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Production of Probiotic Drink Using *Pussy willow* and *Echium amoenum* Extracts

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Abstract

Background and Objective: Nowadays, due to the lack of lactose and cholesterol, demand for consumption of non-dairy probiotic products is increasing. Probiotic drinks mixed with medicinal plant have great beneficial effect on human health. The main problems of non-dairy probiotic drinks are lack of nutrients for the growth of probiotics and bad taste of the product. The aim of this study was to produce a probiotic medicinal plant drink with favorable physicochemical, viability and sensory properties.

Material and Methods: Probiotic drink prepared by *Pussy willow* and *Echium amoenum* extract (0.5 % w v^{-1}, for each extract or together), *Lactobacillus casei* and *Lactobacillus rhamnosus* (10^8 CFU ml^{-1}), individually and their combination. Glucose and whey powder (0.2%) were used as a source of nutrition for the probiotics, and apple juice (20 and 30%) was added to improve the taste of drink. The level of glucose was adjusted to reach the brix of 13 g100 g^{-1}. Ascorbic acid (0.05%) was used to improve micro-aerophilic conditions. The pH, acidity, glucose and viability of probiotic bacteria as well as the sensory properties of the prepared drink were investigated during 28 days at 4° C.

Results and Conclusion: Based on the results, the treatment containing *L. casei*, *Pussy willow*, *Echium amoenum* and 30% apple juice due to the highest probiotic viability and the treatment containing *Lactobacillus rhamnosus*, *Pussy willow*, *Echium amoenum* and 30% apple juice because of higher total acceptance score, proper pH and acidity values were selected as the best treatments.

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1. Introduction

Probiotics are known as living microorganisms that in sufficient amounts balance the microbial flora of host. It has been documented that foods containing probiotic microorganisms help the survivability of indigenous intestinal microbes and balance its micro flora, thereby providing many health benefits [1]. The current highly marketed probiotic products are commonly milk and yogurt, which have some limitations in consumption by people for having high blood cholesterol content [2,3]. In recent years, demand for non-dairy probiotic products has been growing leading to production of products including probiotic drinks. Fruit- and vegetable-based drinks are rich in functional nutrients including minerals, vitamins, fiber and antioxidants [2]. In addition, most juices contain oxygen inhibitors such as ascorbic acid, which improve micro-aerophilic conditions for the growth of probiotics [4]. The problems of non-dairy probiotic drinks are undesirable taste and lack of sufficient nutrients for the growth of probiotic microorganisms. By using juices with strong flavors and fragrances such as mango and pineapple juices, unfavorable taste of non-dairy probiotic drinks could be covered [5]. Sugar and whey could be used in order to enhance the nutrients of probiotics [4,6]. Apple and apple juice are known as health promoters due to their bioactive components such as polyphenols, pectin and organic acids [7]. Pereira et al. investigated probiotic apple juice fermented by *Lactobacillus* (*L.*) *casei*. The results showed that *L. casei* grows during the refrigerated storage. Viable cell counts were higher than 8 log CFU ml^{-1} throughout the storage period (42 days) [8]. A mixed juice of apple, pear and raspberry was inoculated with *L. rhamnosus*, and its viability was measured during storage at 2-7° C for 2-4 w under the consumption conditions. Their results showed good viability of *L. rhamnosus* [9].

Whey is one of the most important compounds for promoting the growth of probiotics. Since the amount of essential amino acids in whey proteins is higher than casein, probiotics possessing proteolytic activity (e.g. Lactobacillus) use directly nitrogenous sources. The addition of whey protein concentrate improves the survivability of Lactobacillus. It is known that free amino nitrogen provides the nutrients required by probiotics and activates decarboxylase for Lactobacillus [6]. Another important growth promoter for probiotics is glucose. Marhamatizadeh et al. studied the manufacture of probiotic apple and orange drink with *L. acidophilus* and Bifidobacterium (*B.*) *bifidum.* Milk, maltose, lactose and glucose were then added to the drink. The results depicted that glucose and lactose had significant effect on its extended storage life [10]. The amount of viable cell count of probiotics in the product should be 10^6-10^7 (CFU g^{-1}) to be able to exert the healthful effect [11].

L. casei is an important type of probiotics. It is also used for industrial production of lactic acid derived from whey by cell immobilization on supports such as agar and polyacrylamide. This bacterium illustrates suitable vancomycin resistance and the highest viability in dairy fermented products. *L. rhamnosus* is another important probiotic widely used in food products for its acid resistance in the digestive system [12]. The most commonly used probiotic bacteria include *L. casei*, *L. rhamnosus*, *L. acidophilus* and *L. plantarum* [13]. According to Champagne, *L. casei, L. rhamnosus* and *L. plantarum* enjoy better viability in vegetables containing drinks during fermentation [14].

Today medicinal plants, including *Pussy* (*P.*) *Willow* and *Echium* (*E.*) *amoenum* are in wide use, and because of negative side effects of chemical medicines, people are increasingly interested in herbs [9]. *Pussy willow* contains salicin mostly in its bark. The *P. willow* extract may reduce blood sugar and have a laxative effect. It may enhance the function of heart and nervous system and reduce pain and cerebral disorders [15]. Human body is unable to synthesize essential fatty acids, so they should be supplied through foods and supplements. Iranian *E. amoenum* oil may be introduced as a potential source of fatty acids such as alpha-linolenic acid (ALA) and gama-linolenic acid (GLA) [16]. Jahandideh et al. studied an *E. amoenum*-based drink fermented by four strains of Lactobacillus. The results revealed that *E. amoenum* extract was a suitable medium for the growth of lactic bacteria and production of functional drinks [17].

Unfortunately, the information about the survival of probiotic microorganisms in ideal substrate and the sensory properties of non-dairy drinks, especially medicinal plants drink are not sufficient. Therefore, the objective of this study was to produce a probiotic medicinal plant drink using *P. willow* extract, *E. amoenum* extract, glucose, ascorbic acid, whey powder and apple juice through inoculating with *L. casei* and *L. rhamnosus.* The pH, acidity, glucose and viability of probiotic bacteria and the sensory properties of the prepared drink were investigated during 28 days at 4° C.

