Food Chemistry 194 (2016) 304–311

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Application of the Kombucha 'tea fungus' for the enhancement of antioxidant and starch hydrolase inhibitory properties of ten herbal teas

Mindani I. Watawana [a], Nilakshi Jayawardena [a], Candy Choo [b], Viduranga Y. Waisundara [a,*]

[a] National Institute of Fundamental Studies, Hantane Road, Kandy 20000, Sri Lanka
[b] Temasek School of Applied Science, Temasek Polytechnic, 21, Tampines Avenue 1, Singapore 529757, Singapore

ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form 20 July 2015
Accepted 11 August 2015
Available online 12 August 2015

Keywords:
Acetobacter aceti
Antioxidant activity
Brettanomyces claussenii
Starch hydrolases
Zygosaccharomyces bailii

ABSTRACT

Ten herbal teas (*Acacia arabica*, *Aegle marmelos* flower, *A. marmelos* root bark, *Aerva lanata*, *Asteracantha longifolia*, *Cassia auriculata*, *Hemidesmus indicus*, *Hordeum vulgare*, *Phyllanthus emblica*, *Tinospora cordifolia*) were fermented with the Kombucha 'tea fungus'. The pH values of the fermented beverages ranged from 4.0 to 6.0 by day 7, while the titratable acidity ranged from 2.5 to 5.0 g/mL ($P < 0.05$). Gallic acid had statistically significantly increased ($P < 0.05$) in almost all the samples by day 7. The Oxygen radical absorbance capacity assay indicated 5 of the Kombucha beverages to have statistically significant increases ($P < 0.05$) by day 7. The α-amylase inhibitory activities ranged from 52.5 to 67.2 μg/mL in terms of IC_{50} values following fermentation, while the α-glucosidase inhibitory activities ranged from 95.2 to 196.1 μg/mL. In conclusion, an enhancement of the antioxidant and starch hydrolase inhibitory potential of the herbal teas was observed by adding the tea fungus.

1. Introduction

Kombucha or 'tea fungus' is a fermented beverage with origins in Asia and gained rapid popularity among the rest of the world. It is a symbiotic growth of bacteria and osmophilic yeast strains in a thick jelly membrane which is cultured in sugared black tea (Jayabalan, Malini, Muthuswamy, Swaminathan, & Yun, 2010). The substrate is incubated statically under aerobic conditions, usually for a minimum of 7 days at 20–28 °C (Jayabalan, Marimuthu, & Swaminathan, 2007). The fermentation length could be different, even up to 60 days. However, to obtain a pleasantly sour beverage, the fermentation is terminated when the titratable acidity (TA) reaches 4.0–4.5 g/L – a level which has been confirmed as acceptable by longtime consumers of the Kombucha beverage and is known as the optimal consuming acidity (Reiss, 1994). Kombucha tea is considered as the ultimate therapeutic agent in countless diseases such as rheumatism, intestinal disorders, and cancer (Dufresne & Farnworth, 2001). While the benefits outweigh the side-effects, the beverage is nevertheless advised to be cautiously administered to immune-compromised individuals due to possibilities of pathogenic contamination (Smolinske, 2005). This includes some allergic reactions and an uncomfortable stomach as a result of consuming Kombucha tea by people with acid sensitivities and renal insufficiencies (Kovacevic et al., 2014). It was discovered that acute renal failure may occur with lactic acidosis and hyperthermia due to consumption of this beverage (Kole, Jones, Christensen, & Gladstein, 2009). Tea provides the necessary nitrogen sources for the 'tea fungus' culture (Sreeramulu, Zhu, & Knol, 2000). Regardless of the content of caffeine in green tea (about 5%) which is higher than black tea (2%) and provides a much higher nitrogen amount for tea fungus culture, black tea is the traditional and most dominant substrate for Kombucha fermentation (Chen & Liu, 2000). Given this requirement, some studies have been able to successfully demonstrate the preparation of Kombucha beverage from the addition of the tea fungus to various types of plant-based powders which are essentially not of *Camellia sinesis* origin (Lee et al., in press; Liu, Hsu, Lee, & Liao, 1996; Watawana, Jayawardena, & Waisundara, in press). Considering the therapeutic properties of many of the other herbal teas available in the marketplace, whether this antioxidant potential can be enhanced by natural or artificial means has been the subject of many studies. A previous study by Lee et al. (in press) was able to successfully demonstrate the enhancement of the antioxidant activities, polyphenol contents and starch hydrolase inhibitory activities in Kombucha beverages fermented for 7 days. This particular study focused on the analysis of these parameters on different types of teas of *C. sinesis* origin as well as *Aspalathus linearis* or Rooibos tea. It was observed that the amount of phenolic compounds had increased in this instance. Given this evidence as well

* Corresponding author.
E-mail address: viduranga@gmail.com (V.Y. Waisundara).

http://dx.doi.org/10.1016/j.foodchem.2015.08.033
0308-8146/

as the study by Liu et al. (1996) and Watawana et al. (in press) whether the tea fungus is able to enhance the therapeutic potential of herbal beverages in general is an aspect worth exploring further.

