World Journal of Food Science and Technology
2023; 7(3): 67-76
http://www.sciencepublishinggroup.com/j/wjfst
doi: 10.11648/j.wjfst.20230703.14
ISSN: 2637-6016 (Print); ISSN: 2637-6024 (Online)

Changes in Physicochemical, Phytochemical and Microbiological Parameters During Fermentation of "Kargasok" Tea

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To cite this article:

Daoudou Bakari, Tchoing Hyacinthe, Yaya Garga, Bayoï James Ronald, Djoulde Darman Roger, Tatsadjieu Ngoune Leopold. Changes in Physicochemical, Phytochemical and Microbiological Parameters During Fermentation of "Kargasok" Tea. *World Journal of Food Science and Technology*. Vol. 7, No. 3, 2023, pp. 67-76. doi: 10.11648/j.wjfst.20230703.14

Received: July 8, 2023; **Accepted**: July 25, 2023; **Published**: August 5, 2023

Abstract: "Kargasok" is an artisanal drink with a tangy, slightly sparkling flavour obtained by fermenting sweet tea (green or black tea). This drink is highly prized for its therapeutic properties and a source of income to the producers but, its quality attributes and shelf life are still unknown during the fermentation time. This study aimed to evaluate the influence of fermentation time on the quality attributes and shelf life of the "Kargasok" tea. The sweet tea was prepared and fermentation was initiated using "Kombucha" as starter for two weeks. Physicochemical, phytochemical, microbiological and sensory parameters were followed every two days until the fourteenth day using the referenced methods. pH varied from 3.35 to 3; total acidity from 1.35gl^{-1} to 3.22gl^{-1}; soluble solids content from 9.08°Brix to 10.15°B; total sugar content between 10.43 gl^{-1} and 2.83 gl^{-1}; alcohol content from 0.12gl^{-1} to 5.89gl^{-1} and conductivity from 1080µs/cm to 1326 µscm^{-1}. The polyphenol and flavonoid contents were 4.55gL^{-1} and 22.78gL^{-1} respectively; an average tannin content of 14.50 gl^{-1}; a scavenging activity (DPPH) of 68.38g EqTl^{-1} with ferric reducing power (FRAP) of 59.55gl^{-1} were recorded. Despite the presence of yeasts and moulds, the beverage displayed an acceptable hygienic quality. This drink was more appreciated from the eighth day of fermentation. Results above suggest that "Kargasok" could be used as potential functional beverage.

Keywords: "Kargasok" Tea, Physicochemical, Hygienic Quality, Processing, Fermentation

1. Introduction

Traditional beverages can be defined as fermented extracts prepared by the populations, brewed or not according to ancestral processes specific to every locality [1]. "Kargasok" tea is a traditional beverage obtained by fermenting sweet tea. "Kargasok" tea also called kombucha is considered as a miraculous medicine for curing almost all diseases, even Cancer [2]. In the Far North of Cameroon there is a mosaic of traditional beverages, including "Kargasok" tea which is a traditional fermented beverage obtained through a symbiotic culture of bacteria and yeasts. This beverage with therapeutic properties is highly appreciated by the local population. The production of "Kargasok" is a source of income for producers and consumers greatly benefit from its virtues. Like many traditional drinks, the conditions of its production do not guarantee to consumers a healthy product with good nutritional and sensory qualities. So far, there exist no relevant data

recorded on the microbiological, physicochemical parameters of this drink during its fermentation. These parameters may vary during the fermentation with a greater influence on it proximate composition and the quality of the final product. Little knowledge of the evolution of the parameters could facilitate the management of the manufacturing steps of this beverage on a semi-industrial scale. Therefore, this study is aimed to evaluate the influence of fermentation time on the quality attributes and shelf life of this indigenous drink.

2. Material and Methods

2.1. Plant Material

The plant material used for the preparation of the drink is green tea. It was purchased from Maroua central market, Cameroon on August 2022. The tea was placed into sterile polyethylene bags, sealed and transported to the laboratory for identification and pre-treatment.

2.2. Ferment Agent

The kombucha ferment used was sourced by a local producer.

2.3. Production of "Kargasok" Tea

The production of the beverage requires two main unit operations: decoction and fermentation. For this purpose, 750 g of green tea were weighed and placed in a clean container with 4L of water. The mixture was boiled at 100°C for 30 - 45 min, and filtered after a cooling stage. The filtrate was mixed with 400 g of sugar and heated at 100°C for 5 to 10 min to allow total dissolving of the sugar. The resulting tea was filtered using a sieve of variable mesh size and diameter, and the filtrate was allowed to cool at room temperature. After cooling, 250 g of ferment previously cleaned were pooled into the tea, and the mixture was covered using a fine cotton fabric or canvas and the container was attached with the rubber so that the air circulates between the surface of the liquid and the outside, essential for the success of the operation. The container was kept in a well-supervised place for fermentation. Finally, 30 mL of fermenting tea were collected every two days during 2 weeks for the physicochemical, phytochemical, microbiological and sensory analyses.

