

## Characteristics of Kombucha Fermentation from Different Substrates and Cytotoxicity of Tea Broth

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**Abstract.** Kombucha is made from sweetened tea fermented by a symbiotic culture of bacteria and yeast consumed worldwide because of its potentially beneficial effects on health. However, there are only few studies on the safety of kombucha consumption that will establish it as a functional beverage. The present study compared, pH, temperature and sugar content of different tea mixtures of black or green tea as nitrogen sources and white and brown sugar as carbon sources in a 30-day fermentation period. A marked decrease in pH was observed throughout fermentation with Green tea-White (GW) sugar mixture showing the lowest recorded pH value of 2.37 on the 14<sup>th</sup> day of fermentation. Temperature is essential in the fermentation process and thus maintained at ambient 29±1°C. Black and green teas with white sugar (7°Brix) showed to have higher sugar level compared to tea mixtures with brown sugar (5°Brix). Brine Shrimp Lethality Assay was carried out to determine cytotoxicity of kombucha. The four substrate combinations have very low LC<sub>50</sub> values with Black tea-Brown sugar (BB) mixture showing to have the lowest in both acute and chronic effects (0.073 ppm and 0.101 ppm, respectively). This indicates safety of kombucha for consumption.

### Introduction

Kombucha is a fermented beverage which is mildly sweet, slightly acidic and moderately bubbly. It is produced by the fermentation of tea and sugar by a symbiotic association of bacteria and yeasts forming a “tea fungus.” The tea fungus is embedded within a cellulosic pellicle with a common household term “scooby,” an acronym for “symbiotic colony of bacteria and yeast” that forms a floating mat in the tea and generates a new layer with each successful fermentation. Kombucha originated in China about 220 BC during the Tsin Dynasty for its detoxifying and energizing properties. In 414 A.D., the physician Kombu brought the tea fungus to Japan from Korea to cure the digestive troubles of the Emperor Inkyo [1]. It was introduced into Russia by oriental merchants and then into Eastern Europe and Europe around the turn of this century [2].

Actual microbial populations in cultures vary but the most abundant bacterial genera based on culture-based analyses of the kombucha microbiota are *Acetobacter* and *Gluconobacter*. These acetic acid bacteria are *Acetobacter xylinum* [3], *A. xylinoides*, *Bacterium gluconicum* [4], *A. aceti*, *A. pasteurianus* [5]. The yeast component generally includes *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii*, *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Brettanomyces bruxellensis*, *B. lambicus*, *B. custersii*, *Candida* and *Pichia* species [3, 5-6]. A culture-independent, high-throughput sequencing analysis of the bacterial and fungal populations of pellicles and the resultant fermented kombucha established that the major bacterial genus present was *Gluconacetobacter* with only trace populations of *Acetobacter* [7]. A prominent *Lactobacillus* population was also identified. The yeast populations were found to be dominated by *Zygosaccharomyces* in the fermented beverage. The symbiotic relationship between bacteria and fungi present in Kombucha inhibits the growth of potential contaminating bacteria [3, 5].

The microbiota in tea fungus requires four basic components for fermentation and respiration: sugar, water, oxygen and tea [8]. Sugar provides the carbon source and water is required for cellular

respiration. Although the yeasts in Kombucha are facultative anaerobes (producing ethanol in the absence of oxygen and oxidize sugar into carbon dioxide and water if oxygen is present), the acetic acid bacteria in Kombucha are strict aerobes and require oxygen. Tea provides necessary nitrogen sources (purine derivatives: caffeine and theophylline) for tea fungus culture [9].

Kombucha has been consumed as functional beverage for its various benefits for human health including stimulation of the immune system, aiding digestion, protection against cancer and cardiovascular diseases, prevention of microbial infections; it is also known for its hypoglycemic and antilipidemic properties and free-radical scavenging activities [10]. These effects have not been proven scientifically yet, but could be attributed to the presence of gluconic acid, glucuronic acid, vitamins, amino acids, micronutrients produced during fermentation [11].

