CHARACTERIZATION OF WATER-TYPES AND THEIR INFLUENCE ON THE ANTIMICROBIAL PROPERTIES OF KOMBUCHA FERMENTS AGAINST BACTERIA AND YEAST

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ABSTRACT

Understanding the relationships between diet, gut microflora, and health is an increasingly important area of research. Recent studies have demonstrated that Kombucha tea provides variable antimicrobial activity against pathogenic microbes. In this study, we tested Kombucha tea for antimicrobial activity against various Gram-positive and Gram-negative bacteria, as well as yeast, using an agar diffusion method. Standard zone of inhibition assays were used to test the hypothesis that variance in antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* may be due to the varying levels of cations, like Ca^{2+}, found in different water-types (well water, artesian water, city water, type-II water and distilled water). Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) results indicated that high cationic (Ca^{2+}, Mg^{2+}, and Na^{+}) content water (well water) resulted in the largest zone of inhibition against *S. aureus*, with a 12.3% difference when compared to low cationic content water (type-II water). *E. coli* maintained a constant zone of inhibition regardless of water-type or batch-type, while *C. albicans* showed no zones of inhibition. Inhibition is either through a synergistic relationship with the pH conditions, the other cations present (Mg^{2+}, Na^{+}, Si, etc.) or a mix of both, as pH in the range of 4.5 – 3 is not enough to inhibit the growth of *S. aureus*. These results indicate that a direct relationship exists between cationic concentrations of water used to prepare Kombucha, and antibacterial activity against *S. aureus*, due to the improved fermentation of the tea with high concentrations of cations. Strong antimicrobial potential exists, particularly against *S. aureus*, which may be useful in determining novel approaches to synthesize antimicrobial drugs. Further study is needed to assess other *S. aureus* strains, as well as to determine how this relationship translates to human microbiota interactions and their microbial metabolic profiles.

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KEYWORDS

- Fermented tea
- Kombucha
- Antimicrobial
- Cation

INTRODUCTION

Kombucha is a fermented tea produced with the help of a symbiotic culture of bacteria and yeast (SCOBY). The genus *Acetobacter* is an aerobic, nitrogen-fixing bacteria which produces acetic acid, gluconic acid, and cellulose. *Saccharomyces* is an aerobic to facultatively anaerobic, single celled yeast that produces ethanol and carbon dioxide. These two microorganisms have synergistic roles and are the most common components of SCOBY. The microbial composition of the SCOBY can vary and is dependent upon the origin of the culture (3). *Acetobacter* produces cellulose, which is seen as a thin white film originating on the top of the tea as early as the first day of fermentation (1). This is when the bacteria and yeast cell mass accumulates to begin the synergetic fermentation process. The synergy of the yeasts cleaving the sugar and bacteria producing the acidic components is what establishes the unique cider-like carbonated characteristics of Kombucha. *Saccharomyces* strains cleave sucrose into glucose and fructose, with the former used to produce ethanol and carbon dioxide. *Acetobacter* strains then oxidize ethanol to acetaldehyde and then into acetic acid (5). Glucose also leads to the production of cellulose and gluconic acid. The acidic levels attained during fermentation (pH 3.0 – 2.5) result in unsuitable growing conditions for most microorganisms, reducing contamination (14). Kombucha has been used as a beneficial health drink for several millennia. It has been suggested that Kombucha aids in digestion, prevents microbial infections, can vitalize the physical body, increase the efficacy of the gastrointestinal tract, and is believed to enhance immunity (8). The popularity of Kombucha in the United States has increased within the last 15 years, with annual reports showing Kombucha sales expected to surpass $500M by the end of 2015 (10). This increase in popularity highlights the importance of the overall health implications of Kombucha. Kombucha has been identified as having antimicrobial properties against *Helicobacter pylori*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Agrobacterium tumefaciens*, *Bacillus cereus*, *Shigella sonnei*, *Salmonella enteritidis* and *Escherichia coli* (8,12,15). No previous study has characterized water-types and identified its relationships with Kombucha ferments relative to its antimicrobial activity.

