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Effect of simultaneous inoculation of commercial yeast starter cultures on Kombucha fermentation

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Abstract. Kombucha – a spontaneously fermented tea beverage, produced by SCOBY (a symbiont of bacteria and yeasts), has become popular in recent years. Its functional properties and features for industrial production and treatment remain largely unknown, however. Our aim of using additional yeast cultures during the primary fermentation was to modify and ultimately improve the sensory properties of the kombucha beverage. During five fermentation experiments the total viable count (TVC) of microbes was determined both in Kombucha and SCOBY samples. The first four fermentation experiments were conducted to stabilize the growth of SCOBYs that were initially of different origin. The last (5^{th}) fermentation contained the simultaneous inoculation of three different active *S. cerevisiae* cultures into the sweetened black tea together with the SCOBY and was followed by a sensory analysis. Two samples remained as control samples without additional yeast starter culture. The yeast starter cultures affected the microbial counts of Kombucha, but the effect on the microbial count of SCOBY was not statistically significant (p >0.05). The Kombucha containing wine yeast culture had the lowest sensory quality, while Kombucha containing brewer's yeast had the most pleasant flavour and aroma. In conclusion, the simultaneous fermentation with commercial yeast cultures had a minor effect on the microbial counts in SCOBY when compared to the fermentation time, but all added cultures clearly modified the taste and aroma properties of the Kombucha drinks.

Key words: kombucha, SCOBY, tea, simultaneous fermentation, *Saccharomyces cerevisiae*.

INTRODUCTION

Kombucha is a beverage obtained by fermentation of sugar-sweetened tea with Symbiotic Culture of Bacteria and Yeasts (SCOBY) (Greenwalt et al., 2000; Jayabalan et al., 2011; Leal et al., 2018; Zhao et al., 2018). Kombucha has become very popular in recent years and both its production and consumption have increased. Especially the production of flavoured Kombucha drinks and the improvement of Kombucha's probiotic properties by the addition of lactic acid bacteria during simultaneous fermentation have recently gained more interest (Cvetković et al., 2019).

Kombucha and SCOBY contain acetic acid bacteria (e.g. from genera *Acetobacter*, *Bacterium*, *Gluconoacetobacter*, *Gluconobacter*, *Halomonas*, *Herbaspirillum*, *Komagataeibacter*), lactic acid bacteria (e.g. *Oenococcus oeni*, *Lactobacillus satsumensis*, *Lactobacillus nagelii*) and yeasts (e.g. from genera *Brettanomyces,*

Candida, *Dekkera*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Mycoderma*, *Mycotorula*, *Rhodotorula*, *Saccharomyces*, *Saccharomycodes*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Zygotorulaspora*, *Pichia*, *Torula*, *Torulaspora, Torulopsis*) (Kozaki et al., 1972; Jankovic & Stojanovic, 1994; Markov et al., 2001; Dogan et al., 2002; Teoh et al., 2004; Jayabalan et al., 2010; Yapar, 2010; Reva et al., 2015; Coton et al., 2017) that live in tight symbiosis. The microbial species in different SCOBYs and Kombuchas may vary and due to this it is not possible to identify a uniform microbial community (Jayabalan et al., 2014). The most important acetic acid bacterium in Kombucha is *Komagataeibacter xylinus*, which synthesizes cellulose during fermentation (Jayabalan et al., 2010). The cellulose appears as a thin film on the surface of the tea solution and cells of other bacteria and yeasts attach to it (Jayabalan et al., 2010).

To make Kombucha, tea leaves are first put into a container, then hot boiled water is added and sucrose is dissolved in this hot tea beverage. Traditionally black tea is used for making Kombucha, but other teas can also be used – e.g. white tea, green tea, oolong tea and different herb teas (thyme, mint, sage, lemon balm, echinacea) (Markov et al., 2001; Malbaša et al., 2002; Markov et al., 2006; Jayabalan et al., 2010; Velićanski et al., 2013; Adzadogo, 2015). The sugared tea beverage is then cooled down to room temperature and SCOBY is finally added to start the fermentation process (Teoh et al., 2004; Malbaša et al., 2005; Vázquez-Cabral et al., 2014; Leal et al., 2018). The container used for growing SCOBY is covered with paper or other suitable material to prevent airborne microbes or insects from getting into the drink (Dutta & Paul, 2019). To obtain a pleasant sour beverage, primary fermentation is stopped once the titratable acidity has been reached 44.5 g L^{-1} (Velićanski et al., 2013).

Fermented Kombucha beverage contains alcohols, aldehydes, ketones, esters, amino acids, tannins, terpenoids, saponins, flavonoids, phenols, alkaloids, CO_2, enzymes, catechins, caffeine and other compounds (Adzadogo, 2015; Kumar & Joshi 2016). It is claimed that the chemical composition, mainly polyphenols and secondary metabolites formed during fermentation of Kombucha, add a therapeutic effect to the beverage (Watawana et al., 2015). Malbaša et al. (2011) and Watawana et al. (2015) state that Kombucha helps in improving digestion, gives relief against arthritis, prevents microbial infections, helps in combating cancer and removes toxic substances from the body.

