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# Development of a beverage from red grape juice fermented with the Kombucha consortium

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**Abstract** Kombucha is a health-promoting fermented beverage traditionally made by fermenting a sweetened tea with a symbiotic culture of yeast species and acetic acid bacteria. The aim of this work was to develop a beverage using red grape juice as an alternative substrate. Grape juice contains various nutrient elements and phytochemicals, such as polyphenols, which possess a wide range of biological activities. We investigated the chemical characteristics and sensory and antimicrobial activities of the fermented grape juice Kombucha beverage. The pH decreased from 3.95 to 2.9 during the fermentation process and remained fairly constant thereafter, and the acetic acid bacteria and yeast counts in the broth increased up to 6 days of fermentation and subsequently decreased. Phenolic and anthocyanin contents and the antioxidant activity of the fermented beverage were higher after fermentation, with the maximum increase observed on the sixth day of fermentation when values were approximately 2.47- and 1.59-fold higher than pre-fermentation values, respectively, as assessed by 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) radical scavenging assays. Fourier transform infrared spectroscopy was used for the qualitative analysis of the grape juice before and after fermentation. Distinct peak variations in the spectral region between 2500 and 1650 cm$^{-1}$ were observed, which matched the appearance of organic acids and changes in phenolic compounds. Fermented juice Kombucha showed antibacterial activity toward all tested bacteria, which can be primarily ascribed to the increased production of acetic acid, but also to the biosynthesis of other metabolites, during the fermentation process. The 6-day fermented juice was the most appreciated by the taste panel based on the overall quality evaluation; with prolongation of fermentation the fermented juice acquired a distinct sour flavor.

## Introduction

Kombucha is a slightly sweet and acidic beverage that is generally prepared by fermenting sweetened black tea with the tea fungus, a symbiotic consortium of acetic acid bacteria and different yeasts known as SCOBY (Kappel and Anken 1993; Sreeramulu et al. 2000). Several lactic acid bacteria (LAB) have been isolated from some Kombucha associations (Greenwalt et al. 2000; Yang et al. 2010; Marsh et al. 2014). The Kombucha fermentation process leads to the formation of a cellulosic pellicle layer floating on the surface of the growth medium.

The composition of yeasts and bacteria in the Kombucha symbiotic consortium is highly variable. The main acetic acid bacteria found in the tea fungus are *Acetobacter xylinum* (Balentine 1997), *Acetobacter aceti*, *Acetobacter pasteurianus* (Liu et al. 1996) and *Gluconobacter oxydans* (Liu et al. 1996; Greenwalt et al. 2000; Kurtzman et al. 2001). In another study, Marsh et al. (2014) indicated that the dominant bacteria in five Kombucha samples from different origins are species of *Gluconacetobacter* and *Lactobacillus*. Many yeast species have also been identified in Kombucha samples, such as *Saccharomyces*,

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*Schizosaccharomyces*, *Zygosaccharomyces*, *Brettanomyces*, *Candida*, *Torulospora*, *Koleckera*, *Pichia*, *Mycotorula* and *Mycoderma* (Jayabalan et al. 2014).

The yeast cells convert the sugar into ethanol via the glycolysis metabolic pathway (Sievers et al. 1995). Acetic acid bacteria convert glucose to gluconic acid and fructose into acetic acid. Acetic acid stimulates the yeast to produce ethanol, and the presence of ethanol facilitates the growth of acetic acid bacteria and thereby the production of acetic acid (Liu et al. 1996). Both ethanol and acetic acid have been reported to have antimicrobial activity against pathogenic bacteria (Liu et al. 1996).

Kombucha beverages are claimed to have a number of curative properties, such as improving general health, increasing longevity and improving gastrointestinal disorders (Dufresne and Farnworth 2000; Jayabalan et al. 2007). The beneficial properties of Kombucha have long been attributed primarily to its acidic composition, such as gluconic acid, glucuronic acid, acetic acid and lactic acid (Pauline et al. 2001). Glucuronic acid is a well-known significant detoxicant. The conjugation of glucuronic acid with undesirable compounds increases the solubility of the latter and facilitates their transport and elimination from the body (Vína et al. 2013). Other components of the Kombucha beverage are sugars, ethyl gluconate, oxalic, saccharic, lactic, 5-ketogluconic acid, water-soluble vitamins (B1, B6, B12 and C), tea components and hydrolytic enzymes (Jayabalan et al. 2014).

The health benefit of Kombucha beverages has also been ascribed to the presence of phenolic antioxidants (Vijayaraghavan et al. 2000), which depend primarily on the phenolic content of the substrate and secondarily on the normal microbiota of the Kombucha culture, which in turn determine the nature of produced metabolites (Jayabalan et al. 2014). These compounds act as antioxidants in the body by scavenging harmful free radicals implicated in degenerative diseases. Black and green teas are the usual substrates used to prepare Kombucha beverages. The search for a fermentation media rich in antioxidants has led to the use of fruit juices, which contain more vitamins and nutrients for the human body (Ayed and Hamdi 2015). Grapes are known to be a potent source of various nutrient elements, such as vitamins, minerals, carbohydrates, edible fibers and antioxidants (Dopico-Garcia et al. 2008; Spacil et al. 2008; Xia et al. 2010). The most powerful antioxidants are in the form of polyphenols, which include phenolic acids, resveratrol, proanthocyanidins and flavonoids such as anthocyanin (Xia et al. 2010). Anthocyanins are the main polyphenolics in red grape varieties, whereas flavan-3-ols are more abundant in white ones (Cantos et al. 2002). Vinson et al. (2000) claimed that the grape juice polyphenols are more powerful antioxidants than ascorbates in human plasma. The antioxidant activity of phenolics may be due to their ability to act as reducing agents by donating hydrogen and/or capturing singlet oxygen,

or as chelators (Burin et al. 2010). Many epidemiological studies have reported that increased dietary intake of natural phenolic antioxidants from grapes correlates with reduced coronary heart disease, neurodegenerative diseases, and cancer (Tsanga et al. 2005; Jung et al. 2006; Shanmuganayagam et al. 2007; God et al. 2007).

