EVALUATION OF THE ANTIMYCOBACTERIAL AND ANTIMYCOLACTONE EFFICACY OF KOMBUCHA

BY

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DECLARATION

I ADIZA ABASS, declare that except for references to other people’s work for which I have acknowledged, the experimental work described in this project was performed by me in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana and Noguchi Memorial Institute for Medical Research under the supervisions of Dr. Lydia Mosi and Dr. Rev. W. S. K. Gbewonyo.

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ABSTRACT

Buruli ulcer is caused by mycolactone, a toxin produced by *Mycobacterium ulcerans*. Drug development for the treatment of the disease and early diagnosis is greatly hampered by the lipid nature of the mycolactone and bacterial resistance to currently used antimycobacterials. Kombucha tea is a health drink consumed worldwide. Research has shown that Kombucha exerts medicinal properties including enhanced wound healing and antimicrobial activity. This study was aimed at characterizing the microbial content of Kombucha tea, assessing Kombucha antimicrobial, antimycobacterial and mycolactone inactivating potential. The antioxidants as well as the phytochemical constituents of the tea were also investigated. Kombucha tea was cultured, and microbial isolates were Gram stained, DNA extracted, followed by PCR with 16S rRNA and fungal ITS primers for identification of bacteria and yeasts, respectively. Resulting amplicons were sequenced and blasted against the PUBMED database for identification based on sequence similarity and homology. Phytochemical analysis was conducted on the Kombucha tea and the total phenolic content were determined. DPPH assay was used for the determination of the antioxidant property of the tea. Different concentrations of the tea were co-incubated with mycolactone for various time points to observe for toxin attenuation. The presence of intact or inactivated mycolactone was detected using TLC and cytotoxicity assays on cultured human fibroblast cell lines. Antimicrobial potency of increasing concentrations of the tea was tested against *S. aureus* and *M. ulcerans* by pre-incubation prior to microscopy and culture to observe morphological changes and viability respectively. Yeasts in the Kombucha tea were identified as *Dekkera bruxellensis*, *Brettanomyces bruxellensis*, *Rhodotorula mucilaginosa* and *Lachancea fermentati* and the bacteria as *Paenibacillus lactis*, *Paenibacillus cineris* *Bacillus licheniformis*, *Lactobacillus amylolyticus* and *Corynebacterium glutamic*. Phytochemicals detected in the tea were saponins, flavonoids, alkaloids, phenols and terpenoids. The antioxidant property of Kombucha was higher as compared to the unfermented
tea and a similar effect was observed with the phenolic content. Unfermented was 2-fold less potent in total phenolic content than Kombucha tea. The tea possessed antimicrobial activity against *S. aureus* but not on *M. ulcerans*. Kombucha was not cytotoxic to the human skin fibroblast cells, however, mycolactone treated with Kombucha tea retained its potency against human fibroblast cell lines and viable bacilli of *M. ulcerans* were observed after Kombucha treatment. Kombucha tea possesses antioxidant and antimicrobial activities but lacks antimycobacterial and antimycolactone activity.
DEDICATION

I dedicate this work to Almighty Allah for seeing me through every step of the way. Secondly, to the Abass family especially, my mum Hajia Habiba Abass and my sister, Rabiatu Abass for their immersed love, care, support and encouragement throughout my life.

Finally, my profound appreciation goes to my dear supervisor, Dr. Lydia Mosi of Biochemistry, Cell and Molecular Biology, for various contributions you have made, this work is dedicated to you.
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<tr>
<td>BCG</td>
<td>Bacillus Calmette Guerin</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BU</td>
<td>Buruli ulcer</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>DNTP</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LJ</td>
<td>Lowenstein Jensen</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>µL</td>
<td>micro litre</td>
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<tr>
<td>µM</td>
<td>micro Molar</td>
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<tr>
<td>µg/ml</td>
<td>microgram per millilitre</td>
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<tr>
<td>M7H9</td>
<td>Middlebrook 7H9</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<td>mM</td>
<td>milli Molar</td>
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</table>
mg/ml  milligram per millilitre
M  Molar
*M. ulcerans*  *Mycobacterium ulcerans*

nm  nanometer

NCBI  National Center for Biotechnology Information

OD  Optical density

PBS  Phosphate Buffer Saline

PCR  Polymerase Chain Reaction

Rf  Retention factor

RPMI  Roswell Park Memorial Institute medium

rRNA  Ribosomal Ribonucleic acid

sp  specie

*S. aureus*  *Staphylococcus aureus*

SCOBY  Symbiotic Community of Bacteria and Yeasts

TLC  Thin Layer Chromatography

TPC  Total phenolic concentration

UV  Ultra violet

Vol  Volume

V  Voltage
CHAPTER ONE

1.0 INTRODUCTION

*Mycobacterium ulcerans* (*M. ulcerans*) is the etiological agent for the disease Buruli ulcer (BU). It is the second most important mycobacteria infection after tuberculosis in Ghana (Amofah *et al.*, 2002). The disease is characterized by massive subcutaneous tissue damage, mainly in the extremities with occasional bone involvement (Debacker, 2004). *M. ulcerans* secretes a macrolide toxin called mycolactone which has been proven to be solely responsible for the pathogenesis of Buruli ulcer (George *et al.*, 1999). Mycolactone has been indicated to be immunosuppressive and immunomodulatory both *in vivo* and *in vitro* (Adusumulli *et al.*, 2005). The absence of pain has contributed to the lack of epidemiological data on the incubation period and the early stages of the disease, as they are often ignored. Thus, most affected individuals report to the health centers only after development of a large persistent ulcer.

Treatment of Buruli ulcer infections seeks to curtail the spread of ulcers and repair existing tissue damage to affected areas. In the early and intermediate stages of the disease, antibiotic drugs including rifampicin and streptomycin or amikacin are highly effective (Sarfo *et al.*, 2010a). However, individuals with large persistent ulcer undergo surgical excision of the ulcer followed by several weeks of antibiotics treatment (Chauty *et al.*, 2007).

The use of Kombucha, a fermented tea as both beverage and medicinal product dates back to the Qin Dynasty around 250 BC in China (Gunther, 1995). The tea is reported to exert a number of medicinal effects including: antimicrobial, anti-inflammatory and antioxidant activities and is reportedly used for wound healing (Gbewonyo, 2014). There was a considerable reduction in CNS infiltration of inflammatory cells and experimental autoimmune encephalomyelitis (EAE) in Kombucha–treated mice as compared to the control (untreated), signifying a substantial anti-inflammatory property of the tea (Marzban *et al.*, 2015). However, there is not
much scientific evidence for the efficacy of Kombucha and its bioactive components. The tea is reported to contain some enzymes, amino acids, polyphenols (Stamets, 1995) and several other organic acids depending on the source of the culture. These organic acids include acetic acid, lactic acid, malic acid, oxalic acid and usnic acid (Velicanski et al., 2007).

Buruli ulcer is mainly endemic in rural areas where access to health care facilities is either very far or non-existent. Thus, the lengthy duration of chemotherapy and expensive hospital stays tend to deter patients from seeking treatment. It is therefore necessary to identify adjunct treatment alternatives that are relatively less expensive, non-invasive and most importantly, readily available at the point of care with minimal professional administration.

In the present study, we characterized the microbial content of Kombucha and assessed the tea’s mycobactericidal potential as well as its potential to modify mycolactone activity. The antioxidant activity and phytochemical constituents of Kombucha tea were also investigated.

1.1. HYPOTHESIS

It has been widely identified that the potent effects of Kombucha tea is due to the secondary metabolites produced during the fermentation process by the symbiotic culture of yeasts and bacteria. Thus, we proposed that not only can Kombucha increase the rate of Buruli ulcer wound healing but also the potent secondary metabolites in the tea may also contribute to elimination of the causative pathogen, *M. ulcerans* and its virulent toxin mycolactone.

1.2. AIM

To identify active compounds in Kombucha with potential antimycobacterial and mycolactone modifying and complete inactivating capacity.
1.3. SPECIFIC OBJECTIVES

- To characterize the bacteria and yeasts strains present in Kombucha
- To determine the phytochemical composition of Kombucha tea
- To evaluate the antioxidant activity of the tea
- To extract mycolactone from *M. ulcerans* and test Kombucha compounds for potency against mycolactone for inactivation or toxin modification
- To determine antimycobacterial activity of the Kombucha tea
CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Buruli ulcer

Buruli ulcer is the third highest recorded mycobacterial infection after tuberculosis and leprosy worldwide (Wansbrough-Jones and Phillips, 2006). It is depicted by dermatological necrotic lesions that result in chronic open sores and ulcerations with sporadic bone engrossment (Walsh et al., 2011). Buruli ulcer presents as painless nodule or papule then progresses into an oedema before evolving into an ulcer (Hayman, 1991; van der Werf et al., 1999; WHO, 2004) as shown in figure 3. All age groups are affected by the disease, however, the burden of Buruli ulcer is most severe with children under 15 years of age (Debacker et al., 2004). The disease is not gender biased since both sexes are equally affected. It is reported to cause significant morbidity in affected individuals, even though lethal infections are really uncommon. The main causative agent for Buruli ulcer is an environmental member of the Mycobacteria family, *Mycobacterium ulcerans*. This bacterium is an acid-fast bacillus, aerobic, non-motile bacterium with thicker cell wall composed of mycolic acid (Ryan and Ray, 2004; Bhamidi, 2009). In addition, these slow growing mycobacteria have replication time of 80 days (Marsollier et al., 2004), a restricted growth temperature range of 28°C to 34°C and typically grows in clumps and cords (Brambilla et al., 2012). *M. ulcerans* possesses a 174 Kb plasmid that encodes for a toxin macrolide, mycolactone (Fig. 1), which is responsible for the pathogenesis of the disease (Stinear et al., 2004). The bacterial toxin has immunosuppressive as well as necrosis ability (George et al., 1999).
2.1.1. Mycolactone

