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Microbial Properties Evaluation of a Yogurt Whey-Based Orange Beverage Fermented with Kombucha as a Starter Culture

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ABSTRACT

Background: This study aimed to evaluate microbial counts of beverage samples fermented using kombucha, including total acetic acid bacteria (AAB), total lactic acid bacteria (LAB), yeasts, and total microbial count (TMC).

Methods: A fermented orange beverage was produced using yogurt whey at three different concentrations (0 %, 10 %, and 20 % v/v) and kombucha at four different concentrations (1 %, 2 %, 3 %, and 4 % w/v) as the starter culture. Next, the microbial counts of the produced samples were evaluated at the end of the incubation and during cold storage at 4 ℃. The results were expressed as log CFU/mL.

Results: The results indicated a significant increase in the viability of AAB, TMC, and yeasts with increasing yogurt whey concentrations in the samples ($p < 0.05$). Furthermore, increasing the kombucha percentage in beverage samples resulted in the viability increment of TMC, AAB, LAB, and yeasts. Over time, the number of yeasts and viability of LAB increased in the samples. Moreover, the viability of TMC and AAB increased in the samples from days 7 to 10 and decreased from days 10 to 14 ($p < 0.05$).

Conclusion: Increasing yogurt whey and kombucha percentages affect microbial counts of beverage samples fermented using kombucha.

1. Introduction

Yogurt whey, a greenish-yellow liquid, is a by-product of concentrated yogurt production, containing lactose, lactic acid, small amounts of soluble protein, water-soluble vitamins (especially group B vitamins), and minerals [1]. Due to its high-quality protein and rich essential amino acid content, yogurt whey offers significant nutritional value [2]. Kombucha is a traditional beverage obtained from the fermentation of sweat tea by the strong commensalism of LAB and yeasts. This drink has been widely used around the globe for its preventive and therapeutic properties. Kombucha contains ethanol, carbon dioxide, high concentrations of glucuronic acid, acetic and lactic acids, and some other metabolites. Sucrose-tea solution is fermented by commensalism of bacteria and yeasts (trapped inside cellulose masses, forming a floating layer on the tea) [3]. Research has shown that kombucha can be grown on various substrates, such as green and black tea. It has also been shown that other sugar sources, such as lactose, can also be used to cultivate kombucha [4]. Numerous studies have investigated the production of different types of fermented beverages based on milk or whey using kombucha [3, 5-19]. Additionally, drinks based on soy whey have been produced [20]. It is worth noting that the content range of kombucha compounds, including ethanol (< 5.5 g/l), glucuronic acid (0.04-2.23 g/l), acetic acid (0-1.65 g/l), and lactic acid (0.85-5.25 mg/mL), is different [21-27]. However, no report is available on the fermentation of yogurt whey with kombucha and evaluating microbial properties. Therefore, this research aims to evaluate the microbial counts of specific microorganisms in the final product, namely a yogurt whey-

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based orange beverage fermented with kombucha as a starter culture, as these microorganisms significantly influence the quality of the beverage.

2. Materials and Methods

2.1 Materials

Yogurt whey was supplied by Pegah Company (Zanjan, Iran). The Kombucha culture and orange concentrate were obtained from the Research Institute of Agricultural and Engineering (Tehran, Iran) and Noosh Company (Mazandaran, Iran). White sugar from Golestan Company (Iran) and E 440 pectin from Canrose Company (Japan) were also prepared for this study. Other chemicals and culture media were purchased from Merck Company (Germany).