Scoby has been passed from home to home to produce an inexpensive supply of kombucha and to make it readily available for consumption. Despite its recognized health benefits there is also some evidence of toxicity associated with it [1]. It is possibly from contamination during home preparation where cases of serious adverse effects related to kombucha drinking arise. Factors such as fermentation substrates, fermentation time and incorporation of other probiotics such as lactic acid bacteria change the chemical and microbial components and bioactivities during tea fungus fermentation [11-13]. These factors also influence the antioxidant property of kombucha [10, 14].

Despite the wide popularity of kombucha consumption for its therapeutic benefits, more studies on the safety aspects of kombucha are needed to qualify it as a functional beverage and establish its stand as a substitute for carbonated drinks [15]. The present study monitored the pH, temperature and sugar content of kombucha prepared from black tea and white sugar (BW), green tea and white sugar (GW), black tea and brown sugar (BB) and green tea and brown sugar (GB) in a 30-day fermentation time. Cytotoxicity of kombucha produced from these four fermentation substrates were determined by brine shrimp lethality assay to determine the LC<sub>50</sub>.

## Materials and Methods

**Tea Fungus Starter Culture.** Tea fungus, containing both the upper cellulosic pellicle (scoby) and the lower fermented tea broth, was from a local culture. Several batches of kombucha were harvested from this culture since activation of tea fungus was done at about every two weeks.

**Preparation of Sweetened Tea.** Sweetened tea broth for kombucha fermentation was prepared using black tea and green tea (Lipton Yellow Label and Lipton Clear Green, 2 grams/tea bag). Both were from dried leaves of *Camelia sinensis* L. Organic cane white and brown sugar were used as sweeteners. The following mixture combinations were made: black tea and white sugar (BW), green tea and white sugar (GW), black tea and brown sugar (BB) and green tea and brown sugar (GB). Distilled water (1.6 liters) was boiled together with three tea bags for a total of 20 minutes, stirring and pressing the bags every 5 minutes. After removal of the tea bags, 110 grams of sugar was dissolved in the hot concoction. The mixture was allowed to cool (below 32°C) and three 1 liter jars were dispensed each with 500 ml of the tea. The three jars served as replicates for each mixture combination.

**Preparation of Kombucha Culture.** Tea broth (118 ml) from a previously fermented culture was inoculated to the 1.6 liter sweetened tea. About 20 grams (wet weight) of cellulosic pellicle fragments (scoby), also from a previously fermented culture, was then inoculated to each 500 ml of the above mixture combinations. The jars were covered with clean cheesecloth fixed with the screw cover of the jars. The fermentation was carried out under ambient temperature (29±1°C) for 30 days.

**Physicochemical Analysis.** Analyses of pH, temperature and sugar content were measured for the tea fungus mother culture, before adding and after adding the mother culture to the sweetened tea. Subsequent readings of the kombucha were done every two days of fermentation until day 30.

Measurements of pH and temperature (Celcius) were done using a table top electronic pH meter (Sartorius PB 11) while the sugar content was analyzed using a hand-held refractometer (Brix). Values were expressed as means of three replicate readings  $\pm$  the standard deviation.

