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Potential Probiotic Properties of Kombucha Tea Beverage and it's Beneficial Effects on Human Health

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A B S T R A C T

Fermentation is considered to be one of the most ancient methods of different food preservation techniques. During fermentation various biochemical changes take place and may affect the nutritional composition and consequently the properties of the final product, like the bioactive compounds from plants in food and beverage industries. Sugared tea fermentation with a colony of live bacteria and yeast, i.e. SCOBY (symbiotic colony of bacteria and yeast), yields fermented tea named Kombucha tea which is consumed worldwide. Kombucha tea has shown its refreshing and beneficial properties on human health. Several research findings from past decade concerning Kombucha tea have been made and reports claiming that Kombucha Tea has potential anti-microbial activity against a spectrum of organisms, can promote antioxidant activity, and improvement of the Immune system. The beneficial effects of Kombucha are attributed to the presence of bioactive compounds that act synergistically. Bacteria belong to the genus *Lactobacillus* sp., *Acetobacter* sp., *Gluconobacter* sp., and the Yeasts of the genus *Saccharomyces*, *Brettanomyces* are present in Kombucha. This study aimed at isolation, *identification*, physiological and biochemical characterization of probiotics present in Kombucha. Nevertheless, Kombucha tea could be easily recognized as a beverage which is able to replace the consumption of carbonated beverages due to its possession of health benefits and therapeutic properties. Existing reports have suggested that the protective effects of kombucha tea are as good as those of black tea, however, more studies on kombucha tea and its composition are needed before final conclusions can be made.

Introduction

Probiotics are live bacteria and yeasts that are thought to be beneficial in preventing several health conditions. It has been reported that certain dietary factors, such as lactic acid bacteria, oligosaccharides, amino acids, and polyphenols, may be promising ingredients for the future development of functional foods (Shimizu, 2012). However, the bioavailability and efficacy of these compounds at levels that are scientifically achievable in typical eating patterns should be revised (Kaur and Singh, 2017).

Different factors such as unhealthy lifestyle, stress, and environmental pollution influence the excessive synthesis of reactive oxygen species. The disturbance in homeostasis caused by free radicals

leads to the formation of oxidative stress and damage to the structures of the human organism (Blokhina *et al.,* 2003; Jakubczyk *et al.,* 2020; Chandrakala *et al.,* 2019; Kim and Adhikari, 2020). To maintain the balance between the production and removal of reactive oxygen species, it is important to work for easily accessible sources of antioxidants (Blokhina *et al.,* 2003). Antioxidants complement everyday diet like tea, coffee fruits vegetables, spices and herbs, contributing to good health.

Kombucha tea

Kombucha tea is also one of the potential probiotics. Kombucha originated in Northeast China (historically referred to as Manchuria) around 220 B.C. and was initially prized for its healing properties. Its name is reportedly derived from Dr. Kombu, a Korean physician who brought the fermented tea to Japan as a curative for Emperor Inkyo.

Fermentation

Kombucha is a slightly sweet and acidic refreshing fermented beverage created by using the symbiotic cultures of bacteria and yeast i.e., SCOBY (Symbiotic Cultures of Bacteria and Yeasts). SCOBY, when added to sugared tea (10%), initiates fermentation at room temperature for a period of 7–14 days, resulting in the formation of various new bioactive compounds.

A floating cellulosic pellicle layer and the sour liquid broth are the two portions of kombucha tea. Out of various tea types e.g., red, black or yellow tea, black tea and white sugar (saccharose) are considered the finest ingredients that condition the proper content of the drink as well as its healthy properties.

The different variables of the fermentation process, like time, temperature and sucrose concentration, will determine the final concentration of organic substances such as acids and pH of final product i.e., Kombucha tea. Organic acids produced during fermentation diminished the tea's pH value, which leads to a lack of oxygen induced by the acidity. As A result, the number of possible pathogenic microbial cells, if any, diminishes, resulting in a safe beverage for consumption, despite having a microbial origin. Kombucha fermentation is a combination of alcoholic, lactic, and acetic acid fermentation. This because of the presence of several yeasts and bacteria coexisting in the medium. The main acids present are acetic, gluconic, tartaric, malic, and in less proportion citric acid. All these acids are responsible for its characteristic sour taste (Jayabalan *et al.,* 2007).

Materials and Methods

Preparation of Kombucha

The kombucha starter cultures (which generally consists of *Acetobacter*, *Gluconobacter*, *S. cerevisiae*), were obtained from a commercial source, consists of sour broth and cellulosic layer (SCOBY floating on the liquid surface) (Figure.1). One hundred grams of sugar (100.0 g/L, 10.0%), eight grams of tea (8.0 g/L, 0.8%) and 1 liter of hot, distilled water (90 °C) were mixed.

The solution was infused for 10 min in a sterile conical flask. After cooling (30°C), the tea decoction was filtered and kept in clean glass bottles for its fermentation under aseptic conditions at 28 ± 1°C for 14-30 days. The kombucha obtained was filtered and analyzed.

Isolation and screening of probiotic microorganisms from Kombucha Tea

Pour plate technique was used to isolate the organisms. Samples were directly and diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS (Man, Rogosa and Sharpe) agar. The plates were incubated at 37 °C for 3 days under anaerobic conditions. The purified isolates were examined according to their colony morphology and different biochemical reactions.

Identification of microorganisms present in Kombucha tea

Gram Staining

A thin smear of each of the pure 24 h old culture was prepared on clean grease-free slides was treated with Crystal Violet, Gram's Iodine, Acetone followed by Saffranine, as per the standard Gram staining protocol (Figure 2).

Biochemical Analysis

Selected isolates were subjected to different morphological, biochemical and physiological tests. colony characteristics including colony size, shape, consistency, etc. were recorded. Physiological tests like bile salt tolerance, NaCl tolerance, growth at different temperature and low pH tolerance and biochemical tests including catalase, oxidase, MR-VP, citrate utilization, bile esculin test, hemolytic activity and various sugar fermentation tests were performed.

