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ORIGINAL RESEARCH PAPER

SURVIVAL OF DIFFERENT PROBIOTIC STARTERS IN BLENDS OF APPLE JUICE AND VEGETABLE EXTRACTS CONTAINING PREBIOTICS

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Abstract: Viabilities of three different probiotic starters (LGG, LA-5, and ABT-2) inoculated to the formulations (F1-9) based on blends of apple juice and some vegetable extracts containing fructooligosaccharide and lactitol were monitored during fermentation and cold storage. Storage quality and stability of formulations in terms of physicochemical and antioxidant properties and sensory evaluation were investigated. F7 (Apple Juice + Cucumber extract + Carrot extract; 1 : 1 : 1, v/v/v) was the most successful among the formulations in terms of probiotic growth with more than 7 logs CFU·mL^{-1} for all starters after fermentation at 37 °C for 24 h. F8 (Apple Juice + Cucumber extract (1 : 1, v/v) + 2 % FOS + 4 % Lactitol) come second for only LGG starter. Among the starters, LGG strain maintained its required viability with more than 6 logs CFU·mL^{-1} in the formulations (F7-9) during cold storage. The viabilities of LA-5 and ABT-2 with more than 7 logs at the beginning of the storage periods constantly decreased below 1 log and 3 logs, respectively. Antioxidant properties of the probiotic beverages did not change significantly during the fermentation and storage period. F9 (Apple juice + Cucumber extract + Carrot extract (1 : 1 : 1, v/v/v) + 2 % FOS + 4 % Lactitol) was the most preferred formulation compared to all other formulations.

Keywords: *antioxidants, consumer liking, non-dairy probiotic beverage, prebiotics, storage quality*

INTRODUCTION

Recently, consumers paid attention to functional foods to regulate their diet. Functional foods can contain probiotics, prebiotics, sterols, and other functional nutrients that provide health benefits in addition to meeting basic nutrient requirements [1]. The probiotic food industry is one of the fastest-growing sectors in the functional food industry. Probiotics are microorganisms that can grant health benefits to the host when consumed in adequate amounts [2] and they can be marketed as food, dietary supplement, or medicine. Lactobacillus sp. (*L. acidophilus, L. plantarum, L. rhamnosus,* and *L. casei*) and Bifidobacterium sp. (*B. adolescentis, B. lactis, B. bifidum, B. infantis, and B. longum*) are the predominant probiotics in the market [3]. They show many health benefits such as balancing colonic microflora, decreasing cholesterol levels, relieving the symptoms of lactose intolerance and constipation, stimulating the immune system, enhancing the absorption of minerals, and they have anti-carcinogenic, anti-hypertensive, and anti-mutagenic effects [1]. Probiotics generate antibacterial compounds such as bacteriocins, diacetyl hydrogen peroxide, and organic acids that slow down the growth of pathogenic bacteria in the intestine [4]. Probiotic-based foods should contain at least 10^6 - 10^7 CFU·mL^{-1} of product at the moment of consumption to have these healthcare benefits [5].

Probiotic microorganisms are generally incorporated in dairy products that have an appropriate biochemical environment and rich nutritional values, so dairy-based probiotic products are on the market for a long time [3]. However, probiotic dairy products cannot be consumed by people who have lactose intolerance or milk allergy and people with cholesterol-restricted diets. The increasing number of people who follow a vegan diet is also another limiting factor in the consumption of dairy products. Although dairy-based products are very good for probiotic cultures, non-dairy based functional products are being investigated to overcome the limitations and to increase the number of probiotic foods in the markets [6].

Non-dairy based functional products that are produced from fruits, vegetables, cereals [7], and soybean have recently gained importance, and their market share has increased. Vegetable and fruit juices come into prominence because of their nutritional contents (vitamins, minerals, fibers, bioactive compounds) which are released from cellular content by mechanical processes and promote the growth of probiotic cultures [1]. Non-dairy probiotic beverages also include various other ingredients, mainly, prebiotics as non-digestible food ingredients. Prebiotics stimulate the growth or activity of one or more microorganisms, improve the functionalities of products so they benefit host health. One of the most widely used prebiotics in functional foods is inulin which is a plant-derived polysaccharide, and its breakdown product is fructooligosaccharides (FOS) [8].

Fruits and vegetables are suitable environments to incorporate probiotics. However, the behavior of microorganisms and interaction with these matrices is a complex process that is influenced by probiotic cultures and their metabolism, presence of nutrients, antimicrobial compounds, acidity, *p*H of the environment, and oxygen levels [9]. To investigate these factors and obtain information about non-dairy probiotic beverages, some fruit, and vegetables such as grapes [10], mango [11], sugarcane [12], pomegranate [13], and black carrot [14] were studied.

The key purpose of this study is to develop non-dairy probiotic beverage formulations based on blends of apple juice and some vegetable extracts containing various prebiotic active ingredients (fructooligosaccharide and lactitol) using three different probiotic starters LGG (*Lactobacillus rhamnosus*), LA-5 (*Lactobacillus acidophilus*), and ABT-2 (*Bifidobacterium lactis, Lactobacillus acidophilus,* and *Streptococcus thermophilus*), and investigate the storage quality and stability of formulations in terms of probiotics viability, physicochemical and antioxidant properties, and sensory evaluation.

MATERIALS AND METHODS

Materials

LGG (*L. rhamnosus*), LA-5 (*L. acidophilus*), and ABT-2 (*Bifidobacteriun lactis, L. acidophilus,* and *S. thermophilus*) lyophilized starter cultures were obtained from Chr. Hansen (Denmark). The apple juice concentrate used to develop fruit-based probiotic beverage formulations was obtained from the TAMEK fruit juice factory (Turkey). Spinach, cucumber, carrot, and watermelon were purchased from the local market and stored at 4 °C until the extraction process. Fructo-oligosaccharide and lactitol were obtained from DuPont Danisco (Denmark) and Baolingbao Biology Co., Ltd. (China), respectively.

Preparation of fruit and vegetable extracts

Fruits and vegetables were washed, and their extracts were prepared using a juicer. Extracts were filtered from 4-fold cheesecloth and sterilized (121 °C, 15 min). Sterile extracts (cucumber, spinach, watermelon, and carrot) were stored at -22 °C for further application.

