SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 18, NUM. 9 2022
doi: 10.14808/sci.plena.2022.091502

Evaluation of antibacterial activity of black and green tea kombucha

Avaliação da atividade antibacteriana de kombucha de chá preto e verde

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(Recebido em 06 de abril de 2022; aceito em 01 de agosto de 2022)

Currently, the search for natural antimicrobial agents has increased. Thereat, plant extracts, products of fermentative processes, and microbial growth have been tested. Kombucha is a fermented beverage that resulted from the fermentation of the infusion of *Camellia sinensis*. There are reports about biological activities, such as anti-inflammatory, digestibility, antioxidant, antimicrobial, and others. The study aimed to evaluate the antibacterial activity of black and green tea kombucha against eight foodborne pathogenic microorganisms. The antibacterial activity was analyzed using the agar well diffusion method. Each kombucha consisted of three treatments: natural, neutralized, and filtered. The solutions of acetic acid (4% $v.v^{-1}$) and kanamycin (30 $\mu g.L^{-1}$) were used as a control. The black and green tea kombucha beverages, not neutralized, showed antibacterial activity against *Escherichia coli* serotypes. Black and green tea kombucha did not show antibacterial activity against *Staphylococcus aureus*, *Shigella Flexneri,* and *Salmonella Typhimurium*. The inhibition may be attributed to acetic acid production during the fermentation process. Therefore, it appears that kombucha may have a potential antimicrobial agent.
Keywords: *Camellia sinensis*, fermentation, antimicrobial.

Atualmente a busca por agentes antimicrobianos naturais tem aumentado. Com isso, extratos de plantas, produtos de processos fermentativos e crescimento microbiano têm sido testados. Kombucha é uma bebida fermentada resultante da fermentação da infusão de *Camellia sinensis*. Existem relatos sobre suas alegadas atividades biológicas, tais como: anti-inflamatória, digestibilidade, antioxidante, antimicrobiana e outras. O estudo objetivou avaliar a atividade antibacteriana de kombucha de chá preto e chá verde contra oito micro-organismos patogênicos de origem alimentar. A atividade antibacteriana foi analisada utilizando-se o método de difusão por poço em ágar. Cada kombucha consistiu de três tratamentos: natural, neutralizada e filtrada. As soluções de ácido acético (4% v/v^{-1}) e canamicina (30 $\mu g.L^{-1}$) foram usadas como controle. As kombuchas de chá preto e chá verde não neutralizadas demonstraram atividade antibacteriana contra os sorotipos de *Escherichia coli* analisados. Kombucha de chá preto e chá verde não apresentaram atividade antimicrobiana contra *Staphylococcus aureus*, *Shigella Flexneri* e *Salmonella Typhimurium.* A inibição pode estar atribuída à produção de ácido acético durante o processo de fermentação. Portanto, verifica-se que kombucha pode ser um potencial agente antimicrobiano.
Palavras-chave: *Camellia sinensis*, fermentação, antimicrobiano.

1. INTRODUÇÃO

Natural antimicrobials are compounds capable of inhibiting the growth of microorganisms [1, 2]. The characterization and use of these substances can significantly decrease the demand for new drugs, microbial multidrug resistance, and represent lower risks of toxicity to the organism. These characteristics are of interest to the pharmaceutical and cosmetic sectors [1, 3-6]. Various compounds are formed during the fermentation process, such as organic acids, bacteriocins, and peptides.

Kombucha is a fermented beverage of *Camellia sinensis* infusion. In the process, the microorganisms change sucrose to ethanol, cellulose, acetic acid, glucuronic acid, gluconic acid, and others [7-11]. The kombucha consumption is related to its functional properties and health-promoting effects: antimicrobial property [7, 11-14], antioxidant [11, 15-19], anticarcinogenic activity [11, 20], anti-diabetic [21-23], hepatoprotective [24-26], and hypocholesterolemic [16].

Polyphenols are present in kombucha composition through tea leaves. These compounds result from the secondary metabolism of plants being responsible for the protection of the plant body against thermal and water stress, ultraviolet radiation, attacks by herbivores, and others. The polyphenols are found in fruits and vegetables, in addition, to drinks derived from them, such as juice, tea, and wine [27]. They can be divided into two large groups, such as flavonoid and non-flavonoid [27-30].

The flavonoids group includes flavonols, flavones, flavanones, anthocyanins, flavanols, and isoflavones. Non-flavonoid compounds are phenolic acids derived from benzoic acid (gallic acid and protocatechuic acid) and cinnamic acid (coumaric acid, ferulic acid, caffeic acid, and others) [27]. The antimicrobial activity of polyphenols has been widely investigated. Compounds such as flavanols, flavan-3-ols, and tannins have shown a broad spectrum of action and higher antimicrobial activity when compared to other polyphenols. They have also been able to suppress microbial virulence factors, such as reduction of ligand adhesion in the host, inhibition of biofilm formation, and neutralization of bacterial toxins, in addition to demonstrating synergism with antibiotics [27]. Due to the antimicrobial properties, there are proposals for the development of food preservatives from polyphenols [27].