Few studies have used materials other than tea as an alternative substrate for Kombucha (Yavari et al. 2010; Velićanski et al. 2014; Ayed and Hamdi 2015). The aim of this study was to evaluate the potential use of red grape juice for Kombucha fermentation. As the proprieties of the beverage are attributed to their antioxidant proprieties, we investigated the effects of fermentation time on polyphenol and total anthocyanin content and antioxidant activity. We also evaluated the sensory properties of the fermented juice and determined its antimicrobial activity against Gram-negative and Gram-positive pathogenic microorganisms.

## Material and methods

### Sample

Red grapes from *Vitis Vinifera* were bought from the supermarket. In the laboratory, grapes were removed from the stems and washed, following which juice was extracted by a juice extractor (Moulinex ZU5008; Groupe SEB, Écully, France). The grape juice was then pasteurized at 75 °C for 5 min and subsequently cooled at room temperature before processing.

The characteristics of the juice were as follows: total soluble solids, $18.2 \pm 0.9$ degrees Brix (°Br); pH, $3.95 \pm 0.13$; total acidity, $25.9 \pm 1.5$ meq/L; total anthocyanin content, $276 \pm 21$ mg/L; total phenol content, $2159.5 \pm 12.6$ mg galic acid equivalents (GAE)/L.

### Kombucha consortium

The Kombucha consortium was purchased from a supplier of traditionally prepared Russian black tea. *Zygosaccharomyces* and *Saccharomyces* were determined to be the most dominant yeast species, according to the criteria of Sievers et al. (1995). The acetic acid bacteria of the beverage were classified as *Acetobacter xylinus*, *Acetobacter pasteurianus* and *Gluconobacter oxydans* according to the criteria of Du Toit and Lambrechts (2002).

*Lactobacillus* sp and *Lactococcus lactis* subsp were isolated from Kombucha as described by Han (2007) and identified as *Lactobacillus plantarum* and *L. lactis* subsp. *lactis*. Preliminary identification was on the basis of cell morphology and phenotypic properties as described by Thapa et al. (2006).

Carbohydrate fermentation patterns of LAB were determined using the API 50 CHL identification system

(bioMerieux SA, Marcy l'Etoile, France). The APILAB PLUS database identification software (bioMérieux SA) was used to interpret the results.

We first boiled 200 ml water with 20 g saccharose, then added 2.4 g black tea to the solution was added and left it to draw for 5 min. After cooling, the sweet tea was filtered, transferred into a sterile 1-L beaker and inoculated with the Kombucha consortium (3% cellulose pellicles, 10% Kombucha beverage from a previous fermentation). The glass vessel was covered with a cotton cloth and incubated for 15 days at 30 °C in the dark to avoid oxidation of the phenolic compounds.

## Fermentation of grape juice

For fermentation, we poured 500 ml grape juice into a 2-L beaker (diameter 16.3 cm, height 18.8 cm) that has been previously sterilized at 121 °C for 20 min. The grape juice was then inoculated with the Kombucha consortium (3% cellulose pellicles, 10% of Kombucha beverage) and incubated in darkness for 12 days at 30 °C. At the end of the fermentation, the beverage was filtered to remove the cellulose pellicle which had formed and stored in capped bottles at 4 °C. The fermentations were carried out in triplicate.

## Analytical methods

The pH values were measured with a pH meter (model EL20; Mettler-Toledo, Columbus, OH), and the total acidity of fresh and fermented grape juices was determined according to the International Organization of Vine and Wine procedures (OIV 2010). Brix degree (°Br) was used as an indicator of soluble solid content (%). The total soluble solids content of the juice samples were evaluated using a handheld refractometer (RF.5532 Brix hand refractometer; Euromex Microscopen B.V., Arnhem, the Netherlands). The ethanol content (g/100 ml) of the fermented beverages was determined using a commercially available assay kit (R-Biopharm A.G., Darmstadt, German) according to the manufacturer' instructions.

## Determination of total phenolic content

The total phenolic content of each sample was determined according to the method described by Singleton et al. (1999). Briefly, 100 μl of diluted juice was mixed with 6 ml distilled water and 500 μl Folin-Ciocalteu's phenol reagent and the solution allowed to stand for 1 min before 1.5 ml of 20% $Na_2CO_3$ and 1.9 ml of distilled water. After incubation for 30 min at 25 °C, the optical density was measured at 760 nm using a spectrophotometer (model 63200 UV/Vis; Jenway, FelstedDunmow, Essex, UK). Total phenol concentration was expressed as milligram GAE per liter.