Indole production test

Indole production test is used to determine the ability of microorganism to degrade amino acid tryptophan. The presence of indole is detected by adding Kovacs reagent; which produces a cherry red reagent layer.

Tubes of inoculated 5ml of tryptone broth kept for incubation at 37^0C for 48 hours followed by addition of 1ml of Kovac's reagent to each tube including control. Examined the tubes for the colour of the top layer. A red or red-violet colour at the top surface of the tube indicates a positive result while yellow coloration indicates a negative result.

Methyl Red Test

Methyl red test is used to determine the ability of microorganisms to oxidize glucose with production and stabilization of high concentration of acid end product. In this test pH indicator methyl red detects the large concentration of acid product. 5 mm of glucose phosphate broth (1 g glucose, 0.5% KH2PO4, 0.5% peptone and 100 mL distilled water) were inoculated with the isolated test organisms and incubated at 37°C for 48 hours followed by addition of few drops of methyl red solution and colour change was observed. A red color indicates a positive reaction.

Voges -Proskauer Test

The Voges Proskauer test determines the capacity of some organisms to produce non acidic and neutral products such as acetyl methyl carbinol from organic acid that result from fermentation of glucose. 5ml of glucose phosphate broth (1 g glucose, 0.5% KH2PO4, 0.5% peptone and 100 mL distilled water) was inoculated with test organism and incubated at 37^0C for 48 hours, followed by addition of few drops of Barrit's reagent (6% α-naphhtol and 6% sodium hydroxide). Strong red colour within 30 minutes indicates the positive result.

Citrate Utilization Test

The test is used to detect the ability of an organism to use citrate as a sole source of carbon and energy. About 2.4 g of citrate agar was dissolve in 100 mL of distilled water. 10 mL of citrate medium was dispensed into test tubes.

Further tubes were inoculated with 24 hour old test culture and incubated at 37^0C for 48 hours. A change from green to blue indicates utilization of the citrate i.e., positive result.

TSI agar test

The Triple Sugar Iron (TSI) test is used to determine carbohydrate fermentation and H2S production in bacteria. TSI agar was inoculated with inoculation needle by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the slant. Further inoculated slants were kept for incubation at 37^0C for 18 to 24 hours.

Oxidation Fermentation (OF) Test

Of test is used to determine the microbe's ability to oxidize or ferment a specific carbohydrate. Inoculate the test organism by stabbing the agar to approximately 1/4 inch from the bottom followed by application of sterile mineral oil, sterile melted paraffin, or sterile melted petrolatum to one of each duplicate tubes. Tighten the cap of the overlaid tube, and loosen the cap of the non-overlaid tube. Incubate both tubes aerobically at 37ºC. for up to 14 days. Examine tubes daily for colour change.

Nitrate reduction test

Nitrate Reduction test is used to determine the ability organism to produce an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO_3).

A well isolated colony was inoculated in nitrate broth (peptone 10 g, KNO_3 10 g in 1000 ml distilled water) and incubated at 37°C for 24 to 48 hours. After incubation, add few drops of sulphanilic acid and few drops of α-naphthylamine. The appearance of red color observed after 5–10 minutes that indicates the positive result.

Gelatin liquefaction test

This test is used to determine the ability of an organism that produce gelatinases. Inoculate the gelatin deep with 4 to 5 drops of a 24-hour broth culture and incubated at 35-37°C in ambient air for up to 14 days. Alternatively, inoculate the gelatin deep from a 24-hour-old colony by stabbing four or five times, 0.5 inch into the medium. Remove the gelatin tube daily from the incubator and place at 4°C to check for liquefaction. Daily gelatin tube was removed from the incubator and place at 4°C to check for liquefaction.

Catalase test

Catalase test is used to determine the presence of catalase enzyme. A small quantity of 24 h old culture was transferred into a drop of 3% hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicates the positive result.

Oxidase test

$$2H_2O_2 \rightarrow 2\,H_2O + O_2$$

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain.

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick a colony of the test organism and smeared on the filter paper. If the organism is oxidase producing, the phenylenediamine in the reagent will be oxidized to a deep purple.

Sugar fermentation

Sugar fermentation test was carried out to determine the ability of organisms to ferment sugars with production of acid and gas. Sugar indicator broth was prepared using peptone water medium containing 1% fermentable sugar and 0.01% phenol red.

About ten milliliters of sugar broth was dispensed into each of the test tubes, Durham tube which would trap the gas if produced was inverted carefully.

The test tubes were autoclaved and inoculated with a loopful of 24 h old culture of the test organisms after then incubated for 2-7 days at 36±1°C and observed daily for acid and gas production. Yellow coloration indicates acid production while gas production was indicated by displacement of the medium in the Durhamtube (Figure 5).

Hemolysis test

Selected medium called blood agar plate medium is used to study hemolysis. Under sterilized conditions inoculation of organisms was done and incubated for24 hours at 37°C.

Antibiotic disc diffusion

To evaluate the antibiotic susceptibility of the *Acetobacter* sps, *Lactobacillus* sps, *Brettanomyces* sps, and Yeast, the method of Patel *et al.,* (2009) was used. The fresh culture of Kombucha tea, was streaked densely on Mueller-Hinton agar by a sterile cotton swab. Paper discs impregnated with streptomycin, tetracycline, trimethoprim, amoxicillin, ceftriaxone and chloramphenicol were loaded on the plates. The diameters of the clear zones were measured after incubation for 48 h at 37°C (Figure 4**)**.

