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Enhancement of Antioxidant Activity and Fermentation Kinetics of Kombucha using Graviola Cheong: A Comparative Study

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ABSTRACT

Aims: The study focuses on the fermentation of kombucha using graviola fruit infusion/Cheong, this study aimed to develop a layered fermentation approach for kombucha production through secondary fermentation utilizing an ancient Korean fermentation syrup known as Cheong including comparative studies on its antioxidant properties and bioactivity against standard kombucha.

Study design: This study employed an experimental design to evaluate the effects of incorporating graviola cheong during secondary fermentation, comparative studies were performed against standard kombucha and Graviola Cheong-infused kombucha subjected to quantitative and qualitative analysis, including pH, specific gravity, brix, titratable acidity, total phenolic content, antioxidant properties and microbial analysis, including sensory evaluation of the layered kombucha.

Place and Duration of Study: Padmashree institute of Management and Sciences Department of Food, between January 2025 and February 2026

Methodology: Primary fermentation was carried out using SCOBY (symbiotic culture of bacteria and yeast) for 7days in room temperature. During secondary fermentation, Cheong prepared using 50% Graviola concentrated with 50% sugar is introduced to create an additional layered fermentation.

Results: The layered fermentation demonstrated significant improvements in antioxidant activity, enhanced sensory complexity compared to standard kombucha formulations. The incorporation of Cheong increased the bioactive compound retention and developed a unique flavor profile while maintaining high shelf life of the fermented product and microbial stability.

Conclusion: This study demonstrates the potential of layered fermentation as an innovative approach for developing value-added kombucha beverages with improved functional and commercial applications in the fermented beverage industry.

Kombucha, Layered fermentation

1. INTRODUCTION

Annona muricata is a member of the Annonaceae family and is a fruit tree with a history of usage in traditional medicine. *Annona muricata* is locally referred to as soursop or graviola and is an evergreen plant that is mostly distributed in tropical and subtropical regions around the world. *Annona muricata* is mainly a heart-shaped fruit, with its leaves, roots, and seeds known to have beneficial properties in traditional medicine. The fruit of *Annona*

muricata is exclusively used in the preparation of syrups, candies, beverages, ice creams and shakes. *muricata* has also been used in brewing wine in several countries such as South America, Indonesia etc. (Soheil Zorofchian Moghadamtousi et; al.,2015).

A wide array of ethnomedicinal activities is studied and employed for different parts of *Annona muricata*, and indigenous communities in Africa and South America extensively use this plant in their folk medicine. Numerous investigations have substantiated these activities, including anticancer, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic activities. Recent studies have shown antidepressant properties in the extracts of *Annona muricata* leaves and fruit pulps. Phytochemical studies reveal that annonaceous acetogenins are the major constituents of *Annona muricata*. Recent studies have identified more than 100 annonaceous acetogenins that are generally characterised as a family of natural products with antitumor, anti-diabetic and antidepressant activities. Roots, leaves, barks, fruits and seeds of Graviola have been widely used in alternative medicine for many purposes. (Soheil Zorofchian Moghadamtousi et; al.,2015).

In the Peruvian Andes for example, the Graviola leaves are used to combat parasites and treat diabetes. Reports in the literature indicate that seventy-four of these bioactive compounds exhibit a variety of anticancer effects in preclinical cell culture and animal model systems. Several dozen annonaceous acetogenins have been studied. In addition, extracts from fungi (*Periconia* sp.) collected on *A. muricata* that contain bioactive compounds have been tested for their anticancer properties and other health benefits. Despite the documented nutritional and therapeutic properties of *Annona muricata*, its application in fermented functional beverages remains limited. Kombucha, a tea fermented probiotic beverage, provides an ideal medium for incorporating bioactive fruit derivatives. In this study, a layered fermentation technique involving the incorporation of Graviola cheong during secondary fermentation was observed to develop a differentiated functional beverage with enhanced antioxidant and physicochemical properties.

1.1 Background of study

The global demand for functional foods has surged as consumers increasingly seek dietary options that offer therapeutic benefits beyond basic nutrition. Within this genre fermented beverages have emerged as a premier market. **Kombucha**, a sparkling beverage produced through the fermentation of sweetened tea by a **Symbiotic Culture of Bacteria and Yeast (SCOBY)**, is famous for its probiotic properties, essential organic acids, and metabolic enzymes that support gut health and mobility. *Annona muricata* has considerable attention in nutritional science due to its exceptional phytochemical profile, which is primarily potent with antioxidant and anti-inflammatory properties, primarily attributed to its elevated concentrations of bioactive secondary metabolites. These phytochemicals actively scavenge reactive oxygen species (ROS) and downregulate cellular inflammatory pathways, presenting significant utility in mitigating oxidative stress-mediated chronic conditions. Despite these profound therapeutic attributes, the commercial, industrial, and clinical exploitation of soursop is severely restricted by its intrinsic physiological and agronomic vulnerabilities. It is considered to be strictly seasonal and exceptionally short post-harvest shelf life, making it susceptible to rapid microbial spoilage, enzymatic degradation. Furthermore, conventional thermal preservation methodologies such as pasteurisation or dehydration frequently induce irreversible thermal degradation that can affect bioactive components and their functional properties.

1.2 Botanical Description and Distribution

Annona muricata, also known as guanabana, paw-paw, sirsak, or soursop, belongs to the family Annonaceae, which comprises 130 genera and 2300 species. *Annona Muricata* is widely distributed throughout the subtropical and tropical regions of the world across North and South America, including South Asian countries such as India, Malaysia, and Indonesia. Graviola is an evergreen terrestrial erect tree growing up to 5-8 Meters in height, featuring an open, roundish canopy with dark and glossy leaves.