Preparation of formulations

Nine different formulations as a non-dairy based beverage were produced (Table 1) using apple juice concentrate, sterilized vegetable extracts, fructooligosaccharide (FOS, DuPont Danisco, Denmark), and lactitol (Baolingbao Biology Co., Ltd., China). 12 °Brix apple juice was prepared from apple juice concentrate (65-72 °Brix). Prepared formulations (F1-F9) were pasteurized (90 °C, 3 min) in a water bath (Memmert WNB 10, Germany) before probiotic inoculation.

Table 1. *Formulations (Fs)*

F1	Apple Juice + Spinach extract + Water (2:1:1 v/v/v)
F2	Apple Juice + Spinach extract (1:1 v/v)
F3	Apple Juice + Cucumber extract (1:1 v/v)
F4	Apple Juice + Spinach extract (1:1 v/v) + 2 % FOS
F5	Apple Juice + Cucumber extract (1:1 v/v) + 2 % FOS
F6	Apple Juice + Cucumber extract + Watermelon extract (1:1:1 v/v/v)
F7	Apple Juice + Cucumber extract + Carrot extract (1:1:1 v/v/v)
F8	Apple Juice + Cucumber extract (1:1 v/v) + 2 % FOS + 4 % Lactitol
F9	Apple Juice + Cucumber extract + Carrot extract (1:1:1 v/v/v) + 2 % FOS + 4 % Lactitol

Probiotic culture preparations and inoculations

Starter cultures were activated in MRS broth (Merck, Germany) at 37 °C for 18 hours in an incubator (Memmert IN55, Germany) and adjusted to 0.5 McFarland (~ 1.5×10^8 CFU·mL^{-1}) at 620 nm in a spectrophotometer. Starter cultures (0.5 McFarland) were centrifuged (4 °C, 5000 rpm, 5 min.) and the medium was removed. The pellet cultures were dissolved in the same volume of 0.85 % NaCl (Sigma-Aldrich, Germany) solution. 1 mL of 0.5 McFarland starter culture was adjusted to ~ 1.5×10^6 CFU·mL^{-1} with 0.85 % NaCl solution [15]. 1 mL of starter culture (~1.5×10^6 CFU·mL^{-1}) was inoculated into 9 mL of pasteurized formulations. All prepared formulations were incubated (Memmert IN55, Germany) at 37 °C for 24 hours. After incubation, all formulations were stored at 4 °C for 4 weeks (Figure 1).

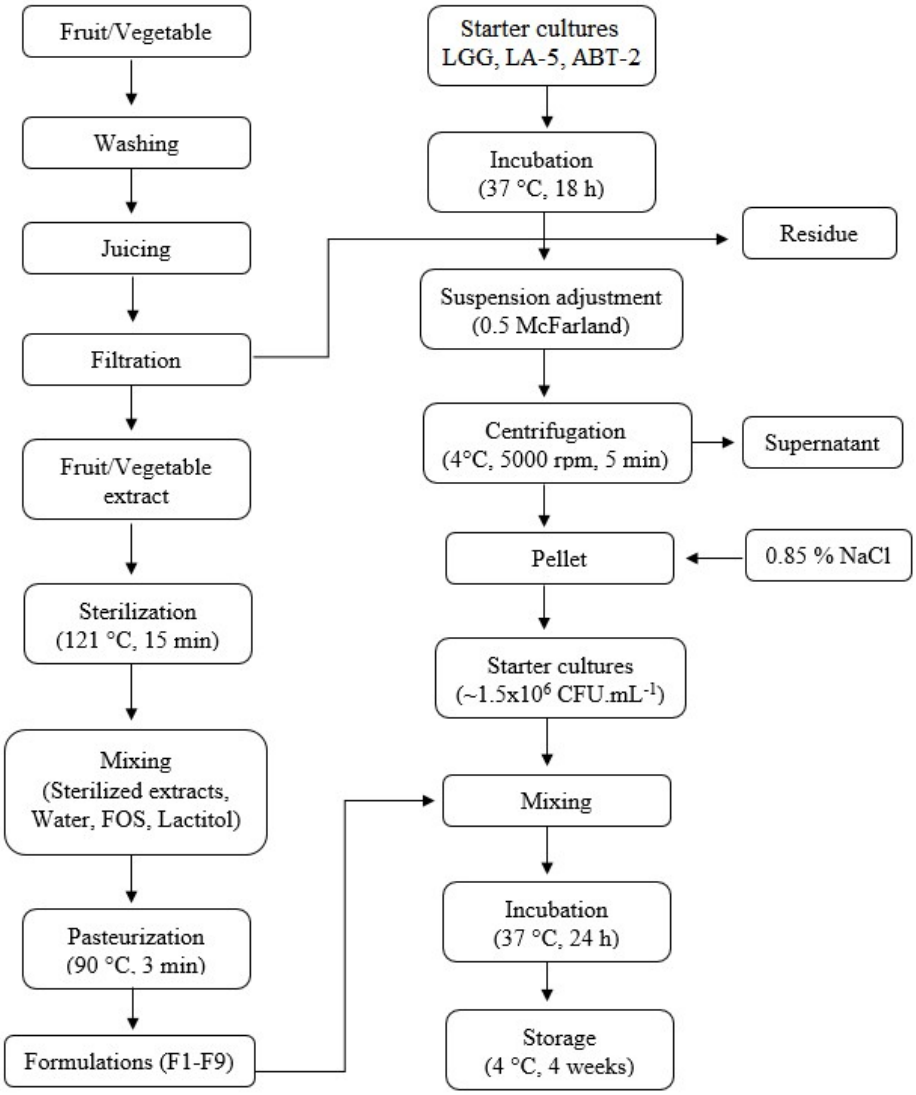

Figure 1. *Flow chart of the preparation of probiotic formulations*

The microbial growth curves of inoculated probiotics were spectrophotometrically monitored. 200 µL inoculated and control samples were pipetted into a 96-well

microtiter plate that was coated with a film (Platemax™) and was incubated in a spectrophotometer (Thermo Scientific, Multiskan™ GO Microplate Spectrophotometer, USA) at 37 °C for 24 hours. The turbidity readings of samples were made hourly at 620 nm.

Enumeration of microorganism

The microorganisms were counted during incubation (0 and 24. hours) and storage (7, 14, 21, and 28 days). After serial dilution of the microorganisms with 0.1 % peptone water (*p*H 7,2; Merck, Germany), double layer MRS Agar (de MAN, ROGOSA, and SHARPE, Merck, Germany) were poured on 1 mL diluted samples that were used for detection of *L. rhamnosus, L. acidophilus, B. bifidum,* and *L. acidophilus*. M17 agar (Merck. Germany) was used for the enumeration of *S. thermophilus*. Colonies were evaluated after incubation at 37 °C for 48 - 72 h [16].