2. Materials and Methods

2.1. Materials

L. rhamnosus (PTCC 1637) and *L. casei* (PTCC 1608) were purchased from Iranian Research Organization for Science and Technology (Tehran, Iran). To produce the probiotic drink, apple juice concentrate manufactured by Behnoush Iran Co. (Tehran, Iran) with brix 69 (g 100 g^{-1}) was used. The glucose (Brix 80.08 g 100 g^{-1}) used in this study was obtained from Glucozan Company (Tehran, Iran). Whey powder was obtained from Maybe Company (Turkey). All chemical materials and media were purchased from Merck (Germany) *P. willow* and *E. amoenum* extract were supplied from Iran Golab (Kashan, Iran).

2.2. Methods

2.2.1. Lactic Acid Bacteria Cultures

The strains were added to MRS-broth, and the test tubes containing both strains were incubated. *L. casei* and *L. rhamnosus* were incubated at 30 and 37°C, respectively, for 48 h. Then microbial suspension was prepared to obtain a bacterial dilution. To do so, MRS–broth containing the bacteria was centrifuged (1792× g, 20 min). The bacteria were enumerated by pour plate method [18].

2.2.2. Preparation of Probiotic Drink

Probiotic drink was prepared by the method of Marhamatizadeh et al. with some minor variations [10]. Apple juice concentrate (in 20 and 30% concentrations) with no preservatives was mixed with distilled water. Glucose was added to all the treatments to bring the brix of the product to 13 g 100 g^{-1}. Next, whey powder (0.2% w w^{-1}) and ascorbic acid (0.05% w w^{-1}) were added to all the treatments at the same concentrations, and then *Pussy willow* and *E. amoenum* extracts (0.5% w v^{-1}, each or together) were added (level of extracts accepted by consumers considering the pre-tests conducted by Iran Behnoush Iran Co). Finally, all the treatments were pasteurized at 95° C for 5 min and cooled down to 4°C followed by 10^8 CFU ml^{-1} or (100%) inoculation of *L. casei,* and *L. rhamnosus* individually, and (10^4+10^4) CFU ml^{-1} or (50%+50%) inoculation of *L. casei,* and *L. rhamnosus* in combination. After inoculation of the medicinal plant drink with probiotic bacteria, the samples were incubated at 37° C for 48 h.

2.2.3. Analysis

The pH value and acidity (g 100 g^{-1}) were measured according to Daneshi et al. method [19]. Glucose (mg dl^{-1}) was measured by glucose kit (Pars Azmoon, Iran) by the GOD-PAP method (enzymatic colorimetric test) using

spectrophotometer (Lange Hack, USA) [20]. Sensory test including total acceptance was conducted by 9-point hedonic method on 28 days by a group of trained panelists including 10 members [21]. Probiotic bacteria were enumerated by pour plate method with the use of MRS agar according to the method of Nematollahi et al. [22]. Mold and yeast were measured according to Alexopoulos and Mims [23].

2.2.4. Treatments Design

The treatments were performed in full factorial design (FFD). Three variables including A: kind of microorganism(s) treatment (in 3 subgroups (levels) including A1: 100% *L. rhamnosus*, A2: 100% , *L. casei* and A3: 50% *L. rhamnosus* + 50% *L. casei*), B: kind of extract treatment (in 3 levels including B1: 100% *Pussy willow*, B2: 100% *E. amoenum* and B3: 50% *Pussy willow* + 50% *E. amoenum*), and C: apple juice concentration (in 2 levels including C1: 20% of product and C2 : 30% of product) were selected based on our preliminary study. Consequently, 18 treatments (3×3×2) were developed by Minitab 14 software for variable evaluation. In addition, 6 control treatments (without probiotic bacteria) were compared with the FFD developed treatments. All treatments (18 runs) and controls (6 runs) are shown in Table 1.

2.2.5. Statistical Analysis

The data obtained from the measurements were subjected to analysis of variance (ANOVA) to determine the significant differences among the samples, and the values were compared using the Tukey's test defined at $p \leq 0.05$. All measurements were carried out in triplicate and reported as the mean±SD. The data analysis was performed using MINITAB 14 (MINITAB Inc., State College, PA and USA).

Table1. Experimental design for the production of probiotic medicinal plant drink

Run	Kind of microorganism	Kind of extract	Apple juice concentration
1	A1	B1	C2
2	A1	B2	C2
3	A1	B3	C2
4	A2	B1	C2
5	A2	B2	C2
6	A2	B3	C2
7	A3	B1	C2
8	A3	B2	C2
9	A3	B3	C2
10	A1	B1	C1
11	A1	B2	C1
12	A1	B3	C1
13	A2	B1	C1
14	A2	B2	C1
15	A2	B3	C1
16	A3	B1	C1
17	A3	B2	C1
18	A3	B3	C1
19	-	B3	C2
20	-	B1	C2
21	-	B2	C2
22	-	B3	C1
23	-	B1	C1
24	-	B2	C1

· A1*: L. rhamnosus* (100%): inoculated with 10^8 (CFU ml^{-1})
A2*: L. casei* (100%): inoculated with 10^8 (CFU ml^{-1})
A3*:* L. *rhamnosus* + *L. casei* (50%+ 50%): inoculated with (10^4+ 10^4) CFU ml^{-1}
B1: 100% *P. willow* (0.5% of product)
B2: 100% *E. amoenum* (0.5% of product)
B3: 50% *Pussy willow* +50% *E. amoenum* (0.5% of product)
C1: Apple juice concentration (20%)
C2: Apple juice concentration (30%)
0.2% whey, 0.05% ascorbic acid have been added to all samples.
Glucose has been added to all samples to bring the brix of the product to 13 g100 g^{-1}