1.2.1 Ethnomedicinal Uses

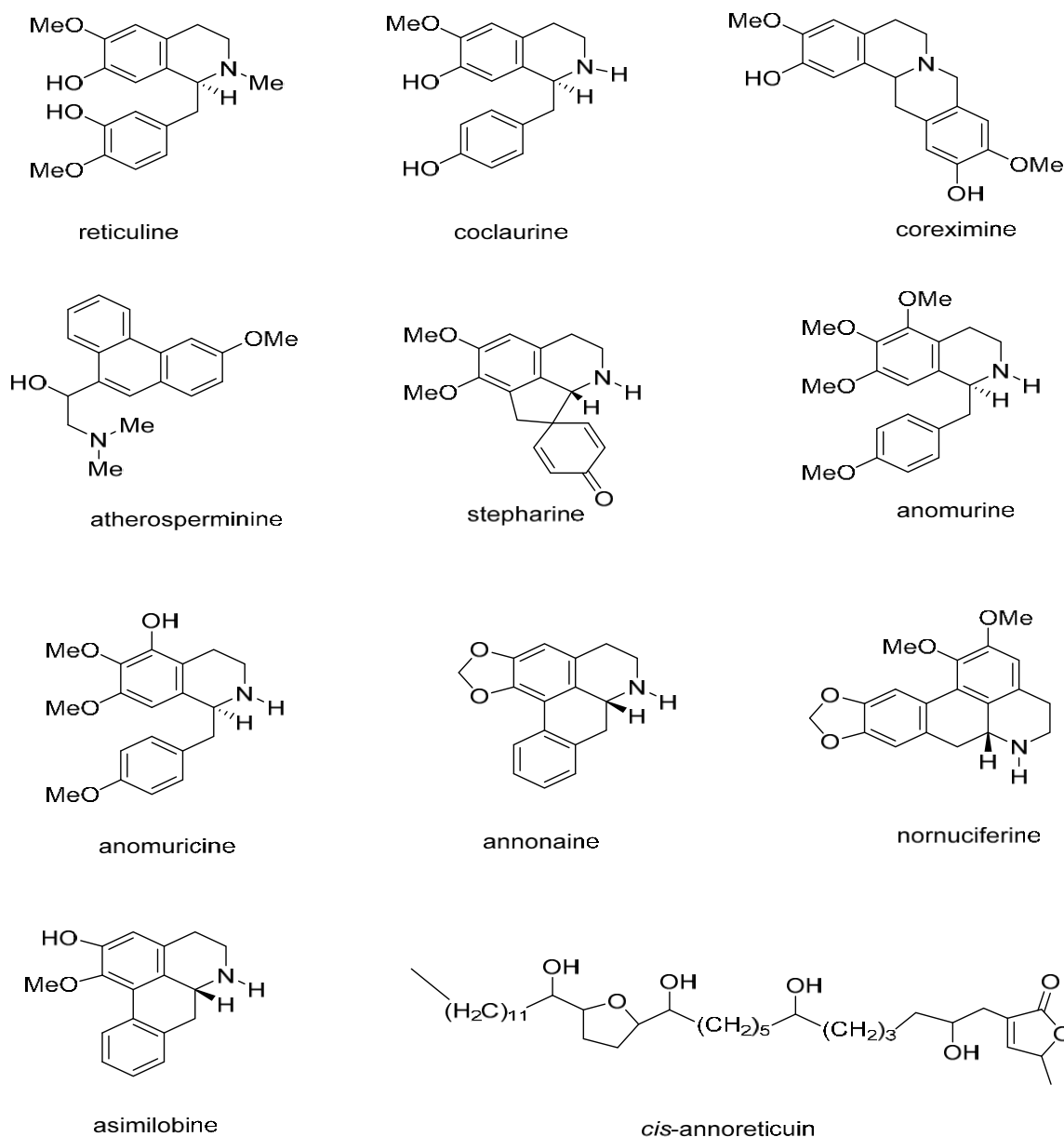
Annona muricata, including other *Annona* species such as *A. squamosa* and *A. reticulata*, are mainly used in traditional medicine against several non-communicable diseases such as cancer, diabetes, parasitic infections, arthritis, insomnia, headaches, etc.

In tropical Africa, it is used as an astringent, insecticide, and piscicide to treat coughs, pain, and several skin diseases. In India, the fruit and flower are employed as remedies against catarrh, while the root-bark and leaves are believed to have antiphlogistic and anthelmintic activities. In Malaysia, the crushed leaf mixture of *A. muricata* together with *A. squamosa* and *Hibiscus rosa-sinensis* is used as a juice on the head to protect against fainting. In South America and tropical Africa, including Nigeria, leaves of *A. muricata* are deployed as an ethnomedicine against tumors and cancer.

1.2.2 Annonaceous Acetogenins

AGEs are a unique class of C-35/C37 secondary metabolites derived from long-chain (C-32/C34) fatty acids in the polyketide pathway. They are usually characterized by a combination of fatty acids with a 2-propanol unit at C-2 that forms a methyl-substituted α , β -unsaturated γ -lactone. Since the discovery of uvaricin from *Uvaria accuminata* in 1982, more than 500 AGEs have been identified from different parts of the plants in the Annonaceae family [73,74]. Due to the special structures and extensive biological activities, AGEs have attracted significant scientific interest in recent years. Various biological activities have been reported for AGEs, including antimalarial, antiparasitic and pesticidal activities.

Picture 1 : Representative chemical structures of bioactive compounds reported in *Annona muricata*, including alkaloids and annonaceous acetogenin-related constituents



2. MATERIAL AND METHODS

2.1 Glassware & Instruments

Standard laboratory glassware, including conical flasks, beakers, test tubes, volumetric flasks, pipettes, burettes, and measuring cylinders, was used throughout the study. All glassware were washed with detergent, rinsed with distilled water, and oven-dried at 80 °C prior to use to avoid contamination. Major instruments used are listed below,

- Hot air oven (Lab Line, India; calibrated at 60 ± 2 °C)
- Magnetic stirrer
- Vacuum oven
- Analytical balance (Shimadzu AY220, Japan; ± 0.1 mg precision)
- UV–Visible spectrophotometer (Shimadzu UV-1800, Japan; 1 cm cuvette)
- Water bath (Remi WB-12, India)
- Hydrometer
- pH Meter
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All equipment was calibrated prior to experimentation

2.2 Collection & Preparation of fruit sample

Fresh and ripe *Annona muricata*, which is devoid of visible damage to avoid microbial infestation, were collected from a farm located in Bengaluru, Karnataka, during the month of March. Fruits were washed thoroughly under running water to remove dust, foreign particles, followed by rinsing with distilled water. The outer covering of the fruit was removed and pulp was extracted followed by removal of the seeds. The deseeded pulp is layered with sugar for storage purposes until infused. SCOBY starter culture was purchased from Earthy leaf. The outer covering of the fruit was removed and pulp was extracted followed by removal of the seeds. The deseeded pulp is layered with sugar for storage purposes until infused. Raw materials such as tea, sugar was purchased in Reliance fresh Bengaluru.