Antimicrobial assessment

The inhibitory effect of *Lactobacillus* spp., *Acetobacter* spp., Yeast and *Brettanomyces* spp., strains on selected clinical reference strains was determined by the well-diffusion method. For the agar well diffusion assay, an overnight culture of the indicator strain (*Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas*) was used to inoculate to Muller Hinton agar growth media at 37 °C (approximately 10^6 cells ml^{-1} of indicator strains were overlaid onto Muller Hinton agar plates). Wells of 5 mm diameter were cut into agar plates and 50 µL of *Lactobacillus* culture supernatant fluid that probably containing antibacterial activity was added to each well. Inhibitory zone of *Lactobacillus* spp., *Acetobacter* spp., Yeast and *Brettanomyces* spp., were checked after 24-hour incubation at 37 °C.

Probiotic tolerance test for pH, Salt concentration, Bile and Temperature

Tolerance test for pH

For the determination of optimal pH for growth, 100 µL of fresh culture of isolated organisms was inoculated into MRS broth containing test tubes with varying pH ranging from 2.0, 3.0 and 6.0. The inoculated broth was incubated in anaerobic condition for 24 h at 37°C and 48hrs. After incubation, growth of the bacteria was measured using a spectrophotometer at 560 nm against uninoculated control broth.

Tolerance Test for Bile Salts

Growth rate of bacterial cultures was determined in MRS broth containing different levels (0.05, 0.15, and 0.3 %) of bile salts. Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h and 48hrs incubation, under anaerobic condition. Then optical density of each sample was measured using a spectrophotometer at 560 nm

Tolerance test for Temperature

Tolerate to temperature: the isolates where tested to tolerate the temperature (15°C, 37°C, 45°C)the isolates were inoculated in MRS broth and incubated at 15°C and 37°C temperature readings are taken at 24hrs, 48hrs for turbidity by using colorimeter at 570 nm by using uninoculated broth as blank.

Tolerance test for NaCl

For the determination of NaCl tolerance, isolated organisms were grown in MRS broth containing nine test tubes that were adjusted with different concentrations of NaCl (3.5 % and 6.5%). After autoclaving for 15 min in 15 lbs pressure at 121°C, each test tube was inoculated with 10ul over night culture of isolated organisms and incubated anaerobically at 37°C for 24 h and 48hrs. After 24 hrs and 48hrs incubation, the bacterial growth was measured using a spectrophotometer at 560 nm.

Anti-Oxidant activity determination by Reducing Power assay

The reducing power of the of the Kombucha tea was measured according to method of Liu *et al.*, Solutions of the samples at different concentrations (0.2–0.04 mg/mL) were mixed with 2.5 mL of 0.2 mol/L Phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) Potassium ferro cyanide K3(Fe (CN)6) solution. The mixture was incubated for 20 min at 50°C in a water bath. After cooling, 2.5 mL of

10% (w/v) Cl3CCOOH solution was added and centrifuged at 3000 ×g for 10 min. A 2.5 mL aliquot of the upper layer was combined with 2.5 mL of deionized water and 0.5 mL of 0.1% (w/v) Ferric Chloride (FeCl3) solution. After allowing the mixture to stand at room temperature for 10 min, the absorbance was determined at 707 nm.

Anti-inflammatory activity determination

Inflammation is the reaction process of living tissues to stimuli evoked by inflammatory agents such as physical injuries, heat, microbial infections, and noxious chemical irritations. Hence, the ability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity. The capacity of different plant parts of Kombucha tea to inhibit protein denaturation of albumin which was ranging from 43.33 ± 0.002% to 70.58 ± 0.004% inhibition in this assay had therefore provided another evidence for its promising anti-inflammatory properties. In the current study, diclofenac sodium, routinely used NSAIDs. The assay was carried out by adopting the methods described by Kumari *et al.*,. The Kombucha tea and positive standards (ibuprofen or diclofenac) were prepared at a concentration of 0.1% each (1.0 mg/ml). A reaction vessel for each mixture was prepared consisted of 200 µl of egg albumin, 1400 µl of Phosphate buffered saline, and 1000 µl of the test extract. Distilled water instead of extracts was used as a negative control. Afterward, the mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, their absorbances were measured at 660 nm and the data were processed by Spectra Manager system. The inhibition percentage of protein denaturation was calculated using the following formula:

$$\% \text{ Denaturation inhibition} = (1-D/C) \times 100\%$$

Where

D is the absorbance reading of the test sample, and
C is the absorbance reading without test sample (negative control).

Results and Discussion

Isolation and screening of probiotic microorganisms from Kombucha

Identification of bacteria through bacteriological and biochemical tests: The four isolates were grown in Man, Rogosa and Sharpe (MRS) medium at pH 6.5.

Among all the isolates which were produced, small, irregular and round shape with shiny whitish cream or brownish colored which were morphologically similar to *Lactobacillus* spp,.

Pale to off white, circular, raised, convex, smooth round colony which were similar to *Acetobacter* spp., Off-white, milky, raised, even and glossy which were morphologically similar to *Brettanomyces* spp., Creamy to white color, fluffy, and smooth margin which were morphologically similar to Yeast.

Then all isolates were examined under Compound microscope to observe their microscopic features. Some isolates were found to be gram positive, short and medium rod shaped non-spore forming bacterium (Fig. 1) which indicate them to be member of *Lactobacillus* spp. (Thamaraj and Shah, 2003).

Some isolates were Gram positive ovoid, ellipsoidal, frequently cylindrical to elongate which indicate them to be member of *Brettanomyces* spp. Some isolates were Gram negative bacilli, Ellipsoidal, rods, squat bacilli, roundish, which indicate them to be member of *Acetobacter* spp., Some isolates were Gram positive Round with entire elevation, raised margin, smooth and surface glistening texture which were morphologically similar to Yeast.

In addition, some biochemical tests such as catalase test, oxidase test, indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, citrate utilization test and carbohydrate fermentation patterns were performed as delineated by Bergey's manual systematic bacteriology.