Kombucha is described as a refreshing and slightly sweet-sour beverage whose taste is similar to that of effervescent apple cider (Jayabalan et al., 2014; Kumar & Joshi, 2016). After a short fermentation time, the beverage should acquire a pleasant fruity and sour taste, which tends to become slightly vinegary during prolonged fermentation (Reiss, 1994; Jayabalan et al., 2014). The taste of Kombucha depends on the concentration levels of residual sugar, carbon dioxide and organic acids (especially the concentration of acetic and gluconic acid) (Leal et al., 2018). Acetic acid gives the tea beverage an astringent and sour taste and gluconic acid gives it a mild taste (Chen & Liu, 2001). As a result of CO_2 formation, gas bubbles are formed in the tea solution (Mukadam et al., 2016).

Flavourings are usually added to Kombucha after primary and secondary fermentation have taken place in aerobic conditions or in a sealed or air-locked vessel (Bleam et al., 2016). Secondary fermentation may be carried out with or without SCOBY (Dutta & Paul, 2019). Whereas in an open vessel the new SCOBY is formed and a

domineering growth of acetic acid bacteria can be observed, in a sealed vessel more alcohol and lactic acid are produced (Bleam et al., 2016). To improve the probiotic properties of Kombucha lactic acid bacteria are used for the simultaneous fermentation (Nguyen et al., 2015; Cvetković et al., 2019).

There is a lack of scientific research about the effect of simultaneous fermentation on the sensory properties and microbial loads of Kombucha. Traditionally the taste properties of Kombucha are altered or improved during secondary fermentation by adding for example different juices to Kombucha. Modification of sensory properties with yeasts during primary fermentation would be an easier and more time-efficient method, however, especially in a commercial context, i.e. the food industries. Accordingly, the aim of this study was to evaluate the effect of different commercial *Saccharomyces cerevisiae* starter cultures on the microbial abundance of both SCOBY and Kombucha as well as on the sensory properties of the fermented drinks.

MATERIALS AND METHODS

Materials

SCOBYs used in the experiments were originally obtained from two different households in Estonia: one was grown in green tea (TF 1, TF referring to 'tea fungus') and the second was grown in black tea (TF 2).

For simultaneous Kombucha fermentation the *Saccharomyces cerevisiae* commercial starter cultures for dry wine (Enovini dry wine yeast, Browin, Poland), dry cider (Dry cider yeast Ciderini Dry, Browin, Poland) and ale-type beer (BrewGo- O2, Browin, Poland) were obtained from the local market in Tartu, Estonia.

Preparation of black tea

According to proportions suggested by AL-Kalifawi & Hassan (2013), tea solutions containing 5 g of black tea (Ceylon Jambo Grand black tea, Ranfer, Sri Lanka), 0.5 L of hot water and 50 g of sucrose (Polski Cukier, Poland) were prepared in eight sterilized 720 mL glass jars for each of the five fermentation test series. Tea leaves were removed after 30 minutes of soaking and the solutions were then left to cool in closed jars at room temperature (20–24 °C).

Growing SCOBYs

Four circular pieces with a diameter of 2.8 cm each were cut out of both TF 1 and TF 2 'mother' SCOBYs (Fig. 1, a) obtained from two different households. These samples were marked as TF 1.0, TF 1.1, TF 1.2, TF 1.3 and TF 2.0, TF 2.1, TF 2.2, TF 2.3, respectively. All pieces of SCOBYs were then inoculated in pre-made black tea solutions and grown at room temperature (20–24 °C) in glass jars (Fig. 1, b), covered with sterile gauze and fixed with a rubber band in a room with restricted access. In the following experiments, the daughter cultures of the SCOBYs from the previous test series (TS) were used. Before inoculation into the sugared tea beverage, an additional 10 g piece of the daughter SCOBY was taken for microbiological analysis.

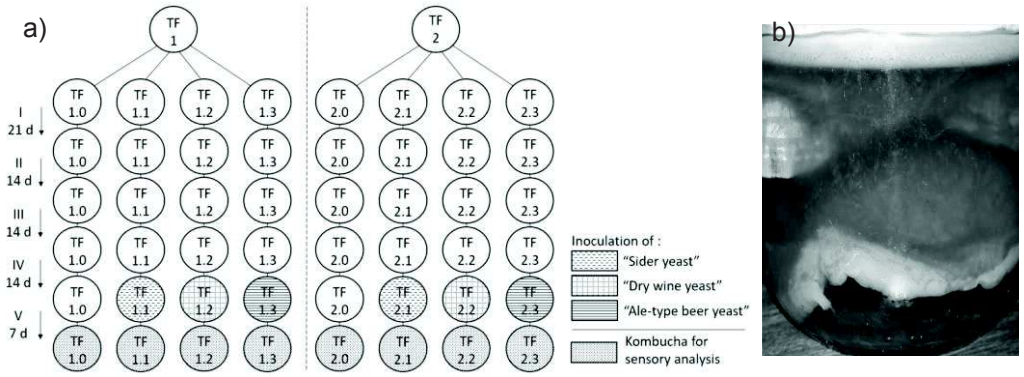

Figure 1. a) Scheme of SCOBY sampling through the five test series of fermentation. TF-SCOBY; TF 1 and TF 2 – 'mother' SCOBYs previously grown in green or black tea, respectively; TF 1.0–1.3 and TF 2.0–2.3 – daughter SCOBYs originating either from TF 1 or TF 2, respectively, I–V – five test series, d – fermentation time in days.b) Photo of SCOBY growing in sugared black tea, taken by M. Abel.

The fermentation time varied through the different test series. Initially it was 21 days in the first test series for initial growth activation and then 14 days in the next three experiments that were performed for growth stabilization of SCOBYs. Shortening the duration of the fermentation process to 7 days for the final (V) test series prior to sensory analysis was important as the acidity levels of the Kombucha drink may become potentially harmful for consumers if its fermentation lasts more than 10 days (Nummer, 2013).