## Total anthocyanin content

The total anthocyanin concentration was estimated by a pH differential absorbance method (Randazzo et al. 2016). For each sample, Briefly, a 2-ml aliquot of each sample was added to 10 ml of sodium acetate buffer (0.4 M, pH 4.5) or to 10 ml of potassium chloride (0.025 M, pH 1.0) buffer. After the solutions were allowed to rest for 15 min, the absorbance was measured with a spectrophotometer (model 63200 UV/Vis; Jenway). The results were expressed as milligrams cyanidin-3- glucoside per liter of sample and obtained using the following equation:

$$\text{cyanidin}-3-\text{glucoside(mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times l),$$

where $A = (A_{\lambda 520} - A_{\lambda 700})_{pH\ 1} - (A_{\lambda 520} - A_{\lambda 700})_{pH\ 4.5}$; MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol) extinction; DF is the dilution factor; $\varepsilon$ is the molar absorption coefficient of cyanidin-3- glucoside (26,900 $M^{-1}$ $cm^{-1}$); l is the path length (1 cm)

## Total antioxidant activities

### 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay

This method is based on the reduction of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Zafra-Rojas et al. (2013). Briefly, 100 μl of the sample (at a dilution of 1:50 in water) was mixed with 500 μl of ethanolic solution (7.4 mg/100 ml) of stable DPPH. After a 1-h reaction time, the solution was stirred and centrifuged at 3000 rpm for 10 min, and absorbance was measured at 520 nm.

### 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay was performed according to the procedure described by Delgado-Andrade et al. (2005). The radical cation $ABTS^+$ was produced by the reaction of 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulfate under the conditions of darkness and room temperature for 16 h before use. The $ABTS^+$ solution was then diluted with deionized water to an absorbance of $0.70 \pm 0.10$ at 734 nm, and 15-μl aliquots of juice samples diluted tenfold were each mixed with 950 μl $ABTS^+$ solution; each mixture was then allowed to stand for 6 min and the absorbance was then measured.

A 6-hydroxy-2, 5, 7,8-tetramethylchroman-2-carboxylic acid (Trolox) standard calibration curve was constructed. The results were expressed as millimoles of Trolox equivalents (TE) per liter of fermented juice.

The equation used for calculating the scavenging capacity of the juice was:

$$DPPH\ or\ ABTS\ radical\ scavenging\ activity\% = \left[(Ac-As)\Big/Ac\right] \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

## Fourier transform infrared spectroscopy studies

The structural characterization of the samples was examined by Fourier transform infrared spectroscopy (FTIR) using a Nicolet IR 200 FT-IR spectrophotometer (Thermo Scientific, Madison, WI). FTIR spectra were recorded over the range of 500–4000 cm$^{-1}$.

## Microbiological analysis

The growth rates of yeasts and acetic acid bacteria were determined using potato dextrose agar (Merck KGaA, Darmstadt, Germany) and Kneifel medium (30 g/L yeast extract, 1 ml/L bromocresol green (2.2%), 20 g/L agar; OIV 2010), respectively. The culture medium was supplemented with 20 ml/L 95% ethanol, 100 mg/l natamycin to inhibit the growth of yeasts and 12.5 mg/L penicillin to eradicate the growth of LAB after autoclaving. Blue and green colonies indicated the presence of *Acetobacter* and *Gluconobacter*, respectively. For the enumeration of acetic acid bacteria and yeasts in the broth samples, 1-ml aliquots were used to determine the growth pattern. Samples after a series of decimal dilution (prepared with 0.1% sterile peptone water) were spread on each of the two media for enumeration of the desired microorganisms (Chen and Liu 2000).

LAB were enumerated on MRS agar (Oxoid Ltd., London, UK) supplemented with 100 μg of cycloheximide per milliliter to suppress the growth of yeasts (Bae et al. 2006). Colonies that grew on the surface of the plates and were both Gram positive and Catalase negative were counted as LAB.

For the enumeration of microorganisms in cellulose pellicles, 180 ml of sterile peptone water (0.1%) was added to 20 g bacterial cellulose sample and the sample homogenized in a blender for 9 min. The suspension obtained was used for the enumeration of bacteria and yeasts (Chen and Liu 2000).

The colonies were counted after 3 days of incubation at 30 °C. Results were expressed as colony-forming units per milliliter or gram.

## Antimicrobial activity

Antimicrobial activities were determined using the well diffusion method and tested with the following bacteria: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 10541, *Bacillus cereus* ATCC 11778, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 10536.

Samples used for the determination of antimicrobial activity were unfermented juice, 12-day fermented beverage and neutralized fermented juice (with 0.1 M NaOH). Fermented samples were filtered through a sterile microfilter (diameter 0.22 μm) to remove cells.

The bacterial strains were grown on Mueller–Hinton slants at 37 °C for 24. After incubation, the cells were first washed from the agar surface and then suspended in sterile physiological solution. The bacterial suspensions were adjusted to a concentration of 10$^7$ CFU/ml. A 1-ml aliquot of this suspension was homogenized with 19 ml of melted Mueller–Hinton agar and the agar suspension poured into petri dishes. Three wells (diameter 9 mm) were cut into the agar media, and 100 μl of juice was then poured into the wells. Inoculated plates were incubated at 37 °C for 24 h, and zones of inhibition were measured. Chloramphenicol (30 μg) was used as a reference control to evaluate the tested strains' susceptibility. The evaluation of antimicrobial activities of samples was carried out in three repetitions.

## Descriptive analysis

Sensory evaluation tests were performed by a panel of 20 trained members, and a descriptive analysis was conducted on three samples: fresh juice and 6- and 12-day fermented juices.

The panelists first participated in four 1-h training sessions, during which time descriptors were developed. The final descriptors were chosen by the panelists after discussions during training. After the training period, samples were evaluated in duplicate. In all cases, samples were presented randomly and were served at an ambient temperature in coded clear plastic glasses. Potable water was available for rinsing the mouth between test samples.

