COMPARISON OF PHYSICOCHEMICAL PROPERTIES AND ANTI-MICROBIAL ACTIVITIES OF TEA AND COCOA-BASED KOMBUCHA.

A RESEARCH PROJECT REPORT SUBMITTED

BY

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DECLARATION

I, Senanu Richmond Adzadogo, do declare that the experimental work described in this project report was done by me in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon under the supervision of Rev. Dr. W. S. K. Gbewonyo.

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Supervisor.
DEDICATION

This project is dedicated to God Almighty, whose blessing and mercies has brought me this far.

This work is also dedicated to my supervisor, who encouraged and guided me throughout the work.

I finally dedicate this project to my family and all MSc. and MPhil. Biochemistry students for their support and encouragement.
ACKNOWLEDGEMENT

I thank God Almighty for granting me and my supervisor good health, strength, protection throughout the period of this project. It is by His Grace that this project was successful.

Secondly, I wish to express my profound gratitude to my supervisor Rev. Dr. W. S. K. Gbewonyo for his fatherly guidance and support throughout the period of the project.

I am also grateful to all lecturers of the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon for their contribution to the successful completion of my study and to the laboratory technicians for their assistance during this project.

Finally I will like to acknowledge all my colleagues for their support during this project work.
ABSTRACT

Kombucha tea is a slightly sweet, slightly acidic refreshing beverage consumed worldwide. Kombucha is a symbiosis of the Genera *Acetobacter* and *Gluconobacter*, with *Acetobacter xylinum* as a characteristic species, and various yeasts such as genera of *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces* and *Pichia* depending on the source. The tea fungus broth is composed of two portions, a floating cellulosic pellicle layer and a sour liquid broth. Black and green tea are known to be the best substrate for kombucha preparation.

Black tea and cocoa powder served as the substrate in this study. The aim of the study was to compare the physical and chemical as well as the antimicrobial properties of tea and cocoa-based kombucha. The study revealed that tea kombucha is more acidic compared with cocoa kombucha, both tea and cocoa kombucha have similar phytochemicals. Tea and cocoa kombucha were found to inhibit the growth of *Candida albican* and *Shigella* spp, confirming its antimicrobial activity. The presence of phytochemicals and antimicrobial properties support the school of thought that kombucha drink provide a maximum health benefit to humans.
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CHAPTER ONE

1.0 INTRODUCTION

Kombucha tea also known as Kargasok Tea, Tea Fungus, Haipao and Manchurian Mushroom is a slightly sweet, slightly acidic refreshing beverage consumed worldwide. Kombucha is fermented by a symbiosis of microbes of the genera *Acetobacter* and *Gluconobacter*, with *Acetobacter xylinum* as a characteristic species, and various yeasts such as genera of *Brettanomyces*, *Zygosaccharomyces*, *Saccharomyces* and *Pichia* depending on the source (Mayser et al., 1995).

The yeasts ferment the sugar in the cultivation medium to ethanol, which is further oxidised by the acetic acid bacteria to acetic acid. The result is a reduced pH of the medium. The final product is a sour, slightly carbonated, acidic beverage, comprised of sugars, organic acids, tea components, vitamins, and minerals. Many flavour compounds, including alcohols, aldehydes, ketones, esters and amino acids have been identified. The tea fungus is composed of two portions; a floating cellulosic pellicle layer and a sour liquid broth.

The main metabolites identified in the fermented beverage are acetic acid, lactic acid, gluconic acid, glucuronic acid and the alcohol and glycerol (Blanc, 1996; Chen & Liu, 2000; Jayabalain et al., 2007). The changes that occur in the beverage are due to the metabolic activity of the bacterial and yeast (‘tea fungus’) in the medium. Some of the changes that occur include changes in phenols, minerals, total acidity and vitamin content (Malbasa et al., 2006; Chen & Liu, 2000).
Figure 1: Representation of set-up showing tea and cocoa kombucha fermentation process. The beverage has been tagged to be a prophylactic agent and exhibit some health benefits. These health benefits include antimicrobial, antioxidant, anti-inflammatory and liver detoxification properties (Jayabalan, 2010; Malbasa et al., 2008; Greenwalt et al., 1998). These beneficial properties are attributed to tea polyphenols, gluconic acid, lactic acid glucuronic acid, vitamins, amino acids, antibiotics and other micronutrients produced at the fermentation stage (Jayabalan 2010).

A study on black and green tea used in kombucha preparation revealed its potency in reduction of cancers of all kinds and has antioxidant and antimicrobial activities (Cabrera et al., 2003).
Antimicrobial activity of kombucha tea is attributed to acetic acid as well as antibacterial agents such as bacteriocins produced during the fermentation. Gluconic acid is known to be useful in detoxification of the liver, gluconates and vitamins contribute to the antioxidant and anti-inflammatory properties of the beverage (Sreeramulu et al., 2001).

1.2 JUSTIFICATION FOR THE STUDY

Early reports on kombucha suggest the beverage has anti-microbial activity against a spectrum of disease causing microbes, also concentrates of unfermented tea components had anti-microbial properties. Phytochemicals present in the beverage is reported to inhibit microorganisms. Steinkraus et al. (1996) reported that antimicrobial activity of kombucha was attributable to the acetic acid content. Kombucha was introduced in Ghana in the mid 1980’s and its popularity has increased ever since, this can be attributed to the numerous health benefits associated with this all important beverage. However, there is very little scientific report on kombucha brewed in Ghana. Furthermore, current development of antibiotic resistance by these human pathogens calls for the search into new antimicrobial substances from various sources, hence this study.