2.4. Physicochemical Analyses

The pH was determined directly according to the method [3], using a portable ATC portable pH meter (Eco Testr, Singapore) after calibration with pH 4 and pH 6.8 buffers. The reading was taken when the equilibrium potential between the electrodes was reached, the soluble solids (°Brix) and the conductivity were determined using a portable refractometer (RHW-25ATC) and a multifunctional portable conductivity meter type "e-1 TDS&EC" respectively by the method described by Daoudou [3]. The density was evaluated by the ratio of the density of the sample to that of water. The titratable acidity was determined by titration using NaOH (0.1 N) in the presence of phenolphthalein according to the method described by Muyanja [4]. Total sugars and alcohol content were evaluated using the methods described by Dubois [5].

2.5. Phytochemical Analysis

The determination of phenolic compounds and antioxidant properties of "Kargasok" tea during its fermentation was done using spectrophotometry. The total polyphenols were determined according to the Folin-Ciocalteu method as described by Mahmoudi [6], the flavonoid content was evaluated in the presence of aluminum chloride (AlCl3) according to the method of Dukic -Bozin Mimica [7] and the tannin content was determined in the presence of acidified vanillin according to the method described by Kouamé [8]. Similarly, the reducing power (FRAP) and the scavenging activity (DPPH) were determined by the methods described by Benzie [9] and Sun [10] respectively.

2.6. Microbiological Analysis

The counting of the microbial flora present in the different samples was carried out after 10-fold serial dilution of undiluted beverage (10^{-1} to 10^{-8}) using sterile distilled water followed by plating on specific solid medium. Depending on the microorganism sought, the diluted samples were inoculated into specific culture media and incubated at the optimal growth temperature of each microorganism. At the end of incubation, only plates with 33 to 330 colonies were considered [11].

Total mesophilic aerobic flora: This microbial flora was enumerated in Plate Count Agar (PCA) after 24 hours of incubation at 30°C.

Total coliforms were counted in Mac Conkey medium after incubation for 24 hours at 37°C.

Faecal coliforms were enumerated in MacConkey agar after 48h of incubation at 45°C.

Faecal streptococci were determined using Slanetz and Bartley agar containing 0.4% sodium azide, and the colonies were counted after 24h of incubation at 37°C.

Yeasts and moulds were counted using Sabouraud-Chloramphenicol medium after incubation for 2 to 5 days at 30°C.

Salmonella and *Shigella* were enumerated in two phases, an enrichment phase in Muller-Kauffmann medium for 24 hours at 37°C and a seeding phase of the medium enriched on the pre-poured SS medium. Incubation was carried out at 37°C for 48 h.

2.7. Sensory Analysis

Sensory analysis represents all the methods, tools and instruments that make it possible to evaluate the organoleptic qualities of a product, i.e. The characteristics which involve the senses of human beings: taste, smell, sight, touch and hearing [12]. It makes it possible to systematically describe and quantify all human perceptions.

2.8. Statistical Analysis

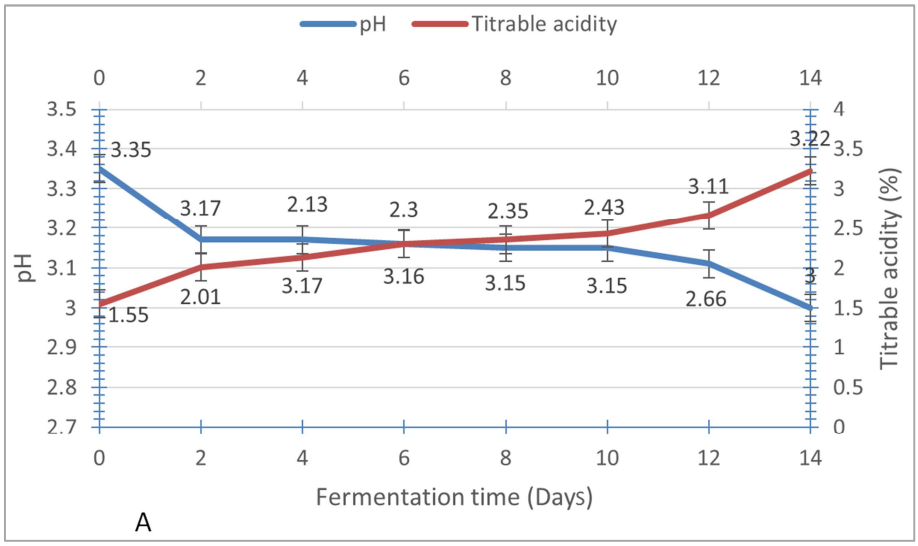
Results were organized using Microsoft Office/Excel 2013