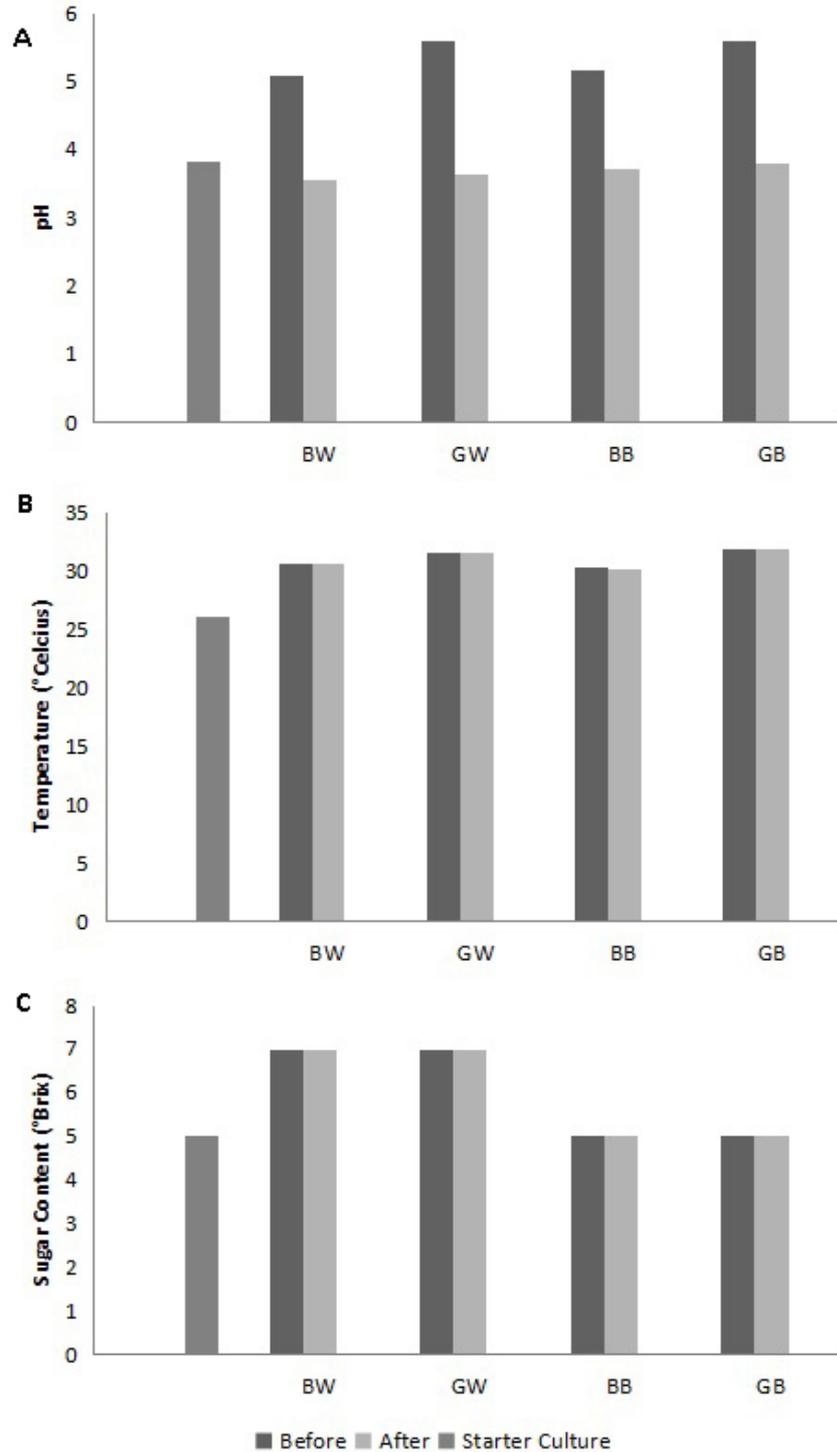
**Cytotoxicity Assay.** The brine shrimp lethality assay was carried out as describe by Guevarra et al. [16] with some modifications. Eggs of brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a shallow rectangular dish (12.3 in x 4 in x 7.6 in) half-filled with sterilized filtered sea water (FSW). The dish was divided into two compartments by a plastic divider punched with several 2 mm holes and under constant aeration for 48 hours. Brine shrimp eggs were then sprinkled into one of the compartment provided with a cover. After hatching, free swimming nauplii were collected from the lighted compartment of the hatching chamber and used for the assay. Ten nauplii were drawn using a glass capillary and placed in each vial containing 4.5 ml of sterilized filtered sea water. A volume of 0.5 ml of three concentrations (100 ppm, 10 ppm and 1 ppm) of Kombucha fermented from three different substrate combinations (BW, GW, BB and GB) were added to each vial to make a total volume of 5.0 ml. A set of three tubes per test dose and of the control (FSW) were prepared. Test and control vials were maintained at room temperature for 24 hours under the light and surviving nuplii were counted after 6 hours (acute) and after 24 hours (chronic) using a hand-held magnifying lens. Determination of LC<sub>50</sub> was based on the percent mortality using probit analysis method by Finney [17].

