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Comparison of hardaliye produced by different starters: Back-slopping and kombucha

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ABSTRACT

During traditional hardaliye production by fermentation from grape juice, mustard seeds are insufficient to inhibit yeast activities and alcohol formation. Chemical preservatives are used for the production of hardaliye of standard quality in traditional and industrial production. Today, consumers prefer natural products that do not contain chemical preservatives and additives. For this reason, in this study, considering that different production techniques should be tried in order to prevent alcohol formation, hardaliye production was carried out with two different methods as back-slopping (BH) and addition of kombucha mushrooms (KH). These methods were tried for the first time on hardaliye. Fermentation continued for 7 days (d) and storage for 14 d. During fermentation, pH and reducing sugar, L* and a* values of samples decreased, while phenolic compounds' concentration, viscosity, and b* values increased. The pH continued to drop during storage. No significant changes were observed in reducing sugar contents. During storage, phenolic content of KH sample decreased and viscosity and L* values increased. The titratable acidity increase was greater in BH sample compared to the KH sample. While 5.5% alcohol formation was observed in the BH sample on the 7th day of fermentation, no alcohol formation was detected in the KH sample At the end of fermentation and storage, Total mesophilic aerobic bacteria (TMAB), yeast, *Lactobacillus* spp. and lactic streptococci numbers were found to be higher in BH sample than in KH sample. According to the results of the research, thanks to the metabolic activities of the kombucha mushroom microorganisms and their symbiotic association, natural fermentation takes place without any preservative chemicals in KH and more durable hardaliye production is provided compared to BH.

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1. Introduction

There are two main fermentation methods in the fermentation of foods. The first is the spontaneous fermentation that occurs spontaneously without external intervention with the microorganisms present in the natural structure of the raw material or in the processed environment, as was the case in the period when fermentation was discovered. Another fermentation method is culture dependent fermentation and can be performed by adding a known starter culture (i) to the raw material. Fermented products such as kefir, kombucha and natto can be given as an example to this method (Rezac et al., 2018; Dimidi et al., 2019). Another way of culture-dependent fermentation is the back-slopping method (ii). Fermentation is initiated by taking a small amount of pre-fermented food and adding it to the raw material. For example, fermented products such as sourdough bread, beer, various cheeses, natto and tempeh can be produced by back-slopping method (Marco et al., 2017; Dimidi et al., 2019). This method is preferred over spontaneous fermentation to reduce fermentation time and minimize the risk of unsuccessful fermentation (Harris, 1998; Leroy and De Vuyst, 2004).

Hardaliye is a traditional beverage of the Thrace Region of Türkiye, produced from grape juice. It is a non-alcoholic, acrid, characteristic drink produced as a result of lactic acid fermentation with the addition of mustard seeds and cherry leaves to dark-colored and fragrant grapes (Faikoğlu, 2012). Mustard seeds are preferred to prevent yeast activities and alcohol fermentation during fermentation, and for the same purpose 0.1% benzoic acid is used. It has also been observed that 0.1% benzoic acid and sorbic acid mixtures are used in traditional productions. In addition to giving the local drink its name, mustard seeds also contribute to the unique taste and smell of hardaliye. The leaves of the cherry trees grown in the same region are used to give aroma to hardaliye (Aşkın and Atik, 2016; Aydogdu et al., 2016; Arici et al., 2017). In a study conducted by Arici et al. (2017) to determine lactic acid bacteria in hardaliye samples, it was determined that 23 isolates (46%) were *Lactobacillus plantarum*, 20 isolates (40%) were *Lactobacillus pentosus*, 4 isolates (8%) were *Lactobacillus brevis* and the remaining 3 isolates (6%) were *Lactobacillus collinoides*.

Kombucha; It is an acidic and comforting beverage that is formed by fermenting sweetened tea with the addition of a cellulosic film layer that is formed as a result of the symbiotic association of yeast and bacteria. The cellulosic layer formed by yeast and bacteria is called SCOBY (Symbiotic Culture of Bacteria and Yeast) (Sreeramulu et al., 2000). The botanical nomenclature of kombucha was made by Lindau in 1965 as *Medusomyces gisevii* (Hesseltine, 1965). In the cellulosic film layer formed by the symbiotic