This toxin is composed of a 12-membered macrolide core with an ester linked to two highly unsaturated acyl side chains (Stinear et al., 2004). The lactone core is produced by two polyketide synthases encoded by genes mlsA1 and mlsA2, whereas the fatty acid acyl chain is synthesized by the mlsB gene (Stinear et al., 2004). The macrolide core structure is conserved across the several mycolactone congeners (Fig. 2) from the diverse M. ulcerans sub-lineages whereas there are differences observed in the side chains (Scherr et al., 2013). M. ulcerans from different geographical locations produces different mycolactone congeners which are directly proportional to their degree of toxicity (Mve-Obiang et al., 2003). The strains of M. ulcerans in Africa produce mycolactone A/B which is the most potent toxin, whereas the Australian isolate makes the less toxic mycolactone C. The Chinese isolates also produce mycolactone D (Pidot et al., 2008). Furthermore, M. ulcerans strains isolated from fish and frog were observed to produce both the variants mycolactone E and F (Scherr et al., 2013).
A study by George et al. (1999) revealed that intra-dermal injection of mycolactone into guinea pigs produces Buruli ulcer characteristics similar to human infections, whereas the spontaneous *M. ulcerans* mycolactone negative mutants are avirulent. This macrolide exotoxin has been proven to act on several mammalian cell types including dendritic cells (Coutanceau et al., 2007), adipocytes (Dobos et al., 2001), macrophages (Sarfo et al., 2010; George et al., 2000), fibroblast (George et al., 1999), keratinocytes (Bozzo et al., 2010) and monocytes (Houngbedii et al., 2011).

**Figure 2: Mycolactone variations represented in naturally occurring isolates**

Source: Pidot et al., 2008
The disease starts as a painless nodule (A) or a papule (B), then progresses into an oedema (C) before evolving into an ulcer (D). The gross pathology of the disease associated with the virulence factor, mycolactone (En et al., 2008) includes apoptosis and or necrosis, impaired cytokine production, skeletal and cytoskeletal rearrangement.

**Figure 3: Stages of Buruli ulcer**

Source: WHO, 2004
2.1.2. Epidemiology of the disease

Buruli ulcer has been identified in thirty countries worldwide (Fig. 4) among inhabitants living near swamps, river, lakes or wetlands in Australia, West and Central Africa, Asia and South America (Walsh et al., 2008; Duker et al., 2006). Nevertheless, the cases reported from the African countries are the most severe (Stinear et al., 2007; WHO, 2000).

Figure 4: Global distribution of reported cases of Buruli ulcer as of 2015

Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Buruli_2015.png

South Peru has reported fifteen cases since 1996 with French Guiana recording 42 cases from 1969-2011 (Faber et al., 2015). Ghana is the second most endemic country with 1,048 BU cases after Cote d’Ivoire with 2,670 BU cases (WHO, 2012). The Ministry of Health in Ghana revealed through their 2012 annual report that 60% of the country’s BU cases were from the Ashanti region.
2.1.3. Transmission pattern

The mode of transmission of *M. ulcerans* is still a mystery, but it is believed to occur with direct contact from the environment (Merritt *et al.*, 2010). Fortunately, person-to-person transmission is uncommon and only one case has been reported (Debacker *et al.*, 2002). The bacterial DNA has been detected in several aquatic organisms including; odonates, naucorids, coleopterans, mosquitoes, small fishes and snails (Eddyani *et al.*, 2004; Johnson *et al.*, 2007; Marsollier *et al.*, 2002). Furthermore, *M. ulcerans* DNA was observed on inanimate objects like soil and water filtrates (Marsollier *et al.*, 2004; Williamson *et al.*, 2008) and in wild animals such as koalas and possums (Johnson *et al.*, 2005; Portaels *et al.*, 2001).

Several attempts to culture the bacteria from soil, water, insects and the environment at large were all to no avail until recently when Portaels and her colleagues in 2008 isolated and characterized *M. ulcerans* from an aquatic insect (*Gerridae*) after passage of the insect homogenate through mice. In spite of all these research efforts, the mode of transmission of this bacterium is still not well understood. Three hypotheses have been proposed concerning the transmission. One of the hypothesis states that *M. ulcerans* could be introduced into humans through pre-existing wound which has been tested and confirmed by Williamson *et al.* (2014).

The second hypothesis suggests that thorns of leaves or edges of twigs or insects contaminated with *M. ulcerans* from the environment can serve as an inoculation route of infection through cuts incurred during movement. Work by Marsollier *et al.* in 2002 tested that hypothesis. However, their studies had issues of reproducibility and in addition (i) the insects were not African species (ii) the *M. ulcerans* strains were also not African strains and finally (iii) the data did not specify the exact location of the bacterium in the insect.

Amoeba has also been hypothesized as a possible reservoir due to the ability of most mycobacteria to survive and replicate even in the amoeba cyst stage. Adekambi *et al.* (2006)
were able to isolate environmental mycobacteria from free living amoeba specifically *A. castellani*. Moreover, the higher prevalence of amoeba in Buruli ulcer endemic areas as opposed to non-endemic areas in Benin led to the hypothesis that amoeba might be a possible reservoir for *M. ulcerans* (Eddyani et al., 2008).

### 2.1.4. Mode of Diagnosis, Treatment and Control

The painless nature of Buruli ulcer as well as the absence of epidemiological data on the incubation period has made the early stages of the disease to be often ignored. Thus, individuals with the disease tend to report to the hospitals only at the ulcerative stage of the disease. Some of the methods used in the diagnoses of the disease include:

(i) Microbiological staining with fluorescent auramine-rhodamine or Ziehl-Neelsen, thus, presence of clumped acid fast extracellular bacilli signals a positive test.

(ii) Culturing on Lowenstein Jensen slant and Middlebrook 7H9, 7H10 and 7H11 agar medium for 4 to 6 weeks (Portaels et al., 2008). The observation of light yellow pigment indicates the presence of the bacteria.

(iii) Polymerase chain reaction targeting;

(a) multicopy insertion sequence 2404 and 2606 primers (Ross et al., 1999; Stinear et al., 1999). The amplification of 492 and 332-bp regions of *IS2404* and *IS2606* respectively suggest the presence of *M. ulcerans*.

(b) Nine loci of the variable number tandem repeats (Ablordey et al., 2005).

(iv) Loop-Mediated Isothermal Amplification (LAMP) test (de Souza et al., 2012). The visual observation of a turbid solution and intensity of the fluorescence observed is indicative of the amount of *M. ulcerans* DNA present.

Treatment of the early and the intermediate stages of the disease require the use of antimycobacterial drugs such as rifampicin and streptomycin or amikacin which have proven
to be highly effective (Silva et al., 2009; Teellken et al., 2003). The drugs are usually administered as a combined therapy by daily injection of streptomycin and oral administration of rifampicin for eight weeks (Chauty et al., 2007). Patients who report to the health facilities with large ulcers undergo surgical excision of the ulcer followed by antibiotic treatment for the eight weeks’ period. Unfortunately, the extension of the surgery usually destroys healthy normal cells and mostly does not prevent the disease relapse (Merritt et al., 2010). Notwithstanding the setbacks, the use of combinations therapy has proved a worthy approach to drug therapy (Espey et al., 2002). Also, the BCG vaccine has been shown to provide significant protection against M. ulcerans in children with BU (Portaels et al., 2002).

In Ghana, the annual financial and economic cost for Buruli ulcer treatment was $121,189 and $143,609 respectively with the main cost driver in both cases being the patient. Also, the annual wound dressing cost per capita was $1616 for the financial cost (Asare and Aikins, 2014). Thus, the long hospital stays, the expensive costs for treatment and lack of vaccine against this disease deter people from seeking Buruli ulcer treatment.
2.2. History of Kombucha Tea

Kombucha is a fermented tea which originated in China 220 BC during the Tsin Dynasty and is known for its detoxifying and energizing properties (Roche, 1998). This tea is known by the names, Kombucha (Japan), Tee Kvas (Russia), Kargasok tea (Kargasok), Olinka (Bohemian and Moravian monasteries) and Cha Gu (China). Thus, from the many names, it can be noted that its origin lies in the East-China, Japan and Russia (Günther, 1995). This beverage was popularly known as the “Godly Tsche” throughout the Chinese Qin Dynasty (221-206 BCE) as the “tea with magical powers that enables people to live forever” (Tietze, 1995). Other reports profess that doctor Kombu in 414 AD sent the tea fungus to Japan from Korea to treat the Japanese Emperor’s digestive troubles (Dufrense and Farnworth, 2000). Kombucha tea was later introduced into European countries from China by the Portuguese and Dutch explorers as a medicinal herb (Hollman et al., 1996) and then extended across the Pacific, India, Far East, Germany and other part of the globe for the treatment of many medical conditions. However, consumption of this beverage became famous in Ghana in the early 1980’s and was named “Agboomdon” by the Gas as a reflection of its identity as tea of “Immortality”. Dufresne and Farnworth (2000) and Allen (1998) provided a comprehensive review on Kombucha and its health effects to enable some understanding of the complexity of the mechanisms involved. Also, a scientific review by Thomson (2006) showed the acceptability and the widespread use of Kombucha tea. A more recent comprehensive review on the microbiology, composition, fermentation, beneficial effects and the toxicity of Kombucha has been presented by Jayabalan et al. (2014).
2.2.1. Microbiological composition of the tea fungus

Kombucha is a sweetened black tea fermented by a mixture of yeast and bacteria forming what looks like a mat known as the SCOBY (Symbiotic Community of Bacteria and Yeast) on the surface. A report by Bauer-Petrovska and Petrushevska-Tozi (2000) established that the symbionts composition of the tea depends on the bacteria species and the wild yeast types as well as the existing local climatic and geographical conditions. The bacteria usually present in this tea include, *Acetobacter xylinum* (Balentine, 1997), *Acetobacter xylinoides*, *Bacterium gluconicum* (Reiss, 1994), *Acetobacter aceti*, *Acetobacter pasteurianus* (Liu et al., 1996). The yeasts were identified as *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *Brettanomyces lambicus*, *Brettanomyces custersii*, *Candida* and *Pichia* species (Balentine, 1997; Liu et al., 1996; Mayser et al., 1995) in a thick jelly membrane.