To evaluate aroma intensity, sweetness, acidity, color and overall acceptability, we used a scoring range of 1–9, where 1 indicated extreme dislike; 2, great dislike; 3, moderate dislike; 4, slight dislike; 5, neither liking nor dislike; 6, slight liking; 7, moderate liking; 8, great liking; 9, extreme liking.

## Statistical calculations

All of the data were presented as the average of triplicate measurements ± standard deviation (SD). Statistical treatment of the data was carried out using one-way analysis of variance. The significance level was set at $p < 0.05$. SPSS for Windows, version 16.0 (IBM Corp., Armonk, NY) was used for the analyses.

# Results and discussion

## Changes in chemical and microbiological parameters during fermentation

This study was carried out to explore the suitability of red grape juice as raw material for Kombucha fermentation. The changes observed in the concentrations of yeast, acetic acid bacteria and LAB during fermentation, based on cell counts, are presented in Fig. 1. The yeasts and acetic acid bacteria were able to grow by assimilating the sugar present in the juice, as demonstrated by their increasing cell numbers in the broth up to day 6 of culture and their decreasing number thereafter. Chen and Liu (2000) reported that the creation of an anaerobic and starved environment during the fermentation period are responsible for the decrease in viable cell density observed during fermentation. In our study, the evolution of the *Gluconobacter* growth curve during the fermentation followed the same trend as that of *Acetobacter*.

The number of viable yeast cells was tenfold higher than that of viable acetic acid bacteria cells because the metabolic activity of acetic acid bacteria is lower than that of yeasts, as reported by Goh et al. (2012). Our results are contrary to those of Chen and Liu (2000) who found a higher number of viable acetic acid bacteria cells than viable yeast cells in the broth of various tea fungus samples.

LAB were present in only low numbers in the Kombucha consortium, with the majority identified as *Lactobacillus plantarum* and *Lactococcus lactis* subsp *lactis* by preliminary identification on the basis of cell morphology and phenotypic properties, as described by Thapa et al. (2006). During fermentation the proportion of LAB increased from 1.3 $10^2$ to 5.7 $10^3$ CFU/ml within 2 days and thereafter decreased. This result differs from that of Marsh et al. (2014) who reported the lowest proportion of LAB was on day 3 of fermentation and
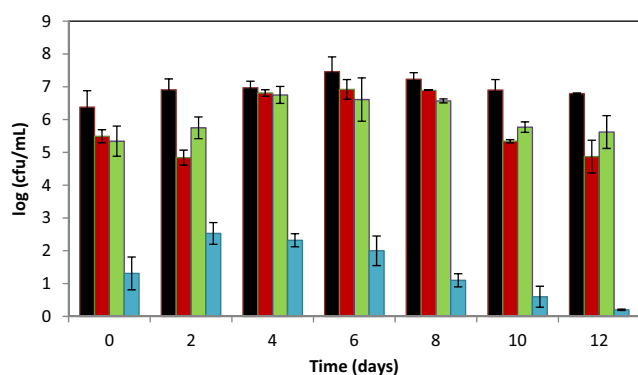
the highest on day 10. Yang et al. (2010) showed that LAB might support the growth of acetic acid species.

The cell concentrations of bacteria and yeasts in the pellicle were lower than those in the broth (unreported data). This observation is in agreement with the results reported by Chen and Liu (2000), Goh et al. (2012) and Marsh et al. (2014). These authors found that viable counts of acetic acid bacteria in the broth were generally higher because most acetic acid bacteria were entrapped within the floating cellulosic matrix, rendering their exact enumeration difficult. To the contrary, Reiss (1994) found that the cell concentration of acetic acid bacteria in the upper pellicle portion was higher than that in the liquid broth due to a greater oxygen supply.

As shown in Table 1, the pH value of the fermented juice decreased simultaneously with the increase in organic acids. Similar results have been reported in previous studies (Sievers et al. 1995; Jayabalan et al. 2007; Ayed and Hamdi 2015). The decrease in pH can be beneficial in terms of preventing the chemical degradation of polyphenols and retaining beverage color. Under acidic conditions, anthocyanins retain their chemical structure and become more stable (Torskangerpoll and Andersen 2005). Vīna et al. (2014) attributed the beneficial properties of Kombucha beverage primarily to its acidic composition (.

Previous studies have proven that the major organic acids produced during fermentation are acetic acid, gluconic acid and glucuronic acid (Jayabalan et al. 2014). In fact, in the Kombucha system, yeasts convert glucose and fructose into ethanol via glycolysis (Jayabalan et al. 2014). Acetic acid bacteria use glucose to produce gluconic acid and ethanol to produce acetic acid, with the latter being the predominant organic acid produced by acetic acid bacteria (Jayabalan et al. 2010). Glucuronic acid is considered to be one of the important components found in Kombucha beverage due to its detoxifying action through conjugation to the xenobiotics



**Fig. 1** Changes in the number of total yeast (*black bars*), *Gluconobacter* (*red bars*), *Acetobacter* (*green bars*) and lactic acid bacteria (*blue bars*) during grape juice fermentation by the Kombucha consortium. Data are presented as the average of triplicate measurements ± standard deviation (SD). *CFU* Colony-forming unit