2.2 The preparation method of fermented orange drink based on yogurt whey using kombucha as the starter culture

Water, yogurt whey, and orange concentrate were added to the pectin and sugar mixture. The admixture was then heated to 60 ℃ using a water bath (Shimadzu, Iran). Next, for complete homogenization, they were mixed using a mechanical mixer (Starlux-SL-133, China) at 500 rpm speed. Pasteurization was performed at 90 ℃ for 5 min in a water bath. Various treatments were cooled down to 25 ℃, kombucha was inoculated, and the samples were held in an incubator at 25 ℃ for 7 days until a pH of 4.2. Then, to reduce the fermentation speed, kombucha was separated from the drink in aseptic conditions, and the treatments were stored at 4 ℃ for 7 days for microbial analysis. Therefore, the drink was produced from 0 to 7 days of fermentation in an incubator (25 ℃) and stored in a refrigerator (4-8 ℃) from days 7 to 14. Table 1 shows different treatments used in this research.

2.3 Microbial analysis

Serial dilutions of samples were prepared in aseptic conditions using sterile saline containing 0.89 % NaCl. In this study, de Man Rogosa Sharpe (MRS) Agar was used for counting total LAB, and Petri plates were incubated for 24 hours at 37 ℃ [17]. Potato dextrose agar (PDA) was used for total yeast count, and Petri plates were incubated for three days at 25 ℃ [28]. For total AAB count, a medium containing 1 % yeast extract, 2 % $CaCO_3$, and 1.8 % agar (calcium carbonate agar) was prepared and sterilized. Then, 3 % ethanol (95 %) was added to the mixture. Afterward, the Petri dishes were incubated for three days at 30 ℃ [28]. Plate count Agar (PCA) was used for counting TMC, and Petri plates were incubated for 24 h at 37 ℃ [18]. Colonies were evaluated for all microorganisms at the end of the incubation (day 7) and during cold storage (days 10 to 14). In the end, the results were expressed as log CFU/mL.

2.4 Statistical analysis

A completely randomized factorial design was employed for microbial data. For each treatment, 3 replications were considered. The averages of experimental treatments were compared using least squares means (LSMs). After conducting experiments and collecting data, data analysis was carried out for the mentioned designs using the SPSS software.

Table 1. Treatments used in this research

Treatments	Yogurt Whey (V/V)	Kombucha (W/V)	Pectin (W/V)	Sugar (W/V)	Water (V/V)	Orange concentrate (W/V)
T1	0	1	0.5	7	84.5	7
T2	0	2	0.5	7	83.5	7
T3	0	3	0.5	7	82.5	7
T4	0	4	0.5	7	81.5	7
T5	10	1	0.5	7	74.5	7
T6	10	2	0.5	7	73.5	7
T7	10	3	0.5	7	72.5	7
T8	10	4	0.5	7	71.5	7
T9	20	1	0.5	7	64.5	7
T10	20	2	0.5	7	63.5	7
T11	20	3	0.5	7	62.5	7
T12	20	4	0.5	7	61.5	7

3. Results and Discussion

Gluconic and acetic acids are produced by AAB using glucose and ethanol, respectively. With increasing the kombucha content, the total AAB of samples without yogurt whey increased at the end of the incubation (i.e., day 7) and cold storage (i.e., day 14) ($p > 0.05$) and was in the range of 5.63-5.93 and 7.56-8.14 log CFU/mL, respectively (Table 2). At a concentration of 10 % of yogurt whey, with increasing the kombucha content (1-4 %), the total AAB of samples at the end of the incubation and cold storage enhanced ($p > 0.05$) and was at the range of 5.53-5.96 and 8.51-8.98 log CFU/mL, respectively. At a concentration of 20 % of yogurt whey, with increasing the kombucha content (1-4 %), the total AAB of samples increased at the end of the incubation and storage ($p > 0.05$) and was at the range of 5.67-6.38 and 8.70-9.38 log CFU/mL, respectively. As can be seen, the AAB count is more affected by the increase in yogurt whey percentage than the increase in kombucha content.