Antimicrobial activity assessment

In this study, the selected 4 isolates were examined according to their antibacterial activity against different pathogenic bacteria such as *E.coli*, *Pseudomonas* spp., *Klebsiella* spp., and *Staphylococcus* spp. The comparison of their inhibition (in mm) against 4 tests pathogens is shown in Table 3.

The experimental results showed that the highest inhibitory activity of isolate *Acetobacter* spp., was demonstrated against *Pseudomonas* spp., and lowest zone of inhibition was against *Klebsiella* spp., after 24 hours of incubation. The highest diameter of inhibition zone of isolate *Brettanomyces* spp., was showed against *Staphylococcus* spp. and *Pseudomonas* spp., and lowest zone (20.10±1.00 mm) against *Klebsiella* spp., after 24 h of incubation. Similarly, the highest diameter of inhibition zone of isolate *Lactobacillus* spp., was showed against *Staphylococcus* spp. and lowest zone against *Pseudomonas* spp.,. And finally, in isolate Yeast highest zone was observed against *E.coli* and lowest zone against *S. aureus* after 24 h incubation.

Antibiotic Disc Diffusion Assay

Sugar fermentation

In this study, *Acetobacter* spp, were able to produce acid in glucose, fructose, lactose, but no gas production in fructose, maltose, and galactose. *Brettanomyces* spp. were able to produce acid in glucose, sucrose, galactose, maltose, and fructose and produce gas only in glucose. *Lactobacillus* and Yeast were able to ferment the 6 sugars indicating that they are able to grow in variety of habitats utilizing different type of carbohydrates. The summarized results of all bacteriological and biochemical tests are presented in Table 2.

Hemolysis Test

After incubation under optimum conditions the blood agar plates show hemolytic zone present in *Acetobacter* sps. and *Brettanomyces* sps. while hemolytic zone was not seen in case of *Lactobacillus* sps. and Yeast.

Tolerance for pH

This method used to evaluate the viability of the cells under acidic stress in gastric conditions. The pH tolerance capacity of the isolates *Lactobacillus* spp., *Acetobacter* spp., *Brettanomyces* spp., and Yeast at 37◦C for 48hrsis shown in Figure 6. The best growth of the isolates was observed at pH 6.5. However, moderate growths of both the isolates were observed at pH 3.0.

Tolerance Test for Bile Salts

The effects of bile on the growth of probiotic strains present in Kombucha were examined using methods modified from those of Gilliland and Walker (1990) and Tsai *et al.*, (2007). A series of bile concentrations were employed in this study considering the fluctuation of bile concentration at different times. Broth with 0% bile concentration serves as a control of the study. Although the bile concentration of the human gastro intestinal tract varies, and the staying time is suggested to be 48 h (Prasad *et al.*, 1998). Strains were detected in 0.3% 0.15%, and 0.05% bile salt concentrations during 48 hours. According to the results all of the isolates are resistant to 0.3%, 0.15%, and 0.05% bile salt. *Lactobacillus* spp., *Brettanomyces* spp., are more tolerant than *Acetobacter* spp., Yeast. All of the isolates are also able to grow in 0.3% bile salt as they survive (Figure 7).

Tolerance for salt NaCl

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. In this study, the results showed that *Lactobacillus* spp., isolated from Kombucha were able to tolerate 3.5% of NaCl and optimal growth was observed at 6.5% NaCl. *Acetobacter* spp., were able to tolerate 6.5% of NaCl and optimal growth was observed at 3.5% NaCl. *Brettanomyces* spp., were able to tolerate 6.5% of NaCl and optimal growth was observed at 3.5%

NaCl. The growth rate was decreased for *Acetobacter* spp., and Brettanomyes spp., with the increasing level of salt concentration (Figure 9).

Tolerance test for Temperature

Subjecting the strains to different temperature increase resulted in a strong tolerance in viability (Fig 7). Fig 7showed that the *Lactobacillus* spp., and *Acetobacter* sp., have consistent tolerance at 15^{O}C for 24 hours and 15^{O}C at 48hrs.And temperature down shifts when compared to *Brettanomyces* sps., and Yeast. *Brettanomyces* spp., and *Lactobacillus* spp., were tolerant to 15°C temperature, but showed general progressive loss to temperature increase (Figure 8).

Detection of Antioxidant properties by reducing power assay

The antioxidant activities of the two samples and the standard solutions (L-Ascorbic Acid), as reflected in their reducing power, are presented in Figure 10. In this assay, the reducing powers of the two samples and three positive controls were concentration-dependent. The reducing power of L-Ascorbic Acid is just as strong as those of Kombucha tea. Based on this result, as the L-Ascorbic Acid can more easily transfer electrons to the reactive radicals and convert them to more stable, nonreactive species, same way Kombucha also works as a strong antioxidant.

Detection of Anti -inflammatory properties

In clinical setting, major pharmacological agents used for the anti-inflammatory and pain-relief management are NSAIDs due to their capacity in inhibiting protein. However, this type of drugs is associated with adverse effects on gastrointestinal tract leading to the formation of gastric ulcers and may result in cardiovascular complications as well. Kombucha tea had been documented to be used as a natural remedy for gastric ulcer. Indeed, this provides another added value for the species to be considered as a potential candidate for anti-inflammatory agent, deliberating the expectation that the risk of developing gastric ulcers could be minimized considering its ethnopharmacological use.

Kombucha beverage is a source of bioactive components, such as polyphenols and glucuronic acid. The beneficial outcomes of kombucha consumption are attributed to the synergistic effect between these components, making it a drink with potential beneficial health properties (when elaborated under adequate sterile conditions).

