation, enhancement of immune function and anti-tumour activity, binding to potential food carcinogens including toxins in meat products, and reduction of bacterial enzymes that hydrolyse precarcinogenic compounds such as β-glucuronidase (Geier et al. 2006; Wollowski et al. 2001).

The abundance and quantity of intestinal bacteria present in the gut microbiota can be affected by the consumption of certain foods. To give an example, soluble fibre increases the ratio of intestinal *Bacteroides fragilis*, such as *B. acidifaciens*, and the production of immunoglobulin. This could improve the immune function of the gut, thereby protecting against intestinal pathogens and reducing the incidence of inflammatory bowel disease. Good sources of soluble fibre include *Hordeum vulgare*, *Plantago indica* and *Glycine max* products such as milk (Nakajima et al. 2020). Data processed by scientific research has shown that lactoferrin, commonly found in bovine milk, slightly promotes the growth of *Bifidobacterium* spp., however according to this research it promotes the growth of *Lactobacillus acidophilus* even more (Kim et al. 2004). *Clostridium perfringens* spores usually germinate in raw or cooked foods under anaerobic conditions; after ingestion, these vegetative cells sporulate, allowing the production of *C. perfringens* enterotoxin, which causes human disease (Grass et al. 2013). According to a previous study, high intake of dairy products in healthy adults may reduce the incidence of *F. nucleatum* and prevent CRC development (Narii et al. 2023).

Lactic acid bacteria (LAB) and their probioactive cellular substances have many beneficial effects in the gastrointestinal tract as well. LAB releases various enzymes into the intestinal lumen and have potential synergistic effects on digestion and alleviate symptoms of intestinal malabsorption. Consumption of fermented dairy products with LAB may induce anti-tumour effects. These effects are attributed to inhibition of mutagenic activity, a decrease in several enzymes involved in the formation of carcinogens, mutagens, or tumour-promoting substances (Kumar et al. 2010).

Stimulant beverages may also have a potential effect on a healthy gut microbiota, but their exact effects on this environment are being further investigated. Some studies have identified the potential benefit in the consumption of stimulant drinks to promote healthy intestinal microflora by promoting beneficial bacteria. To give an example in a case study of Jaquet et al. (2009) it was shown that the greatest increase in bifidobacteria occurred in healthy individuals with lower initial populations of this bacteria after coffee consumption. The metabolic activity of bifidobacterial species also increased in some cases after coffee consumption. Although these results cannot be directly linked to product consumption, they suggest that coffee consumption may prove useful in increasing bifidobacterial numbers or metabolic activity.

4. Stimulant beverages

Stimulant beverages are infusions, decoctions and macerates from plants or parts of plants that are cultivated for their chemical constituents having stimulating effect on the central nervous system. They have a significant amount of secondary metabolites e.g., caffeine, nicotine, theobromine, theophylline, and flavonoids (Vossen & Wessel 2000).

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine) are one of the most consumed stimulant substances in the whole world (Anthonisen et al. 2001). Naturally occurring methylxanthines such as caffeine, theophylline or theobromine are widely consumed in foods (Janitschke et al. 2021). Although there are more than 100 species of plants that contain these alkaloids there is only a small number of these highly consumed sources of methylxanthines in the world such as: coffee (*Coffea* spp.), tea (*Camelia sinensis*), maté (*Ilex paraguariensis*), cocoa (*Theobroma cacao*), guaran seeds (*Paulinia cupana*) and cola seeds (*Cola nitida*) (Anthonisen et al. 2001).

People's interest in stimulant beverages such as coffee and tea may have initially had religious reasons. However, these stimulants soon gained widespread popularity at all levels of society in and around the countries where they were first cultivated, and eventually in Europe and America (Vossen & Wessel 2000). According to Statista (2023) tea and coffee make up for 4.38 % of all non-alcoholic beverages for 2022. From the six examples mentioned above, the focus was mostly on coffee, tea, maté, and rooibos (*Aspalathus linearis*). All the beverages used for this thesis come from different plants and different regions of the world, so the following paragraphs are giving an overview of the characteristics of these beverages and their origins.

4.1. *Aspalathus linearis*

4.1.1. Botany

A. linearis (Rooibos) is an erect to spreading, highly variable shrub or shrublet up to 2 m hight belonging to the Fabaceae family with its origin in the Cedarberg Mountains in the Western Cape region of South Africa, where it is commonly cultivated for its commercial use as an herbal tea or tisane (Mckay & Blumberg 2007). Leaves are without

petiole and palist and often times occur in dense clusters. They are variously flat to rounded and end in spines (Leistner 2000). The flowers are of typical "pea" form and borne in short clusters. They are yellow and 6.5 mm long. The seedpod is lanceolate, downy, 1.5 cm long, contains a single tiny, hard, yellow, kidney-shaped seed which is flung out when the pod splits open (Morton 1983).

4.1.2. Consumption and uses

To consume rooibos tea, it is recommended to use 1 teaspoon of rooibos tea per cup, pour boiling water over it, let it infuse for a few minutes (to achieve the desired strength) and strain. Excess cooled tea can be reheated if necessary. The tea will not become cloudy, bitter, lose flavour or colour. For serving iced rooibos tea, it is suggested to use 2 teaspoons per cup of boiling water, boil, strain and add ice. If you wish to preserve as much ascorbic acid as possible, you can prepare cold rooibos tea simply by filling a tea strainer, hooking the chain over the rim of a glass of water and letting it sit in the refrigerator until desired. The infusion can be drunk as is or diluted to taste. In some countries, for example, it is diluted with milk. For young people it is much preferable to caffeinated soft drinks (Morton 1983; Cheney & Scholtz 1963).