3. Results and Discussion

3.1. pH and Acidity

Results from pH and acidity measurement on 0, 7, 14, 21 and 28 days are shown in Tables 2 and 3, respectively. During the storage time, the acidity increased and pH decreased in all the treatments but the change of pH was not statistically significant. The trend of these changes in the control treatments (without probiotics) was milder than the probiotic samples. The reason could be sugar consumption by the probiotic bacteria resulting in more acid production and increased acidity. A significant difference in pH and acidity was clearly seen amongst all the probiotic samples with different probiotic species and formulation. Treatment 18 had the lowest pH and the highest acidity during storage time, possibly due to the different ability of the probiotic cultures to metabolize glucose. Mousavi et al. investigated the fermentation of pomegranate juice by *L. casei*, *L. delbrueckii*, *L. plantarum* and *L. paracasei*, and concluded that variation of pH during storage period was not insignificant ($p>0.05$) [24]. Yoon et al. produced tomato probiotic drink by using *L. acidophilus*, *L. plantarum*, *L. casei* and *L. delbrueckii*, and reported that *L. plantarum's* consumption of sugar is faster than that of other species, thus it produces more acid [25]. Another reason for pH drop and acidity increase in T18 can be its higher glucose content that is used more by probiotic bacteria, leading to more bacterial activity and acid production, thereby increasing acidity and reducing pH. Similarly, Karbasi et al. fermented date syrup by *L. rhamnosus* and *L. acidophilus*, and concluded that pH was dropped and acidity was increased over 50-h fermentation [26]. Probiotic bacteria can extend the shelf life of product through increase of acidity and production of antimicrobial compounds such as organic acid, hydrogen peroxide, and other bacteriocins. The probiotic bacteria existing in the intestine produce organic acids, which lead to increasing acidity and reducing pH with inhabitation effect on pathogenic bacteria [27].

Table 2. Results of pH in probiotic medicinal plant drinks and controls during storage

	Days				
Treatments	0	7	14	21	28
1	3.50 ± 0.19^{abcdA}	3.42 ± 0.19^{abcdeA}	3.32 ± 0.18^{abcdefgA}	3.27 ± 0.18^{abcdeA}	3.24 ± 0.18^{abcdeA}
2	3.45 ± 0.29^{abcdA}	3.38 ± 0.28^{abcdeA}	3.31 ± 0.28^{abcdefgA}	3.24 ± 0.27^{abcdeA}	3.21 ± 0.27^{abcdeA}
3	3.42 ± 0.09^{abcdA}	3.32 ± 0.09^{abcdeA}	3.23 ± 0.09^{bcdefgA}	3.20 ± 0.09^{bcdeA}	3.18 ± 0.08^{bcdeA}
4	3.42 ± 0.09^{abcdA}	3.35 ± 0.09^{abcdeA}	3.29 ± 10.29^{abcdefgA}	3.21 ± 0.09^{abcdeA}	3.15 ± 0.08^{cdeA}
5	3.41 ± 0.14^{abcdA}	3.33 ± 0.14^{abcdeA}	3.25 ± 0.25^{abcdefgA}	3.19 ± 0.13^{bcdeA}	3.16 ± 0.13^{bcdeA}
6	3.34 ± 0.09^{abcdA}	3.24 ± 0.09^{abcdeA}	3.19 ± 0.19^{defgA}	3.17 ± 0.08^{cdeA}	3.15 ± 0.08^{cdeA}
7	3.39 ± 0.04^{abcdA}	3.28 ± 0.04^{abcdeAB}	3.23 ± 0.04^{bcdefgAB}	3.20 ± 0.04^{bcdeB}	3.18 ± 0.04^{bcdeB}
8	3.40 ± 0.19^{abcdA}	3.28 ± 0.18^{abcdeA}	3.20 ± 0.18^{defgA}	3.19 ± 0.18^{bcdeA}	3.13 ± 0.17^{deA}
9	3.29 ± 0.09^{abcdA}	3.19 ± 0.09^{deA}	3.12 ± 0.08^{gA}	3.10 ± 0.08^{eA}	3.09 ± 0.08^{eA}
10	3.35 ± 0.04^{abcdA}	3.28 ± 0.04^{abcdeA}	3.22 ± 0.04^{cdefgA}	3.19 ± 0.04^{bcdeA}	3.17 ± 0.04^{bcdeA}
11	3.34 ± 0.14^{abcdA}	3.23 ± 0.13^{bcdeA}	3.18 ± 0.13^{efgA}	3.15 ± 0.13^{deA}	3.13 ± 0.13^{deA}
12	3.32 ± 0.18^{abcdA}	3.21 ± 0.18^{cdeA}	3.16 ± 0.17^{fgA}	3.14 ± 0.17^{eA}	3.11 ± 0.17^{deA}
13	3.32 ± 0.09^{abcdA}	3.26 ± 0.09^{abcdeA}	3.21 ± 0.09^{defgA}	3.15 ± 0.08^{deA}	3.11 ± 0.08^{deA}
14	3.33 ± 0.04^{abcdA}	3.25 ± 0.04^{abcdeAB}	3.20 ± 0.04^{defgAB}	3.17 ± 0.04^{cdeAB}	3.12 ± 0.04^{deB}
15	3.31 ± 0.18^{abcdA}	3.20 ± 0.18^{cdeA}	3.11 ± 0.17^{gA}	3.06 ± 0.17^{eA}	3.03 ± 0.17^{eA}
16	3.23 ± 0.13^{cdA}	3.18 ± 0.17^{deA}	3.09 ± 0.13^{gA}	3.07 ± 0.13^{eA}	3.05 ± 0.12^{eA}
17	3.24 ± 0.18^{bcdA}	3.19 ± 0.18^{deA}	3.13 ± 0.17^{gA}	3.11 ± 0.17^{eA}	3.09 ± 0.17^{eA}
18	3.22 ± 0.18^{dA}	3.17 ± 0.17^{eA}	3.08 ± 0.17^{gA}	3.05 ± 0.17^{eA}	3.02 ± 0.17^{eA}
19	3.83 ± 0.05^{aA}	3.81 ± 0.05^{aA}	3.80 ± 0.05^{aA}	3.76 ± 0.05^{aA}	3.74 ± 0.05^{aA}
20	3.82 ± 0.16^{abA}	3.80 ± 0.16^{abA}	3.79 ± 0.16^{abA}	3.74 ± 0.15^{abA}	3.71 ± 0.15^{abA}
21	3.82 ± 0.10^{abA}	3.80 ± 0.10^{abA}	3.78 ± 0.10^{abcA}	3.73 ± 0.10^{abA}	3.71 ± 0.10^{abA}
22	3.75 ± 0.21^{abcdA}	3.73 ± 0.21^{abcdeA}	3.72 ± 0.21^{abcdefA}	3.70 ± 0.20^{abcdA}	3.65 ± 0.20^{abcdA}
23	3.80 ± 0.05^{abcdA}	3.75 ± 0.05^{abcdA}	3.74 ± 0.05^{abcdeA}	3.70 ± 0.05^{abcdA}	3.69 ± 0.05^{abcA}
24	3.81 ± 0.05^{abcA}	3.77 ± 0.05^{abcA}	3.75 ± 0.05^{abcdA}	3.71 ± 0.05^{abcA}	3.69 ± 0.05^{abcA}