**Table 1** The changes in pH values, total acidity and ethanol during grape juice fermentation by the Kombucha consortium

| Length of fermentation (days) | pH | Total acidity (meq/L) | Ethanol (g/100 ml) |
|---|---|---|---|
| 0 | $3.95 \pm 0.13$ | $25.9 \pm 1.5$ | $0 \pm 0.0$ |
| 2 | $3.37 \pm 0.01$ | $35.1 \pm 5.2$ | $0.11 \pm 0.01$ |
| 4 | $3.2 \pm 0.01$ | $49.2 \pm 3.3$ | $0.39 \pm 0.01$ |
| 6 | $3.18 \pm 0.02$ | $62.2 \pm 5.1$ | $0.52 \pm 0.03$ |
| 8 | $2.9 \pm 0.00$ | $77.4 \pm 0.3$ | $0.67 \pm 0.01$ |
| 10 | $2.92 \pm 0.07$ | $90.1 \pm 4.2$ | $0.45 \pm 0.04$ |
| 12 | $2.91 \pm 0.01$ | $104.2 \pm 3.2$ | $0.29 \pm 0.01$ |
|  | $P < 0.01$[a] | $P < 0.01$[a] | $P < 0.01$[a] |

Data are presented as the average of triplicate measurements ± standard deviation

[a] Change between baseline (day 0) and day 12 of fermentation is significant at $P < 0.01$ for all three parameters

(Vīna et al. 2014). Jayabalan et al. (2007) established that maximum D-glucuronic acid concentration was obtained after 12 days of fermentation on sweetened black tea. Yavari et al. (2010, 2011) found that the highest level of glucuronic acid produced on cherry juice and grape juice was achieved after 14 days of fermentation and that grape juice was the best substrate for glucuronic acid production by Kombucha culture. Yavari et al. (2011) found that the products obtained on grape juice had a glucuronic acid concentration of between 34 and 178 g/L, which may be considered as an advantage compared to the production of glucuronic acid on other substrates.

pH stabilization was observed from day 8 of fermentation onward despite the increase in organic acid content. One possible explanation is the dissociation of carbon dioxide and the formation of the amphiprotic hydrocarbonate anion $HCO_3$. This anion easily reacts with the hydrogen ions from the organic acids present, preventing further changes in the pH, thus contributing to the buffer character of the medium (Chen and Liu 2000; Jayabalan et al. 2008). The low pH and high acidity may provide some protection against invasive contaminants (Greenwalt et al. 2000).

Changes in ethanol production in the fermented beverage are shown in Table 1. Ethanol content increased to a maximum on day 8 of fermentation and reached a value of about 0.67 g/L, followed by a slow decline. A similar observation was also made by Chen and Liu (2000) who explained the ethanol production decrease by the low pH value and the decreasing low sugar concentration in the broth as fermentation proceeded.

The health-promoting activities of Kombucha beverage are also attributed to their phenolic content and their ability to act as antioxidants. Following the initiation of the fermentation process, the total phenolic content of the fermented juice increased significantly ($P < 0.05$), showing a 40% increase by day 6 (Fig. 2). The observed changes in total phenolic content during fermentation are comparable with those reported previously in investigations on the traditional Kombucha beverage of black tea (Chu and Chen 2006; Jayabalan et al. 2007). These authors observed that total phenol content increased

linearly during fermentation and explained this increase by both enzyme synthesis by the Kombucha consortium and by acid hydrolysis.

*Gluconbacter* has been identified as the key bacterial strain which enhances the bioaccessibility of polyphenols and the antioxidant activity of the beverages (Dufresne and Farnworth 2000). However, we observed a slight decrease in phenolic content from the 10th day onwards in our study. This phenomenon can be explained by the polymerization of some of the phenolic compounds to molecules of higher molecular weight, leading to the detection of lower polyphenol content. This observed decrease in phenolic content is in agreement with the results reported by Chu and Chen (2006).

The evolution of monomeric anthocyanins during the fermentation was characterized by an increase up to day 6 (Fig. 2), followed by a decline. Two different processes have been proposed to explain this evolution pattern: (1) monomeric anthocyanins combine with other phenolic compounds (Somers and Evans 1977), and (2) monomeric anthocyanins are adsorbed onto microorganism cells (Morata et al. 2003). In addition to their direct role on color, anthocyanins also contribute to the taste and chemical characteristics of the beverage (Mazza and Brouillard 1987; Cai et al. 1990; Liao et al. 1992).

The health benefits of Kombucha are also attributed to its antioxidant activity. Antioxidants are known to prevent many disorders and metabolic diseases caused by free radicals (Jayabalan et al. 2014). In our study, we used two radical scavenging assays (ABTS, DPPH) to determine the antioxidant activity of the fermented juice. The results of these assays are shown in Fig. 3. The DPPH and ABTS radical scavenging activity increased by 55.7 and 38.1%, respectively, after 6 days of fermentation, then stabilized. Similar trends have been reported by many authors (Chu and Chen 2006; Jayabalan et al. 2008; Malbaša et al. 2011). Malbaša et al. (2011) and Ayed and Hamdi (2015) observed a significant increase in DPPH radical scavenging activity of tea and cactus pear juice during fermentation, with average values of 48.7 and 18.1%, respectively.