4.1.3. Chemistry

During the fermentation process, aspalathin, a dihydrochalcone found in rooibos tea, is oxidized to dihydroisoorientine (Bramati et al. 2003). The C-glycosylflavones isoorientin, orientin, isovitexin and vitexin are also degraded but to a lesser extent. Nothofagin, a dihydrochalcone structurally similar to aspalathin, is also degraded (Joubert & Ferreira 1996). Other predominant flavonoids found in rooibos tea are rutin, isoquercetin and hyperoside, quercetin, luteolin and chrysoeriol. In red rooibos tea phenolic acids such as caffeic, ferulic, p-coumaric, p-hydroxybenzoic, vanillic and protocatechuic acid was found (Rabe et al. 1994). Due to the known high polyphenol content of rooibos, it may potentially act as a prebiotic in the intestines, facilitating the improvement of chronic inflammatory gastrointestinal conditions (Pretorius et al. 2022).

4.2. *Camellia sinensis*

The beverage called tea comes from the *C. sinensis* plant. There are 4 main types of tea which include black tea, green tea, oolong tea and white tea. Each of these are produced differently depending on the method of processing. However, in this bachelor thesis only black and green tea was used.

4.2.1. Botany

There are two major types of tea plants both belonging to the Theaceae family: *Camellia sinensis var. sinensis* which is evergreen, multi-stemmed shrub up to 3 m tall with small (less than 10cm) dark green leaves, it is native to China and can grow in other Asian countries with mild cold climates as well. Due to the unique aroma and taste of this variety, it is mainly used to produce green tea. The second main variety is *Camellia sinensis var. assamica*, which is evergreen tree up to 10-15 m with one main stem. It has large (15-20 cm) leaves and was discovered in the southwestern part of China and India and is also imported to other countries with semi-tropical climates (Vossen & Wessel 2000). Due to its high catechin and tannin content, this variety is mainly used for black tea production (Li et al. 2013). Flowers are axillary, single or in racemes 2-4 cm across, very fragrant with 5-7 sepals and petals. Usually, they are white or pale pink. Fruit is a subglobose capsule, 1.5-2 cm across, thick-walled and woody, brownish green. There are 1-2 seeds per capsule which are globose or flattened on one side (Vossen & Wessel 2000).

4.2.2. Consumption and uses

Green and black tea account for about 20% and 78% of the worldwide tea consumption (Li et al. 2013). Approximately three billion kilograms of tea are being produced and consumed annually (Yang & Landau 2000). The way of consuming black and green tea is known worldwide. The typical method of consumption is to pour over 1 teaspoon of tea with boiling water, steep for about 3 minutes and then strain. Black tea is typically served with milk and honey in some countries while green tea is in most cases served alone. People consume it for refreshment especially during breakfast or after breakfast and it is the cheapest drink consumed worldwide. Two or three cups of tea is the mostly consumed amount of tea (Naveed 2014).

4.2.3. Chemistry

Tea polyphenols, known as catechins, typically make up 30-42% of the dry weight of brewed green tea (Shahidi & Naczk 2003). Catechins are characterised by a di- or tri-hydroxyl substitution of the B ring and a meta-5,7-dihydroxy substitution of the A ring. There are four main catechin structures, (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC). EGCG is the major catechin in tea and may account for 50-80% of the total catechin in tea. Catechin, gallocatechin, epigallocatechin digallate, epicatechin digallate, 3-O-methyl EC and EGC, catechin gallate and gallocatechin gallate are present in smaller amounts. Flavonols are also present in tea, including quercetin, kaempferol, myricitin and their glycosides. A typical tea beverage, prepared at a ratio of 1 g leaf to 100 ml of water with a three-minute infusion, typically contains 250-350 mg of tea solids, which consist of 30-42 % catechins and 3-6 % caffeine (Mukhtar & Ahmad 1999).

4.3. *Coffea* spp.

For this bachelor's thesis only two types of coffee beans were used which included arabica and robusta coffee beans.

4.3.1. Botany

Coffea arabica is an evergreen, semi-deciduous shrub, or small tree, often multi-stemmed, up to 4-5 m tall and in cultivation cut to 1.8-2.5 m belonging to the Rubiaceae family. It is native to the southwestern and south-eastern montane forests of Ethiopia (Senbeta 2020). Leaves are petiolate with petioles up to 2 cm long, dark green above, lighter green below. Flowers are in axillary clusters, hermaphrodite, fragrant and creamy white. The fruit is ovoid-ellipsoidal and green at first, but red at maturity. The seeds are ellipsoid, 8-12 mm long and there are 2 per fruit. *C. canephora* is a larger tree, up to 8-12 m tall, with longer leaves up to 15-30 cm with corrugated surface, belonging to the Rubiaceae family with its origin in the East African Great Lakes region (Kusolwa et al. 2019). The flowers are white and more abundant. Fruits are smaller, 8-16 mm long and in general, *C. canephora* is more vigorous than *C. arabica* and shows much higher polymorphism. (Vossen & Wessel 2000).