Table 2. Changes in the AAB counts (log10 CFU / mL) in beverage samples at the end of the incubation (day 7) and during cold storage (days 10 to 14)

Storage Time (Day) / Treatment	7	10	14
T1	5.63 ± 0.15^{eC}	6.73 ± 0.09^{gB}	7.56 ± 0.15^{jA}
T2	5.81 ± 0.15^{dC}	7.01 ± 0.07^{efB}	7.27 ± 0.02^{iA}
T3	5.89 ± 0.10^{cdC}	7.04 ± 0.04^{deB}	8.03 ± 0.07^{hA}
T4	5.93 ± 0.15^{cC}	7.10 ± 0.10^{cdB}	8.14 ± 0.04^{gA}
T5	5.53 ± 0.02^{fC}	6.76 ± 0.15^{gB}	8.51 ± 0.10^{fA}
T6	5.71 ± 0.06^{eC}	6.95 ± 0.04^{fB}	8.79 ± 0.03^{dA}
T7	5.91 ± 0.09^{cdC}	7.08 ± 0.02^{cdB}	8.92 ± 0.02^{cA}
T8	5.96 ± 0.01^{cC}	7.13 ± 0.07^{cB}	8.98 ± 0.08^{cA}
T9	5.67 ± 0.05^{eC}	7.03 ± 0.03^{deB}	8.70 ± 0.08^{eA}
T10	5.97 ± 0.07^{cC}	7.13 ± 0.08^{cB}	8.93 ± 0.01^{cA}
T11	6.24 ± 0.03^{bC}	7.27 ± 0.07^{bB}	9.10 ± 0.09^{bA}
T12	6.38 ± 0.09^{aC}	7.85 ± 0.12^{aB}	9.38 ± 0.05^{aA}

* Different capital letters have significant differences in rows and different small letters have significant differences in columns ($p < 0.05$).

* Yogurt Whey (%) = YW; Kombucha (%) = K

* T1: YW = 0, K = 1; T2: YW = 0, K = 2; T3: YW = 0, K = 3; T4: YW = 0, K = 4; T5: YW = 10, K = 1; T6: YW = 10, K = 2; T7: YW = 10, K = 3; T8: YW = 10, K = 4; T9: YW = 20, K = 1; T10: YW = 20, K = 2; T11: YW = 20, K = 3; T12: YW = 20, K = 4.

Increased survival of AAB with increasing the concentrations of kombucha and yogurt whey and changes in the survival of these bacteria over time can be attributed to the multiplication of acid-resistant bacterial strains [29]. This enhancement can also be related to the positive effect of nutrients in yogurt whey on the survival of these bacteria. Sreeremula *et al.* [29] reported rapid growth of the number of AAB over 4 days through kombucha fermentation and its decrease until day 6. They also observed a secondary growth of AAB after day 12. Likewise, in the present study, the AAB count increased. These researchers concluded that except AAB, other bacteria barely grow on kombucha. Through fermentation of whey with kombucha, Suciati *et al.* [17] reported that using kombucha does not significantly affect AAB counts. Increased survival of AAB with increasing levels of kombucha was also reported by Nurliyani *et al.* [18]. The presence of LAB in kombucha is known to assist the growth of AAB to increase cellulose production and induce the growth of Gluconoacetobacter in kombucha. Both at the end of the incubation and the end of cold storage, the samples containing yogurt whey with a concentration of 20 % had a total LAB higher than beverages containing yogurt whey with a concentration of 10 % (Table 3). With increasing the kombucha content, the total LAB of samples without yogurt whey at the end of the incubation and cold storage increased ($p > 0.05$) and was at the range of 6.76-7.42 and 6.32-6.72 log CFU/mL, respectively.