association of bacteria and yeasts of Kombucha mushroom, Gram-negative aerobic bacilli belonging to the *Acetobacteraceae* family (*Acetobacter xylinum, A. xylinoides, A. aceti, A. pasteurianus, Bacterium gluconicum* and *Gluconobacter oxydans*), yeasts (*Saccharomyces cerevisiae, S. ludwigii, Zygosaccharomyces bailii, Z. rouxii, Z. kombuchaensis* sp.*nov., Schizosaccharomyces pombe, Torulaspora delbrueckii, Brettanomyces bruxellensis, B. lambicus, B. custerii, Candida krusei, C. albicans, Kluyveromyces africanus, Pichia membranaefaciens, Kloeckera apiculata, Torulopsis* sp., *Dekkera* sp.) and lactic acid bacteria (*Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Bifidobacterium* sp.) were detected (Jarrell et al., 2000; Kurtzman et al., 2001; Goh et al., 2012; Velicanski et al., 2014). Bacteria in kombucha fermentation mainly produce acetic acid, gluconic acid and cellulose (Greenwalt et al., 2000). It has been reported that ethanol and acetic acid, which are produced as a result of the symbiotic association of yeasts and bacteria during fermentation, have antimicrobial activity against pathogenic bacteria (Liu et al., 1996). Today, kombucha has been tested with new substrates and some of the new substrates have been reported to stimulate fermentation better and complete fermentation in a shorter time compared to the original kombucha tea (Vitas et al., 2013).

In a study, it was determined that the number of lactic acid bacteria (LAB) and species diversity in the product produced by the back-slopping method was higher than in self-fermentation (Wirawati et al., 2019). The back-slopping method has positive effects on flavor and textural stability (Kim et al., 2018).

In the production of hardaliye, alcohol formation can be observed in the later stages of storage. In order to prevent this, hardaliye productions with different starters have been tried in recent years. In this study, physical, chemical and microbiological properties of hardaliye produced with different starters were investigated during production and storage. As a starter culture, kombucha mushrooms and back-slopping were used for the first time in the production of hardaliye.

2. Materials and methods

2.1. Materials

The research materials consist of hardaliye samples produced in the laboratory, using traditionally obtained hardaliye (back-slopping) and kombucha mushrooms as starters. The hardaliye obtained by the back-slopping method was named "BH", and the hardaliye produced by adding kombucha mushrooms was named "KH". Grape juice produced from Öküzgözü grapes used in production from Şarköy Mursallı Agricultural Development Cooperative, kombucha mushroom from Shaman's Secret A.Ş., mustard seeds and cherry leaves to be used in aromatization of hardaliye from Arpaş Arifoğlu Pazarlama Dağıtım ve Ticaret A.Ş., hardaliye which has completed the fermentation required in the production of traditional hardaliye by the back-slopping method was obtained from Karlıbağ Hardaliye A.Ş.

2.2. Methods

2.2.1. The production of hardaliye

Two different methods were used in the production of hardaliye. In two different methods, fermentation was continued until the pH of the hardaliye samples decreased to 3.10-3.25. Some physical, chemical and microbiological analyzes were applied to the produced hardaliye samples on the 0^{th} day when fermentation started, on the 7^{th} day when fermentation was completed, and on the 14^{th} day of storage at +4 ° C. It was added ground mustard seeds (1% of total weight of grape juice+hardaliye whose fermentation was completed or SCOBY) and cherry leaf (1% of total weight of grape juice+hardaliye whose fermentation was completed or SCOBY) to grape juice (90%). Then pasteurization was applied (72 °C, 20 min). After the cooling process (22-25 °C), hardaliye (10%), whose fermentation was completed, was added to the first sample where the first method will be applied. SCOBY (10%) was added to the sample where the second method will be applied. Fermentation of both samples took 7 d at 23 °C. After the samples were filtered using a 1 mm diameter mesh strainer, and then bottled in glass bottles. They were stored at 4°C for 14 d (Roussin, 1996).

2.2.2. Physical and chemical analyses

HANNA pH211 model pH meter was used to measure the pH of the samples (Wirawati et al., 2019). In the acidity analysis, 10 mL of the sample was taken, and 10 mL of distilled water was added. After the mixture became homogeneous, 0.5 mL of phenolphthalein indicator was added and titrated with 0.1 N NaOH until the pH reached 8.1 (Mbaeyi-Nwaoha and Ajumobi, 2015). Luff-Schoorl method was used to determine the reducing sugar content of hardaliye samples (Cemeroğlu, 2004). In alcohol analysis, 2.9 mL of Glycine buffer solution was poured into the tubes containing NAD, 0.1 mL of distilled water was placed on it and after 0.1 mL of sample was added, reading was taken at 340 nm in the spectrophotometer (Shimadzu Corporation UV- 1208, Japan) (Boehringer-Mannheim, 1989). The amount of ethanol (mg/dL) was calculated from the absorbance value read according to the formula below.