4.3.2. Consumption and uses

Coffee is the leading worldwide beverage after water (Butt & Sultan 2011). Its global consumption has grown by 67.9% in the last 26 years (Torga & Spers 2020). The dried seeds ("beans") are roasted, ground, and made into one of the two most popular drinks in the world. In its homeland, Ethiopia, it has been used as masticatory since ancient times. Cooked in butter, it can be used to prepare rich pancakes. Coffee is widely used as a flavouring agent in ice cream, candies, confectionery, and liqueurs. In Arabia, fermented drink made from the pulp is used as well (Orwa et al. 2020).

4.3.3. Chemistry

Coffee contains several biologically active substances such as caffeine, diterpenes, chlorogenic acids and melanoidins, which may have an effect on human health (Godos et al. 2014). Green coffee is composed predominantly by carbohydrates (60 % of dry matter), including soluble and insoluble polysaccharides (cellulose, arabinogalactan and galactomannan), which are slightly higher in robusta coffee, followed by oligosaccharides (stachyose and raffinose), disaccharides (sucrose) and monosaccharides (glucose, galactose, arabinose, fructose, mannose, mannitol, xylose and ribose) (Mazzafera 1999; Hu et al. 2001). The lipid content of green coffee accounts for 8-18 % of its dry matter and is significantly higher in Arabica coffee than in Robusta coffee. The lipid fraction of coffee is 75% triglycerides. Other lipids include sterols (stigmasterol, sitosterol), fatty acids (linoleic, linolenic, oleic, palmitic, stearic, arachidic, lignoceric and behenic) and in coffee wax, pentacyclic diterpenes (kafestol, kahweol) and fatty acyl tryptamides (Kurzrock & Speer 2001). Proteins, peptides and free amino acids make up 9-16% of the dry matter of green coffee beans. The main amino acids, both protein-bound and free, are asparagine, glutamic acid, alanine, aspartic acid, and lysine (Hu et al. 2001).

4.4. *Ilex paraguariensis*

4.4.1. Botany

Maté (*I. paraguariensis*) usually also known as Yerba Maté or Brazilian tea, is an evergreen tree belonging in the Aquifoliaceae family with its origin in Paraguay, Argentina, Brazil, Colombia, Ecuador, and Uruguay (Small & Catling 2001). In the wild

it is up to 18 m tall tree, in culture it is cut down to a 3-6 m tall multi-stemmed and heavily branched shrub. Leaves are alternate, cordate, petiole 1 cm long and dark green. Flowers are small, pedunculate with 4 white petals. The fruit is a reddish to blackish globose drupe, 0.5-0.8 cm in diameter, each containing a single seed. (Vossen & Wessel 2000).

4.4.2. Consumption and uses

Production of this potion is estimated at around 1,4 million tonnes per year, of which just under 5 % is exported to other countries, while the vast majority is destined for local consumption as a national product, e.g., around 80 % in Brazil (Cardozo Junior & Morand 2016). The drink is prepared by compacting a quantity of maté, previously moistened with water. Hot, but not boiling, water is poured over the wall of the container made of gourd or 'cuia'. The infusion is drunk by suction through a silver tube or 'bomb', which has a flattened perforated disc at the end which is immersed in the infusion and serves as a filter. For the same batch of mate, hot water can be added more than twice until the drink loses its flavour (Mazzafera 1997). The best tea from this plant is only from young leaves, but sometimes young shoots are also used (Small & Catling 2001). Maté is also used medicinally as a diuretic, depurative and general tonic to relieve mental and physical fatigue. In Europe, maté is used for weight loss because it reduces appetite. Extracts of mate are also used to flavour other products such as liqueurs, ice creams, and desserts (Vossen & Wessel 2000).

4.4.3. Chemistry

Polyphenols come from the parent plants and are considered their main bioactive component. The number of polyphenolic compounds in yerba mate is higher than in green tea and similar to that found in red wine (Gugliucci et al. 2009; Gugliucci & Bastos 2009). Polyphenols in yerba mate include caffeic acid, caffeine, caffeoyl derivatives, caffeoylshikimic acid, chlorogenic acid, feruloylquinic acid, kaempferol, quercetin, quinic acid, rutin and theobromine (Carini et al. 1998; Chandra & De Mejia 2004; Atoui et al. 2005; Bravo et al. 2007), with caffeoyl derivatives accounting for approximately 10% of dry weight (Filip et al. 2001).

Although the aforementioned plants and the substances they contain have been described in previous research, they have never been tested together with CRC-related bacteria especially not using the kinetics we used for this bachelor thesis.

5. Aims of the Thesis

The aim of this thesis is to evaluate *in vitro* effects of stimulant beverages on growth of representatives of bacterial pathogens associated with CRC risk.

The specific objectives of the thesis are as follows:

a) Determination of minimum inhibitory concentrations (MICs) of stimulant beverages against CRC-causing aerobic and anaerobic bacteria

b) Monitoring of growth-inhibitory or supporting action of stimulant beverages on growth kinetics and length of lag phase of *E. coli* and *S. bovis*

6. Materials and Methods

6.1. Plant materials

Tea (including rooibos) and coffee samples were purchased from Herbs Life (Sokolov, CZ) and Manu Café (Ludgeřovice, CZ), respectively. Maté was purchased from Salvia Paradise (Zaječov, CZ). All the samples were grounded to mild powder using an electric mill GM 100 (Retsch, Haan, DE) and used for the extraction. Four grams of the powder were poured over 200 ml of boiling distilled water and left leaching for 5 minutes. During the leaching process the samples were stirred using the laboratory magnetic stirrer (RH basic 2, IKA, Staufen, DE) and afterwards poured through a sieve into a volumetric beaker. After the beverages completely cool down, they were filtered through filter paper. Twenty millilitres of homogenized liquids were lyophilized by freeze-dryer (Coolsafe, Labogene, Lillerød, DK) for 75 hours. Dried residues were then diluted in 100% dimethyl sulfoxide (DMSO) (Penta, Prague, CZ) to obtain stock solution of the final concentration 51.2 mg/ml while laboratory shaker (GFL, Burgwedel, DE) was used to dissolve the samples into DMSO. Diluted samples were afterwards stored in Eppendorf Tubes 2.0 ml at -20°C until their use.