Table 3. Changes in the LAB counts (log10 CFU / mL) in beverage samples at the end of the incubation (day 7) and during cold storage (days 10 to 14)

Storage Time (Day) Treatment	7	10	14
T1	6.76 ± 0.15^{jB}	8.05 ± 0.09^{fA}	6.32 ± 0.15^{gC}
T2	7.07 ± 0.15^{ghB}	8.18 ± 0.06^{efA}	6.37 ± 0.02^{gC}
T3	7.14 ± 0.10^{fB}	8.35 ± 0.05^{deA}	6.62 ± 0.07^{fC}
T4	7.42 ± 0.15^{eB}	8.36 ± 0.10^{deA}	6.72 ± 0.04^{fC}
T5	6.88 ± 0.10^{iC}	8.29 ± 0.15^{deA}	8.07 ± 0.10^{bB}
T6	7.10 ± 0.06^{fgC}	8.39 ± 0.04^{dA}	6.08 ± 0.03^{bB}
T7	7.46 ± 0.07^{eC}	8.82 ± 0.08^{bA}	7.79 ± 0.02^{cB}
T8	7.67 ± 0.01^{cB}	9.00 ± 0.07^{aA}	7.45 ± 0.08^{dC}
T9	7.01 ± 0.05^{hC}	8.60 ± 0.05^{cA}	7.22 ± 0.04^{eB}
T10	7.60 ± 0.07^{dC}	8.62 ± 0.06^{cA}	7.80 ± 0.01^{cB}
T11	7.88 ± 0.03^{bC}	8.64 ± 0.04^{bcA}	7.95 ± 0.02^{bB}
T12	8.27 ± 0.09^{aB}	8.68 ± 0.08^{bcA}	8.24 ± 0.05^{aC}

* Different capital letters have significant differences in rows and different small letters have significant differences in columns ($p < 0.05$).

* Yogurt Whey (%) = YW; Kombucha (%) = K

* T1: YW = 0, K = 1; T2: YW = 0, K = 2; T3: YW = 0, K = 3; T4: YW = 0, K = 4; T5: YW = 10, K = 1; T6: YW = 10, K = 2; T7: YW = 10, K = 3; T8: YW = 10, K = 4; T9: YW = 20, K = 1; T10: YW = 20, K = 2; T11: YW = 20, K = 3; T12: YW = 20, K = 4.

Changes in LAB counts during storage time could be due to high acidity, low pH, and production of metabolites such as organic acids and reduction of sugars. In this study, the LAB count increased until the 10 th day of fermentation and then decreased. Research has shown that LAB lacks the tolerance to reduced pH and elevated acidity of fermented products and loses its viability completely after 2 weeks of storage [30]. Some compounds produced during fermentation by kombucha (e.g., ethanol, lactic and acetic acids, and massive proteins) have shown antimicrobial activity combined with decreased pH. At low pH values, lactic acid is in toxic form for most bacteria and yeasts. Also, polyphenols and tannins of tea are inhibitors against numerous Gram-positive and Gram-negative bacteria [17, 28, 29, 31]. Besides, LAB in the natural flora of kombucha may break down the proteins in yogurt whey by protease production and convert them to bioactive peptides, some having antimicrobial activity. In this respect, Coskun and Kayisoglu [28] reported that lactococcus numbers decrease in the kombucha after day 10 of fermentation. Suciati *et al.* [17], through fermentation of whey by kombucha, have reported the presence of some LAB including lactobacillus, lactococcus, bifidobacterium, and leuconostoc in the kombucha layer. This finding justifies the elevated number of LAB in the samples with higher kombucha levels. According to Gramza-Michalowska *et al.* [32], the level of nitrogen-containing compounds is directly relevant to the higher biomass efficiency in kombucha. The explanation is that the microbial flora of kombucha develops with continuous sugar consumption. The noteworthy point is that many compounds produced during the fermentation stage cannot be consumed. Furthermore, the lack of sufficient amounts of sugar for consuming the microbial flora of kombucha leads to the loss of kombucha activity. This phenomenon unbalances the maintenance of soluble compounds. Consequently, the natural flora of kombucha does not have enough nutrients available, resulting in the death of the microbial flora in the solution. At the end of the incubation and cold storage, with increasing the levels of kombucha, the yeast range of 7.61-7.94 and 8.85-10.38 log CFU/mL, respectively, was observed in beverage samples without yogurt whey (Table 4). Also, the TMC range corresponding to these samples (Table 5) was 4.40-7.63 and 6.21-6.93 log CFU/mL, respectively. The yeast and TMC-count change trends in samples containing 10 and 20 % yogurt whey were similar to LAB and AAB.