MATERIALS AND METHODS

WATER-TYPES

Five water types were selected on the basis of cationic concentration: 1.) Well water taken from a private well located in Prescott, WI USA; 2.) Artesian water purchased from a local Minnesota based company – Artesian Fresh (LeRoy, MN USA); 3.) City water taken from a tap located in Hennepin County, Minneapolis, MN USA; 4.) Type-II water; 5.) Distilled water from Millipore water purification systems. Water-types were analyzed for the major cations on a Thermo Scientific iCAP 6500 Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) at the University of Minnesota's Analytical Geochemistry Laboratory. Triplicate measures were taken on each water sample, with the mean of each cation determined. High levels of Ca^{2+}, Mg^{2+}, and Na^{+} were identified in well water and

artesian water, comparative to World Health Organization drinking water guidelines (7). Silicon also appears with interesting values, but due to the higher levels of Ca^{2+}, Mg^{2+}, and Na^{+} relative to Si, and due to the biological role of these cations within cellular processes, this study focused on these cations, primarily Ca^{2+}.

KOMBUCHA CULTURE (SCOBY)

Kombucha cultures were purchased from Anahata Balance at Organic-Kombucha.com (Buckley, MI USA). The primary strains used in these cultures were *Acetobacter xylinum* and *Saccharomyces boulardii*. The composition of the disks was specially constructed by Anahata Balance to fit the fermentation containers used, to prevent cross-contamination. Each culture was then selected to ferment in only one water-type. A total of five cultures were assessed, one for each water-type in the experimental batches (as control batches did not have any cultures).

PREPARATION OF TEA FERMENTS

The recipe provided by Anahata Balance (Fig. 1) was used to prepare solutions. Kombucha was prepared by infusing 75.0g of organic sucrose (10% w/v) with 3.30g of hibiscus tea (0.44% w/v) into 750mL of boiling water in a 1L beaker, and allowed to steep for 20 min. Organic sucrose and hibiscus tea were purchased from The Wedge food co-op (Minneapolis, MN USA). The contribution of the tea's antimicrobial activity to Kombucha ferments is shown to be insignificant (13) and as such, hibiscus tea was selected. Five experimental Batches (EB) and five Control Batches (CB) were prepared, each relative to the corresponding water-type. CBs were identical to the EBs except no Kombucha culture was added (only water, sugar, and tea). After homogeneity of the sugar tea solutions in the EBs and CBs, the tea solutions were cooled to ambient temperature (20°C) to allow for the addition of Kombucha cultures to the EBs, in order to prevent harming the cultures. Necessary pH sensor measurements were taken to ensure all EBs and CBs started the fermentation process at pH 4.5. EBs and CBs were then placed into a sterilized Thermolyne Type 4200 Incubator at 30°C for 8 – 9 days (pH dependent) until a pH of approximately 3.0 was reached in the EBs (as no change in pH would be noted in CBs). CBs pH was then adjusted to 3.0 with 1.0M acetic acid, which later acts as the pH controls. All pH determinations for the EBs and CBs were measured using a Vernier pH Sensor and Vernier LabQuest Palm Pilot. EBs and CBs were covered with Parafilm and then placed in a walk-in cooler for later experimentation.

EXPERIMENTAL TEST MICROORGANISMS

Two bacterial species were chosen to test against the antimicrobial activity of Kombucha. One Gram-positive bacteria: *Staphylococcus aureus ATCC 27661* and a Gram-negative bacteria: *Escherichia coli ATCC 11303*. Both were provided by MCTCs Department of Microbiology teaching stocks. *S. aureus* and *E. coli* were cultivated on Nutrient agar (NA) at 37°C for 24h.

A common human pathogenic yeast strain was chosen to test against the antimicrobial activity of Kombucha. *Candida albicans* SN76 was provided by Dr. Kirkpatrick at the University of Minnesota.

OPTICAL DENSITY DETERMINATION

Optical density (OD) determinations were measured with a Vernier Spectro Vis-plus at 600nm, on a Vernier LabQuest

Instructions: To make a 1 gallon batch of Kombucha tea

A. Bring 1 gallon of distilled or filtered water to a boil. After the water starts to boil, slowly add 1 to 1 ½ cups of organic cane sugar.
Stir & cover solution, then simmer for another 10 min. or until sugar is completely dissolved. Tea/sugar solution may be boiled in metal or glass container. It is suggested not to use non-organic sugar, honey, or maple syrup, agave, etc. when brewing kombucha. Alternative sugars may not provide the kombucha yeast cultures with the correct food source and is not recommended.