Thus, Kombucha is composed of two portions made up of a floating cellulosic pellicle layer and a sour liquid broth (Fig. 5).
The presence of these different strains of bacteria in SCOBY, inhibit potential contaminating bacteria from cohabiting the beverage and this might be due to the production of organic acids, alcohol or other metabolites in the tea (Sreeramulu et al., 2000).

The floating cellulosic pellicle layer (SCOBY) has been proven to be composed of diverse species of bacteria and yeasts. Figure 6 displays the bacteria and yeasts cells in the community.
2.2.2. Preparation and fermentation of Kombucha tea

The tea is prepared by fermenting a sweetened black tea with the tea fungus as the starter culture (Jayabalan et al., 2007). Boiled sugared (sucrose)-water (38 g/l of water) is infused with one threaded sachet of tea bag (2 g tea/litre), then further boiled for 1-2 minutes, transferred into a clean culture container, partially covered and allowed to cool to room temperature. A seed culture (Kombucha mat) is introduced onto the cooled tea and the set-up is incubated at a temperature (30 ± 2°C) for 14 days.

Figure 6: A microscopic image of Kombucha mat showing the yeast and bacteria cells coexisting as the SCOBY
Source: http://microbialfoods.org/science-digested-microbial-diversity-kombucha/
During fermentation, ethanol formed from the sugars is converted into organic acids. Below is an example of the fermentation of sucrose with its end-product,

\[
\text{Sucrose} \quad \xrightarrow{\text{Invertase}} \quad \text{Glucose + Fructose} \quad \xrightarrow{\text{Zymase}} \quad \text{Ethanol} \quad \xrightarrow{[O_2]} \quad \text{Organic acids}
\]

The yeast in Kombucha converts sucrose into glucose and fructose and produce ethanol (Reiss, 1994). The bacteria convert the monosaccharides to acids, Acetobacter for example converts glucose to gluconic acid and fructose to acetic acid. Cellulose is synthesized by the bacteria through stimulation of related xanthines and caffeine in the tea infusion (Dufresne and Farnworth, 2000). The presence of acetic acid in the tea stimulates the yeasts to produce ethanol which incites the growth of acetic acid bacteria which produces acetic acid that is further metabolized by other enzymes to generate organic acids (Liu et al., 1996). Both the ethanol and the acetic acid exert antimicrobial activity against pathogenic bacteria thus preventing contamination of the tea fungus (Liu et al., 1996).

### 2.2.3. Chemical composition of Kombucha

Apart from acetic and glucuronic acids, other acids present in the Kombucha beverage include: usnic acids, malic acids, lactic acids, oxalic acids, glucaric acids, butyric acids, acetiuconic acids (Jayabalan et al., 2007; Tietze, 1995).

One of the natural antibiotics present in Kombucha is usnic acid which is effective against Gram positive bacteria like *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* (Lautwerwein et al., 1995). Glucuronic acid conjugates toxins produced by the liver are eliminated by the help of glucaric acid (Roussin, 2003). Oxalic acid present in the tea acts
as an effective preservative (Tietze, 1995). Furthermore, butyric acid protects the human cellular membranes and links with gluconic acid to strengthen the walls of the gut in order to combat yeast infections like candidiasis (Tietze, 1995). Lactic acid aids in balancing acids and alkaline in the body, prevents bowel decay and constipation and finally assist in blood circulation (Tietze, 1995). A report by Greenwalt et al. (1998) and Tietze (1995) indicated that acetic acid acts as a powerful preservative that is capable of inhibiting harmful bacteria. Gluconic acid which is produced by the bacteria in Kombucha can be broken down to caprylic acid which is of great benefit to sufferers of candidiasis and thrush (Tietze, 1995). Vitamin B-complex (B, B, B, B, and B), folic acid, vitamin C and antibiotic substances are also part of the metabolites of Kombucha fermentation (Blanc, 1996).

Tea is said to be a strong antioxidant due to the presence of catechins, theaflavin, thearubigins and flavonols in it (Hakim et al., 2003). In the black tea, theaflavin and thearubigins are the major polyphenolic derivatives present (Jayabalan et al., 2007). Green tea on the other hand contains major polyphenolic derivatives such as (-)-epicatechin, (-)-epicatechin-3- gallate, (-)-epigallocatechin and (-)-epigallocatechin-3-gallate (Jayabalan et al., 2007) (Fig. 7). The antioxidant properties of the tea polyphenols provide health benefits. An instance was a report of the reduction of total and low density lipoprotein cholesterol in mildly hypercholesterolemic adults after consumption of black tea (Davies et al., 2003). The tea preparation is also reported to have inhibitory activity against tumorigenesis (Hakim et al., 2003). Black tea is known to be the most preferred substrate for the preparation of Kombucha, although green tea can be used (Reiss, 1994). This suggests that the beneficial effects of Kombucha tea may be attributed to the presence of tea polyphenols as well as the other components obtained after fermentation. Some organic acids present in Kombucha are also shown in Figure 8.
Figure 7: Chemical structures of some polyphenols in green tea
Source: Zaveri, 2006

Figure 8: Organic acid contained in Kombucha tea
Source: https://www.hindawi.com/journals/jchem/2015/591869/fig2/
2.2.4. Health benefits of Kombucha

Kombucha is known to exert medicinal properties which include its being a detoxification and prophylactic agent towards the treatment and prevention of all forms of arthritis, aiding in digestion and gut health (Blanc, 1996; Sreeramulu, et al., 2000), prevention of paracetamol induced hepatotoxicity (Pauline et al., 2001) and being an immune booster (Srihari and Satyanarayana, 2012). It is also known to reverse chromate-induced changes in albino rats (SaiRam et al., 2000).

The medicinal properties of this beverage may be ascribed to the presence of tea polyphenols, gluconic acids, glucuronic acids, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during fermentation (Vijayaraghavan et al., 2000). Additionally, antimicrobial activity has been reported on the tea against various pathogens including \( Staphylococcus aureus \), \( Escherichia coli \) (Greenwalt, 1998), \( Pseudomonas aeruginosa \), \( Listeria sp \), \( Yersinia enterocolitic \), \( Salmonella enteritidis \), \( Bacillus cereus \) and \( Helicobacter pylori \) (Guttapadu and Sreeramulu, 2000).

The beverage has also been reported to exhibit anti-mutagenic and anti-inflammatory property (Halder and Bhaduri, 1998; Sangsrichan and Ting, 2010). The free radical scavenging and antioxidant activities of Kombucha have been reported by Jayabalans et al., (2008) and Malbasa et al. (2011). Furthermore, the administration of the tea has been observed to significantly improve lipid peroxidation and oxidative stress suggesting that Kombucha can assist in the repair of damage likely to be caused by environmental pollutants (Gharib et al., 2009). Work by Ofori et al. (2015) demonstrated that Kombucha tea protects against arsenic-induced protein peroxidation in rats.

A preliminary study to the current project showed that Kombucha exerts some anti-inflammatory activity against lesions created in rat oesophageal tracts. It was found that
Kombucha stimulated the production of interleukin-10, tumour necrosis factor-alpha among others (Ofori, 2008). It was further demonstrated that Kombucha administered orally and topically to rats enhanced wound healing better than unfermented tea (Larbie, 2012). In a recent study, the healing ability of Kombucha on skin lesions and muscles was found to be comparable to commercial healing product (Brosin of Bayer). It was shown that Kombucha could decrease the inflammation process quickly, initiate formation of basal matrix and cell proliferation continuously while at the same time helping with compaction and tissue remodelling without delays in the injured tissue (Rosales-Cortes et al., 2015).

A study by Aloulou et al. (2012) demonstrated that Kombucha tea had hypoglycaemic and lipidemic activities that were enough to alleviate alloxan-induced diabetes in rats. Thus, they concluded that Kombucha could be a potential candidate to be used as supplement for treatment and the prevention of diabetes.

The Oncological Research Unit of Russia and the Russian Academy of Sciences in Moscow in 1951 revealed that daily consumption of this tea makes an individual more resistant to cancer. They also indicated that the wonder beverage may improve the immune system as well as prevent cancer (Dufrense and Farnworth, 2000).

Several research proved Kombucha exhibit a number of prophylactic and curative properties and also some of its biological activities have been investigated. The tea also exerts antimicrobial activity against some pathogenic organisms and shown to have wound healing property. However, the effect of Kombucha on mycolactone as well as mycobacteria has not been reported. Thus, this present study was aimed at characterizing the microbial content of Kombucha tea, assess the tea’s mycobactericidal potential and also modify mycolactone to interfere with its action. Also, the antioxidant and the phytochemical constituents of Kombucha tea were determined.
CHAPTER THREE

3.0. METHODOLOGY

3.1. Characterization of Kombucha microorganisms

3.1.1. Preparation of Kombucha tea

Kombucha tea was prepared as described by Gbewonyo (2014). A four and half litre solution of sucrose (approx. 38.5g/l) was prepared using filtered boiled water. One Lipton tea bag/litre (i.e. 5 tea bags for 5 litres Kombucha) was infused for 2 minutes. The sugared tea was then transferred into a container and allowed to cool to room temperature and then seeded with 500 µl (0.5 L) of a previous ferment containing a ‘baby mat’ (composed of symbiotic community of bacteria and yeasts (SCOBY)). The container was then covered with linen and allowed to ferment for 14 days. Tea without SCOBY served as control. The extracts were filtered, frozen, freeze-dried and kept at 4 °C for subsequent assays.