The extent of the antioxidant activity in the Kombucha beverage would appear to depend on many factors, such as



**Fig. 2** The changes in total phenolic content (*blue line/blue diamonds*) and total monomeric anthocyanin content (*red line/red squares*) during the fermentation of red grape juice by the Kombucha consortium. Data are presented as the average of triplicate measurements ± SD. *GAE* Galic acid equivalents
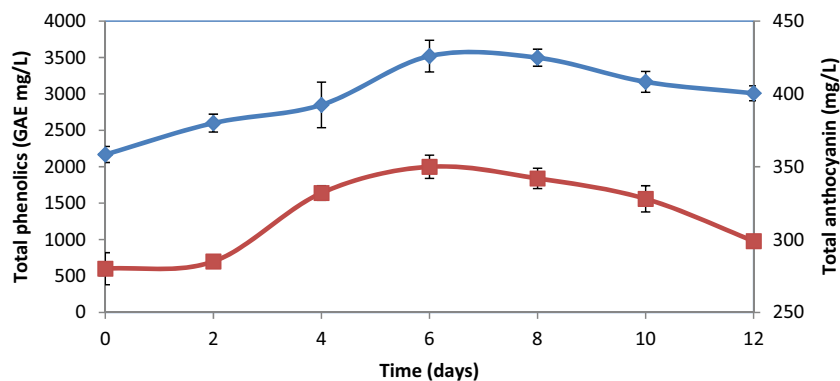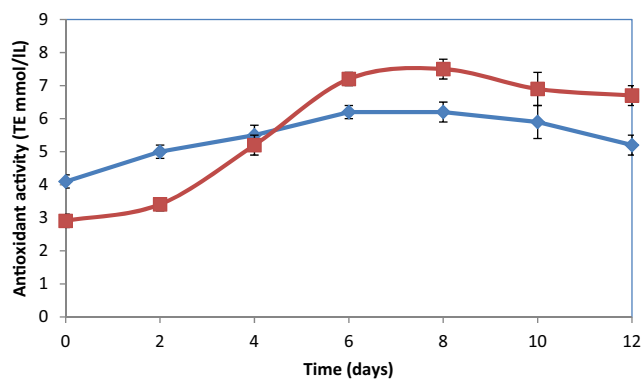
**Fig. 3** The changes in 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; *red line/red squares*) and 2,2-diphenyl-1-picrylhydrazyl (ABTS; *blue line/blue diamonds*) scavenging abilities during the fermentation of red grape juice by the Kombucha consortium. Data are presented as the average of triplicate measurements ± SD. *TE* Trolox (6-hydroxy-2, 5, 7,8-tetramethylchroman-2-carboxylic acid) equivalents

fermentation time, type of substrate, and the normal microbiota of the Kombucha culture, which in turn determines the nature of the secondary metabolites which develop during the fermentation process (Jayabalan et al. 2014). We considered the scavenging effects to be attributable to the sum of the antioxidant capabilities of many compounds present in the grape juice and to the synergistic effects between certain metabolic produced during the fermentation process. Maier et al. (2009) claimed that the antioxidant activity could be a synergic effect among several compounds, rather than a single one. Singletary et al. (2007) indicated that anthocyanins have strong free radical scavenging and antioxidant activities, which in contrary to Arnous et al. (2002) who states that the anti-radical activity is due to the flavan-3-ols, and not to the anthocyanins.

This increase in antioxidant activities may be also due to the peptides released by yeasts during autolysis. Alcaide-Hidalgo et al. (2007) showed that peptides released by *S. cerevisiae* during autolysis under wine conditions presented antioxidant activities. The presence of LAB probably played also a role in the enhancement of these activities because of their proteolytic system. Alcaide-Hidalgo et al. (2007) also found that the sequential inoculation of *Oenococcus oeni* under conditions similar to those in wine enhanced DPPH scavenging activity through the release and/or production of peptides with biological activity derived from nitrogen compounds after the autolysis of *S. cerevisiae*.

The total content of polyphenols and monomeric anthocyanins was positively correlated to the antioxidant activity of the beverage before and after fermentation. A high polyphenol content is generally associated with high antioxidant activity (Sun et al. 2015). However, Dani et al. (2009) suggested that the antioxidant capacity of phenolics has a concentration saturation limit, and that above this limit the activity cannot increase further with phenolic concentration.

Grape juice and fermented grape juice were further subjected to FTIR spectrophometric analysis, and the functional groups of the components were separated based on their peak ratio. FTIR spectroscopy is an analytical technique that provides structural information on molecular features of a large range of compounds. FTIR spectrometry has been previously used in the analysis of a number of foods, including wine (Fernández and Agosin 2007).

In our study, the FTIR spectrum of grape juice (Fig. 4a) showed a peak at 3366 $cm^{-1}$, which can be associated with C–H stretching vibrations and with O–H stretching of alcohol, phenol or carboxyl groups (COOH). The peak at 2940 $cm^{-1}$ is due to C–H stretching vibration of aliphatic compounds. The principal bands in the 2000–1500 $cm^{-1}$ region are due to C=C and C=O– stretching. The peaks at 1664 and 1424 $cm^{-1}$ were assigned to symmetrical and asymmetrical stretching vibration, respectively, for the carboxyl ion (COO⁻), indicating the existence of carboxylic acid, ester or carbonyl groups. The deformation vibration of the C–C bonds in the phenolic groups adsorb in the region between 1500 and 1400 $cm^{-1}$. The 1364 $cm^{-1}$ absorption band can be attributed to the O–H in plane deformation in polyphenols. IR absorption due to the presence of sugar functional groups is within the range of 1262 and 1065 $cm^{-1}$.

The bands between 1542 and 965 $cm^{-1}$ correspond to the vibration of the C–O, C–C, C–N and C–H bonds in this region. This area provides important information regarding organic compounds such as sugars, organic acids, alcohols and aromatic compounds.

The bands of peaks at 1098 and 995 $cm^{-1}$ are generally assigned to OH deformation and C–O stretching in phenolic, to C–H deformation of CH2 and CH3 groups and to COO⁻ anti-symmetric stretching. The peaks between 670 and 900 $cm^{-1}$ correspond to C–H deformation vibration of aromatic compounds. The identification of the principal absorption bands in the FTIR spectra and their corresponding assignments are based on published values (Zhang et al. 2010; Agatonovic-Kustrin et al. 2013; Park et al. 2015). The spectrum of the fermented juice (Fig. 4b) differed greatly from that of the grape juice before fermentation in the wavelength regions between 2500 and 1650 $cm^{-1}$, matching the appearance of organic acids and changes in phenolic compounds.