B. Remove tea solution from heat. Add 6 tea bags (6 teaspoons) worth of green tea or black tea and allow solution to steep for 10 minutes. After 10 min. remove tea bags, cover, and allow solution to cool to room temperature. Always keep tea solution securely covered with a tight weave cloth.

C. Transfer tea solution to a glass container for brewing the Kombucha culture, never allow metal or ceramic to have long term contact with the culture. *IMPORTANT NOTE: ADD KOMBUCHA S.C.O.B.Y. MUSHROOM and STARTER LIQUID ONLY AFTER TEA HAS COOLED TO ROOM TEMPERATURE.*
Notes: Make sure not to fill the brewing jar to full (it should be level with the straight side of the jar not up into the rounded edge. Make sure that the smoother white side of mushroom faces upwards, it may float or sink, and this is acceptable. However, by gently placing the culture on top of the tea it should stay towards the top. If it does sink, the culture should start to rise to the surface as gases are forming in the fermenting tea. Securely cover container with cloth (the culture needs to breath) and allow the inoculated tea solution to set undisturbed in a warm place out of direct sunlight.

D. Tea will be ready to drink after 5 to 14 days of fermentation, depending on temperature (around 75 –85 deg F is ideal) and amount of sugar added (more sugar = more time). If checking pH of a finished batch of tea, it should be around 3; a reading of 4 is to high and 2 too low. Checking pH can greatly reduce the chance of contamination of the culture and is highly recommended.

E. To make new batch of tea, make sure to save 10–15% of the old tea solution (as a starter to lower pH below 4.6) and one or two layers of the kombucha mushroom. When it is time for a new batch, just follow the directions above to start the process again.

Figure 1. Kombucha Mushroom Tea Culture Starter Recipe (AnahataBalance.com)

Palm Pilot. Distilled water was used for calibration and for blank samples. A McFarland Standard of 0.5 was prepared using ASM McFarland Standards SOP 5.14.1 (2). Bacterial and yeast colonies were transferred to 10mL of sterile distilled water and vortexed. After vortexing, OD_{600} determinations were measured until each sample reached the target density at or around 0.20. Each run included 2 blanks, 2 standards, 1 *S. aureus*, 1 *E. coli*, and 1 *C. albicans*. One run per zone of inhibition assay, three runs total, were performed.

AGAR DIFFUSION METHOD

The antimicrobial activity of Kombucha was determined using an agar diffusion method. Kombucha EBs and CBs were removed from the cold storage and allowed

to equilibrate to ambient temperature (20°C). Agar plates (100mm) were purchased "pre-poured" from Teknova (Hollister, CA USA). Mannitol Salt Agar (MSA) plates were used for *S. aureus* inoculations, Luria broth (LB) agar plates were used for *E. coli* cultivation, and Yeast Mannitol (YM) agar plates were used for *C. albicans*. Agar plates were inoculated using a spreading technique to achieve lawn growth throughout with use of sterile cotton wool swabs instead of glass spreaders. Thirty Millipore absorbent disks (25mm) were then sorted appropriately, three disks per EB water-type, and three disks per CB water-type (hence 30 total) with 1mL of the appropriate correlating batch. This was to ensure duplicate and triplicate measurements were taken. All appropriate general lab practices were followed to prevent cross-contamination of one batch to another during disk saturations. Once disks became saturated they were placed in the center of the inoculated agar plate, one disk per plate (3 inoculated plates per batch type, 30 total). Plates were then covered and placed into a NorLake Scientific Incubator at 37°C for 72 h. Agar plates were then removed from the incubator and zone of inhibitions were measured with a standard metric ruler. Measurements included the diameter of the clearings end-to-end, disregarding the disk in the center. All zone of inhibition assays were carried out in triplicate and analyzed statistically using 1-way ANOVA ($p<0.05$).