The composition of *Camellia sinensis* tea is similar to that found in the leaves. The content of phenolic compounds found in the leaves can reach 30% of the dry weight of the plant [31]. The chemical composition of the leaves is affected by several factors, such as edaphoclimatic conditions, preparation, and conditioning of the plant material. The catechin monomers are the most abundant flavonols in green tea. For black tea, the most abundant phenolic compounds are theaflavins and thearubigins [32-35].

Catechin, present mainly in green tea (*C. sinensis*), has antibacterial activity. For *in vitro* tests, it was able to inhibit the growth of several bacteria (*Campilobacter jejuni, Clostridium perfringes Escherichia coli, Streptococcus mutans,* and *Vibrio cholerae*) [27]. The flavonols, tannins, and non-flavonoids showed antimicrobial activity [33, 36-40]. The antimicrobial activity of phenolics can be attributable to both direct actions against microorganisms and the suppression of microbial virulence factors.

Studies on the mechanism of action of phenolic compounds against microorganisms suggest that compounds such as flavonol, flavan-3-ol, and flavolan classes damage the cytoplasmic membrane [41-43]. The flavan-3-ols and isoflavones can inhibit nucleic acid synthesis. This action can occur by inhibition of topoisomerase and/or dihydrofolate reductase [44-47]. Also, demonstrating inhibition of energy metabolism by inhibiting ATP synthase [48]. Currently, the search for antimicrobial agents from natural sources has increased, such as natural compounds from plant extracts. Thus, we aimed to evaluate the antibacterial activity of black and green tea kombucha by using the agar well diffusion method against foodborne pathogens.

2. MATERIAL AND METHODS

2.1 Kombucha fermentation

The inoculum (fermented broth and cellulosic pellicle) used for experiments was provided by an artisanal producer of Uberlândia, Minas Gerais, Brazil (18º 55' 07" S; 48º 16' 38" W). Fermentation was performed on infusion made by dissolving 1.5% (w.v^{-1}) of black or green tea leaves in water for 15 minutes [9, 49-52]. After boiling, 8.0% (w.v^{-1}) of sugar was added, and the infusion was filtered in a paper filter. The sweetened infusion was cooled at 25°C, and the inoculum was added with 10% (v.v^{-1}) fermented broth and 5% (w.v^{-1}) cellulosic pellicle. Processes were carried out in sterile glasses bioreactors (volume 3 L and diameter 12 cm). The bioreactors (glass flaskes) were filled up to 50% of full capacity and covered with cotton gauzes. Fermentation was carried out for 15 days at 28 °C ± 2 °C.

2.2 Microbial enumeration

The microbial population was monitored at the end of the fermentation. The samples were tenfold diluted in 0.1% peptone saline water and spread plated in MYP (Manitol Yeast Peptone) for acetic acid bacteria (AAB) and in GYMP (Glucose, Yeast Extract, Malt Extract, and Peptone) with 0.05% chloramphenicol for yeast enumeration [53]. All plates were incubated at 30 °C for five days; the relative humidity was maintained at 90% [53].

2.3 pH and Total Titratable Acidity

Total titratable acidity (TTA) was performed by titration with NaOH solution (0.1 mol. L^{-1}) [54]. The method for determining total titratable acidity is to neutralize the total acids present in the sample by using a base. The results were expressed as NaOH 0.1 mol.L^{-1} kombucha. The pH measurements were done at 25 °C using a pHmeter with a combined electrode (MS Tecnopon MPA 210) previously calibrated with pH 4.0 and 7.0 buffer solutions [55].

2.4 Preparation of antibacterial extracts

The antibacterial extract used for the test were: kombucha beverages (black and green tea), filtered kombucha, neutralized kombucha, acetic solution (4% v.v^{-1}), and kanamycin solution (30 µg.L^{-1}). For preparing filtered antibacterial extract, both kombucha beverages were filtered, separately, in milipore membrane pore size 0.22 µm (Kasvi, São José dos Pinhais-PR) [12]. The neutralized extract was prepared by neutralizing kombucha beverages with 1 mol. L^{-1} of NaOH until pH 6.5 [7]. The acetic acid solution was prepared to dilute 0,4 mL of acetic acid P.A (Labsynth) in 10 mL sterile distilled water. For kanamycin solution, the kanamycin (Himedia) was dissolved in distilled water and filtered in PES millipore membrane pore size 0.22 µm (Kasvi) [14].