## Antimicrobial activity

Seven microorganisms were tested for their sensitivity to unfermented, fermented and neutralized fermented grape juice by the Kombucha consortium. The antimicrobial activity was determined by the agar well-diffusion method (Table 2). The grape juice was inactive against all tested microorganisms. These observations are in agreement with the findings of Rhodes et al. (2006) who found that grape juice was not active
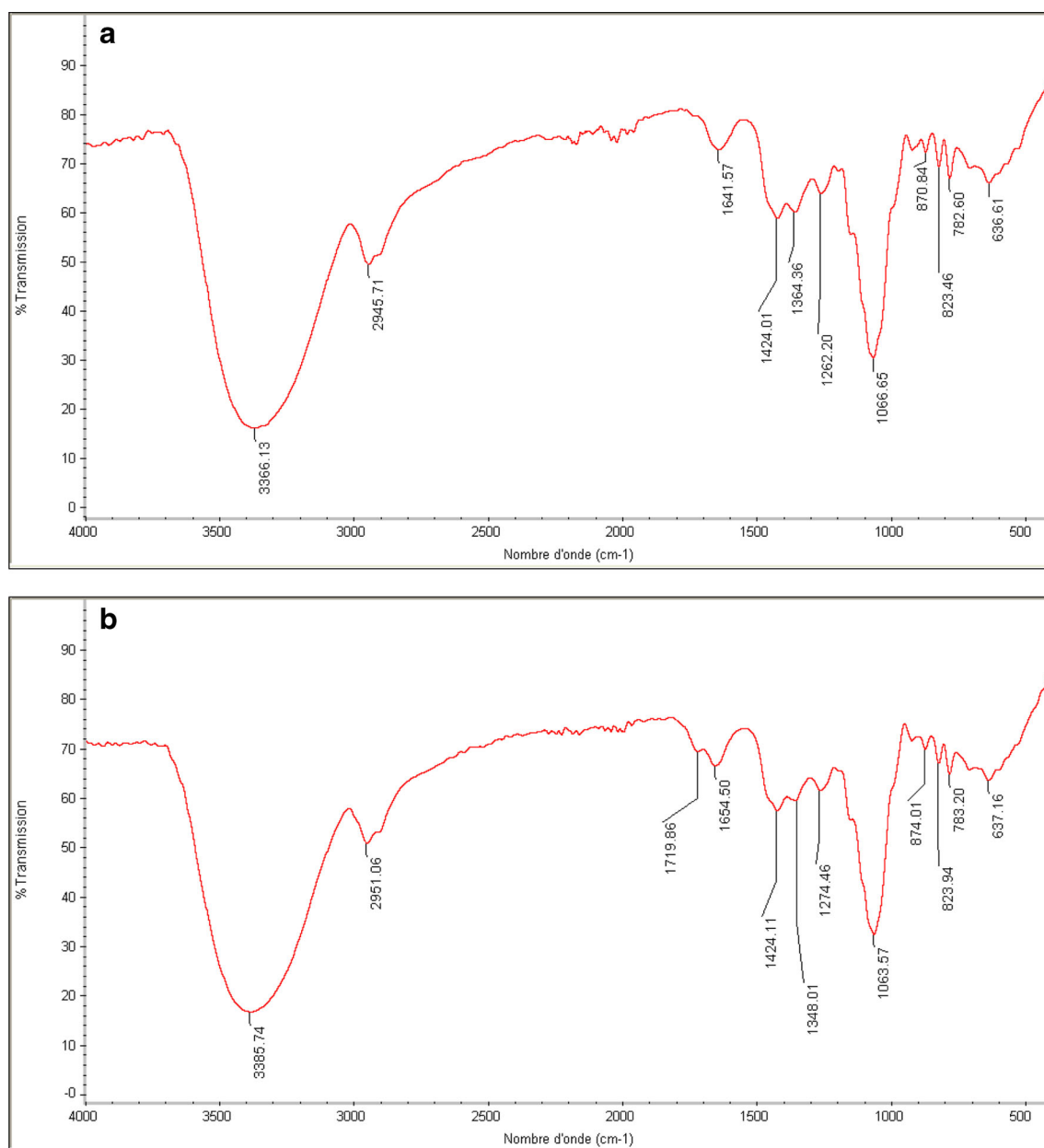
**Fig. 4** Fourier transform infrared spectra for the grape juice before (**a**) and after (**b**) fermentation by the Kombucha consortium

**Table 2** Antimicrobial activity of unfermented, fermented and neutralized fermented grape juice determined by the agar well-diffusion method

| Test bacterium | Unfermented juice[a] | Fermented juice[a] | Neutralized fermented juice[a] | Chloramphenicol[a] | $P$ |
|---|---|---|---|---|---|
| *Escherichia coli* ATCC 10536 | 0 | 2.7 ± 0.7 | 0 | 3.1 ± 0.1 | <0.01 |
| *Pseudomonas aeruginosa* ATCC 9027 | 0 | 2.8 ± 0.3 | 0 | 3.2 ± 0.5 | <0.01 |
| *Klebsiella pneumoniae* ATCC 10031 | 0 | 2.8 ± 0.2 | 0 | 3.4 ± 0.1 | <0.01 |
| *Staphylococcus aureus* ATCC 6538 | 0 | 3.0 ± 0.4 | 1.22 ± 0.32 | 3.1 ± 0.2 | <0.01 |
| *Enterococcus faecalis* ATCC 10541 | 0 | 2.2 ± 0.1 | 0 | 3.3 ± 0.2 | <0.01 |
| *Bacillus cereus* ATCC 11778 | 0 | 2.9 ± 0.1 | 0 | 2.1 ± 0.1 | <0.01 |
| *Staphylococcus epidermidis* ATCC 12228 | 0 | 2.2 ± 0.3 | 0 | 3.2 ± 0.2 | <0.01 |

Values presented in table are the diameter of the halo zone in centimeters, including the diameter of the well (9 mm)well

against *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*.

