Evaluation of the antimycobacterial and antimycolactone efficaciousness of kombucha

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Abstract. Abass A, Mosi L, Gbewonyo WSK. 2018. Evaluation of antimycobacterial and antimycolactone efficaciousness of kombucha. Bioteknologi 15: 80-91. This study was intended at distinguishing the microbial substance of kombucha tea, appraising kombucha antimicrobial, antimycobacterial and mycolactone deactivating potential. The antioxidants, as well as the phytochemical constituents of the tea, were also examined. Kombucha tea was cultured, and microbial isolates were Gram stained, DNA extracted, followed by PCR with 16S rRNA and fungal ITS primers for recognition of bacteria and yeasts, successively. Resulting amplicons were sequenced and blasted against the PUBMED database for recognition based on sequence alikeness and homology. Phytochemical analysis was carried out on the kombucha tea and the total phenolic substances were ascertained. DPPH assay was utilized for the ascertainment of the antioxidant characteristic of the tea. Dissimilar concentrations of the tea were co-brooded with mycolactone for numerous time points to examine for poison weakening. The existence of undamaged or deactivated mycolactone was discovered utilizing TLC and cytotoxicity assays on cultured human fibroblast cell lines. Antimicrobial efficacy of elevated concentrations of the tea was tried out against S. aureus and M. ulcerans by pre-hatching before microscopy and culture to examine morphological alterations and survival successively. Yeasts in the kombucha tea were recognized as Dekkera bruxellensis, Brettanomyces bruxellensis, Rhodotorula mucilaginosa and Lachancea fermentati and the bacteria as Paenibacillus lactis, Paenibacillus cineris Bacillus licheniformis, Lactobacillus amylolyticus and Corynebacterium glutanic. Phytochemicals discovered in the tea were saponins, flavonoids, alkaloids, phenols and terpenoids. The antioxidant characteristic of kombucha was higher contrasted with the fresh tea and a semblant impact was perceived with the phenolic substance. Fresh was 2-fold less efficacious in total phenolic substance than kombucha tea. The tea had antimicrobial liveliness against S. aureus but not on M. ulcerans. Kombucha was not cytotoxic to the human skin fibroblast cells, however, mycolactone administered with kombucha tea preserved its efficacy against human fibroblast cell lines and sustainable bacilli of M. ulcerans were perceived after kombucha medication. Kombucha tea has antioxidant and antimicrobial livelinesses but lacks antimycobacterial and antimycolactone liveliness.

Keywords: Antimycobacterial, antimycolactone, kombucha

INTRODUCTION

Mycobacterium ulcerans (M. ulcerans) is the etiological agent for the ailment of Buruli ulcer (BU). It is the second most significant mycobacteria contagion after tuberculosis in Ghana (Amofah et al. 2002). The ailment is identified by massive subcutaneous tissue breakdown, mainly in the terminuses with periodic bone entanglement (Debacker, M. ulcerans releases a macrolide poison called mycolactone which has been evidenced to be individually liable for the pathogenesis of Buruli ulcer (George et al. 1999). Mycolactone has been shown to be immunosuppressive and immunomodulatory both in vivo and in vitro (Adusumulli et al. 2005). The unavailability of pain has participated in the lack of epidemiological data on the hatching period and the early phases of the ailment, as they are often disregarded. Thus, most affected people inform the health centers only after growth of a large tenacious ulcer. Medication of Buruli ulcer contagions tries to decrease the extent of ulcers and heal existing tissue breakdown to affected areas. In the early and intermediate phases of the ailment, antibiotic drugs comprising rifampicin and streptomycin or amikacin are highly efficacious (Sarfo et al. 2010a). However, people with large tenacious ulcer experience surgical excision of the ulcer followed by several weeks of antibiotics medication (Chauty et al. 2007).

The utilization 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 denounced to exert a number of medicinal effects comprising: antimicrobial, anti-inflammatory and antioxidant livelinesses and is reportedly utilized for wound mending (Gbewonyo 2014). There was a considerable reduction in CNS infiltration of inflammatory cells and experimental autoimmune encephalomyelitis (EAE) in kombucha-administered mice as compared to the control (untreated), signifying a substantial anti-inflammatory characteristic of the tea (Marzbian et al. 2015). However, there is not much scientific evidence for the efficaciousness of kombucha and its bioactive components. The tea is denounced 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 medication. It is, therefore, necessary to recognize adjunct medication 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 identified the microbial substance of kombucha and assessed the tea’s mycobactericidal potential as well as its potential to alter mycolactone liveliness. The antioxidant liveliness and phytochemical constituents of kombucha tea were also investigated.