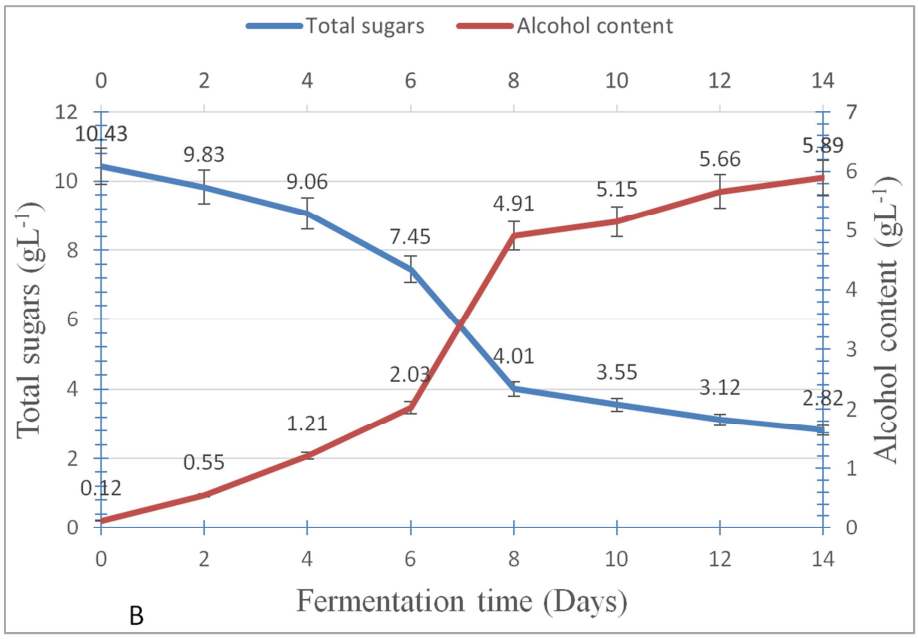
and processed with STATGRAPHICS Centurion 16.1 software. The comparison of the means was carried out by ANOVA then the test of multiple comparison of the significant differences (HSD) of Turkey was used to discriminate the pairs of means significantly different. The mean values were considered to be statistically different at the probability threshold $p \leq 0.05$. At the end of the analyses, the results obtained were presented as Mean ± standard deviation.

3. Results

3.1. Changes in Physicochemical Parameters During Fermentation of the Tea Beverage

Figure 1 below shows the evolution of the physicochemical parameters of the drink samples during fermentation. The physicochemical parameters evaluated are: pH, glucose content, total acidity and refractometric solids content (ESR) Brix degree and density have significantly dropped in pH as observed from 3.35 on the first day of fermentation to 3 on the last day (Figure 1A). Like the pH, the total sugars (10.43 gL^{-1} to 2.83 gL^{-1}) Figure 1B, the degree of Brix (9.08°B and 10.15°B) Figure1C and the density (1.05 to 0.93) Figure 1E decreased significantly during fermentation. Conversely to these parameters, the titratable acidity, the degree of alcohol and the conductivity increase significantly during the fermentation with respectively 1.55 to 3.22gL^{-1}, from 0.12 gL^{-1} to 5.89gL^{-1} and 1080 to 1326 µScm^{-1}, respectively Figure 1A, Figure 1B and Figure 1D.