In this study, ten plant-based herbal teas were fermented by addition of the 'tea fungus'. All these herbs had similar taste and color perceptions as black tea, thereby, providing assuring that the fermented product did not result in any adverse sensory properties. In addition, the selection of herbs was also based with the intention of widening the application of the tea fungus to other types of herbal teas which maybe more readily available depending on the locality and vegetation. Identification of the dominant bacteria and yeast species were carried out as well as the changes to the overall population of bacteria and yeast in the fermented broth as well as the pellicle which formed as a result of the fermentation process. Apart from investigating quality parameters such as pH and TA, one aspect of this study was to investigate the enhancement of the antioxidant potential and changes to the phenolic compounds which are responsible for these antioxidant effects characterized to be common across all ten beverages as compared with their unfermented counterparts. Another aspect of this study was the investigation of the starch hydrolase inhibitory activities of the beverages. The incorporation of starch hydrolase inhibitors into the diet has been known to retard the absorption of glucose through inhibition of α-amylase and α-glucosidase. Inhibitors of these enzymes can delay starch digestion, causing a reduction in the rate of glucose absorption into the bloodstream and consequently blunting postprandial plasma glucose rise in diabetic patients. Whether the ten Kombucha-fermented herbal teas contained any starch hydrolase inhibitory potential as well as the enhancement of this existing potential through addition of the 'tea fungus' was also investigated in this study. The beverages and their analytical parameters were monitored for a period of 7 days, which was the minimum duration by which the beverage could be consumed without the presence of metabolic artefacts resulting from prolonged fermentation according to literature (Chu & Chen, 2006; Jayabalan, Marimuthu, et al., 2008). Although the fermentation process can continue up to 60 days, it was imperative to investigate whether the therapeutic potential had already set in by the least duration. In addition, the scientific evidence to date pertaining to an increase in the antioxidant activity was reported when the fermentation had been carried out for 7 days rather than extensive periods (Jayabalan, Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008).

2. Materials and methods

The dominant bacterial strain present in the tea fungal mat used for the study was verified and authenticated as *Acetobacter aceti* while the dominant yeast components were identified as *Zygosaccharomyces bailii* and *Brettanomyces claussenii* using DNA sequencing as per the method by Marsh, O'Sullivan, Hill, Ross, and Cotter (2014). Black tea dust was obtained from Watawala Plantations, Sri Lanka and was used as the control sample. Table S1 (Supplementary Information) lists the edible plants which were used chosen for the study for the preparation of the herbal teas, based on their evaluated antioxidant and starch hydrolase inhibitory properties and popularity among consumers as documented in authoritative literature and market surveys (Arul, Miyazaki, & Dhananjayan, 2005; Fernando, Wickramasinghe, Thabrew, Ariyananda, & Karunanayake, 1991; Isabelle et al., 2010; Karalliadde & Gawarammana, 2008; Khopde et al., 2005; Madsen, 2007; Pari & Latha, 2002). Dried powders of the plants were obtained from the Ayurveda Medicinal Hall in Kandy, Sri Lanka. The authentication of the powders were carried out by comparison with herbal standards using identification HPLC-MS and NMR. All other reagents, chemicals and HPLC standards used for the study were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.1. Preparation of Kombucha teas and determination of the pH and TA

One gram each of plant powders were added to 100 mL of boiling water and infused for 5 min after which they were filtered through a sterile sieve. Sucrose (10%) was dissolved in each beverage and the preparation was left to cool to room temperature at 24 ± 3 °C. The cooled teas were aseptically inoculated with the freshly grown tea fungus for 7 days. The fermentation was carried out at 24 ± 3 °C. Sampling was carried out only once per day in order to avoid contamination. The fermented teas were centrifuged at 7240*g* for 10 min prior to the assays and analyses. The pH values were measured with an electronic pH meter (Orion model 290A), while the TA was measured according to the method by Chen and Liu (2000).

2.2. Quantification of changes to the dominant bacteria and yeast population in the broth and enumeration of bacteria and yeast population in the broth and pellicle

Changes to the dominant bacteria and yeast population in the broth were determined according to the method by Markov, Malbaša, Hauk, and Cvetković (2001). The evaluation was carried out only in the broth since only the broth is typically consumed and not the pellicle, thus assessment of the microbial composition in the broth could be deemed as relatively more important than the pellicle. Enumeration of the overall population of bacteria and yeast in the broth and pellicle of the fermented beverages were determined according to the method by Chen and Liu (2000). Approximately 20 g of sample was withdrawn and placed in a sterile plastic bag and 180 mL of 0.1% sterile peptone water was added. The samples were then homogenized in a blender (Stomacher Lab Blender 400, Seward Medical Ltd., London, UK) for 10 min. The suspension obtained was used for the enumeration of bacteria and yeasts. Both figures were expressed as colony-forming units per mL (cfu/mL).

2.3. Determination of the total phenolics content and antioxidant activity

The method by Huang, Ou, Hampsch-Woodill, Flanagan, and Deemer (2002) was used for determining the total phenolics content using the Thermo Scientific Multiskan FC Microplate Reader. Results were expressed as milligrams of gallic acid equivalents (GAE) per milliliters (mg GAE/mL). The Oxygen Radical Absorbance Capacity (ORAC) assay was carried out according to Prior et al. (2003) using a Thermo Scientific Multiskan FC Microplate Reader and the values were expressed as micromoles of trolox equivalents (TE) per milliliters (μmol TE/mL). As for the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging activity assay, 100 μL of each of the beverages were mixed with 1 mL of 0.1 mM DPPH in ethanol and 450 μL of 50 mM Tris–HCl buffer (pH 7.4). The solution was incubated at room temperature for 30 min and reduction of DPPH radicals was measured by reading the absorbance at 517 nm. The antioxidant activity was calculated as % DPPH radical scavenging activity using the following equation:

$$\%\,\text{DPPH Radical Scavenging Activity} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Extract}} \times 100}{\text{Abs}_{\text{Control}}}$$

Scavenging ability of superoxide radical (O_2^-) was assessed by the method described by Lee, Kim, Jim, and Jang (2002) and calculated by using the following formula:

$$\%\,\text{Superoxide Scavenging Activity} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Extract}} \times 100}{\text{Abs}_{\text{Control}}}$$

2.4. Determination of the α-amylase and α-glucosidase inhibitory activities

The α-amylase inhibitory activity was evaluated according to the method by Liu, Song, Wangm, and Huang (2011) while the α-glucosidase inhibitory activity was carried out according to the method by Koh, Wong, Loo, Kasapis, and Huang (2009). Acarbose was used as the positive control for both assays and the data were expressed as IC_{50} (mg/mL).

