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# Insights into the fermentation biochemistry of Kombucha teas and potential impacts of Kombucha drinking on starch digestion

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ABSTRACT

The biochemistry of Kombucha fermentation was analyzed using green and black teas (GTK and BTK). Sucrose disappeared linearly in Kombucha mediums during the two week fermentation period for GTK (2.3 $g.day^{-1}.L^{-1}$) but only during the first week for BTK (5.0 $g.d^{-1}.L^{-1}$). The produced glucose and fructose formed disappeared faster in BTK than in GTK and in both fermentation mediums glucose was preferred to fructose as the carbon source. Ethanol, acetate equivalents and cellulose linearly increased during the fermentation (0.17, 0.35 and 0.50 $g.d^{-1}.L^{-1}$ in GTK, and 0.15, 0.46 and 0.63 $g.d^{-1}.L^{-1}$ in BTK, respectively). Likewise, the disappearance of glucose + fructose in Kombucha first linearly increased during the first stages of fermentation before it dropped at day 9 for GTK and at day 12 for BTK, suggesting a change of the carbon source. The protein fraction (<0.5 g/L) transiently increased during the fermentation of teas and so did a 60-kDa protein band in SDS-PAGE. In both GTK and BTK, only slight changes were observed in total phenolics and in the main tea flavanols. Nevertheless, the theaflavins moderately increased (more than 50% at day 15) and the thearubigins markedly decreased (more than 2-fold at day 15). The Kombucha beverage was able to strongly inhibit starch hydrolysis by porcine pancreatic alpha-amylase and the inhibition potency increased during fermentation progress. The active compounds were suspected to be monomeric and/or oligomeric phenolic compounds. After drinking a bowl of Kombucha beverage, the concentration of these phenolics could be sufficient for inhibiting, to a certain extent, pancreatic alpha-amylase in the small intestine, with a possible impact on starch digestion and on net absorbed glucose.



## 1. Introduction

Kombucha is a traditional beverage consumed in various parts of the world and especially in Asia. Its earliest known use originated with Dr. Kombu in 220 BC for curing the digestive troubles of Japan's emperor. Many health promoting effects of Kombucha have been claimed (Greenwalt, Steinkraus, & Ledford, 2000), but adverse effects attributed to this beverage have been reported as well (Ernst, 2003). Kombucha is prepared under aerobic conditions by fermenting sweetened black or green tea with tea fungus. The product is a slightly sweet and carbonated acidic beverage resulting from numerous compounds that are produced by the symbiotic culture of bacteria and yeasts (Liu, Hsu, Lee, & Liao, 1996; Teoh, Heard, & Cox, 2004). The Kombucha fermentation process also leads to the formation of cellulose pellicles floating at the surface of the growth medium, due to the activity of some strains such as *Acetobacter xylinum* (Johnsy, Ramana, Sabapathy, & Bawa, 2005).

Sucrose is hydrolyzed by Kombucha invertases into glucose and fructose, which are used as a carbon source of the symbiotic system. The main metabolites identified in the fermented beverage are acetic, lactic, gluconic and glucuronic acids, and the alcohols ethanol and glycerol (Blanc, 1996; Chen & Liu, 2000; Jayabalan, Marimuthu, & Swaminathan, 2007). Acetic acid is dominant in the beverage and was able to stimulate ethanol production by yeast and, in turn, ethanol stimulated acetic acid production (Liu et al., 1996). Kombucha beverage also contains plenty of other substances, such as phenolic compounds, which constitute about one third of the dry mass of tea (Dufresne & Farnworth, 2001). Tea is generally added at a concentration of about 10 g/L in the fermentation medium. Flavonoids are the most abundant, especially the flavanols epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC) (Friedman et al., 2005). The content and composition of the phenolic fraction (phenolics) in Kombucha vary considerably depending on the variety of tea and on their own processing procedures. During black tea manufacture, the catechin group undergoes oxidations and other chemical reactions forming the dimeric theaflavins (TFs) and the oligomeric thearubigins (TRs) (Peterson et al., 2004). The subtle combination of the contents of TFs and TRs results in a typical astringency, brightness, mouth feel (thickness) and color of the black tea beverage (Obanda, Owuor, Mang'oka, & Kavoi, 2004). Kombucha beverage also contains several nitrogen-containing compounds, such as amino acids,

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methylxanthine alkaloids (caffeine, theophylline and theobromine) and proteins.

Many studies have been devoted to Kombucha fermentation and they have provided much data on the chemical composition of the Kombucha beverage, but it is rather difficult to build a clear overview of the biochemistry of this fermentation process.

We followed several biochemical markers of Kombucha fermentation of green and black teas for a period of two weeks. The metabolism of carbon was targeted using sucrose, glucose, fructose, cellulose, ethanol and total acetic acid equivalents. Caffeine and proteins were followed as markers of nitrogen metabolism, whereas the phenolic fraction was probed as total phenolics, TRs, TFs and the monomeric compounds previously reported to be present in Kombucha beverage (EGCG, GCG, ECG, catechin (C) and epicatechin (EC). Concomitantly, Kombucha samples were tested on the activity of porcine pancreatic alpha-amylase. The aims of the study were to get a general overview of the Kombucha fermentation biochemistry and to address the impact of frequent consumption of Kombucha beverage on starch digestion.