The objectives of this research were (i) To identify the bacteria and yeasts strains present in kombucha. (ii) To ascertain the phytochemical content of kombucha tea. (iii) To evaluate the antioxidant liveliness of the tea. (iii) To extract mycolactone from M. ulcerans and test kombucha compounds for efficacy against mycolactone for inactivation or poison modification. (iv) To ascertain antimycobacterial liveliness of the kombucha tea.

MATERIALS AND METHODS

Characterization of kombucha microorganisms

Preparation of kombucha tea
Kombucha tea was set up as explained by Gbewonyo (2014). A four and half liter solution of sucrose (approx. 38.5g/l) was set up utilizing sifted boiled water. One Lipton tea bag/L (i.e. 5 tea bags for 5 L kombucha) was infused for 2 minutes. The sugared tea was then poured into a container and left to cool to room temperature and then seeded with 500 µL (0.5 L) of a previous ferment containing a ‘baby mat’ (compiled of symbiotic community of bacteria and yeasts (SCOBY)). The container was then suffused with linen and left to ferment for 14 days. Tea without SCOBY served as control. The extracts were sifted, frozen, freeze-desiccated and stored at 4 °C for subsequent assays.

Culturing of kombucha isolates
Five milliliters of the kombucha broth and residue were centrifuged at 845 g utilizing a microcentrifuge for 10 minutes. The obtained pellets were serially liquefied (1:10, 1:100 and 1:1000) and inoculated on LB agar plates before hatching 37°C for a period of 48 hours. Weighted section of the mat was also homogenized in 5 mL PBS and administered as explained above. Unique colonies were sub-cultured again for pure isolates of the microorganisms.

Microscopy-Gram staining
Smears of the colonies established on the LB plates were produced on glass slides. The slides were left to air desiccate, then passed through flame to heat fix. They were then suffused with two drops of crystal violet for 60 seconds and rinsed with water. Two drops of Lugol’s iodine were supplemented for 60 seconds and rinsed with water afterward. Decolourizer (alcohol) was then supplemented and rinsed immediately. Finally, safranin was utilized to flood the slides for 2 minutes followed by rinsing and air desiccating of the slides. The slides were then observed under oil immersion (100X) utilizing light microscope (Leica, Wetzlar, Germany).

DNA extraction
DNA was extracted from the kombucha isolates utilizing the procedure of Guanidine Hydrochloride. The kombucha isolates were adjourned in PBS solution before the start of the extraction. About 450 µL of lysis buffer was supplemented along with an eyeballed amount of glass beads (150-212 microns) and bead beat for 15 minutes. It was then brooded for 20 minutes in a water bath at 65 °C then centrifuged at 5600 g for 2 minutes. Potassium acetate was supplemented to 0.5 mL Eppendorf tube containing 400 µL of the supernatant. The blend was stored 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 blend was poured into a spin filter and centrifuged for 2 minutes at 5600 g. Flow through was discarded. Rinse solution was then supplemented, spun and the flow through discarded. Ethanol was supplemented, spun and flow through discarded. The spin filter was spun again and then poured into a fresh tube. Finally, 100 µL of the elution buffer was supplemented, brooded for 10 minutes and spun at the same speed and time to elute the DNA.

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 compiled of (5 µL of 10X PCR buffer, 0.5 µL of 10 mM dNTP, 0.5 µL of 10 µM forward primer (51 AGGAGGTGATCACAACCCGA 31) and 0.5 µL of 10 µM reverse primers (51 AACCTGGAAGGTTGGGAT31), 0.13 µL of Taq polymerase and 15.87 µL of PCR water. About 2.5 µL of each DNA template was supplemented to the master mix to make a total reaction volume of 25 µL. The amplification was performed utilizing a thermocycler (Applied Biosystems, California, U.S.A). An overall reaction of 35 cycles was performed with the first phase 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 compiled 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 (51 TCCTCGCTTATTGATATGC 31) and ITS 5 (51 GGAAGTAAAAGTCTGGTAACAGG 31) primers and 9 µL of PCR water. About 2.5 µL of the DNA template was supplemented to the master mix to make a reaction volume of 25 µL. The amplification was performed utilizing a thermocycler. The PCR thermal profile protocol utilized 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 utilizing a digital camera.