ABV- Control ABV= ΔA340
ΔA340 x 223=…..mg dL^{-1} ethanol
ABV= Absorbance Value

The color of the grape juice and hardaliye samples used in the production of hardaliye was determined using the Konica Minolta Chroma Meter CR-5 color measuring device. Hardaliye samples were placed in the glass container of the device and their readings were performed. The results were given as L*, a* and b* values (Utoiu et al., 2018). The total amount of phenolic substances was determined according to the method reported by Cemeroğlu (2007). The commonly used Folin-Ciocalteu reagent was used to calculate the total phenolic content. Total phenolic content was calculated as gallic acid equivalent (GAE) using the gallic acid calibration curve (Cemeroğlu, 2007). Viscosity analysis of grape juice and hardaliye samples was carried out using TA Discovery HR-20 rheometer device. Viscosity values were expressed as Pa.s.

2.2.3. Microbiological analyses

The dilutions were prepared into tubes containing 9 mL of 0.85% sterile physiological water and 0.1 mL of the dilutions

were dispersed on the surface of agar plate using the spread-plate method. For total aerobic mesophilic bacteria count, PCA Agar medium was used and the colonies (cfu mL^{-1}) formed after 48 h of incubation at 30 °C were counted (Temiz, 2002). PDA Agar medium was used for total yeast and mold count, incubation lasted 5 d at 25 °C (Bergmann et al., 2010; Maturin and James 2001; Tournas et al., 2001). Mold growth was observed morphologically. For *Lactobacillus* spp. enumeration, MRS Agar medium was used and incubation was continued at 37 ° C under anaerobic conditions for 72 h (De Man et al., 1960; Bergmann et al., 2010) M17 Agar medium was used for lactic streptococci counting, incubation continued for 72 h in aerobic environment at 37 °C (Bergmann et al., 2010).

2.2.4. Statistical analysis

Hardaliye samples were analyzed in triplicate. The results obtained were evaluated by using the JMP 5.0.1 (SAS Institute) program and applying binary ANOVA analysis. Significant differences between results were determined by Tukey's multiple comparison test, with a grade of $P<0.05$.

3. Results and discussions

3.1. Physicochemical properties

During the fermentation, the pH decrease was 0.27 unit in hardaliye produced by back slopping method and 0.21 unit in hardaliye produced with kombucha mushrooms ($P<0.05$). The decrease during storage was less than during fermentation ($P<0.05$). Ayed et al., (2017) stated in their research that the pH values decreased during the fermentation of grape juice with kombucha mushrooms. While the pH value was 3.95 on the first day of that study, it was measured as 3.18 on the 6th day. It is seen that the values obtained in that study are similar to the values of the KH samples in this study. In the study of Coşkun et al. (2018), the pH values of hardaliye produced from different grape varieties varied between 3.33 and 3.73 at the end of fermentation. In this study, the pH decreases were higher than in the study of Coşkun et al. (2018) because benzoic acid or sorbic acid was not used as a preservative in hardaliye produced by the back slopping method. Mustard seeds do not show sufficient effect in preventing alcohol formation. In order to prevent the formation of alcohol, preservatives must be used together with mustard seeds. When the results of the study of blueberry tea carried out using sucrose, glucose, fructose carbon sources are examined; pH values were measured as 3.30, 3.27 and 3.09, respectively on the 0th day of fermentation, and as 3.01, 2.94 and 2.91 on the 8th day, respectively (Tarhan, 2017). Based on these studies (Arici and Coskun, 2001; Tarhan, 2017), it is thought that the acidity development ability of kombucha culture is similar to hardaliye.

Although there is a partial relationship between pH and total acidity, the degree of this relationship varies according to the type of acid formed (Amerine et al., 1965). While pH is important for assessing the ability of a microorganism to grow in a particular food, titratable acidity is a better indicator than pH of how organic acids in the food affect flavor (Tyl and Sadler, 2017). The total acidity of the BH sample increased during production and storage. In the KH sample, there was no increase in the total acidity value during fermentation, but an increase was observed during storage (Figure 1). In a study conducted without using preservatives; in the sample using 1% mustard seeds and starter culture *(L. plantarum*), the total acidity (as tartaric acid) value on the 7th day of fermentation was measured as 6.10 g L^{-1} (Gürbüz, 2018). It is seen that the values are close to the total acidity (as tartaric acid) value on the 7th day of the BH sample in this study.