3.1.2. Culturing of Kombucha isolates

Five millilitres of the Kombucha broth and residue were centrifuged at 845 g using a micro centrifuge for 10 minutes. The pellets obtained were serially diluted (1:10, 1:100 and 1:1000) and inoculated on LB agar plates before incubating 37 °C for a period of 48 hours. Weighted section of the mat was also homogenized in 5 ml PBS and treated as described above. Unique colonies were sub-cultured again for pure isolates of the microorganisms.

3.1.3. Microscopy- Gram staining

Smears of the colonies formed on the LB plates were made on glass slides. The slides were allowed to air dry, then passed through flame to heat fix. They were then covered with two drops of crystal violet for 60 seconds and washed with water. Two drops of Lugol’s iodine
were added for 60 seconds and washed with water afterwards. Decolourizer (alcohol) was then added and washed immediately. Finally, safranin was used to flood the slides for 2 minutes followed by washing and air drying of the slides. The slides were then viewed under oil immersion (100X) using light microscope (Leica, Wetzlar, Germany).

3.1.4. DNA extraction

DNA was extracted from the Kombucha isolates using the Guanidine Hydrochloride method. The Kombucha isolates were suspended in PBS solution before the start of the extraction. About 450 µl of lysis buffer was added along with an eyeball amount of glass beads (150-212 microns) and bead beat for 15 minutes. It was then incubated for 20 minutes in a water bath at 65 °C then centrifuged at 5600 g for 2 minutes. Potassium acetate was added to a 1.5 ml Eppendorf tube containing 400 µl of the supernatant. The mixture was kept in a -20°C freezer overnight and then centrifuged at 5600 g for 30 minutes the next day. Guanidine hydrochloride was then pipetted into fresh tubes containing known volumes of the supernatant. The mixture was transferred to a spin filter and centrifuged for 2 minutes at 5600 g. Flow through was discarded. Wash solution was then added, spun and the flow through discarded. Ethanol was added, spun and flow through discarded. The spin filter was spun again and then transferred into a fresh tube. Finally, 100 µl of the elution buffer was added, incubated for 10 minutes and spun at the same speed and time to elute the DNA.

3.1.5. PCR and gel electrophoresis of DNA from bacterial and yeasts isolates

For bacterial isolates, the 16S rRNA gene was amplified. The PCR master mix was composed of (5 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTP, 0.5 µl of 10 µM forward primer (5¹ AGGAGGTGATCCAACCGCA 3¹) and 0.5 µl of 10 µM reverse primers (5¹ AACTGGAGGAAGGTGGGGGAT3¹), 0.13 µl of Taq polymerase and 15.87 µl of PCR water. About 2.5 µl of each DNA template was added to the master mix to make a total reaction
volume of 25 µl. The amplification was carried out using a thermocycler (Applied Biosystems, California, U.S.A). An overall reaction of 35 cycles was carried out with the first stage being the denaturation of the template DNA for 1 minute at 94 °C, followed by primer annealing at 60 °C for another minute and the extension of the primer for 2 minutes at 72 °C.

With the yeast samples, the fungal 18S rRNA gene was amplified. The PCR reaction was composed of 12.5 µl of Top Taq 2X master mix (containing the Taq polymerase, the DNTPs and the reaction buffer), 0.5 µl each of ITS 4 (5' TCCTCCGCTTATTGATATGC 3') and ITS 5 (5' GGAAGTAAAAGTCGTAACAAGG 3') primers and 9 µl of PCR water. About 2.5 µl of the DNA template was added to the master mix to make a reaction volume of 25 µl. The amplification was carried out using a thermocycler. The PCR thermal profile protocol used had the initial denaturation at 94 °C for 5 minutes, then 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 50 °C for 1 minute and extension at 72 °C for 1 minute 15 seconds. The last step is the final extension at 72 °C for 7 minutes.

Seven microliters of the PCR amplicons together with 3 µl of loading dye were electrophoresed on 1% agarose gel containing ethidium bromide in Tris-borate EDTA buffer at 100 V for 45 minutes. The amplicons were run along a 100-base pair molecular weight ladder, the negative (only PCR master mix with water as template) and positive control (known bacterial and yeast DNA). The gel was photographed under UV light illumination using a digital camera.

3.1.6. DNA Sequencing and Nucleotide blast analysis in the Genbank

The PCR amplicons were shipped to Cambridge University to be sequenced using the Sanger method. The nucleotide sequences obtained were blasted with the NCBI database and aligned to closely related strains. The sequence with the highest identity (98% and above) and lowest E-value (0.00) was selected as the identified species or genus of the isolates.
3.2. Antioxidant activity

Phytochemical analysis was carried to determine the presence of saponins, tannins, terpenoids, flavonoids, alkaloids and phenols in the tea using standard methods (Jack and Okorosaye-Orubite, 2009; Waterman, 1993). Concentration of Kombucha used was 100 mg/ml.

3.2.1. Test for Saponins

About 3 ml of distilled water and 3 ml of Kombucha was vigorously shaken together for 2 minutes. The presence of a foam layer indicates the presence of saponins.

3.2.2. Test for Flavonoids

Approximately 3 ml of 1 M NH₃ was added to 3 ml of Kombucha and allowed to stand at room temperature for 2 minutes. After the stipulated time, 2 ml of concentrated H₂SO₄ was added to the mixture and the observation of an intense yellow precipitate showed the presence of flavonoids.

3.2.3. Test for Alkaloids

Kombucha of volume 2 ml was heated until all the solvent in which it was dissolved was lost. It was cooled to room temperature and 5 ml of 2 M HCl was added. After complete dissolution, 3 drops of Wagner reagent were added and the appearance of a reddish flocculation designates the presence of alkaloids.

3.2.4. Test for Phenols

About 1 ml of Kombucha was mixed with 1 ml of 5 % FeCl₃ solution and the presence of phenols was indicated by the formation of deep blue/ back colour in the tube.
3.2.5. Test for Terpenoids

A 3 ml volume of Kombucha was mixed to 1 ml of chloroform after which 3 drops of concentrated sulphuric acid was added. Formation of a reddish-brown precipitate at the interphase indicated the presence of terpenoids.

3.2.6. Test for Tannins

About 2 ml of 5 % FeCl$_3$ was added to 2 ml of Kombucha after which the observation of a green precipitate signals the presence of tannins.

3.2.7. DPPH analysis

The free radical scavenging activity of Kombucha was evaluated as described (Anim et al., 2016). Approximately, 100 µl of each of the different concentrations of Kombucha (2-fold serial dilutions from 40 mg/ml to 0.625 mg/ml) was added to 100 µl of 0.5 mM of DPPH dissolved in methanol. The mixtures were incubated for 20 minutes in the dark at room temperature. The absorbance of the resulting solution was read at 517 nm. A freshly BHT solution (5 mg/ml) and unfermented tea were used as standard and control, respectively.

To calculate the percentage DPPH activity

\[
\% \text{ Antioxidant Activity} = \left( \frac{\text{Absorbance of the control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100
\]

3.2.8. Total phenolic content

The total phenolic content of Kombucha was estimated as described (Anim et al., 2016). About 10 µl of 5 mg/ml of gallic acid was added to 0.79 ml of distilled water in an Eppendorf tube. Folin Ciocalteau reagent (50 µl) was added to each of the different concentrations of the Kombucha and mixed thoroughly. The solutions were then incubated in the dark for 8 minutes.
after which 150 µl of Na$_2$CO$_3$ was added and further incubated for 2 hours at room temperature. Afterwards the absorbance was read at a wavelength of 760 nm. A standard calibration curve for gallic acid (Figure 1, Appendix) was used to calculate the total phenolic content of Kombucha and unfermented tea and were expressed as gram/100 g GAE (gallic acid equivalent).

### 3.3. Determination of antimicrobial activity of Kombucha tea

An overnight culture of *S. aureus* at log phase in LB broth was serially diluted ten folds in distilled water and the optical density (OD) at 600nm was taken. Freeze dried Kombucha of concentration 120 mg/ml was freshly prepared and serially inoculated into 100 µl of *S. aureus* broth culture containing 2.03x10$^6$ cells/ml to make 7 different concentrations of the tea (100 mg/ml, 80 mg/ml, 60 mg/ml, 40 mg/ml, 20 mg/ml, 10mg/ml and 5 mg/ml). Kombucha-treated *S. aureus* was incubated for 24 hours after which their OD was recorded. About 50 µl of each treated culture was then sub-cultured on Mueller Hinton agar media to determine the presence of viable *S. aureus* post treatment. The assay was performed in triplicates.

The calculated volumes used in the assay are shown on Table 3A in the Appendix section

### 3.4. Evaluation of antimycobacterial activity of the Kombucha tea

Middlebrooks H9, H10 and Lowenstein Jensen media were used to culture *M. ulcerans* (obtained from Dr. Heather Williamson of the Tennessee University). Six weeks old *M. ulcerans* culture was serially diluted to 1:10 in distilled water and the OD$_{600\text{nm}}$ was taken. The bacteria were inoculated with the different concentrations (100 mg/ml, 50mg/ml, 20 mg/ml, 10 mg/ml and 5 mg/ml) of Kombucha for a period of seven days. The Kombucha -*M. ulcerans* treated samples were acid fast stained and cultured on LJ slants to investigate the presence of
viable mycobacteria post treatment. The assay was performed in triplicates using amikacin as control.