2.5 Antibacterial activity

2.5.1 Bacteria strains

Strains of *Escherichia coli* (*E. coli*) 25922, enteropathogenic *E. coli*- EPEC (CDC 0126), enterohaemorrhagic *E. coli* - EHEC (CDC EDL-933), enteroinvasive *E. coli* - EIEC, enterotoxigenic E. coli - ETEC (H10407), *Salmonella Typhimurium* (ATCC 14028), *Shigella Flexneri* (ATCC 12002) e *Staphylococcus aureus* (ATCC 33591) were grown in nutrient broth at 35 °C. All microorganisms were provided by the Collection of Cultures of Mycology Laboratory, Department of Microbiology - UFMG (Minas Gerais, Brazil). Microorganisms kept Brain Heart Infusion (BHI) broth with 10% (v.v^{-1}) glycerol at -20 °C until use. All strains were evaluated for sensitivity to kanamycin solution (30µg. L^{-1}) [14]. The microorganisms used are standard strains and are commonly involved in processes of food poisoning.

2.5.2 Antibacterial tests

Bacteria cell concentration was standardized by optical density (OD) measurement in 0.5 (1.5×10^8 cells. mL^{-1}) of the standard McFarland scale [56]. Well Diffusion Method was used to assess the antibacterial activity of Kombucha [12, 13]. Müller Hinton agar were distributed in sterile Petri dishes, separately. After plates were set, a hole of 8 mm bores was punched aseptically with a sterile borer. The wells were filled up 50 µL antibacterial extract (see item 2.4). Before the test, all plates were stored in the refrigerator overnight for complete absorption of the extracts. After storage, the agar plates were inoculated in superficies with 100 µl of bacterial suspension adjusted at 0.5 McFarland scale. The inoculated Petri dishes were incubated at 36°C for 24h. After incubation, the diameter of the inhibition zone was measured.

Measurements were carried out, in triplicate, with a digital pachymeter (Mitutoyo, 500-19730B). All experiments were performed in duplicate. The results were analyzed by analysis of variance (Three Way Anova) and means were compared by Scott-Knott test at a 5% significance level.

3. RESULTS AND DISCUSSION

3.1 Characterization of antibacterial extracts

The pH values for kombucha beverages at final fermentation time (15 days) were pH 3.5 (black tea) and 3.4 (green tea). Total Titratable Acidity was 18 $g.L^{-1}$ for black tea and 15 $g.L^{-1}$ for green tea. The population of AAB and yeast was 10^6 CFU. mL^{-1} for both Kombucha. Filtered Kombucha showed the absence of microorganisms, pH ranged from 3.4 (black) to 3.6 (green), and TTA was 16.5 g. L^{-1} for black Kombucha and 13 $g.L^{-1}$ for green Kombucha. The pH for neutralized kombucha beverages varied from 6.7 (black) to 6.8 (green), and TTA was 1.5 g. L^{-1} for black Kombucha and 1.0 g. L^{-1} for green Kombucha. Also, the microbial population was reduced with neutralization: for yeast, the counts ranged from 10^3 $CFU.mL^{-1}$(green) to 10^4 $CFU.mL^{-1}$ (black), and for AAB, the counts varied from 10^4 $CFU.mL^{-1}$(green) to 10^5 $CFU.mL^{-1}$ (black). The pH for Acid Acetic Solution was 2.6. The filtration hasn't modified the pH of Kombucha [57]. However, the neutralization increased the pH, as expected. The same behavior was observed for TTA; the addition of NaOH modified the acidity of the kombucha beverage, which showed low values.

3.2 Antibacterial activity

The inhibition zone diameter of kombucha extracts against selected bacterial strains is shown in Table 1. Concerning Gram-Negative bacteria, the highest efficacy of Kombucha was found against enterotoxigenic *E. coli* ETEC (H10407). Whereas, *Salmonella* Typhimurium, *Shigella* Flexneri, and *Staphylococcus aureus* showed high resistance for kombucha beverages of black and green tea (Table 1).

E. coli was the most inhibited pathogen by both fermented beverages. For kombucha beverages, the inhibition zone diameter for E. coli ranged from 14.93 mm to 17.88 mm for black tea and 15.11 mm to 17.3 mm for green tea kombucha. Filtered Kombucha showed similar behaviour, the inhibition zone varied from 14.16 mm to 17.52 mm. However, for *Salmonella* Typhimurium, *Shigella* Flexneri and *Staphylococcus aureus* the filtered kombucha didn't show potential of inhibition.

Among the serotypes analyzed for *E. coli* the natural kombucha and filtered kombucha didn't statistical difference ($p<0.05$) for inhibition zone diameter, except for EPEC serotype.