Physico-Chemical analysis

pH, titratable acidity, and total soluble solids (TSS)

A *p*H meter was used to sample *p*H levels (Ohaus Starter 3100, USA). For the determination of titratable acidity, the sample taken from the formulations was titrated with 0.1 N NaOH until they reached *p*H 8.1. Titration acidity of the samples was calculated as g lactic acid·100 mL^{-1} [17]. TSS of samples was measured as °bx using a digital refractometer (Atago PAL1 Pocket Refractometer, Japan) in the room [18].

Color

A color measuring device (Minolta CR 400/CR 410) was used to measure the color values of the samples (*L**, *a**, and *b**), and the values were expressed as per CIE Lab system [18].

Total phenol content

The Folin-Ciocalteau method [19] was used to determine the total phenolic content of samples. 100 µL of the sample, 900 µL of distilled water, 5 mL of 0.2 N Folin-Ciocalteau solution and 4 mL of 7.5 % sodium carbonate solution were added to the test tube respectively and vortexed. The samples were incubated at room temperature for 2 hours in the dark and the absorbance values were read at 765 nm using a spectrophotometer (Thermo Scientific, Multiskan™ GO Microplate Spectrophotometer, USA). The results were expressed in "mg GAE (gallic acid equivalent)·L^{-1}".

CUPRAC (Copper Reducing Antioxidant Capacity)

The method suggested by Apak [20] was used. 20 µL of the sample, 1 mL of 0.01 M copper solution, 1 mL of 7.5×10-3 M neocuproin, 1 mL of 1 M, *p*H 7.0 ammonium acetate, 1080 µL of distilled water were added to the test tube respectively. The samples were incubated at room temperature for 30 min in the dark and the absorbance values were read at 450 nm using a spectrophotometer (Thermo Scientific, Multiskan™ GO Microplate Spectrophotometer, USA). The results were expressed as "mg TE (Trolox equivalent)·L^{-1}".

Sensory properties

Sensory properties (color, taste, general) of F7, F8 and F9 formulations were evaluated using a 7-point hedonic scale (7 = like extremely and 1 = dislike extremely) [21]. Sensory properties of formulations evaluated by panelists (15 males and 32 females) between the ages of 20 - 40.

Statistical analysis

All analyses were carried out in duplicate and three parallels. Statistical Analysis Systems Version 9 (SAS Institute Inc, Cary, NC, USA) with the MIXED procedure was used to analyze the data. Significant differences among the LS means were determined by the Tukey *post hoc* test.

RESULTS AND DISCUSSION

Microbial growth and viability during the fermentation process and storage period

Eight formulations (F1-8; Table 1) inoculated with 3 different probiotic cultures were initially produced as a non-dairy probiotic juice (Figure 1). Probiotic loads of formulations before and after fermentation (24 h, 37 °C) were given in Table 2. As seen in Table 2, the initial probiotic counts were adjusted to approximately ~5 logs CFU·mL^{-1} for all formulations (F1-F8). After fermentation, LGG strain maintained its viability in all formulations with the population ranged from 5.57 ± 0.02 to 8.11 ± 0.07 log CFU·mL^{-1} compared with other probiotic cultures (LA-5 and ABT-2). LGG showed the highest viability in F7 formulation (8.11 ± 0.07 log CFU·mL^{-1}), followed by F8 (7.90 ± 0.01 log CFU·mL^{-1}) and F5 (7.77 ± 0.01 log CFU·mL^{-1}) formulations, respectively. While LGG count slightly increased in F2, F4, and F6 formulations, LGG maintained its initial count in F1 and F3 formulations. On the other hand, LA-5 and ABT-2 strains only survived in F7 formulation and they increased their count to 7.50 ± 0.05 log CFU·mL^{-1} and 6.95 ± 0.02 log CFU·mL^{-1}, respectively. No colonies from LA-5 and ABT-2 were observed in other formulations. In addition, M17 Agar was used as a medium for the selective enumeration of *Streptococcus thermophillus* in the ABT-2 inoculated formulations. The highest vitality of *S. thermophillus* was also observed in F7 with 6.77 ± 0.06 CFU·mL^{-1} after the fermentation process, followed by F8 (Table 2). Microbial population curves also confirmed the growth of probiotic strains with the increased absorbance (Figure 2). When the growth in F7 compared with growth in MRS Broth that supports the growth of lactic acid bacteria, the adaptation of LGG strain to the formulation medium was longer than its adaptation to MRS broth because of the acidic environment of formulations (*p*H 4.56 - 5.07). ABT-2 strain adapted to both F7 and MRS broth similarly. However, ABT-2 counts in F7 were lower than the counts in MRS broth after the fermentation process. The counts of LA-5 were quite similar both in F7 formulation and MRS broth after fermentation, but the lag phase of LA-5 was very long compared to the lag phase of other strains.

Table 2. *LGG, LA-5, and ABT-2 counts (log CFU·mL^{-1}) in different formulations before and after fermentation.*

Fs	LGG		LA-5		ABT-2		ABT-2 M17	
	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours
F1	5.11±0.03	5.57±0.02	5.26±0.02	≤ 1	4.60±0.05	≤ 1	4.67±0.01	≤ 1
F2	5.19±0.06	7.10±0.01	5.19±0.01	≤ 1	4.63±0.02	≤ 1	4.78±0.01	≤ 1
F3	5.26±0.03	5.92±0.10	5.19±0.03	≤ 1	4.45±0.02	≤ 1	4.70±0.02	≤ 1
F4	5.11±0.18	7.08±0.04	5.15±0.02	≤ 1	4.64±0.02	≤ 1	4.85±0.04	≤ 1
F5	5.25±0.03	7.77±0.01	5.22±0.04	≤ 1	4.59±0.08	≤ 1	4.91±0.05	≤ 1
F6	5.21±0.03	6.33±0.01	5.20±0.04	≤ 1	4.60±0.10	≤ 1	4.87±0.04	4.85±0.01
F7	5.15±0.02	8.11±0.07	5.20±0.03	7.50±0.05	4.58±0.03	6.95±0.02	4.82±0.03	6.77±0.06
F8	5.29±0.03	7.90±0.01	5.14±0.05	≤ 1	4.72±0.01	≤ 1	4.75±0.07	5.23±0.08
MRS Broth	5.41±0.01	8.52±0.02	5.17±0.02	7.34±0.05	4.60±0.02	7.49±0.01	4.82±0.08	7.34±0.04