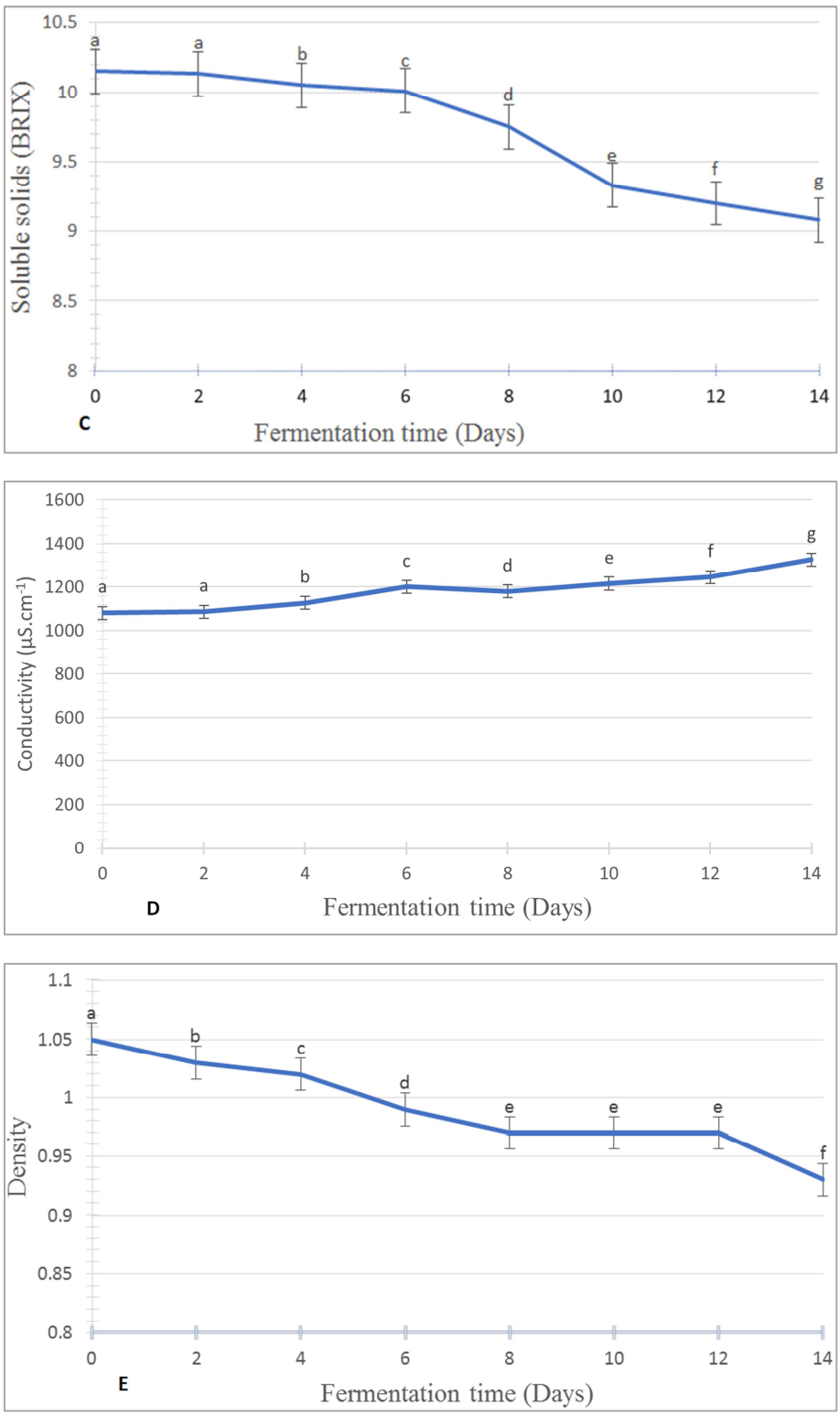
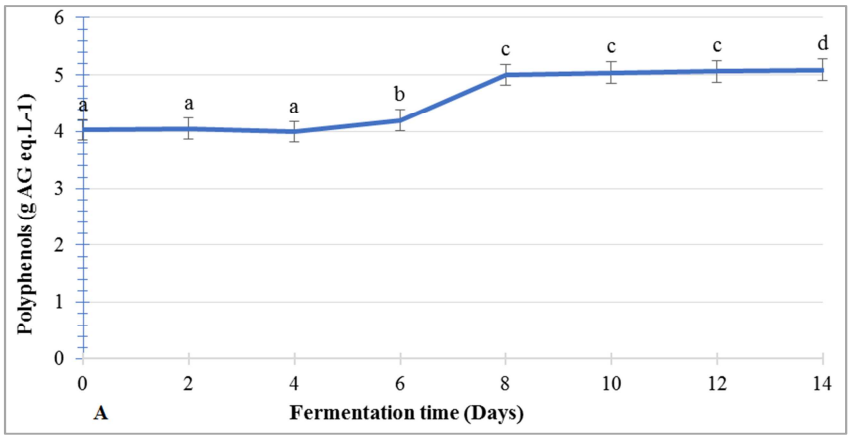


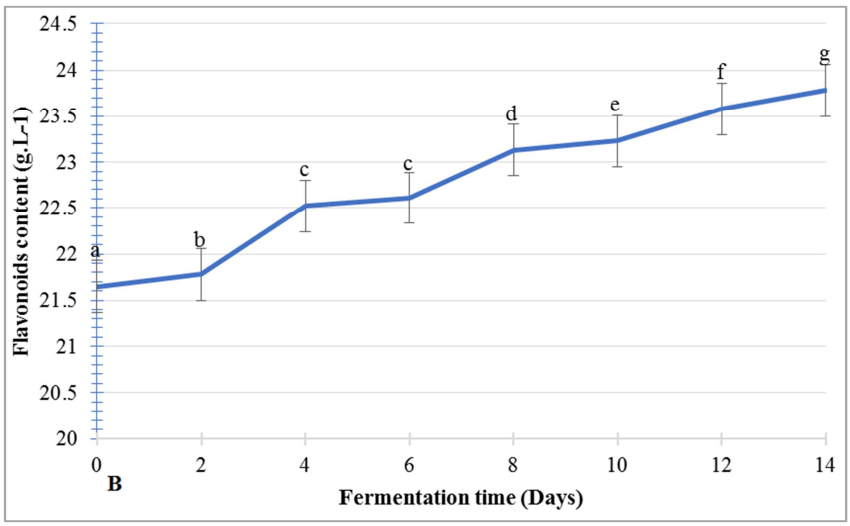
Figure 1. *Trends of pH and titratable acidity (A), total sugars and alcohol content (B), soluble solids content (C), conductivity (D) and density (E).*