DNA sequencing and nucleotide blast analysis in the Genbank
The PCR amplicons were shipped to Cambridge University to be sequenced utilizing the Sanger procedure. The obtained nucleotide sequences 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 picked as the recognized species or genus of the isolates.

Antioxidant liveliness
Phytochemical analysis was performed to ascertain the existence of saponins, tannins, terpenoids, flavonoids, alkaloids, and phenols in the tea utilizing standard methods (Jack and Okorosay-Orubite 2009; Waterman 1993). Concentration of kombucha utilized was 100 mg/mL.

Saponins
About 3 ml of purified water and 3 ml of kombucha was vigorously shaken together for 2 minutes. The existence of a foam layer shows the existence of saponins.

Flavonoids
Approximately 3 ml of 1 M NH3 was supplemented to 3 ml of kombucha and left to stand at room temperature for 2 minutes. After the stipulated time, 2 ml of concentrated H2SO4 was supplemented to the blend and the examination of an intense yellow precipitate showed the existence of flavonoids.

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 supplemented. After complete dissolution, 3 drops of Wagner reagent were supplemented and the existence of reddish flocculation designates the existence of alkaloids.

Phenols
About 1 ml of kombucha was blended with 1 ml of 5% FeCl3 solution and the existence of phenols was shown by the formation of deep blue/ back color in the tube.

Terpenoids
A 3 ml volume of kombucha was blended to 1 ml of chloroform after which 3 drops of concentrated sulphuric acid was supplemented. Formation of a reddish-brown precipitate at the interphase shown the existence of terpenoids.

Tannins
About 2 ml of 5% FeCl3 was supplemented to 2 ml of kombucha after which the examination of a green precipitate signals the existence of tannins.

DPPH analysis
The free radical scavenging liveliness of kombucha was evaluated as explained (Anim et al. 2016). Approximately, 100 µL of each of the dissimilar concentrations of kombucha (2-fold serial dilutions from 40 mg/mL to 0.625 mg/mL) was supplemented to 100 µL of 0.5 mM of DPPH dissolved in methanol. The blends were brooded for 20 minutes in the dark at room temperature. The absorbance of the generating solution was read at 517 nm. A freshly BHT solution (5 mg/mL) and fresh tea were utilized as standard and control, successively. To calculate the percentage DPPH liveliness:

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

Total phenolic substance
The total phenolic substance of kombucha was appraised as explained (Anim et al. 2016). About 10 µL of 5 mg/mL of gallic acid was supplemented to 0.79 ml of purified water in an Eppendorf tube. Folin Ciocalteau reagent (50 µL) was supplemented to each of the dissimilar concentrations of the kombucha and blended thoroughly. The solutions were then brooded in the dark for 8 minutes after which 150 µL of Na2CO3 was supplemented and further brooded 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) was utilized to calculate the total phenolic substance of kombucha and fresh tea and was expressed as gram/100 g GAE (gallic acid equivalent).

Determination of antimicrobial liveliness of kombucha tea
An overnight culture of S. aureus at log phase in LB broth was serially liquefied ten folds in purified water and the optical density (OD) at 600nm was taken. Freeze desiccated kombucha of concentration 120 mg/mL was freshly set up and serially inoculated into 100 µL of S. aureus broth culture containing 2.03x10⁸ cells/mL to make 7 dissimilar 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-administered S. aureus was brooded for 24 hours after which their OD was recorded. About 50 µL of each administered culture was then sub-cultured on Mueller Hinton agar media to ascertain the existence of sustainable S. aureus post medication. The assay was carried out in triplicates.