The amount of reducing sugar in hardaliye samples decreased with fermentation and increased slightly during storage (Table 1). Arici and Coskun (2001) reported that these reductions in reducing sugar during fermentation are due to the microorganisms in the environment using reducing sugar as a substrate and breaking it down into lactic acid, ethyl alcohol, CO_2 and some other organic acids. The increase in the amount of reducing sugar in Hardaliye samples on the 14th day of storage was associated with the release of sugar bound to anthocyanidins as a result of the degradation of anthocyanins, as stated by Coskun et al. (2012). It was determined that the amount of reducing sugar in the KH sample at the end of the fermentation (7th day) as in the hardaliyes (17.5-16.88%) produced by Coskun et al. (2012). The amount of reducing sugar on the 7th day of the BH sample in this study seems to be close to the values of hardaliye (8.81 g L^{-1}-10.91 g L^{-1}) produced by Faikoğlu (2012) with different grape varieties.

Alcohol was not detected at the beginning of Hardaliye production (Table 1). While 5.5% alcohol formation was observed at the end of fermentation (7th day) in the BH sample, no alcohol formation was observed in the KH sample during production and storage. Alcohol could not be detected in hardaliye samples produced in laboratory by Coşkun et al. (2018). Researchers detected alcohol (maximum 6%) in hardaliye samples collected from the people of Kırklareli, as they may be in the advanced stages of storage. Sometimes producers prefer producing hardaliye containing some alcohol and do not use chemical preservatives other than mustard during the first few days of fermentation. It is seen that the alcohol content of those samples is close to the amount of alcohol on the 7th day of the BH sample in this study. In the study of Ayed et al. (2017), changes in ethanol were observed during kombucha fermentation in grape juice. In that study, the amount of ethanol was measured as 0.52 g 100 mL^{-1} on the 6th day, and 0.29 g 100 mL^{-1} on the 12th day. In the study of Tarhan (2017), as a result of kombucha fermentation carried out by using different carbon sources in coffee and various herbal and fruit teas, alcohol was not detected as in the KH sample in our study. This has been associated with the conversion of ethanol produced by yeasts to acetic acid, where the main thing in kombucha fermentation is acetic acid fermentation.

In the BH sample, while a rapid decrease is observed in the amount of reducing sugar during fermentation, there is a simultaneous increase in the amount of alcohol. This situation can be explained as the microorganisms in the environment using reducing sugar as a substrate and breaking it down into lactic acid, ethyl alcohol, CO_2 and some other organic acids (Arici and Coşkun, 2001). Alcohol could not be detected in the KH sample during production and storage.

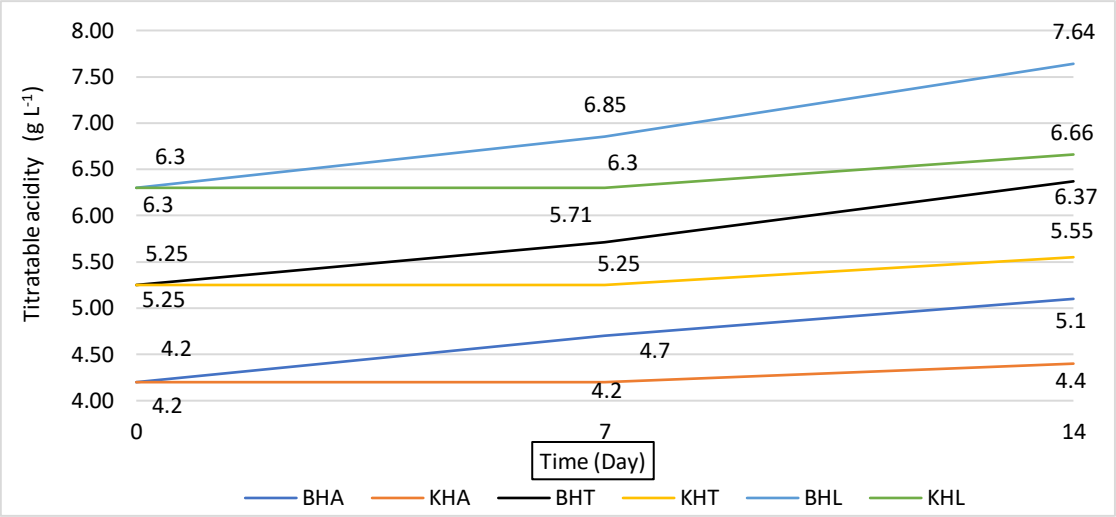

Figure 1. Titratable acidity (g L^{-1}) values of BH and KH samples. *BHA:BH acetic acid, KHA: KH acetic acid, BHT:BH tartaric acid, KHT: KH tartaric acid, BHL: BH lactic acid, KHL: KHL lactic acid*