2.5. High performance liquid chromatography (HPLC) determination of the phenolic compounds

A Shimadzu LC2010 HPLC system (Kyoto, Japan) equipped with an SPD-M10AVP diode array detector (Kyoto, Japan) and a phenomenex Luna C-18(2) column (4.6 mm i.d. × 25 cm, 5 μm was used for the quantification of gallic acid, vanillic acid, gallocatechin, catechin and ellagic acid. A gradient profile using two solvents was applied following the method by Wijeratne, Abou-Zaid, and Shahidi (2006) with a few modifications. The solvents used were as follows: Solvent A: 8% aqueous formic acid and solvent B: acetonitrile/methanol (10:90, v/v). A flow rate of 0.9 mL/min was maintained. The gradient was as follows: 0 min – 20% B; 7 min – 35% B; 14 min – 45% B; 21 min – 65% B; 25 min – 85% B; 32 min – 95% B. The wavelengths of the diode array detector were set at 260, 280 and 320 nm for monitoring of the phenolic compounds. The concentrations of all the compounds in the extracts were quantified using standard curves and expressed as micrograms per mL (μg/mL).

2.6. Statistical analysis

IBM SPSS Statistics version 21.0 released in 2012 (IBM Corp., Armonk, NY, USA) for Windows was used for the statistical analyses. Results were calculated and expressed as mean ± standard error mean (SEM) of ⩾3 independent analyses. *P* values of >0.05 were considered to be significant.

3. Results

The initial values of all the analytical parameters prior to the fermentation process are indicated as day 0 in Tables 1–3, Supplementary Information Tables S1–S3 and Figs. 1–3.

3.1. Mat formation and changes to the color and clarity of the beverages

Observations for the mat formation and changes in color and clarity are provided in Table S2 of the Supplementary Information. The progressive darkening of color and loss of clarity of the fermented teas had been observed in previously conducted studies as well, where the changes were associated with the microbial transformation of polyphenols (Chu & Chen, 2006; Jayabalan et al., 2007). In this respect, AA, AM-F, AM-RB, ALa, ALo and CA were observed to become darker with the continuation of the fermentation process, thus, providing evidence as to the biotransformation of polyphenols into smaller phenolic compounds. However, although, HI, HV, PE and TC were not observed to have a darkening in color, their individual polyphenol contents were nevertheless able to prove this transformation process as observed in Fig. 2 and Table 3.

Table 1
Changes to the composition of the overall population of bacteria and yeast present in the broth prior to fermentation (day 0) as well as on day 1 and day 7.

Herbal tea	Microbes	Days		
		0 (cfu/mL)	1 (cfu/mL)	7 (cfu/mL)
Black tea	Bacteria	$2.0 \pm 0.1 \times 10^6$	$8.1 \pm 0.1 \times 10^7$	$8.5 \pm 0.1 \times 10^8$
	Yeast	$1.5 \pm 0.2 \times 10^6$	$6.0 \pm 0.3 \times 10^8$	$8.2 \pm 0.1 \times 10^9$
AA	Bacteria	$2.9 \pm 0.1 \times 10^6$	$8.6 \pm 0.2 \times 10^6$	$3.9 \pm 0.3 \times 10^9$
	Yeast	$1.5 \pm 0.2 \times 10^6$	$8.9 \pm 0.1 \times 10^6$	$9.2 \pm 0.1 \times 10^9$
AM-F	Bacteria	$3.6 \pm 0.2 \times 10^6$	$4.9 \pm 0.2 \times 10^7$	$6.5 \pm 0.2 \times 10^9$
	Yeast	$2.1 \pm 0.2 \times 10^6$	$7.5 \pm 0.3 \times 10^6$	$5.6 \pm 0.2 \times 10^{10}$
AM-RB	Bacteria	$3.9 \pm 0.1 \times 10^6$	$6.5 \pm 0.3 \times 10^6$	$9.1 \pm 0.1 \times 10^{10}$
	Yeast	$6.9 \pm 0.2 \times 10^6$	$6.6 \pm 0.2 \times 10^7$	$9.5 \pm 0.1 \times 10^{10}$
ALa	Bacteria	$3.5 \pm 0.1 \times 10^6$	$6.6 \pm 0.2 \times 10^7$	$7.5 \pm 0.2 \times 10^9$
	Yeast	$5.3 \pm 0.1 \times 10^6$	$9.3 \pm 0.2 \times 10^5$	$9.6 \pm 0.1 \times 10^{10}$
ALo	Bacteria	$3.4 \pm 0.2 \times 10^6$	$6.6 \pm 0.2 \times 10^6$	$9.8 \pm 0.1 \times 10^{10}$
	Yeast	$5.1 \pm 0.2 \times 10^6$	$5.5 \pm 0.1 \times 10^7$	$1.8 \pm 0.1 \times 10^{10}$
CA	Bacteria	$1.9 \pm 0.2 \times 10^6$	$2.5 \pm 0.1 \times 10^6$	$3.6 \pm 0.1 \times 10^{10}$
	Yeast	$1.2 \pm 0.2 \times 10^6$	$8.8 \pm 0.2 \times 10^6$	$9.2 \pm 0.1 \times 10^{10}$
HI	Bacteria	$3.6 \pm 0.2 \times 10^6$	$5.9 \pm 0.1 \times 10^6$	$3.9 \pm 0.1 \times 10^{10}$
	Yeast	$5.3 \pm 0.1 \times 10^6$	$6.5 \pm 0.2 \times 10^7$	$9.0 \pm 0.1 \times 10^{10}$
HV	Bacteria	$4.1 \pm 0.1 \times 10^6$	$3.5 \pm 0.3 \times 10^6$	$4.8 \pm 0.1 \times 10^{10}$
	Yeast	$2.5 \pm 0.1 \times 10^6$	$4.4 \pm 0.3 \times 10^5$	$8.0 \pm 0.2 \times 10^{10}$
PE	Bacteria	$1.2 \pm 0.2 \times 10^6$	$3.9 \pm 0.2 \times 10^6$	$7.1 \pm 0.1 \times 10^9$
	Yeast	$3.2 \pm 0.1 \times 10^6$	$5.0 \pm 0.1 \times 10^7$	$8.0 \pm 0.2 \times 10^9$
TC	Bacteria	$1.9 \pm 0.2 \times 10^6$	$3.2 \pm 0.1 \times 10^6$	$2.3 \pm 0.2 \times 10^9$
	Yeast	$4.1 \pm 0.1 \times 10^6$	$8.3 \pm 0.2 \times 10^6$	$1.9 \pm 0.1 \times 10^{10}$

Table 2
Changes to the composition of the overall population of bacteria and yeast present in the pellicle on day 1 and day 7.