Measuring the pH

During all experiments, the pH value was measured in sugared tea solutions before fermentation and in Kombucha (TFL 1.0–1.3 and 2.0–2.3, TFL referring to 'tea fungus liquid' according to the TF that was grown in it) after fermentation periods of 21, 14 or 7 days with a pH meter (SevenGo pro, Mettler Toledo, Switzerland).

Addition of starter cultures for simultaneous Kombucha fermentation

At the beginning of the last (V) test series, *S. cerevisiae* starter cultures were added to the sugared black tea solutions in duplicates (Table 1, Fig. 1, a) together with the SCOBY according to instructions by the manufacturer. 1 g of yeasts were previously dissolved in sterile distilled water, pre-incubated and added to the black tea.

Yeast cultures were not added to samples TF 1.0 and TF 2.0 that remained control samples.

Table 1. *S. cerevisiae* starter cultures used for simultaneous Kombucha fermentation

Starter culture	Pre-incubation*	Samples
Yeast for making dry cider 'Ciderini dry'	20 min, 25 °C	TF 1.1/ TF 2.1
Yeast for making dry wine 'Enovini'	20 min, 30 °C	TF 1.2/ TF 2.2
Yeast for making ale-type beer 'BrewGo- O2'	10 min, 35 °C	TF 1.3/ TF 2.3

*Pre-incubation conditions of yeasts were conducted according to manufacturer's instructions.

Sensory analysis of Kombucha

Sensory analysis of Kombucha fermented for 7 days (containing wine, beer and cider yeast starter cultures as well as control samples) was carried out at the end of the last (V) test series. Ten randomly selected untrained assessors took part in the sensory analysis.

20 mL of each sample (TFL 1.0, TFL 1.1, TFL 1.2, TFL 1.3, TFL 2.0, TFL 2.1, TFL 2.2, and TFL 2.3) was given for each assessor. During the sensory analysis appearance, aromatic and taste properties, consistency and acceptability of the samples were assessed.

A descriptive method was used for the sensory analysis. The properties to be evaluated were selected by the authors through external observation, prior tasting and based on literature sources (Gramza-Michałowska et al., 2016; Neffe-Skocińska et al., 2017). During the evaluation, the assessors had the opportunity to point out additional tastes and aromas of each Kombucha beverage under review and graded predetermined properties of a beverage on an unstructured linear scale with a length of 10 cm. The grades on the linear scale ranged from 0 to 10 with 0 being the lowest and 10 being the highest grade. One point on the linear scale corresponded to 1 cm. The intensity of colour, aroma and taste was rated on a scale of not perceptible to intense. Flocculation and gaseousness were evaluated based on the presence of flakes and bubbles in the liquid. The acceptability of the beverages was evaluated on a scale 0–5 where 0 was considered as 'unfit for consumption' and 5 was considered as 'very pleasant' The evaluation results were averaged for each of the eight samples.

Enumeration of bacteria and yeasts

For the microbial analysis of Kombucha, five ten-fold serial dilutions of a 1 mL sample were performed in 9 mL of 0.1% sterile peptone water. For the microbial analysis of SCOBY, a 10 g sample was placed in a sterile plastic bag with 90 mL of 0.1% sterile peptone water. The samples were then homogenized in a Stomacher®400 Circulator (Seward Ltd., England) at 300 rpm for 10 min, after which the ten-fold serial dilutions up to 10^{-7} were finally prepared.

In order to enumerate the total viable count of bacteria and yeasts, 1 mL of dilutions were pour plated in duplicates in either PCA (Milk Plate Count Agar LAB 115, LabM Ltd., England) or in SDA (Sabouraud Dextrose Agar, Biolife, Italy), respectively. The PCA plates were then incubated for 72 h at 30 °C and SDA plates for 5 days at 25 °C. After the incubation, the colonies on the growth media were counted, averaged and transformed into $\log_{10}$-scale, displayed as log CFU mL^{-1} (or g^{-1}).

Additionally, the decimal dilutions of Kombucha and SCOBY were spread plated using a sterile 10 µl inoculation loop on Violet Red Bile Agar LAB 31 (LabM Ltd., England) followed by an incubation for 72 hours at 37 °C for the inspection of the presence of *coli*-like bacteria.

Statistical analysis

Microsoft Office Excel 2013 was used to analyse the data collected during the experiments. To analyse variations in the observed parameters among the samples ANOVA (Analysis of Variance) and Student's *t-test* were used, where a *P* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The pH value of Kombucha

Monitoring the pH value of Kombucha during the fermentation process is important, because if the pH has not reached ≤ 4.2 within 7 days, the Kombucha is either contaminated or the fermentation temperature is too low (Nummer, 2013). In both cases, the fermentation process must be restarted with a new SCOBY. If the pH of Kombucha drops below 2.5, it is unsafe for consumers, because long-term fermentation will raise the amount of acetic acid to a level which may adversely affect their health (Nummer, 2013). pH values of all Kombucha samples tested during experiments (Table 2) were safe for consuming, however.