6.2. Microorganisms and media

The antibacterial activity was determined against 6 representatives of both Gram-positive/- negative and aerobic/anaerobic bacteria. Standard American Type Culture Collection (ATCC) and Czech Collection of Microorganisms (CCM) strains namely *B. fragilis* ATCC 25285, *C. septicum* ATCC 12464, *E. coli* ATCC 25922, *S. bovis* ATCC 33317, *F. necrophorum* CCM 5981 and *P. anaerobius* CCM 3790 were obtained from Oxoid (Basingstoke, UK) and CCM (Brno, CZ).

Mueller-Hinton broth (MHB) was used as growth medium for aerobic group of bacteria and Wilkins-Chalgren broth (WCB), together with Brain Heart Infusion (BHI) broth were used for anaerobic bacteria. All media were purchased from Oxoid (Basingstoke, UK). Buffered forms of broth were used for testing. Into one litre of distilled water, 0.2 g KCl, 6.1 g of Tris Base and 8.0 g of NaCl was added and then the mixture was stirred properly on the laboratory magnetic stirrer (RH basic 2, IKA, Staufen,

DE). The pH determined by pH meter (Eutech Instruments pH 510, Chromservis, Prague, CZ) was adjusted to 7.6. Vertical steam sterilizer (Tuttnauer 3870ELV, NL) was used for sterilization of the growth media, which were stored in fridge in 4°C until using.

6.3. Determination of the minimal inhibitory concentrations (MIC)

For the effective assessment of *in vitro* antimicrobial activity, the specific broth microdilution method using 96-well microtiter plates was employed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, modified by Cos et al. (Cos et al. 2006), where MICs [μg/ml] were assessed. The MIC was defined as the lowest concentration of an antimicrobial compound that inhibits visible growth of the microorganism after overnight incubation and is thus an important indicator of resistance of microorganisms to antimicrobials (Andrews 2001). All 6 beverages were dissolved in DMSO, and 2-fold diluted in appropriate growth media (100 μL) in a range of 512, 256, 128 and 64 μg/ml using automated pipetting platform Freedom EVO 100 (Tecan, Männedorf, CH) for assessment of aerobic and anaerobic bacteria, respectively. All bacterial cultures were diluted to contain 1.5×10^8 CFU/mL by densimeter (DENSI-LA-METER II, Erba Lachema, Brno, CZ) and microtiter plates were subsequently inoculated with the suspension. Microplates inoculated with *E. coli* and *S. bovis* were then incubated for 24 h at 37 °C. The plates inoculated with *B. fragilis*, *C. septicum*, *F. necrophorum* and *P. anaerobius* were prepared and incubated in Whitley A35 Anaerobic Workstation (Don Whitley Scientific, West Yorkshire, UK). Bacterial growth was determined by the absorbance measurement by Cytation 3 Imaging Reader (BioTek, Vermont, USA) at 405 nm. The lowest DMSO diluted samples concentration showing at least ≥80 % reduction of microbial growth compared to the positive growth control was considered as MIC. Ciprofloxacine and tetracycline (Sigma-Aldrich, Prague, CZ) previously dissolved in DMSO (Sigma-Aldrich, Prague, CZ) and ethanol (Lach-Ner, Neratovice, CZ) respectively, were tested as positive antibiotic control in concentration range of <0.0625 to >32 μg/ml All tests were performed as three independent experiments each carried out in triplicate.

6.4. Monitoring of growth kinetics

For monitoring of bacterial growth kinetics, the protocol of broth microdilution method described above was used. However, only *E. coli* ATCC 25922 and *S. bovis* ATCC 33317 were used for kinetic assay. Stimulant beverages samples were 2-fold diluted in MHB (for *E. coli*) and BHI (for *S. bovis*) in ranges of 64-512 μg/ml, whereas ciprofloxacine and tetracycline were prepared in ranges of 0.125-32μg/ml. During 24 h of incubation, absorbance measurements by Cytation 3 Imaging Reader (BioTek, Vermont, USA) were performed every hour and the regular orbital shaking conditions were selected. The lag time was defined as the time at which the extrapolated slope of the exponential phase intercepts a horizontal line, extrapolated from the starting inoculum concentration. The lag phase values at each concentration of tested agent were calculated by Gen5 Image+ 3.11 software (BioTek, Vermont, USA). Subsequently, the difference between the growth control and the individual concentrations was calculated based on these values. Growth curves plotting the time dependence of optical density at wavelength of 405 nm were constructed using Microsoft Excel (Microsoft corporation, Washington, USA) for stimulant beverages showing the most significant prolongation and shortening of calculated lag times.