It is apparent that its consumption can protect against the development of CVDs, mainly due to its polyphenol content that inhibits the oxidation of LDL, regulates cholesterol metabolism, and prevents high blood pressure by promoting smooth muscle relaxation. Based on our test we can conclude that the microbes isolated from Kombucha tea; *Acetobacter* spp., *Brettanomyces* spp., *Lactobacillus* spp., and yeasts are Methyl Red positive that is the microorganism has ability to produce acid as end product from glucose fermentation. *Acetobacter* spp., and *Lactobacillus* spp., is Voges Proskauer positive that is the microorganism has ability to produce acetyl methyl carbinol. Only *Acetobacter* spp., is Citrate positive that is use citrate as sole source of carbon. *Acetobacter* spp., *Brettanomyces* spp. showed alkaline slant and acidic butt means indication of dextrose fermentation only. *Lactobacillus* spp. showed alkaline slant and alkaline butt that is absence of carbohydrate fermentation and are fermentative means metabolize glucose by fermentation and *Acetobacter* metabolize glucose oxidatively. *Acetobacter* spp., and *Brettanomyces* spp., is positive for nitrate test that is has ability to reduce nitrate to nitrite. *Acetobacter* is positive for Gelatin liquefication test that is has ability to produce gelatinase. In this study, the selected 4 isolates were examined according to their antibacterial activity against different pathogenic bacteria such as *E.coli*, *Pseudomonas* spp., *Klebsiella* spp., and *Staphylococcus* spp. The comparison of their inhibition (in mm) against 4 tests pathogens is shown in Table 3.

Table.1

Organisms	Glucose	Lactose	Sucrose	Maltose	Galactose
Acetobacter	A+/G+	A+/G+	A-/G+	A+/G-	A-/G-
Brettanomyces	A+/G+	A-/G-	A+/G-	A+/G-	A+/G-
Lactobacillus	A+/G	A+/G+	A+/G+	A+/G+	A+/G+
Yeast	A+/G+	A-/G-	A+/G+	A+/G+	A+/G+