Data expressed as "mean ± standard deviation" (n=2).
Detection limit ≤ 1 $\log_{10}$ CFU·mL^{-1}

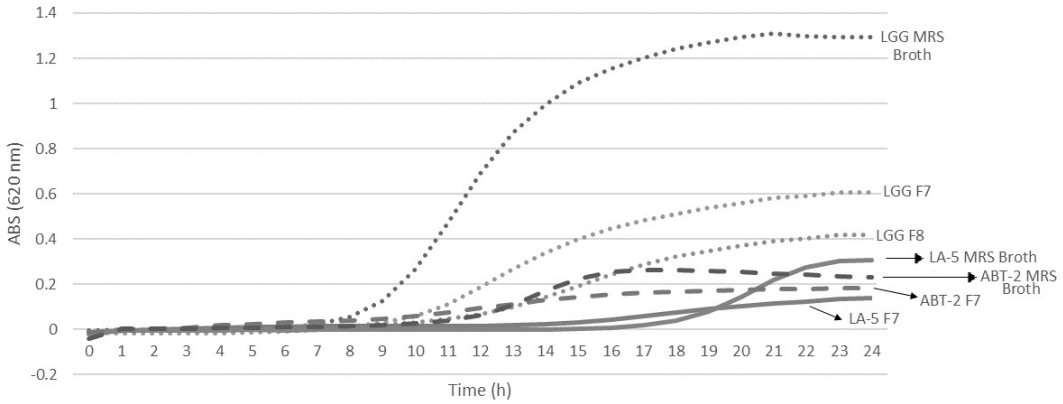

Figure 2. *Growth of LGG, LA-5, and ABT-2 during fermentation (37°C, 24 h)*

According to the results obtained so far, F7 formulation (apple juice + cucumber extract + carrot extract; 1 : 1 : 1, v/v/v) seems to be capable of promoting the growth of probiotic strains during the fermentation process. *L. acidophilus* utilizes sugars such as glucose, fructose, sucrose, lactose, and galactose in order [22]. Carrot juice (pH 6.2 ± 0.5) contains sucrose, glucose, and fructose as a carbon source, carotenoids which promote the growth of probiotics, and some vitamins and minerals that could also contribute to growth [23]. By evaluating these preliminary data, F7 and F8 formulations for LGG and F7 formulation for LA-5 and ABT-2 were selected as proper mediums for the determination of viability during cold storage. In addition, F9 formulation (apple juice + cucumber extract + carrot extract (1 : 1 : 1 v/v/v) + 2 % FOS + 4 % lactitol) was also designed for cold storage study to evaluate synergistic effect of FOS and lactitol.
The strains favored carrot extract when compared with F7 and F8 formulations. F8 formulation contains FOS and lactitol which can promote the growth of *Bifidobacterium* and *Lactobacillus* [24]. However, LA-5 and ABT-2 could not survive during fermentation. Nazzaro [25] investigated the effects of inulin and FOS on the growth of *L. rhamnosus* and *L. bulgaricus* which were inoculated in carrot juice. They reported that, despite adding FOS to the carrot juice, the strains reached the same counts (~ 9

logs) both in carrot juice with and without FOS after incubation and 4 weeks storage (4 °C). Carrot juice was also an appropriate medium for the growth of Bifidobacterium strains (*B. lactis* Bb-12, *B. lactis* B7.1 and B3.2). Their count reached ~ 10^8 CFU·mL^{-1} in carrot juice after incubation (37 °C, 24 h) [23].

The viability of probiotic strains was evaluated before and after fermentation and after 7, 14, 21, and 28 days of storage at 4 °C (Table 3). Probiotic strains were inoculated into F7 and F9 formulations to evaluate the count during storage. In addition, LGG also was inoculated into F8 formulation because when F3 and F8 formulation were compared, the addition of FOS and lactitol increased LGG counts from 5.92 ± 0.10 to 7.90 ± 0.01 logs CFU·mL^{-1} during fermentation.

Table 3. *LGG, LA-5, and ABT-2 counts (log CFU·mL^{-1}) in different formulations during fermentation (37 ºC, 24 h) and cold storage (4 °C)*

Strain	Formulation	0 h	24 h	7 days	14 days	21 days	28 days
LGG	**F7**	5.79±0.22	8.32±0.05	8.10±0.09	8.05±0.06	8.24±0.00	8.20±0.14
	F9	5.77±0.23	8.30±0.06	8.15±0.12	8.15±0.05	8.17±0.06	8.13±0.02
	F8	5.72±0.34	7.68±0.20	7.33±0.17	7.36±0.09	7.29±0.26	7.30±0.08
	MRS Broth	5.84±0.34	8.82±0.02	8.31±0.15	7.61±0.92	5.45±0.06	3.95±0.73
LA-5	**F7**	5.69±0.57	7.79±0.07	6.53±0.59	4.52±0.61	2.38±0.87	0.85±0.15
	F9	5.66±0.63	7.56±0.01	6.60±0.34	5.02±0.35	3.69±0.37	1.13±0.04
	MRS Broth	5.77±0.52	6.77±0.59	5.56±0.26	4.73±0.16	2.35±0.07	0.77±0.08
ABT-2	**F7**	5.37±0.70	7.76±0.06	6.64±0.61	5.12±0.65	3.88±0.19	2.89±0.19
	F9	5.48±0.58	7.55±0.16	6.71±0.41	5.51±0.12	4.50±0.17	3.04±0.04
	MRS Broth	5.48±0.54	6.83±0.04	5.62±0.16	5.07±0.68	3.14±0.29	2.50±0.35
ABT-2 (M17)	**F7**	5.35±0.61	7.77±0.09	7.30±0.13	6.42±0.70	6.42±0.12	5.56±0.44
	F9	5.37±0.59	7.57±0.11	7.19±0.08	6.22±0.47	5.75±0.84	5.13±0.39
	MRS Broth	5.32±0.67	7.06±0.38	6.67±0.60	5.45±1.02	4.62±0.62	3.35±0.65

Data expressed as "mean ± standard deviation" (n=2).