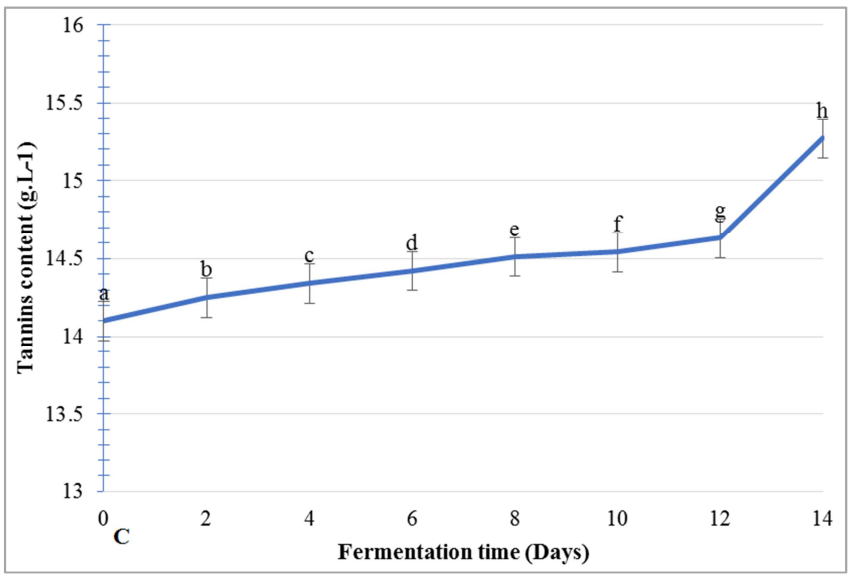
3.2. Changes in Phytochemical Parameters During Fermentation of the Tea Beverage

Figure 2 below shows the evolution of the phytochemical parameters of the drink samples during fermentation. The polyphenols content in "Kargasok" tea is approximately stable for the fourth days and begins to increase at the sixth day and then stabilizes from the eighth day (Figure 2A). The flavonoids content increases significantly during fermentation from 21.65 to 23.78 gL^{-1}. On day zero and day two, the variation in the flavonoid content varies very little, but on the fourth day we observe a notable increase in this content which is stabilized until the sixth day and then a gradual increase on the eighth

day until the last day of the fermentation (Figure 2B). The tannin content increases significantly during fermentation. This content ranges from 14.1gL^{-1} to 15.27gL^{-1} (Figure 2C). The antioxidant activity increases significantly during the fermentation of "Kargasok" tea, it ranges from 60.15 to 65.11gEqTL^{-1} (Figure 2D). A stability of the antiradical activity is observed until the sixth day of fermentation and a clear increase on the eighth day until the fourteenth day. A significant increase in the reducing activity is observable as it ranges from 56.44 to 62.76 g EqTL^{-1} (Figure 2E). The reducing activity is constant the first two days, then a slight increase on the fourth day and then there is a clear progression from the sixth day to the fourteenth day.







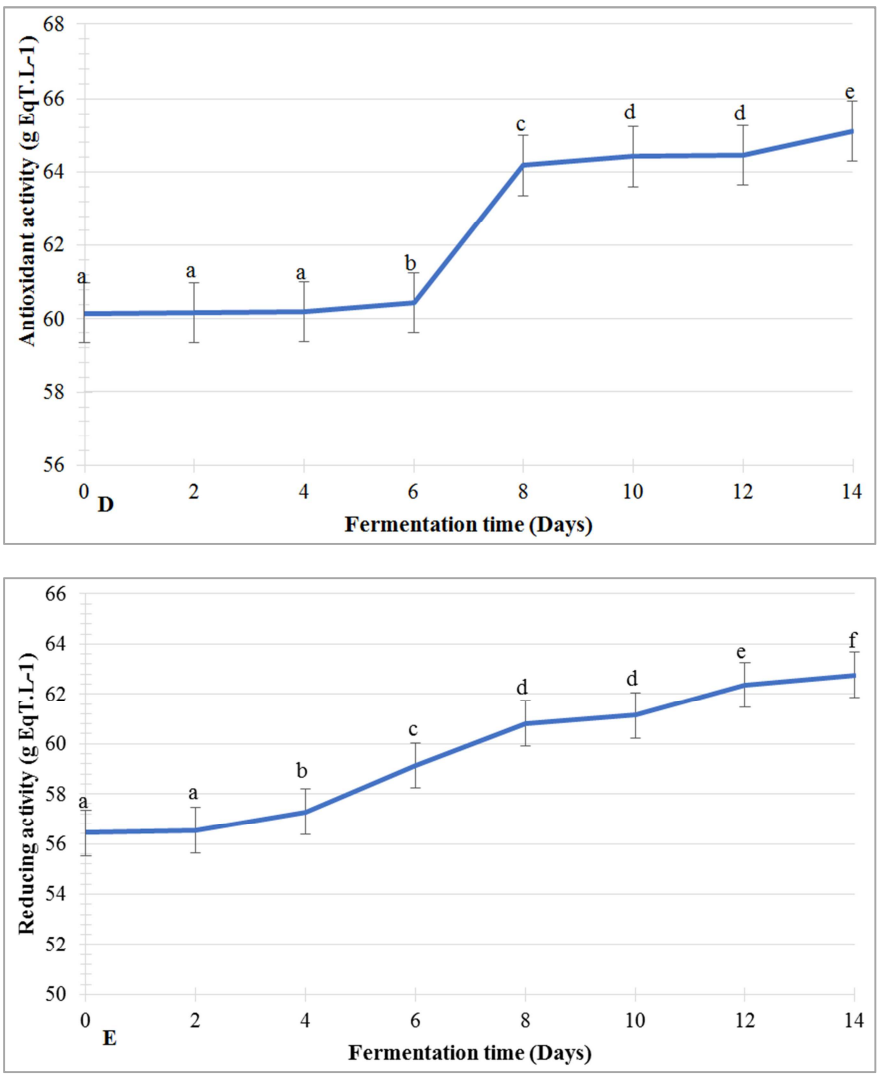


Figure 2. *Total polyphenol content (A), flavonoid content (B), tannin content (C), antiradical activity (D) and iron reducing activity (E).*