Herbal tea	Microbial species	1 (cfu/mL)	7 (cfu/mL)
Black tea	Bacteria	$2.0 \pm 0.1 \times 10^5$	$6.6 \pm 0.1 \times 10^7$
	Yeast	$1.2 \pm 0.2 \times 10^5$	$1.9 \pm 0.1 \times 10^7$
AA	Bacteria	$3.7 \pm 0.2 \times 10^6$	$2.2 \pm 0.1 \times 10^8$
	Yeast	$1.1 \pm 0.2 \times 10^5$	$1.6 \pm 0.2 \times 10^7$
AM-F	Bacteria	$1.5 \pm 0.1 \times 10^6$	$2.6 \pm 0.1 \times 10^8$
	Yeast	$3.5 \pm 0.2 \times 10^5$	$3.9 \pm 0.2 \times 10^7$
AM-RB	Bacteria	$1.1 \pm 0.1 \times 10^6$	$1.5 \pm 0.1 \times 10^8$
	Yeast	$1.5 \pm 0.2 \times 10^5$	$5.5 \pm 0.2 \times 10^7$
ALa	Bacteria	$6.6 \pm 0.2 \times 10^6$	$7.5 \pm 0.1 \times 10^8$
	Yeast	$1.5 \pm 0.1 \times 10^5$	$9.2 \pm 0.1 \times 10^7$
ALo	Bacteria	$2.1 \pm 0.2 \times 10^6$	$9.5 \pm 0.1 \times 10^8$
	Yeast	$1.6 \pm 0.2 \times 10^5$	$1.9 \pm 0.1 \times 10^7$
CA	Bacteria	$2.8 \pm 0.1 \times 10^6$	$2.8 \pm 0.1 \times 10^8$
	Yeast	$5.5 \pm 0.1 \times 10^5$	$4.8 \pm 0.2 \times 10^7$
HI	Bacteria	$3.9 \pm 0.1 \times 10^6$	$3.9 \pm 0.1 \times 10^8$
	Yeast	$4.9 \pm 0.1 \times 10^5$	$5.9 \pm 0.3 \times 10^7$
HV	Bacteria	$4.4 \pm 0.2 \times 10^6$	$7.5 \pm 0.1 \times 10^8$
	Yeast	$4.7 \pm 0.1 \times 10^5$	$5.5 \pm 0.1 \times 10^7$
PE	Bacteria	$3.5 \pm 0.1 \times 10^6$	$6.6 \pm 0.2 \times 10^8$
	Yeast	$2.2 \pm 0.1 \times 10^5$	$2.9 \pm 0.1 \times 10^7$
TC	Bacteria	$3.1 \pm 0.1 \times 10^6$	$2.5 \pm 0.1 \times 10^8$
	Yeast	$6.6 \pm 0.1 \times 10^5$	$1.9 \pm 0.2 \times 10^7$

3.2. Changes in population of viable yeasts and acetic acid bacteria

The distribution of the initial microbial compositions added to the beverages and their subsequent changes in the broth are

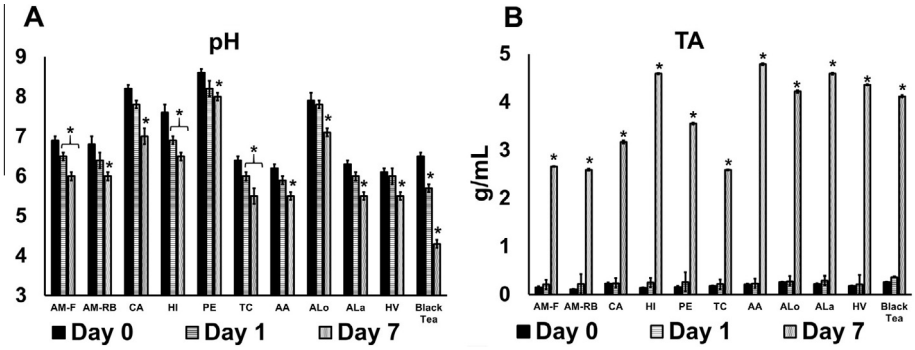

Fig. 1. Changes in the (A) pH and (B) titratable acidity values of the herbal beverages. Error bars represent the SEM. $^{*}P < 0.05$ versus the value at day 0.