This can be explained as a symbiotic association of yeasts and acetic acid bacteria found in kombucha mushrooms. While glucose in grape juice is converted to gluconic acid by acetic acid bacteria in the environment, fructose is converted to ethanol by yeasts. Ethanol produced by yeast is converted to acetic acid by acetic acid bacteria (Arıkan, 2018). Acetic acid bacteria (AAB) are well-known microorganisms found in fruits such as grapes (Valera et al., 2011). Since the alcohol formed in hardaliye is broken down by acetic acid bacteria and the conversion of hardaliye into vinegar is in the later stages of storage, it was thought that the number of AABs transferred from grapes to mustard is less than that of kombucha. According to the Turkish Food Codex Communiqué on Non-Alcoholic Beverages (Communiqué No: 2007/26), it is stated that the amount of ethyl alcohol that may arise from the nature of production in the beverages covered by the Communiqué should be at most 3.0 g L^{-1} (approximately 0.4% v/v) (TGK 2007). In this study, the alcohol content of the BH sample is well above the specified limit. In this respect, it is very important to prevent the formation of alcohol during the fermentation of hardaliye.

The highest amounts of phenolic substances were determined as 792.33 mg GAE L^{-1} in BH sample and 830.19 mg GAE L^{-1} in KH sample on the 7th day of fermentation ($P<0.05$). The phenolic content of hardaliye samples stored at +4 ° C for 14 days decreased by 7.6% in BH samples and 6.6% in KH samples ($P<0.05$) (Table 1). Total phenolic content was determined in the range of 368-2727 mg L^{-1} in twenty-three hardaliye samples collected from local people by Coşkun et al. (2018).

In the study of Gündüz et al. (2019), the total phenolic content of grape juice was determined as 1515.27 mg L^{-1}. In that study, the total phenolic content of hardaliye produced by fermenting grape juice was determined as 2029.20 mg GAE L^{-1} in homemade hardaliye and 2193.08 mg GAE L^{-1} in commercial hardaliye. As a result of the processing of grape juice into hardaliye, the total amount of phenolic compounds increased significantly. In this study, an increase of 6.7% in the BH sample and 11.7% in the KH sample was observed in the total amount of phenolic substances with fermentation (Table 1). In a study examining the changing parameters of grape juice during fermentation with kombucha, it was observed that 6 d after the start of fermentation, the total phenolic content of grape juice increased by 40% with fermentation (Ayed et al., 2017). *Gluconobacter* has been identified as a key bacterial species that increases the bioavailability of polyphenols and the antioxidant activity of beverages (Dufresne and Farnworth 2000). However, in Ayed et al. (2017), a slight decrease in phenolic content was observed from the 10th d. This phenomenon can be explained by the polymerization of some phenolic compounds into higher molecular weight molecules, which leads to the detection of lower polyphenol content. A similar situation was observed in this study as well. Phenolic substances can be hydrolyzed by the microflora (Ozcan et al., 2021). The difference in the amount of phenolic substances in KH and BH samples during fermentation and storage can be explained by the research they have done by Çam and Yıldırım (2018). In that research; they suggested that the selection of appropriate cultures for fermentation may affect the phenolic profile and phenolic substance content of the product formed at the end of fermentation, that mixed cultures should be used to obtain a good phenolic profile, and that the phenolic profile of the product can be controlled by using different cultures.

The desired red color in grape juices is due to the presence of anthocyanins. Anthocyanin content of grapes varies depending on many factors such as grape variety, maturity, harvest year, environmental conditions, etc. (Mazza and Francis, 1995). The color values of the hardaliye samples in this study are given in Table 1. It was determined that there were statistically significant losses in the brightness level of both hardaliye samples during the fermentation ($P<0.05$). While no significant change was observed in the BH sample during storage ($P>0.05$), the change in the KH sample was found to be statistically significant ($P<0.05$). It was thought that the increase in the microorganism counts during fermentation caused a decrease in the L* value by increasing the turbidity in the grape juice. A similar result was obtained in the study of Bayram et al. (2015). Watawana et al. (2016) suggested in their study with kombucha that the decrease in L* value was

due to the degradation of color pigments and polyphenolic components as a result of the decrease in pH due to fermentation and microorganism growth.