1.3 AIM

To compare the physicochemical properties and antimicrobial activity of tea and cocoa- based kombucha.
1.3.1 SPECIFIC OBJECTIVES

- To determine pH and total acid in tea and cocoa kombucha.
- To determine phytochemicals content in tea and cocoa kombucha.
- To evaluate antimicrobial activity of tea and cocoa kombucha.
CHAPTER TWO

2.0. LITERATURE REVIEW

2.1 History of kombucha

Kombucha was first used in East Asia for its healing benefits. Kombucha originated in northeast China (Manchuria) where it was prized during the Tsin Dynasty (“Ling Chi”), about 220 B.C., for its detoxifying and energizing properties (Battikh et al., 2012; Jarrell et al., 2000; Greenwalt et al., 2000). In 414 A.D., a physician Kombu brought the tea fungus to Japan and used it to cure the digestive problems of the Emperor Inkyo. As trade routes expanded, kombucha found its way first into Russian (as Cainiigrib, Cainii kvass, Japonskigrib, Kambucha, Jsakvasska) and into other eastern European areas. It appeared in Germany (as Heldenpilz, Kombuchaschwamm) around the turn of the 20th century. During World War II, this beverage was again introduced into Germany and in the 1950’s it arrived in France and also in France-dominated North Africa where its consumption became quite popular.

Today, kombucha is sold worldwide in retail food stores with different flavors and kombucha culture is sold in several online shopping websites. A kombucha journal is electronically published by Gunther W. Frank and available worldwide in thirty languages (Dufresne and Farnworth 2000; Hartmann et al., 2000). As a result of its beneficial effects on human health and its ease in home preparation, the popularity of kombucha expanded just as many other traditional beverages.
2.2 MICROBIOLOGY AND BIOCHEMISTRY OF KOMBUCHA

Ilmara et al. (2003) stated that, if kombucha is used as a therapeutic substance or functional beverage, it should be defined and standardized with regard to its microbiological and chemical composition. The exact microbiological composition depends on the source of the tea fungus culture (Sreeramulu et al., 2000). A better understanding of the microbial ecology of the fermentation will facilitate the development of combinations of bacterial yeast strains to provide a product with taste that is predictable and with constant quality (Teoh et al., 2004).

The microbial composition of tea fungus (starter culture) has been well studied and documented. This is known to encompass a symbiotic relationship between yeast and acetobacter that are involved in the fermentation of the tea (Malbasa et al., 2006). Yeasts are fungi whose vegetative growth predominantly result from budding or fusion and do not form their sexual states within or upon a fruiting body. Yeast plays a vital role in the production of fermented foods and alcoholic and non-alcoholic beverages. They can be beneficial and harmful. Yeast also produce a wide range of useful secondary metabolites such as vitamins and antibiotics.

Kombucha tea is prepared by inoculating the kombucha culture (tea fungus) into a sugared tea broth for fermentation (Chen and Liu, 2000), producing a beverage with high nutritive value and medicinal properties (Loncar et al., 2000). The amounts of tea, sugar, and tea fungus differ in different places. An optimum fermentation time is required for the production of kombucha. Longer fermentation produces high levels of acids (like mild vinegar) which may pose potential risks when consumed (Sreeramulu et al., 2000). Acetic acid, ethanol, and gluconic acid are the major components of the liquid broth (Roussin, 1996). Other minor constituents such as lactic acid, glucuronic acid, phenolic acid, groups of vitamin B and enzymes are also present (Blanc, 1996).
During the fermentation process, yeast cells hydrolyze sucrose into glucose and fructose;

\[
\text{C}_{12}\text{H}_{22}\text{O}_{11} + \text{H}_2\text{O} \xrightarrow{\text{yeast}} \text{C}_6\text{H}_{12}\text{O}_6 + \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{ethanol} + \text{CO}_2
\]

The product of the yeast hydrolysis produces ethanol (CH₃CH₂OH) and carbon dioxide (CO₂) as metabolites. Acetic acid bacteria converts glucose into gluconic acid and fructose into acetic acid (Loncar et al., 2006). The primary metabolites of ethanol and acetic acid behave as catalyzing agents; yeasts are stimulated to produce ethanol by acetic acid, whereas ethanol stimulates the growth of acetic acid bacteria and their production of acetic acid (Liu et al., 1996). Fructose is utilized to a lesser degree and remains part of the fermented liquid (Greenwalt et al., 1998). Secondary metabolism of Acetobacter may also occur, this involves the oxidation of glucose to gluconic acid (Abd EL-Salam, 2012);

\[
\text{C}_6\text{H}_{12}\text{O}_6 \xrightarrow{\text{Oxidation}} \text{C}_6\text{H}_{12}\text{O}_7.
\]

Glucose molecules are also oxidizes to glucoronic acid (GlcUA).

Both GlcUA and gluconic acid are found in the fermented kombucha drinks. Many different compounds, significant for human health protection, are produced during fermentation of Kombucha. A range of organic acids apart from glucuronic acid, gluconic acid as mentioned above produced in the fermented kombucha includes lactic, acetic, succinic acid, butyric, malic...
and usnic acid, also are vitamins, in particular group B complex vitamins and folic acids (Bauer-Petrovska and Petrushevska-Tozi, 2000) and vitamin C. Free amino acids and different active enzymes are also present.

Also Ahmed and Dirar (2005) in their study reported that during the course of the kombucha fermentation, yeast population declined in the beginning and then increased towards the end of fermentation. On the other hand bacteria began growing from the start and dominated the fermentation process, but their number dropped sharply at the end of fermentation. The organic acids produced throughout the fermentation and the corresponding decrease in pH value play a vital role in preventing the symbiotic culture from becoming contaminated by undesirable microorganisms not contained in the tea fungus (Greenwalt et al., 1998).

The tea fungus broth compose of a floating cellulosic pellicle layer a jelly-like membrane and a sour liquid broth. The floating jelly-like membrane, called a zoogoleal mat, is where the cell mass of the bacteria and yeasts are attached (Jayabalan et al., 2010). The cellulose is a secondary metabolite of the fermentation, similar in structure to a ‘mother of vinegar’. Tea fungus utilizes sugar as it carbon source, forming a new jelly-like membrane during fermentation (Jayabalan et al., 2010). The composition and exact diversity of the microbiological presence depends on the source of the Kombucha culture (Sreeramulu et al., 2000).