LGG counts increased during fermentation from ~5 to 8.32 ± 0.05 log CFU·mL^{-1} and 8.30 ± 0.06 log CFU·mL^{-1} for F7 and F9 formulations, respectively. Although F9 formulation also contains 2 % FOS + 4 % lactitol, which is different from F7 formulation, LGG counts were the same for both after fermentation. For F8 formulation, the count was determined as 7.68 ± 0.20 log CFU·mL^{-1} after fermentation. The count of LGG increased from 7.68 ± 0.20 to 8.30 ± 0.06 due to the addition of carrot extract. LGG strain maintained its count (~ 8 log CFU·mL^{-1}) constantly during cold storage. After 28 days of storage, LGG formulations provide the required viability as a probiotic product which must contain at least 6 logs CFU·g^{-1} probiotic microorganism [26]. The count of LGG inoculated into orange juice was determined as 7.90 ± 0.2 log CFU·mL^{-1} after storage at 4 ºC for 12 weeks [27].

Though LA-5 and ABT-2 strain increased their count to approximately 8 log CFU·mL^{-1}, decreases were observed up to ~ 1 log CFU·mL^{-1} for LA-5 strain and ~ 3 log CFU·mL^{-1} for ABT-2 strain during cold storage. LA-5 and ABT-2 strains lost their viabilities about 80 % and 40 %, respectively, during storage. Whereas *Streptococcus thermophillus* loads in the ABT-2 inoculated formulations slightly decreased after storage. The decreases in the count of viable bacteria may result from the acidic environment of beverage, presence of oxygen, and low levels of nitrogenous compounds. The *p*H of F7-F9 formulations ranged from 3.90 ± 0.01 to 4.02 ± 0.02 after fermentation (Table 4).

Probiotic cells need more energy to maintain their intracellular *p*H below *p*H 4.5. Using energy for *p*H adaptation causes a lack of ATP which is required for the other vital functions, so cell death occurs [28]. The count of *L. acidophilus* inoculated into orange juice significantly ($P < 0.05$) decreased from ~ 7 log CFU·g^{-1} to ~ 5 log CFU·g^{-1} after storage at 4 ºC for 4 weeks [29].

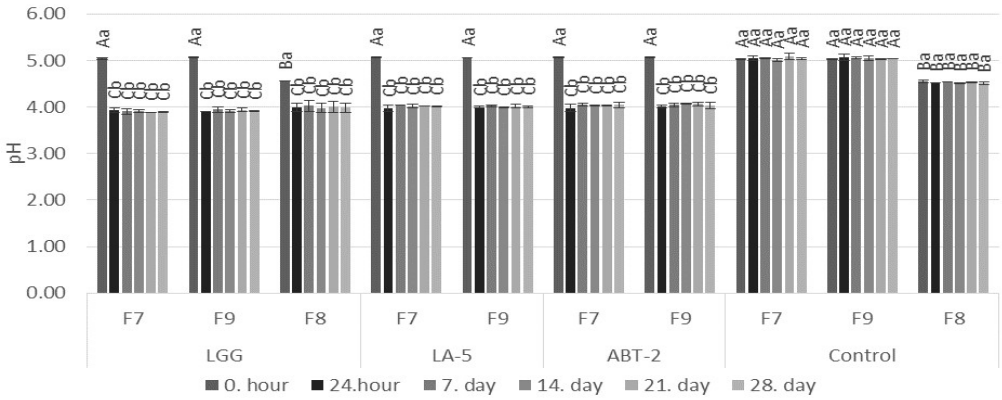

Figure 3. *pH values of selected formulations during fermentation and cold storage*
Capital letters (A, C) indicate the significant differences between formulations on the same day ($P < 0.05$). Lower case letters (a, b) indicate the significant differences between different days of the same formulations ($P < 0.05$)

Physicochemical parameters of formulations during the fermentation process and cold storage

Physicochemical parameters such as *p*H, TA, TSS, the color, TPC, CUPRAC of selected formulations during fermentation, and cold storage were determined to evaluate the stability of formulations.

*p*H has been reported [30] to be one of the limiting factors for the increase in lactic acid bacteria load. The other factors affecting the growth are the chemical composition of the fruit juices, the presence of nutrient and inhibitory compounds in the medium, and the adaptation ability of the strains to the medium [31]. Changes in *p*H values of formulations both before and after the fermentation process and during storage were given in Figure 3. According to the results, there is no significant difference between the initial *p*H values of F7 and F9 formulations and their controls which range between 5.03 ± 0.02 and 5.07 ± 0.01 ($P > 0.05$). The initial *p*H values of F8 formulation (4.56 ± 0.01) and control of F8 (4.54 ± 0.03) were lower than the other formulations ($P < 0.05$) because of the addition carrot extract (*p*H ~ 6.5) increased the initial *p*H values of F7 and F9 formulations.

The optimum *p*H range for the development of lactic acid bacteria has been reported as 5.5 – 6.0 [32]. Although the initial *p*H values of formulations were below the reported optimum *p*H, a significant decrease was observed in the *p*H of the formulations after fermentation at 37 °C, for 24 hours ($P < 0.05$) and were determined in the range of 4.02 ± 0.02 - 3.90 ± 0.01. The *p*H reduction in the product is important because it positively affects shelf life and prevents further food contamination during storage [33]. Similarly, the *p*H of apple juice inoculated with *L. plantorum* NCIMB 8826 decreased from 5.05 ± 0.02 to 4.32 ± 0.05 during 72 hours of fermentation at 30 °C [34]. The *p*H reduction

during the fermentation process indicates the activity of microorganisms and their metabolism products, mainly, organic acids [7]. During fermentation, lactic acid bacteria convert malic acid into D- and L-lactate and CO_2 [35]. Rhamnosus is a heterofermentative facultative anaerobic bacterium and may have the ability to produce acetic acid as well as lactic acid [7]. *L. acidophilus*as an obligate homofermentative one produces mainly lactic acid [1]. Unlike Lactobacilli, Bifidobacteria ferment the glucose (1 mol) to lactic acid (1 mol), and acetic acid (1.5 mol) through the Bifidus pathway [36]. Thus, lactic acid and acetic acid contents of the formulations mainly increased according to the inoculated starter types and the *p*H values of formulations decreased during fermentation.