## 2. Materials and methods

### 2.1. Preparation of Kombucha and sampling

Green and black teas were manufactured from *Camellia sinensis* by the Tunisian Office Trade. Kombucha tea was prepared as previously reported (Jayabalan et al., 2007). Briefly, sucrose was solubilized in deionized water at 100 g/L and, after boiling, green or black tea was added at 12 g/L and allowed to infuse for 5 min. Then, the mixture was filtered using a sterile sieve and 200 mL of the filtrate was poured into a 500-mL sterilized glass flask. Thereafter, 10% (v /v) of an antecedent Kombucha culture as well as 3% (m/v) of cellulosic pellicle were added to the cooled Kombucha medium. Finally, the flasks were covered with sterile gauze and fermentation was allowed to proceed without light at 24 °C. After 3, 6, 9, 12 and 15 days of fermentation, one flask of GTK and one flask of BTK were withdrawn from the incubator. The floating pellicle was removed to determine the cellulose mass. The liquor was centrifuged at 10000 rpm for 10 min and passed through a 0.45 μm filter and, after pH measurement the beverage was stored at −20 °C until use. The flask representing the time zero of the fermentation kinetics was that inoculated with tea fungus and immediately stored.

### 2.2. Analyses of the biochemical compounds

All of the analyses were carried out in triplicate and all the chemicals used were of analytical grade. Total acidity was measured according to Delanoë, Maillard, and Maisondieu (1996) and expressed as total acetic acid equivalents. The cellulose floating pellicle was removed from the liquid surface and rinsed with distilled water, then dried to constant mass at 75 °C and weighed. The protein content was determined according to the method of Bradford (1976) and to that of Lowry, Rosebrough, Farr, and Randall (1951). In the latter case, measurements were also done after precipitation of tannins using polyvinyl pyrrolidone material. SDS-PAGE analysis was achieved using 12% polyacrylamide gel and silver nitrate staining procedure. Before loading on the polyacrylamide gel, the samples were dialyzed overnight against deionized water and concentrated using a speed vacuum system. Sucrose, glucose and fructose were measured using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described elsewhere (Al Kazaz, Desseaux, Marchis-Mouren, Prodanov, & Santimone, 1998). Quantification was achieved using the Chromeleon program (Dionex Corp., Sannyvale, CA), based on appropriate calibration curves for glucose, fructose and sucrose. Ethanol content was determined using K-ETOH enzymatic kit (Megazyme, International Ireland Limited). The total phenolic compounds were determined using Folin–Ciocalteu's reagent according to the method described in Chu and Chen (2006) and expressed as mg of gallic acid equivalent based on calibration with gallic acid. The contents of the thearubigins and the theaflavins were determined according to the method described in Thanaraj and Seshadri (1990). The contents of caffeine, catechin, epicatechin, gallocatechin gallate, epicatechin gallate and epigallocatechin gallate were determined by reversed phase HPLC, using Waters Alliance™ System equipped with a Waters 2690 XE separation module and a Waters 996 diode array detector (Waters, Saint Quentin Falavier). The phenolic compounds were extracted as previously described in Djeridane et al. (2008). Separation was carried out at room temperature on a Nucleodur RP-18 column (125 mm x 4.6 mm; 3 μm) from Macherey–Nagel at flow rate of 0.8 ml.min$^{-1}$. Solvent A is $H_2O$ containing 0.1% orthophosphoric acid (pH = 2.6) and solvent B is methanol. The following separation gradient was used: 0 min: 20% B, 12 min: 25% B, 13 min: 30% B, 40 min: 85% B and 57–65 min: 20% B. Quantification was achieved based on calibration with the commercial flavanols and caffeine using Millinium Software.

### 2.3. Amylase assay

Amylase activity was determined according to the method of Dygert, Li, Florida, and Thoma (1965). Activity measurement was performed in 20 mM sodium phosphate, pH 6.9, containing 6 mM NaCl 6 and 1 mM $NaN_3$. Porcine pancreatic alpha-amylase (PPA) was routinely purified to homogeneity in our Laboratory. The reaction medium contained 0.09 % soluble potato starch and 0.3 nM of PPA, to which Kombucha beverage diluted in phosphate buffer was or was not added. The incubation periods were 0, 1, 3 and 5 min at 37 °C.

### 2.4. Statistical analysis

The data were statistically analyzed by means of ANOVA tests and multiple range tests using STATGRAPHICS Software, version 5. The difference was considered as significant at $p<0.05$.