## Results and Discussion

**Fermentation Characteristics.** The changes in pH, temperature and sugar content before and after inoculation of the starter culture are presented in Fig. 1. The pH of the starter culture was 3.82 while all tea mixtures have pH of 5.0. It dropped to 3.56-3.80 immediately after inoculation with the starter culture. There was no observed increase in temperature before and after inoculation. The starter culture was slightly cooler at 26°C since it has stayed in room temperature for fermentation while all tea mixtures were freshly brewed. Sugar content of tea mixtures with white sugar were higher (7°Brix) than tea mixtures with brown sugar (5°Brix). There was no change in the sugar content of all tea mixtures before and after inoculation with starter culture which as a sugar content of 5°Brix.

The main constituents of green tea leaves belong to the polyphenol group accounting for 25±35% on a dry weight basis [3]. These compounds contribute to the bitterness, astringency and sweet aftertaste of tea beverages [2].

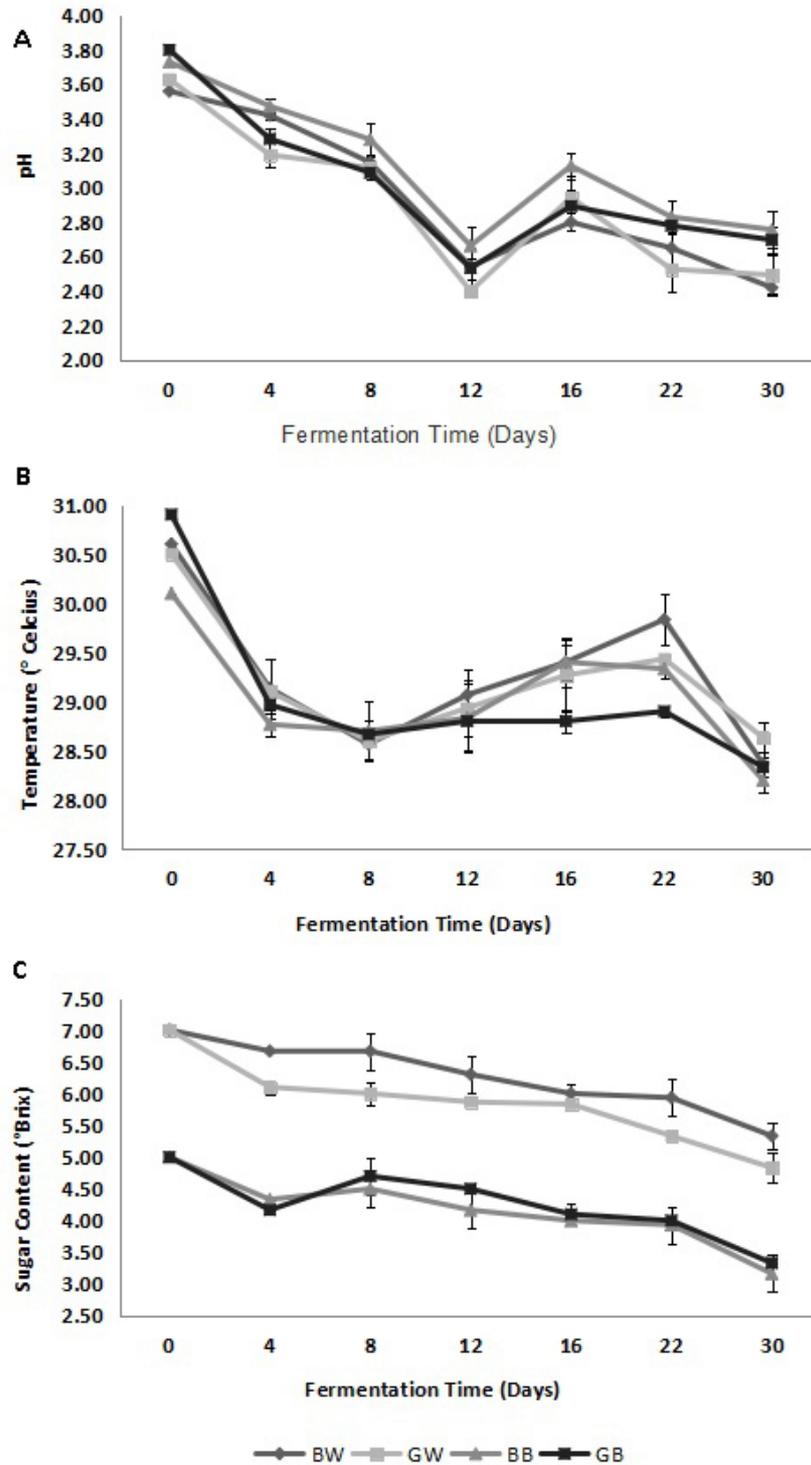
Tea contains also flavonols, mainly quercetin, kaempferol, myricetin, and their glycosides. In black tea, the oxidation of polyphenols during processing leads to the formation of catechins and gallic acid complexes [3]. Tea contains many amino acids, but theanine, specific to the tea plant, is the most abundant, accounting for 50% of the total amino acids [2]. Sucrose is the most common carbon source in kombucha fermentation. Its considerable amount stays largely unfermented during the process. Investigations showed that 34.06% of sucrose stays unfermented after 7 days, and after 21 days this value is 19.28% [1].