The initial total acidity of the formulations was 0.18 ± 0.01 g Lactic acid·100 mL^{-1} as seen in Figure 4. Significant increases were observed in the acidity of all formulations after fermentation ($P < 0.05$) and the results varied from 0.32 ± 0.05 to 0.51 ± 0.01. No changes were observed in control formulations ($P > 0.05$). While initial TSS values were between 6.50 ± 0.60 and 6.95 ± 0.15 °bx for F7 formulation, they ranged between 11.50 ± 0.10 and 11.93 ± 0.02 °bx for F8 and F9 formulations because of the addition of FOS and lactitol (Figure 5). Even if there was no significant difference in TSS values before and after fermentation ($P > 0.05$) expected, increases of lactic acid were seen because of the consumption of sugars in the formulations by strains. There was also no significant difference in TSS values during cold storage for any of the formulations ($P > 0.05$).

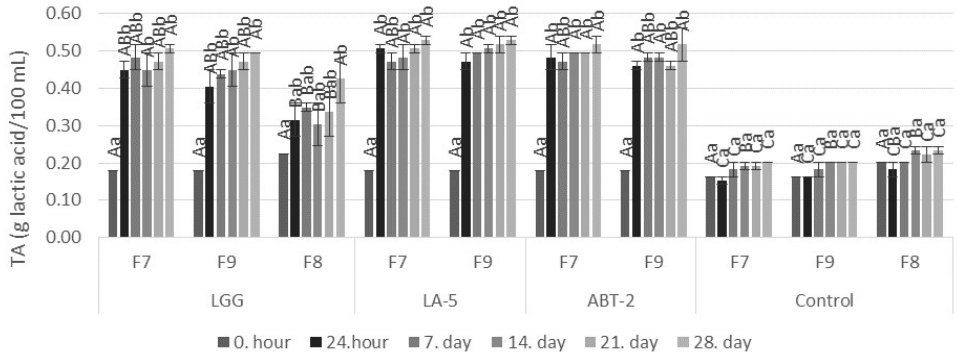

Figure 4. *Total acidity (%) of selected formulations during fermentation and cold storage*

Capital letters (A, C) indicate the significant differences between formulations on the same day ($P < 0.05$). Lower case letters (a, b) indicate the significant differences between different days of the same formulations ($P < 0.05$)

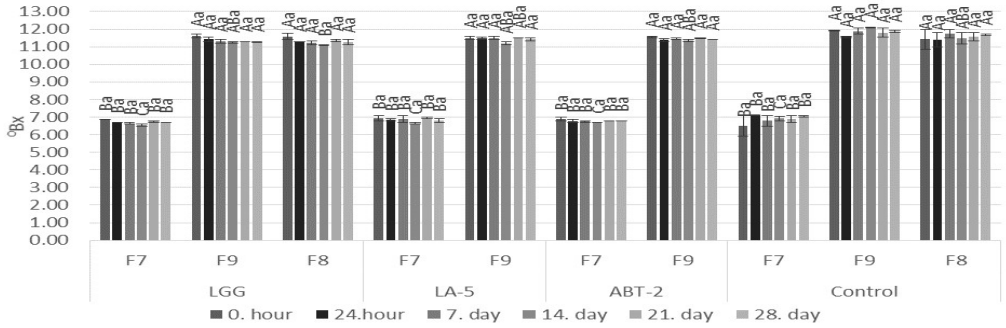

Figure 5. *Total soluble solids (°bx) of selected formulations during fermentation and cold storage*

Capital letters (A, C) indicate the significant differences between formulations on the same day (P < 0.05). Lower case letters (a, b) indicate the significant differences between different days of the same formulations (P < 0.05)

Color is one of the important quality criteria for beverages and changes in color values have a considerable effect on consumer appreciation. The effects of the fermentation and storage period on the color values (L^*, a^*, and b^*) of the formulations are given in Figures 6, 7, and 8, respectively. It was found that L^* (lightness) values did not change during the fermentation process ($P > 0.05$), but the decrease of L^* value was observed as the storage time increased for all formulations and their controls. The fixed effects of storage time as a statistical factor on L^* values were significant ($P < 0.0001$). The lowering of lightness in all formulation during cold storage could be a result of the browning processes due to the activation of particular oxidases, such as polyphenol oxidase occurring during fermentation in the presence of trace oxygen [37] a^* values (red color) did not change during fermentation ($P > 0.05$) and there are non-significant increases during storage for all formulation and their controls ($P > 0.05$).

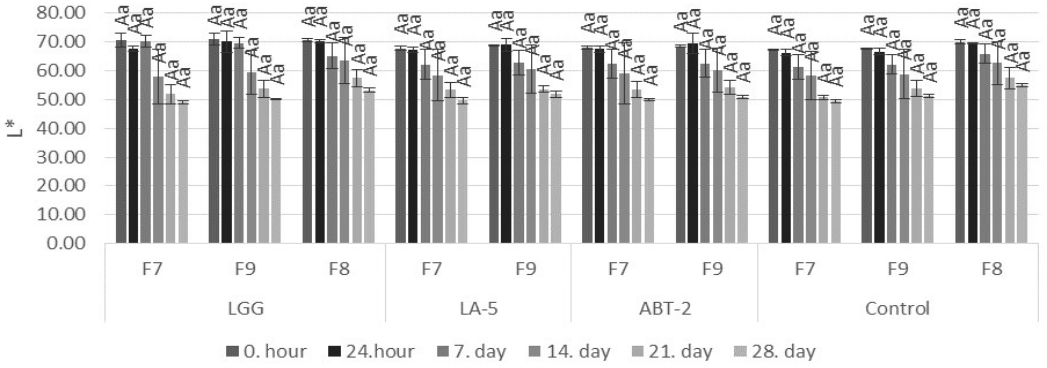

Figure 6. *L* values of selected formulations during fermentation and cold storage*

Capital letters (A, C) indicate the significant differences between formulations on the same day (P < 0.05). Lower case letters (a, b) indicate the significant differences between different days of the same formulations (P < 0.05)

Also, our results show that b^* values (yellow color) did not change during the fermentation process and storage ($P > 0.05$). In general, the increase of a^* value in the fermented products may be due to the build-up of destructed bacteria cells [38]. Similar results were observed by da Costa [39]. L^* value of the orange-based beverage

produced by *L. paracasei* and oligofructose decreased, however, *a** value of the beverage increased during cold storage. Color values of acaí based smoothies inoculated with L. *acidophilus* LA3 were observed during 28 days of storage at 4 ºC. *L** values decreased significantly on day 14 of storage for the acaí based smoothies ($P < 0.05$). While *a** value decreased, the *b** value increased during storage ($P < 0.05$) [40].