## 3. Results and discussion

### 3.1. Biochemical changes during Kombucha fermentation

#### 3.1.1. Sucrose, glucose and fructose

At day zero, the sucrose concentration was 110 and 106 g/L in GTK and BTK, respectively (Fig. 1a). This corresponded to the sucrose added (100 g/L) and the sucrose already present in the fermentation starter (10% in volume). The sucrose linearly disappeared with time during the two weeks of fermentation for GTK but only during the first week for BTK. The velocities of sucrose disappearance ($R^2>0.99$) were 5.0 g.L$^{-1}$.d$^{-1}$ in BTK and 2.3 g.L$^{-1}$.d$^{-1}$ in GTK. No additional disappearance of sucrose was observed in BTK during the second week of fermentation. In previous studies, sucrose was found to linearly disappear during the first week in BTK with a velocity of about 10 g.L$^{-1}$.d$^{-1}$ (Blanc, 1996) and 15 g.L$^{-1}$.d$^{-1}$ (Sreeramulu, Zhu, & Knol, 2001). In another study, sucrose linearly disappeared for up to four weeks of Kombucha fermentation with a velocity of 2.1 g.L$^{-1}$.d$^{-1}$ (Chen and Liu (2000). The velocity of sucrose disappearance here obtained for BTK was in-between those of the studies mentioned above, setting the velocity of sucrose consumption at the first stages of Kombucha fermentation in the interval 2–15 g.L$^{-1}$.d$^{-1}$. In large part, the discrepancy between these velocity values is most likely related, to the nature and abundance of Kombucha microflora, in conformity with their respective invertase activities. With regard to GTK, it exhibited sucrose consumption quite distinct from that of BKT (Fig. 1a). Indeed, sucrose continuously disappeared during the two weeks of fermentation and the velocity of sucrose disappearance was lower than that of BTK. This could be explained by a lower invertase activity in GTK than in BTK resulting from partial or total elimination of invertase inhibitors during black tea manufacture.

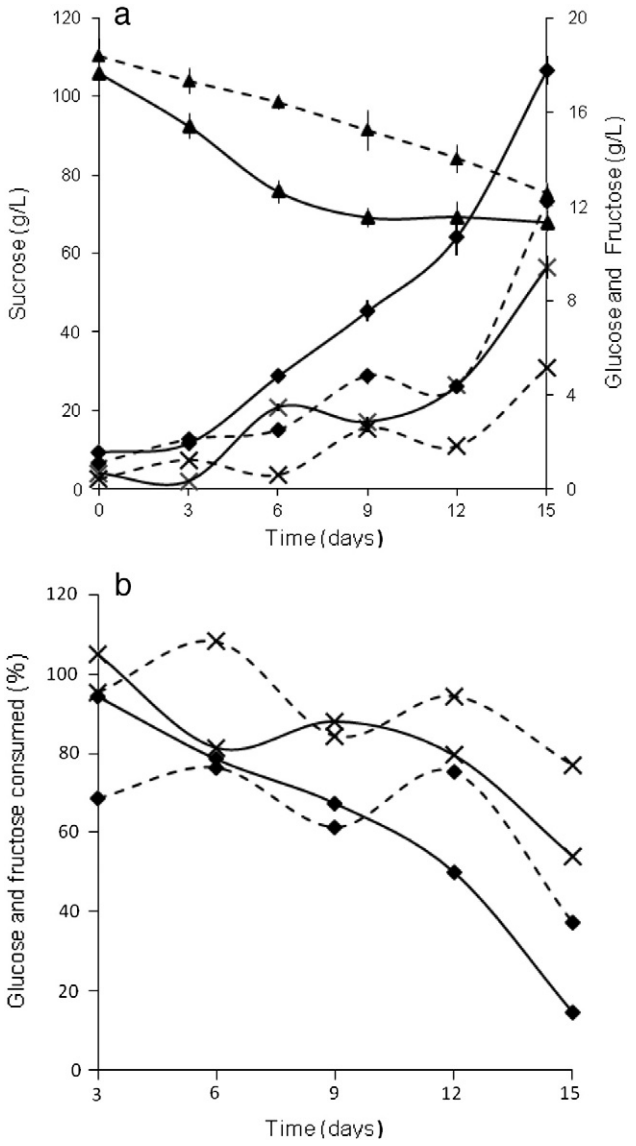


**Fig. 1.** Changes in the contents of sucrose, glucose and fructose during Kombucha fermentation of green tea (dotted lines) and black tea (full lines). (a): disappearance of sucrose (▲) and appearance of fructose (♦) and glucose (×); (b): fraction (%) of glucose and fructose consumed during Kombucha fermentation.

Fig. 1a also shows that the concentrations of glucose and fructose in BTK were higher than those in GTK throughout the two week fermentation period. At day 15, the concentrations of glucose and fructose in GTK were 5.2 and 12.2 g/L, respectively; those in BTK were 9.4 g/L and 17.8 g/L, respectively, indicating that glucose and fructose were consumed faster in GTK than in BTK. The concentrations of glucose and fructose at day 15 in BTK were close to those (10 g/L and 18 g/L, respectively) reported by Chen and Liu (2000). It also appeared that the consumption of glucose and fructose decreases with time, the glucose disappearance being more important than that of fructose (Fig. 1b). This suggests that glucose is preferred to fructose as a carbon source by Kombucha microflora, unless a substantial isomerization of glucose into fructose occurs in the fermentation medium. In line with this, Seto, Kojima, Tonouchi, Tsuchida, and Yoshinaga (1997) have reported that *Acetobacter* strains, which are abundant in Kombucha medium, consumed preferentially glucose to fructose as carbon source.