The calculated volumes used in the assay are shown on Table 2A in the Appendix section.

3.4.1. Acid fast stain

Smears of the mixtures of Kombucha and M. ulcerans were made on glass slides and allowed to air dry, then passed through a flame to heat fix. The slides were then covered with carbol fuchsin reagent for 5 minutes then washed with water. Slides were tilted to dry before the addition of the decolourizer for 5 minutes. Each slide was washed with distilled water after which it was flooded with methylene blue for 2 minutes. The slides were then washed with water, air-dried and viewed under oil immersion (100X) using a light microscope (Leica, Wetzlar, Germany).

3.5. Determination of effect of Kombucha on mycolactone inactivation or modification.

Mycolactone A/B used in this assay was a kind gift from Yoshito Kishi lab, Harvard University (Cambridge, USA). About 12.5 µl of 40 µg/ml mycolactone was incubated with Kombucha concentrations of 100 mg/ml, 50 mg/ml, 10 mg/ml and 1 mg/ml to make a mycolactone working concentration of 10 µg/ml. The mixtures were incubated for 24 and 48 hours respectively. Approximately, 50 µl of the treated mycolactone was spotted on 0.20 mm silica gel thin layer chromatography (TLC) plates with UV fluorescent indicator. Each plate was run with controls of Kombucha only and mycolactone only with a solvent system: chloroform:methanol:water in the ratio 90:10:10. The plates were air dried and viewed under UV light of 254 nm.

The calculated volumes used in the assay are shown on Table 1A in the Appendix section.
3.6. Cytotoxicity assay

3.6.1. Human skin fibroblast culture

The human skin fibroblast cells used in this experiment were obtained from the Clinical Pathology Department of the Noguchi Memorial Institute for Medical Research, University of Ghana. The fibroblasts were cultured in RPMI 1640 medium/ supplemented with 10% foetal bovine serum and 4% L-glutamine at 37 °C under the atmosphere of 5% CO₂.

3.6.2. Effects of Kombucha tea treated mycolactone on human skin cells

About 100 µl of the skin fibroblasts in RPMI were seeded at a concentration of $1 \times 10^4$ cells/well in 96 well plate. The cells were incubated with the mycolactone treated with the different concentrations of the Kombucha (100 mg/ml, 50 mg/ml, 10 mg/ml and 1 mg/ml) for a period of 24 and 48 hours, respectively. Human skin fibroblast cells treated with only mycolactone and Kombucha served as controls for this experiment. After incubation, the cells were viewed under inverted microscope (Carl Zeiss, Gottingen, Germany) for morphological changes.
CHAPTER FOUR

4.0. RESULTS

4.1. Characterization of Kombucha microorganisms

4.1.1. Isolation of microorganisms in Kombucha

The various portions of Kombucha tea were cultured and the microbial isolates were identified and further characterized. Serial dilution of the various portion of Kombucha tea showed that, 1:1000 dilutions had distinct and fewer isolates as compared to 1:100 and 1:10 respectively. The latter dilutions exhibited more of “mat-like” colonies. The mat and residue portions of the tea recorded the highest number of isolates as compared with the broth. The mat had the most yeasts isolates than the residue and broth. Also, the residue contained more bacteria isolates than that of the mat and the broth. However, the broth recorded the least number of bacteria and yeasts isolates. Furthermore, it was observed that the isolates from the mat and residue grew faster than that of the broth. Also, some of the isolates exhibited entire margin with raised elevation. Again, most of the isolates from the broth and residue appeared creamy whereas that of the mat had whitish colonies on the LB agar plates. Figure 9 shows the bacterial and yeasts isolates that were present in the Kombucha tea.

Figure 9: Plates showing sub-cultures of the unique colonies from the Kombucha tea.
A: Isolates obtained on the mat culture, B: Isolates obtained from the broth culture, C: Isolates from the residue culture
4.1.2. **Microscopy**

The isolates were Gram stained to ascertain the morphology and the Gram reaction of the microorganisms obtained from the tea. Figures 10 A and C demonstrates Gram positive rod shaped bacteria while Figure 10 B shows an oval yeast shape. Furthermore, the isolates D and F are Gram negative bacteria with rod shape while the isolate E are bacteria exhibiting coccobacillus morphology. The morphologies of the various isolates obtained from the tea are indicated by the arrows in the figure below.

![Gram stain reaction and morphology of isolates from Kombucha tea.](image)

Figure 10: Gram stain reaction and morphology of isolates from Kombucha tea.
4.1.3. DNA extraction of isolates from Kombucha

The DNA of the isolates were extracted using the guanidine hydrochloride method and then run on an agarose gel. Figure 11 represents a gel electropherogram of the extracted DNA of Kombucha tea isolates indicating successful DNA extraction by the presence of bands in all the sample wells.

![DNA bands](image)

**Figure 11: Gel electropherogram of DNA extracted from Kombucha isolates.** MW: Molecular weight marker (100 bp), Sample A-N: DNA from Kombucha isolates.

4.1.4. PCR and Gel electrophoresis

Identification and further characterization of the isolated bacteria was carried out on the DNA samples using Polymerase Chain Reaction. For bacterial identification, primers targeting the 16S ribosomal DNA gene whereas ITS 4 and 5 fungal primers (18S rDNA) were used to amplify the internal transcriber space of the fungal isolates. The PCR amplicons obtained were run on 1% agarose gel (Fig. 12 and 13). All the isolates were successfully amplified giving a molecular band size of approximately 350 and 450 bp as expected for the bacterial and fungal genes target, respectively.
Figure 12: Gel electropherogram of Kombucha bacterial isolates using 16S rRNA PCR amplification. MW: Molecular weight maker (100 bp), Kombucha bacterial samples: A-M, N: Uncultured Kombucha tea, O: Positive control, Q: Negative control

Figure 13: Gel electropherogram of Kombucha yeasts isolates using ITS 4 (18S rRNA) PCR amplification. Kombucha yeasts samples 1-13, MW: DNA ladder (100 bp), N: Negative control, P: Positive control
4.1.5. Microorganisms in Kombucha tea

After the gel electrophoresis, the PCR amplicons were sent to Cambridge University for sequencing using the Sanger method. Bacteria and Archaea 16S ribosomal as well as ITS fungal RNA sequences for species or genus assignment were used. The organism with the highest sequence identity (98%) and lowest E-value (0.000) was selected as the identified species or genus of the isolates. Table 1 displays the bacterial and yeasts isolates that might be present in the Ghanaian Kombucha tea.

Table 1: Characteristics of representative bacteria and yeasts isolates in Kombucha tea

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>Yeasts Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample</strong></td>
<td><strong>Portion of Kombucha tea</strong></td>
</tr>
<tr>
<td>A</td>
<td>Broth &amp; Residue</td>
</tr>
<tr>
<td>F</td>
<td>Residue &amp; Broth</td>
</tr>
<tr>
<td>I</td>
<td>Mat</td>
</tr>
<tr>
<td>K</td>
<td>Broth</td>
</tr>
</tbody>
</table>
4.2. Biochemically active compounds in Kombucha tea

Kombucha concentration of 100 mg/ml used for the phytochemical analysis and it revealed the presence of saponins, flavonoids, alkaloids, phenolic compounds and terpenoids but absent of tannins in the tea.

Table 2: Phytochemical constituents of Kombucha tea

<table>
<thead>
<tr>
<th>Saponin</th>
<th>A whole layer of foam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>+++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
</tbody>
</table>

+: colour intensity  -: Absent

4.2.1. Antioxidant activity of Kombucha tea

The antioxidant property of the Kombucha beverage was determined by the DPPH method. The free radical scavenging ability of Kombucha tea was measured together with BHT. BHT was used as the standard positive control and unfermented tea as normal control. The plot of concentration against % antioxidant gave a curve from which the effective concentration (EC$_{50}$) was calculated (Fig 14).
Figure 14: Graphs showing the antioxidant properties of (A) BHT (B) Kombucha tea and unfermented tea. It can be seen from Figure 14 (A and B) that as the concentration increases, the mean (%) antioxidant activity of BHT, Kombucha and unfermented tea increases rapidly and then peaked around antioxidant activity of around 80%.

The effective concentration (EC$_{50}$) of Kombucha and unfermented tea were found to be 0.77 mg/ml and 2.27 mg/ml, respectively as compared to 0.06 mg/ml obtained for the standard BHT.

4.2.2. Total phenolic content of Kombucha tea

The total phenolic content (TPC) of Kombucha and unfermented tea were evaluated and expressed (mg/100 gGAE) using the Gallic acid calibration curve (Appendix Fig 1). It was observed that the total phenolic content of both tea increased with increasing concentration (Fig 15). Also, the TPC for Kombucha and unfermented tea at a concentration of 2.5 mg/ml was 1,330 and 638 mg/ml, respectively; and at 5 mg/ml, Kombucha recorded a TPC of 1,771 mg/ml whereas unfermented tea had 1,047 mg/ml.
Figure 15: Total phenolic content of Kombucha and unfermented tea. Concentration 1: 2.5 mg/ml; Concentration 2: 5 mg/ml. Results are means± SEM of n=3 for both Kombucha and unfermented tea. The p-values between the two concentration of Kombucha and tea are statistically significant (p<0.0001).