Evaluation of antitycobacterial liveliness of the kombucha tea
Middlebrooks H9, H10, and Lowenstein Jensen media were utilized to culture M. ulcerans (gotten from Dr. Heather Williamson of the Tennessee University). Six weeks old M. ulcerans culture was serially liquefied to 1:10 in purified water and the OD600 nm was taken. The bacteria...
were inoculated with the dissimilar concentrations (100 mg/mL, 50 mg/mL, 20 mg/mL, 10 mg/mL and 5 mg/mL) of kombucha for a period of seven days. The kombucha - M. ulcerans administered samples were acid-fast stained and cultured on LJ slants to examine the existence of sustainable mycobacteria post medication. The assay was carried out in triplicates utilizing amikacin as control.

Acid fast stain
Smears of the blends of kombucha and M. ulcerans were produced on glass slides and left to air dry, then passed through a flame to heat fix. The slides were then suffused with carbol fuchsin reagent for 5 minutes then rinsed with water. Slides were tilted to dry before the supplement of the decolorizer for 5 minutes. Each slide was rinsed with purified water after which it was inundated with methylene blue for 2 minutes. The slides were then rinsed with water, air-desiccated and observed under oil immersion (100X) utilizing a light microscope (Leica, Wetzlar, Germany).

Determination of impact of kombucha on mycolactone inactivation or modification.
Mycolactone A/B utilized 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 brooded 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 blends were brooded for 24 and 48 hours successively. Approximately, 50 µL of the administered 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 desiccated and observed under UV light of 254 nm.

Cytotoxicity assay
Human skin fibroblast culture
The human skin fibroblast cells utilized in this experiment were gotten 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.

Effects of kombucha tea administered mycolactone on human skin cells
About 100 µL of the skin fibroblasts in RPMI were seeded at a concentration of 1x10^4 cells/well in 96 well plate. The cells were brooded with the mycolactone administered with the dissimilar 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, successively. Human skin fibroblast cells administered with only mycolactone and kombucha served as controls for this experiment. After hatching, the cells were observed under inverted microscope (Carl Zeiss, Gottingen, Germany) for morphological alterations.

RESULTS AND DISCUSSION
Characterization of kombucha microorganisms
Isolation of microorganisms in kombucha
The numerous portions of kombucha tea were cultured and the microbial isolates were recognized and further identified. Serial dilution of the numerous portion of kombucha tea showed that, 1:1000 dilutions had distinct and fewer isolates as compared to 1:100 and 1:10 successively. 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 perceived 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 1 shows the bacterial and yeasts isolates that were present in the kombucha tea.

Figure 1. Plates showing sub-cultures of the unique colonies from the kombucha tea. A: Gotten Isolates on the mat culture, B: Gotten Isolates from the broth culture, C: Isolates from the residue culture
**Microscopy**

The isolates were Gram stained to determine the morphology and the Gram reaction of the microorganisms gotten from the tea. Figures 2.A and C demonstrates Gram-positive rod shaped bacteria while Figure 2.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 numerous isolates gotten from the tea are shown by the arrows in Figure 2.

**DNA extraction of isolates from kombucha**

The DNA of the isolates was extracted utilizing the procedure of guanidine hydrochloride and then run on an agarose gel. Figure 3 shows a gel electropherogram of the extracted DNA of kombucha tea isolates representing successful DNA extraction by the existence of bands in all the sample wells.

**PCR and Gel electrophoresis**

Identification and further characterization of the segregated bacteria were performed on the DNA samples utilizing Polymerase Chain Reaction. For bacterial recognition, primers targeting the 16S ribosomal DNA gene whereas ITS 4 and 5 fungal primers (18S rDNA) were utilized to amplify the internal transcriber space of the fungal isolates. The gotten PCR amplicons were run on 1% agarose gel (Figure 4 and 5). 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, successively.
Microorganisms in kombucha tea

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

Biochemically active compounds in kombucha tea

Kombucha concentration of 100 mg/mL utilized for the phytochemical analysis and it showed the existence of saponins, flavonoids, alkaloids, phenolic compounds and terpenoids but absent of tannins in the tea (Table 2).

It can be seen from Figure 6 (A and B) that as the concentration escalates, the mean (%) antioxidant liveliness of BHT, Kombucha and fresh tea escalates rapidly and then peaked around antioxidant liveliness of around 80%.

The effective concentration (EC50) of kombucha and fresh tea were found to be 0.77 mg/mL and 2.27 mg/mL, successively as compared to 0.06 mg/mL gotten for the standard BHT.