*3.1.2. Total acidity, pH, ethanol, cellulose and proteins*

The initial pH of sweetened tea infusion was 5.5 and it dropped immediately to 3.8 after the inoculation of the fermentation broth. At day 15 it further decreased during fermentation down to 2.6 and 2.7 for GTK and BTK, respectively, (Fig. 2a). No marked difference was observed between BTK and GTK regarding pH change, but total acidity expressed as g of acetic acid equivalent was higher in BTK than in GTK. Acetic acid equivalents appeared linearly with time at 0.35 and 0.45 $g.d^{-1}.L^{-1}$ in GTK and BTK, respectively ($R^2 > 0.96$). At day 15, the concentration of acetic acid equivalents was 5.4 g/L in GTK and 8.0 g/L in BTK. Similar acetic acid concentration was reported by Jayabalan et al. (2007) but in this study the concentration of acetic acid in GTK was higher than that in BTK (9.5 and 6.2 g /L, respectively, at day 15). It seems that the level of acetic acid, the major short organic acid in Kombucha, is at 5–10 g/L in Kombucha teas, depending on the content and/or activity of the residing acetic bacteria. It is also worth noting that the level of total acidity between the two Kombucha teas is easier to distinguish than did pH. Actually, pH values should not be considered as significant since carbon dioxide produced during fermentation may perform buffering effects.

Ethanol concentration increased during Kombucha fermentation, earlier and more rapidly in BTK as compared to GTK (Fig. 2b). The velocities of ethanol formation ($R^2 > 0.95$) were found to be 0.15 and 0.17 $g.d^{-1}.L^{-1}$ in GTK and BTK, respectively. At day 15, ethanol concentration was about 2 g/L and 3 g/L in GTK and BTK, respectively. Until now studies have shown that under similar experimental conditions the concentration of ethanol in this beverage was in-between 1.7 and 5.5 g/L (Blanc, 1996; Chen & Liu, 2000; Lončar, Djurić, Malbaša, Kolarov, & Klašnja, 2006). These data could significantly impact the dietary habits of populations which forbid alcohol consumption.

As for the protein fraction, before fermentation took place, the concentration as determined based on Bradford assay, was 0.32 g/L and 0.47 g/L in GTK and BTK, respectively (Fig. 2c). These values were in the range of those previously reported (Graham, 1992; Nagao et al., 2005). The concentration of proteins increased thereafter up to +40% at days 6 for GTK and day 12 for BTK, (Fig. 2c). The same behavior was observed using SDS-PAGE analysis. Two major bands of approximately 60 kDa appeared and subsequently disappeared in GTK and BTK mediums. The transient increase of proteins occurred earlier in GTK as compared to BTK. Seven-fold concentration was necessary to get sufficient band intensity using silver nitrate staining, while no protein bands were detected for the same samples using coomassie blue staining . The protein fraction was also analyzed using Lowry and micro BCA methods, which gave higher values for protein fraction: up to 3 g/L, data not shown). At such a protein concentration, the proteins would have been detected in SDS-PAGE without concentration. Consequently, polyvinyl pyrrolidone was used to eliminate tannins and other Kombucha phenolics. In this case, the protein concentration obtained using Lowry method were closer to those obtained using the Bradford method (data not shown). This indicates that Kombucha phenolics interfeere in the quatification of proteins using micro BCA and Lowry methods. According to Graham (1992), green tea contains 5% amino acids on dry mass basis, which under the present experimental conditions corresponds to about 100 mg nitrogen/L. Caffeine, which was quantified at the same time as the phenolic monomers (see Fig. 3c,d), remained quite unchanged in BTK (0.24 g/L) but its concentration in GTK decreased from 0.17 to 0.08 g/L during the two weeks of fermentation. In the latter case, 24 mg nitrogen/L were provided at day 15. The nitrogen demand for protein neosynthesis in GTK and BTK observed in Fig. 2c was approximately 25 mg/L based on a conversion factor of 6.2. This was quite lower than the amino acid nitrogen pool, and nitrogen availability could be higher in GTK due to inputs from caffeine.

Cellulose pellicle became thicker during Kombucha fermentation and the dry mass linearly increased with time, up to 7.2 g/L for GTK and 8.9 g/L for BTK at day 15 (Fig. 2d). The calculated velocities