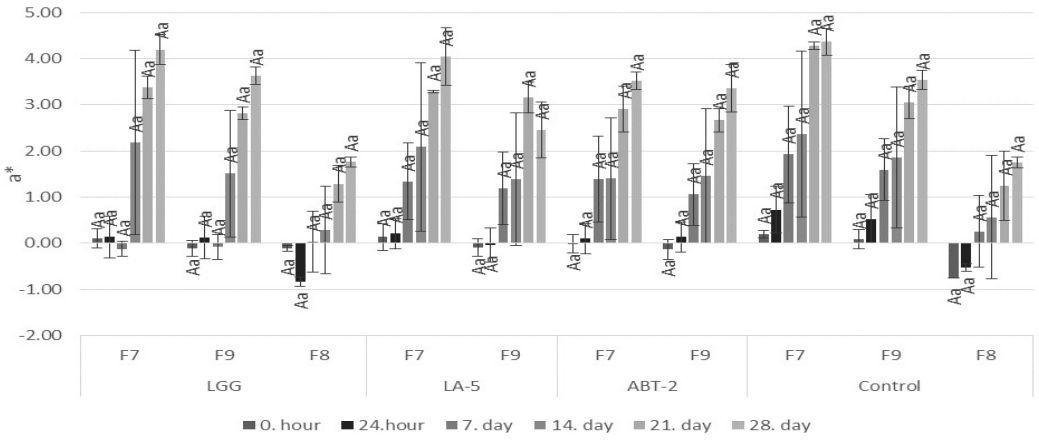

Figure 7. *a* values of selected formulations during fermentation and cold storage*
Capital letters (A, C) indicate the significant differences between formulations on the same day ($P < 0.05$). Lower case letters (a, b) indicate the significant differences between different days of the same formulations ($P < 0.05$)

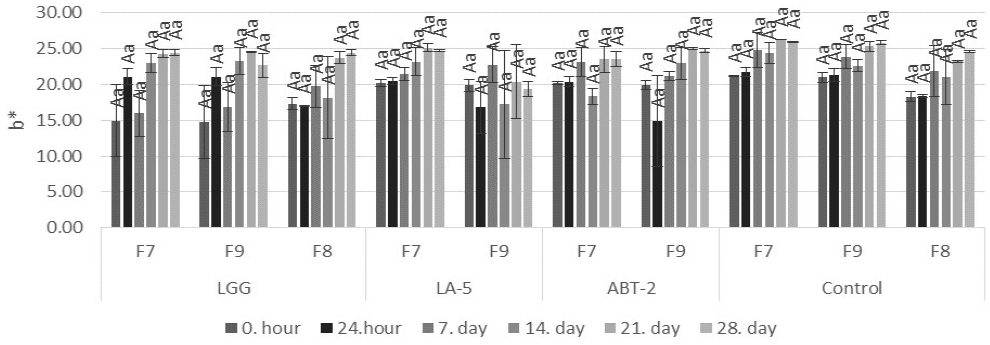

Figure 8. *b* values of selected formulations during fermentation and cold storage*
Capital letters (A, C) indicate the significant differences between formulations on the same day ($P < 0.05$). Lower case letters (a, b) indicate the significant differences between different days of the same formulations ($P < 0.05$)

Antioxidant properties of the samples were investigated using total phenolic (Figure 9) and CUPRAC (Figure 10) analysis before and after fermentation and during storage. When the results of the inoculated formulations were examined, it was found that there is no significant change during fermentation ($P > 0.05$) in both total phenolic content and CUPRAC analysis. Finding no significant change in antioxidant properties after fermentation may be due to a short fermentation time (24 h). Yan [41] observed that the antioxidant activity of blueberry pomace liquid inoculated with Lactobacillus strains (*L. rhamnosus* GG, *L. plantarum*-1, and *L. plantarum*-2) did not increase significantly during fermentation ($P > 0.05$). The CUPRAC values of the mango slurry inoculated with *L. casei* also did not change significantly during fermentation (48 h, 37 °C) [42].

Although it seems like there were increases in antioxidant properties of all formulations (F7-9) over storage, no significant change was observed in terms of total phenol and CUPRAC values in all formulations ($P > 0.05$; Figures 9 and 10). Similarly, Mauro [43] inoculated *L. reuteri* into a blueberry and carrot blend and determined the total phenolic content and antioxidant activities of samples during 28 days of storage. Although total phenolic content increased from 1122.7 ± 9.21 to 1209.8 ± 3.03 mg GAE·L^{-1}, they found non-significant differences ($P > 0.05$). While the ABTS+ value increased during storage ($P < 0.05$), the DPPH value did not show significant differences ($P > 0.05$). On the other hand, total phenolic contents of orange juice and orange juice mixed with nettle (*Urticadioica* L.) inoculated with LGG ATCC 53103 were monitored for 28 days [44]. Samples had the maximum phenolic content (1211 ± 135.10 mg·GAE·L^{-1} and 1225 ± 19.09 mg·GAE·L^{-1}) on the 8th day of storage and decreased significantly afterward ($P < 0.05$). Mantzourani [45] also outlined that the total phenolic content of pomegranate juice fermented by *Lactobacillus Plantarum* ATCC 14917 reached its maximum level at 2 weeks of cold storage. The reason for these increases in phenolic content might be due to the release of phenolic compounds from plant tissues that decompose during fermentation [46]. Moreover, lactic acid bacteria could minimize the formation of reactive oxygen species due to their enzymatic and non-enzymatic antioxidant mechanisms [47]. Some bacteria can produce *β*-galactosidase which catalyzes the release of phenolic compounds from bound sugars, so the antioxidant activity increases [45].