Total phenolic substance of kombucha tea

The total phenolic substance (TPC) of kombucha and fresh tea were evaluated and expressed (mg/100 gGAE) utilizing the Gallic acid calibration curve. It was perceived that the total phenolic substance of both teas escalated with elevating concentration (Figure 7). Also, the TPC for kombucha and fresh tea at a concentration of 2.5 mg/mL was 1,330 and 638 mg/mL, successively; and at 5 mg/mL, kombucha recorded a TPC of 1,771 mg/mL whereas fresh tea had 1,047 mg/mL.

Table 1. Characteristics of representative bacteria and yeasts isolates in kombucha tea

<table>
<thead>
<tr>
<th>Sample</th>
<th>Portion of kombucha tea</th>
<th>Gram reaction</th>
<th>Shape</th>
<th>Putative organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Broth &amp;Residue</td>
<td>Positive</td>
<td>Rods</td>
<td>Paenibacillus cineris</td>
</tr>
<tr>
<td>F</td>
<td>Residue &amp; Broth</td>
<td>Negative</td>
<td>Long rods</td>
<td>Paenibacillus lactis</td>
</tr>
<tr>
<td>I</td>
<td>Mat</td>
<td>Positive</td>
<td>Long rods</td>
<td>Bacillus licheniforms</td>
</tr>
<tr>
<td>K</td>
<td>Broth</td>
<td>Positive</td>
<td>Rods</td>
<td>Corynebacterium glutamic</td>
</tr>
<tr>
<td>L</td>
<td>Residue</td>
<td>Negative</td>
<td>Short fat rods</td>
<td>Lactobacillus amyloyticus</td>
</tr>
<tr>
<td>N</td>
<td>Kombucha tea</td>
<td>Variable</td>
<td>Rods and ovals</td>
<td>Uncultured Bacterial spp</td>
</tr>
<tr>
<td>Yeasts isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mat</td>
<td>Positive</td>
<td>Oval</td>
<td>Rhodotorula mucilaginosa</td>
</tr>
<tr>
<td>6</td>
<td>Broth &amp;Residue</td>
<td>Positive</td>
<td>Oval</td>
<td>Lachancea fermentati</td>
</tr>
<tr>
<td>11</td>
<td>Mat</td>
<td>Positive</td>
<td>Oval</td>
<td>Dekkera bruxellensis</td>
</tr>
<tr>
<td>13</td>
<td>Mat &amp; Broth</td>
<td>Positive</td>
<td>Oval</td>
<td>Brettanomyces bruxellensis</td>
</tr>
</tbody>
</table>

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>

Note: +: colour intensity; -: absent
Figure 7. Total phenolic substance of kombucha and fresh tea. Concentration 1: 2.5 mg/mL; Concentration 2: 5 mg/mL. Results are means± SEM of n=3 for both kombucha and fresh tea. The p-values between the two concentration of kombucha and tea are statistically considerable (p<0.0001).

Table 3. Colony forming units (CFU)/mL of S. aureus prior and post kombucha medication

<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>57</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 S. aureus</td>
<td>323</td>
<td>6.46 x10^6</td>
</tr>
</tbody>
</table>

Antioxidant liveliness of kombucha tea

The antioxidant characteristic of the kombucha beverage was ascertained by the DPPH procedure. The free radical scavenging ability of kombucha tea was measured together with BHT. BHT was utilized as the standard positive control and fresh tea as normal control. The plot of concentration against % antioxidant gave a curve from which the effective concentration (EC50) was counted (Figure 6).

Antimicrobial liveliness of kombucha tea

S. aureus (within the log phase) was brooded with the dissimilar 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. aSu.reus administered with kombucha concentration of 5 mg/mL had complete 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 accretion 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 medication. However, 100 mg/mL kombucha concentration significantly impeded the accretion of the bacteria with only 7.4 x10^5 CFU of the S. aureus remaining after kombucha medication as compared with the amikacin medication having 1.5 x10^4 CFU (Table 3; Figure 7).