The main acetic acid bacteria and yeast found in kombucha are Acetobacter xylinum, A. xylinoides, A. aceti, A. pasteurianus, Bacterium gluconicum, Gluconobacte roxydans (Sreeramulu et al., 2000; Dufresne and Farnworth. 2000) and yeasts like Schizosaccharomyces pombe, Kloeckera apiculata, Saccharomycodes ludwigii, Saccharomyces cerevisiae, Zygosaccharomyces bailii, Brettanomyces bruxellensis, B. lambicus, B. custersii and Pichia
species (Dufresne and Farnworth, 2000). All Acetobacters trains over-oxidize ethanol to acetic acid and then finally to carbon dioxide and water (Kadere et al., 2008).

2.3 SUBSTRATES FOR KOMBUCHA BEVERAGE

Though green and oolong tea can be used for kombucha preparation, black tea and white sugar are considered the finest substrates. Gbewonyo (2013) also reported that cocoa powder and white sugar can be used as a substrate for the preparation of kombucha.

2.4 COMPOSITION OF BLACK TEA

Composition of black tea solid extract includes, Polyphenolic compounds including catechins in monomeric, dimeric and oligomeric form, other flavonoids (including myricetin, quercetin and kaempferol, etc.); amino acids (including L-theanine); methylxanthines, carbohydrates, organic acid, and minerals (Sanderson., 1972; Millin., 1987; Graham., 1984). Black tea is also considered a dietary source of antioxidant nutrients like carotenoids, tocopherols, minerals such as Cr, Mn, Se, or Zn, and certain phytochemicals (Goldberg, 2003). These compounds enhance beneficial health effects of consumption of black tea.

2.4.1 Polyphenols

Polyphenols simply refer to a categorization of compounds composed of many phenolic groups, hence the name poly-phenol. These compounds are plant metabolites produced as a defense against other animals and are the most abundant compounds in tea (Harbowy and Balentine, 1997).
Most phenolic compounds found in black tea consist of more than one benzene ring, with each containing at least one hydroxyl group (–OH). Research interest in black tea has been primarily due to the presence of the flavonoids. The most common subclasses of flavonoids in black tea are non-ketone polyhydroxy polyphenol compounds the flavan-3-ols (catechins in monomeric, dimeric or oligomeric forms) and flavones (quercetin, myricetin, etc.), (Balentine et al., 1997).

The fermentation process during manufacturing of black tea allows the leaves to undergo enzymatic oxidation causing polymerization of flavan-3-ols (monomeric catechins) to a large extent, resulting in formation of dimeric (theaflavins) and oligomeric (thearubigins) forms.

Phenolic acids such as gallic acid and cinnamic acid esters of quinic acid are also found in tea but insignificantly in lower concentrations. Enzyme-catalyzed oxidation and polymerization of tea catechins occurs in the crushing of the tea leaves during manufacture of black tea (Harbowy and Balentine, 1997). This process results in the formation of dimeric catechins (theaflavins) and oligomers known as "thearubigins".
The polyphenolic group that is most reactant during the enzymatic fermentation of fresh green leaves to black tea leaves are the catechins (Balentine et al., 1992). Studies suggest that crude catechins may be useful in the prevention of some bacterial plant diseases (Kodama et al., 1991).

2.4.2 Amino acid (l-Theanine)

l-Theanine (γ-glutamylethylamine) is another important compound unique for tea accounting for almost 50% of its amino acid content and responsible for its unique “bready” taste. Studies suggest doses similar to those found in a cup of tea did induce changes in alpha waves. Alpha waves occur in the brain and they are associated with relaxation. It is known for considerable neuroprotective effects, cognition enhancing properties and assists in brain function development (Kimura et al., 2007).
Schallier et al. (2013) reported that L-theanine has the potential to reduce the damage caused by stroke. L-theanine treatment reduced Aβ1-42 levels and the accompanying Aβ1-42-induced neuronal cell death in the cortex and hippocampus of the brain. L-theanine also significantly reduces oxidative protein and lipid damage and increases the elevation of glutathione levels in the brain (Song et al., 2012). The positive effects of L-theanine on memory might result in the reduction of macro-molecular oxidative damage preventing neuronal apoptosis that is important for learning and memory capabilities.

Figure 3: Structure of an L-theanine.

2.4.3 Methylxanthine

Methylxanthines are molecular compounds found in black tea that act as cardiac stimulants and smooth muscle relaxants. The most commonly known form of methylxanthines is caffeine, however other methylxanthines found in trace amounts in tea is theophylline. Some of the effects caused by caffeine are influenced by theophylline content in tea. Theophylline induces psychoactivity, it also has vasodilator effect. Theophylline in tea can act as bronchodilators and can stimulate respiratory system and act inotrope in patients with pulmonary disease and provide them short-term relief. This adds to the enormous evidence that tea can make a contribution to a healthy lifestyle. (Varnam et al., 1994)
2.5 COMPOSITION OF COCOA POWDER

Cocoa powder, a by-product from the industrial preparation of chocolate, can also serve as a substrate for kombucha preparation (Gbewonyo, 2013). So far, no literature is known on cocoa kombucha. Cocoa powder contains compounds such as purines, alkaloids, catechins, proanthocyanin, anthocyanin, leucoanthocyanins and organic acid, which contribute to the taste of cocoa powder (Belitz et al., 2009). In recent years, much research has been focused on cocoa polyphenols, especially the flavonoids and its function as potent antioxidant in human health. Cocoa powder is very rich in sources of phenolic phytochemicals and is reported of having higher antioxidant capacity than tea and red wine (Lee et al., 2003). Cocoa powder also contains copper, zinc, magnesium and manganese. Main classes of polyphenolic compounds identified in cocoa are compounds such as simple phenols, benzoquinones, phenolic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, phenylpropenes, coumarines, chromones, naphtoquinones, xanthones, stilbenes, anthraquinones, flavonoids, lignans and lignins. Some of the beneficial effects of polyphenols includes anti-microbial, anti-carcinogenic, anti-atherogenic,
Anti-ulcer, anti-thrombotic, anti-inflammatory, immune modulating, vasodilatory and analgesic effects (Wollgast et al., 2000).