During the fermentation, a significant decrease of 3.67 units in the a* value of the BH sample and 9.63 units in the a* value of the KH sample was detected (P<0.05). Although there was an increase in the a* value of the KH sample and a decrease in the a* value of the BH sample during storage, these changes were not found to be statistically significant (P>0.05) (Table 1). When KH and BH samples were compared, the difference in a* values determined on the 7th day of production and 14th day of storage was found to be statistically significant (P<0.05). The color tone of the hardaliyes produced using red grape juice is red at the beginning and it is desired to have less yellow tone. On day 0, b* values were measured as 33.69. The differences between b* values of hardaliye samples during production and storage were significant (P<0.05). No significant change was observed in the b* value of the KH sample during production and storage (P>0.05). The highest b* value in the BH sample was measured as 36.57 on the 7th day, and then a slight decrease was observed during storage and was measured as 36.05 on the 14th day of storage. The changes in the b* value of the BH sample during production and storage were significant (P<0.05).

Table 1. Physicochemical properties of hardaliye samples during fermentation and storage.

Properties	Samples	Fermentation time (day)		Storage time(14th day)
		0	7	14
pH	BH	3.41 ± 0^{Aa}	3.14 ± 0.01^{Bb}	2.97 ± 0.16^{Ca}
	KH	3.41 ± 0^{Aa}	3.2 ± 0.01^{Ba}	3.00 ± 0.06^{Ca}
Reducing sugar (%)	BH	24.96 ± 1.59^{Aa}	7.93 ± 0.32^{Bb}	8.16 ± 0.45^{Bb}
	KH	24.96 ± 1.59^{Aa}	$16.45\pm1,32^{Ba}$	16.8 ± 1.42^{Ba}
Ethyl alcohol (%)	BH	0 ± 0^{B1}	$5.5\pm0,3^{A}$	5.45 ± 0.24^{A}
	KH	0±0	0±0	0±0
Phenolic compound (mg GAE L^{-1})	BH	743.52 ± 1.79^{Ba}	792.33 ± 6.84^{Ab}	732.52 ± 6.28^{Cb}
	KH	743.52 ± 1.79^{Ca}	830.19 ± 7.36^{Aa}	775.15 ± 7.56^{Ba}
Viscosity (Pa.s)	BH	2.05 ± 0.02^{Ba}	3.82 ± 1.46^{Aa}	3.79 ± 1.28^{Ab}
	KH	2.05 ± 0.02^{Ca}	2.77 ± 0.055^{Ba}	5.62 ± 0.43^{Aa}
Color				
L*	BH	32.81 ± 0.61^{Aa}	26.04 ± 2.7^{Bb}	26.65 ± 2.32^{Bb}
	KH	32.81 ± 0.61^{Aa}	30.12 ± 2.44^{Ba}	33.76 ± 1.65^{Aa}
a*	BH	52.73 ± 0.10^{Aa}	49.06 ± 0.94^{Ba}	48.93 ± 0.93^{Ba}
	KH	52.73 ± 0.10^{Aa}	43.1 ± 0.29^{Bb}	45.76 ± 2.81^{Bb}
b*	BH	33.69 ± 0^{Ca}	36.57 ± 0.51^{Aa}	36.05 ± 0.01^{Ba}
	KH	33.69 ± 0^{Aa}	33.00 ± 1.50^{Ab}	32.21 ± 1.49^{Ab}

There is no statistically significant difference between the values shown with the same lowercase letters in each column (P>0.05). There is no statistically significant difference between the values shown with the same capital letters in each row (P>0.05).

Tarhan (2017) investigated the differences in the growth of kombucha mushroom with the use of various sugar sources in different plants-fruits and coffee. In the study, it was determined that the b* value in all carbon sources of pomegranate tea was not affected by fermentation. In the same study, it was determined that there was a decrease in the L* and b* values of only xylose sugar samples due to fermentation in blueberry and rosehip teas. While no significant difference was observed in the b* value during fermentation and storage in the KH sample in this study, a difference was detected in the BH sample. Watawana et al. (2016), in their study with kombucha, thought that the reason for the changes in a* value and the decrease in L*, b* value was the decrease in pH due to fermentation and the degradation of color pigments and polyphenolic components by microorganisms growing in the environment. Color stability is highly dependent on pH and anthocyanin structure (Torskangerpoll and Andersen, 2005).