3.3. Microbial Quality of the Tea Beverage During the Fermentation

Microbial quality of the tea-based drink samples during the fermentation is presented in table 1. According to Table 1, there is a total absence of mesophilic spore-forming bacteria, faecal streptococci, faecal coliforms and salmonella in "Kargasok". According to the results, the number of yeast colonies decreases significantly during fermentation, ranging from 58 to 17 colonies.

Table 1. *Results of microbiological analysis.*

Microorganisms (cfu. mL^{-1})	Days								Standard AFNOR (cfu.mL^{-1})
	0	2	4	6	8	10	12	14	
Mesophilic spore-forming bacteria	0	0	0	0	0	0	0	0	$<10^6$
Fecal coliforms	0	0	0	0	0	0	0	0	$<10^2$
Total coliforms	0	0	0	0	0	0	0	0	$<10^3$
Fecal streptococci	0	0	0	0	0	0	0	0	$<10^2$
Salmonella	0	0	0	0	0	0	0	0	0
yeast	580	400	370	300	270	230	200	170	$<10^4$
Moulds	0	0	0	0	200	350	430	450	$<10^3$

3.4. Sensory Quality of the Tea-Based Concoction During Fermentation

The results of the sensory analysis of the tea beverage are summarised in Table 2. The results obtained show a significant increase in acidity during fermentation, ranging from 1.21±0.41 to 5±0. During the first six days of fermentation, the acidity is not strong enough but from the

eighth day it goes from 3.14 ± 0.77 to reach a score of 5 ± 0. The alcohol content also increases significantly during fermentation ranging from 1±0 to 4.21±0.32. The alcohol level is low the first six days and increases significantly from the eighth day from 3.92±0.26d to 4.21±0.32 on the last day of fermentation (day 14). The sugar level decreases significantly during the fermentation of "Kargasok" tea because it ranges from 4.07±0.91 to 1.14±0.36. The sweet taste is very high the first six days of fermentation but from the eighth day the sugar level drops significantly to reach 1.14 ± 0.36 on the fourteenth day. The bitterness of the drink increases significantly during the fermentation process (1±0 to 3.07±0.47). The bitter taste is weak the first six days but from the eighth day the degree of bitterness increases to reach a stable score of 3.07 ± 0.47. The smell of the drink is increasingly strong during fermentation (2.9±0.73 to 4.9±0.26). The first six days of fermentation the drink does not smell strong enough (2.9±0.73) but from the eighth day it becomes more and more pungent (4.9±0.26). The color of the drink becomes darker and darker (2.92±0.7 to 4.21±0.42) over time due to the combined action of water, alcohol and fermentative microorganisms which lyse the leaves into microelements thus darkening the drink during fermentation. The acceptability of the drink increases progressively during the fermentation, it ranges from 2.19±0.30 to 3.76±0.19. It is greater from the eighth day to the fourteenth day of fermentation.

Table 2. *Trends of sensory attributes of the tea beverage during the fermentation.*

Sensory attributs		Fermentation time (days)								
		0	2	4	6	8	10	12	14	Mean
Taste	Acidity	1.21±0.41^{a}	2±0.55^{b}	2.14±0.36^{b}	3.14±0.77^{c}	4.78±0.42^{d}	4.85±0.36^{d}	4.92±0.26^{d}	5±0^{d}	3.5±1.53
	Alcohol	1±0^{a}	2.14±0.53^{b}	2.14±0.53^{b}	3.14±0.86^{c}	3.92±0.26^{d}	4.07±0.47^{d}	4.14±0.36^{d}	4.21±0.32^{d}	3.09±1.22
	Sweetness	4.07±0.91^{a}	3.21±0.69^{b}	3.14±0.66^{b}	3.07±0.61^{b}	1.28±0.46^{c}	1.14±0.36^{c}	1.14±0.36^{c}	1.14±0.36^{c}	2.27±1.27
	Bitterness	1±0^{a}	1.85±0.36^{b}	2.42±0.64^{c}	2.42±0.64^{c}	3.07±0.47^{d}	3.07±0.47^{d}	3.07±0.47^{d}	3.07±0.47^{d}	2.5±0.84
Odour		2.9±0.73^{a}	2.9±0.73^{a}	2.9±0.73^{a}	2.9±0.73^{a}	4.9±0.26^{b}	4.9±0.26^{b}	4.9±0.26^{b}	4.9±0.26^{b}	3.9±1.13
Colour		2.92±0.7^{a}	2.92±0.7^{a}	2.92±0.7^{a}	2.92±0.7^{a}	3.92±0.26^{b}	4.07±0.47^{b}	4.14±0.36^{b}	4.21±0.42^{b}	3.5±0.81
Acceptability		2.19±0.30^{a}	2.66±0.39^{b}	2.61±0.30^{b}	2.94±0.38^{c}	3.65±0.12^{d}	3.69±0.19^{d}	3.72±0.16^{d}	3.76±0.19^{d}	3.15±0.64