As exhibited in Table 3, the CFU of S. aureus per ml alleviates with elevating kombucha concentration. Thus, higher concentrations of the tea were perceived to significantly impede the accretion of the bacteria better than the lower concentrations (Figure 8).
Antimycobacterial liveliness of kombucha tea

About $3 \times 10^5$ concentration of *M. ulcerans* was administered with dissimilar concentrations of kombucha and brooded for seven days after which the blends were acid fast stained to discover the existence of complete mycobacteria. The *M. ulcerans* were pink with complete rods representing the existence of acid fast bacilli whereas kombucha non-mycobacteria were perceived as blue rods (Figure 9). We did not examine any differences in the morphology of the *M. ulcerans* administered with the dissimilar concentrations of kombucha.

Detection of sustainable *M. ulcerans* after kombucha medication

We further cultured the kombucha administered *M. ulcerans* on LJ slants to discover sustainable bacterial cells. For all the dissimilar medication concentrations, we perceived accretion of *M. ulcerans* prompting that the tea had no bactericidal liveliness against the mycobacteria. The arrows in Figure 11 represents light yellow colonies on LJ slants which shows the existence of *M. ulcerans* after medication with kombucha.

Thin Layer Chromatography profile of mycolactone administered with kombucha tea

About 12.5 µL of mycolactone was co-brooded with the numerous concentrations of kombucha for 24 and 48 hours and the generating blend was run on a TLC plate to examine for mycolactone modification. We perceived a protruding band at the retention factor (rf) for mycolactone in all the administered fraction that was commensurable to the mycolactone only control (Figure 12). We also examine a protruding band closed to the solvent front in all the administered fraction which was commensurable to the kombucha only control indicative of unresolved constituents in the kombucha tea.
Cytotoxicity profile of mycolactone administered with kombucha tea

To further ensure our examination of the impotence of kombucha to inactivate mycolactone, we inoculated and cultured human skin fibroblasts with the kombucha administered mycolactone for 24 to 48 hours successively (Figure 16). The cells lines were administered with kombucha only, and as exhibited in Figure 14, kombucha only showed no cytotoxic characteristics on the cultured fibroblasts. However, medication of the cell with 10 μg of mycolactone disclosed the characteristic rounding and shrinkage of the fibroblast, consistent with the widely published cytotoxic effects of mycolactone on cultured cells (Figure 15). Furthermore, the cell lines were administered with a 24 and 48 hours pre-brooded blend of 10 μg mycolactone and 100 mg or 50 mg of kombucha successively to ascertain whether the kombucha had debilitated the mycolactone liveliness. After 24 hours of medication on the fibroblasts, it was perceived that the cells had rounded and removed from the plates as was seen in the mycolactone control medication (Figure 15).

Discussion

Kombucha is a sugared black tea (*Camellia sinensis*) fermented with symbiotic association of bacteria and yeasts (tea fungus). The tea is regarded as traditional medicinal potion with myriad health characteristics, among which include anti-cancer (Jayabalan et al. 2011), antidiabetic (Hiremath et al. 2002; Aloulou et al. 2012), immune booster (Ram et al. 2000), liver detoxification (Loncar et al. 2014), and many others. This study explored the effect of mycolactone on the fibroblast and evaluated the impact of kombucha as an inactivator of mycolactone.
2000) and the medication of high cholesterol (Yang et al. 2009). Also, the tea has been exhibited to exert antibactericidal liveliness, thus we sorted out to examine this characteristic on M. ulcerans. The objectives of this study were to identify the microbial diversity of kombucha as well as investigate its antioxidant liveliness and phytochemical constituents. We also evaluated the tea’s mycobactericidal potential and also its capability to alter and impede the liveliness of mycoflectone, a poison known to be the main virulence factor in Bu contagions. The recognized bacteria in the tea in our study utilizing the 16S rRNA target specific loci BLAST comprised: Corynebacterium glutamic, Bacillus licheniformis, Lactobacillus amylolyticus, (lactis and cineris) and unidentified bacterial species. The yeasts recognized were Dekkera bruxellensis, Lachancea fermentati, Brettanomyces bruxellensis and Rhodotorula mucilaginosa. Strains of Paenibacillus spp have been segregated from soil, water, and food. One unique characteristic is their capability to degrade proteins, polysaccharide and polyaromatic hydrocarbons (Daane et al. 2002). Some strains of Paenibacillus spp present antimicrobial characteristic by generating antibacterial compounds such as polymyxin M (Martin et al. 2003) and fusaricidin-type antifungal agent (Beatty and Jensen 2002). Lactobacillus amylolyticus have been segregated from beer malt and beer wort (Bohak et al. 1998) prompting they perform remarkable role in fermentation process. Marsh and his colleagues (2014) also denoted that 30% of the bacterial content of their kombucha samples appertains to the genus Lactobacillus. This same genus has been segregated from Chinese kombucha (Wu et al. 2004; Zhang et al. 2011). Thus, findings from this study which presents the existence of Lactobacillus in Ghanaian kombucha, espouses the denounced role of these bacteria in fermentation. The bacteria Corynebacterium glutamic has been employed in the generation of glutamic acid by submerged fermentation (Hermann 2003; Ahmed et al. 2013) while Bacillus licheniformis has been segregated from kombucha with very high acetic acid substance (Roussin 2007). Furthermore, all the bacterial isolates gotten from this study appertain to the Phylum Firmicutes and in accordance with Chakravorty et al. (2016), 11.2% of the kombucha bacteria are members of this phylum. Seto et al. (2006) have also denounced the existence of Firmicutes in kombucha from UK, Ireland and Canada. The diversity perceived in the microbial content in all kombucha worldwide is usually because of many factors such as, initial culture utilized, type of tea utilized, type of sugar utilized, brewing time, type of water utilized, culturing temperature and many more (Teoh et al. 2004). The present study focused on recognizing bacterial isolates present in the kombucha tea, thus, the reads gotten after the BLAST search could not recognize the types of bacteria that were not cultured. Marsh et al. (2014) have denounced that most Gluconacetobacter species have been tagged 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 utilized in this study.