1- The results were expressed as mean ± SD.
2-$^{a-g}$ Means shown as small letters in each column are significantly different ($p\leq 0.05$).
3- $^{A-B}$ Means shown as capital letters in each row are significantly different ($p \leq 0.05$).
4- Design of treatments and controls is shown in Table 1.

Table 3. Results of acidity (g100 g^{-1}) in probiotic medicinal plant drinks and controls during storage

Treatments	Days				
	0	7	14	21	28
1	0.134 ± 0.007^{fB}	0.143 ± 0.008^{fB}	0.168 ± 0.009^{hAB}	0.191 ± 0.010^{iA}	0.193 ± 0.010^{gA}
2	0.134 ± 0.003^{fC}	0.151 ± 0.004^{efC}	0.175 ± 0.004^{ghB}	0.199 ± 0.005^{ghiA}	0.210 ± 0.005^{fgA}
3	0.140 ± 0.002^{efD}	0.161 ± 0.003^{efC}	0.182 ± 0.003^{fghB}	0.201 ± 0.004^{efghiA}	0.210 ± 0.004^{efgA}
4	0.140 ± 0.005^{fD}	0.180 ± 0.007^{deC}	0.209 ± 0.008^{cdefBC}	0.228 ± 0.009^{defghAB}	0.248 ± 0.010^{cdeA}
5	0.140 ± 0.005^{fD}	0.181 ± 0.007^{deC}	0.203 ± 0.008^{defgBC}	0.238 ± 0.010^{cdeAB}	0.255 ± 0.0010^{bcdA}
6	0.149 ± 0.004^{defD}	0.193 ± 0.005^{cdC}	0.212 ± 0.006^{cdefBC}	0.236 ± 0.006^{cdefAB}	0.240 ± 0.006^{defA}
7	0.166 ± 0.002^{bcdeD}	0.231 ± 0.003^{abC}	0.260 ± 0.003^{abB}	0.268 ± 0.003^{abcAB}	0.276 ± 0.003^{abcdA}
8	0.179 ± 0.010^{bcB}	0.194 ± 0.010^{cdB}	0.220 ± 0.012^{cdeAB}	0.251 ± 0.014^{bcdA}	0.263 ± 0.014^{bcdA}
9	0.172 ± 0.004^{bcdC}	0.199 ± 0.005^{cdB}	0.221 ± 0.006^{cdeB}	0.257 ± 0.007^{bcdA}	0.264 ± 0.007^{bcdA}
10	0.156 ± 0.002^{cdefD}	0.178 ± 0.002^{deC}	0.192 ± 0.002^{efghB}	0.192 ± 0.002^{hiB}	0.209 ± 0.002^{fgA}
11	0.158 ± 0.006^{cdefB}	0.177 ± 0.007^{deAB}	0.196 ± 0.008^{efghA}	0.201 ± 0.008^{fghiA}	0.207 ± 0.008^{fgA}
12	0.171 ± 0.009^{bcdB}	0.214 ± 0.012^{bcAB}	0.225 ± 0.012^{cdeA}	0.232 ± 0.013^{cdefgA}	0.250 ± 0.014^{cdA}
13	0.170 ± 0.004^{bcdD}	0.212 ± 0.006^{bcC}	0.235 ± 0.006^{cdBC}	0.257 ± 0.007^{abcdAB}	0.278 ± 0.007^{abcA}
14	0.169 ± 0.002^{bcdE}	0.217 ± 0.003^{bcD}	0.234 ± 0.003^{bcdC}	0.251 ± 0.003^{bcdB}	0.270 ± 0.003^{abcdA}
15	0.180 ± 0.010^{bcC}	0.230 ± 0.013^{abBC}	0.271 ± 0.015^{aAB}	0.281 ± 0.015^{abAB}	0.291 ± 0.016^{abA}
16	0.186 ± 0.007^{abC}	0.217 ± 0.009^{bcBC}	0.238 ± 0.010^{abcAB}	0.268 ± 0.011^{abcA}	0.271 ± 0.011^{abcdA}
17	0.174 ± 0.009^{bcC}	0.211 ± 0.011^{bcBC}	0.239 ± 0.013^{abcAB}	0.257 ± 0.014^{bcdAB}	0.272 ± 0.015^{abcdA}
18	0.210 ± 0.011^{aB}	0.253 ± 0.014^{aAB}	0.271 ± 0.015^{aA}	0.294 ± 0.016^{aA}	0.307 ± 0.017^{aA}
19	0.079 ± 0.001^{gC}	0.080 ± 0.001^{hBC}	0.082 ± 0.001^{iABC}	0.084 ± 0.011^{jAB}	0.085 ± 0.001^{hA}
20	0.079 ± 0.003^{gA}	0.081 ± 0.003^{hA}	0.083 ± 0.003^{iA}	0.084 ± 0.003^{jA}	0.085 ± 0.003^{hA}
21	0.079 ± 0.002^{gB}	0.081 ± 0.002^{hAB}	0.085 ± 0.002^{iAB}	0.089 ± 0.002^{jAB}	0.089 ± 0.002^{hA}
22	0.099 ± 0.005^{gA}	0.112 ± 0.006^{gA}	0.116 ± 0.006^{iA}	0.119 ± 0.006^{jA}	0.121 ± 0.006^{hA}
23	0.095 ± 0.001^{gC}	0.108 ± 0.001^{ghB}	0.112 ± 0.001^{iAB}	0.115 ± 0.001^{jA}	0.118 ± 0.001^{hA}
24	0.095 ± 0.001^{gD}	0.108 ± 0.001^{ghC}	0.112 ± 0.001^{iBC}	0.117 ± 0.001^{jAB}	0.119 ± 0.001^{hA}

1- The results were expressed as mean±SD.
2-$^{a-j}$ Means shown as small letters in each column are significantly different ($p\leq 0.05$).
3- $^{A-E}$ Means shown as capital letters in each row are significantly different ($p \leq 0.05$).
4- Design of treatments and controls is shown in Table 1.