7. Results and discussion

7.1. Antibacterial susceptibility testing

The growth-inhibitory effect of stimulant beverages was determined *in vitro* for six representatives of both aerobic and anaerobic bacteria. As a result, all samples of stimulant beverages produced no inhibitory activity (MIC > 512 μg/ml) against *B. fragilis*, *C. septicum*, *E. coli*, *F. necrophorum, P. anae*robius and *S. bovis*. As a positive antibiotic control, ciprofloxacine and tetracycline were used. Both tested antibiotics showed different levels of activity against tested bacteria with MICs from <0.0625 to >32 μg/mL (Table 1).

Table 1. *In vitro* growth -inhibitory effect of antibiotics on bacteria associated with colorectal cancer

Microorganisms	Minimum inhibitory concentration (μg/ml)	
	Ciprofloxacine	Tetracycline
Bacteroides fragilis	2	>32
Clostridium septicum	0.5	0.125
Escherichia coli	<0.0625	4
Fusobacterium necrophorum	1	>32
Peptostreptococcus anaerobius	0.5	>32
Streptococcus bovis	8	8

According to scientific research of Reygaert & Jusufi (2013), green tea extract may have inhibitory effects on the growth of several strains of *E. coli* causing urinary tract infections. In this research, the concentrations ranging from 2500 to 4000 μg/ml of the extract were found to be effective against the strains tested. Nevertheless, since the extract concentrations used in the previous study were several times higher than those used in our experiments, it can be assumed that the MIC values of stimulant beverages are outside the range of concentrations assayed in this study (64-512 μg/ml). Similarly, Prado Martin et al. (2013) discovered that *I. paraguariensis* methanolic and ethanolic extracts did not inhibit growth of *E. coli* ATCC 25922 at the concentrations ranging from 780 to 25000 μg/ml.

7.2. Bacterial growth kinetics

Since the values of MICs were not detected in the first experiment, the effect of stimulant beverages on CRC associated aerobic microorganisms (*E. coli* and *S. bovis*) were determined by monitoring of bacterial growth kinetics. The results presented as standard culture growth curves showed both inhibitory and supportive effects of the stimulant beverages on the growth kinetics of bacteria assayed. In the case of *E. coli*, *I. paraguariensis* was the only sample producing significant extension of the lag time (20 minutes) of its growth at the highest concentration tested (512 μg/ml). Growth curves showing the effect of *I. paraguariensis* at the concentration of 512 μg/ml on the growth of *E. coli* is shown in the Figure 1. In contrast, *A. linearis*, *C. arabica* and *C. canephora* reduced lag time growth of *E. coli* by 30, 25 and 27 minutes at the concentration of 64, 128, and 256 μg/ml respectively. In general, the effects of stimulant beverages on *E. coli* growth were much more significant than on *S. bovis.* The effect of stimulant beverages on growth lag phase duration of *E. coli* and *S. bovis* are shown in Tables 2 and 3.

There is not a large number of studies that measure the effects of stimulant beverages on bacterial growth using bacterial growth kinetics. However, there are some studies monitoring the effects of various natural products such as tea and coffee extracts on the growth of bacteria which are not tested in this study (Becerril et al. 2011).Based on this finding, this bachelor's thesis is probably the first report reporting the effects of stimulant beverages on bacteria associated/causing CRC. Based on our results, we can speculate that the stimulant beverages may have both inhibitory and supportive effects on CRC development. Some of these beverages, such as *A. linearis* or *C. arabica* and *C. canephora,* may promote the development of CRC, while others, such as *I. paraguariensis,* may inhibit its development.

Table 2. The effect of stimulant beverages on growth lag phase duration of *E. coli*

Plant species	Growth control	Stimulant concentration (µg/ml)/Lag time (min)							
		Measured value ± standard deviation				Calculated [a]			
		64	128	256	512	64	128	256	512
A. linearis	100 ± 7.08	71±2.76	80 ± 6.87	80 ± 4.64	75 ± 8.29	-30	-21	-21	-26
C. sinensis var. sinensis	100 ± 7.08	82 ± 6.49	85 ± 12.71	98 ± 3.16	86 ± 7.21	-19	-16	-3	-15
C. sinensis var. asamica	100 ± 7.08	107 ± 6.99	100 ± 4.51	102 ± 2.78	100 ± 6.11	6	-1	1	-1
C. arabica	100 ± 7.08	105 ± 1.09	76 ± 0.70	81 ± 8.90	91 ± 9.82	4	-25	-20	-10
C. canephora	100 ± 7.08	104 ± 0.21	76 ± 8.16	74 ± 7.92	82 ± 0.46	3	-25	-27	-19
I. paraguariensis	100 ± 7.08	115 ± 6.11	96 ± 8.32	107 ± 2.28	121 ± 2.30	14	-5	6	20

Footnotes: [a] = difference between the growth control and the individual concentrations of lag time

Table 3. The effect of stimulant beverages on growth lag phase duration of *S. bovis*

