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Effects of sugar sources and fermentation time on the properties of tea fungus (kombucha) beverage

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Abstract

Recently, fermented foods have been developing huge demand among modern consumers due to their health benefits and pleasant flavour. The objective of the present work was to evaluate the effects of fermentation time and different sugar sources on the physicochemical and antioxidant activities of kombucha tea. The sugar sources selected were white refined sugar (WRS), coconut palm sugar (CPS) and molasses sugar (MS). The fermentation substrate was boiled black tea, 10% (w/v) of each sugar, 3% (w/v) of tea fungus (SCOBY) and 10% (v/v) of previously fermented kombucha tea (back slope fermentation). The mixture was incubated in the dark at 24±3°C for 14 days. The sugar and organic acid contents were determined by HPLC, while the antioxidant active was determined by the DPPH and FRAP methods. Results demonstrated significantly higher biomass formation, glucose and sucrose content for kombucha tea fermented with WRS, while kombucha tea fermented with MS showed higher organic acid contents. Moreover, kombucha tea fermented with CPS exhibited the highest antioxidant activity and total phenolic content, followed by those fermented with MS and WRS. The present work demonstrated that kombucha tea fermented with CPS is recommended to be consumed as functional beverage for health benefits and prevention of oxidation related diseases. In addition, CPS and MS are good sugar alternatives to sucrose and other sugars frequently used in kombucha fermentation.

Introduction

The dramatic increase in the prevalence of chronic diseases due to lifestyle and diet has led consumers to look for new way of health improvement via functional foods and nutraceuticals. Kombucha or fungus tea is a sweetened tea inoculated with tea fungus, and allowed to change the taste into pleasantly sour through fermentation. Fermentation generally has positive effects on humans' health as a result of compounds produced during the fermentation by a number of microorganisms. Organic acids are the main metabolites produced by kombucha fermentation which act as an active ingredient to impart beneficial health effects such as antioxidant activities (Chu and Chen, 2006; Jayabalan *et al*., 2010; Malbaša *et al*., 2011). In previous studies, kombucha fermented tea prevented oxidative stress when tested on albino rats induced with chromate (VI) besides anti-hepatotoxicity that is caused by paracetamol and carbon tetrachloride (Sai *et al*., 2000; Pauline *et al*., 2001).

Molasses sugar (MS) and coconut palm sugar (CPS) have a unique taste and nutritional values that may influence the kombucha fermentation system and give beneficial effects to consumers. Molasses is not only attractive because of its low price but also due to the presence of other components such as minerals, organic compounds and vitamins (Rodrigues *et al*., 2006). In addition, another complex system of carbon sources to be worth focusing is a sugar produced from the sap of cut flower buds of coconut palm that is known as coconut palm sugar. It is a traditional sweetener in South and South-East Asian regions, and rich in a number of micronutrients, minerals, vitamins and amino acids. To the best of our knowledge, there is very limited research on the application of inexpensive alternative of carbon source for kombucha fermentation. Therefore, the objective of the present work was to evaluate the

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effects of different sugar sources and fermentation times on the physicochemical and antioxidant activity of kombucha tea.

Materials and methods

Materials

Black tea (Sabah Tea, Sabah, Malaysia) was selected, while the starter culture was obtained from Microbial Technology Laboratory, UPM. White refined sugar (WRS) used was of food-grade (*Gula Prai*, Malayan Sugar Manufacturing Company Berhad, Malaysia). Coconut palm sugar was obtained from a shop located in Malacca, Malaysia. Molasses was supplied by Central Sugars Refinery Sdn. Bhd., Malaysia. Unless otherwise stated, all chemicals used were either of HPLC- or analytical-grade.

Preparation of kombucha tea

The tea fermentation was carried out to produce kombucha tea by using commercialised starter culture. Briefly, preparation of 100 mL was done by mixing 77 mL black tea (1.2%, w/v) that was boiled in water for 5 min and filtered through a sterile sieve, 10% (w/v) of each sugar was dissolved in the hot tea and left to cool, the cooled tea was inoculated with 3% (w/v) tea fungus (SCOBY) and 10% (v/v) kombucha tea broth (cultivated in the same medium for 14 d). The containers were covered and fermented in the dark at 24±3°C for 14 d (Jayabalan *et al*., 2010). At the end of the fermentation, the samples were dried by oven for further analysis.