Table 2. pH of Kombucha during five series of fermentation

TS (d)*	1.0	1.1	1.2	1.3	Mean[1]	2.0	2.1	2.2	2.3	Mean[2]
I (21)	2.66	2.67	2.86	2.71	2.73 ± 0.08	2.88	2.84	2.79	3.01	2.88 ± 0.08
II (14)	2.79	3	2.93	2.89	2.90 ± 0.08	2.69	2.82	2.83	2.73	2.77 ± 0.06
III (14)	2.91	2.8	2.84	2.84	2.85 ± 0.04	2.87	2.91	2.82	2.94	2.89 ± 0.05
IV (14)	2.88	2.94	3.05	2.79	2.92 ± 0.09	3.03	2.96	2.95	3.03	2.99 ± 0.04
V (7)	3.03	3.05	3.02	3.07	3.04 ± 0.02	3.39	3.17	3.06	3.12	3.19 ± 0.12

*test series (duration in days); [1]averaged values of TFL 1.0–1.3 Kombucha samples, originating from TF 1 'mother' SCOBY ± standard deviation; [2]averaged values of TFL 2.0–2.3 Kombucha samples, originating from TF 2 'mother' SCOBY ± standard deviation.

The average pH of the initial sugared black tea solutions was 6.77, which is consistent with earlier scientific studies where their pH ranged from 6.62–7.8 (Blanc, 1996; Lončar et al., 2000; Velićanski et al., 2013; Gramza-Michałowska et al., 2016). The pH of the fermented solution, Kombucha, in the current study was 2.66–3.39 which in turn is in good concordance with previously reported pH ranges of 2.2–3.4 (Teoh et al., 2004; Gramza-Michałowska et al., 2016; Coton et al., 2017).

The analysis of the results showed that the history of previous growth conditions of SCOBY (samples TF 1.0–1.3 vs samples TF 2.0–2.3 originally grown in sweetened green or black tea, respectively) did not have a significant effect on the overall pH of the fermented solution ($P > 0.05$). Comparison of the pH differences of samples TFL 1.0–1.3 and TFL 2.0–2.3 in different test series revealed that the pH of samples TFL 2.0–2.3 in TS II was statistically significantly lower than that of samples TFL 1.0–1.3 ($P < 0.05$). There were no statistically significant differences ($P > 0.05$) on the pH of Kombucha in the remaining test series, including the last fermentation (TS V) where the yeast starter cultures were included, however. Compared to other samples where the starter cultures were added, the pH was lowest in samples that contained wine yeast starter cultures, i.e. samples TFL 1.2 and TFL 2.2 (for which the pH was 3.02 and 3.06 respectively).

Similar to previous studies by Lončar et al. (2000), Velićanski et al. (2013) and Gramza-Michałowska et al. (2016), the pH of the Kombucha was found to be most affected by the length of time of the fermentation - the longer the fermentation time (7 *vs* 14 *vs* 21 days), the lower the pH value ($P < 0.001$).

Microbial dynamics in SCOBY and Kombucha

As SCOBYs used in experiments were of different origin, the first four fermentation series were conducted to stabilize the growth of SCOBYs. It was important to observe whether and how the SCOBY previously grown in green tea would adapt to the new growth conditions in black tea. To monitor the change in microbial loads over time, the total viable count (TVC) of bacteria and yeasts was determined in all Kombucha ($n = 48$) and SCOBY ($n = 48$) samples used during the experiments. An additional goal was to observe how inoculation with yeast starter cultures affected the microbial growth in SCOBY and Kombucha during the last test series.

The initial values of bacteria in two SCOBYs before fermentation experiments were 7.15 log CFU g^{-1} in 'mother' TF 1 and 5.53 log CFU g^{-1} in 'mother' TF 2. There was a statistically significant ($P < 0.05$) decrease in bacterial loads in the two following fermentation series in samples taken from TF 1 (TF 1.0–1.3 in TS I and II) possibly due to the stress caused by the replacement of the green tea with the black tea (Table 3). The number of bacteria in this SCOBY line was back to the initial level (6.15–7.26 log CFU g^{-1}) by the end of the 3rd fermentation in sugared black tea solution. Such dynamics in bacterial counts was not seen in TF 2.0–2.3 samples and neither in TVC of yeasts where the initial numbers in TF 1 and TF 2 were 5.2 and 6.11 log CFU g^{-1}, respectively. Overall, the TVCs of microbes in SCOBY during the various stages of the fermentation process were similar with bacteria ranging between 5.28–8.81 log CFU g^{-1} and yeasts ranging between 5.08-8.04 log CFU g^{-1}. Our results were somewhat lower for bacteria, but well within the range of previous findings in which the counts of bacteria and yeasts in SCOBY were 7.90–9.11 log CFU g^{-1} (Coton et al., 2017) and 6.32–7.40 log CFU g^{-1} (Chen & Liu, 2001), respectively.

Table 3. Number of bacteria (B) and yeasts (Y) in SCOBY samples (TF 1.0–2.3) during five fermentation test series (I-V)

Sample	Microbes	Total viable count (log CFU g^{-1})*				
		I	II	III	IV	V
TF 1.0	B	5.43	5.80	7.26	7.18	6.68
	Y	5.62	5.56	7.11	7.20	6.65
TF 1.1	B	5.69	6.53	7.20	< 5.48	6.57
	Y	5.66	6.65	7.15	< 5.48	6.38
TF 1.2	B	5.90	5.98	5.83	7.72	7.95
	Y	5.92	5.82	5.74	7.73	7.85
TF 1.3	B	5.28	6.36	6.15	6.52	6.99
	Y	5.08	6.28	6.15	6.57	7.00
TF 2.0	B	6.68	7.23	5.72	6.04	8.11
	Y	5.65	7.18	5.75	5.91	8.04
TF 2.1	B	7.61	6.89	6.66	8.04	6.89
	Y	7.66	6.92	7.63	7.99	6.83
TF 2.2	B	5.72	6.15	6.86	7.61	8.81
	Y	5.58	6.08	6.88	7.64	7.92
TF 2.3	B	5.90	6.59	< 5.48	5.80	7.11
	Y	5.90	6.52	< 5.48	5.82	6.94

*values < 5.48 refer to possible errors during the cultivation and were excluded from the analysis.