4.3. Antimicrobial activity of Kombucha tea

*S. aureus* (within the log stage) was incubated with the different concentrations of Kombucha (5 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml and 100 mg/ml) and cultured for 24 hours. *S. aureus* treated with Kombucha concentration of 5 mg/ml had intact Gram positive cocci after staining as seen with the untreated *S. aureus*. The concentrations 80, 60, 40, 20 and 10 mg/ml of Kombucha partially inhibited bacteria growth leaving behind 1.34 x10^6, 1.78 x10^6, 2.28 x10^6, 2.80 x10^6 and 3.34 x10^6 colony forming units (CFU) of the bacteria post treatment. However, 100 mg/ml Kombucha concentration considerably hampered the growth of the bacteria with only 7.4 x10^5 CFU of the *S. aureus* remaining after Kombucha treatment as compared with the amikacin treatment having 1.5 x10^4 CFU (Table 3 and Figure 15).
Table 3: Colony forming units (CFU)/ml of *S. aureus* prior and post Kombucha treatment

<table>
<thead>
<tr>
<th>Kombucha concentration (mg/ml)</th>
<th>Number of colonies</th>
<th>Number of CFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>37</td>
<td>7.4 x10^5</td>
</tr>
<tr>
<td>80</td>
<td>67</td>
<td>1.34 x10^6</td>
</tr>
<tr>
<td>60</td>
<td>89</td>
<td>1.78 x10^6</td>
</tr>
<tr>
<td>40</td>
<td>114</td>
<td>2.28 x10^6</td>
</tr>
<tr>
<td>20</td>
<td>140</td>
<td>2.80 x10^6</td>
</tr>
<tr>
<td>10</td>
<td>172</td>
<td>3.34 x10^6</td>
</tr>
<tr>
<td>5</td>
<td>231</td>
<td>4.62 x10^6</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin (30 µg/ml)</td>
<td>15</td>
<td>1.5 x10^4</td>
</tr>
<tr>
<td>Untreated <em>S. aureus</em></td>
<td>323</td>
<td>6.46 x10^6</td>
</tr>
</tbody>
</table>

As shown in Table 3, the CFU of *S. aureus* per ml decreases with increasing Kombucha concentration. Thus, higher concentrations of the tea were observed to considerably hamper the growth of the bacteria better than the lower concentrations (Fig. 16).
Figure 16: A plate of *S. aureus* incubated with varying Kombucha concentrations. A: *S. aureus* inoculated with 100 mg/ml Kombucha; B: *S. aureus* inoculated with 80 mg/ml Kombucha; C: *S. aureus* inoculated with 60 mg/ml Kombucha; D: *S. aureus* inoculated with 40 mg/ml Kombucha; E: *S. aureus* inoculated with 20 mg/ml Kombucha, F: *S. aureus* inoculated with 10 mg/ml Kombucha, G: Untreated *S. aureus* culture, H: *S. aureus* inoculated with amikacin (30 µg/ml). It can be observed that Kombucha tea of 100 and 80 mg/ml concentration considerably hampered the growth of the bacteria.

4.4. Antimycobacterial activity of Kombucha tea

About $3 \times 10^5$ concentration of *M. ulcerans* was treated with different concentrations of Kombucha and incubated for seven days after which the mixtures were acid fast stained to detect the presence of intact mycobacteria. The *M. ulcerans* were pink with intact rods indicating the presence of acid fast bacilli whereas Kombucha non-mycobacteria were observed as blue rods (Fig 17). We did not observe any differences in the morphology of the *M. ulcerans* treated with the different concentrations of Kombucha.
Figure 17: Acid fast stain reactions of (A) Kombucha tea microbes (B) *M. ulcerans*

Figure 18: Different concentrations of Kombucha incubated with *M. ulcerans*. A: 1000 mg/ml Kombucha with Mu; B: 100 mg/ml Kombucha with Mu; C: 50 mg/ml Kombucha with Mu; D: 10 mg/ml Kombucha with Mu; E: 1 mg/ml Kombucha with Mu.
4.4.1 Detection of viable *M. ulcerans* after Kombucha treatment

We further cultured the Kombucha treated *M. ulcerans* on LJ slants to detect viable bacterial cells. For all the different treatment concentrations, we observed growth of *M. ulcerans* suggesting that the tea had no bactericidal activity against the mycobacteria. The arrows in Figure 19 shows light yellow colonies on LJ slants which indicates the presence of *M. ulcerans* after treatment with Kombucha.

![Figure 19: Cultures of *M. ulcerans* with Kombucha on LJ slants.](image)

*Mu:* *M. ulcerans* cultures only, *A:* 100 mg/ml Kombucha with *Mu;* *B:* 50 mg/ml Kombucha with *Mu;* *C:* 10 mg/ml Kombucha with *Mu;* *D:* 10 mg/ml Kombucha with *Mu;* *E:* 1 mg/ml Kombucha with *Mu.* Growth was observed in all the Kombucha treated *M. ulcerans* as compared to the *M. ulcerans* only.
4.5. Thin Layer Chromatography profile of mycolactone treated with Kombucha tea

About 12.5 µl of mycolactone was co-incubated with the various concentrations of Kombucha for 24 and 48 hours and the resulting mixture was run on a TLC plate to observe for mycolactone modification. We observed a prominent band at the retention factor (rf) for mycolactone in all the treated fraction that was comparable to the mycolactone only control (Fig 20). We also observe a prominent band closed to the solvent front in all the treated fraction which was comparable to the Kombucha only control indicative of unresolved constituents in the Kombucha tea.

Figure 20: TLC plate showing mycolactone and Kombucha bands under UV light (254 nm). Solvent system: chloroform:methanol:water in the ratio 90:10:10. A: Mycolactone (10 µg/ml) only; B: Mycolactone (10 µg/ml) inoculated with 100 mg/ml Kombucha; C: Mycolactone (10 µg/ml) inoculated with 50 mg/ml Kombucha; D: Mycolactone (10 µg/ml) inoculated with 10 mg/ml Kombucha; E: Mycolactone (10 µg/ml) inoculated with 1 mg/ml Kombucha; F: Only Kombucha.
4.5.1. Cytotoxicity profile of mycolactone treated with Kombucha tea

To further confirm our observation of the inability of Kombucha to inactivate mycolactone, we inoculated and cultured human skin fibroblasts with the Kombucha treated mycolactone for 24 to 48 hours respectively (Fig 24). The cells lines were treated with Kombucha only, and as shown in Fig 22, Kombucha only exhibited no cytotoxic properties on the cultured fibroblasts. However, treatment of the cell with 10 µg of mycolactone revealed the characteristic rounding and shrinkage of the fibroblast, consistent with the widely published cytotoxic effects of mycolactone on cultured cells (Fig. 23). Furthermore, the cell lines were treated with a 24 and 48 hours pre-incubated mixture of 10 µg mycolactone and 100 mg or 50 mg of Kombucha respectively to determine whether the Kombucha had attenuated the mycolactone activity. After 24 hours of treatment on the fibroblasts, it was observed that the cells had rounded and detached from the plates as was seen in the mycolactone control treatment (Fig. 23).

Figure 21: Human skin fibroblast cells incubated in RPMI medium
The cell lines were large, flat, elongated (spindle shape)
Figure 22: Human fibroblast cells treated with only Kombucha tea (control) at (A) 24 hours (B) 48 hours.

Figure 23: Human skin fibroblast cells treated with 10 µg mycolactone concentration. Cell lines treated with mycolactone (10 µg) at (A) 24 hours and (B) 48 hours
Figure 24: Human skin fibroblast inoculated with mycolactone treated with different concentrations of Kombucha. A: 100 mg/ml Kombucha + 10 µg mycolactone; B: 50 mg/ml Kombucha + 10 µg mycolactone
CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Kombucha is a sugared black tea (Camellia sinensis) fermented with symbiotic association of bacteria and yeasts (tea fungus). The tea is considered as traditional medicinal beverage with numerous health properties, among which include anti-cancer (Jayabalan et al., 2011), anti-diabetic (Aloulou et al., 2012; Hiremath et al., 2002), immune booster (Ram et al., 2000), liver detoxification (Loncar et al., 2000) and the treatment of high cholesterol (Yang et al., 2009). Also, the tea has been shown to exert antibactericidal activity, thus we sorted out to investigate this property on M. ulcerans. The aims of this study were to characterize the microbial diversity of Kombucha as well as investigate its antioxidant activity and phytochemical constituents. We also assessed the tea’s mycobactericidal potential and also its ability to modify and interfere with the activity of mycolactone, a toxin known to be the main virulence factor in Bu infections.

The bacteria identified in the tea in our study using the 16S rRNA target specific loci BLAST included: Corynebacterium glutamic, Bacillus licheniformis, Lactobacillus amylolyticus, (lactis and cineris) and unidentified bacterial species. The yeasts identified were Dekkera bruxellensis, Lachancea fermentati, Brettanomyces bruxellensis and Rhodotorula mucilaginosa. Strains of Paenibacillus spp have been isolated from soil, water and food. One unique characteristic is their ability to degrade proteins, polysaccharide and polyaromatic hydrocarbons (Daane et al., 2002). Some strains of Paenibacillus spp demonstrate antimicrobial property by producing antibacterial compounds such as polymyxin M (Martin et al., 2003) and fusaricidin-type antifungal agent (Beatty and Jensen, 2002). Lactobacillus amylolyticus have been isolated from beer malt and beer wort (Bohak et al., 1998) suggesting they play significant role in fermentation process. Marsh and his colleagues (2014) also
reported that 30% of the bacterial composition of their Kombucha samples belongs to the genus Lactobacillus. This same genus has been isolated from Chinese Kombucha (Wu et al., 2004; Zhang et al., 2011). Thus, findings from this study which demonstrates the presence of Lactobacillus in Ghanaian Kombucha, supports the reported role of these bacteria in fermentation. The bacteria Corynebacterium glutamic has been employed in the production of glutamic acid by submerged fermentation (Hermann, 2003; Ahmed et al., 2013) while Bacillus licheniformis has been isolated from Kombucha with very high acetic acid content (Roussin, 2007). Furthermore, all the bacterial isolates obtained from this study belong to the Phylum Firmicutes and according to Chakravorty et al. (2016), 11.2% of the Kombucha bacteria are members of this phylum. Seto et al. (2006) have also reported the presence of Firmicutes in Kombucha from UK, Ireland and Canada. The diversity observed in the microbial composition in all Kombucha worldwide is usually due to factors such as, initial culture used, type of tea used, type of sugar used, brewing time, type of water used, culturing temperature and many more (Teoh et al., 2004). The present study focused on identifying bacterial isolates present in the Kombucha tea, thus, the reads obtained after the BLAST search could not identify the types of bacteria that were not cultured. Marsh et al. (2014) have reported that most Gluconacetobacter species have been labeled as uncultured bacterial species after blasting 16S rRNA reads in NCBI database. This might be the reason for the non-detection of these species in the Kombucha used in this study.