**Figure 1.** Physicochemical characteristics such as pH (A), temperature (B) and sugar content (C) of tea fungus mother culture, before adding mother culture to the sweetened tea and after inoculation of the starter culture to the sweetened tea.

Traditional substrate for the kombucha fermentation is black or green tea extract sweetened with 5% to 8% sucrose.

In the first two days of fermentation, there was a decrease in pH value with the highest observed difference of 0.48 units in Green tea-Brown sugar (GB) mixture (Fig. 2A). A slight increase was observed in Black tea-White sugar (BW) and Black tea-Brown sugar (BB) during day 4 of fermentation and another on day 12 to day 16 of fermentation. After this period, the pH value decreases consistently and reached 2.42- 2.76 on day 30. Green tea-White sugar mixture showed the lowest recorded pH value of 2.37 on day 14.



**Figure 2.** Changes in pH values (A), temperature (B) and sugar content (C) during 30-day Kombucha fermentation from different substrate combinations. Error bars are standard deviations around means of three replicates.

The temperatures of freshly brewed tea mixtures started at a range of 30.10°C-30.90°C in all tea mixtures and lowered down to the range of the ambient fermentation temperature (29±1°C) from day 2 to day 30 of fermentation (Fig. 2B).

Black tea-White sugar (BW) and Green tea-White sugar (GW) mixtures showed higher sugar content compared with Black tea-Brown sugar (BB) and Green tea-Brown sugar (GB) all throughout the 30-day fermentation time (Fig. 2C). The lowest sugar content recorded on day 30 was that of Black tea-Brown sugar (BB) mixture at 3.17 °Brix.

The yield and properties of cellulose produced in kombucha after fermentation for 14 days in the presence of different amounts of black tea and sucrose as nitrogen and carbon sources showed that 8.7g/L black tea and 100g/L sucrose produced highest weight of bacterial cellulose wherein its production increased with the increase of surface area and depth of the broth [18]. Temperature was essential factor on growth, where the pellicle was formed at range (20°C - 50°C) and higher temperature over 50°C depressed the bacterial cellulose formation.

Residual sucrose concentration decreased linearly with time thus the desired quality or composition of kombucha can be obtained through the proper control of fermentation time [19]. Acidity also increase steadily with increasing fermentation time due to the accumulation of acetic acid and gluconic acid [12].