The yeasts recognized in the present study have historically related to kombucha fermentation. D. bruxellensis have been termed the spoilage yeasts and are related with the fermentation of wine and beer and also increasing the flavour of lamic beer (DeKeersmaecker 1996). It is also the major and preferred ethanol generating microbe than S. cerevisiae in ethanol generating industries (Liberal et al. 2007; Passoth et al. 2007). Brettanomyces spp have 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 segregated from kombucha after seven days’ fermentation by Chakravorty et al. (2016). Teoh and colleagues (2004) denounced the existence of Rhodotorula mucilaginosa in kombucha from South Wales. Tea is a popular beverage consumed around the world and it is known to hold 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 disclosed existence of saponins, flavonoids, alkaloids, and terpenoids. Flavonoids have been exhibited to decrease the risk of cardiovascular ailment and lower blood pressure (Erdman et al. 2008). Furthermore, the high antioxidant liveliness usually recorded for kombucha is because of the existence of polyphenols in the tea (Jayabalan et al. 2014). The antimicrobial liveliness of kombucha may be partly because of the existence of these phytochemicals in the tea such as flavonoids which are familiar to show pharmacological liveliness (Bhattacharya et al. 2016). Dissimilarities in phytochemical constituents have been perceived among kombucha from several sources which might be because of dissimilarities in preparation and fermentation process of the beverage (Blanc 1996).