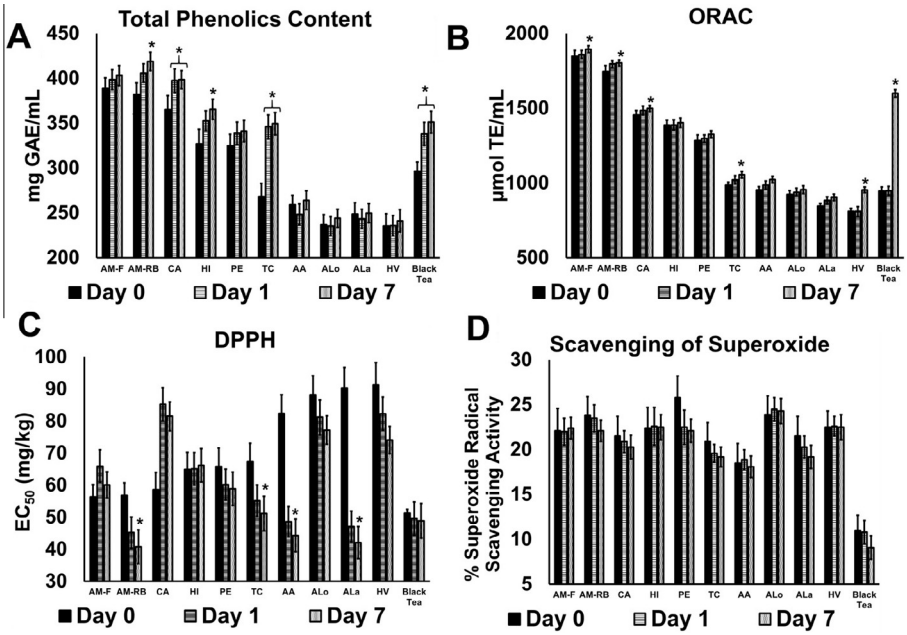

Fig. 2. Changes in the (A) total phenolics content, (B) ORAC, (C) DPPH EC_{50} and (D) superoxide scavenging activities of the herbal beverages. Error bars represent the SEM. $^{*}P < 0.05$ versus the value at day 0.

reported in Table 1. Changes to the composition of the overall population of bacteria and yeast present in the pellicle on days 1 and 7 are shown in Table 2. Both tables indicate the increase in the overall bacteria and yeast population in all beverages. The increase was indicative of the microbes' successful livelihood in the beverages, thus, confirming their ability of thrive in the environments in similar fashion to black tea. The highest number of mixed cultures of acetic acid bacteria and yeasts was achieved with the black tea control sample at the end of the fermentation process. As per Table S3 (Supplementary Information), the population of *A. aceti*, *B. claussenii* and *Z. bailii* increased over time in all of the broths of the fermented beverages. Overall, according to Tables 1 and 2 and S3 (Supplementary Information), the cell concentrations of both bacteria and yeasts in the broth were generally higher than those in cellulosic pellicles. In addition, the cell concentrations of bacteria were higher than the yeasts in both the broth as well as the pellicle. This observation was also visible when comparing the individual cell concentrations of *A. aceti* versus the combined cell concentrations of *B. claussenii* and *Z. bailii*. It was observed that the increase in the concentrations of both bacteria and yeasts in the herbal beverages were comparable with the black tea samples, although the microbial count at the end of fermentation of all beverages were slightly lower in comparison to the black tea control. The versatility of the 'tea fungus' zoogleal mat may be confirmed from these results alone, where its ability to carry out fermentation on any type of beverage substrate other than *C. sinesis*-based tea can be assured.

3.3. pH and TA

As per Fig. 1A, all the beverages had a statistically significant decrease ($P < 0.05$) in the pH values on day 7. AM-F, HI and TC had statistically significant decreases ($P < 0.05$) in the pH from day 1 itself. The overall decrease in pH of all the Kombucha samples would have been due to the increased concentration of organic acids produced during the fermentation process. All teas had an initial pH value of between 6.0 and 8.0 prior to fermentation, while the pH of the teas were between 4.0 and 6.0 on day 7 at the end of the fermentation period. The low pH of the Kombucha beverages prepared in this study could be beneficial in terms of maintaining the bioactivity of the phenolic compounds and ascertaining their protection from undergoing chemical degradation (Zhu, Zhang, Tsang, Huang, & Chen, 1997). As per Fig. 1B, the TA of all the teas had initial values ranging from 0.1 to 0.5 g/mL. On day 1, the values

Table 3
Changes in the polyphenol contents (gallic acid, vanillic acid, gallocatechin, catechin and ellagic acid) in the broth of the herbal tea beverages when fermented with the Kombucha tea prior to fermentation (day 0) as well as on day 1 and day 7 expressed as mean ± SEM in μg/mL.