Tea origin and pH partially determine the rate of product evolution in kombucha broth, and tea may inhibit the growth of the microbial consortia. Cell counts of the kombucha broth peaked at pH 3 on day 3 after inoculation, indicating that the acid concentration of the peak may provide a strong environmental filter for the microbes [20].

**Cytotoxicity Assay.** Determination of LC<sub>50</sub> was based on the percent mortality of brine shrimp after 6 hours (acute) and after 24 hours (chronic) using probit analysis method (Figs. 3 and 4). All tea mixtures showed low cytotoxicity against brine shrimp with LC<sub>50</sub> values ranging from 0.241-0.073 ppm after 6 hours (Table 1) and 0.860-0.101 ppm after 24 hours (Table 2). Black tea-Brown sugar mixture consistently has the lowest LC<sub>50</sub> in both acute and chronic effects.

Kombucha tea has been claimed by kombucha drinkers all over the world to have many beneficial effects on human health. Nonhuman studies regarding antimicrobial, antioxidant, hepatoprotective, and anticancer properties of kombucha tea have been carried out. Tea polyphenols which includes (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and theaflavin (TF) have been reported to possess various biological activities [2].

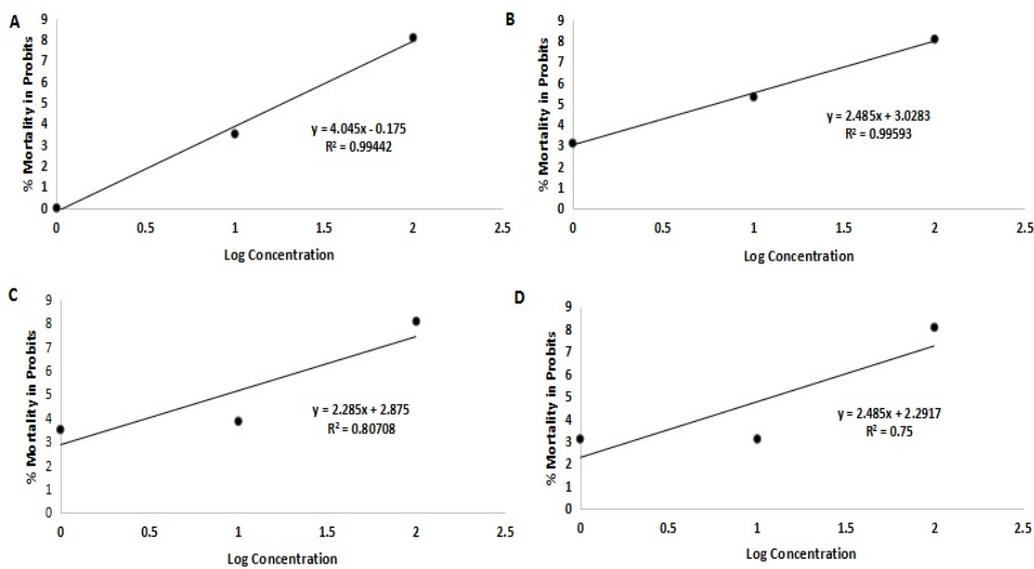
Treatment of human cancer cell lines A549 and Hep-2 with Kombucha tea showed a dose dependent inhibition of growth as a result of cytotoxicity. The antimicrobial activity of kombucha tea against a spectrum of Gram-positive and Gram-negative organisms was also reported [21]. In rat models, low density cholesterol, [triglycerides and homocysteine levels elevated by fat diet decreased during intake of kombucha for 60 days [22]. Furthermore, kombucha was found efficient as hepatoprotective and curative against carbon tetrachloride-induced toxicity in male albino rat model.

**Table 1.** Toxicity (LC<sub>50</sub>) of Kombucha from different substrate combinations using brine shrimp lethality assay after 6 hours.