Viscosity values of BH and KH samples at the beginning of fermentation were measured as 2.048 Pa.s. The increase in viscosity of the BH sample during fermentation was greater than that of the KH sample. During storage, a statistically insignificant (P>0.05) decrease was observed in the BH sample. In the KH sample, however, the increase continued (P<0.05) (Table 1). In study conducted by Watawana et al. (2016), coconut water with kombucha mushrooms was fermented for 7 days. As in this study, they reported that the viscosity increased due to fermentation. This may be due to the secretion of exopolysaccharides formed during fermentation (Zhao et al., 2015; Vivek et al. 2019). Exopolysaccharides (EPS) from LAB have been reported to be used as stabilizing, viscosity modifying and gelling agents in foods (Ahmed et al., 2013; Altay et al. 2013). Exopolysaccharides can be produced by many microorganisms other than LAB (Ergene and Avcı, 2016).

3.2. Microbiological properties

Grape juice, mustard seed and cherry leaf mixture used as substrate in this study was pasteurized to prevent microbial contamination from raw materials. TMAB was not found at the beginning of fermentation after heat treatment (Table 2). In the BH sample, the TMAB count was 5.58 log cfu mL^{-1} at day 7. It was determined as 4.49 log cfu mL^{-1} after 14 d of storage at +4 °C and a significant decrease was observed during storage ($P<0.05$). In the KH sample, it was determined as 4.55 log cfu mL^{-1} on the 7th day, and 3.1 log cfu mL^{-1} with a significant decrease on the 14th day of storage ($P<0.05$). In the study of Arici and Coskun (2001) the total bacterial count of hardaliye samples produced by the traditional method was at least (pH 3.21) 2.04 log cfu mL^{-1}, at most (pH 4.12) 5.9 log cfu mL^{-1} average (pH 3.58) was determined as 4.9 log cfu mL^{-1}. The results of that study appear to be similar to those of this study. The decrease in TMAB numbers of KH and BH samples during storage may be due to the increase in acidity in the products. As a matter of fact, Arici and Coskun (2001) suggested in his study that the low pH of hardaliye may cause a lethal and/or inhibitory effect against many microorganisms.

Yeast was not detected in the product consisting of pasteurized grape juice, mustard seed and cherry leaf mixture at the beginning of fermentation (Table 2). Mold growth was not observed in Hardaliye samples in analyzes made during production and storage. While yeast growth was observed during fermentation, a decrease was observed in the number of yeast during storage (Table 2). Gürbüz (2018), in his study on hardaliye, stated that the optimum growth temperature of yeasts is between 20-30 ºC, and suggested that a decrease in the number of yeasts can be observed depending on the temperature when the hardaliye samples are stored at +4 ºC. In this study, we can associate the decrease in yeast numbers of KH and BH samples with temperature during storage at +4 ºC (Table 2). In the BH sample, an increase in the amount of ethyl alcohol and a decrease in the amount of invert sugar were observed with the growth of yeast during fermentation. Yeast growth was lower in the KH sample than in the BH sample. While an increase was observed in the number of yeast during fermentation in the KH sample, the amount of reducing sugar decreased less than in the BH sample. Alcohol formation was not observed. This is thought to be due to the symbiotic association of yeasts and acetic acid bacteria during KH fermentation. Glucose in grape juice is converted to gluconic acid by acetic acid bacteria in the environment, while fructose is converted to ethanol by yeasts. Ethanol produced by yeast is converted to acetic acid by acetic acid bacteria (Arıkan, 2018). The fact that the number of acetic acid bacteria transferred from grapes to mustard may be less than that of kombucha, suggested that the ethyl alcohol content of the BH sample may have been higher than that of KH.

Table 2. Microbiological properties of hardaliye samples during fermentation and storage.