The yeasts identified in the present study have historically associated with Kombucha fermentation. D. bruxellensis have been termed the spoilage yeasts and are associated with the fermentation of wine and beer and also enhancing the flavour of lambic beer (DeKeersmaecker, 1996). It is also the major and preferred ethanol producing microbe than S. cerevisiae in ethanol producing industries (Liberal et al., 2007; Passoth et al., 2007). Brettanomyces spp has been branded producers of acetic acid and acetic acid esters under anaerobic conditions (Gancedo
and Senano, 1989; Teoh et al., 2004). *Lachancea fermentati* has also been isolated from Kombucha after seven days’ fermentation by Chakravorty et al. (2016). Teoh and colleagues (2004) reported the presence of *Rhodotorula mucilaginosa* in Kombucha from South Wales. Tea is a popular beverage consumed around the world and it is known to contain phytochemicals such as flavonoids, catechins, saponins, amino acids, aromatic compounds (Dufrense and Farnsworth, 2001), epigallocatechin and polyphenols (Hakim et al., 2003; Jayabalan et al., 2007). In this study, the phytochemical analysis of Kombucha tea revealed presence of saponins, flavonoids, alkaloids and terpenoids. Flavonoids have been shown to reduce the risk of cardiovascular disease and lower blood pressure (Erdman et al., 2008). Furthermore, the high antioxidant activity usually recorded for Kombucha is due to the presence of polyphenols in the tea (Jayabalan et al., 2014). The antimicrobial activity of Kombucha may be partly due to the presence of these phytochemicals in the tea such as flavonoids which are known to exhibit pharmacological activity (Bhattacharya et al., 2016). Differences in phytochemical constituents have been observed among Kombucha from several sources which might be due to differences in preparation and fermentation process of the beverage (Blanc, 1996).

Antioxidants are popularly known to be substances that scavenge free radicals and reactive molecules that cause multiple chain reactions leading to cell damage and cell death (Sheibs et al., 2013). Kombucha tea has been reported to demonstrate antioxidant properties due to the presence of phytonutrients such as polyphenols and flavonoids in the beverage (Chan et al., 2011; Bancirova, 2010). An indication of the potential efficacy of Kombucha as an antioxidant is demonstrated by its free radical scavenging abilities. In this study, Kombucha and normal tea exhibited an EC$_{50}$ of 0.77 mg/ml and 2.27 mg/ml, respectively as compared to BHT with 0.06 mg/ml. Thus, Kombucha is 3 times more potent than unfermented tea and 12-fold less potent than BHT. The total phenolic content of Kombucha and unfermented tea at 2.5 mg/ml
was 1330 and 638 mg/ml; whereas that at 5 mg/ml was 1771 and 1047, respectively, thus, making Kombucha tea about 2-fold more potent in terms of phenolic content as compared to unfermented tea. It was noticed that the phenolic content of Kombucha tea increased with increasing concentration as seen with most antioxidant studies on Kombucha. Similar work reported by Velicanski et al. (2014) for a traditional Kombucha after seven days of fermentation gave an antioxidant ability of approximately 90 µl/ml and a total phenolic content of 575 µg/ml. Furthermore, Chakravorty et al. (2016) also reported a comparable antioxidant capacity of Kombucha with an IC₅₀ of 0.95 mg/ml for 100 mg/ml of Kombucha tea. These researchers also suggested that the observation of high antioxidant activity might be due to the presence of the yeast Lachancea fermentati. It is therefore not surprising that high antioxidant activity was obtained for Kombucha tea in this study which also contained Lachancea fermentati. Biotransformation of complex phenolic compounds to small molecules is suggested to be mediated by some enzymes within the SCOBY (Symbiotic Community of Bacteria and Yeasts) used in fermenting the tea. This might account for the general increase in the total phenols recorded in Kombucha beverage (Srihari and Satyanarayana, 2012). Also, the diverse microbiota, variation in culture duration and origin of starter of Kombucha might also lead to the observed differences in the radical scavenging abilities of Kombucha from different geographical sources (Chu and Chen, 2006).

In support of the antimicrobial activity of Kombucha, the tea showed dose-dependent bactericidal effect on S. aureus. Kombucha at 100 mg/ml considerably inhibited the bacteria growth as compared to the treatment with the standard antibiotics (amikacin) (table 3 and Fig 16). This suggests that the tea possess compounds which are toxic to bacteria. Antimicrobial efficacy of Kombucha against some pathogenic organisms has previously been shown (Greenwalt et al., 1998). Indeed, work in our laboratories demonstrated that Kombucha tea possess growth inhibitory activity against S. aureus and Methicillin Resistant S. aureus (Ofori,
This antimicrobial activity has been attributed to the presence of organic acids, proteins and catechins in the tea. Acetic acid has been known to inhibit the growth of many Gram negative and Gram positive bacteria (Sreeramulu et al., 2000) and also some Mycobacterium spp (Cortesia et al., 2014). In addition, the isolation of B. bruxellensis from Kombucha which is known to be producers of acetic acid and acetic acid esters further support the tea antibacterial activity (Gancedo and Senano, 1989; Teoh et al., 2004).

Kombucha tea did not show antmycobacterial effects towards M. ulcerans, after co-incubation, as acid fast bacilli were seen with no morphological changes (Fig 18), and bacterial growth was observed on LJ slants after three weeks of culture (Fig 19). Possibly, it could be that Kombucha tea was ineffective against M. ulcerans because of the thick cell wall of the pathogen which is mainly composed of mycolic acids. On the other hand, most of the active components of Kombucha tea are polar compounds (hydrophilic), thus limiting permeation into the bacteria. Research by Cortesia et al. (2014) reported the efficacy of 6% acetic acid in killing of M. tuberculosis after 30 minutes of exposure. Therefore, since the acetic acid components of Kombucha is known to partly account for the tea antimicrobial property, these acids could be purified and further tested on M. ulcerans to investigate whether they might possess any antmycobacterial activity.

Data on the investigation into the ability of Kombucha to chemically modify mycolactone suggested that the different concentrations of Kombucha could not inactivate the toxin even after 48 hours of incubation (Fig 20). To further confirm this observation, an in vitro assay on human skin fibroblast cells was conducted. Our data showed that cells treated with only Kombucha displayed similar growth and morphological characteristics to that of the control (untreated human skin fibroblast cells) (Fig 21 and 22) indicating that the tea only is not cytotoxic to the cell line used. Furthermore, the cells treated with the Kombucha-mycolactone mixtures were rounded in morphology and also detached from the tissue culture flask after 24
hours of incubation (Fig 24). The same effects were observed with the skin cells treated with only mycolactone (Fig 23). Pronounced effects were observed and cell death was recorded after 48 hours of incubation. Our data agrees with the report by George et al. (1999 and 2000) that mycolactone caused cytopathic effects on mouse fibroblast cell line after 24 hours of incubation. Thus, suggesting that Kombucha tea was did not modify mycolactone as we hypothesized.

5.2. CONCLUSION

The microbial composition of Kombucha tea comprises diverse bacteria and yeasts. The outcome of this study suggests that Kombucha possesses valuable phytochemicals that are released into the tea which probably lead to the considerable phenolic content and antioxidant activity. Kombucha tea was not cytotoxic on the human skin fibroblast cell lines. However, it exerted antimicrobial effect on S. aureus but not on M. ulcerans. Kombucha also possessed no antimycolactone activity. Further work needs to be carried out to determine the roles of the various microorganisms to the characteristics associated with Kombucha tea. Since Kombucha tea is known to have wound healing ability, animal studies should be conducted to investigate the in vivo effect on Buruli ulcer lesions.