Kombucha tea physicochemical properties

The kombucha tea was analysed to determine the effects of the sugars on the fermentation profile. The pH was measured by a calibrated electric pH meter (JENWAY 3505, Essex, England); the total soluble solid (TSS) was measured using a hand refractometer (Atago N1, Tokyo, Japan); and the colour was determined by chromameter (Minolta CR300, Osaka, Japan). In addition, proximate analysis including moisture, ash and crude protein were determined according to the standard methods of AOAC (2002).

The pellicle of each kombucha was rinsed with distilled water, dried and the yield percentage of biomass was calculated by using the following equation:

$$\text{Increase in mass} = M_1 - M_0$$

where M_1 = final mass, and M_0 = initial mass.

Kombucha tea compositions

Sugar analyses

The kombucha tea was passed through a membrane filter (0.45 µm) and subjected to analyses of fructose, glucose, sucrose and maltose by using HPLC (Agilent 1200, Germany). Briefly, 6 µL sample was manually injected into a HPLC system equipped with a refractive index (RI) detector (JASCO RI-4035, Tokyo, Japan) using isocratic pump (Shimadzu LC-6A, Japan) and LiChroCART® column (250 mm × 4.6 mm, 5 µm) (Merck, Germany). The mobile phase was a mixture of acetonitrile (75%) and deionised water (25%). The flow rate and column temperature was maintained at 1 mL/min at room temperature. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound using Brown chromatography software. The concentrations of natural sugars were quantified from standard curves. All samples were analysed in duplicate.

Organic acid analyses

The kombucha tea was passed through a membrane filter (0.45 µm) and 6 µL sample was injected into a HPLC system (Agilent 1200, Germany) to determine the organic acids namely tartaric acid, malic acid, acetic acid, and succinic acid equipped with an ultraviolet detector and Hypersil Gold C18 column. The mobile phase was H_3PO_4 solution at 6×10^{-3} mol/L (pH 2.1) under a flow rate of 1.0 mL/min at room temperature, and at a wavelength of 210 nm.

Kombucha tea antioxidant activities

Free radical scavenging activity

The free radical scavenging activity of kombucha tea was evaluated by the DPPH assay following the method described by Chu and Chen, (2006). The kombucha tea (0.025 mL) was mixed with 0.6 mL 1 mM DPPH solution in 96-well micro-titre plate and incubated in the dark at room temperature for 30 min before the absorbance was determined at 517 nm using a spectrophotometer (Shimadzu, UVmini-1240, Tokyo, Japan). The control was water and DPPH solution. The scavenging capacity of kombucha was calculated as

$$\textit{Scavenging activity (\%)} = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100$$

where A_{blank} = control reading, and A_{sample} = sample reading.

Ferric reducing antioxidant power assay (FRAP)

The antioxidant activity was measured by ferric reducing antioxidant power (FRAP) assay as described by Benzie and Strezo (1999). Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 µM, pH 3.6), a solution of 10 µM TPTZ in 40 µM HCl, and 20 µM FeCl3 at 10:1:1 (v/v/v). The reagent (300 µL) and kombucha tea (10 µL) were mixed thoroughly in 96-well micro-titre plates and incubated in the dark for 30 min, after which the absorbance was measured at 593 nm by using a spectrophotometer (Shimadzu, UVmini-1240, Tokyo, Japan).

Total phenolic content

The total phenolic content of kombucha tea was determined according to the method of Chu and Chen (2006). Briefly, kombucha tea (0.05 mL) was mixed with 2 mL2% sodium carbonate, and incubated for 2 min. Next, 0.1 mL Folin–Ciocalteu reagent was mixed with the solution and incubated for 30 min, after which the absorbance was measured at 750 nm by using a spectrophotometer (Shimadzu, UVmini-1240, Tokyo, Japan).

Statistical analysis

The obtained data were analysed by one-way ANOVA using MINITAB 16 (Minitab Inc. USA) with Tukey's Multiple Comparison to determine the significant differences ($p < 0.05$) among means of all samples/treatments.