Properties	Samples	Fermentation time (day)		Storage time (14th day)
		0	7	14
TMAB (log cfu mL^{-1})	BH	0±0^{C}	5.58±0.06^{Ab}	4.49±0.49^{Ba}
	KH	0±0^{C}	4.55±0.02^{Ab}	3.1 ±0.14^{Bb}
Total yeast (log cfu mL^{-1})	BH	0±0^{C}	5.00±0.07^{Aa}	4.65±0.19^{Ba}
	KH	0±0^{C}	3.7±0.11^{Ab}	2.69±0.15^{Bb}
Total mold (log cfu mL^{-1})	BH	<DL	<DL	<DL
	KH	<DL	<DL	<DL
Lactobacillus spp. (log cfu mL^{-1})	BH	0±0^{C}	5.44±0.05^{Aa}	4.32±0.19^{Ba}
	KH	0±0^{C}	4.15 ±0.13^{Ab}	3.86 ±0.49^{Aa}
Lactic streptococci (log cfu mL^{-1})	BH	0±0^{C}	4.75±0.06^{Aa}	3.91±0.05^{Ba}
	KH	0±0^{C}	3.9±0.09^{Ab}	2.97±0.09^{Bb}

There is no statistically significant difference between the values shown with the same lowercase letters in each column ($P>0.05$). There is no statistically significant difference between the values shown with the same capital letters in each row ($P>0.05$). DL: Less than detection limit (10 cfu g^{-1})

In the BH and KH samples, on the 7th day of the fermantation, *Lactobacillus* spp. development was observed and the numbers were determined as 5.44 and 4.15 log cfu mL^{-1}, respectively. *Lactobacillus* spp. numbers decreased at the end of storage. In the study of Arici and Coskun (2001), the change in the number of lactic acid bacteria during the fermentation period (7 d) of hardaliye produced by inoculating different mustard seeds with different *Lactobacillus* ssp. was investigated. The number of lactic acid bacteria on the 7th day of fermentation in hardaliye with the addition of 1% black mustard seeds and *Lb. sanfansisco* was found to be 5.46 log cfu mL^{-1}. The number of *Lactobacillus* determined is similar to the results of the BH sample in this study. It is seen that the LAB number of the KH sample is low compared to that study. This may be because the number of lactic acid bacteria in SCOBY added to grape juice for the KH sample was lower than that of hardaliye added to the grape juice for the BH sample. Also, there may be a difference in the fermentation metabolism in the KH sample to which SCOBY was added. In a study investigating the metabolic activity of kombucha in milk and its ability to be a functional beverage; milk products fermented with kombucha were stored at +4 °C for 30 d at the end of fermentation and their biochemical and microbiological changes in this process were investigated. It has been reported that the number of *Lactobacillus* spp. decreased in the first 10 d of storage (Şarkaya, 2019). It was

observed that the *Lactobacillus* spp. numbers of the KH sample in this study also decreased during storage.

On the 7th day of fermentation, the streptococcal numbers of Hardaliye samples were determined as 4.75 log cfu mL^{-1} in BH sample and 3.91 log cfu mL^{-1} in KH sample. A statistically significant decrease occurred in both samples at the end of 14 d of storage ($P<0.05$) as in the study of Şarkaya (2019). In their study, Akarca and Tomar (2020) performed kombucha fermentation with red and purple vegetables for 21 days. On the 7th day of fermentation, the *Streptococcus* spp. numbers were determined as 3.75 log cfu mL^{-1} in the kombucha sample produced with red carrots, and 3.63 log cfu mL^{-1} in the kombucha sample prepared with red beet. The number of *Streptococcus* spp. on the 7th day of fermentation in the KH sample in this study is similar to the study.

4. Conclusion

When the results obtained were evaluated, it was determined that hardaliye, a fermented product with high nutritional value, can be produced with kombucha mushrooms without using preservatives. It has been observed that yeast growth cannot be prevented only with mustard seeds during BH production and alcohol will form in the product. Back-slopping production method should not be preferred in hardaliye production without using preservatives. It is thought that the preference of vigorous fermentation in the production of hardaliye with kombucha mushrooms will positively affect the total phenolic content and color values of the product. There are few studies on the production of hardaliye without preservatives. There is no legal standard for this traditionally produced product. In this context, studies on hardaliye are of great importance for a legal regulation to be made about hardaliye in the future. Physical, chemical and microbiological similarity of hardaliye produced using kombucha mushroom to hardaliye produced using preservative should be investigated, studies should be carried out on the identification of the microflora of hardaliye produced with kombucha mushroom.

Compliance with Ethical Standards

Conflict of Interest

As the author of article declare that there are no conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

Ayşenur Pekcan: Validation, Writing - original draft, Methodology, Investigation, Formal analysis, Data curation. **Fatma Coşkun**: Writing - original draft, Methodology, Investigation, Conceptualization, Validation, Review and editing. **Ömer Öksüz**: Methodology, Investigation, Conceptualization, Validation, Writing - original draft, Review and editing, Visualization, Data

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