Plant species	Growth control	Stimulant concentration (µg/ml)/Lag time (min)							
		Measured value ± standard deviation				Calculated [a]			
		64	128	256	512	64	128	256	512
A. linearis	75 ± 5.13	66 ± 0.71	71 ± 1.4	72 ± 2.08	62 ± 5.74	-9	-4	-3	-13
C. sinensis var. sinensis	75 ± 5.13	68 ± 0.76	70 ± 1.39	61 ± 1.58	56 ± 3.7	-7	-5	-14	-19
C. sinensis var. asamica	75 ± 5.13	72 ± 0.81	72 ± 1.67	70 ± 1.55	73 ± 8.68	-3	-3	-5	-2
C. arabica	75 ± 5.13	65 ± 1.50	65 ± 1.00	66 ± 1.81	67 ± 1.93	-10	-10	-9	-8
C. canephora	75 ± 5.13	67 ± 0.41	67 ± 0.99	68 ± 1.77	68 ± 3.21	-8	-8	-7	-7
I. paraguariensis	75 ± 5.13	68 ± 1.68	68 ± 1.39	71 ± 0.4	75 ± 2.96	-7	-7	-4	0

Footnotes: [a] = difference between the growth control and the individual concentrations of lag time

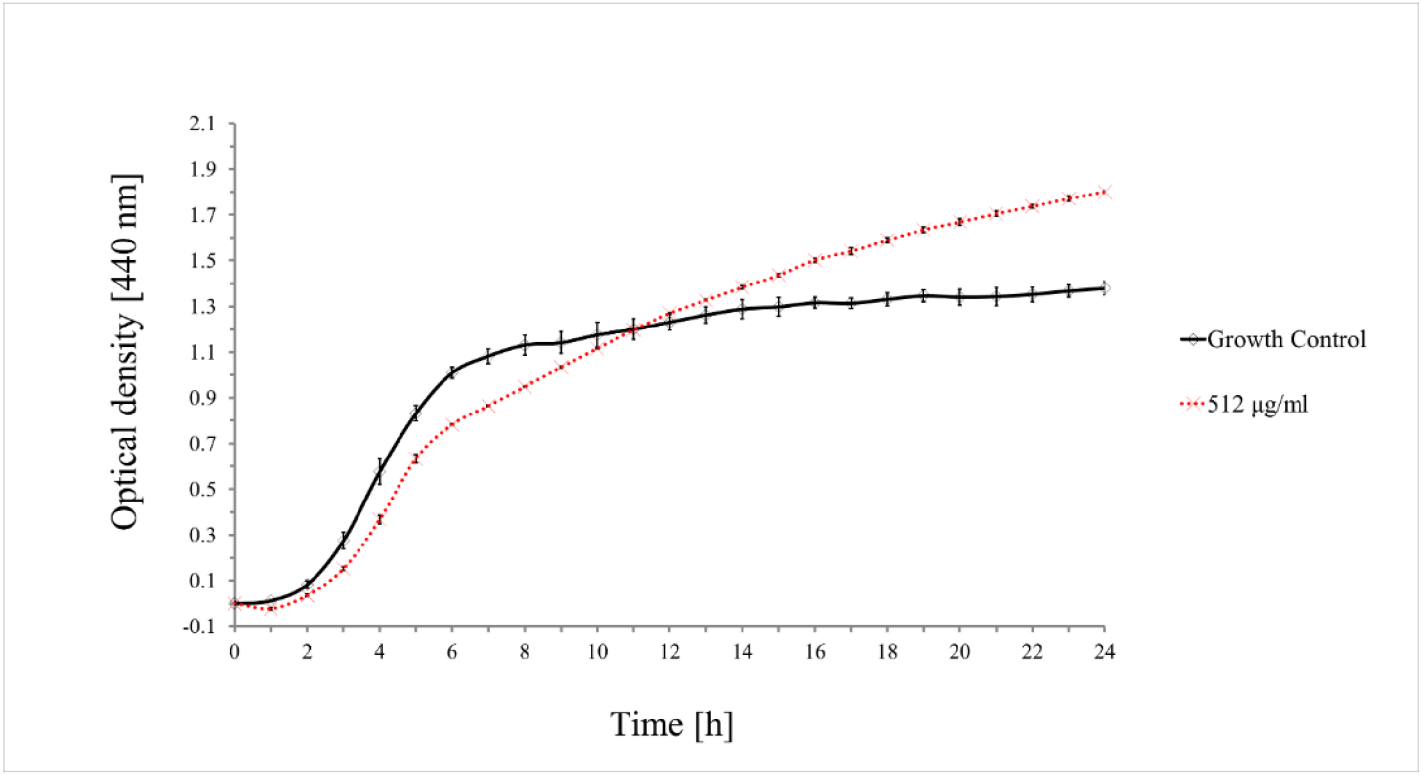

Figure 1. Growth curves of *E. coli* in the presence of *I. paraguariensis* at the concentration of 512 µg/ml