Herbal tea	Polyphenol	Days		
		0 (μg/mL)	1 (μg/mL)	7 (μg/mL)
Black tea	Gallic acid	23.5 ± 1.3	29.4 ± 2.1	38.6 ± 2.8*
	Vanillic acid	16.5 ± 1.1	26.1 ± 3.2*	28.5 ± 2.3*
	Gallocatechin	16.9 ± 1.3	18.5 ± 1.0	19.2 ± 1.2
	Catechin	3.2 ± 0.3	3.6 ± 0.1	3.5 ± 0.1
	Ellagic acid	4.1 ± 0.6	5.9 ± 0.2*	6.1 ± 0.1*
AM-F	Gallic acid	13.5 ± 1.1	16.5 ± 1.3*	16.9 ± 1.2*
	Vanillic acid	12.5 ± 1.1	15.2 ± 1.2*	15.9 ± 1.3*
	Gallocatechin	26.1 ± 2.0	34.6 ± 2.1*	35.9 ± 2.2*
	Catechin	9.2 ± 0.9	13.1 ± 1.0*	14.2 ± 1.1*
	Ellagic acid	12.5 ± 1.1	16.9 ± 1.2*	19.5 ± 1.1*
AM-RB	Gallic acid	14.2 ± 1.3	14.6 ± 1.1	17.4 ± 1.1*
	Vanillic acid	10.1 ± 1.3	18.4 ± 1.0*	19.2 ± 1.2*
	Gallocatechin	27.2 ± 1.9	37.2 ± 2.5*	38.1 ± 2.0*
	Catechin	10.1 ± 0.8	14.0 ± 1.1*	18.3 ± 1.3*
	Ellagic acid	14.1 ± 1.0	18.7 ± 1.1*	20.2 ± 1.0*
CA	Gallic acid	23.6 ± 1.1	26.9 ± 1.0*	28.9 ± 1.0*
	Vanillic acid	13.2 ± 1.2	16.5 ± 1.3*	17.4 ± 1.0*
	Gallocatechin	9.3 ± 0.7	13.6 ± 0.8*	13.4 ± 1.0*
	Catechin	8.2 ± 0.3	12.4 ± 1.0*	13.6 ± 1.5*
	Ellagic acid	9.6 ± 0.1	14.7 ± 0.3*	16.2 ± 1.1*
HI	Gallic acid	19.2 ± 1.2	28.3 ± 1.1*	30.2 ± 1.3*
	Vanillic acid	18.2 ± 2.1	18.3 ± 1.9	18.2 ± 1.4
	Gallocatechin	12.3 ± 1.0	16.2 ± 1.1*	17.2 ± 1.3*
	Catechin	6.3 ± 0.2	6.4 ± 0.5	6.2 ± 0.4
	Ellagic acid	6.2 ± 0.1	6.3 ± 0.1	6.8 ± 0.1*
PE	Gallic acid	8.7 ± 0.8	9.5 ± 0.2*	10.4 ± 0.1*
	Vanillic acid	13.2 ± 0.4	16.2 ± 1.1*	18.2 ± 1.0*
	Gallocatechin	26.1 ± 1.2	29.1 ± 1.0*	30.2 ± 1.0*
	Catechin	23.9 ± 1.0	34.1 ± 1.1*	35.6 ± 1.2*
	Ellagic acid	9.3 ± 0.4	16.3 ± 0.9*	15.2 ± 1.1*
TC	Gallic acid	9.5 ± 0.3	16.5 ± 1.0*	18.2 ± 1.0*
	Vanillic acid	18.9 ± 1.0	18.2 ± 1.1	19.3 ± 1.2
	Gallocatechin	21.3 ± 2.0	26.3 ± 2.1*	28.5 ± 2.0*
	Catechin	16.2 ± 1.2	18.3 ± 1.1*	19.2 ± 1.1*
	Ellagic acid	9.3 ± 0.6	12.6 ± 0.9*	14.3 ± 0.8*
AA	Gallic acid	3.4 ± 0.1	4.9 ± 0.3*	5.9 ± 0.1*
	Vanillic acid	4.3 ± 0.2	6.8 ± 0.4*	7.2 ± 0.1*
	Gallocatechin	5.3 ± 0.1	6.9 ± 0.2*	7.9 ± 0.1*
	Catechin	4.2 ± 0.3	8.4 ± 1.2*	9.0 ± 0.3*
	Ellagic acid	6.1 ± 0.3	9.7 ± 0.1*	11.2 ± 0.2*
ALo	Gallic acid	13.8 ± 1.2	14.2 ± 1.1	15.9 ± 1.2
	Vanillic acid	9.3 ± 1.0	13.6 ± 1.1*	14.9 ± 1.1*
	Gallocatechin	15.9 ± 1.1	19.8 ± 1.2*	21.5 ± 1.0*
	Catechin	12.3 ± 1.2	16.4 ± 1.1*	17.8 ± 1.0*
	Ellagic acid	6.5 ± 1.0	6.9 ± 1.2	6.9 ± 1.1
ALa	Gallic acid	16.9 ± 1.2	19.5 ± 1.3*	21.0 ± 1.1*
	Vanillic acid	18.2 ± 1.1	19.6 ± 1.0	20.1 ± 1.1
	Gallocatechin	10.2 ± 0.9	12.3 ± 1.1	13.6 ± 1.1*
	Catechin	3.9 ± 0.2	4.5 ± 0.1*	4.6 ± 0.1*
	Ellagic acid	5.2 ± 0.2	6.1 ± 0.3*	6.6 ± 0.2*
HV	Gallic acid	6.9 ± 0.1	8.5 ± 0.1*	8.9 ± 0.2*
	Vanillic acid	8.5 ± 0.1	13.6 ± 0.2*	13.6 ± 0.3*
	Gallocatechin	6.1 ± 0.9	6.9 ± 0.2	7.6 ± 0.1*
	Catechin	5.2 ± 0.2	5.3 ± 0.1	5.6 ± 0.1*
	Ellagic acid	4.9 ± 0.2	6.8 ± 0.4*	6.9 ± 0.1*

* $P < 0.05$ versus the value of each tea at day 0.

were still within this range. However, on day 7, the values ranged between 2.5 and 5.0 g/mL, where all beverages had statistically significant increases ($P < 0.05$). Given the optimum consumable acidity level of 4.0–4.5 g/L, only AA and HI did not have TA levels falling within this range by day 7. Thus, according to the TA reference levels as indicated by Reiss, it may be assumed that all of the Kombucha beverages were acceptable for consumption (Reiss, 1994).

3.4. Total phenolics content and changes in the tea polyphenol quantities

The total phenolics content are shown in Fig. 2A. AM-F and AM-RB had the highest total phenolics contents prior to fermentation with HV being the lowest. On day 1, the highest total phenolics contents were still observed in AM-F and AM-RB. Statistically significant increases ($P < 0.05$) in the total phenolics contents on day 1 itself were observed only in CA, TC and the black tea control samples. However, by day 7, statistically significant increases ($P < 0.05$) in the total phenolics contents were observed in AM-RB, CA, HI, TC and the black tea control samples. AM-RB had the highest total phenolics content by day 7. Despite the overall increase in the total phenolics contents in AM-RB, CA, HI, TC and the black tea following the initiation of the fermentation process, the individual polyphenol contents were present in varied quantities. Changes in the gallic acid, vanillic acid, gallocatechin, catechin and ellagic acid quantities are shown in Table 3. Representative HPLC diagrams of the 10 fermented herbal beverages indicating the peaks pertaining to these phenolic compounds are shown in Fig. S1 of the Supplementary Information. Quantities of these phenolic compounds were reflective of the total phenolics contents displayed in Fig. 2A. Out of all the phenolic compounds, gallic acid was observed to statistically significant increase ($P < 0.05$) in all of the samples except ALo by the end of the fermentation period. Vanillic acid was observed to have the least statistically significant increase ($P < 0.05$) as compared with the rest of the phenolic compounds analyzed.