Figure 1 *Annona muricata* fruit before sample collection

2.3 Preparation of Tea for Kombucha fermentation

2.3.1 Materials required

- Black tea leaves
- Granulated sugar
- Distilled water
- SCOBY (Symbiotic Culture of Bacteria and Yeast)
- Starter kombucha liquid
- Glass fermentation jars
- Muslin cloth and rubber bands

2.3.2 Fermentation of Kombucha

Kombucha fermentation involves primary fermentation, which starts with the preparation of sweetened tea, conventionally prepared from green/ oolong tea, yet black tea is commonly used in kombucha tea preparation. About 6.5 gms of Black tea powder is infused in 1000ml boiled water at 90°C for 30 minutes. The tea leaves are further filtered, followed by the addition of 40-60 g of sugar. The tea infusion is cooled to 20 °C followed with addition of SCOBY. Optimum fermentation occurs at 20-30 °C but is dependent on SCOBY composition. Around 2-3 days after fermentation, the formation of new tea fungus/ Daughter culture is observed and further carried out for 14 days in dark, clean conditions at room temperature. SCOBY can also be defined as a symbiotic culture of bacteria and yeast, where the yeast metabolises monosaccharides, producing ethanol and CO₂. The Bacteria in SCOBY, mainly Acetic acid bacterial/ *Acetobacter aceti*, convert ethanol to organic acids or acetic acid, leading to a gradual decrease in pH and giving kombucha its characteristic tangy flavour.

The secondary fermentation of kombucha is optional and can enhance additional flavors, antioxidant properties and bioactivity of the kombucha. Hence, secondary fermentation was carried out using Cheong, which is a traditional Korean fermented syrup prepared by layering fruit with sugar, preserving its bioactive compounds and shelf stability of the fruit. The kombucha is infused with *Annona muricata* cheong on the 14th day of primary fermentation.



Figure 2 Kombucha fermentation setup

2.4 Physio-Chemical Analysis

2.4.1 pH Value

The pH value of kombucha was recorded during the fermentation period to observe changes in acidity levels and to monitor the fermentation process and sensory acceptability of the kombucha. Trials were performed to optimize the process with respect to changes in acidity, flavour profile, and sensory acceptability before and after secondary fermentation using graviola infusion/Cheong. The pH value of the fermentation brew was determined using a pH meter at room temperature in triplicate once every 24 hours until the completion of fermentation, including the layered kombucha samples. The pH meter was calibrated using a standard buffer solution at pH 4.0 and 7.0 buffer solution.

2.4.2 Specific Gravity

To systematically monitor sugar consumption and fermentation processes, specific gravity was evaluated at 48-hour intervals during sample collection from the fermentation vessel/chamber. Each collected sample was filtered and refrigerated to 15-20°C. Once cooled, the sample is further agitated vigorously, also known as the degassing of CO₂. At 20.0°C, the sample was transferred into a measuring cylinder, where the glass hydrometer was submerged into the sample. The values were recorded and further calculated. The specific gravity values were recorded to track real-time fermentation kinetics.

2.4.3 Total soluble solids (Brix)

To calculate the total soluble solids variations in different production stages using a refractometer, Total soluble solids were systematically evaluated for the standard kombucha sample, Layered kombucha, and Graviola cheong. The soluble sugar concentration was measured. The observed differences reflected formulation characteristics and changes in fermentation.

2.5 Proximate analysis

The moisture and ash content of the sample were analysed according to IS 3752 - 2005 (Reaffirmed 2020). The fat and protein contents were determined using Soxhlet extraction and the Kjeldahl method, respectively. The carbohydrate composition was determined by calculating the remaining per cent after all other components and reducing sugars were estimated by the DNS method.

2.6 Titratable Acidity

The titratable acidity of kombucha was estimated on days 1, 7, 14, 30, and 45 of kombucha fermentation by titrating 10ml of kombucha sample with 1-2 drops of phenolphthalein reagent against 0.1N NaOH; the titrate values were taken in triplicate, and the average value of acidity was calculated using the formula.

$$\text{Titratable acidity \%} = \frac{\text{Volume of NaOH} \times \text{Normality of NaOH} \times \text{Equivalent weight (acetic acid)}}{\text{Volume of sample}}$$

2.7 Estimation of Vitamin C (Ascorbic Acid)

Ascorbic acid content was determined by iodometric titration according to standard methods, with minor modifications. Standard iodine solution was prepared by dissolving 5.0 g of potassium iodide (KI) and 0.268 g of potassium iodate (KIO₃) in distilled water, acidifying with 3 M sulfuric acid (H₂SO₄), and diluting to a final volume of 500 ml. For analysis, 10 mL of kombucha sample (or L-ascorbic acid standard solution) was transferred to a conical flask, followed by the addition of 1–2 drops of 1% (w/v) soluble starch indicator. The mixture was titrated against the prepared iodine solution until the persistent appearance of a bluish-black end-point. Sampling was carried out across primary fermentation on days 1, 7, 14, 30, 45. Graviola Fruit infusion (cheong) was introduced

on day 7 for secondary fermentation and enhancement of bioactive compounds and antioxidant properties, and subsequent measurements were taken on days 30 and 45 to evaluate the shelf-life stability of vitamin C and associated bioactive compounds. All determinations were performed in triplicate and vitamin C concentrations were expressed as mg/100 mL of sample using standard stoichiometric calculations.

2.8 Antioxidant property

2.8.1 Total phenolic content

The total phenolic content of standard kombucha and graviola-infused layered kombucha was determined by using the Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent was prepared by dissolving 10ml Folin-Ciocalteu Reagent into 90 ml distilled water and Gallic acid was used as a standard. A 0.01 ml sample was added to 10ml of methanol, and a 0.5 ml sample aliquot was mixed with 2.5 ml of Folin-Ciocalteu reagent and incubated for 5 min. Then, 2.0 ml of 7.5% sodium carbonate solution was added. The mixture was mixed and stored at room temperature in the dark for 2 h. The absorbance was measured at 765 nm using a spectrophotometer.

2.8.2 Determination of DPPH Radical Scavenging Activity

The antioxidant activity of the samples was measured in terms of hydrogen-donating or radical-scavenging ability using the DPPH reagent. First, a 0.2 mM DPPH solution was prepared by dissolving 0.0078 g in 100 mL of ethanol. Then, 0.01 g of the extract was added to 10 mL of ethanol to obtain a concentration of 1 mg/ml. Approximately 2 mL of the sample aliquot was added to 2 mL of DPPH in ethanol and shaken well before incubation in the dark for 30 min. The absorbance of the solution was measured at 517 nm using a spectrophotometer. Finally, the percentage inhibition was calculated.

$$\text{Inhibition \%} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.9 Sensory Evaluation

The sensory analysis was performed on taste, aroma, colour, appearance and overall acceptance of the layered kombucha with base kombucha by 50 untrained students using a 9-point hedonic scale: 1 being the least likeable; 9 being the most likeable. The kombucha samples were coded with three-digit random numbers and presented on a tray for individual booths of the panelists; further observations were recorded and documented. In this sensory evaluation, water was provided to each panelist between samples as a palate cleanser.

3. RESULTS AND DISCUSSIONS

3.1 Physio-chemical Analysis

3.1.1 pH Value

The initial pH of the fermentation tea was 4.96, and a gradual decrease in pH was observed throughout the fermentation, indicating an increase in acidity as a result of the effective breakdown of sugars into alcohol and the further production of organic acids by SCOBY. The pH value is mainly contributed by Acetobacter and Gluconobacter bacteria due to acetic acid production. According to Jayabalan et al. (2014), the pH decreases from 5.44 to 3.0 after 5 to 7 days of fermentation.

