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Production of probiotic Mango and Orange juices: Evaluation of qualitative properties and viability of probiotic *Lactobacillus acidophilus* PTCC 1643

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ABSTRACT

This study aimed to evaluate the viability of microencapsulated *Lactobacillus acidophilus* PTCC 1643 with soy protein isolate, xanthan gum and fructooligosaccharide as wall materials by freeze-drying after inoculation into two mango and orange juices and to investigate the physicochemical properties of the juices during storage. Tests were performed to evaluate the viability of probiotic bacterium, determine acidity, pH, Brix, total sugar, formalin index, microbial properties, and sensorial evaluation of the juice. Evaluation of the results with two-way ANOVA analysis without repetition and LSD analysis for comparison of the samples using Excel 2019 software showed that the viability of bacterium in mango and orange juice samples did not have a significant difference. Also, based on the time results, there was a considerable difference in the average viability of probiotic bacterium inoculated in the juice sample during storage. The low storage temperature of the samples, low pH and high acidity prevented the growth of bacterium, so it limited their growth and ultimately decreased the microbial population during the storage period. Also, based on the results obtained, the survival rate of microencapsulated bacterium with xanthan gum, soy protein isolate and fructooligosaccharide was higher than that of fruit juice samples with free bacterium. In fact, due to the multi-cationic and physical structure of the wall materials that create a coating layer around the bacterium, they strengthen the microcapsule wall and protect the bacterium. Based on the results of the sample and time, no significant difference was observed in the physicochemical properties of the fruit juices including acidity, pH, total sugar, formalin index of the average of the samples during their shelf life and the type of fruit juice. In terms of sensorial evaluation of fruit juices over time and depending on the type of fruit juice, no significant difference was observed between the average of the orange juice and mango juice samples studied.

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1.Introduction

In recent decades, the development of functional foods such as probiotic products have increased. Probiotics are defined as live microorganisms that can positively affect the host when consumed in adequate amounts. Probiotics have benefits such as maintaining the natural gut microflora, enhancing the immune system, and reducing blood cholesterol levels [1, 2]. The most common probiotics introduced into functional foods are Lactobacillus species, which are recognized as beneficial microorganisms in the gut and play an important role in preventing pathogen colonization and regulating the host's immune response. *Lactobacillus acidophilus* exhibits antimicrobial effects due to the formation of organic acids and bacteriocins [3]. It is also resistant to bile acid and has an antibiotic effect on gut pathogens [4, 5]. Microencapsulation is one of the most common techniques used to enhance the survival of probiotics. Encapsulation traps an active agent (core materials) within another compound (wall materials) and produces particles in the nanometer or micrometer range. The wall or coating materials used in microencapsulation methods must be edible, safe, and biodegradable. These compounds can be carbohydrates, proteins, resins, and lipids [6,7].

Xanthan gum is a heteropolysaccharide composed of units of D-glucose, D-mannose, and D-glucuronic acid derived from bacteria and fungi. Xanthan gum is produced through the aerobic fermentation of the pure culture medium of *Xanthomonas campestris* [8]. This hydrocolloid has become one of the most successful hydrocolloids due to its high performance, especially in environments such as acidic conditions, high salt concentrations, and high shear stress [9]. Among all food proteins, soy protein isolate is an important dietary protein with significant potential that acts as a carrier for insoluble biological agents through complexation due to its hydrophobic surface nature. Soy protein isolate is used in the food industry due to its functional properties, non-toxic nature, low cost, easy availability, and high nutritional value [6]. Additionally, this compound is widely used in many food products such as processed meat, nutritional beverages, infant formula, and dairy product substitutes due to its emulsifying and gelling capabilities [10]. The main protein components in soy protein isolate are glycinin and β- β–conglycinin. Glycinin consists of one acidic subunit and one basic subunit connected by a single disulfide bridge. β-conglycinin is composed of three subunits: α, 'α, and β [11]. Fructo-oligosaccharides are fructose oligomers linked to glucose or fructose molecules, containing a maximum of ten sugar units. Fructo-oligosaccharides are highly soluble in water and are considered low-calorie carbohydrates. Recommended dosage levels of fructo-oligosaccharides range from 2% to 50% (W/W) for various food formulations. Fructo-oligosaccharides are short-chain carbohydrates and currently belong to the prebiotic category. These carbohydrates, with prebiotic properties, show high resistance to digestion and absorption by the gastrointestinal tract, leading to a reduction in calorie content [12, 13].

In recent years, numerous studies have been conducted by various researchers on the encapsulation of probiotics. These include the encapsulation of *Lactobacillus acidophilus* with rice bran protein wall and malto dextrin [14], the encapsulation of *L.*

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acidophilus with whey protein isolate and lactose wall [15], the encapsulation of *L. acidophilus* with alginate and whey protein isolate wall [16], and the encapsulation of *Lactobacillus plantarum* with soy protein wall [17].