Fig.1 Formation of mother Kombucha Tea

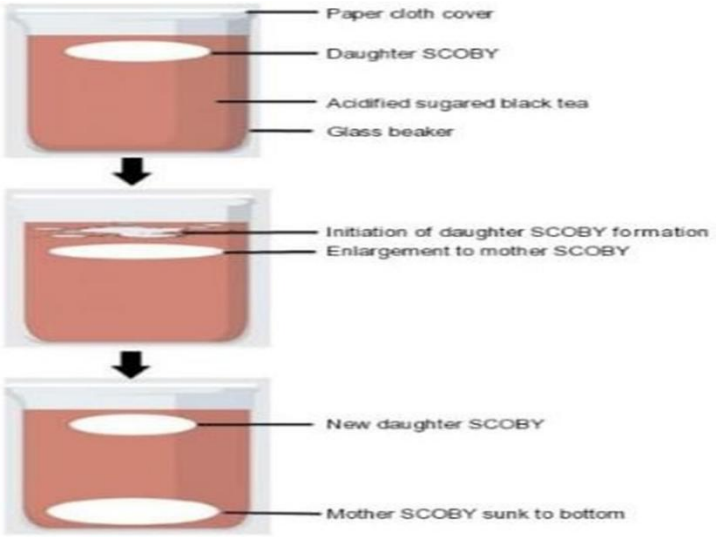

Fig.2

Indole Positive

MR Positive **VP Positive**

Citrate Positive

Fig.3

Fig.4 Gelatin Hydrolysis Test

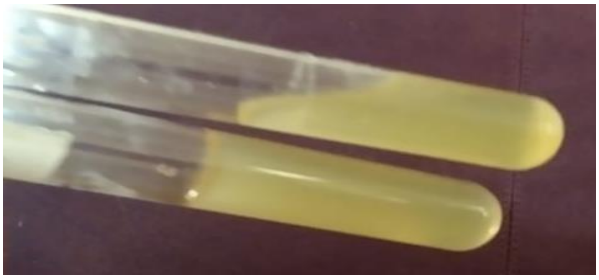

Fig.5 Catalase test

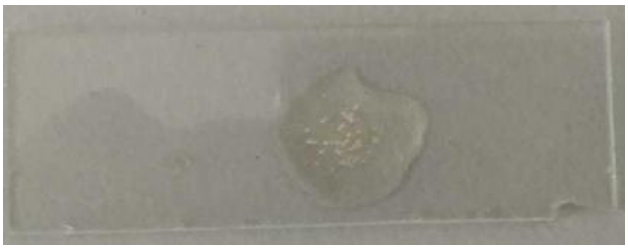

Fig.6 OxidaseTest

Fig.7

Fig.8

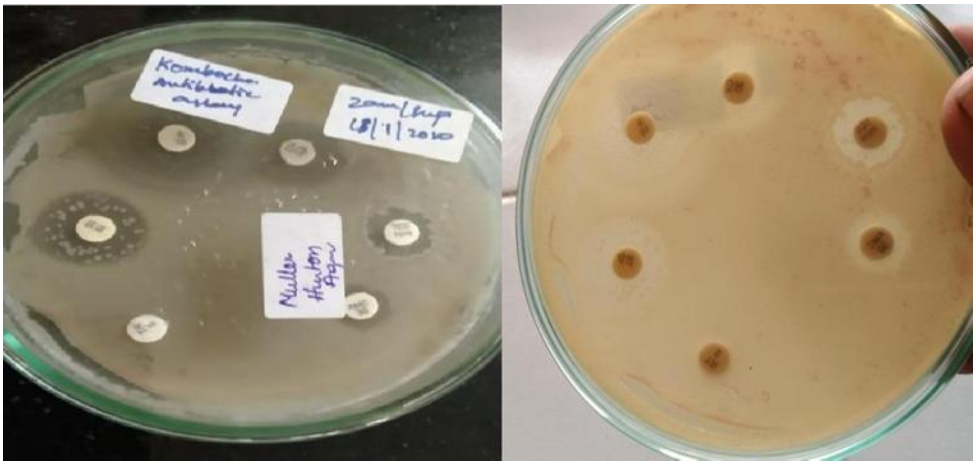

Fig.9 Anti-Oxidant activity determination by Reducing Power assay

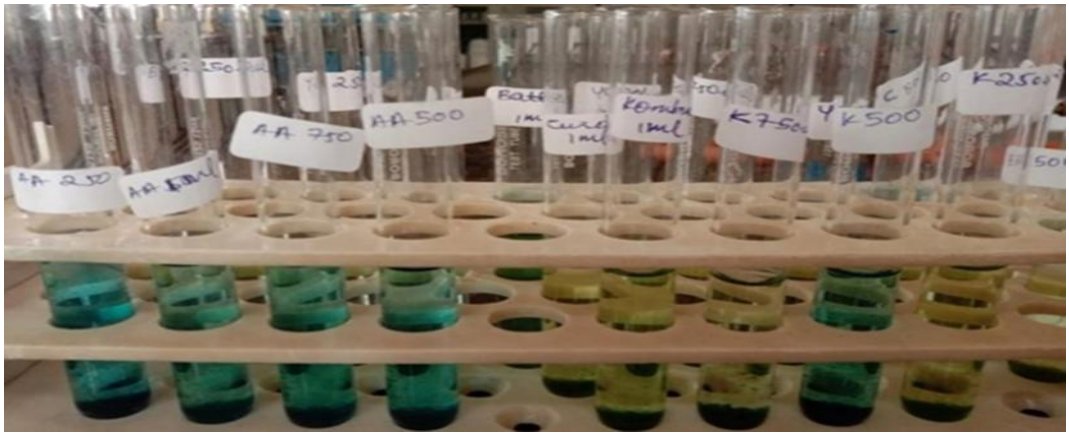

Fig.10 Anti-inflammatory Assay

Fig.11

	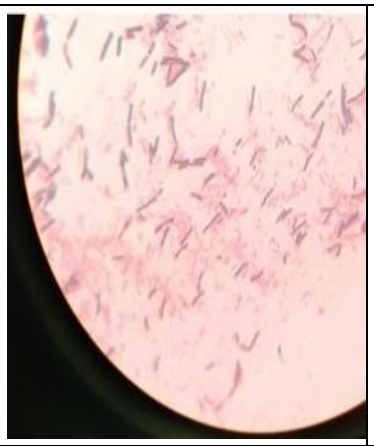	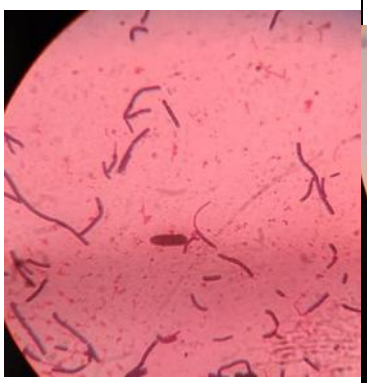	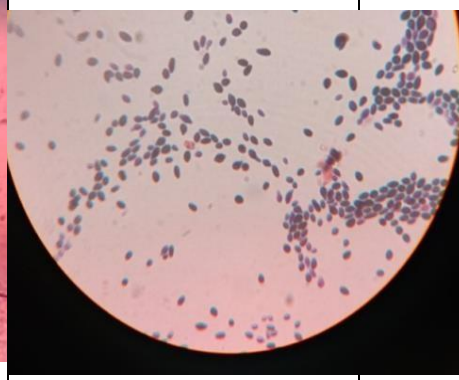
***Lactobacillus* sps.**	***Acetobacter* sps**	***Acetobacter* sps.**	***Brettanomyces* sps.**

Fig.12 Cultural and Biochemical Analysis result

Test	Acetobacter spp.	Brettanomyces spp.	Lactobacillus spp.	Yeast
Colony Characters	Pale to off white, circular, raised, convex, smooth	Off- white, milky, raised, even and glossy	small, creamy white, shiny, convex, opaque	Creamy to white color, fluffy, and smooth margin
Colony Morphology	Ellipsoidal, rods, squat bacilli, roundish	ovoid, ellipsoidal, frequently cylindrical to elongate	Spherical white sticky colonies	ovoid, ellipsoidal
Gram's Nature	Gram negative bacilli	Gram positive ovoid	gram positive, short and medium rod shaped non-spore forming bacterium	Gram positive
Catalase Test	+	+	_	_
Oxidase test	_	+	_	_
IMViC	_+_+	_+_ _	_+_+	_+_ _
TSI	Alkaline Slant Acidic Butt	Alkaline Slant Acidic Butt	Alkaline Slant Acidic Butt	-
OF test	F	F	F	F
Gelatin Liquefaction test	+	_	_	_
Nitrate Broth test	+	+	_	_
Hemolysis	+	+	_	_

Fig.13

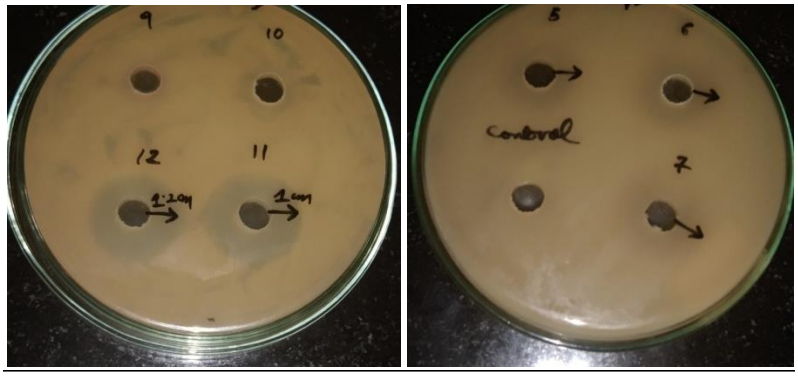

Fig.14

RESULT OF ANTIBACTERIAL ACTIVITY

Antibacterial Test	E. coli	Pseudomonas	Klebsiella	Staphylococcus
Acetobacter	-	2cm	0.5 cm	1cm
Bretano-myces	-	1	0.5cm	1 cm
Lactobacillus	-	1.5 cm	-	2 cm
Yeast	12 cm	-	-	8 cm