Also it is believed that the decrease in pH was caused by organic acids such as acetic acid or gluconic acid produced by microorganisms, and this result was similar to that of [Sreeramulu et al. \(2000\)](#) , [Tanticharakunsiri et al. \(2020\)](#) , and [Aung and Eun \(2021\)](#). During secondary fermentation, the base kombucha resulted in further decrease in fermentation, whereas graviola-infused layered kombucha observed a temporary increase

in pH following the addition of *Annona muricata* (graviola) Cheong suggested that the fruit infusion altered the fermentation matrix through the fruit's biomass. By the end of fermentation, the pH had decreased to 2.80.

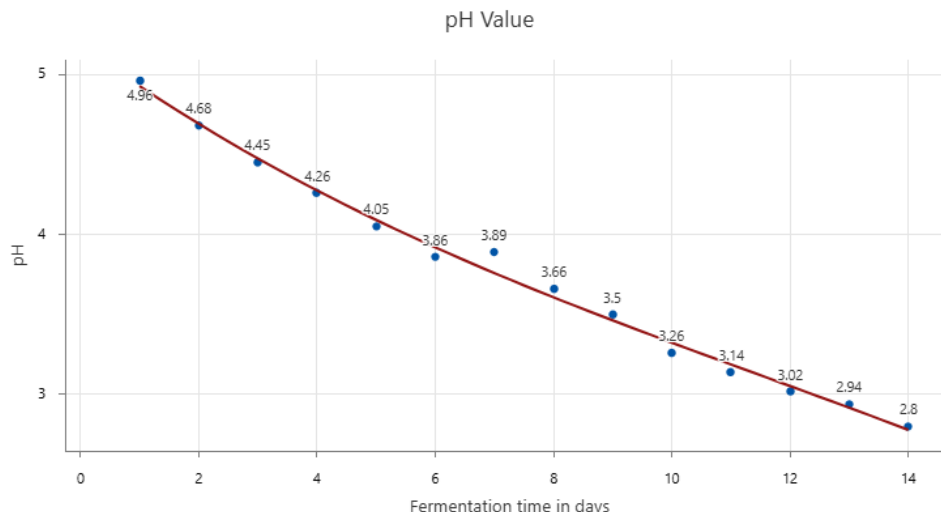


Figure 3 Changes in pH during fermentation

3.1.2 Specific Gravity

The specific gravity of the initial tea brew of 1.028 was recorded as the original gravity of the fermentation media, indicating a high number of dissolved sugars. A gradual drop in specific gravity was observed as a result of ethanol production by yeast and the bacterial conversion of ethanol into organic acids present in the SCOBY. The specific gravity increased temporarily on the seventh day of fermentation due to the addition of *Annona muricata* (graviola) Cheong, which contributed to the introduction of additional sugars and dissolved fruit infusions. As fermentation progressed, the specific gravity decreased, indicating the utilization of additional sugars in secondary fermentation. By the end of fermentation, the specific gravity dropped to 1.018.

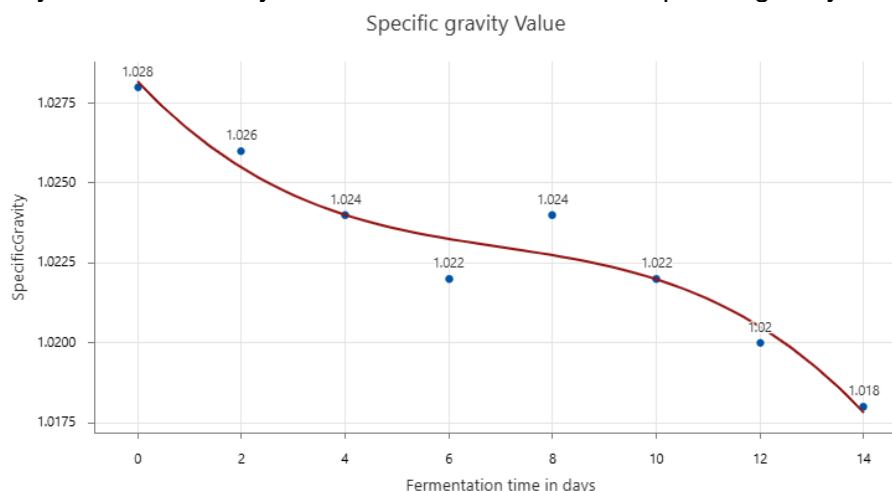


Figure 4 Changes in specific gravity during fermentation

3.1.3 Total soluble solids

Total soluble solids (TSS, °Brix) were determined to evaluate changes in soluble sugar concentration during kombucha fermentation and secondary infusion with *Annona muricata* (Graviola) cheong. The graviola cheong exhibited the highest TSS due to its high sucrose concentration, while the standard kombucha showed comparatively lower values as fermentable sugars had already been utilised during primary fermentation. Following the addition of cheong during secondary fermentation, the TSS of the graviola-infused kombucha increased temporarily due to the incorporation of fruit-derived soluble solids and sugars. As fermentation progressed, a gradual decline in TSS was observed, reflecting the continued utilization of fermentable sugars by the microbial consortium.

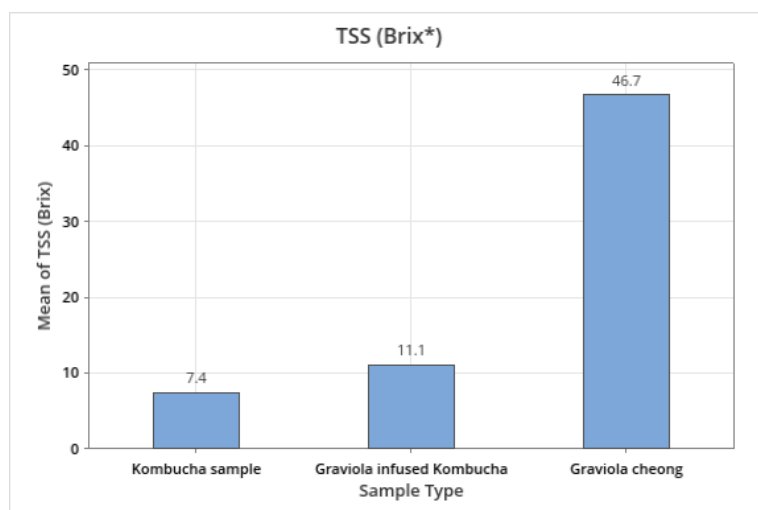


Figure 5 Comparison of TSS in kombucha samples

3.2 Proximate Analysis

According to the data given in the table, Graviola-infused kombucha has a higher ash content than base kombucha due to its high inorganic residual compounds, including high fiber content. The crude protein content in both graviola-infused kombucha and base kombucha showed no significant difference, as did the total fat content. However, the final Kombucha formulation exhibits a total sugar concentration of 6.05%, with a 10% (v/v) Graviola infusion/Cheong addition of 50:50 fruit and sucrose infusion. Chromatographic analysis reveals that reducing sugars (glucose and fructose) account for only 1.59% of the total matrix, indicating a mature primary kombucha. Fermentation where residual simple sugars were largely depleted by the SCOBY, combined with the natural monosaccharide payload introduced by the graviola infusion/cheong during secondary fermentation.