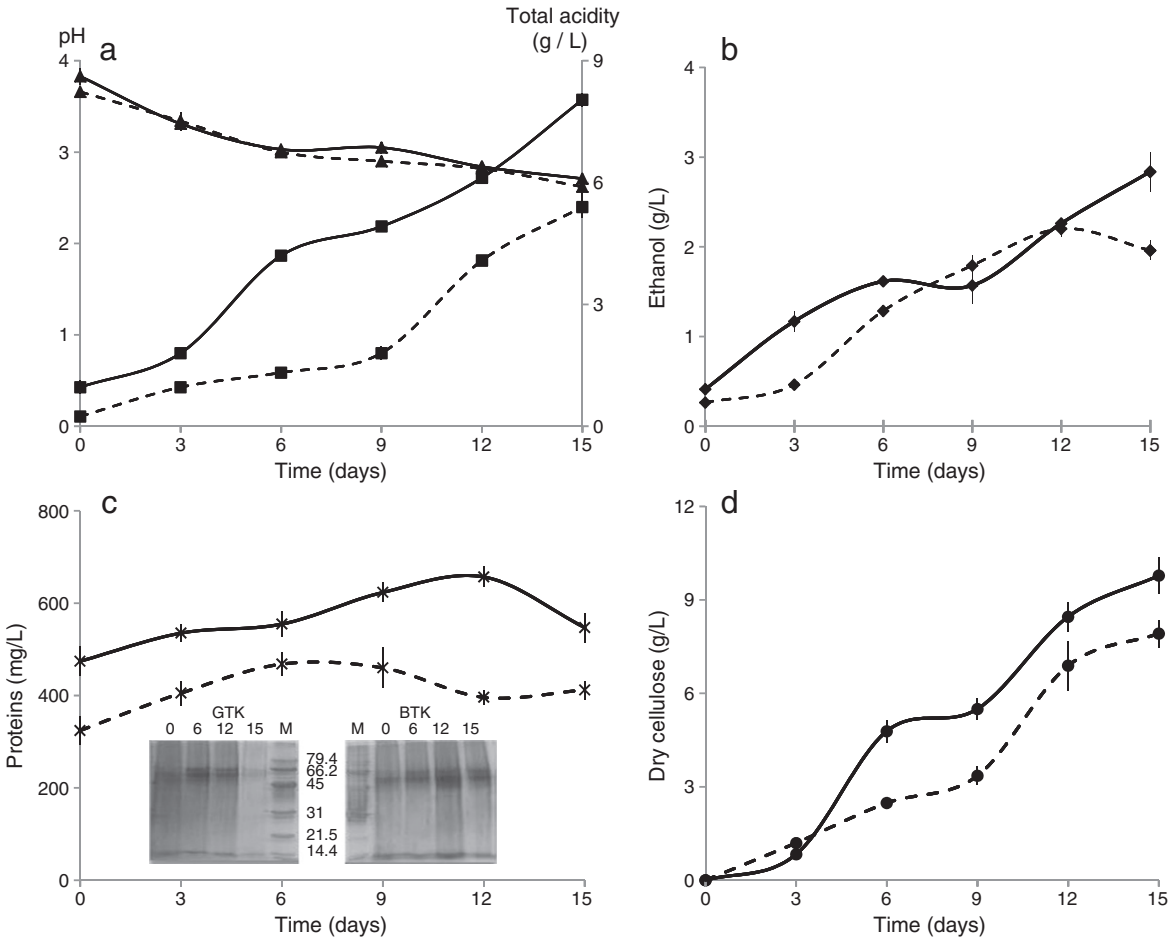


**Fig. 2.** Changes in pH and total acidity (a), ethanol (b), proteins (c) and dry cellulose (d) during Kombucha fermentation of green tea (dotted lines) and black tea (full lines). The insets in (c) show SDS-PAGE of BTK and GTK at the indicated times. Before loading on the gel, the samples were concentrated 7 fold, except day 15 of GTK which was concentrated only 4 fold.

($R^2 > 0.95$) were 0.50 and 0.63 $g.d^{-1}.L^{-1}$, respectively. In GTK, the formed cellulose corresponded to about 35 % of the mass of the glucose that disappeared up to day 9 and to about 50% in the following days of the fermentation (Table 1). Conversely, in BTK, the corresponding percent values increased from 11% at day 3 to 87% at day 15 (Table 1). Thus, it seems that the part of the consumed glucose that is used for cellulose synthesis increases during the fermentation development, more markedly in BTK than in GTK. Since the formed cellulose at day 15 in BTK corresponded to 87% of the disappeared glucose, this suggests that there could not be enough glucose for forming other Kombucha metabolites (acetic acid, ethanol…). Actually, fructose could be converted to glucose within the symbiotic system via isomerization reactions and polyol pathway, which made the relationship between cellulose and glucose as stated above rather speculative. In any case, the present data throw some light on the subtle differences between BTK and GTK regarding the use of carbon sources.

### 3.1.3. Phenolics

At day zero, the concentration of total phenolics, expressed as g gallic acid equivalents, was 0.78 g/L in GTK and 1.01 g/L in BTK (Fig. 3a). Thereafter, phenolics moderately increased during the two weeks of fermentation (+39% for GTK and +12% for BTK). At day 15, total phenolics of GTK was 1.08 g/L and that of BTK was 1.12 g/L. Similarly, Jayabalan, Subathradevi, Marimuthu, Sathiskumar, and Swaminathan (2008) who used 12 g of green or black tea per litter reported moderate increases in Kombucha phenolics: +19% of total phenolics in GTK (0.85-1.25 g/L) and +17% in BTK (0.60-0.72 g/L) at fermentation day 18.