RESULTS AND DISCUSSION

CATIONIC RELATIONSHIPS

Results of the ICP-OES water-type analysis are shown in Table 1. High levels of Ca^{2+} (104 ppm), Mg^{2+} (56 ppm) and Na^{+} (77 ppm) were observed in well water samples relative to the other water-type samples (Fig. 2). Of the 11 major cation concentrations determined in all five water-types, the three cations noted above (Ca^{2+}, Mg^{2+}, Na^{+}) demonstrated the largest variance throughout; in addition a gradient trend of Ca^{2+} concentration levels was observed in the water-types and seemed to correlate with the zone of inhibition measurements in the *S. aureus* plates. Due to Ca^{2+} exhibiting the highest concentration levels, this study will focus on that cation.

Every metal cation has its own unique system to support prokaryotic and eukaryotic cellular regulation and growth requirements. Of the most abundant cations required for these conditions (Ca^{2+}, Mg^{2+}, and Na^{+}), it is calcium that plays the universal role as a messenger, transmitting signals from the cell surface to the cytoplasm (6). Calcium is also associated with the regulation of many types of cellular processes relative to cell differentiation, transport, motility, gene expression, metabolism, cell cycle effects, cell division, and pathogenesis (11).

To date, a number of bacterial surface proteins have been implicated in various calcium transport mechanisms (11). In *S. aureus*, this includes a biofilm-associated protein (Bap), a *S. aureus* surface protein essential for biofilm formation. Studies have shown that *S. aureus* biofilm matrices are assembled by Bap proteins (16). Under controlled conditions, Bap proteins are also shown to become inhibited by

Table 1: Results of the ICP-OES analysis on the water-types evaluated in this study (+/- SD).

	Water type				
	Well Water	Artesian Water	City Water	Type-II Water	Distilled Water
Al (ppb)	4.3 ± 1.0	6.3 ± 0.8	8.8 ± .0.8	1.0 ± 1.5	6.5 ± 0.6
Ba (ppb)	42.5 ± 0.8	105.0 ± 1.2	7.2 ± 0.3	0.3 ± 0.5	0.0 ± 0.5
Ca (ppb)	103716.7 ± 231.4	72122.0 ± 214.3	22332.0 ± 54.4	117.9 ± 3.7	-1.7 ± 11.2
Fe (ppb)	13.1 ± 0.9	1.0 ± 0.9	10.0 ± 0.9	1.6 ± 0.7	0.0 ± 1.2
K (ppb)	1201.8 ± 11.7	1219.0 ± 7.8	2561.1 ± 9.9	4.0 ± 0.6	5.0 ± 4.6
Mg (ppb)	56462.0 ± 53.3	23091.0 ± 166.1	6518.4 ± 21.3	7.8 ± 0.7	3.1 ± 8.7
Mn (ppb)	8.3 ± 0.2	4.8 ± 0.1	0.3 ± 0.1	5.6 ± 0.1	0.2 ± 0.2
Na (ppb)	77140.0 ± 145.8	3546.2 ± 28.2	16581.3 ± 36.7	9.0 ± 71.8	-26.3 ± 9.1
P (ppb)	4.4 ± 3.8	0.2 ± 3.8	281.1 ± 5.6	2.3 ± 1.6	-0.1 ± 2.4
Si (ppb)	8398.4 ± 45.7	5778.7 ± 32.4	4352.1 ± 8.6	5.0 ± 1.4	59.0 ± 4.0
Sr (ppb)	88.2 ± 0.6	121.7 ± 0.8	54.9 ± 0.2	0.2 ± 0.0	0.2 ± 0.5

calcium treatments, thus preventing any Bap-mediated biofilm formation (4) and allowing S. *aureus* to become susceptible to any number of exposures that can lead to cellular complications. As the concentration of calcium increases, so do the zones of inhibition by S. *aureus*. This result seems to be a response specific to the calcium concentrations seen on the ICP-OES in the water-types, and not pH alone, as the CBs were used as pH controls. S. *aureus*, when exposed to the CBs, saw zero detectable inhibition, which tells us that a pH of 3.0 does not inhibit S. *aureus*. Calcium could be playing a direct role, either through a synergistic relationship with the pH or with the other cations present (Mg^{2+} & Na^{+}) or a combination of these effects.