Sample	Moisture Content (%)	Ash Content (%)	Total Fat Content (%)	Crude Protein Content (%)	Carbohydrate Content (%)	Crude Fibre Content (%)	Total Sugar (%)
Test method	FSSAI manual for analysis of fruit 2016	IS3752-2005 (Reaffirmed 2020)	SMSLA/FD/SOP /042-2024	AOAC 22 nd edition 2001.11.2023	IS1656-2022	IS2026 (part1)-1982 (Reaffirmed 2020)	SMSLA/LS /SOP/019-2024
Base kombucha	96.63	0.15	<10	<10	5.46	0.11	4.20
Graviola infused kombucha	93.95	0.56	<10	<10	6.05	0.259	5.50

Table 1. Proximate composition of base kombucha and graviola-infused kombucha

Rather than relying on thermal pasteurisation or chemical additives, this concentrated sucrose matrix functions as a natural preservative and may contribute to improved stability and nutrient retention.

3.2.1 Determination of Reducing Sugar Content (DNS Method)

The reducing sugar content of the fermented kombucha samples was determined using the 3,5-dinitrosalicylic acid (DNS) colorimetric method. The method is based on the reduction of 3,5-dinitrosalicylic acid by reducing sugars under alkaline conditions to form 3-amino-5-nitrosalicylic acid, producing an orange-red coloured complex. Briefly, 0.5 mL of appropriately undiluted sample and 1:10 diluted sample was mixed with 3 mL of DNS reagent and heated in a boiling water bath for 5 min. After cooling to room temperature, 0.5 mL of distilled water was added, and the absorbance was measured at 540 nm using a UV–Visible spectrophotometer. A calibration curve was prepared using glucose as the reference standard (0–1.0 mg mL⁻¹). All analyses were performed in triplicate, and the reducing sugar concentration was determined from the calibration curve and expressed as mg glucose equivalent (GE) mL⁻¹ of sample.

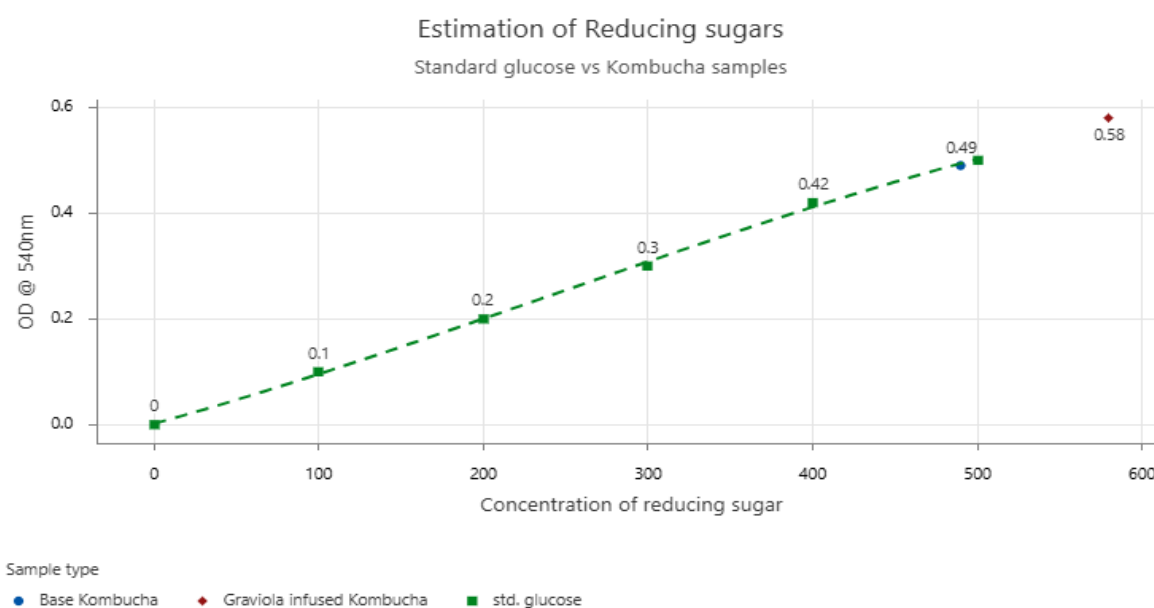


Figure 6 Determination of Reducing sugar using DNS

3.3 Titratable Acidity



Figure 7 Showing before and after titration

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The titratable acidity was estimated on fermentation days 1,7 and 14, followed by analysis of titratable acidity post-bottling on days 30 and 45, mainly to observe the stability of acidity of the fermented kombucha. Titratable acidity progressively increased during primary fermentation, from 0.162% on Day 1 to 0.342% on Day 7, reached a maximum of 0.98% on day 14 post-secondary fermentation by the addition of graviola infusion/Cheong. The increase in titratable acidity indicates progressive accumulation of organic acids as fermentation proceeded, corresponding with the reduction in pH observed during the fermentation period. This increase may be attributed to the metabolic activity of the SCOBY consortium, in which yeast metabolizes fermentable sugars and produces ethanol, followed by the conversion of ethanol and other substrates into organic acids by acetic acid bacteria. The titratable acidity declined following bottling and storage on day 30 to 0.781% and further declined to 0.498% on day 45. The reduction in titratable acidity during storage may be associated with changes in the organic acid profile and continued microbial metabolism. The decrease in acidity was accompanied by relatively stable pH values during the later storage period.