Results and discussion

Physicochemical properties of kombucha tea

The fermentation of plant-based materials heavily depends on the sugar type and content in the raw substrate. In the present work, the effect of three sugar sources on the physicochemical properties and antioxidant activities of kombucha tea was evaluated. The pH significantly ($p < 0.05$) decreased in kombucha tea fermented with white refined sugar (WRS) and molasses sugar (MS) after 14 d fermentation, while less pH reduction was observed for the coconut palm sugar (CPS) sample (Table 1). The total soluble solid (TSS) showed high drop in the WRS sample, and very slight drop in the CPS and MS samples. The utilisation of different sugars by the starter culture of kombucha resulted in the formation of several organic acids which eventually decreased the tea pH (Jayabalan *et al*., 2014). On the other hand, the TSS might have decreased in correlation with the fermentation period as many nutrients have been utilised for the microorganisms' growth and metabolism.

Table 1: The pH, total soluble solid and *L**, *a**, and *b** values of kombucha tea fermented with different sugar sources.

Source of sugar	pH		TSS (°Brix)		*L** value		*a** value		*b** value	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
WRS	5.23 ± 0.06^{a}	2.83 ± 0.09^{c}	10.07 ± 0.12^{a}	7.00 ± 0.20^{ab}	34.94 ± 1.33^{a}	35.39 ± 3.15^{c}	10.86 ± 0.68^{a}	6.63 ± 0.52^{b}	8.68 ± 1.36^{a}	18.55 ± 2.53^{a}
MS	5.11 ± 0.02^{b}	3.83 ± 0.02^{a}	7.80 ± 0.2^{c}	6.87 ± 0.12^{b}	20.61 ± 0.51^{c}	95.37 ± 0.32^{a}	3.82 ± 1.50^{b}	2.36 ± 1.92^{c}	1.63 ± 0.53^{b}	1.06 ± 0.06^{c}
CPS	4.89 ± 0.02^{c}	3.36 ± 0.04^{b}	8.73 ± 0.12^{b}	7.33 ± 0.12^{a}	24.36 ± 1.94^{b}	63.42 ± 2.87^{b}	11.86 ± 1.40^{a}	20.00 ± 0.86^{a}	10.36 ± 0.21^{a}	7.24 ± 1.61^{b}

WRS = white refined sugar, CPS = coconut palm sugar, MS = molasses. Values with different superscript in a column represent significant difference ($p < 0.05$)

Table 2: Biomass yield, proximate composition, sugar content and organic acid content of kombucha tea fermented with different sugar sources.

Source of sugar	Biomass			Proximate composition			Natural sugars concentrations			Organic acids concentrations			
	Initial mass (g)	Final mass (g)	Biomass yield (g)	Moisture (%)	Ash (%)	Crude protein (%)	Fructose (g/mL)	Glucose (g/mL)	Sucrose (g/mL)	Tartaric acid (g/mL)	Malic acid (g/mL)	Acetic acid (g/mL)	Succinic acid (g/mL)
WRS	15.21 ± 0.01^{a}	116.53 ± 0.10^{a}	101.31 ± 0.09^{a}	92.59 ± 1.09^{b}	0.23 ± 0.01^{b}	0.07 ± 0.02^{a}	0.13 ± 0.05^{b}	3.88 ± 0.23^{a}	83.00 ± 5.04^{a}	2.84 ± 0.01^{c}	1.03 ± 0.01^{c}	2.37 ± 0.04^{c}	0.26 ± 0.01^{c}
CPS	15.2 ± 0.03^{b}	49.40 ± 0.02^{b}	34.18 ± 0.00^{b}	92.69 ± 0.25^{b}	0.04 ± 0.02^{c}	0.05 ± 0.02^{a}	1.06 ± 0.16^{b}	2.84 ± 0.27^{b}	60.46 ± 5.99^{b}	3.35 ± 0.01^{b}	1.91 ± 0.00^{b}	8.27 ± 0.01^{b}	0.87 ± 0.00^{b}
MS	15.41 ± 0.02^{b}	43.22 ± 0.18^{c}	27.81 ± 0.18^{c}	94.38 ± 0.10^{a}	0.60 ± 0.04^{a}	0.13 ± 0.04^{a}	2.42 ± 0.71^{a}	1.36 ± 0.40^{c}	28.86 ± 8.85^{c}	7.58 ± 0.01^{a}	5.06 ± 0.02^{a}	11.62 ± 0.05^{a}	4.93 ± 0.02^{a}