Saw et al. produced tropical fruit drink using *L. acidophilus, L. casei, L. delbrueckii* and *L. bulgaricus,* and showed greater pH value drop at lower concentrations of the drink [28]. Also Guo et al. reported that water-based probiotic products showed greater and faster drop in pH value. Additionally, pH drop and acidity increase depend on the used probiotic species and are associated with the higher rate of growth in lactic fermentation, as the combination of *L. casei* and *L. rhamnosus* resulted in greater pH drop and acidity increase [29]. In agreement with our results, Jahandideh et al. produced an *E. amoenum*-based fermented drink using *L. paracasei, L. acidophilus, L. delbrueckii* and *L. plantarum*. The results showed that *L. paracasei* caused the most significant changes in pH and acidity [17].

3.2. Glucose Content

Glucose content was measured during 28 days at a 4ºC, and the results are shown in Table 4. The glucose content decreased during the storage time in all treatments. Samples inoculated with probiotics showed more dramatic decrease in glucose content than blanks, due to the use of glucose by the probiotic bacteria. In addition, bacterial strain has effect on glucose usage. Mousavi et al. fermented pomegranate drink using *L. casei, L. delbrueckii, L. plantarum* and *L. paracasei,* and measured the glucose content. The results revealed that *L. plantarum* and *L. delbrueckii* decreased the pH value at the initial hours of fermentation, and the consumption of glucose obviously increased [24]. Also Jahandideh et al. worked on a fermented drink based on *E. Amoenum* by *L. paracasei,*

L. acidophilus, *L. delbrueckii* and *L. plantarum.* Their results showed that all strains consumed glucose followed by fructose and saccharose as carbon source [17]. Wang et al. reported that glucose is an excellent energy source for Lactobacillus and Bifidobacteria [30]. Kun et al. studied fermentation of carrot juice by Bifidobacteria (*B. lactis* BB-12, *B. bifidum.* B 7.1, *B. bifidum.* B 3.2). During the fermentation, glucose and saccharose contents decreased significantly. However, fructose content did not change significantly [31]. According to the results, the highest and the lowest glucose contents were found for T22 (control) and T9, respectively. This could be due to different ability of the microorganisms in sugar consumption. Tantipaibulvut et al. investigated the fermentation of Roselle (belonging to Okra family) by lactic acid bacteria (*L. casei and L. plantarum*). They found out that glucose was more suitable than galactose as a carbon source for fermentation, since it showed faster acid production [32]. Thus, it could be said that the presence of glucose has a significant impact on the activity of probiotics.

Table 4. Results of glucose content (mg dl^{-1}) in probiotic medicinal plant drinks and controls during storage

Treatments	Days				
	0	7	14	21	28
1	9300 ± 395^{efghA}	8470 ± 359^{defAB}	7720 ± 328^{bcdB}	7520 ± 319^{cB}	7240 ± 307^{cB}
2	9240 ± 392^{fghA}	8410 ± 357^{defAB}	7660 ± 325^{bcdB}	7370 ± 313^{cB}	7200 ± 305^{cB}
3	9430 ± 133^{defghA}	8610 ± 122^{defB}	7950 ± 112^{bcdC}	7570 ± 107^{cC}	7530 ± 106^{cC}
4	9250 ± 523^{fghA}	8450 ± 478^{defAB}	7780 ± 440^{bcdAB}	7390 ± 418^{cB}	7120 ± 403^{cB}
5	9200 ± 390^{fghA}	8390 ± 356^{defAB}	7720 ± 328^{bcdB}	7330 ± 311^{cB}	7060 ± 300^{cB}
6	9300 ± 263^{efghA}	8470 ± 240^{defAB}	7790 ± 220^{bcdBC}	7380 ± 209^{cC}	7100 ± 201^{cC}
7	8250 ± 117^{hA}	7450 ± 105^{fB}	7110 ± 101^{dBC}	7000 ± 99^{cC}	6940 ± 98^{cC}
8	8610 ± 478^{ghA}	7760 ± 439^{efA}	7410 ± 419^{dA}	7140 ± 404^{cA}	6990 ± 395^{cA}
9	8060 ± 228^{hA}	7400 ± 209^{fAB}	6890 ± 195^{dB}	6780 ± 192^{cB}	6650 ± 188^{cB}
10	11080 ± 157^{abcdA}	10390 ± 147^{bcB}	9020 ± 128^{bC}	7840 ± 111^{cD}	7790 ± 110^{cD}
11	10940 ± 464^{abcdeA}	9700 ± 412^{bcdAB}	8780 ± 373^{bcBC}	7300 ± 310^{cCD}	7250 ± 308^{cD}
12	10530 ± 596^{bcdefA}	8610 ± 487^{defB}	7790 ± 441^{bcdB}	6830 ± 386^{cB}	6760 ± 382^{cB}
13	10940 ± 309^{abcdeA}	8750 ± 247^{defdB}	6950 ± 197^{dC}	6830 ± 19^{cC}	6730 ± 190^{cC}
14	10940 ± 155^{abcdeA}	8890 ± 126^{cdefdB}	6950 ± 98^{dC}	6800 ± 96^{cC}	6720 ± 95^{cC}
15	10390 ± 588^{cdefA}	8890 ± 503^{cdefA}	6970 ± 394^{dB}	6800 ± 385^{aB}	6720 ± 380^{cB}
16	10670 ± 453^{abcdefA}	9300 ± 395^{cdA}	6910 ± 293^{dB}	6800 ± 288^{cB}	6710 ± 285^{cB}
17	10670 ± 604^{abcdefA}	9160 ± 518^{cdeAB}	8060 ± 456^{bcdBC}	6820 ± 386^{cC}	6710 ± 380^{cC}
18	10120 ± 572^{cdefgA}	9300 ± 526^{cdAB}	7650 ± 433^{cdBC}	6820 ± 386^{cC}	6710 ± 380^{cC}
19	11150 ± 158^{abcA}	11060 ± 156^{abA}	10940 ± 155^{aA}	10790 ± 153^{bA}	10730 ± 152^{bA}
20	11240 ± 477^{abcA}	11200 ± 475^{abA}	11110 ± 471^{aA}	11020 ± 468^{abA}	10970 ± 465^{abA}
21	11160 ± 316^{abcA}	11050 ± 313^{abA}	10950 ± 310^{aA}	10900 ± 308^{abA}	10790 ± 305^{bA}
22	12330 ± 696^{aA}	12260 ± 694^{aA}	12230 ± 692^{aA}	12130 ± 686^{abA}	12080 ± 683^{aA}
23	12170 ± 172^{abA}	12130 ± 172^{aA}	12060 ± 171^{aA}	11980 ± 169^{abA}	11910 ± 168^{abA}
24	12320 ± 174^{aA}	12150 ± 172^{aA}	12060 ± 171^{aA}	11980 ± 169^{aA}	11930 ± 169^{abA}