Theaflavin values were found to be 0.25% and 0.37% at day zero in GTK and BTK, respectively, whereas thearubigin values were 4.4% and 6.1%, respectively. TFs and TRs were higher in BTK than in GTK, due to advanced processing in black tea as compared to green tea. The content of TRs was more than ten-fold higher than that of TFs, which is in agreement with Peterson et al. (2005). Fig. 3b shows the changes in TFs and TRs starting from day zero and expressed in percent basis. TFs increased more than 50% at day 15, whereas TRs decreased more than two-fold. It is therefore possible that a part of TRs was converted to TFs during Kombucha fermentation. Jayabalan et al. (2007) reported about 12% increase of TRs in both GTK and BTK at fermentation day 18, but TFs did not change. Actually, data on the absolute contents of TFs and TRs in tea and tea derivatives are scarce due to limited availability in pure standards.

The sum of the phenolic monomers before fermentation took place was 0.69 g/L in GTK and 0.31 g/L in BTK (Fig. 3c,d). These values were in agreement with the data of literature (Dufresne & Farnworth, 2001; Graham, 1992) and show advanced stages of oxidation/polymerization processes in BT as compared to GT. The sum of monomers corresponded to 88% and 31% of total phenolics in GTK and BTK, respectively, (Fig. 3a). EGCG was the major phenolic compound (0.39 g/L and 0.15 g/L in GTK and BTK, respectively), whereas catechin was present in GTK (0.14 g/L) but absent in BTK. Conversely, EC was higher in BTK, possibly due to catechin isomerization forming epicatechin during black tea processing. At day 15, the sum of phenolic monomers was 0.66 g/L in GTK (5% decrease) and 0.27 g/L in BTK (12% decrease). Focusing on the individual changes in the phenolic monomers during Kombucha fermentation, at day 15 in GTK, there was a disappearance of 48 mg EGCG/L and of

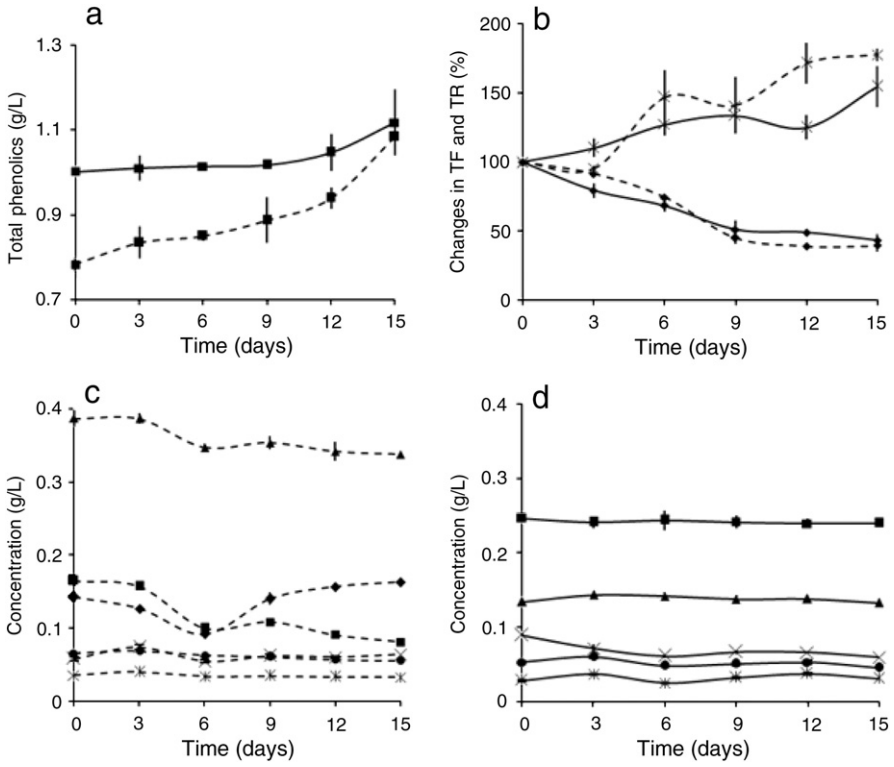


**Fig. 3.** Changes in phenolic compounds during Kombucha fermentation of green tea (dotted lines) and black tea (full lines).(a):content of total phenolics; (b): changes in the contents of theaflavins (×) and thearubigins (♦); (c) and (d): changes in the concentrations of monomeric phenolics and caffeine; EGCG (▲), caffeine (■), GCG (✳), C (♦), EC (×), ECG (●).

10 mg ECG/L and an appearance of 6 mg EC/L and of 20 mg C/L. The small amounts of neo-formed EC, could have resulted from ECG (hydrolysis) or from C (isomerization), whereas the increase of C at the later stages of fermentation may be linked to degradation of TRs (Fig. 3b). Similarly, the decrease in the EGCG pool would have been used to form TFs. In BTK, the sole significant change was that of EC (−34% at day 15: a disappearance of 30 mg/L). It is possible that in BTK EC was converted to TFs during Kombucha fermentation, in which case the concentration of TFs at day 15 in BTK could be about 30 mg/L. Consequently, TRs should be more than 300 mg/L. All these suppositions agree with the changes observed in the individual phenolics, but they must be considered with due caution as the system is complex and some of the changes were not sufficiently different from the quantification limits of the analytical method used, for instance those of EC and ECG.