Cocoa is rich in polyphenols such as (+)-catechin, (–)-epicatechin, and oligomers of these monomeric base units, namely procyanidins, and anthocyanidins. It has been reported that flavonols (epicatechin and catechin) are predominant compounds in cocoa powder (Natsume et al., 2002).

Apart from polyphenols, cocoa is also rich in methylxanthines, namely caffeine, theobromine, and theophylline (Rios et al., 2003; Greer et al., 2001). The amount of theobromine in cocoa and cocoa product is higher compared to caffeine in cocoa (Rios et al., 2003; Greer et al., 2001; Tuabert et al., 2007). Theobromine is a psychoactive compound without diuretic effects.

Although most studies indicate that the health benefits of cocoa or cocoa products are attributable to polyphenols (Cooper et al., 2008), cocoa and its products are not only rich in polyphenols, but are also rich in methylxanthines (caffeine, theobromine, and theophylline) and proteins. Cocoa peptides are generally responsible for the flavor precursor formation.
Cocoa beans contain four types of proteins, namely albumins, globulins, prolamin, and glutelin. The health properties of cocoa and cocoa products are not solely dependent on their polyphenol contents, but also on other components such as methylxanthines (caffeine and theobromine), peptides, and minerals. Steinberg et al.(2003) showed that minerals are one of the important components in cocoa and cocoa products. Although cocoa and tea contain some similar compounds, the amount of each compound present might vary. The variation in the composition might be a contributing factor to the differences in the physicochemical and antimicrobial properties of tea and cocoa kombucha.

2.6 REPORTED HEALTH BENEFIT OF KOMBUCHA

Many are the beneficiary effects of kombucha reported by individual and various research institutions. Among all the traditional fermented foods and beverages none is more intriguing than kombucha. It has attained worldwide recognition as an alleged panacea for practically all chronic diseases that affect man (Ahmed and Dirar, 2005). Its curative effects have been cited in popular scientific journals and magazines.

Reported effects of kombucha from tea drinkers (Dufresne and Farnworth 2000;Teoh et al., 2004), reveals that kombucha detoxifies blood; reduces cholesterol level; reduce atherosclerosis by regeneration of cell walls; reduces blood pressure; reduces inflammatory problems; alleviates arthritis, rheumatism and gout symptoms; promotes liver functions, normalizes intestinal activity, balances intestinal flora, cures hemorrhoids; reduces obesity and regulates appetite, prevents bladder infection and reduces kidney calcification, stimulates glandular systems, protects against diabetes and increases body resistance to cancer. It has an antibiotic effect
against bacteria, and yeasts, enhances the immune system and stimulates interferon production, relieves bronchitis and asthma, reduces menstrual disorders and menopausal hot flashes, improves hair, skin and nail health, reduces an alcoholic’s craving for alcohol, reduces stress and nervous disturbances and insomnia, relieves headaches, improves eyesight, counteracts aging, enhances general metabolism as well as demonstrating interesting sensory properties.

The beneficial effects of kombucha tea are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during the fermentation (Jayabalan et al., 2008).

Glucuronic acid is a major component of Kombucha beverage due to its detoxifying action and claimed health effects (Loncar et al., 2000; Jayabalan et al., 2007). It is claimed to prevent chronic degenerative cardio-vascular, neurodegenerative diseases and cancer (Dufresne et al., 2000; Sreeramulu et al., 2000). Glucuronic acid is well-known in the prophylaxis of human health and is considered to be the main therapeutic agent in kombucha as it functions in the liver as detoxicant (Loncar et al., 2000). UDP-glucuronosyltransferases (UGTs) - the family of enzymes responsible for the glucuronidation have many isoforms and broad substrate specificity that allows conjugating GlcUA with many foreign and natural compounds. Glucuronidation is an important process for detoxication and excretion of exogenous chemicals - xenobiotics, as well as, for biotransformation of endogenous reactive metabolites, such as bilirubin, oxidized fatty acids and excess of steroid hormones. The conjugation of GlcUA with undesirable compounds results in the decreased toxicity due to their increased solubility which further facilitates transport and elimination from the body.

Currently kombucha is alternately praised as “the ultimate health drink” or damned as “unsafe medicinal tea” (Blanc, 1996; Hartmann et al., 2000). There are many conceptions and
misconceptions regarding the health benefits and toxicity of kombucha beverage. Though it is claimed to be beneficial for many medical ailments, very little and in some cases no clinical evidence is available to support these claim.

2.7 ANTIMICROBIAL PROPERTY OF KOMBUCHA

An antimicrobial agent is a substance that kills or inhibits the growth of microbes such as bacteria, fungi and viruses. Antimicrobial drugs either kill microbes (microbicidal) or prevent the growth of microbe (microbistatic). Antimicrobial screening methods are influenced by several factors such as extraction method, inoculation volume, culture composition, pH, and incubation temperature as well as the potency of the active ingredients. One of the possible strategies for finding new anti-effective drug may involve the search for compounds with structures widely different from those in common use.

Kombucha tea shows remarkable antimicrobial activity against pathogenic microorganisms and this activity is largely attributable to acetic acid as the major antimicrobial agent in kombucha tea (Greenwalt et al., 1998) and antibiotics present in the kombucha tea (Chen and Liu, 2000; Jayabalan et al., 2007). Many reports reveal that the polyphenols or tannins extracted from tea inhibit a broad spectrum of Gram-positive and negative bacteria (Zhu et al., 2000). Additionally, Sreeramulu et al. (2001) also reported that the metabolites produced by the bacteria and/or yeasts during the fermentation of kombucha tea are responsible for its antimicrobial activity. Kombucha exerts antimicrobial properties against Salmonella typhimurium, Staphylococcus aureus, Helicobacter pylori (Greenwalt et al., 1998), Shigella sonnei, Salmonella enteritidis and Escherichia coli (Greenwalt et al., 1998; Sreeramulu et al., 2001).
However, Yokihiko and Watanabe (1989) found that *Clostridium botulinum* spores were killed when inoculated into tea drinks. This investigation demonstrates that the inhibitory effects observed could have been due to the catechin content of the tea. It was later determined that most of the bactericidal activity of tea itself may be attributed to the polyphenols, specifically catechins (Ahn *et al*., 1991).