1- The results were expressed as mean ± SD.

2-$^{a-h}$ Means shown as small letters in each column are significantly different ($p\leq 0.05$).

3- $^{A-D}$ Means shown as capital letters in each row are significantly different ($p \leq 0.05$).

4- Design of treatments and controls is shown in Table 1.

3.3. Viability

The results of viability of microorganism in probiotic medicinal plant drinks are shown in Table 5. There is a great challenge to find suitable microorganism and matrix for the growth of probiotics in non-dairy products. Probiotic viability depends on the type of probiotic bacteria, incubation temperature, food formulations, presence of live competitors, pH, oxygen levels, inhibitors, storage time, and temperature [2]. The number of initially inoculated probiotic bacteria (10^8 CFU ml^{-1}) increased during the incubation time (48 h, 37°C); therefore, significant differences were observed between the viability of probiotic bacteria in the medicinal plant drink on day 0. These differences may be due to the ability of probiotic microorganisms to grow on the medium during the incubation time. The highest and the lowest viabilities after 28 days of storage were observed on T6 (8.62 log CFU ml^{-1}) and T18 (7.70 log CFU ml^{-1}), respevtively, with 2.28 and 0.75 log cycle decreases as compared to day 0. This could be due to the more resistance of *L. casei* to the acidic medium as compared to *L. rhamnosus*, as well as the appropriate medium of *E.* extract for the growth of *L. casei*. This finding supports the idea of Jahandideh et al., who reported that *E.* extract is a suitable medium for the growth of *L. paracasei* [17]. Moreover, Fazeli et al. observed that the viability of *L. casei* was greater than *L. acidophilus*, *L. fermentum* and *L. plantarum* in watermelon drink [33].

Decrease of the viability was due to the consumption of glucose by the probiotic bacteria, resulting in increased acidity and reduced viability. It is to be noted the viability of probiotics after 28 days of storage was within the effective range (10^6 CFU ml^{-1}) in all the probiotic medicinal plant drinks; hence, it can be concluded that the *Pussy willow* and *E. amoenum* extracts were favorable media for the growth of *L. casei* and *L. rhamnosus*, and also for the resistance of *L. casei* and *L. rhamnosus* to acidic conditions. Consistent with our results, Sheehan et al. studied the resistance of lactic bacteria to acid as well as their resistance to the drink media. They examined the survivability of five species of Lactobacillus and Bifidobacterium in orange juice (pH 3.65), pineapple (pH 3.40) and cranberry (pH 2.5), and reported that there were wide differences between probiotic strains on acid resistance [18].

Champagen et al. stated that *L. rhamnosus* can grow properly in the mixture of different fruits [14]. In agreement with the results of the current study, Mousavi et al. observed good survivability of *L. rhamnosus* and *L. gasseri* in orange and tomato drinks after four weeks [34]. Malganji et al. investigated the pasteurized grape drink inoculated with three species of lactic acid bacteria (*L. delbrueckii*, *L. plantarum and L. rhamnosus*) separately. Based on their results, *L. rhamnosus* and *L. delbrueckii* displayed longer survival time than *L. plantarum* during the cold storage [11].

Table 5. Viability results of probiotics (log CFU ml^{-1}) in probiotic medicinal plant drink during storage