### 3.2. Assessment of carbon utilization during Kombucha fermentation

The consumption of carbon sources (glucose + fructose) was followed during fermentation, along with the formation of the three products (cellulose + acetic acid equivalents + ethanol). In both GTK and BTK, the three products appeared linearly throughout the two week fermentation period (Table 2). At the same time, the consumption of the substrates (glucose + fructose) linearly increased with time up to day 9 for BTK and to day 12 for GTK (Table 2). At these linear phases, the sum of the three products was about half of the mass of consumed substrates (glucose + fructose). The remaining half of the disappeared substrates could have been used for the synthesis of low and high molecular mass Kombucha constituents (noncellulosic polysaccharides, organic acids and alcohols, etc.). This suggests there was a change in the choice of carbon source that occurred at day 9 for BTK and at day 12 for GTK. At day 15, the mass of the three products in BTK was higher than that of the disappeared substrates, meaning that what has been the left over (about 7 g/L) could have been satisfied by alternative carbon sources. Potential candidates as alternative carbon sources may be tea constituents (noncellulose carbohydrates, amino acids, organic acids and alcohols, among other chemicals)that formed and deformed during Kombucha fermentation. The symbiotic system likely contributed in large parts to this carbon mass balance, since the missing 7 g/L represents as much as 56% of dry mass of the initially added tea (12.5 g/L).

**Table 1**
Changes in cellulose/glucose mass ratio (%) during Kombucha fermentation of green and black tea.

| Cellulose/glucose mass ratio (%) | | |
|---|---|---|
| Time (days) | GTK | BTK |
| 3 | $34.1 \pm 7.4^{a}$ | $10.8 \pm 0.0^{a}$ |
| 6 | $34.0 \pm 1.7^{a}$ | $35.5 \pm 4.4^{b}$ |
| 9 | $36.8 \pm 0.6^{a}$ | $31.0 \pm 3.6^{b}$ |
| 12 | $49.7 \pm 3.9^{b}$ | $52.8 \pm 4.1^{c}$ |
| 15 | $52.3 \pm 2.6^{b}$ | $86.7 \pm 0.1^{d}$ |

Data are expressed as mean ± standard deviation. Different letters in the same column indicates significant difference at $p<0.05$.

**Table 2**
Assessment of carbon utilization during Kombucha fermentation of green and black teas regarding consumption of glucose and fructose (substrates) and formation of cellulose, acetic acid equivalents and ethanol (products).

| Time (days) | GTK | | BTK | |
|---|---|---|---|---|
| | Substrates (g/L) | Products (g/L) | Substrates (g/L) | Products (g/L) |
| 3 | $5.7 \pm 1.5^{a}$ | $2.5 \pm 0.1^{a}$ | $13.4 \pm 0.8^{a}$ | $3.8 \pm 0.15^{a}$ |
| 6 | $11.4 \pm 1.3^{b}$ | $4.9 \pm 0.0^{b}$ | $24.1 \pm 1.2^{b}$ | $10.1 \pm 0.2^{b}$ |
| 9 | $14.4 \pm 1.42^{c}$ | $6.8 \pm 0.3^{c}$ | $28.6 \pm 2.1^{c}$ | $11.4 \pm 0.5^{b}$ |
| 12 | $22.7 \pm 0.2^{d}$ | $12.6 \pm 0.6^{d}$ | $23.7 \pm 0.6^{b}$ | $16 \pm 0.4^{c}$ |
| 15 | $20.6 \pm 0.8^{d}$ | $14.7 \pm 0.6^{e}$ | $13 \pm 0.4^{a}$ | $19.9 \pm 1^{d}$ |

Data are expressed as mean ± standard deviation. Different letters in the same column indicates significant difference at $p<0.05$.

### 3.3. Effects of Kombucha beverage on pancreatic alpha-amylase activity

Fig. 4a shows that the Kombucha samples diluted 500 times inhibited porcine pancreatic alpha-amylase and that the inhibition potency increases during the progression of fermentation. BTK exhibited slightly more potent PPA inhibition. The inhibition potency at the end of fermentation (day 15) was dose-dependent in the range 250–2000 dilution fold (Fig. 4b). Based on this, the $IC_{50}$ (inhibitor concentration that performs 50% enzyme inhibition) was close to the concentration of the sample diluted 1000 fold, which performed 42% inhibition in the case of GTK and 55% inhibition in the case of BTK (Fig. 4b). Previous reports showed that salivary alpha-amylase is inhibited by monomeric phenolic compounds (Hara & Honda, 1990; Piparo et al., 2008). Thus, the inhibition of PPA observed in the present study could be due to the phenolic fraction of Kombucha beverage. We therefore tested pure EGCG, GCG and ECG on the activity of PPA and found that the three phenolics are effective PPA inhibitors (data not shown). Total phenolics in Kombucha teas were about 6 mM gallic acid equivalent (Fig. 3a) in which the phenolic monomers represented two thirds in GTK and one third in BTK (Fig. 3c,d). Consequently, if PPA inhibition by Kombucha was entirely due to phenolics, the $IC_{50}$ should be 6 μM or less.