Interestingly, there was no statistically significant difference between the total bacterial counts of samples TF 1.0–1.3 when compared to the total bacterial counts of samples TF 2.0–2.3 in the respective test series, suggesting that the SCOBY, previously grown in green tea (TF 1.0–1.3), adapted rapidly to the new growth conditions. Yet, there was a significant variance ($P < 0.05$) in the counts of bacteria and yeasts that grew slowly with each fermentation and was most significant after the final fermentation, however. Nevertheless, there was no statistical difference ($P > 0.05$) in counts between the last two fermentations. This could be explained with either the effect of added yeast starter cultures or alternatively with the shorter fermentation period at the last fermentation.

In Kombucha the TVC of bacteria and yeasts was, like in SCOBY samples, similar to each other: 3.56–6.26 and 3.72–6.08 log CFU mL^{-1}, respectively (Table 4). In previous studies the TVC of bacteria and yeasts in Kombucha has been higher, ranging between 4.11–7.90 log CFU mL^{-1} in bacterial (Jayabalan et al., 2007; Coton et al., 2017) and 5.97–7.90 log CFU mL^{-1} in yeasts counts (Chen & Liu, 2001).

Table 4. Number of bacteria (B) and yeasts (Y) in Kombucha samples (TFL 1.0–2.3) during five fermentation experiments (I-V)

Sample	Microbes	Total viable count (log CFU mL^{-1})				
		I	II	III	IV	V
TFL 1.0	B	3.75	5.00	5.15	5.08	5.51
	Y	3.72	4.93	5.11	4.95	5.52
TFL 1.1	B	4.64	5.43	4.65	4.76	6.23
	Y	4.63	5.51	4.64	4.72	6.08
TFL 1.2	B	4.57	4.48	4.04	5.69	> 5.48
	Y	4.48	4.52	4.00	5.67	> 5.48
TFL 1.3	B	4.04	6.26	4.71	4.61	6.15
	Y	3.96	5.36	4.79	4.61	6.04
TFL 2.0	B	4.69	5.30	4.11	3.56	5.60
	Y	4.59	5.26	4.66	3.97	5.32
TFL 2.1	B	4.41	4.78	4.72	5.18	6.04
	Y	4.57	4.88	5.04	5.15	5.80
TFL 2.2	B	4.64	5.08	5.11	5.15	> 5.48
	Y	4.61	4.11	5.49	5.18	> 5.48
TFL 2.3	B	4.65	4.75	4.48	4.18	4.32
	Y	4.71	4.71	4.81	4.32	4.49

*values > 5.48 refer to possible errors during the cultivation and were excluded from the analysis.

As in SCOBY samples, the dynamics of microbial counts during the five test series in Kombucha (TFL) 1.0–1.3 and 2.0–2.3 samples was similar ($P > 0.05$). It was also noted by Kaewkod et al. (2019) that the total counts of bacteria and yeast cells in Kombucha prepared from either green or black tea were not significantly different.

There was a significant increase ($P < 0.05$) in microbial loads in the Kombucha samples when yeast starter cultures were added when compared to the previous two (TS III and IV) and the first (TS I) fermentation. In addition, there was a statistically significant increase ($P < 0.05$) in counts of bacteria and yeasts at the end of the second fermentation. Again, these results could be explained by the effect of the shortened

fermentation time from 21 to 14 and then to 7 days and/or with the simultaneous fermentation with added yeast cultures.

Sensory analysis of Kombucha

In this study the effect of different *S. cerevisiae* starter cultures on the organoleptic properties of Kombucha was evaluated by sensory analysis. For that purpose, first initial four fermentations were carried out to stabilize the entire fermentation process by giving time for SCOBY, obtained from different households, to adapt to the new growth environment and treatments. To ensure the safety of the assessors during the consumption of Kombucha, absence of *coli*-like bacteria both in Kombucha and in SCOBY was tested prior to the first and then for the two last fermentation(s). The commercial yeast cultures were added together with SCOBY to the black tea solutions to start the 5th fermentation experiment (TS V). To our knowledge, Kombucha fermentation has not yet been carried out in this way before. To improve the probiotic properties of Kombucha, lactic acid bacteria have been added to the black tea solution during the first fermentation with SCOBY (Nguyen et al., 2015; Cvetković et al., 2019); the flavour of the Kombucha has also been improved by means of the second fermentation.

During the sensory analysis, the appearance, taste, aroma and overall acceptability of the Kombucha drinks were evaluated. The results show that yeasts added to the black tea solutions had different effects on the properties of the Kombucha. The characteristics of the taste were most influenced by the different yeasts. Samples TFL 1.2 and TFL 2.2 to which wine yeast was added, had the most vinegary and sour taste ($P < 0.05$). This sensory result was supported by their low pH values (pH was 3.02 and 3.06 respectively). In contrast, samples TFL 1.3 and TFL 2.3, to which brewer's yeast was added, had a much sweeter taste than the rest of the samples (with the exception of sample TFL 2.0, which was assessed to be the sweetest sample) ($P < 0.05$) and thus a less sour and vinegary taste than the samples with cider and wine yeasts (TFL samples 1.1, 2.1 and 1.2, 2.2) ($P < 0.05$).