Marked inhibition was only associated with the fermented beverage. The clear zones appearing around the wells indicate bacteriostatic activity against tested bacterial strains (Table 2). A higher effect was observed against *S. aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *E. coli* and *B. cereus*.

The results suggest that the antibacterial activity was not due to polyphenolics, but rather to metabolites produced during fermentation. Acetic acid is known to be the main antimicrobial agent in Kombucha tea (Liu et al. 1996; Sreeramulu et al. 2000). Steinkraus et al. (1996) showed that the antimicrobial activity of Kombucha against *Helicobacter pylori*, *E. coli*, *S. aureus*, and *Agrobacterium tumefaciens* was attributable to their acetic acid content. Acetic acid, as well as other organic acids, can influence antimicrobial activity by two primary mechanisms: cytoplasmic acidification and accumulation of the dissociated acid anion to toxic levels (Mani-López et al. 2012).

The neutralized beverage showed bacteriostatic activity only against *S. aureus*, but this activity was much lower than that of its corresponding fermented beverage. This result is contrary to that reported by Sreeramulu et al. (2000) who found that traditional Kombucha exerts antimicrobial activity against *E. coli*, *Shigella sonnei*, *Salmonella enteriditis* and *Salmonella typhimurium* at neutral pH and after thermal denaturation. These findings suggest that the antimicrobial activity of fermented juice is not only due to its acidity or organic acid content, but also to other biologically active compounds or metabolites biosynthesized during the fermentation process.

### Sensory analysis

The sensory scores were based on the 9-point hedonic scale ratings assigned by the panelists. The sensory profile of the fermented juice is based on the marks given for each attribute by the whole panel. Figure 5 shows the spider charts for fresh and fermented juices.

Fermented juices had a sharp odor, which varied greatly depending on the duration of the fermentation period. After 12 days of fermentation, the smell received a score of <4 and was considered to be unacceptable by the taste panel. The 6-day fermented juice was perceived by the taste panel as being less sweet than the fresh juice sample. However, the analyzed taste descriptor did not significantly differ ($P > 0.05$).

The acid taste of the 12-day fermented sample was more intense than that of the other samples and judged unacceptable by the taste panel. The prolongation of fermentation to more than 6 days turned the pleasant and mild beverage into a kind of vinegar with a pronounced sourish taste. Reiss (1994) reported that teakwass was a refreshing beverage with a fruit-like taste when produced within 6–10 days of incubation; prolongation of fermentation yielded a distinct vinegar-like
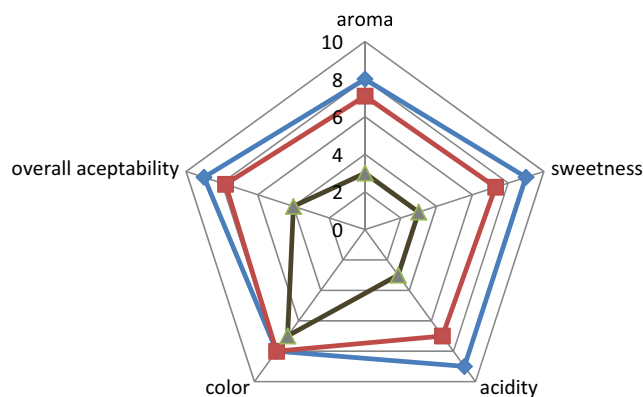


**Fig. 5** Sensory evaluation of color, odor, acidity, sweetness, taste and overall acceptability of fresh *(blue line/blue diamonds)* and fermented *(red line/red squares* 6-day fermentation, *black line/gray triangles* 12-day fermentation)* red grape juice. Scores ranged from 1 to 10, with 1 indicating extreme dislike and 9 indicating extreme liking

flavor. The fermented juices had a lighter color than fresh juice, with the change in color caused by chemical modifications of the phenolic compounds, particularly anthocyanins.

The 6-day fermented juice received an overall assessment of acceptability by the taste panel after smelling and tasting. It had a good appearance and high acceptability, contrary to the 12-day fermented juice which had a vinegary taste. The time required to obtain Kombucha beverage with optimum good sensory characteristics was 6 days.

### Conclusions

These findings support the possibility of developing a grape juice Kombucha beverage with a high added value and functional properties. Six days of fermentation appeared to be sufficient to improve the nutritional and sensory characteristics of the beverage and to reduce the production of unwanted compounds, such as organic acids which give the beverage a vinegary taste.

During the fermentation, valuable compounds were generated which gave the beverage a strong antioxidant character. Furthermore, the beverage showed an antimicrobial activity against the tested Gram-positive and Gram-negative pathogenic bacteria. However, the neutralized beverage showed a bacteriostatic activity only against *Staphylococcus aureus*. We therefore conclude that the antimicrobial activity is not only due to the acidity of the beverage, but also to other metabolites biosynthesized during the fermentation process.

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