Bacteria and yeast strains present in kombucha beverage form a powerful symbiosis that can inhibit the growth of potential contaminating bacteria (Jayabalan *et al*., 2010). Adams and Moss (2000) reported that, the low pH of kombucha contributes enormously to its safety aspects, because most food borne pathogens do not survive such pH.

### 2.8 ANTIBIOTIC RESISTANCE

The success of antimicrobials against pathogens is one of the remarkable achievement of medical science in the past decades. However, infectious diseases account for the major cause of morbidity and mortality in Sub-Saharan Africa and Ghana is no exception. Antibiotics are widely and inappropriately used in Ghana resulting in antibiotic resistance. Newman and colleagues (2006) studied bacterial isolates from various clinical specimens in Ghana. They recorded high resistance rate for tetracycline, co-trimoxazole, ampicillin and chloramphenicol. In Zimbabwe a study on antibiotic resistance also shows a high resistance for ampicillin, co-trimoxazole and trimethoprim/sulphamethoxazole (Mbanga *et al*., 2010). Kibret and Abera (2011) reported high resistance rate to tetracycline in Ethiopia. It is also reported in Nigeria that Gram-negative isolates showed high resistance to ampicillin (90%) and co-trimoxazole (85%) (Clarence *et al*., 2007). Large quantities of assorted antimicrobials are now available to developing countries due
to economic development and technological advances. This remarkable achievement is accompanied by poor practices that promote drug resistance (Beitha, 2008). A major public health challenge confronting microbiologists, clinicians, biochemist, drug development expert and public health specialists is the prevalence of antibiotic resistance in most known human pathogens.

It has become clear over the last decade that antibiotics are losing their effectiveness as pathogens evolve resistance against them. In recent years multiple drug resistance in human pathogenic microorganisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. This situation has led scientists to search for new antimicrobial substances from various sources, for novel antimicrobial chemotherapeutic agents. Cocoa and tea kombucha can also be evaluated as alternative antimicrobial agents due to their reported antimicrobial potential.

2.9 PHYTOCHEMICALS

Phytochemicals are a large group of plant-derived compounds hypothesized to be responsible for much of the disease protection conferred from diets high in fruits, vegetables, beans, cereals, and plant-based beverages such as tea and wine (Arts and Hollman, 2005).

Phytochemicals are basically divided into two groups, primary and secondary metabolites. The most important of these bioactive constituents of plant are flavonoids, alkaloids, phenols, tannins, steroids, terpenoids, carotenoid and glycosides. They are organic substances and could be obtained from both primary and secondary metabolic processes.
The aim of this present study was to compare the physicochemical and antimicrobial activities of tea and cocoa-based kombucha. Comparing the potential antimicrobial activities of the kombucha prepared from black tea and cocoa powder against a number of human pathogens may be very healthful. As resistance to antimicrobial agents has become increasingly an important global health problem, these findings might be very promising and could be useful as an alternative to current synthetic antimicrobial drugs. The organisms Staphylococcus aureus, Candida albicans, Shigella spp, used in this study were chosen because they are common human pathogens.
CHAPTER THREE
MATERIALS AND METHODS

3.0 MATERIALS

Black tea (Lipton, Ghana); sucrose (St. Louis refined sugar, France), cocoa powder and starter culture were provided by Rev. Dr. W. S. K. Gbewonyo of the Department of Biochemistry, Cell and Molecular Biology, Legon. The organisms used, S. aureus, C. albicans, Shigella spp, were obtained from the Department of Biochemistry Cell and Molecular Biology, University of Ghana, Legon.

3.1 METHODS

3.1.1 Preparation of kombucha tea

Sweetened black tea was prepared by infusing one bag of black tea (Lipton) into boiled distilled water (1 L) containing sucrose (37.7g) for 3 min. The sweetened tea was allowed to cool to room temperature. Previous starter culture kombucha was used to inoculate the sweetened black tea. The set-up was then allowed to ferment for 14 days, after which the tea kombucha was harvested and stored in a refrigerator.

3.1.2. Preparation of cocoa kombucha.

Cocoa kombucha was prepared according to the method of Gbewonyo (2013).
3.1.3. Freeze drying of samples

Two hundred milliliters of both tea and cocoa kombucha samples (fermented and unfermented) were freeze-dried at the School of Agriculture in the College of Basic and Applied Sciences of the University of Ghana, to obtain a dried residue of the tea and cocoa samples. The weights of freeze-dried samples were obtained. Each sample was reconstituted in distilled water to give a concentration of 1g/ml which served as the stock solution kept in a falcon tube and stored in a refrigerator until ready for use.

3.1.4. Determination of pH

Aliquots of 50 ml each of freshly prepared broth of cocoa and tea kombucha were transferred into a 100 ml beaker and pH of each sample determined using a pH meter.

3.1.5. Determination of total acid content.

The titratable acid contents of tea and cocoa kombucha samples were determined by the volumetric method. A standardized NaOH (aq) (1.0M) was titrated against 10 ml of each of tea and cocoa kombucha samples. The concentration of total acid present in each sample was then calculated (Greenwalt et al., 1998).

3.1.6 Qualitative analysis of sugars in tea and cocoa kombucha.

The stock samples were applied to TLC silica gel 60 F$_{254}$ plates with fluorescent indicator. Solution of (0.02g/ml) fructose, glucose and sucrose were used as standards. Plates were run using a solvent system combination of chloroform, methanol and acetonitrile (1: 1: 0.5 V/V).
The plate was developed using 10% H$_2$SO$_4$ and heated at 110 °C. Retention factor (Rf) was then calculated for both kombucha samples and the standard sugars used.

### 3.2. Phytochemical analysis

Fermented and unfermented samples were screened for saponins, tannins, alkaloids, terpenoids and flavonoids.