Most of the beverages tested had sweet, sour and vinegary taste (Fig. 2). Traces of mould and a slight bitterness were also perceived in all of the samples. Honey flavours were also detected. Previously, Neffe-Skocińska et al. (2017) noted the taste of tea, lemon and sour tastes in their study, a sensory notion supported by Gramza-Michałowska et al. (2016), who noted that sweet, sour and citrus flavours were most evident in Kombucha. In addition, Gramza-Michałowska et al. (2016) also found that their Kombucha samples had a slight taste of tea and beer.

The overall acceptability of Kombucha (as part of the sensory evaluation) was considered low (in average ± SD, the rating was 2.19 ± 1.44) (Fig. 2). Most of the samples (27.9%) were evaluated with a grade 1 (on a scale of 0–5). The most acceptable Kombucha beverage was sample TFL 1.3 to which brewer's yeast was added ($P < 0.05$, but the difference was not statistically significant when compared to samples TFL 2.0 and 2.3), while TFL samples 1.2 and 2.2, to which wine yeast was added, had the lowest acceptability ($P < 0.05$). Samples TFL 1.2 and TFL 2.2 were described as being too sour and vinegary in taste. Gramza-Michałowska et al. (2016) found that traditionally fermented Kombucha samples evaluated in their experiment were of moderate acceptability.

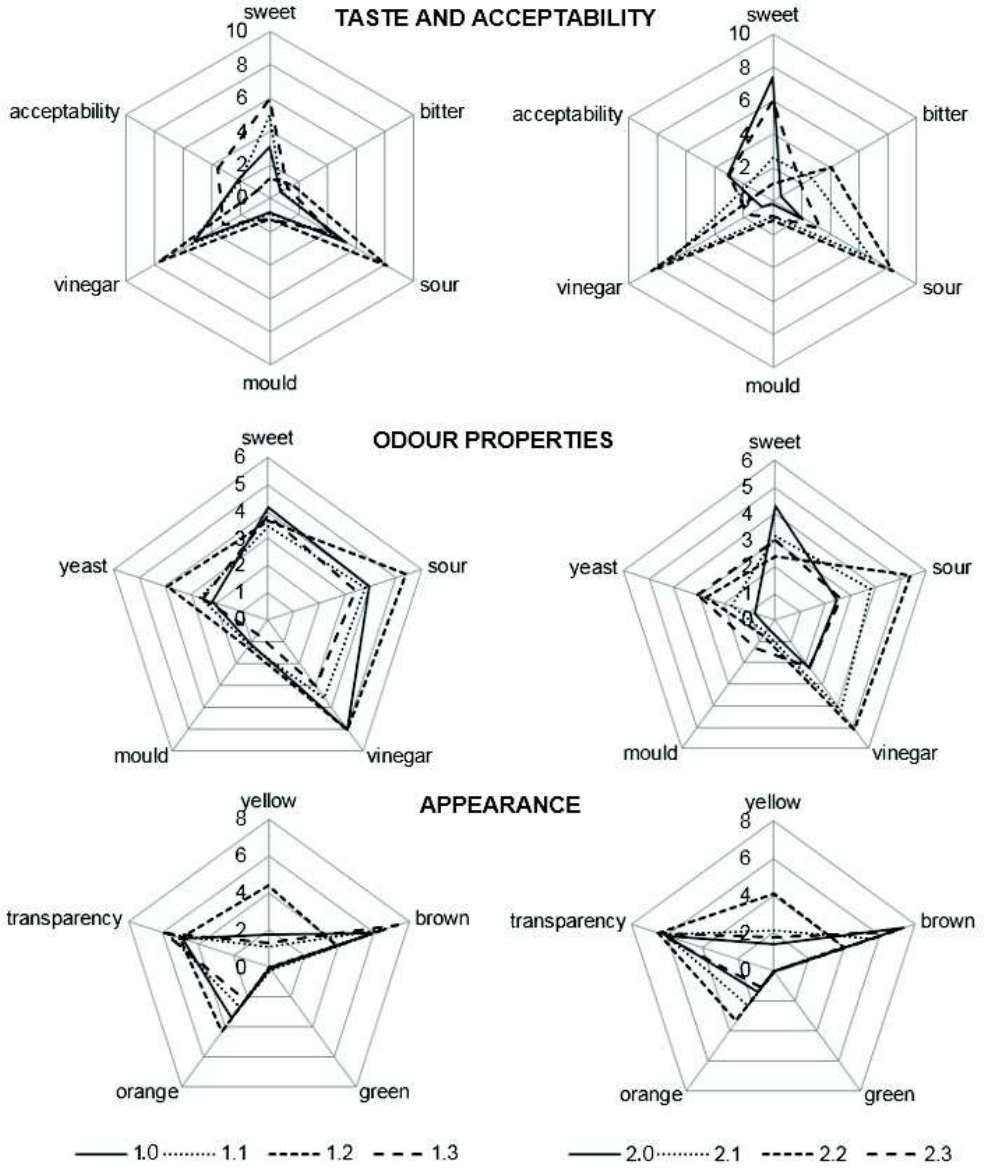

Figure 2. Taste and acceptability, odour properties and appearance of Kombucha samples. TFL 1.0 & 2.0 (control samples), TFL 1.1 & 2.1 (added cider yeast), TFL 1.2 & 2.2 (added wine yeast) and TFL 1.3 & 2.3 (added brewer's yeast). Kombucha drinks in first column originate from SCOBY previously (pre experiments) grown in sugared green tea (TF 1) and drinks in second column originate from SCOBY always grown in sugared black tea (TF 2).