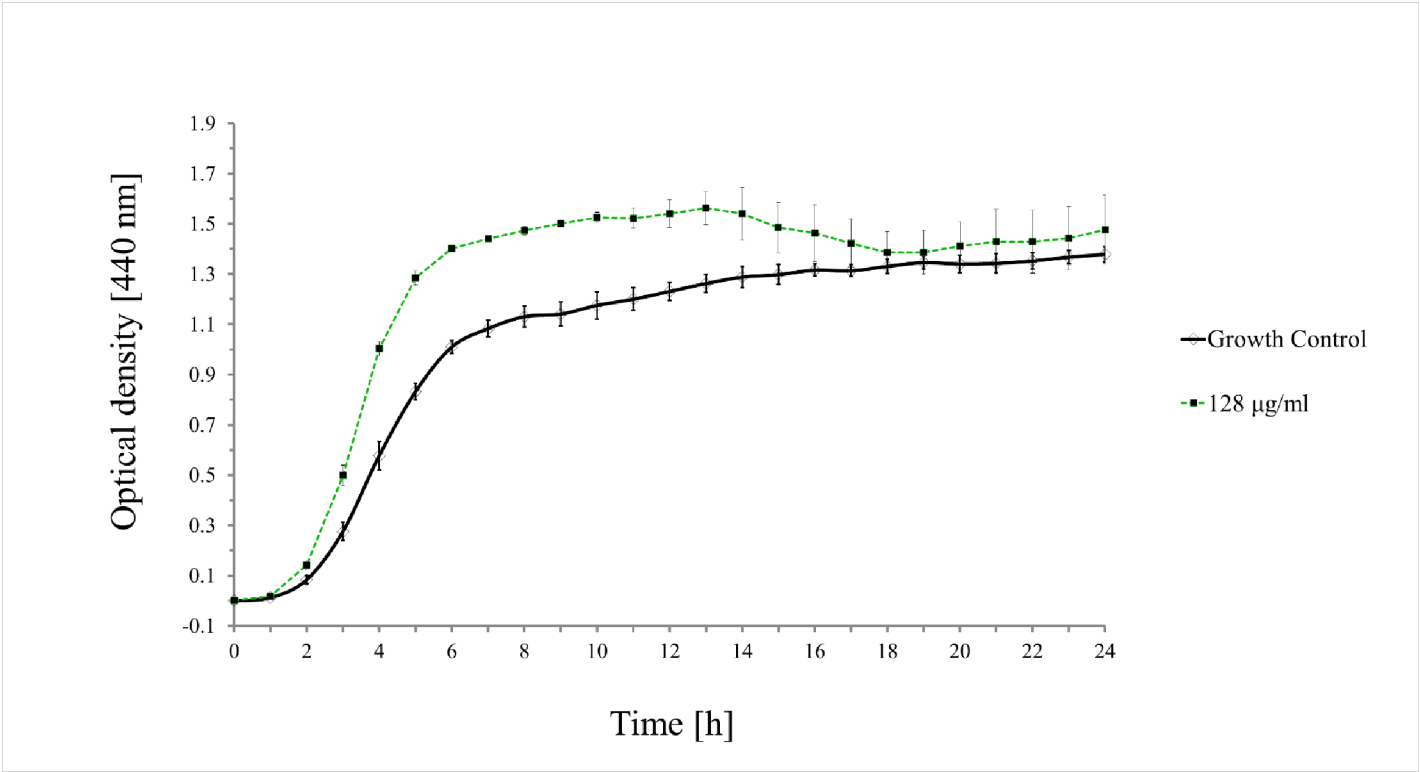

Figure 2. Growth curves of *E. coli* m in the presence of *A. linearis* at the concentration of 128 µg/ml