Table 4. Changes in the yeast count (log10 CFU / mL) in beverage samples at the end of the incubation (day 7) and during cold storage (days 10 to 14)

Storage Time (Day) Treatment	7	10	14
T1	7.61 ± 0.15^{hC}	8.42 ± 0.02^{jB}	8.85 ± 0.15^{gA}
T2	7.73 ± 0.15^{gC}	8.72 ± 0.06^{iB}	9.16 ± 0.04^{fA}
T3	7.82 ± 0.10^{fC}	8.86 ± 0.10^{hB}	9.95 ± 0.07^{cA}
T4	7.94 ± 0.15^{eC}	9.19 ± 0.02^{gB}	10.38 ± 0.04^{aA}
T5	7.83 ± 0.02^{fC}	8.90 ± 0.15^{hB}	9.62 ± 0.10^{eA}
T6	7.96 ± 0.06^{eC}	9.15 ± 0.04^{gB}	9.65 ± 0.03^{eA}
T7	8.07 ± 0.07^{dC}	9.34 ± 0.02^{fB}	9.86 ± 0.07^{dA}
T8	8.12 ± 0.01^{cC}	9.39 ± 0.07^{eB}	9.93 ± 0.03^{cdA}
T9	8.15 ± 0.05^{bcB}	10.08 ± 0.05^{dA}	10.35 ± 0.03^{aA}
T10	8.18 ± 0.07^{bB}	10.18 ± 0.08^{cA}	10.23 ± 0.01^{bA}
T11	8.25 ± 0.03^{aC}	10.31 ± 0.07^{bA}	8.60 ± 0.07^{hB}
T12	8.27 ± 0.09^{aC}	10.38 ± 0.12^{aA}	8.86 ± 0.05^{gB}

* Different capital letters have significant differences in rows and different small letters have significant differences in columns ($p < 0.05$).

* Yogurt Whey (%) = YW; Kombucha (%) = K

* T1: YW = 0, K = 1; T2: YW = 0, K = 2; T3: YW = 0, K = 3; T4: YW = 0, K = 4; T5: YW = 10, K = 1; T6: YW = 10, K = 2; T7: YW = 10, K = 3; T8: YW = 10, K = 4; T9: YW = 20, K = 1; T10: YW = 20, K = 2; T11: YW = 20, K = 3; T12: YW = 20, K = 4.

Based on the obtained results, since kombucha is a combination of bacterial and yeast microbial flora, with increasing the amount of kombucha, the number of yeasts

will increase accordingly. The increase in yeast growth with increasing yogurt whey can be attributed to the positive effect of nutrients in the yogurt whey on the survival of yeasts. Over time, the number of yeasts in the sample increased ($p < 0.05$). Yeasts convert sucrose to fructose and ethanol during the fermentation process of kombucha and are one of the microorganisms that comprise the microbial flora of the kombucha layer. Total yeasts in fermented kombucha with 7 % lactose as a substrate and a 2-day fermentation period were reported to be 6.68 CFU/mL. This value is higher than the total yeasts of fermented kombucha with whey, probably due to the difference in the fermentation medium [17]. Sreeyaamulu *et al.* [29] reported an increase in the number of yeasts during kombucha fermentation until day 4 and a considerable decrease in their number until day 6. They also observed a secondary growth of yeasts after day 12. In this regard, the changes in the number of yeasts were very similar to those of AAB. It has also been reported that in the kombucha drink, microorganisms (lactobacillus, lactococcus, yeasts, and AAB) increased until day 7 of fermentation and decreased thereafter. The reduction in the number of yeasts is attributed to the pH reduction [28], suggesting the decrease in the number of yeasts after day 10 of fermentation. The difference between our study and other researchers' study may be the difference in the culture used (SCOBY or symbiotic culture of bacteria and yeasts, a variable microbiological composition), the culture rate, the amount of sugar, and the fermentation conditions. The increase in TMC could be due to the presence of natural flora in kombucha. The increase in TMC (Table 5) in even levels of kombucha in the samples having higher yogurt whey could be attributed to the positive effect of nutrients in yogurt whey on the viability of total bacteria. In this context, Suciati *et al.* [17], through fermentation of whey by kombucha, reported that TMC in the final product is proportional to the general microbial conditions of the initial whey. However, it was shown that bacteria and yeasts in symbiotic culture can continue their activities [28].