3.5. ORAC, DPPH EC_{50} and superoxide scavenging activities

The ORAC, DPPH EC_{50} and superoxide scavenging activities are shown in Fig. 2B–D, respectively. AM-F and AM-RB had the highest ORAC values with HV being the lowest. With the exception of AM-F, AM-RB, CA, TC and HV, none of the remaining Kombucha beverages had statistically significant increases in the ORAC values at day 1 or 7. Given the high ORAC values of AM-F and AM-RB, the maintenance of their antioxidant potential during the fermentation process could be of therapeutic importance. The DPPH EC_{50} values appear to have complemented the ORAC values with the exceptions of AM-RB, TC, AA and ALa, where their DPPH EC_{50} values were observed to have statistically significant increases ($P < 0.05$) on day 7. As for the superoxide scavenging activities, the trends were not as clear as the ORAC and DPPH EC_{50} values. There was a better correlation between the total phenolics content and the ORAC values rather than the DPPH EC_{50} and superoxide scavenging values of all teas on all days of analysis ($R^2 = 0.985$ for ORAC vs. $R^2 = 0.745$ DPPH EC_{50} and $R^2 = 0.632$ for superoxide scavenging potential). Compounds exhibiting good antioxidant activity by one method have been known to demonstrate good antioxidant activity by the other methods, and likewise for compounds with low activity (Prior & Cao, 2000). However, the ORAC values may have had a better correlation with the total phenolics content since the phenolic compounds present in the beverages may have been better scavengers of peroxyl radicals which are generated during the assay. Nevertheless, in the instance of HI, it was observed that the increase in the polyphenol contents did not necessarily result in an increased ORAC value. Similarly, in the instance of HV, despite not having a statistically significant increase in the polyphenol contents, the ORAC values of this extract had a statistically significant increase ($P < 0.05$) by day 7. For both HI and HV, it is possible that the antioxidant potential may not extend only from the polyphenols but from other categories of antioxidant compounds as well.

3.6. Starch hydrolase inhibitory activities

The starch hydrolase inhibitory activities are shown in Fig. 3. On day 1, only CE, HI, PE and AA had statistically significant increases ($P < 0.05$) in the α-amylase inhibitory activities, whereas by day 7, all the beverages had statistically significant increases ($P < 0.05$). As for the α-glucosidase inhibitory activities, AM-F, AM-RB, CA, HI, PE, AA, ALa and HV had statistically significant increases ($P < 0.05$) during both day 1 and 7. The observed changes in the α-amylase and α-glucosidase inhibitory activities are of therapeutic value since the ability of the beverages to inhibit this enzyme had increased. In addition, the fermentation process was able to enhance the α-amylase inhibitory activity better than the α-glucosidase inhibitory activity. This is an important aspect, given that α-amylase is required for the subsequent reactions of α-glucosidase (Chu & Chen, 2006). Polyphenols existing in edible plants have been identified to exhibit starch hydrolase inhibitory activities (Koh et al., 2009). Thus, given the copious amounts of polyphenols observed to be present in the beverages, it may be hypothesized that a significant portion of the starch hydrolase inhibitory activities was owed due to the presence of this category of therapeutic compounds. From the results, it was observed that the α-amylase inhibitory activity in particular had increased with the fermentation process. This characteristic may yet again have been owed to the increase in the total phenolics content and the increase of the presence of compounds with α-amylase inhibitory activities as a result of the fermentation process (Koh et al., 2009). Overall, it was noteworthy that the α-amylase and α-glucosidase inhibitory activities of all 10 beverages in this study prior to and following fermentation were comparable with values reported of other types of plant-based food products with verified starch hydrolase inhibitory activities (Fossum & Whitaker, 1974; Gujral, Haros, & Rosell, 2004).

4. Discussion

Previous studies had reported an increase in the total phenolics content of Kombucha beverages prepared using black tea samples following fermentation (Jayabalan, Marimuthu, et al., 2008; Jayabalan, Subathradevi, et al., 2008; Jayabalan et al., 2007). The hypothesis for this increment was explained by Blanc (1996), where the enzymes (phytases, in particular) liberated by bacteria and yeast in the tea fungus consortium were identified as capable of liberating polyphenol compounds from the cellulosic backbone. Thus, this biochemical reaction would have resulted in an increase in polyphenols in the soluble fraction of the fermented beverage. It has also been identified that phytases liberated by bacteria and yeast during Kombucha fermentation are able to cause the degradation of complex polyphenols to small molecules which in turn results in the increase of total phenolic compounds. This explanation has also been brought forward by Jayabalan et al. (2007), Jayabalan, Marimuthu, et al. (2008) and Jayabalan, Subathradevi, et al. (2008). The catechins evaluated in this study using HPLC had been previously observed to undergo increases in the studies by Chen and Liu (2000), Jayabalan, Marimuthu, et al. (2008) and Jayabalan, Subathradevi, et al. (2008) as well. These three studies had also carried out the fermentation process for 7 days. According to Haslam (2003), this biotransformation process is typically known to result in a darkening of color – an observation which was qualitatively recorded in Table S2 (Supplementary Information) of this study as well. Generally, polymerisation of phenolics (resulting in a darker color) may also result in larger particles settling to the bottom, and thus a lighter supernatant (Crozier, Del Rio, & Clifford, 2010). However, this phenomenon was not observed in these fermented beverages. In contrast, studies conducted on Kombucha beverages prepared through fermentation with the tea fungus for 1–2 months indicated a decrease in the total phenolics content and the antioxidant potential (Jayabalan, Subathradevi, et al., 2008). These changes were attributed to the partial oxidation of polyphenols to form macromolecular compounds, which were still able to provide a noteworthy radical scavenging activity. However, the antioxidant activity of such teas were observed to be short-lived and the teas were had contained unwanted metabolites as a result of the extended fermentation process (Blanc, 1996).