Treatments	Days				
	0	7	14	21	28
1	9.40 ± 0.39^{bcdeA}	9.04 ± 0.38^{bcAB}	8.69 ± 0.36^{bAB}	8.30 ± 0.35^{aAB}	7.93 ± 0.33^{aB}
2	8.91 ± 0.54^{bcdeA}	8.67 ± 0.49^{bcA}	8.40 ± 0.47^{bA}	8.17 ± 0.46^{aA}	7.92 ± 0.44^{aA}
3	10.14 ± 0.28^{abA}	9.61 ± 0.27^{abcAB}	9.03 ± 0.25^{abBC}	8.52 ± 0.24^{aCD}	7.96 ± 0.22^{aD}
4	10.16 ± 0.28^{abA}	9.75 ± 0.27^{abAB}	9.39 ± 0.26^{abABC}	8.94 ± 0.25^{aBC}	8.61 ± 0.24^{aC}
5	10.11 ± 0.42^{abcA}	9.73 ± 0.41^{abAB}	9.30 ± 0.39^{abAB}	8.91 ± 0.37^{aAB}	8.52 ± 0.36^{aB}
6	10.90 ± 0.30^{aA}	10.70 ± 0.30^{aA}	9.97 ± 0.28^{aAB}	9.25 ± 0.26^{aBC}	8.62 ± 0.24^{aC}
7	9.18 ± 0.13^{bcdeA}	8.88 ± 0.12^{bcAB}	8.62 ± 0.12^{bBC}	8.36 ± 0.11^{aC}	8.20 ± 0.11^{aC}
8	9.00 ± 0.50^{bcdeA}	8.70 ± 0.49^{bcA}	8.44 ± 0.47^{bA}	8.18 ± 0.46^{aA}	8.02 ± 0.45^{aA}
9	10.00 ± 0.28^{abcdA}	9.56 ± 0.27^{abcAB}	9.14 ± 0.25^{abABC}	8.74 ± 0.24^{aBC}	8.30 ± 0.23^{aC}
10	8.69 ± 0.12^{deA}	8.57 ± 0.12^{bcAB}	8.43 ± 0.11^{bAB}	8.22 ± 0.11^{aAB}	8.18 ± 0.11^{aB}
11	8.78 ± 0.37^{cdeA}	8.58 ± 0.36^{bcA}	8.42 ± 0.35^{bA}	8.23 ± 0.34^{aA}	8.11 ± 0.34^{aA}
12	9.60 ± 0.54^{abcdeA}	9.21 ± 0.52^{bcA}	8.85 ± 0.50^{abA}	8.51 ± 0.48^{aA}	8.11 ± 0.45^{aA}
13	8.77 ± 0.24^{cdeA}	8.56 ± 0.24^{bcA}	8.35 ± 0.23^{bA}	8.16 ± 0.23^{aA}	7.96 ± 0.22^{aA}
14	8.76 ± 0.12^{cdeA}	8.55 ± 0.12^{bcAB}	8.34 ± 0.11^{bABC}	8.15 ± 0.11^{aBC}	7.95 ± 0.11^{aC}
15	9.74 ± 0.55^{abcdeA}	9.53 ± 0.53^{abcA}	9.32 ± 0.52^{abA}	8.95 ± 0.50^{aA}	8.51 ± 0.48^{aA}
16	8.55 ± 0.36^{eA}	8.45 ± 0.35^{bcA}	8.30 ± 0.35^{bA}	8.15 ± 0.24^{aA}	7.80 ± 0.33^{aA}
17	8.65 ± 0.48^{deA}	8.75 ± 0.49^{bcA}	8.59 ± 0.48^{bA}	8.45 ± 0.47^{aA}	8.20 ± 0.46^{aA}
18	8.45 ± 0.47^{eA}	8.35 ± 0.47^{cA}	8.26 ± 0.46^{bA}	8.10 ± 0.12^{bA}	7.70 ± 0.13^{bA}

1- The results were expressed as mean ± SD.
2-$^{a-e}$ Means shown as small letters in each column are significantly different ($p\leq 0.05$).
3- $^{A-D}$ Means shown as capital letters in each row are significantly different ($p \leq 0.05$).
4- Design of treatments and controls is shown in Table 1.

3.4. Sensory Evaluation

The results of the total acceptance of sensory evaluation on 28 days at 4° C are illustrated in Figure. 1. As shown, the highest and the lowest scores for total acceptance were found for T1 and T24 (control), respectively. This shows that there is a significant difference, may be due to the development of a pleasant sour taste, which was accepted by the panelists. Nematollahi et al. reported that type of the applied probiotic strains and fruit juices may cause different sensory properties of the fruit drink [22].

According to the pre-tests conducted in Behnoush Iran Co, the determined pH range was 3.1±0.1. Since the pH value of control samples was within 3.7-3.8 range, the probiotic drink sample with desirable pH was accepted by the panelists. It means that bacteria have a positive role in the development of a pleasant sour taste. In agreement with our results, Luckow and Delahunty evaluated the consumer acceptance for the odor, texture, aroma and taste of probiotic black grape juice. To sum up, they preferred probiotic juice to control [35]. In contrast, Luckow and Delahunty studied the effect of functional compounds (probiotic, prebiotic, vitamins and minerals) on the aroma, taste and flavor of probiotic orange drink. The sensory properties of four probiotic orange drink samples and seven control samples were measured by 100 trained panelists. The produced probiotic drink was perceived as possessing dairy and medicine odor, and the consumers preferred ordinary orange juice [36]. Also Krasaekoopt and Kitsawa studied the sensory parameters of probiotic orange and grape drink. The results illustrated that 80% of the consumers accepted the produced probiotic orange and grape drink. However, less than 20% of the consumers did not like the drink for its unsuitable mouth feel being, in agreement with our results [37].

3.5. Mold and Yeast

Mold and yeast test was conducted during the storage time. Only molds and yeasts can cause problems in these products. However, they can be easily controlled during the pasteurization period before adding probiotics. In this study, no mold and yeast were found in any of the treatments, probably due to proper pasteurization and hygienic conditions during the storage time.

3.6. Significance of each independent variable

As shown in Table 6, components of medicinal plant drink and storage time have significant effect on the variations of pH, acidity, glucose content and viability of probiotics in the medicinal plant drinks ($p \leq 0.05$). According to F factor, the effect of components of drink was more significant than time on pH and viability, and also the effect of storage time on acidity and glucose variations was more significant than the components of samples.