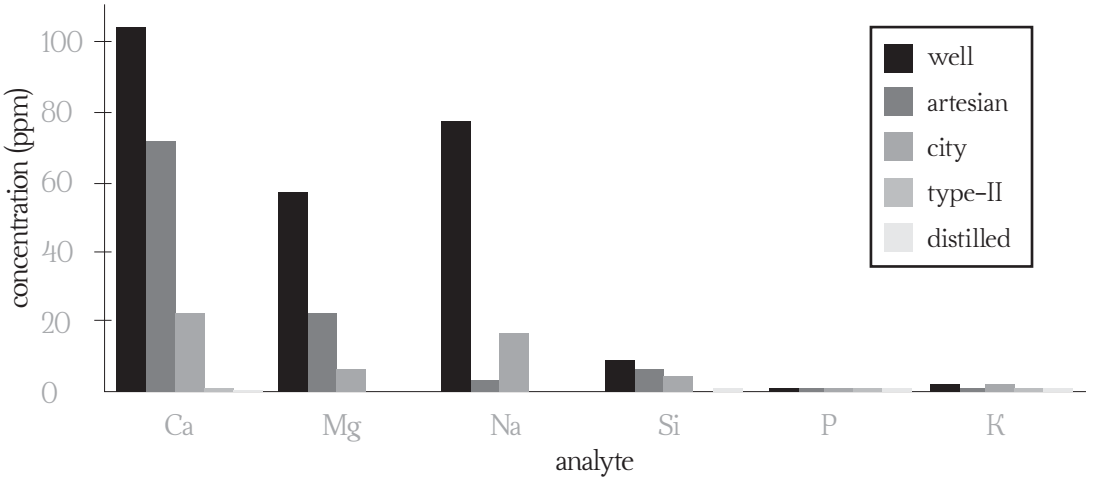
CHANGES IN PH DURING FERMENTATION

The changes in pH during fermentation for the CBs and EBs are shown in Fig. 3. In the first three days of fermentation, CBs observed the greatest change in pH, 4.5 to 3.3, a 1.2 pH difference, while the EBs maintained their initial pH throughout the 8-day period, suggesting no microbial contamination occurred. The initial CBs pH drop could be relative to the high availability of glucose for yeast and bacteria, which ultimately leads to the production of acetic acid and gluconic acid, decreasing the pH. As glucose gets consumed, the availability becomes limiting, thus resulting in a slower change of pH.

ANTIMICROBIAL ACTIVITY OF THE FERMENTS

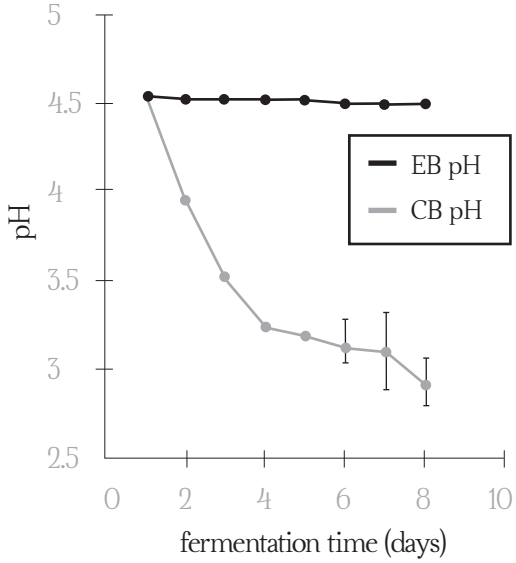
The antimicrobial activity of the EB and CB treatments against bacteria and yeast strains are shown in Figs. 4 and 5. Results indicate that high-ion (Ca^{2+}, Mg^{2+}, Na^{+}) content water (well water) resulted in the largest zones of inhibitions at 35.7 mm for S. *aureus*, with a 12.3 percent difference when compared to near zero ion content water

Figure 2: Comparison of the ICP-OES major cation results relative to water-types.



(type-II water) at 31.3 mm. *E. coli* maintained a constant zone of inhibition regardless of water-type or batch-type, while *C. albicans* showed no zone of inhibition outcomes. Six different sets of the antimicrobial activity of Kombucha EB treatments against S. *aureus*, (Table 2) were used to calculate percent change, with the presumption that well water EBs = 100 percent zone of inhibition. Relative to well water, type-II water had the lowest standard deviation value of +/-2.07. EBs and CBs had zero detectable effect on *C. albicans*, as aggressive growth was seen throughout the plate, even on the absorbent disks. These results indicate a direct relationship may exist between Kombucha prepared in high-Ca^{2+} content water and the antibacterial activity of Kombucha ferments against S. *aureus*. It is also suggestive of further study to determine how this relationship applies to other pathogenic strains. This behavior is indicative of a gradient trend, with an R^2 value of 0.9604, which seems to correlate well with the Ca^{2+} concentration levels identified within these water-types. The *E. coli* strain displayed constant zone of inhibitions regardless of batch-type, with a mean variance of 43-49mm. Both S. *aureus* and *E. coli* results