Antioxidants are popularly famous to be substances that scavenge free radicals and reactive molecules that lead to multiple chain reactions causing cell breakdown and cell death (Sheibs et al. 2013). Kombucha tea has been denounced to show antioxidant characteristics because of the existence of phytonutrients such as polyphenols and flavonoids in the beverage (Chan et al. 2011; Bancirova 2010). An indication of the potential efficaciousness of kombucha as an antioxidant is shown by its free radical scavenging capabilities. In this study, kombucha and normal tea showed an EC50 of 0.77 mg/mL and 2.27 mg/mL, successively as compared to BHT with 0.06 mg/mL. Thus, kombucha is 3 times more efficacious than fresh tea and 12-fold less efficacious than BHT. The total phenolic substance of kombucha and fresh tea at 2.5 mg/mL was 1330 and 638 mg/mL; whereas that at 5 mg/mL was 1771 and 1047, successively, thus, making kombucha tea about 2-fold more efficacious in terms of phenolic substance as compared to fresh tea. It was perceived that the phenolic substance of kombucha tea escalated with elevating concentration as seen with most antioxidant studies on kombucha. Similar work denounced by Velicianski et al. (2014) for a traditional kombucha after seven days of fermentation provided an antioxidant capability of approximately 90 µL/mL and a total phenolic
substance of 575 μg/mL. In addition, Chakravorty et al. (2016) also denounced a commensurable antioxidant capacity of kombucha with an IC_{50} of 0.95 mg/mL for 100 mg/mL of kombucha tea. These researchers also recommended that the examination of high antioxidant liveliness might be because of the existence of the yeast *Lachancea fermentati*. It is consequently not surprising that high antioxidant liveliness was gotten 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) utilized in fermenting the tea. This might account for the general rise 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 cause the perceived dissimilarities in the radical scavenging capabilities of kombucha from dissimilar geographical sources (Chu and Chen 2016). In support of the antimicrobial liveliness of kombucha, the tea exhibited dose-dependent bactericidal impact on *S. aureus*. kombucha at 100 mg/mL significantly impeded the bacteria accretion as compared to the medication with the standard antibiotics (amikacin) (Table 3; Figure 1). This recommends that the tea holds compounds which are poisonous to bacteria. Antimicrobial efficaciousness of kombucha against some pathogenic organisms has previously been exhibited (Greenwalt et al. 1998). Indeed, work in our laboratories demonstrated that kombucha tea holds accretion impediment liveliness against *S. aureus* and Methicillin-Resistant *S. aureus*. This antimicrobial liveliness has been attributed to the existence of organic acids, proteins and catechins in the tea. Acetic acid has been known to inhibit the accretion of many Gram-negative and Gram-positive bacteria (Sreeramulu et al. 2000) and also some *Mycobacterium spp* (Cortesia et al. 2014). In addition, the segregation of *B. bruxellensis* from kombucha which is famous to be generators of acetic acid and acetic acid esters further bolster the tea antibacterial liveliness (Gancedo and Senano 1989; Teoh et al. 2004).

Kombucha tea did not indicate antimycobacterial effects towards *M. ulcerans*, after co-hatching, as acid-fast bacilli were seen with no morphological alterations (Figure 10), and bacterial accretion was perceived on LJ slants after three weeks of culture (Figure 11). Possibly, it could be that kombucha tea was ineffective against *M. ulcerans* due to the bulky cell wall of the pathogen which is mainly compiled of mycolic acids. On the other hand, most of the active components of kombucha tea are polar compounds (hydrophilic), thus restricting permeation into the bacteria. Research by Cortesia et al. (2014) denounced the efficaciousness of 6% acetic acid in killing of *M. tuberculosis* after 30 minutes of revelation. Therefore, since the acetic acid components of kombucha are known to partly account for the tea antimicrobial characteristic, these acids could be distilled and further tested on *M. ulcerans* to examine whether they might hold any antimycobacterial liveliness.

Data on the observation about the capability of kombucha to chemically alter mycolactone recommended that the dissimilar concentrations of kombucha could not inactivate the poison even after 48 hours of hatching (Figure 12). To further ascertain this examination, an *in vitro* assay on human skin fibroblast cells was carried out. Our data indicated that cells administered with only kombucha showed resemblant accretion and morphological characteristics to that of the control (untreated human skin fibroblast cells) (Figure 13-14) representing that the tea only is not cytotoxic to the cell line utilized. Furthermore, the cells administered with the kombucha-mycolactone blends were rounded in morphology and also detached from the tissue culture flask after 24 hours of hatching (Figure 16). The same effects were perceived with the skin cells administered with only mycolactone (Figure 15). Pronounced effects were perceived and cell death was recorded after 48 hours of hatching. Our data agree with the report by George et al. (1999 and 2000) that mycolactone induced cytopathic effects on mouse fibroblast cell line after 24 hours of hatching. Thus, prompting that kombucha tea was did not alter mycolactone as we hypothesized.

**Conclusion**

The microbial content of kombucha tea consists of various bacteria and yeasts. The result of this study recommends that kombucha holds precious phytochemicals that are released into the tea which probably cause the considerable phenolic substance and antioxidant liveliness. Kombucha tea was not cytotoxic on the human skin fibroblast cell lines. However, it exerted antimicrobial impact on *S. aureus* but not on *M. ulcerans*. Kombucha also possessed no antmycolactone liveliness. Further work is necessary to be performed to ascertain the roles of the numerous microorganisms to the characteristics related to kombucha tea. Since kombucha tea is known to have wound mending capability, animal studies should be carried out to examine the *in vivo* impact on Buruli ulcer lesions.

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