Sweet, sour and vinegar scents were the most noticeable fragrances (Fig. 2). In addition, the smell of yeast and apple were also perceived. In earlier studies, the most commonly perceived scents were those of tea, lemon, sour, acetic acid, yeast and beer (Gramza-Michałowska et al., 2016; Neffe-Skocińska et al., 2017).

All samples evaluated showed medium transparency (Fig. 2). The dominant colour of all the samples was brown ($P < 0.05$), but orange and yellow were also perceived. An earlier study also found Kombucha to be brown in colour with amber and yellow (Gramza-Michałowska et al., 2016). The results show that added yeasts did not affect the external properties of the solutions. All samples to which *S. cerevisiae* was added (TFL 1.1, TFL 1.2, TFL 1.3, TFL 2.1, TFL 2.2 and TFL 2.3) were similar in appearance to control samples (TF 1.0 and TFL 2.0).

CONCLUSION

The addition of commercial yeast cultures to Kombucha did not significantly affect the overall microbial count in SCOBY, but it affected the overall microbial count in Kombucha; however the length of time of the fermentation process might play a more essential role in this respect. Therefore, the interplay of different parameters during the simultaneous fermentation process should be further and more thoroughly studied. In this study it was shown that all added commercial yeast cultures modified the sensory properties of the individual Kombucha drink. Detailed research is further needed to explore the precise influence, the possible advantages as well as the optimal conditions for simultaneous inoculation of various microbial strains, including probiotics, to the Kombucha fermentation process.

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REFERENCES

Adzadogo, R.S. 2015. *Comparison of physicochemical properties and anti-microbial Activities of tea and cocoa-based kombucha.* University of Ghana. Department of Biochemistry, Cell and Molecular Biology. 60 pp. http://197.255.68.203/handle/123456789/21380

Al- Kalifawi, E.J. & Hassan, I.A. 2013. Factors influence on the yield of bacterial cellulose of Kombucha (Khubdat Humza). *Baghdad Science Journal* **11**, 1420–1428.

Blanc, P.J. 1996. Characterization of the tea fungus metabolites. *Biotechnology Letters*. **18**, 139–142.

Bleam, W., Goode, R., McKinney, D., Sitzman, B. & Tempes, R. 2016. Teacher's guide for Kombucha: something's brewing. *ChemMatters* 2–22.

Chen, C. & Liu, B. Y. 2001. Changes in major components of tea fungus metabolites during prolonged fermentation. *Journal of Applied Microbiology* **89**, 834–839.

Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G., Deniel, F., Coulloumme- Labarthe, L., Fall, A., Daube, G. & Coton, E. 2017. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiology Ecology* **93**.

Cvetković, D., Ranitović, A., Savić, D., Joković, N., Vidaković, A., Pezo, L. & Markov, S. 2019. Survival of wild strains of lactobacilli during Kombucha fermentation and their contribution to functional characteristics of beverage. *Pol. J. Food Nutr. Sci.* **69**, 407–415.

Dogan, N.M., Beyatli, Y. & Aslim, B. 2002. A study on the production of poly-beta-hydroxybutyrate by some eukaryotic microorganisms. *Turkish Electronic Journal of Biotechnology*, pp. 11–17.

Dutta, H. & Paul, S.K. 2019. *Production and Management of Beverages*. Woodhead publishing, Elsevier, pp. 259–288.

Gramza-Michałowska, A., Kulczyński, B., Xindi, Y. & Gumienna, M. 2016. Research on the effect of culture time on the Kombucha tea beverage's antiradical capacity and sensory value. *Acta Sci. Pol. Technol. Aliment*. **15**, 447–457.

Greenwalt, C.J., Steinkraus, K.H. & Ledford, R.A. 2000. Kombucha, the fermented tea: microbiology, composition, and claimed health effects. *Journal of Food Protection* **63**, 976–981.

Jankovic, I. & Stojanovic, M. 1994. Microbial and chemical composition, growth, therapeutical and antimicrobial characteristics of tea fungus. Food and Agriculture Organization of the United Nations. *Mikrobiologija* **31**, 35–43.

Jayabalan, R., Chen, P.-N., Hsieh, Y.-S., Prabhakaran, K., Pitchai, P., Marimuthu, S., Thangaraj, P., Swaminathan, K. & Yun, S.E. 2011. Effect of solvent fractions of Kombucha tea on viability and invasiveness of cancer cells – Characterization of dimethyl 2-(2-hydroxy-2-methoxypropylidine) malonate and vitexin. *Indian Journal of Biotechnology* **10**, 75–82.

Jayabalan, R., Malbaša, R.V., Lončar, E.S., Vitas, J.S. & Sathishkumar, M. 2014. A review on Kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Comprehensive Reviews in Food Science and Food Safety* **13**, 538–550.

Jayabalan, R., Marimuthu, S. & Swaminathan, K. 2007. Changes in content of organic acids and tea polyphenols during Kombucha tea fermentation. *Food Chemistry*. **102**, 392–398.