#### 3.2.1. Test for Saponins

Equal aliquots of test sample and distilled water were shaken vigorously for 2 minutes, then it was observed for persistent foaming, indicative of presence of saponins presence (Kokate, 1999).

#### 3.2.2. Test for Tannins

Two drops of FeCl$_3$ (5% W/V) were added to 2ml of the aqueous test samples; formation of green precipitate confirms the presence of tannins (Trease and Evans, 1996).

#### 3.2.3. Test for Alkaloids

Two milliliters of each test sample was dried using heat, cooled and 5 ml of 2M HCl was then added to each reaction mixture. Upon complete dissolution of all the residues, 3 drops of Wagner’s reagent was added. The formation of reddish brown flocculation suggested the presence of alkaloids. (Geetha and Geetha, 2014).

#### 3.2.4. Test for Terpenoids.
One milliliters of chloroform was added to 2 ml of the test samples, diluted 1 in 2 with distilled water. Aliquot of concentrated H2SO4 was added gently to the mixture. Observed reddish brown precipitate or ring at the junction of the two solutions suggests the presence of terpenoids (Rimjhim and Kumari, 2014).

3.2.5. Test for flavonoids

Three milliliters of 1M NH₃ was added to an equal volume of the test samples and allowed to stand for 2 minutes after which 2 ml of concentrated H₂SO₄ was added. A yellow precipitate formed indicated the presence of flavonoids (Evans, 1997).

3.2.6 Test for reducing sugar

Three milliliters of Fehling’s solution A and B was added to the test samples and heated at 60°C. Formation of a brick-red color suggests the presence of reducing sugar (Geetha and Geetha, 2014).

3.3. ANTIMICROBIAL ACTIVITY

Media was prepared by dissolving an appropriate mass of nutrient broth and nutrient agar in an appropriate volume of distilled water. The media was then autoclaved and allowed to cool after which it was aseptically poured into sterile petri dishes. The media was allowed to set and refrigerated. Antimicrobial activity of tea and cocoa kombucha were tested against, C. albicans, Shigella spp, S. aureus. Sterile Whatman filter paper discs (6.0 mm diameter) were impregnated with each of the extracts of tea and cocoa (fermented and unfermented). After inoculating the plate with the cultured organisms using cotton swaps, the dried discs impregnated with the samples were placed on the inoculated plates. A positive control was set up using ceftriaxone and fluconazole, and a negative control using unfermented tea and cocoa. The plates were then
incubated at room temperature for 24 hours to allow growth of the organisms. The antimicrobial activity of the kombucha samples was assessed by measuring the diameter of the inhibition zone after 24 hours (Mounyr et al., 2016).
The pH of unfermented black tea and cocoa samples were found to be 5.11 and 6.20 respectively. However fermentation of black tea for 14 days and cocoa for 8 days led to a decrease in the pH values (figure 6). The pH of fermented tea was 3.14, which is about 39% decrease in pH; while the pH of fermented cocoa was 3.37, which is about 45% decrease in pH.

Figure 6: pH of tea and cocoa kombucha.

### 4.2. Total acidity of tea and cocoa kombucha.

The calculated values for the total acid present in tea and cocoa kombucha after titrating against aqueous NaOH solution are shown in Table 1. The total acid concentration in g/L content of kombucha tea after 14 days of fermentation was higher than that for cocoa.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total acid concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tea kombucha</td>
<td>0.126</td>
</tr>
<tr>
<td>Cocoa kombucha</td>
<td>0.087</td>
</tr>
</tbody>
</table>

Table 1: Total acid content of tea and cocoa kombucha

4.3 Phytochemical screening

Results from the phytochemical analysis of tea and cocoa kombucha revealed the presence of tannins, terpenes, flavonoids, reducing sugar and saponins in tea and cocoa kombucha. Alkaloids were found to be absent in both tea and cocoa kombucha (Table 2).
<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
<th>Unfermented</th>
<th>Fermented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tea</td>
<td>Cocoa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tea</td>
<td>Cocoa</td>
</tr>
<tr>
<td>Tannins</td>
<td>Formation of a green ppt.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Reddish-brown ppt formed at the interphase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>Forming persist after shaking vigorously.</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Yellow solution formed, No ppt</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenol</td>
<td>Red colouration formed</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>Brick-red colour formed</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Brown colour of solution persist</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
The results for TLC analysis for sugars using solvent system combination: chloroform, methanol, acetonitrile (1:1:0.5) are shown in Figure 7. The Retention factor (Rf) obtained are also recorded in table 3. The results show that fructose was present in fermented tea, unfermented cocoa while glucose was found to be present in fermented cocoa. Sucrose was detected in unfermented tea.

Fructose (fruc), glucose (glu), sucrose (suc) were used as standards.

Key
1 - unfermented tea, 2 - unfermented cocoa, 3 - fermented tea, 4 - fermented cocoa, fruc - fructose, glu - glucose, suc - sucrose.

Figure 7: TLC plate showing sugars present in fermented and unfermented tea and cocoa.
<table>
<thead>
<tr>
<th>Samples</th>
<th>Retention factor of sugars</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
</tr>
<tr>
<td><strong>Unfermented</strong></td>
<td></td>
</tr>
<tr>
<td>Tea</td>
<td>0.36</td>
</tr>
<tr>
<td>Cocoa</td>
<td>-</td>
</tr>
<tr>
<td><strong>Fermented</strong></td>
<td></td>
</tr>
<tr>
<td>Tea</td>
<td>-</td>
</tr>
<tr>
<td>Cocoa</td>
<td>-</td>
</tr>
<tr>
<td><strong>Standards</strong></td>
<td>0.36</td>
</tr>
</tbody>
</table>

Table 3: TLC analysis of sugars present in tea and cocoa kombucha

4.5 Anti-microbial activity of tea and cocoa kombucha

Freeze-dried samples of tea and cocoa kombucha were tested against *C. albicans*, *Shigella spp* and *S. aureus*. Freeze-dried unfermented tea and cocoa were used as negative control and the antibiotics fluconazole was used as positive control for *C. albicans* and ceftriaxone, was used as positive control for *Shigella spp* and *S. aureus*. Disc diffusion method was used and the diameter of the absorbent disc was 6.0 mm. The inoculated plates were incubated at 37 °C and zones of inhibition were recorded after 42 hours. Figure 8 (i) and (ii) shows the antimicrobial activity of
cocoa kombucha and tea kombucha towards *C. albicans*. Also figure 9 (i) and (ii) shows the antimicrobial activity of cocoa kombucha and tea kombucha towards *Shigella spp.* Figure 10 (i) and (ii) shows the antimicrobial activity of cocoa kombucha and tea kombucha towards *S. aureus*. Figure 11 shows the zones of inhibition by the test samples towards the tested pathogenic microorganisms.