WRS = white refined sugar, CPS = coconut palm sugar, MS = molasses. Values with different superscript in a column represent significant difference ($p < 0.05$)

The L* values significantly ($p < 0.05$) increased in all samples after 14 d fermentation. The a* values of WRS and MS samples were observed to move towards the green spectrum and vice versa for CPS. The b* values significantly increased in WRS sample and slightly decreased in CPS and WS samples. The results indicated high utilisation of sugar which was the primary carbon source for the microorganisms during the fermentation. On the other hand, the lightening of the colour of kombucha tea might have been due to the microbial transformation of polyphenols during the fermentation (Chu and Chen, 2006; Jayabalan *et al*., 2010).

The biomass yield was observed to increase 6-fold for WRS sample, and 3-fold for MS and CPS samples (Table 2). The biomass yield was in correlation with the carbon source in the system, in which the higher the sugar content the higher the biomass yield. Malbasa *et al*. (2008) reported that kombucha fermented with high sucrose concentration (70 g/L) produced the highest amount of biomass (260 g).

The moisture content of MS kombucha was the highest (94.38%) as compared to others. This could be due to the initial state of molasses that was hydrated, while WRS and CPS were in dried form. Water is one of the major components in molasses as it needs to be diluted to dissolve microscopic sugar crystals in the molasses. The ash contents of all kombucha samples were significantly different ($p < 0.05$) where MS yielded the highest (0.60%) followed by WRS and CPS at 0.23% and 0.04%, respectively. The high ash content in MS kombucha might be due to the fact that molasses contains many constituents such as silica, ferric oxide, copper oxide, magnesium and sulphuric acid (Wohryzek, 1914).

Composition analyses of kombucha tea

The sugar analyses showed that MS kombucha contained the highest fructose concentration (2.42 ± 0.71 g/mL), while the glucose concentration of WRS kombucha was the highest (3.88 ± 0.23 g/mL), followed by CPS kombucha (2.84 ± 0.27 g/mL), and MS kombucha (1.36 ± 0.40 g/mL). Generally, the sugar components of CPS were 70.85% sucrose, 3.00% glucose and 2.92% fructose (Purnomo, 2007). Differences in sugar components might possibly be due to the different compositions of the fresh sap used as raw material to produce the CPS (Apriyantono, Wiratna and Husain Nurhayati, 1996).

The organic acid analysis showed that MS kombucha contained the highest amount of all four organic acids. The tartaric acid concentration of MS kombucha was significantly higher (7.58 ± 0.01 g/mL) than that of CPS (3.35 ± 0.007 g/mL) and WRS (2.84 ± 0.01 g/mL). The malic acid concentration of MS kombucha was the highest (5.06 ± 0.02 g/mL), followed by CPS (8.27 ± 0.007 g/mL) and WRS (2.37 ± 0.04 g/mL). The acetic acid concentration of WRS kombucha was the lowest (2.37 ± 0.04 g/mL) while MS kombucha yielded the highest (11.62 ± 0.05 g/mL). The succinic acid concentration of MS kombucha (4.93 ± 0.02 g/mL) was higher than that of CPS (0.87 ± 0.001 g/mL). Previous study reported that the high organic acid content of kombucha tea fermented with molasses could be due to the high content and great number of sugar variety (Dosenovic, 2004). Furthermore, acetic acid was high in the three samples because it is the main metabolites produced by the acetic acid bacteria dominant in the fermentation of kombucha tea.

Antioxidant activity of kombucha tea

The antioxidant activity of fermented foods and beverages is usually very high due to the prevalence of bioactive compounds. The DPPH results and total phenolic contents of the three kombucha tea samples showed significant differences (Table 3).

Table 3: DPPH radical scavenging activities and total phenolic content of kombucha tea fermented with different sugar sources.