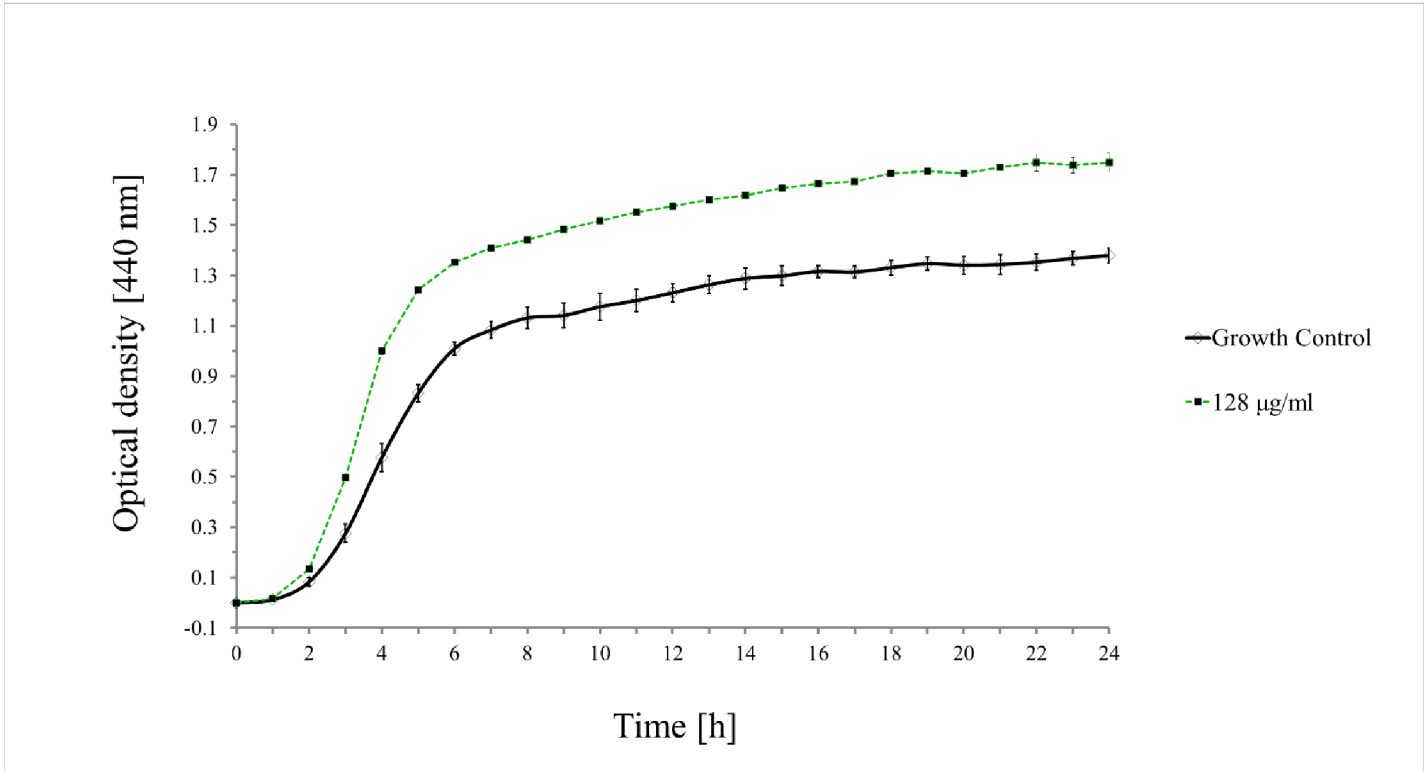

Figure 3. Growth curves of *E. coli* in the presence of *C. arabica* at the concentration of 128 µg/ml

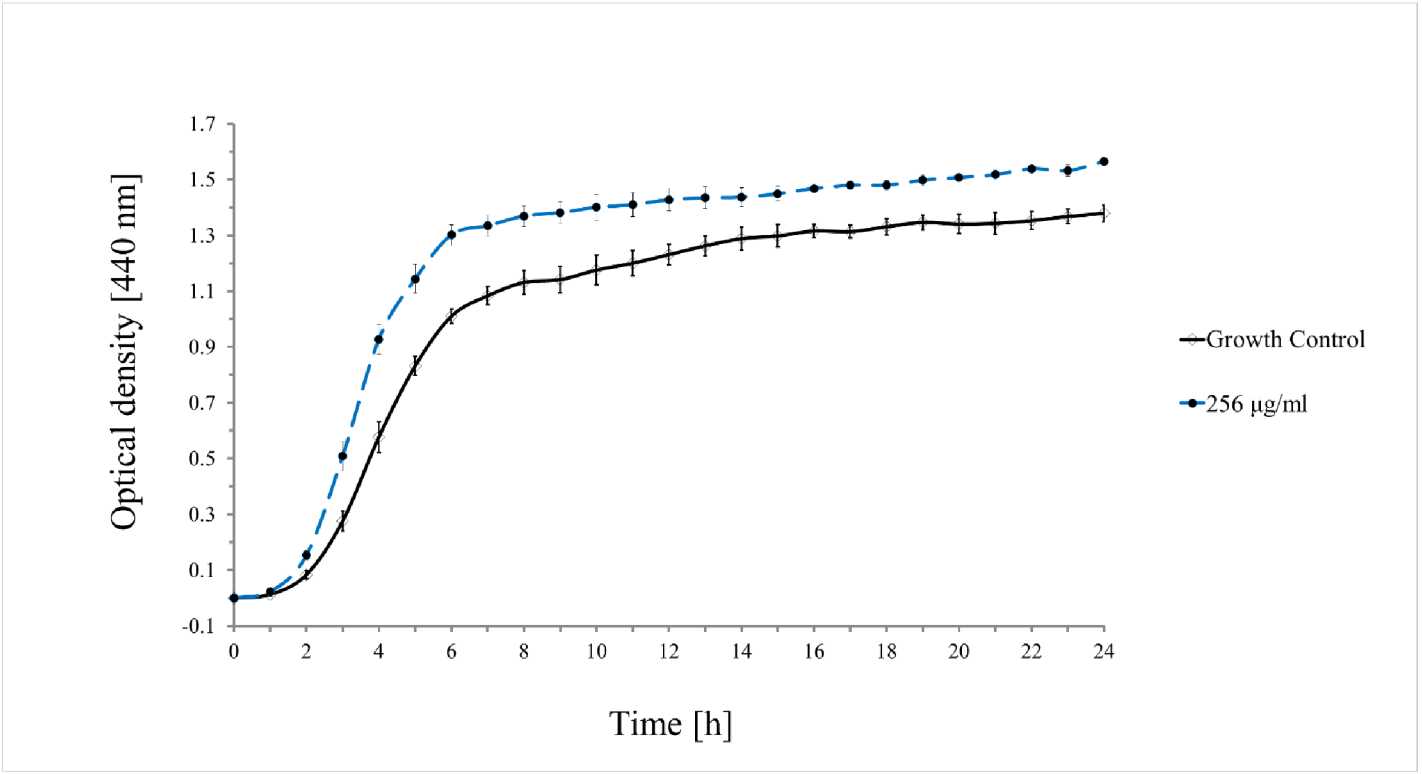

Figure 4. Growth of *E. coli* in the presence of *C. canephora* at the concentration of 256 µg/ml

8. Conclusions

According to the results, we can conclude that some stimulant beverages may affect the growth of certain bacteria that are associated with CRC. Although it was not possible to determine the MICs of stimulant beverages for bacteria associated with the development or occurrence of CRC, the inhibitory or supportive effect of these beverages was detected using bacterial growth kinetics. Data obtained by this method demonstrate that the stimulant beverages samples, *I. paraguariensis* markedly inhibits the growth of *E. coli* by extension of the lag phase. This finding implies that consumption stimulant beverages may have both positive and negative effect on CRC development. It is important to note that further research is needed to test bacterial growth kinetics in other bacterial strains associated with CRC.

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