Figure 8: Antimicrobial activity of cocoa kombucha (i) and tea kombucha (ii) towards *C. albicans*.

Key: A = fluconazole, B = fermented cocoa, C = unfermented cocoa, D = untreated, E = fermented tea, F = unfermented tea.
Figure 9: Antimicrobial activity of cocoa kombucha (i) and tea kombucha (ii) towards Shigella.

Key: B=fermented cocoa, C=unfermented cocoa, D=untreated, E=fermented tea, F=unfermented tea, G=ceftriaxone.
Figure 10: Antimicrobial activity of cocoa kombucha (i) and tea kombucha (ii) towards *S. aureus*.

Key: B=fermented cocoa, C=unfermented cocoa, D=untreated, E=fermented tea, F=unfermented tea, G=ceftiraxone.
Figure 11: Antimicrobial activity on the growth of tested microorganisms.
CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Kombucha is a symbiotic culture of bacterial and yeast. Yeast cell hydrolyze sucrose to glucose and fructose. Acetic acid bacterial convert glucose and fructose into gluconic acid and acetic acid respectively during the fermentation process. These organic acids produced throughout the fermentation of the tea and cocoa kombucha resulted in a corresponding decrease in pH value (Dufresne and Farnworth 2000). This prevents the symbiotic culture from becoming contaminated by undesirable microorganisms not contained in the tea fungus. In this study, a decrease in pH was recorded in both the fermented tea and cocoa kombucha. This result confirms that obtained by earlier studies (Greenwalt et al., 1998; Jayabalann et al., 2007 and Mo et al., 2008; Dufresne and Farnworth 2000). Comparing the pH of tea kombucha with cocoa kombucha, present study showed that tea kombucha was more acidic. This result shows that tea kombucha produced more organic acids compared with cocoa kombucha as reported that, the pH value of kombucha beverage decreases due to the production of organic acids during fermentation (Dufresne and Farnworth 2000). In addition, the study revealed that, the total acid content of tea kombucha is higher compared with cocoa kombucha.

Qualitative test for phytochemicals in tea and cocoa kombucha showed they contain similar phytochemicals. A study by Subhashini, et al., (2010) revealed that cocoa contains the following phytochemicals tannins, saponins, terpenoids and flavonoids. Tea is also known for its richness in tannins, phenols, flavonoids, terpenoids etc. These phytochemicals were detected in both tea and cocoa.
Phytochemical are known to offer protection against diseases in humans. The consumption of flavanol-rich cocoa has been found to improve cerebral blood flow, which is critical for optimal brain function and decreases dementia. The presence of these phytochemicals in both fermented and unfermented tea and cocoa-based kombucha indicates that the health benefits derived from the consumption of tea and cocoa are not lost during the preparation of tea and cocoa kombucha. It can be deduced that the health benefit of kombucha beverage reported by Dufresne and Farnworth (2000) and Teoh et al., (2004) may be attributed to the presence of these phytochemicals.

Yeast cells in the mixture contain enzymes that hydrolyze sucrose into glucose and fructose during the fermentation process. Glucose and fructose were detected in tea and cocoa kombucha by TLC. Sucrose was detected in unfermented tea, fructose was present in fermented tea and unfermented cocoa, glucose was found to be present in fermented cocoa. Loncar et al., (2006) reported that during the fermentation process yeast cells hydrolyze sucrose into glucose and fructose. Glucose produced during the fermentation is used by the yeast to produce ethanol and carbon dioxide as metabolites. Ethanol is then oxidized by the bacteria in the presence of air to acetaldehyde, then to acetic acid (Reiss, 1994; Loncar et al., 2006). The primary metabolites of ethanol and acetic acid act as catalyzing agents; yeasts are stimulated to produce ethanol by acetic acid, whereas ethanol stimulates the growth of acetic acid bacteria and their production of acetic acid (Liu et al., 1996). The acetic acid bacteria also utilize some of the glucose to produce gluconic acid. Fructose is used to a lesser extent, and some remain after the fermentation process (Greenwalt et al., 1998). The presence of the unmetabolized glucose together with the remaining fructose is responsible for sweetness associated with the beverage. Comparatively the study showed that tea kombucha contains fructose and cocoa kombucha contains glucose.
Kombucha beverages exert antimicrobial properties against a spectrum of known human pathogens (Greenwalt et al., 1998; Sreeramulu et al., 2001). Steinkraus et al. (1996) reported that antimicrobial activity of kombucha may be attributed to its acetic acid content.

Fermented cocoa kombucha showed antimicrobial activity towards *C. albicans* with a zone of inhibition of 16mm, unfermented cocoa kombucha gave a zone of 9 mm and the standard (fluconazole) gave a zone of 20 mm. Also fermented tea showed antimicrobial activity on *C. albicans* with an inhibition zone of 17 mm, unfermented tea gave a zone of 13 mm and the standard (fluconazole) gave a zone of 20 mm. The zones of inhibition for cocoa kombucha compared with the standard, cocoa kombucha antimicrobial activity is significant though its zone is not higher than that of the standard. Likewise the zone of inhibition for tea kombucha compared with the standard, tea kombucha antimicrobial activity is significant, it produced a zone not greater than the standard. Fermented tea and cocoa kombucha have the properties that inhibit the growth of *C. albicans*. Comparing tea and cocoa kombucha, tea has a higher antimicrobial activity than cocoa. This might be as a result of tea kombucha having a lower pH than cocoa kombucha. Also, there might be more acetic acid in tea than cocoa kombucha as it is reported by Greenwalt et al. (1998) that antimicrobial activity against pathogenic microorganisms is largely attributable to acetic acid.