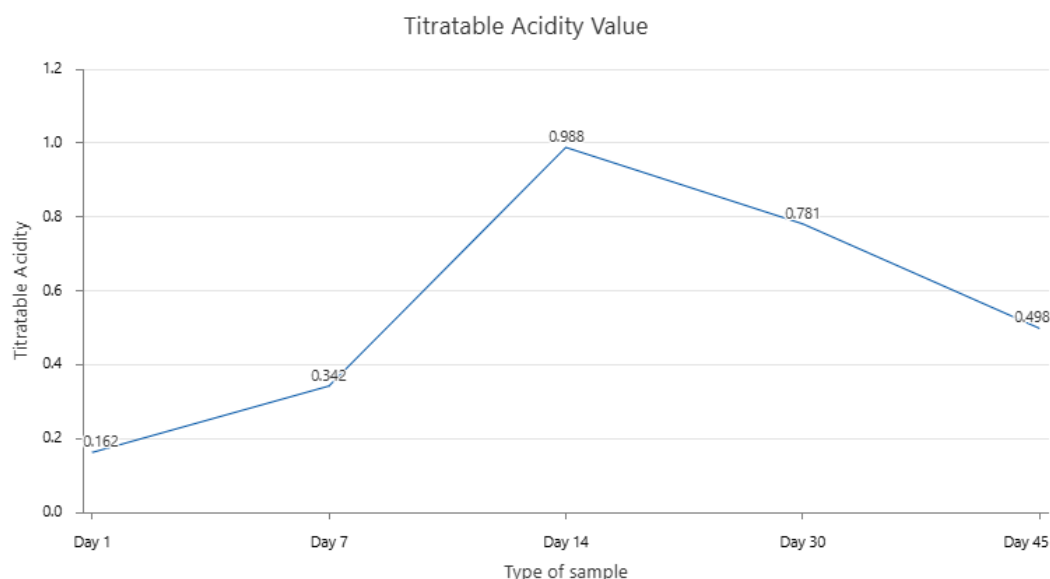


Figure 8 Titratable acidity observation during fermentation

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3.4 Vitamin C (Ascorbic acid) Estimation

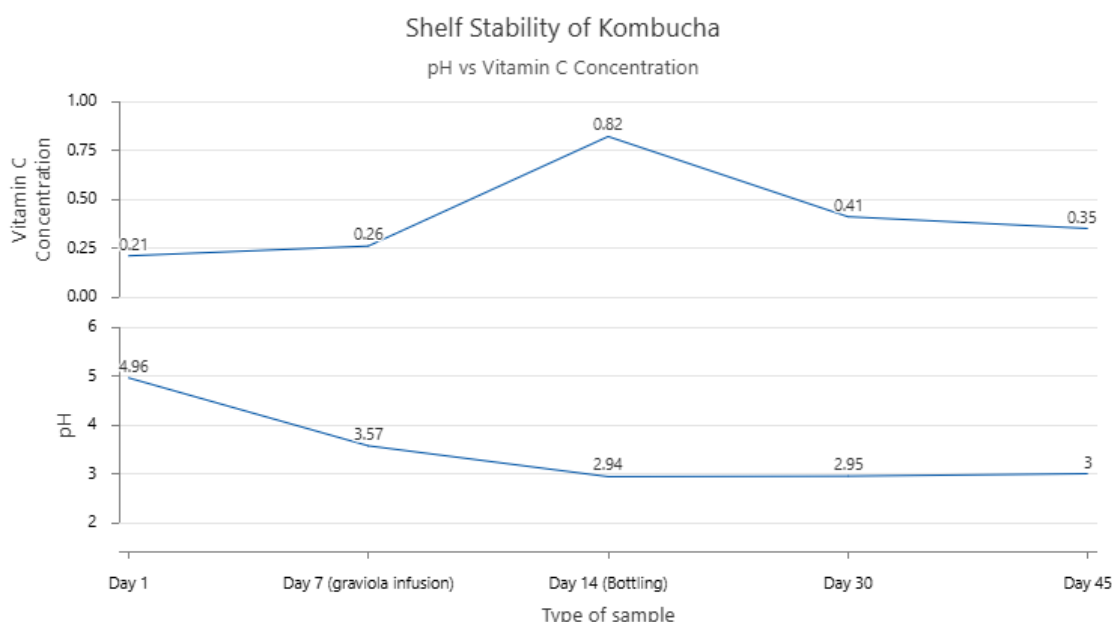


Figure 9 Vitamin C concentration

The vitamin C (Ascorbic Acid) content of base kombucha and graviola-infused kombucha was estimated throughout the fermentation process and refrigerated storage to evaluate the retention of Ascorbic acid and its shelf stability. The initial vitamin C concentration was 0.21 mg/100 mL on Day 1 and later increased to 0.26mg/100 mL on the seventh day of fermentation. Later after the infusion of graviola cheong on the seventh day of fermentation. The vitamin C concentration observed an increase on Day 14 (0.82 mg/100ml), indicating effective incorporation of Vitamin C from graviola cheong infusion into kombucha. During refrigerated storage, the vitamin C concentration gradually decreased to 0.41 mg/100 mL on Day 30 and 0.35 mg/100 mL on Day 45 while being susceptible to oxidative degeneration of ascorbic acid during storage. The kombucha prepared using moringa leaves observed similar decrease in vitamin C concentration with the length of fermentation time Zahra, et al (2022).

3.5 Total phenolic content

The total phenolic content (TPC) of the standard and graviola-infused kombucha samples was determined using the Folin–Ciocalteu colorimetric method and expressed as μg gallic acid equivalents per gram (μg GAE/g) of sample. The standard kombucha exhibited a total phenolic content of 780 μg GAE/g, whereas the graviola-infused kombucha showed a higher value of 980 μg GAE/g. Thus, incorporation of graviola resulted in an approximately 25.6% increase in the measured total phenolic content compared with the standard kombucha. The higher TPC observed in the graviola-infused kombucha may be attributed to the contribution of phenolic compounds naturally present in *Annona muricata* (graviola) fruit, in addition to the phenolic constituents already present in the tea-based kombucha. The increase suggests that incorporation of graviola provided an additional source of phenolic compounds to the beverage matrix. Phenolic compounds in tea, including flavonoids and phenolic acids, contributed an important fraction of its antioxidant constituents. During kombucha fermentation, microbial activity may also influence the transformation and availability of phenolic compounds, contributing to changes in total phenolic content. The higher value observed in the infused formulation may therefore reflect both the contribution of the added graviola infusion/Cheong and changes occurring during fermentation. The TPC value of 980 μg GAE/g in the graviola-infused kombucha indicates an enrichment in phenolic constituents relative to the standard formulation.