In the same row, values with similar lowercase script are not significantly different at *($p<0.05$)*

4. Discussion

It appears from the results above that the pH of the beverage is acidic. This acidity is related to organic acids produced during fermentation by microorganisms, leading to increase in titratable acidity. The figure 1A above indicates a significant drop in pH coupled at the same time with an increase in titratable acidity. The evolution of both parameters follows three phases including two deceleration phases separated by a constancy phase for the pH; with regard to titratable acidity, there are two acceleration phases parted by a stability phase. Idise Okiemute and Emmanuel [13] reported that the evolution of pH is inversely to that of titratable acidity, thus the drop in pH at the end of fermentation is correlated with the increase in titratable acidity. The drop in pH implies that there are reactions that cause this decrease, it is the reaction causing the transformation of glucose into acid. This reaction is induced by fermentative microorganisms which convert sugar with the production of alcohol and acidic metabolites. The kombucha ferment being a biological complex made up of fermenting yeasts and bacteria is therefore responsible for this reaction. During fermentation, a continuous modification of the environment induced by the action of the ferment consisting of yeasts and certain bacteria is observed. These microorganisms use carbon and nitrogen substrates as nutrients and this is accompanied by the production of acid and alcohol metabolites. The total sugar content decreases significantly each two days with a sharp drop on the sixth day. The sugar content ranges from 10.43 gL^{-1} on day zero to 2.83 gL^{-1} on the fourteenth day of fermentation. This decrease could be due to the action of the fermentative microorganisms constituting the kombucha ferment which consume the sugar added during the production of the drink. The alcohol content increases significantly during fermentation as shown in figure 1B. The results give values ranging from 0.12gL^{-1} on the day of production of the drink to 5.89gL^{-1} on the fourteenth day of fermentation. This increase could be justified by the addition of sugar during the production of the drink. Indeed, microorganisms using sugar as nutrients, transform it into alcohol and carbon dioxide. The enzymes involved in the hydrolysis of sugar into alcohol are hydrolases. The degree Brix of "Kargasok" tea decreases significantly depending on the time of fermentation. This decrease indicates a fairly metabolic activity. The values oscillate between 9.08°Brix and 10.15°Brix. There is relative stability until the sixth day, then a deceleration until the fourteenth day. As the beverage to be fermented is a sweetened tea solution, as the reaction progresses, we obtain a beverage taste that gradually becomes acidic and alcoholic. Since sugar constitute the majority fraction of soluble matter at the beginning of the process, their transformation into alcohol would justify the reduction in the degree Brix. The conductivity varies very little the first four days but from the sixth day, we note a gradual increase in the conductivity until the fourteenth day of fermentation. It varies between 1080 and 1326 μScm^{-1}. This low variation on the first four days could be justified by a low content of dissolved matter and its increase on the following days is due to dissolution of the