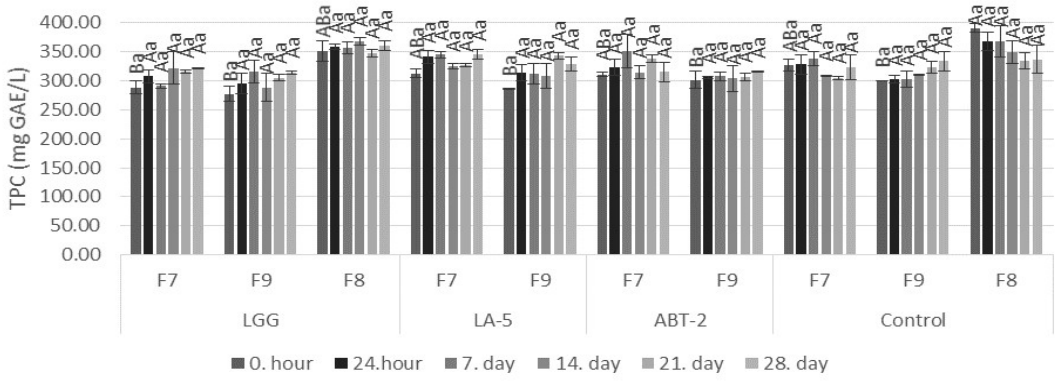

Figure 9. *Total phenolic content (mg·GAE·L^{-1}) of selected formulations during fermentation and cold storage*

Capital letters (A, C) indicate the significant differences between formulations on the same day ($P < 0.05$). Lower case letters (a, b) indicate the significant differences between different days of the same formulations ($P < 0.05$)

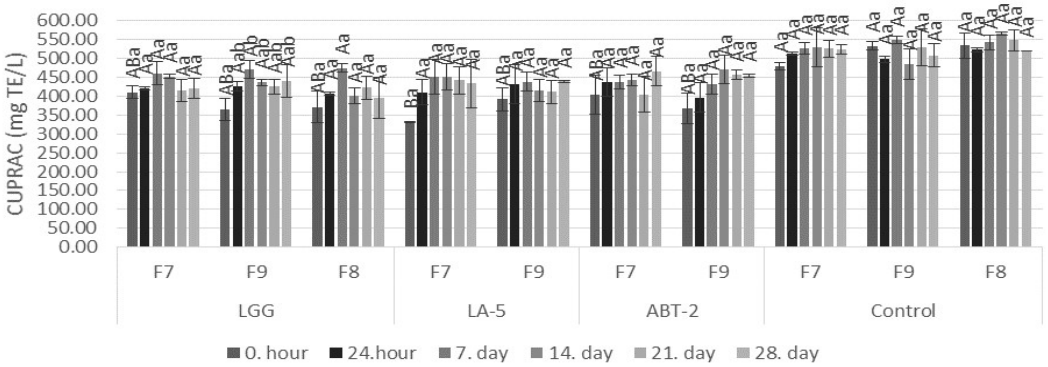

Figure 10. *CUPRAC values (mg TE·L^{-1}) of selected formulations during fermentation and cold storage*

Capital letters (A, C) indicate the significant differences between formulations on the same day (P < 0.05). Lower case letters (a, b) indicate the significant differences between different days of the same formulations (P < 0.05)

Sensory evaluation of formulations

Formulations with commercial potential were identified according to their probiotic viability (above 6 logs CFU·mL^{-1}) and physicochemical properties. After the storage period, only the LGG strain maintained its count (~ 8 logs CFU·mL^{-1}) constantly during cold storage (28 days). Sensory evaluation of F7-9 formulations inoculated with LGG strain was observed to keep customer appreciation (Figure 11). For this purpose, formulations were evaluated in terms of color, flavor, and general acceptability by 47 (15 males and 32 females) panelists between the ages of 20 - 40. The 7-point hedonic scale was used for scoring formulation and the panelists were asked to choose their favorite formulations. As Figure 11 illustrates, the sensorial acceptability of F7-9 inoculated with LGG in terms of color, flavor, and general acceptability did not change significantly (P > 0.05). The color scores varied between 4.56 ± 0.17 to 4.70 ± 0.17, and flavor scores varied between 3.80 ± 0.21 to 4.39 ± 0.21. In terms of general acceptability, F8 formulations received the highest score (4.31 ± 0.17). Only non-significant differences were found between males and females in terms of gender effect on scores (P > 0.05). However, sensorial scores located neither dislike nor liked part of the 7-point hedonic scale.

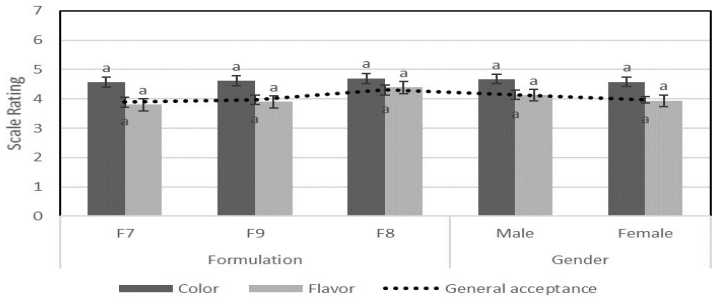

Figure 11. *Sensory evaluation of formulations inoculated with LGG strain using a 7-point hedonic scale*

Lower case letters (a, b) indicate the significant differences between the formulations and gender (P < 0.05)

A similar result was reported by Freire *et al* [48] who formulated a cassava-based beverage using LAB along with yeasts, and the consumers gave the "neither disliked nor liked" score to this beverage. Therefore, the formulations need to be improved for taste with flavorings and sweeteners. Luckow and Delahunty [49] show that tropical fruit juice addition (10 %, v/v), especially pineapple, mango, or passion fruit juices to a probiotic beverage covered the noticeable off-flavors. Among the formulations, the F9 was preferred in the first place with a ratio of 40.4 % and was followed by F8 (36.2 %). It was also indicated that additions of FOS and lactitol to F9 and F8 improved their taste and consequently sensorial perception.

CONCLUSIONS

Non-dairy probiotic beverage formulations (F1-8) containing various fruit and vegetable extracts and prebiotics were developed. The formulations were inoculated with various probiotic strains (LGG, LA-5, and ABT-2). After probiotic fermentation, successful formulations (F7-8) with viability greater than 7-8 logs were identified. A storage study was carried out with these formulations (F7 and F8) and F9 was designed in cold conditions (+4 °C) for 4 weeks. At the end of the storage, only LGG strain survived over 7 - 8 logs. Other strains (LA-5 and ABT-2) could not show enough vitality during cold storage. All formulations preserved their total phenolic content and CUPRAC values during production and storage. The sensory analysis showed that the consumers liked the products in terms of the color attribute but focused on the "neither like it nor not" score in terms of flavor. In this regard, the flavor profiles of these formulations need to be further improved. However, in order to enhance the flavors of these formulations, which are enriched with probiotics and prebiotics, it is suggested that natural flavoring substances should be added to the product formulation.

ACKNOWLEDGEMENT

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