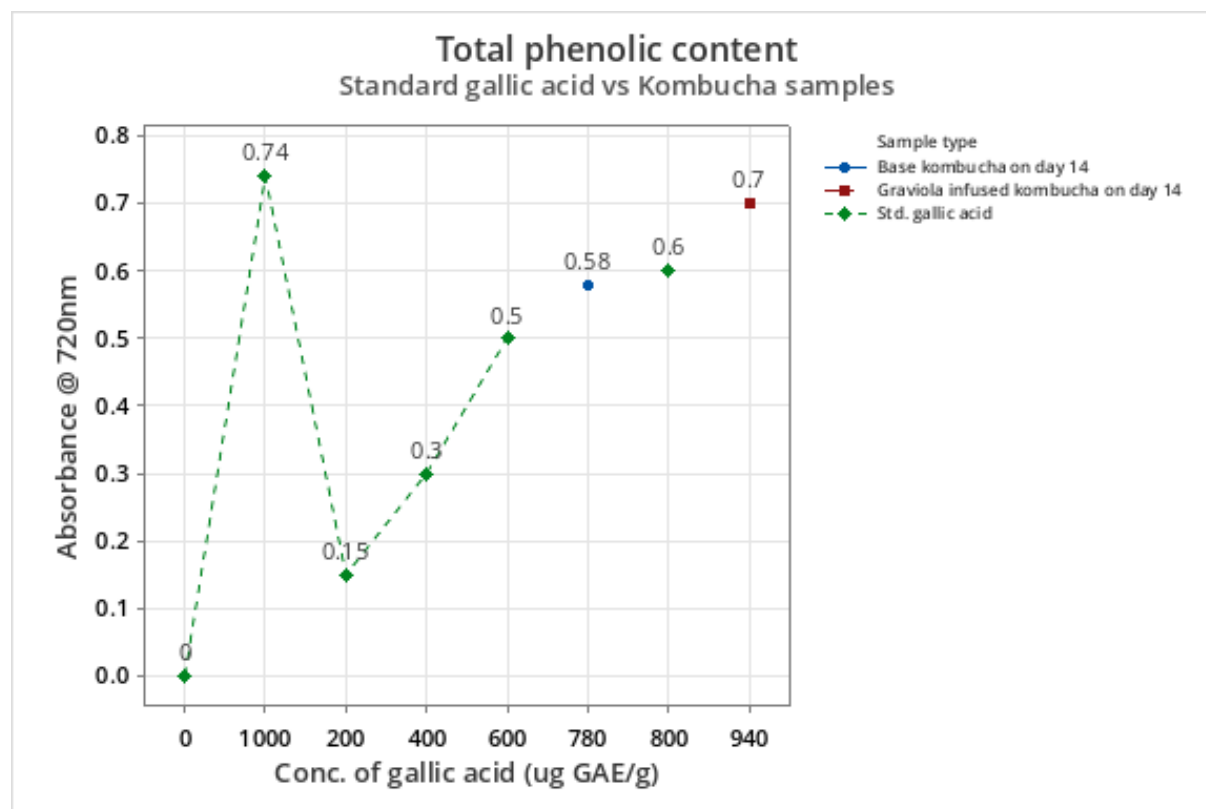


Figure 10 Graphical estimation of TPC

3.6 DPPH Radical Scavenging assay

The DPPH assay was used to evaluate the antioxidant activity of the kombucha samples by measuring their ability to scavenge DPPH free radicals. Antioxidants donate hydrogen atoms or electrons to DPPH radicals, resulting in a decrease in the intensity of the purple colour of the DPPH solution. This reduction is reflected by a

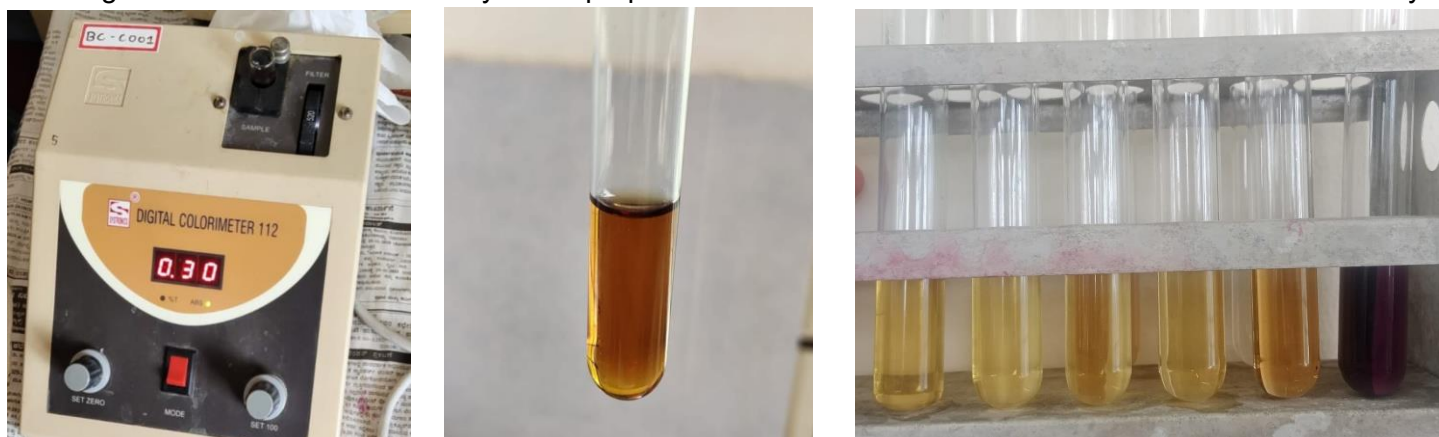


Figure 11 DPPH radicle scavenging assay procedure

lower absorbance value, while a higher percentage of inhibition indicates stronger radical-scavenging activity. The antioxidant activity of kombucha samples was evaluated using DPPH radical scavenging assay at different stages of fermentation. During primary fermentation, the inhibition was observed to be 46% on day 7 of fermentation, which may be attributed to the microbial biotransformation of tea polyphenols and production of antioxidant metabolites by the SCOBY consortium, followed by the addition of graviola cheong. During secondary fermentation, both base kombucha and graviola-infused kombucha observed an increase in inhibition on day 14, with 57% inhibition for base kombucha, indicating a progressive enhancement in antioxidant activity during fermentation. Whereas the *Annona muricata* (Graviola) cheong-infused kombucha exhibited a significantly higher radical scavenging activity of 80% inhibition on Day 14, demonstrating that secondary fermentation with graviola cheong enhanced the antioxidant potential of the beverage. Jayabalan et al. (2014), stated similar increases in DPPH radical scavenging activity during kombucha fermentation have attributed to the enhanced antioxidant capacity during microbial fermentation.