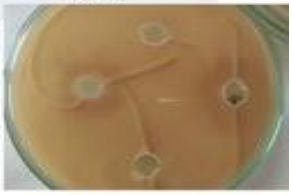

Fig.15

RESULT OF ANTIBIOTIC DISC DIFFUSION

Antibiotic disc diffusion	TR	Te	TCC	S10	AMC	CX
Acetobacter	-	0.8cm	0.6cm	1cm	0.5cm	0.7cm
Lactobacillus	-	-	-	-	-	-

Fig.16

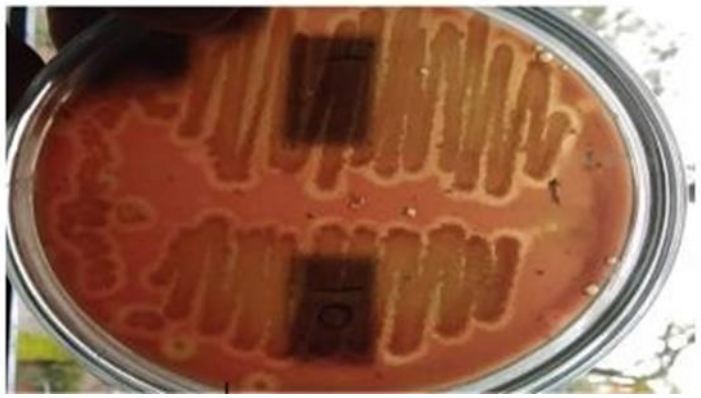

Fig.17

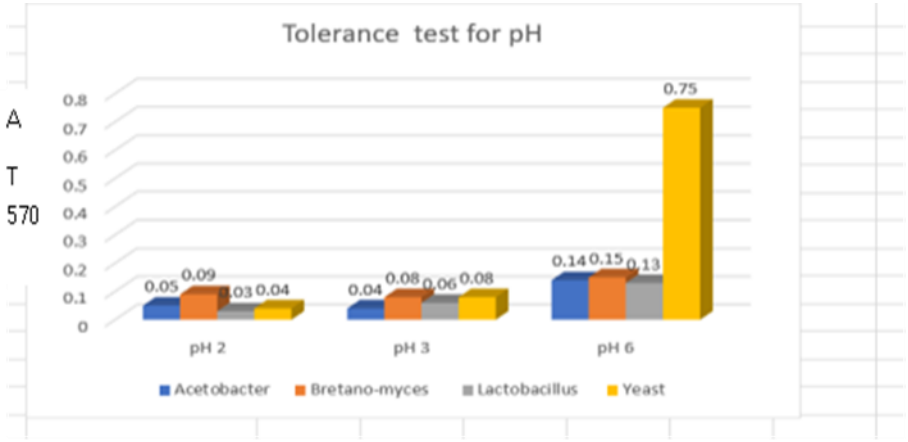

Fig.18

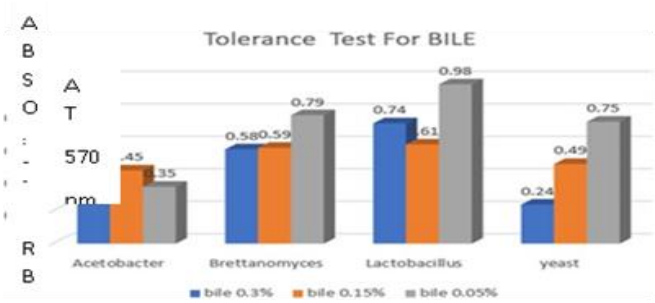

Fig.19

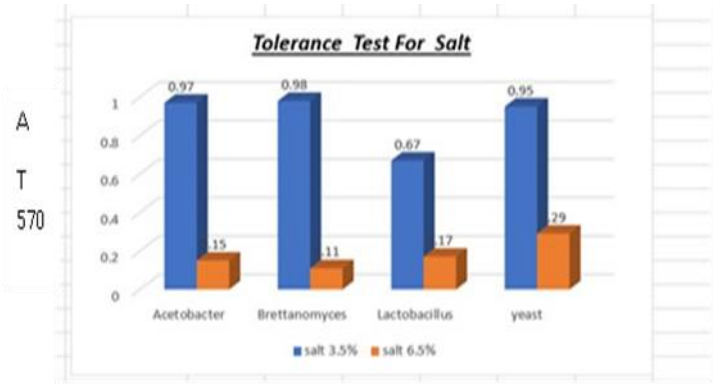

Fig.20

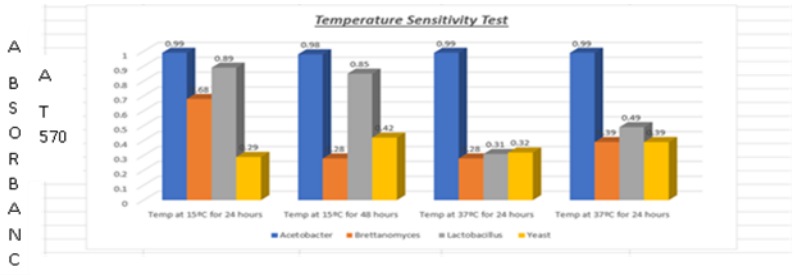

Fig.21

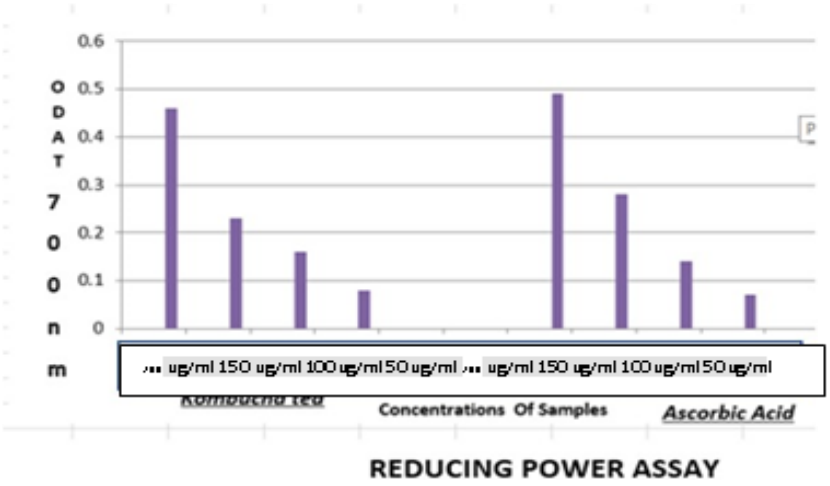

Fig.22

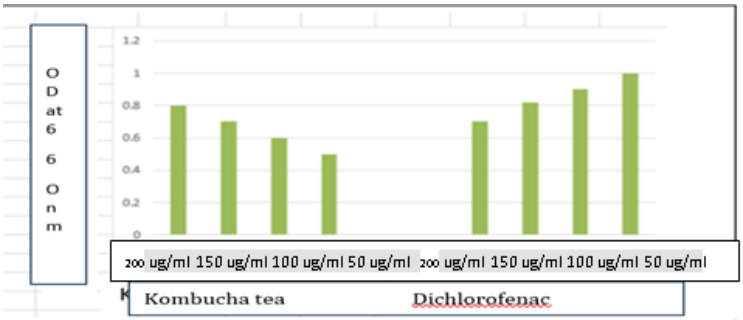

Figure.22. **ANTI INFLAMMATORY ASSAY**

The experimental results showed that the highest inhibitory activity of isolate *Acetobacter* spp., was demonstrated against *Pseudomonas* spp., and lowest zone of inhibition was against *Klebsiella* spp., after 24 hours of incubation. The highest diameter of inhibition zone of isolate *Brettanomyces* spp.was showed against *Staphylococcus* spp. and *Pseudomonas* spp., and lowest zone (20.10±1.00 mm) against *Klebsiella* spp., after 24 h of incubation. Similarly, the highest diameter of inhibition zone of isolate *Lactobacillus* spp., was showed against *Staphylococcus* spp. and lowest zone against *Pseudomonas* spp., And finally, in isolate Yeast highest zone was observed against *E.coli* and lowest zone against S. aureus after 24 h incubation.

Acetobacter spp, *Brettanomyces* spp., showed positive result for Hemolysis.

In this study, Lactobacilli strain were resistant towards Streptomycin, Tetracycline, Trimethoprim, Amoxicilin, Ceftriaxone and Chloramphenicol. *Acetobacter*spp., is susceptible to all the antibiotics except Trimethoprim.

The results obtained in this study reveal that *Acetobacter* spp., *Lactobacillus* spp., *Brettanomyces* spp., and Yeast isolated from Kombucha tea revealed well resistance to temperature, acidic and bile salt stresses, which is consider an important characteristic for probiotics. Being resistant to low pH is one of the major selection criteria for probiotic strains (Quwehand *et al.,* 1999, Çakır, 2003). Since, to reach the small intestine they have to pass through from the stressful conditions of stomach (Chou and Weimer 1999, Çakır, 2003). Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad *et al.*, 1998). For selection the strains resistant to low pH. The time that takes during the digestion in the stomach is 3 hours. So, all the isolates were detected whether they were resistant to pH 3.0 during 24hours and 48 hours.

Kombucha is characterized by high antioxidant potential. In the study it has been shown. The antioxidant activities of the Kombucha tea sample and the standard solutions of L-Ascorbic Acid, as reflected in their reducing power, are presented in Figure 10. The reducing power of L-Ascorbic Acid is just as strong as those of Kombucha tea. Based on this result, as the L-Ascorbic Acid can more easily transfer electrons to the reactive radicals and convert them to more stable, nonreactive species, same way Kombucha also works as a strong antioxidant.