However, unfermented tea did inhibit the growth of the organisms; this might be due to the presence of polyphenols and tannins in tea. Many reports reveal that the polyphenols or tannins extracted from tea inhibit a broad spectrum of Gram-positive and negative bacteria (Zhu et al., 2000). Toda et al. (1991) reported that, unfermented tea at high concentrations (using 20% dry
tea) inhibit *Staphylococcus epidermidis, Salmonella typhi, Salmonella typhimurium, Salmonella enteritidis, Shigella flexneri, Shigella dysenteriae,* and *Vibrio spp.*

Fermented cocoa kombucha showed antimicrobial activity towards *Shigella* with a zone of inhibition of 16 mm, unfermented cocoa kombucha did give a zone of 6 mm and the standard gave a zone of 22 mm. Also fermented tea inhibited the growth of *Shigella* with an inhibition zone of 21 mm, unfermented tea gave a zone of 10 mm and the standard gave a zone of 22 mm. By comparing the zones of inhibition for cocoa kombucha and the standard, cocoa kombucha antimicrobial activity is significant though its zone is not greater than that of the standard. Also the zones of inhibition for tea kombucha compared with the standard, tea kombucha antimicrobial activity is also significant, although it produced a zone not greater than the standard. Fermented tea and cocoa kombucha showed the ability to inhibit the growth of *Shigella.* Tea kombucha tea exhibited a higher antimicrobial activity than cocoa kombucha towards *Shigella.* This might be due to tea kombucha being more acidic with higher acetic acid content than cocoa kombucha as reported by Sreeramulu *et al.* (2000), that the antimicrobial activity of kombucha tea is largely attributable to the presence of organic acids, particularly acetic acid, large proteins, and catechins.

Although the standard showed a high level of inhibition of *S. aureus,* neither fermented or unfermented tea and cocoa showed inhibition toward *S. aureus.*

Comparing the antimicrobial activity of tea and cocoa-based kombucha, the study revealed that tea kombucha appears to have a higher antimicrobial activity than cocoa kombucha. Numerous studies reported that antimicrobial activity of kombucha is attributed to the high acetic acid produced during the fermentation of the beverage (Greenwalt *et al.*, 1998). The low pH of tea
kombucha recorded as compared with that of cocoa kombucha is suggestive of its high acetic acid content, hence the higher antimicrobial activity.

5.2 CONCLUSION

The results obtained suggest tea kombucha is more acidic compared with cocoa kombucha as demonstrated by their pH and total acid content. Tea and cocoa kombucha exhibited significant antimicrobial activity towards *Shigella* and *C. albicans*. The presence of phytochemicals such as phenolics and acetic acid might have contributed enormously to the antimicrobial property. Phytochemicals especially phenols are known for their antimicrobial activity and these are present in both tea and cocoa. This might be the reason why both unfermented tea and cocoa did inhibit the growth of *Shigella* and *C. albicans* although the difference is not significant compared with the standard. The study showed that tea and cocoa-based Kombucha beverage are potential antimicrobial agents.

5.3 RECOMMENDATION

Though kombucha is claimed to be beneficial for several medical ailments, very little or no clinical evidence is available to support these claim, further investigation should be done to verify these claims.

Further studies should be done to determine the organic acid present in cocoa kombucha since there no literature on cocoa kombucha, and as well identify the simple phenols produced during the fermentation period of tea and cocoa kombucha. Also quantitative analysis should be done to separately quantify fructose, sucrose and glucose concentration in the broth.
REFERENCES


APPENDIX

Preparation of 1.0 M NaOH solution

Using the formula, \( m = C \times V \times M \) to determine the mass of NaOH needed to be weighed, where 
C = concentration, \( V = \text{volume} \ (\text{dm}^3) \), \( M = \text{molar mass} \).

A mass of 10.0g of NaOH was weighed using a 50 ml beaker, it was then dissolved with distilled water. The solution was then transferred into a 250 ml volumetric flask with the aid of a funnel, the inside of the beaker and the funnel was repeated rinsed into the 250ml flask. The solution was topped up to the 250ml mark. It was labeled and stored.

Standardization of sodium hydroxide

A mass of 0.563g of Oxalic acid (\( \text{H}_2\text{C}_2\text{O}_4 \)) was weighed. Sufficient volume of distilled water was added for complete dissolution the mixture was transferred into a 250cm\(^3\) of volumetric flask with more distilled water added to reach the 250cm\(^3\) mark on the flask. The actual concentration of solution was calculated to be 0.0250 mol dm\(^3\) using the formula; \( C = \frac{m}{M_r} \). Where \( C \), \( M_r \), \( m \) and \( V \) are the concentration, molar mass, mass of the oxalic acid and volume of distilled water used respectively.

The resultant NaOH solution was then titrated against the prepared oxalic acid solution using phenolphthalein as an indicator. Volume of NaOH that completely neutralized the \( \text{H}_2\text{C}_2\text{O}_4 \) solution was recorded with the concentration of NaOH then calculated using the reaction equation; \( \text{H}_2\text{C}_2\text{O}_4(aq) + 2 \text{NaOH(aq)} \rightarrow \text{Na}_2\text{C}_2\text{O}_4(aq) + 2 \text{H}_2\text{O(l)}. \)
The NaOH solution was then titrated against 10ml each of cocoa and tea kombucha using phenolphthalein as an indicator. The titration was repeated for each sample. The average volume of NaOH that completely reacted with each sample was used to calculate the concentration of the hydrogen ions (H\(^+\)) in each sample using the reaction equation:

NaOH(aq) + HA(aq) → NaA(aq) + H\(_2\)O(l). HA represent the total acid present in the test sample.