solid matter which is accentuated during fermentation. It can be considered that the density of the drink decreases during fermentation, it varies from 1.05 to 0.93. The density drops slightly until the eighth day and remains unchanged until the twelfth, then a deceleration on the fourteenth day. This variation in density during fermentation is explained by the transformation of sugar into alcohol. When the sugar is converted into alcohol and carbon dioxide under the action of yeasts, the density drops because the ethanol obtained is more volatile and the carbon dioxide is released into the air. The polyphenols content in "Kargasok" tea is approximately stable for the fourth days and begins to increase at the sixth day and then stabilizes from the eighth day. The increase in phenolic compounds can be explained by a better extraction by water. Indeed, according to Bourgou [14], water is one of the best solvents for the extraction of phenolic compounds. The increase in total phenolic content by fermentation has also been reported by Peerajan [15] when they carried out a fermentation (15 days) *of Phyllanthus emblica* fruit juice by *Lactobacillus paracasei* HII01. They recorded an increase in the polyphenol content of 10.32 ± 0.28 mg of gallic acid equivalent/ml after 15 days of fermentation. Some bacteria can produce enzymes capable of metabolizing phenolic compounds. Bacteria such as *L. Plantarum* and some *Lactobacillus* spp have several enzymes such as tanase, β-glucosidase, feruloyl-esterase or phenolic acid decarboxylase to metabolize phenolic compounds [16, 17]. Fermentation breaks down the cellulosic walls leading to the release or synthesis of various antioxidant compounds. For example, the biosynthesis of natural antioxidant molecules, such as vitamin C, is induced by fermentation [18]. This increase in phenolic content is also observed in the work of Zerrouki [19] during industrial fermentation of orange juice. The physicochemical and microbiological characteristics (acid pH, type of microorganism) and the time would considerably increase the flavonoid content. Yang [20] identified fermentation which is responsible for modifying the content of bioactive compounds in certain products depending on the type of microorganism and the time of fermentation, which represent important parameters that can affect the concentration and profile of the phenolic compounds. As tannins are part of the large group of polyphenols, their content increases gradually following the influence of physicochemical parameters such as acid pH, the action of water and ethanol. The scavenging activity is correlated with the content of phenolic compounds in the medium as explained by Mbaiogaou [21] that there is a strong correlation between the concentration of total polyphenols and the antioxidant activity of plant extracts. In general, during fermentation, the levels of total polyphenols increased and so did the oxidative activity. The increase is due to the metabolism of phenolic compounds initiated by fermentative microorganisms. This activity correlates with the flavonoid content in the drink. The first six days but with slight variations and increases from the eighth day. The increase in the ferric reducing power during the fermentation would be explained by an increase in the content of antiradical compounds during the fermentation of the drink. The absence of total mesophilic aerobic flora in "Kargasok" could be justified by the respect of hygiene during the preparation but by some chemical parameters such as pH, alcohol content and the presence of bioactive compounds (polyphenols). The acid pH of the very acid medium which does not favour the development of most microorganisms and under the combined action of alcohol, the content of which increases during fermentation. The absence of colonies faecal streptococci indicates that there was no fecal contamination. This result is similar to that obtained by Iberraken [22] in Algeria who also noted an absence of faecal streptococci on blended juice made from orange, carrot and lemon (named OCC). Faecal coliforms do not survive for long outside the body, so their presence in food is a sign of relatively recent contamination [23], their absence at the time of production and the following days would be explained as for the case of faecal streptococci by good compliance with hygiene rules. Or else by the very acid pH of the environment which does not allow the development of these faecal coliforms. Iberraken [22] also obtained an absence of faecal coliforms in juice consisting of orange, carrot and lemon in Algeria. The number of yeast colonies are below the standard set by AFNOR (10^5 $CFUmL^{-1}$). The highest count of yeasts at the beginning is due to the richness of the medium in very essential sugar for their metabolism thus favouring their growth. The decrease in colonies during fermentation is due to the decrease in the sugar level in the drink. It is true that yeasts can resist in very acidic pH but not for such a long period of 14 days causing their decrease. Although ethanol is a product of the transformation of sugar by yeasts, increasing its content in the drink leads to the slowing down of fungal development. The number of mould colonies increases during fermentation according to the data obtained, from 0 to 45 colonies clearly below the standard set by AFNOR (10^5 $CFUmL^{-1}$). No colonies were observed at the first six days of fermentation and an appearance from the eighth day, thus following an increase until the last day of fermentation (day 14). This could be explained by a lack of hygiene during fermentation, the tissue used to cover the "Kargasok" tea could be a vector of contamination during sampling. The deposit of dust from the surrounding environment on the tissue could contaminate the drink during fermentation and when it was opened during for sampling. This value is significantly lower than that obtained by Tlendrebeogo [24] on tiger nut milk sold in the city of Ouagadougou in Burkina Faso which is 1.9 ± 1.0 $x10^5$ $CFUmL^{-1}$. The absence of *Salmonella* in the drink is a sign of compliance with hygiene and the good quality of the water used. Ji-Yeon Lee [25] also noted a complete absence of *Salmonella* in orange juice. Regarding the sensory analysis, this increase of acidity could be due to the conversion by fermentative microorganisms of sugar added into organic acids. The mean, which is 3.5±1.53, is substantially similar to that obtained by Ranaivosoa [2] on the fermented date drink (3.62±1.2). The increase of alcohol is due to the transformation of the added sugar into alcohol by the microorganisms in the kombucha ferment. The mean

value (3.09±1.22) of alcohol is lower than that found by Daoudou [27] on the fermented date drink (3.96±1.21). The mean alcohol value (3.09±1.22) is lower than that obtained by Daoudou [27] on the fermented date drink (3.96±1.21). This significant increase in smell could be due to acids and alcohol produced from sugar by fermentative microorganisms. The average value of 3.9 ± 1.13 is higher than that obtained by Bulembe [26] on carrot juice which is 2.6. The average score of color (3.5 ± 0.81) is lower than that obtained by Bulembe [26] with carrot juice which was 4. The overall acceptability, which is 3.15/5, indicates that the drink is judged as acceptable by the panel.

5. Conclusion

This work investigated changes in the physicochemical, phytochemical and microbiological parameters of "Kargasok" tea during fermentation. We found that some physicochemical parameters such as titratable acidity, alcohol content, and conductivity increased significantly. While other parameters such as pH, total sugar content, soluble solids content, and density decreased. Regarding the phytochemical parameters, we find a significant increase in total polyphenols, flavonoids, and tannin contents as well as antioxidant activity which was improved during the fermentation of "Kargasok" tea. Microbiological analysis showed an absence of microbial flora. The sensory profile showed a significant variation in bitterness, sweetness, acidity and alcohol taste, and smell. The drink was appreciated by the panel from the eighth day of fermentation.

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