5.3. RECOMMENDATIONS

The contributions made by the various isolated bacteria and yeasts in the tea should be investigated. Kombucha is famous for its antimicrobial activity due to acetic acid. The organic acid components could be isolated and further tested on M. ulcerans to investigate whether they might possess any antimycobacterial activity. Kombucha should be combined with the known antimycobacterial drugs to observe whether the tea confers synergistic or additive effects. Animal models should also be used to test for the antimycobacterial efficacy of Kombucha.
5.4. REFERENCES


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4.5. APPENDIX

4.5.1. Concentrations of Kombucha used for the antimycolactone and antimicrobial assays

Table 1A: Concentrations of Kombucha used in the treatment of mycolactone

<table>
<thead>
<tr>
<th>Vol. of Mycolactone (µl)</th>
<th>Vol. of Kombucha (µl)</th>
<th>Initial concentration of Kombucha (mg/ml)</th>
<th>Final concentration of Kombucha (mg/ml)</th>
<th>Vol. of Kombucha + mycolactone (µl)</th>
<th>Top up volume H2O (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>33.33</td>
<td>150</td>
<td>100</td>
<td>45.83</td>
<td>4.17</td>
</tr>
<tr>
<td>12.5</td>
<td>20.83</td>
<td>120</td>
<td>50</td>
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<td>16.67</td>
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<td>12.5</td>
<td>4.167</td>
<td>120</td>
<td>10</td>
<td>16.67</td>
<td>33.33</td>
</tr>
<tr>
<td>12.5</td>
<td>0.417</td>
<td>120</td>
<td>1</td>
<td>12.92</td>
<td>37.08</td>
</tr>
</tbody>
</table>

The total volume of mixture was 50 µl

Table 2A: Concentrations of Kombucha used to incubate with *M. ulcerans*

<table>
<thead>
<tr>
<th>Vol. of <em>M. ulcerans</em> (ml)</th>
<th>Vol. of Kombucha (ml)</th>
<th>Initial concentration of Kombucha (mg/ml)</th>
<th>Final concentration of Kombucha (mg/ml)</th>
<th>Vol. of Kombucha + mycolactone (ml)</th>
<th>Top up volume M7H9 broth (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.67</td>
<td>150</td>
<td>100</td>
<td>0.77</td>
<td>0.23</td>
</tr>
<tr>
<td>0.1</td>
<td>0.42</td>
<td>120</td>
<td>50</td>
<td>0.52</td>
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<tr>
<td>0.1</td>
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<td>120</td>
<td>10</td>
<td>0.18</td>
<td>0.82</td>
</tr>
<tr>
<td>0.1</td>
<td>0.01</td>
<td>120</td>
<td>1</td>
<td>0.11</td>
<td>0.89</td>
</tr>
</tbody>
</table>

The total volume of mixtures was 1 ml
Table 3A: Concentrations of Kombucha used to incubate with S. aureus

<table>
<thead>
<tr>
<th>Vol. of S. aureus (ml)</th>
<th>Vol. of Kombucha (ml)</th>
<th>Initial concentration of Kombucha (mg/ml)</th>
<th>Final concentration of Kombucha (mg/ml)</th>
<th>Vol. of Kombucha + mycolactone (ml)</th>
<th>Top up volume of H₂O (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.833</td>
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<td>100</td>
<td>0.933</td>
<td>0.167</td>
</tr>
<tr>
<td>0.1</td>
<td>0.667</td>
<td>120</td>
<td>80</td>
<td>0.767</td>
<td>0.333</td>
</tr>
<tr>
<td>0.1</td>
<td>0.500</td>
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<td>60</td>
<td>0.600</td>
<td>0.400</td>
</tr>
<tr>
<td>0.1</td>
<td>0.333</td>
<td>120</td>
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<td>0.433</td>
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<tr>
<td>0.1</td>
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<td>0.267</td>
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</tr>
<tr>
<td>0.1</td>
<td>0.083</td>
<td>120</td>
<td>10</td>
<td>0.183</td>
<td>0.817</td>
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<tr>
<td>0.1</td>
<td>0.042</td>
<td>120</td>
<td>5</td>
<td>0.142</td>
<td>0.858</td>
</tr>
</tbody>
</table>

The total volume of mixtures was 1 ml

4.6. Standard calibration curve used to determine the phenolic content of Kombucha

![Graph showing the standard calibration curve for Gallic acid.](image)

\[ y = 0.3076x + 0.0573 \]

\[ R^2 = 0.9995 \]

Figure 1A: Standard calibration curve for Gallic acid

The equation of the line of best fit was used for calculating the phenolic content of Kombucha.
4.7. Solutions preparation

4.7.1. LB Agar

Approximately, 10g of Luria Bertani (LB) broth was dissolved in 500 ml of distilled water together with 7.5g of agar. The solution was autoclaved at 121 °C for 15 minutes, cooled to about 45 °C before the plates were poured and kept in the fridge until ready to use.

4.7.2. PBS Buffer

Respective masses of NaCl (40g), KCl (1g), Na₂PO₄ (7.2g) and KH₂PO₄ (1.2g) were individually weighed into a beaker and dissolved in 450 ml of distilled water. The pH was adjusted to 7.5 and the water was topped to the 500 ml marked and autoclaved.

4.7.3. Composition of the DNA extraction reagent

<table>
<thead>
<tr>
<th>Lysis buffer</th>
<th>1M Tris pH 8.0</th>
<th>0.5M EDTA</th>
<th>0.5M NaCl</th>
<th>SDS</th>
<th>Distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium acetate</td>
<td>Guanidine Hydrochloride</td>
<td>Potassium acetate</td>
<td>Guanidine Hydrochloride</td>
<td>distilled water</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Distilled water</td>
<td>Elution buffer</td>
<td>1M Tris pH 8</td>
<td>Distilled water</td>
<td>5.0M NaCl</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Wash solution</td>
<td></td>
<td></td>
<td></td>
<td>Distilled water</td>
<td></td>
</tr>
</tbody>
</table>
4.8. PCR reagents and solutions used for gel electrophoresis analysis

Table 4A: PCR reaction mix composition for Kombucha bacteria and yeasts isolates

<table>
<thead>
<tr>
<th>Bacterial isolates reaction mix</th>
<th>Yeast isolates reaction mix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent</strong></td>
<td><strong>1X</strong></td>
</tr>
<tr>
<td>PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>10µM dNTPs</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5µM Forward primer</td>
<td>0.5</td>
</tr>
<tr>
<td>2.5µM Reverse primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>15.87</td>
</tr>
<tr>
<td>5U/µl Polymerase</td>
<td>0.13</td>
</tr>
<tr>
<td>DNA Template</td>
<td>2.5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

4.8.2. 1X TAE buffer

To prepare the working solution of 1X TAE buffer, 20 ml of the 50X TAE buffer (Thermo Scientific) was measured and diluted with distilled water to a total volume of 1 L. The pH of this solution was 8.0
4.8.3. Agarose Gel (1%)

To prepare 1% agarose solution, 1 g of agarose was weighed and dissolved in 100 ml 1X TAE buffer.

4.8.4. Ethidium bromide solution (10 mg/mL)

To make 10 mg/mL ethidium bromide solution, 0.1 g ethidium bromide tablet was weighed and dissolved in 10 ml 1X TAE buffer

4.9. Solutions used for the phytochemicals and antioxidant analysis

4.9.1. 5% FeCl\textsubscript{3}

About 5 g of FeCl\textsubscript{3} was dissolved in 100 ml of distilled water in a Falcon tube, covered in with an aluminium foil and stored in the dark room until ready to use.

4.9.2. Wagner reagent

About 2 g I\textsubscript{2} and 6 g KI were dissolved in 100 ml of distilled water.

4.9.3. Preparation of 0.5 mM DPPH solution

Approximately, 1.3 mg of DPPH was dissolved in 7 ml of absolute ethanol to prepare a concentration of 0.5 mM.

4.9.4. Preparation of BHT solution

About 5 mg of BHT was dissolved in 1 ml of absolute ethanol.

4.9.5. Preparation of 5 mg/ml solution of Gallic acid

A 5 mg powder of Gallic acid was weighed into ethanol.
4.9.6. Preparation of 30 ug/ml of amikacin

About 6 µl of 5 mg/ml of stock amikacin was diluted in 994 µl of distilled water to make a working concentration of 30 µg/ml. The resulting solution was mixed thoroughly before sterilized by filtration.

4.10. M. ulcerans culture media preparation

4.10.1. M7H9 broth

About 2.6 g of Middlebrook7H9 powder was weighed and dissolved in 500 ml of distilled water containing 1.11 ml of glycerol. The mixture was stirred, autoclaved and kept at 4°C until ready for use

4.10.2. M7H10 agar

Approximately, 10.56 g of Middle brook powder was dissolved in 500ml of distilled water containing 2.78 ml of glycerol. The resulting solution was autoclaved, plates were poured and kept at 4 °C until ready for use

4.10.3. LJ slants

Lowenstein Jensen powder of mass 2.78 g was weighed and dissolved in distilled water containing 1 ml of glycerol. The solution was autoclaved after which 1 ml of malachite green was added. About 74.55 ml of homogenized eggs was added and 5 ml of the resulting solution was dispensed in tubes and inspissated for 55 minutes.
4.11. Source of reagents and materials used

1. 5X PCR buffer (1nM MgCl$_2$), dNTPs (Inqaba biotech, Pretonia, South Africa)
2. 24-well cell culture plates (Nunc Tm New York, USA)
3. 100 bp DNA ladder (Promega, Madison WI, USA)
4. Agarose (AGTC Bioproducts Ltd, UK)
5. Benchtop 2UV Transilluminator gel photography system (Upland, CA, USA)
6. Camera (Sony, Tokyo, Japan)
7. Centrifuge (Eppendorf, Hamburg, Germany)
8. Eppendorf Mastercycler personal PCR Machine (Eppendorf, Hamburg, Germany)
9. Ethidium bromide (Promega, Madison WI, USA)
10. Glass beads (Sigma-Aldrich, Darmstadt, Germany)
11. Glass slide (frosted slide glass, Thermo Scientific, Waltham, U.S.A.)
13. Incubator (Fisher scientific, Pittsburgh, USA)
14. Inverted microscope (ZEISS Promover, Jena, Germany)
15. Light microscope (Leica, Wetzlar, Germany)
16. Luria Bertani (LB) agar (Oxoid, Hampshire, United Kingdom)
17. Nuclease free water (Hyclone Lab Inc., South Logan, Utah, USA)
18. Primers, 1U GoTaq Polymerase (Thermo Scientific, Waltham, U.S.A)
19. Thermal Cycler (Applied Biosystems,California, U.S.A)
20. Vortexer (Mini vortexer, VMR Scientific Products, Atlanta, U.S.A)
21. Ziehl Neelson kit (Thongsbridge, UK)