Figure 3: After fermentation, CBs were adjusted to a pH of 3.0 for comparative purposes against EBs.



were pH independent for EBs and CBs. We can conclude that something other than the acidic conditions must be inhibiting S. *aureus*. One possibility could be the varying levels of cations, especially Ca^{2+}, in the water-types. Distilled water is the gold standard source of water when making Kombucha, and as such, understanding the

Table 2. Comparison of six different EB treatment sets of the antimicrobial activity against *S. aureus*.

	S. aureus zone-of-inhibition %-change								
Water type	set-1	set-2	set-3	set-4	set-5	set-6	Mean	Standard deviation	Standard error of mean (SEM)
Well water	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
Artesian water	0.00	5.70	0.00	12.50	14.60	7.70	6.75	6.13	1.02
City water	11.1	5.7	13.9	5.4	11.0	5.1	8.7	3.8	0.6
Type-II water	11.1	11.4	13.9	10.2	12.2	7.7	11.1	2.1	0.4
Distilled water	13.9	20.0	19.4	5.4	2.4	2.6	10.6	8.2	1.4

Figure 4. Antimicrobial activity of Kombucha ferments versus controls.

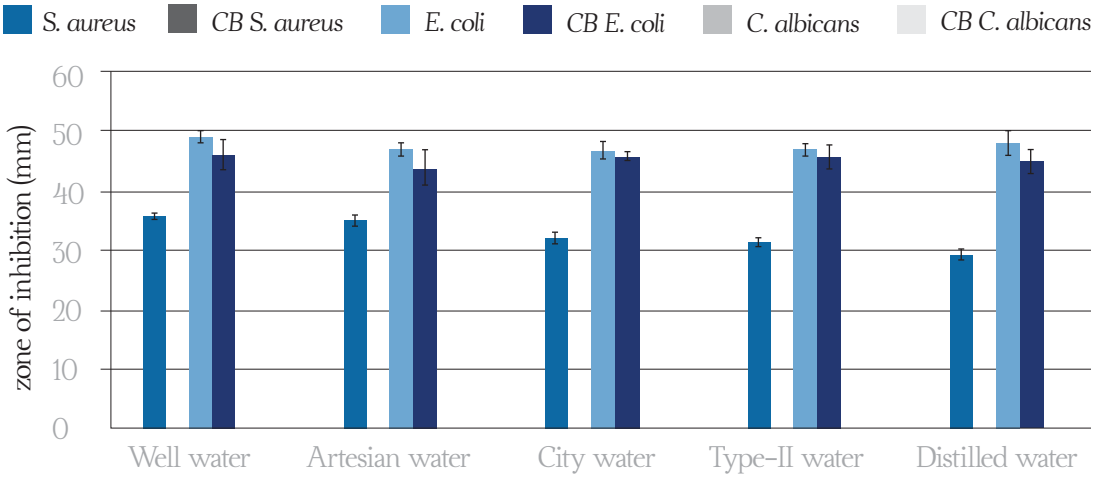
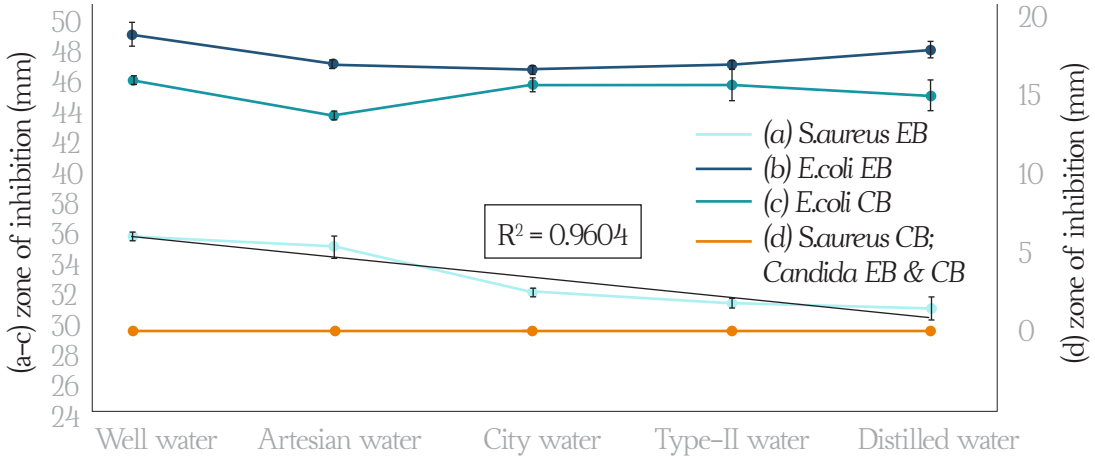