The antimicrobial activity of Kombucha could be attributed to low pH and high TTA (18 $g.L^{-1}$(black tea) and 15 $g.L^{-1}$ (green tea)). The antimicrobial properties of organic acids have been widely studied scientifically [58-61]. In fermented Kombucha, the presence of a high concentration of gluconic acid and acetic could explain the higher inhibition of pathogenic microorganisms [60].

The neutralized beverage (black and green tea) didn't show antibacterial activity for anyone tested microorganisms. Although neutralized Kombucha did not show antibacterial activity, some authors showed that phenolics might act as antimicrobials through phenolic-membrane interaction, DNA gyrase inhibition, and metal sequestering [46, 62, 63]. On the other hand, some studies [7, 12-14] present little effect or absence of antibacterial activity for neutralized fermented beverages. These authors suggest that antibacterial effects are due to organic acids, mainly acetic acid. Also, lower pH is useful to control for pathogenic bacteria. Moreover, the mechanism of action is not entirely known; non-dissociated acetic acid can increase lipid solubility, allowing an increased accumulation of fatty acids in the cell membrane or other cell wall structures.

Table 1: Inhibition zone diameter (mm) for antibacterial extracts.

	Positive Control		**Green Kombucha beverage**			**Black Kombucha Beverage**		
Strains	**Kanamycin (30 µg.L^{-1})**	**AAS**	**Kombucha**	**Filtered**	**Neutralized**	**Kombucha**	**Filtered**	**Neutralized**
***Escherichia coli* (ATTC 25922)**	23.95a	18.89b	15.75c	15.38c	0.0d	15.07c	15.57c	0.0d
EPEC (CDC 0126)	21.25a	18.0b	17.3b	16.23c	0.0d	15.86c	15.65c	0.0d
EIEC	22.5a	21.3a	15.11b	15.46b	0.0c	14.93b	14.16b	0.0c
EHEC (CDC EDL-933)	20.0a	17.65b	17.17b	17.52b	0.0c	16.5b	15.85b	0.0c
ETEC (H10407)	24.3a	20.11b	16.63c	16.73c	0.0d	17.88b	17.75b	0.0d
***Salmonella* Typhimurium *(*ATCC 14028)**	15.0	12.0	0.0	0.0	0.0	0.0	0.0	0.0
***Shigella* Flexneri (ATCC 12002)**	16.2	15.0	0.0	0.0	0.0	0.0	0.0	0.0
***Staphylococcus aureus* (ATCC 33591)**	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Means with equal letters on the same line for each treatment (tea) do not show statistical differences (p <0,05). 0.0= not detected inhibition zone AAS: Acetic Acid Solution (4%).

The positive controls used in this study showed high inhibition for pathogens, except for *Staphylococcus aureus*. The inhibition zone for acetic acid solution ranged from 12.0 mm to 21.5 mm. The effectiveness of pathogen control is for lower pH and high concentration of acetic acid. Also, kanamycin solution inhibited all pathogens. The higher inhibition zone was 24.3 mm for *E. coli* ETEC (H10407), and the lower inhibition zone was 10 mm for *Staphylococcus aureus*.

Notwithstanding, the neutralized Kombucha did not show an antimicrobial effect. The kombucha beverage showed a high inhibition effect for *E. coli* (~15.0 mm) when compared with the acetic acid solution (18.89 mm) and Kanamycin (23.95 mm). The interaction with organic acid and tea catechins work synergistically to inhibit *E. coli* [64-66]. Catechins have a synergistic effect with antibiotics such as amoxicillin, azithromycin, chloramphenicol, gentamicin, levofloxacin, sulfamethoxazole, and others [67]. Catechins and fractions showed efficacy to inhibit the microorganisms, especially *E. coli*. The inhibition occurs by different mechanisms such as damage to the bacterial membrane [65, 68], damage to the cytoplasmic membrane [69], and action of hydrogen peroxide by catechin fractions [70]. The triple interaction between tea, bacterium, and treatments was significant ($p < 0.01$), and therefore the Scott-Knott mean test was applied to assess the alterations within each tea and bacterium.

4. CONCLUSION

Natural kombucha, and filtered kombucha of black and green tea were able to inhibit all the *E. coli* serotypes tested, and the neutralized kombucha didn't show antibacterial potential. Antibacterial activity of kombucha beverages can be related to low pH and organic acids, such as acetic acid and gluconic acid. The presence of catechins might work synergistically with other compounds present in Kombucha. Future research can be done to evaluate the potential of Kombucha as an effective antimicrobial control for emerging multidrug-resistant microorganisms.

5. ACKNOWLEDGMENTS

The authors thank Universidade Federal de Minas Gerais -UFMG, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – finance code 001 for the Doctoral Program Sandwich Abroad (Cosme D. Barbosa) [88881190024/2018–1, 2018].

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