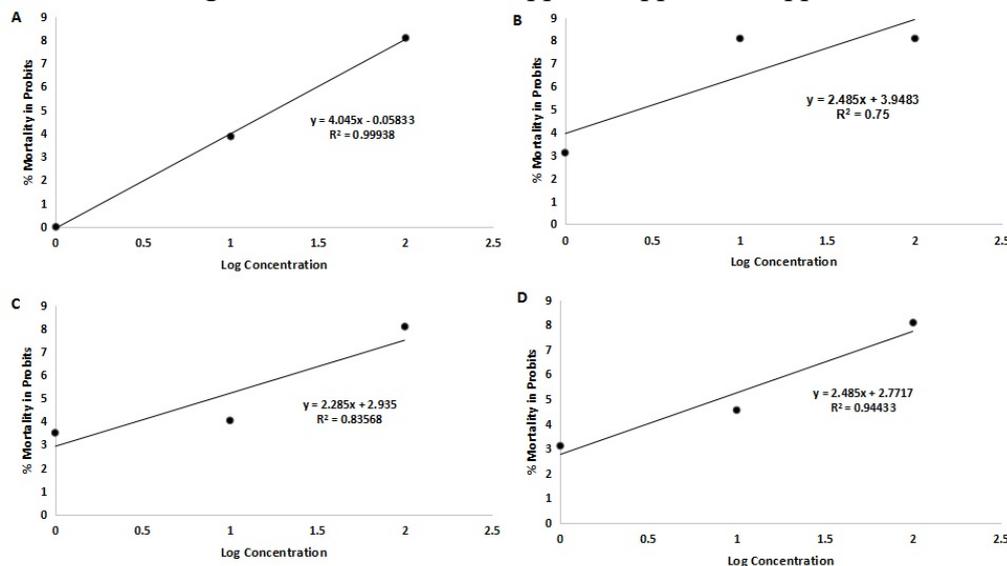
Substrate Combinations	Percent Mortality after 6 hours at 3 levels of Log Concentration			LC <sub>50</sub> [ppm]
	100 ppm	10 ppm	1 ppm	
Black tea and White sugar (BW)	100.00	6.67	0.00	0.241
Green tea and White sugar (GW)	100.00	63.33	3.33	0.232
Black tea and Brown sugar (BB)	100.00	13.33	6.67	0.073
Green tea and Brown sugar (GB)	100.00	3.33	3.33	0.157

**Table 2.** Toxicity (LC<sub>50</sub>) of Kombucha from different substrate combinations using brine shrimp lethality assay after 24 hours.

Substrate Combinations	Percent Mortality after 24 hours at 3 levels of Log Concentration			LC <sub>50</sub> (ppm)
	100 ppm	10 ppm	1 ppm	
Black tea and White sugar (BW)	100.00	13.33	0.00	0.224
Green tea and White sugar (GW)	100.00	100.00	3.33	0.860
Black tea and Brown sugar (BB)	100.00	16.67	6.67	0.101
Green tea and Brown sugar (GB)	100.00	33.33	3.33	0.109



**Figure 3.** Percent mortality of Kombucha transformed into probits using brine shrimp lethality assay after 6 hours. Substrate combinations are BW (A), GW (B), BB (C) and GB (D) and levels of Log concentrations are 100 ppm, 10 ppm and 1 ppm.



**Figure 4.** Percent mortality of Kombucha transformed into probits using brine shrimp lethality assay after 24 hours. Substrate combinations are BW (A), GW (B), BB (C) and GB (D) and levels of Log concentrations are 100 ppm, 10 ppm and 1 ppm.

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## Conclusions

The present study compared the fermentation characteristics of kombucha from different substrates, namely, black tea or green tea as nitrogen sources and white sugar or brown sugar as carbon sources in a 30-day fermentation time. Green tea-White sugar mixture showed the lowest recorded pH value of 2.37 on day 14 while black and green teas with white sugar showed to have higher sugar level compared to tea mixtures with brown sugar. The four substrate combinations showed to have very low LC<sub>50</sub> values in brine shrimp lethality assay exhibiting safety of kombucha for consumption.

## Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgement

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