Figure 5: Zone-of-inhibition results of EB and CB treatments against *S. aureus*, *E. coli* and *C. albicans*



antimicrobial properties of Kombucha in response to the addition of metal ions is still yet unknown and unidentified.

Since no detectable zone of inhibitions were observed in S. *aureus* CB treatments and in *C. albicans* EB and CB treatments, statistical data only represent the remaining treatment scenarios. Because Ca^{2+} concentrations were much higher in well water and artesian water relative to the other water.

CONCLUSION

The antimicrobial effects of Kombucha are reflected as having no inhibition for *C. albicans*, and a constant zone of inhibition for *E. coli* regardless of EB or CB treatments. S. *aureus* displayed the greatest potential, with zone of inhibitions decreasing in size linearly relative to the cationic concentrations measured by the ICP-OES in each of the five water-types. This is indicative that a Ca^{2+} relationship may exist due to the same linear fashion it displayed in its concentration levels decreasing in each of the water-types (well water > artesian water > distilled water). It should also be noted that Mg^{2+} and Si also exhibited linear drops in concentration levels relative to water-types, but due to being less than the Ca^{2+} levels and not playing as much a role within cellular systems as Ca^{2+}, they were not the focus of this paper. Every metal cation has its own unique system to support prokaryotic and eukaryotic cellular regulation and growth requirements. However, it is calcium that plays the universal role as a messenger, transmitting signals from the cell surface to the interior of the cell (6). Calcium is also identified as regulating many types of cell processes relative to cell differentiation, transport, motility, gene expression, metabolism, cell cycle, cell division and pathogenesis (11). Within that, a number of bacteria, surface proteins, many number of these factors have been identified and shown to be involved in various calcium transport mechanisms (11). On S. *aureus* one such component has been identified as a biofilm-associated protein (Bap), a S. *aureus* surface protein that is essential for biofilm formation. One particular study shows that S. *aureus* biofilm matrices assembled by Bap proteins protect the bacteria from antimicrobial treatments (15), and under controlled conditions, these Bap proteins become inhibited by calcium treatments, thus preventing any Bap-mediated biofilm formation (4) and in return allowing S. *aureus* to become susceptible to various environmental stressors. As the concentration of Ca^{2+} increases, so does the zone of inhibitions in S. *aureus*. This result appears to be a response specific to the calcium concentrations measured in the water-types, and not pH alone, as the CBs were used as pH controls, and as such, when S. *aureus* was exposed to the CBs, zero inhibition took place. This tells us that a pH of 3.0 is not enough to inhibit S. *aureus*. There must be a direct role that these cations are having on the Kombucha during fermentation to allow inhibition, possibly calcium, either through synergistic relationships with the Kombucha SCOBY, pH or with the other cations (Mg^{2+}, Na^{+}, Si). No Ca^{2+} control was conducted, so it is difficult to say with certainty how much of a role Ca^{2+} is really having on S. *aureus*. It is suggested that if this study is replicated, that cationic controls be inserted.

S. *aureus* is a major opportunistic human pathogen, able to cause a wide range of

diseases in humans and in animals (6), which is why it is important to further investigate these relationships. Further study is needed to determine how these cations are affecting Kombucha fermentation, how it is interacting with S. *aureus*, and then whether or not the same results apply to other S. *aureus* strains, specifically methicillin resistant S. *aureus* (MRSA).

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