On the other hand, the increase of PPA inhibition potency during progression of Kombucha fermentation was quite intriguing. Except for theaflavins, which are considered as minor tea constituents, there was no other dramatic increase in the content of phenolic compounds. TFs may therefore be suspected as potent inhibitors of alpha-amylase. Mammalian pancreatic and salivary alpha-amylases contain five and six subsites in the active site, respectively, each accommodating one glucose residue (Ajandouz & Marchis-Mouren, 1995; Kandra, Gyémánt, Remenyik, Ragunath, & Ramasubbu, 2003). The best inhibitor candidate would therefore contain five cyclic residues, each mimicking glucose, especially with regard to the number of hydroxyl groups and orientation within the active site.

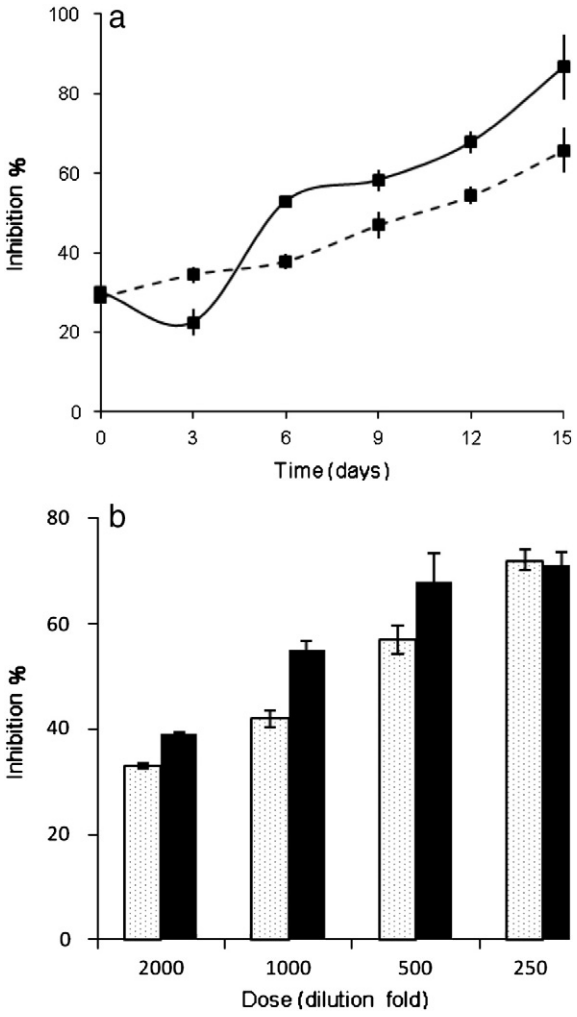


**Fig. 4.** Inhibition of porcine pancreatic alpha-amylase by Kombucha beverage of green tea (dotted lines) and black tea (full lines). (a): time course inhibition during Kombucha fermentation with 500-fold diluted samples; (b): dose-dependent inhibition of PPA by Kombucha beverage (day 15 of fermentation); GTK (□), BTK (■).

## 4. Conclusion

Green and black teas exhibited similar Kombucha fermentation profiles, but specific biochemical behaviors were observed as well. The carbon sources glucose and fructose resulting from sucrose were consumed faster in BTK. The biochemical changes were more rapid in BTK than in GTK and the levels of cellulose, acetic acid equivalents, ethanol, phenolics, caffeine and proteins were higher. The three products cellulose, acetic acid equivalents and ethanol accounted for about a half of the mass of the substrates (glucose + fructose) during a linear phase shorter in BTK (9 days) than in GTK (12 days). The consumption of the substrates thereafter declined in favor of alternative carbon sources, possibly tea carbon or carbon from the symbiotic system. The nitrogen fraction, which was probed to some extent in the present study, also exhibited subtle differences between GTK and BTK regarding both caffeine and proteins. In both fermentation medium, proteins (<0.5 mg/L) transiently increased during fermentation as did a 60-kDa protein band. The transient increase in the protein fraction occurred earlier in GTK than in BTK.

The phenolic fraction was probed at different structural levels: total, oligomeric, dimeric and monomeric. No dramatic changes were observed regarding total phenolics and the detected monomers, although subtle differences between GTK and BTK were observed regarding the changes in individual monomers that were tentatively, but carefully, assigned to the chemical events developing during Kombucha fermentation. Conversely, TFs moderately increased and TRs markedly decreased, during Kombucha fermentation.

Another interesting result of the present study is the ability of Kombucha beverage to inhibit porcine pancreatic alpha-amylase. The inhibition potency increased during Kombucha fermentation and at day 15 the $IC_{50}$ was as low as the concentration of one thousand-diluted Kombucha beverage. The effective inhibitors could be the phenolics, the concentration of which in the one thousand-diluted sample was approximately 1 mg/L. The gallate-containing monomers, including EGCG, GCG and ECG, and possibly TFs, could be the effective PPA inhibitors from Kombucha beverage.

A 200-mL cup of this beverage (BTK or GTK) should contain more than 100 mg of phenolics (Henning et al., 2004), which could inhibit, to some extent, pancreatic alpha-amylase in the small intestine, with possible effects on starch digestion and net absorbed glucose.

The mechanism of PPA inhibition by tea flavanols and the possible impact of consumption of these substances on starch digestion, in line with weight management and diabetes type 2, are currently under investigation.

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