Overall, the therapeutic effect of the Kombucha fermentation has been associated with the presence of polyphenols, compounds produced during the fermentation period, and synergistic action between different compounds (Jayabalan, Marimuthu, et al., 2008; Jayabalan, Subathradevi, et al., 2008). In general, plant polyphenols are a class of chemically diverse secondary metabolites that possess many different biological activities both within the plant and in the animals which eat these plants (Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). The majority of the evidence for such health effects stems from animal studies using either whole plant foods or plant polyphenol extracts. Consequently, both the active chemical moiety and, in many cases, the underlying mechanism of action in humans remain to be determined (Quideau et al., 2011). Although this study was able to confirm the antioxidant potential of ten beverages fermented with the tea fungus, further *in vivo* and clinical studies are warranted for the actual demonstration of their therapeutic potential. According to previous studies, out of the three microbial strains which were determined to be present in the tea fungus, *A. aceti* has been identified as the key bacterial strain responsible for the enhancement of the bioaccessibility of polyphenols and antioxidant activities in the substrates (Chen & Liu, 2000; Jayabalan, Marimuthu, et al., 2008; Jayabalan, Subathradevi, et al., 2008). As a whole, it is known that fermentation is able to promote a progressive polymerization of phenolic compounds to form brown-colored macromolecular

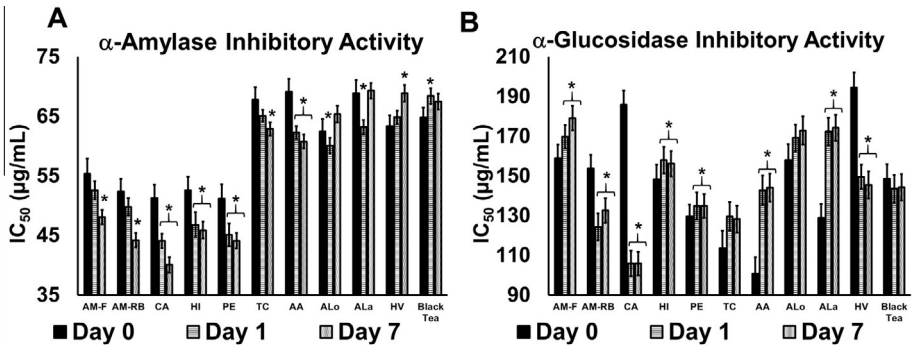

Fig. 3. Changes in the (A) α-amylase and (B) α-glucosidase inhibitory activities of the herbal beverages. Error bars represent the SEM. $^{*}P < 0.05$ versus the value at day 0.

products (Marsh et al., 2014; Rice-Evans, Miller, & Paganga, 1996). In some cases, the oxidation of polyphenols has been known to result in the formation of stable intermediates which exhibit strong antioxidant activity (Fukumoto & Mazza, 2000). Modification in chain-breaking activity and oxygen uptake detected in plant extracts could be related to the progressive oxidation of polyphenols, which leads to the formation of macromolecular compounds with stronger or decreased radical scavenging power (Prior & Cao, 2000). A possible explanation could be that the enzymatic and chemical oxidations of polyphenols present in these plant extracts follow different pathways, which lead to the formation of compounds having markedly contrasting radical scavenging capacities (Fukumoto & Mazza, 2000).

The starch hydrolase inhibitory activities of Kombucha are comparatively less in number than those focusing on antioxidant studies. Only a few studies have been published over the recent years on the ability of the fermented beverage to inhibit α-amylase and α-glucosidase (Lee et al., in press; Watawana et al., in press). The opportunities and challenges for the food industry in the area of evidence-based functional foods with a low glycemic index that may decrease starch digestion rates are on the rise, given the increasing incidence of diabetes throughout the world (Stumvoll, Goldstein, & van Haeften, 2006). Recent warnings on the side effects of anti-diabetic drugs such as Rosiglitazone and Pioglitazone highlight the urgent need of alternative and safer means of blood glucose control, ideally through functional foods which contain bioactive ingredients with the ability to regulate blood glucose concentration toward the normal range (Graham et al., 2010). The importance of beverages such as Kombucha which have demonstrated the properties in support of preventing metabolic diseases such as diabetes could be highlighted in this aspect. Given the ease of preparation of the beverage as well as the economic viability, Kombucha could be promoted as a functional food which could be consumed as a means of supportive therapy for the prevention and containment of disease conditions in association with its demonstrated antioxidant and starch hydrolase inhibitory properties.

5. Conclusions

In conclusion, the study was able to identify the enhancement of the antioxidant and starch hydrolase inhibitory potential of ten fermented herbal teas through the addition of the tea fungus, as a result of the increased bioaccessibility of phenolic compounds. Fermented AM-F and AM-RB were discovered to be the better Kombucha beverages, having the highest antioxidant and starch hydrolase inhibitory activities following fermentation. Although the mechanisms of action of these beverages in the human physiology is yet to be elucidated, this study serves as a platform for the identification and promotion of Kombucha as a functional food which can be easily prepared in households using edible plants. The starch hydrolase inhibitory properties of the beverages were also indicative of its usage to be consumed for the maintenance of health and wellness. It would also be a novelty to investigate the therapeutic potential of Kombucha beverages prepared from other types of herbal tea extracts not studied herein. Systematic sensory evaluations of the novel fermented beverages were not carried out in this study, given that the primary focus was to observe the changes to the antioxidant and starch hydrolase inhibitory potential during the fermentation process. However, during the selection of the beverages, care was taken to identify beverages which carry similar sensory perceptions as black tea, thus, reducing the chances of the resulting fermented beverages possessing adverse gustatory and olfactory properties. Although, the sensory properties of the fermented beverages were qualitatively assessed during the study and were discovered to not harbor off-flavors or off-odors which may displease users, systematic analysis of the sensory aspects of the beverages required to be carried out prior to their being advocated to consumers for consumption.

Conflicts of interest

The authors report no conflicts of interest, financial or otherwise.

Acknowledgements

The financial and analytical support provided by the National Institute of Fundamental Studies, Hantane Road, Kandy, Sri Lanka and the analytical support provided by the Temasek School of Applied Science, Temasek Polytechnic, Singapore are acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.08.033.

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