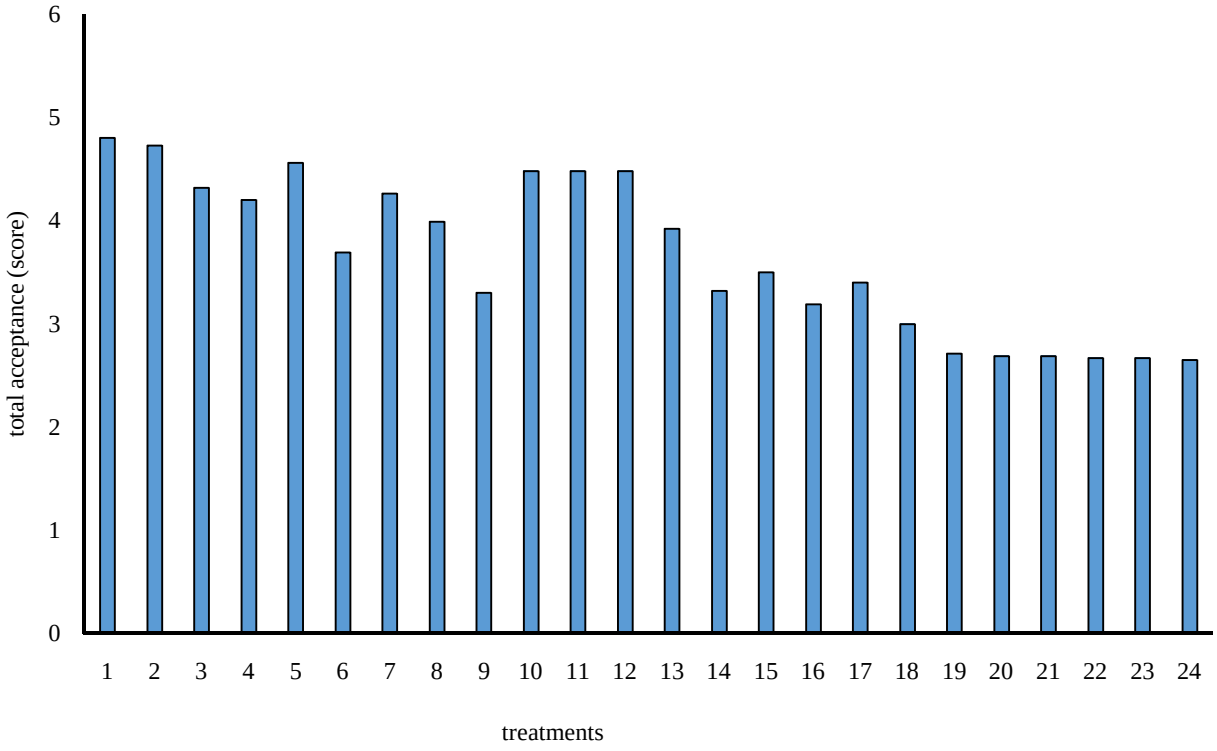

Figure 1. Results of total acceptance in probiotic medicinal plant drinks and controls after 28 days of storage.

Table 6. Determination of significance of each independent variable by the use of p value and F ratio on pH, acidity, glucose and viability

Response variable		Independent variables		
		Components of medicinal plant drinks[1]	Storage time[2]	Interaction
pH	p value	0.000*	0.000*	1
	F ratio	30.36	14.38	0.08
	R^2		86.41	
Acidity	p value	0.000*	0.000*	0.000*
	F ratio	495.03	561.42	7.22
	R^2		99.17	
Glucose	p value	0.000*	0.000*	0.000*
	F ratio	214.76	336.09	6.85
	R^2		98.29	
Viability	p value	0.000*	0.000*	0.005*
	F ratio	1546.28	60.48	1.65
	R^2		99.67	

*Significant differences ($p \leq 0.05$).
1: *E. Amoenum*, *Pussy willow*, apple juice, *L. casei and L. rhamnosus.*
2: 0, 7, 14, 21, 28 d.

4. Conclusion

In this study, a probiotic medicinal plant drink containing apple juice, *P. willow*, *E. amoenum*, glucose and whey powder was produced and stored at 4°C for 28 days, and the parameters including pH, acidity, glucose and viability of *L. casei* and *L. rhamnosus* were investigated. In all the treatments, pH was dropped, acidity was increased, and glucose was decreased during the storage. The results revealed that probiotic treatments had the highest sensory scores throughout the 28 days of storage. The probiotic viable cell of both strains reduced significantly. However, their numbers were $>10^6$ CFU ml^{-1}, a sign of effective dose for exerting healthful effects. It could be said that the produced drink containing apple juice, *P. willow*, *E. amoenum*, glucose and whey powder is a favorable medium for *L. casei* and *L. rhamnosus* to grow. Accordingly, incorporation of medicinal plant extracts and probiotics leads to provide multiple human health effects.

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6. Conflict of Interest

The authors declare that there is no conflict of interest.

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تولید نوشیدنی پروبیوتیکی با استفاده از عصاره های بیدمشک و گل گاو زبان

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چکیده

سابقه و هدف: اخیراً تقاضا برای مصرف فرآورده‌های غیرلبنی پروبیوتیکی، به علت نداشتن لاکتوز و کلسترول، رو به افزایش است. نوشیدنی‌های حاوی گیاهان دارویی اثرات مفید بسیاری بر سلامتی انسان دارند. مشکلات عمده نوشیدنی‌های غیرلبنی پروبیوتیکی نداشتن مواد مغذی برای رشد پروبیوتیک‌ها و طعم بد محصول است. هدف این تحقیق تولید نوشیدنی پروبیوتیک حاوی گیاهان دارویی با خواص فیزیکوشیمیایی، قابلیت زنده‌مانی و حسی مطلوب بوده‌است.

مواد و روش‌ها: هدف از این تحقیق تولید نوشیدنی پروبیوتیک با عصاره گیاهی بیدمشک و عصاره گیاهی گل گاو زبان(برای هر عصاره به تنهایی یا با هم $0/5\%\ w\ v^{-}$)، *لاکتوباسیلوس کازئی*، *لاکتوباسیلوس رامنوسوس* (10^{8} CFU ml^{-1}) به تنهایی و به صورت مخلوط آن‌ها بود. گلوکز و پودر آب پنیر (0/28%)، به عنوان منبع غذایی برای پروبیو تیک‌ها و آب سیب (20 و 30 درصد) برای بهبود طعم نوشیدنی به آن اضافه شدند. میزان گلوکز تنظیم شد تا بریکس به 13 g 100 g^{-1} برسد. آسکوربیک اسید (0/5%) برای بهبود شرایط کم هوا دوست ها (micro-aerophilic) مورد استفاده قرار گرفت. pH، اسیدیته، قند گلوکز، قابلیت زنده‌مانی باکتری‌های پروبیوتیک و خواص حسی در مدت 28 روز نگهداری در دمای 4°C مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: براساس نتایج به دست آمده-تیمار حاوی *لاکتوباسیلوس کازئی*، بیدمشک، گل گاو زبان و 30% آب سیب بیشترین قابلیت زنده‌مانی را داشت و تیمار لاکتو باسیلوس رامنوسوس ، بیدمشک و گل گاو زبان و آب سیب 30% به علت داشتن بیشترین امتیاز پذیرش کلی، pH مناسب و عدد اسیدی مناسب به عنوان بهترین تیمار انتخاب شد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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- *لاکتوباسیلوس کازئی*
- *لاکتوباسیلوس رامنوسوس*
- بیدمشک
- گل گاو زبان

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