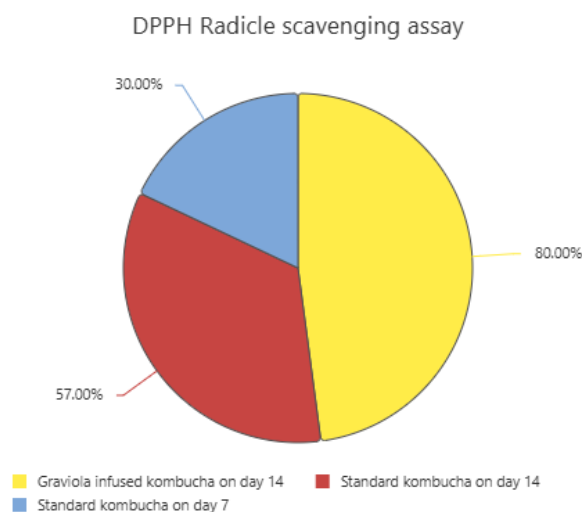


Figure 12 Pie chart with percentage of inhibition

3.7 Sensory Evaluation

Sensory evaluation was conducted to evaluate sensory acceptability between standard kombucha and graviola infused (*Annona muricata*) layered kombucha using a 9-point hedonic scale, where 1 represented “dislike extremely” and 9 represented “like extremely.” A total of 50 untrained student panelists evaluated the samples based on appearance, colour, odour, sourness, sweetness, flavour, and overall acceptability. The sensory profile demonstrated that the layered kombucha generally received favourable scores across the evaluated attributes compared with the standard kombucha. The layered formulation showed improved perception of flavour and sweetness, which may be associated with the incorporation of graviola cheong during secondary fermentation and the resulting balance between fruit-derived sweetness and the characteristic acidity of kombucha. The layered kombucha also exhibited favourable scores for appearance and colour, indicating that the fruit infusion contributed positively to the visual characteristics of the beverage. In comparison, the standard kombucha showed a relatively more pronounced perception of sourness, consistent with its characteristic acidic profile. The odour and overall acceptability scores of both formulations were favourable, suggesting that the incorporation of graviola cheong did not negatively affect consumer acceptance. Overall, the sensory evaluation indicated that the layered fermentation approach produced a more complex and balanced sensory profile, particularly in terms of flavour and sweetness, while maintaining good overall acceptability among the panelists. These findings support the potential of graviola cheong as a secondary fermentation ingredient for improving the sensory characteristics of kombucha.

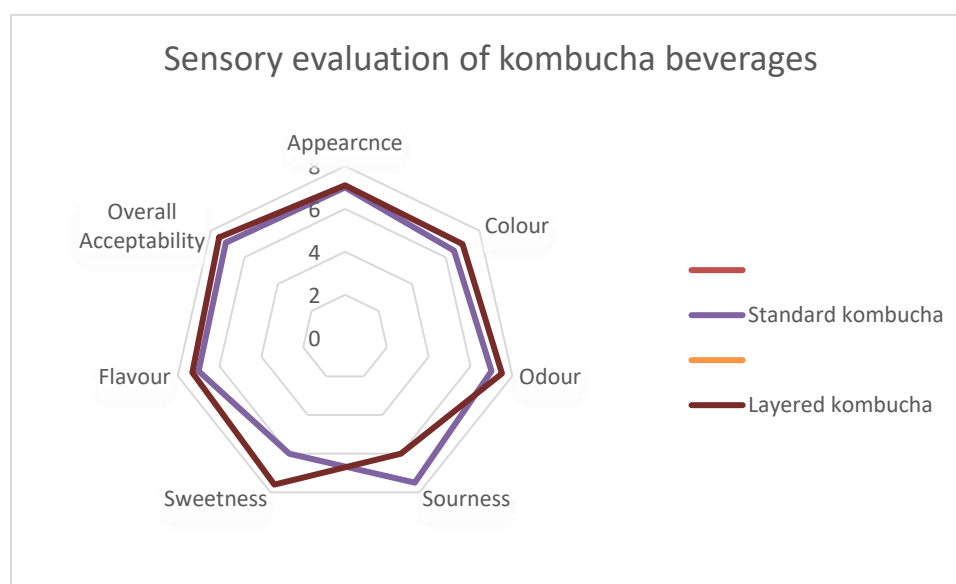


Figure 13 Sensory evaluation comparison between standard kombucha and layered kombucha

4. CONCLUSION

The present study demonstrated the potential of a layered fermentation approach using *Annona muricata* (graviola) cheong as a secondary fermentation substrate for the development of a value-added kombucha beverage with enhanced nutritional, functional, and sensory characteristics. Primary fermentation of black tea using a SCOBY resulted in progressive physicochemical changes associated with microbial activity and substrate utilization, including reduction in pH, specific gravity, and total soluble solids, accompanied by an increase in titratable acidity during active fermentation, reflecting continued microbial metabolism and utilization of fermentable substrates by the SCOBY consortium. The temporary increase in soluble solids and specific gravity following incorporation of graviola cheong further demonstrated the contribution of fruit-derived sugars and soluble constituents to the secondary fermentation matrix, followed by their subsequent utilization during

fermentation. The incorporation of graviola cheong enhanced the functional properties and antioxidant potential of the developed kombucha, as evidenced by the higher DPPH radical scavenging activity observed in the infused kombucha compared with the standard fermented kombucha. The enhanced antioxidant activity is likely associated with the incorporation of naturally occurring phenolic compounds and vitamin C from the fruit infusion, together with continued microbial biotransformation during fermentation. The enhanced antioxidant potential may also be attributed to the combined contribution of tea-derived polyphenols, fruit-derived bioactive compounds, vitamin C, and continued microbial biotransformation during fermentation. Vitamin C analysis further demonstrated that the infused kombucha retained appreciable amounts of ascorbic acid throughout refrigerated storage, indicating satisfactory shelf stability under acidic conditions, although a gradual decline was observed with prolonged storage. Altogether, these findings indicate that the secondary fermentation stage can contribute not only to flavour development but also to the functional characteristics of the final beverage. Unlike conventional kombucha, which is often characterized by pronounced acidity or relies primarily on fruit flavouring/artificial flavours, the layered fermentation developed in this study produced a functional beverage with distinctive sweet-savoury sensory characteristics including enhanced antioxidant potential, bioactive compound contribution, and sensory complexity.

The use of graviola cheong therefore extends the concept of fruit incorporation beyond simple flavour modification by introducing an additional fermentation stage through which fruit-derived constituents interact with the ongoing microbial fermentation process. These findings demonstrate that thoughtful fermentation design, rather than ingredient selection alone, can significantly influence the nutritional quality, functional properties, and sensory experience of modern fermented beverages. This study demonstrates that layered fermentation is more than a flavouring strategy; it represents a novel fermentation design approach capable of enhancing the nutritional profile, antioxidant potential, and sensory characteristics of kombucha through the synergistic interaction of microbial fermentation and fruit-derived bioactive compounds. Overall, the study suggests that graviola cheong is a promising natural ingredient for the development of value-added functional kombucha with enhanced antioxidant properties and improved nutritional quality. Further studies involving comprehensive phytochemical profiling, microbial characterization, and extended shelf-life evaluation are recommended to validate the long-term stability and commercial potential of the developed beverage.

COMPETING INTRESTS

The authors declare that they have no competing interests.

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Declaration of AI Use

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