Source of sugar	DPPH radical scavenging activity (Percent inhibition, %)	Total phenol content (mg GAE/mL)
WRS	29.085 ± 2.481^{b}	4.547 ± 2.600^{b}
MS	32.927 ± 2.552^{b}	12.767 ± 0.313^{a}
CPS	49.265 ± 4.575^{a}	13.460 ± 0.690^{a}

WRS = white refined sugar, CPS = coconut palm sugar, MS = molasses. Values with different superscript in a column represent significant difference ($p < 0.05$)

The CPS kombucha exhibited the highest antioxidant activity (49.266%), while the MS kombucha and WRS kombucha had lower antioxidant activity (32.927%) and (29.085%), respectively. In previous studies, kombucha tea fermented with WRS showed low antioxidant activity (Phillips *et al*., 2009; Nayaka *et al*., 2009). However, in the present work, WRS kombucha showed high antioxidant activity following 14 d fermentation. CPS kombucha had the highest total phenolic content of 13.46 mg GAE/mL followed by WS and WRS kombucha samples. The antioxidant activity was in correlation with the phenolic content of the samples, and is the results of the raw substrate constituents and the metabolites of the fermentation starter cultures (Tabart *et al*., 2007a; Oliveira, 2012). Phenolic compounds are classified as high-level antioxidants because they are able to

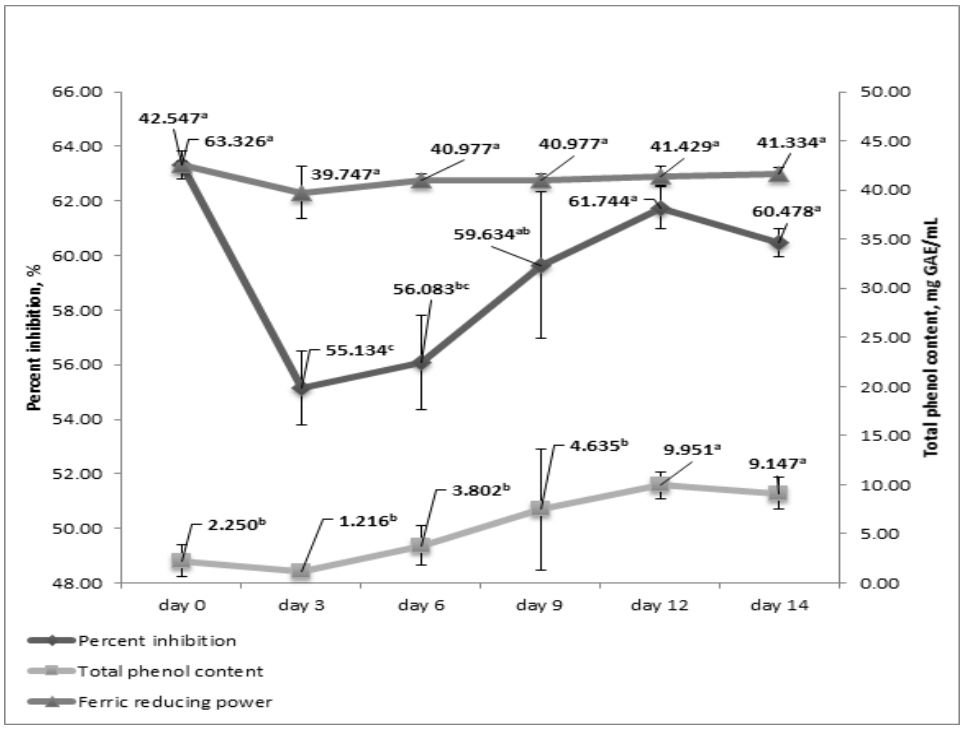

Figure 1: DPPH radical scavenging activity, FRAP antioxidant capacity and total phenol content during fermentation of CPS kombucha tea.

scavenge free-radical and active oxygen species (Jayabalan *et al*., 2010). Several studies reported the direct relation between total phenolic and antioxidant activity (Malbaša *et al*., 2008; Oliveira, 2012). On the other hand, the starter culture enzymes secreted during fermentation will enhance the antioxidant activity of the fermented kombucha in comparison to the raw substrate that has very minimal activity (Jayabalan *et al*., 2008).