Table 5. Changes in TMC counts (log10 CFU / mL) in beverage samples at the end of the incubation (day 7) and during cold storage (days 10 to 14)

Storage Time (Day) / Treatment	7	10	14
T1	4.40 ± 0.15^{jC}	7.70 ± 0.09^{jA}	6.21 ± 0.15^{iB}
T2	5.24 ± 0.15^{iC}	8.09 ± 0.06^{iA}	6.46 ± 0.04^{hB}
T3	5.86 ± 0.10^{hC}	9.19 ± 0.08^{hA}	6.87 ± 0.07^{gB}
T4	7.63 ± 0.15^{fB}	9.37 ± 0.10^{fA}	6.93 ± 0.04^{fC}
T5	7.52 ± 0.02^{gC}	9.22 ± 0.10^{ghB}	9.39 ± 0.10^{aA}
T6	7.74 ± 0.06^{eC}	9.60 ± 0.04^{eB}	9.74 ± 0.03^{bA}
T7	7.99 ± 0.07^{cC}	9.86 ± 0.02^{dA}	9.44 ± 0.02^{cB}
T8	8.09 ± 0.01^{bC}	10.14 ± 0.03^{cA}	8.49 ± 0.08^{eB}
T9	7.84 ± 0.05^{dC}	9.23 ± 0.09^{gA}	8.48 ± 0.07^{eB}
T10	7.96 ± 0.7^{cC}	10.15 ± 0.06^{cA}	9.16 ± 0.01^{dB}
T11	8.14 ± 0.03^{bC}	10.15 ± 0.07^{bA}	9.37 ± 0.02^{cB}
T12	8.29 ± 0.09^{aC}	10.35 ± 0.12^{aA}	9.38 ± 0.09^{cB}

* Different capital letters have significant differences in rows and different small letters have significant differences in columns ($p < 0.05$).

* Yogurt Whey (%) = YW; Kombucha (%) = K

* T1: YW = 0, K = 1; T2: YW = 0, K = 2; T3: YW = 0, K = 3; T4: YW = 0, K = 4; T5: YW = 10, K = 1; T6: YW = 10, K = 2; T7: YW = 10, K = 3; T8: YW = 10, K = 4; T9: YW = 20, K = 1; T10: YW = 20, K = 2; T11: YW = 20, K = 3; T12: YW = 20, K = 4.

4. Conclusion

In this study, a fermented orange beverage was produced based on yogurt whey. For this purpose, yogurt whey was used in 3 different levels of 0, 10, and 20 % (v/v), and kombucha in 4 levels of 1, 2, 3, and 4 % (w/v) as the starter culture. Next, some microbial properties of the produced beverage were evaluated. Based on this research's findings, increasing kombucha and yogurt whey percentages promoted the viability of TMC, total AAB, LAB, and yeasts. Over time, the number of yeasts and viability of AAB rose in the samples. The TMC and LAB counts increased in the samples from days 7 to 10 and decreased from days 10 to 14.

Authors' Contributions

Marzieh Hatami: Formal analysis; Investigation; Visualization; Software; Writing-original draft. Vajiheh Fadaei: Conceptualization; Data curation; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-original draft; Writing-review & editing.

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Conflicts of Interest

The authors declared no conflict of interest regarding the publication of this paper.

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Ethical considerations

The authors declare that data supporting the findings of this study are available within the article. No: 28550417951005.

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