The ability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity. In the current study, diclofenac sodium, routinely used NSAIDs for arthritis had been used as the reference compound anticipated to exert optimally positive inhibition percentage. The anti-inflammatory activity of diclofenac (% inflammatory inhibition) is just as

comparable to Kombucha tea.

Since studies carried out to assess qualitative and quantitative properties of constituents of Kombucha are scattered, scientific research should be carried out to clarify the health beneficial claims and safety aspects, Yet, the importance of studying the safety of Kombucha tea consumption is important as there are only a very few such studies been carried out throughout the years. Since this a popular beverage around the world, investigating the advantages and disadvantages of consuming this beverage in similar capacities can be extremely meaningful. According to literature, there is no evidence about systematic human trials being done using Kombucha tea. This could be an area in which future research could be focused in establishing this beverage as a functional food. Furthermore, the extension of a fermentation process from a laboratory scale to a commercial product is a challenge because of the difficulty of evaluating the factors which may influence the scaling process during cultivation. More scientific research should be done to understand the links between the fermentation and the biological activities of Kombucha tea, establishing it as a functional beverage with clear evidence in the advantages and disadvantages of its consumption. There are a number of parameters and variations to be measured, controlled, and experienced in order to determine the optimum fermentation conditions.

Kombucha, the fermented tea, as a Probiotic has strong antioxidant properties associated with high polyphenol content, particularly flavonoids. Therefore, it should be consumed by people particularly exposed to oxidative stress and also to counter the stress caused in general by unhealthy life styles. Kombucha has very specific anti-inflammatory properties also. The antioxidant and the anti-inflammatory activities of kombucha are diverse and depend on the type and composition of the tea infusion before fermentation and on the content of SCOBY. Therefore, its important to select the Kombucha tea over other beverages for all the beneficial to human health.

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