Jayabalan, R., Sathishkumar, M., Malini, K. & Yun, S.- E. 2010. Biochemical characteristics of tea fungus produced during Kombucha fermentation. *Food Sci. Biotechnol.* **19**, 1–5.

Kaewkod, T., Bovonsombut, S. & Tragoolpua, Y. 2019. Efficacy of Kombucha obtained from green, oolong, and black teas on inhibition of pathogenic bacteria, antioxidation, and toxicity on colorectal cancer cell line. *Microorganisms.* **7**, 700.

Kozaki, M., Kitahara, K & Koizumi, A. 1972. Microorganisms of Zoogloeal Mats Formed in Tea Decoction. *Food Hygiene and Safety Science* **1**, 89–96.

Kumar, V. & Joshi, V. 2016. Kombucha: technology, microbiology, production, composition and therapeutic value. *Intl. J. Food. Ferment. Technol.* **6**, 13–24.

Leal, J.M., Suárez, L.V., Jayabalan, R., Oros, J.H. & Escalante-Aburto, A. 2018. A review on health benefits of Kombucha nutritional compounds and metabolites. *CyTA Journal of Food* **16**, 390–399.

Lončar, E.S., Petrović, S.E., Malbaša, R.V. & Verac, R.M. 2000. Biosynthesis of glucuronic acid by means of tea fungus. *Nahrung*. **44**, 138–139.

Malbaša, R.V., Lončar, E.S. & Kolarov, A. 2002. Tea fungus fermentation on a substrate with iron (II)- ions. *Acta periodica technologica* **33**, 143–149.

Malbaša, R.V., Lončar, E.S., Djurić, M.S., Kolarov, L.A. & Klašnja, M. T. 2005. Batch fermentation of black tea by Kombucha: a contribution to scale-up. *APTEFF*. **36**, 221–229.

Malbaša, R.V., Lončar, E.S., Vitas, J.S. & Čanadanović-Brunet, J.M. 2011. Influence of starter cultures on the antioxydant activity of kombucha beverage. *Food Chemistry* **127**, 1727–1731.

Markov, S.L., Malbaša, R.V., Hauk, M.J. & Cvetković, D.D. 2001. Investigation of tea fungus microbe assotiations. I. The yeasts. *Acta periodica technologica.* **32**, 133–138.

Markov, S., Svetiković & Bukvić, B. 2006. Use of tea fungus isolate as starter culture for obtaining of kombucha. A*nnals of the faculty of engineering hunedoara* **3**, 73–78.

Mukadam, T.A., Punjabi, K., Deshpande, S.D., Vaidaya, S.P. & Chowdhary, A.S. 2016. Isolation and characterization of bacteria and yeast from Kombucha tea. *International Journal of Current Microbiology and Applied Sciences* **5**, 32–4.

Neffe-Skocińska, K, Sionek, B., Ścibisz, I. & Kołoźyn-Krajewska, D. 2017. Acid contents and the effect of fermentation condition of Kombucha tea beverages on physicochemical, microbiological and sensory properties. *CyTA-Journal of Food* **15**, 601–607.

Nguyen, N.K., Dong, N.T.N., Nguyen, H. T. & Le, P.H. 2015. Lactic acid bacteria: promising supplements for enhancing the biological activities of Kombucha. *Springerplus.* **4**, 91.

Nummer, B.A. 2013. Kombucha brewing under the food and drug administration model food code: risk analysis and processing guidance. *Journal of Environmental Health* **76**, 8–11.

Reiss, J. 1994. Influence of different sugars on the metabolism of the tea fungus. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung* **198**, 258–261.

Reva, O.N., Zaets, I.E., Ovcharenko, L.P., Kukharenko, O.E., Shpylova, S.P., Podolich, O.V., Vera, J.-P.& Kozyrovska, N.O. 2015. Metabarcoding of the kombucha microbial community grown in different microenvironments. *AMB Express* **5**, 35.

Vázquez-Cabral, Z., SuI, Y., Wu, H., Zhou, C., Hu, X. & Zhang, J. 2018. Flavour chemical dynamics during fermentation of Kombucha tea. *Emirates Journal of Food and Agriculture* **30**, 732–741.

Teoh, A. L., Heard, G. & Cox, J. 2004. Yeast ecology of Kombucha fermentation. *International Journal of Food Microbiology* **95**, 119–126.

Vázquez-Cabral, B.D., Rocha-Guzmán, N.E., Gallegos-Infante, J.A., González-Herrera, S.M., González-Laredo, R.F., Moreno-Jiménez, M.R. & Córdova-Moreno, I.T.S. 2014. Chemical and sensory evaluation of a functional beverage obtained from infusions of oak leaves (Quercus resinosa) inoculated with the Kombucha consortium under different processing conditions. *Nutrafoods* **13**, 169–178.

Velićanski, A., Cvetović, D. & Markov, S. 2013. Characteristics of Kombucha fermentation on medicinal herbs from Lamiaceae family. *Romanian Biotechnological Letters* **18**, 8034–8042.

Watawana, M.I., Jayawardena, N., Gunawardhana, C.B. & Waisundara, V.Y. 2015. Health, Wellness, and Safety Aspects of the Consumption of Kombucha. *Journal of Chemistry*. 1–11.

Yapar, K., Cavusoglu, K., Oruc, E. & Yalcin, E. 2010. Protective effect of kombucha mushroom (KM) tea on phenol-induced cytotoxicity in albino mice. *Journal of Environmental Biology* **5**, 615–621.