Due to the high antioxidant activity and the phenolic content of CPS kombucha tea, it was further evaluated for the fermentation time optimisation and its effects on the antioxidant activity (**Figure 1**). The results revealed that CPS kombucha tea fermented for 12 days showed higher antioxidant activity and total phenolic contents.

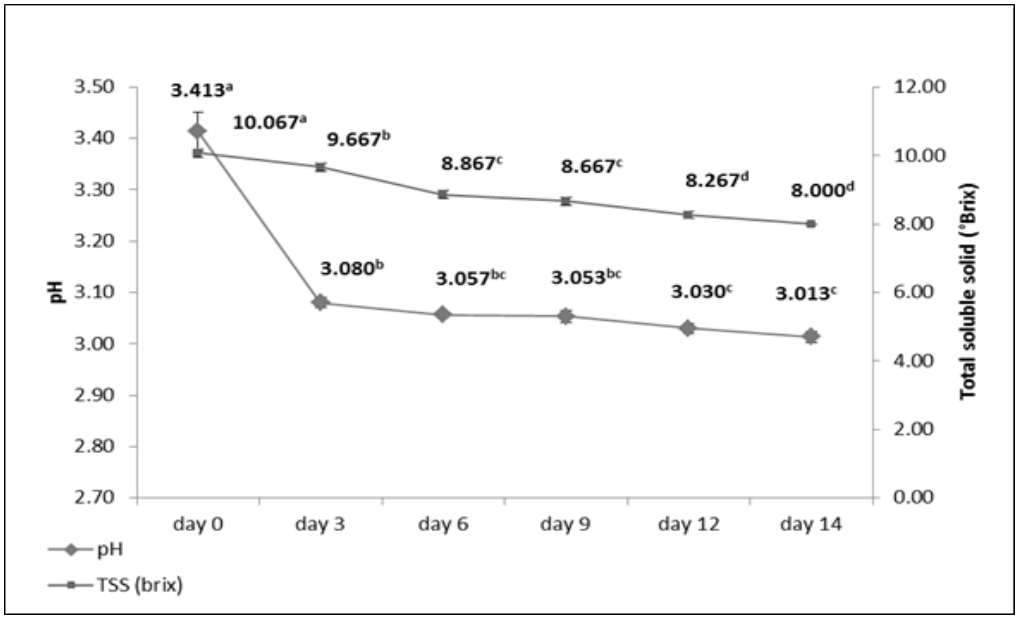

Figure 2: pH values and total soluble solid of samples prepared with CPS. Values that do not share a common superscript in a column represent significant difference ($p < 0.05$).

The pH and TSS of CPS kombucha tea showed significant variation during the 14 days fermentation (Figure 2). The pH of CPS kombucha tea declined rapidly from day 0 to day 3, and then became stationary throughout the remaining days of fermentation. The pH values obtained in the present work were in agreement with previous studies that suggested slower pH changes after three days of fermentation which could be associated with the effect of buffer from the reaction between organic acids and minerals synthesised from the substrate (Loncar *et al*., 2000; Malbaša *et al*., 2011). Interestingly, the prolonged fermentation time will enhance the phenolic content and the antioxidant activity but will not increase the acidic taste and maintain the fermented beverage to be acceptable in the sensory evaluation. In the present work, 12 days of fermentation is suggested due to the higher antioxidant activity and the high potential of health benefits for the consumers. Prolonged fermentation more than 14 days is not recommended as the phenolic content and the antioxidant activity was observed to decrease. On the other hand, the TSS value decreased gradually from day 0 until day 14 of fermentation, indicating the microbial activity in kombucha tea that utilised the nutrients in the substrate.

Conclusion

The kombucha tea fermented with coconut palm sugar (CPS) demonstrated the highest antioxidant activity after 12 days fermentation. The TSS and pH for all the samples decreased in correlation with the fermentation time. The antioxidant activity of the kombucha samples was in correlation with the phenolic content. CPS is an inexpensive source of carbon found to be suitable alternative to the traditionally used sucrose in the preparation of kombucha tea. Further study should be carried out to optimise the fermentation conditions including temperature, starter cultures, and initial pH. Moreover, the metabolites of different sugar sources should be determined and evaluated for health benefits. Most importantly, further study must be done in terms of its quality, safety and sensorial acceptability for human consumption.

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