This research aims to inoculate *L. acidophilus* bacteria encapsulated with a composite wall of xanthan gum, soy protein isolate, and fructo-oligosaccharide into the mango and orange juices and to examine the bacterial viability over storage time. Additionally, the physicochemical properties of the juices, including acidity, pH, Brix, total sugar, formalin index, microbial characteristics, and sensory evaluation of the juices over storage time and depending on the type of juice in terms of microcapsule properties, were conducted.

2. Materials and Methods

2.1. Required materials

Xanthan gum, soy protein isolate, and fructo-oligosaccharide were purchased from Sigma Aldrich, MRS agar and MRS broth were purchased from Merck, Germany. The components of PBS buffer including KH_2PO_4, KCl, NaCl, and Na_2HPO_4, as well as other chemicals and reagents were laboratory-grade, and also were purchased from Merck, Germany. The standard strain *L. acidophilus* PTCC 1643 was purchased from the Industrial Microorganism Collection Center (Tehran, Iran). Mango and orange juices were also purchased with the Lavin brand in 200-milliliter Tetra-Pak packaging (Urmia, Iran).

2.2. Preparation of probiotic bacterium

To prepare the inoculum, 0.5 g of freeze-dried *L. acidophilus* cells were added to 5 mL of MRS broth and incubated anaerobically at 37 °C for 72 h. Then, the cultures were inoculated in 95 mL of MRS broth and incubated under the same conditions until reaching to 12 log CFU/mL of *L. acidophilus* for 72 h. The biomass was harvested using a centrifuge at 4000×g for 10 min at 4 °C. The supernatant was discarded, and the cells were washed and centrifuged twice with sterile buffered saline. Then, a saline suspension was prepared again and adjusted to a solution containing 12 log CFU/mL bacteria for microencapsulation. Optical density at 625 nm was measured using a spectrophotometer (UV-1800PC, Shimadzu Corp., Kyoto, Japan), which provided the number of cells in 12 log CFU/mL. Microbial counting was measured as CFU/mL, and the average of the obtained data was converted to log CFU/mL [18].

2.3. Encapsulation of probiotic bacterium

The encapsulation of bacteria was performed according to the method of Maleki et al. [1]. Initially, the wall materials, including soy protein isolate, xanthan gum, and fructo-oligosaccharide, were mixed and prepared in sterile phosphate buffer at different ratios after dissolution. So that the initial concentration of the xanthan solution, before mixing, was 0.2% (w/v) in the sterile phosphate buffer. The concentration of the soy isolate was 0.5% (w/v) in sterile phosphate buffer, and dissolution was carried out at pH 10. The initial solutions prepared were stored in the refrigerator at 4 °C for 24 h to ensure complete hydration. Then, mixing according to the specified ratios in the plan was carried out using a magnetic stirrer. Next, the fructo-oligosaccharide powder was added to the resulting mixture according to the specified percentage, and stirring was carried out for 10 min using a magnetic stirrer. Under sterile conditions, 10 mL of bacterial suspension (bacterial concentration 7.5×10^9 CFU/mL) were prepared. Then, 40 mL of the gum solution, isolated, and fructo-oligosaccharide were added and mixed with a magnetic stirrer for 10 min. After this step, 2% sterile Tween 80 and 2% sterile glycerol were added to the resulting suspension and

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mixed with a magnetic stirrer for 10 min. Before placing the prepared solution in the freeze dryer, the sample was frozen in a -30 °C freezer. Then, under vacuum conditions, it was placed in the freeze dryer at a temperature of -80 °C. The freeze-drying process took 48 h. The dried microcapsules were collected and stored in sterilized aluminum containers at a temperature of 4 °C [1].

2.4. Preparation of probiotic fruit juice samples

In order to inoculate the encapsulated bacteria into each of the two mango and orange juices, the encapsulated bacteria with a concentration of 2.63 CFU/mL were added to 100 mL of juice (a total of 6 samples of mango and orange juice). Then, the fruit juices were transferred to an incubator at 37 °C for 72 h. This amount, in terms of bacterial count, is equivalent to 0.5 grams of microencapsulated bacteria added to 10 mL of phosphate buffer and stored at 30 degrees for 1 hour. After crushing the added microcapsule in the solution, the encapsulated bacteria were released. Using the prepared serial dilutions, the bacteria were cultured on MRS agar to determine their viability and were transferred to a 37degree Celsius incubator for 72 h. The fruit juices were cultured for both mango and orange juices on the first, tenth, twentieth, and thirtieth days after inoculating the encapsulated bacteria into the juices. Eight control fruit juices were also prepared by inoculating 100 mL of mango and orange juice with pure bacteria. Thus, the total number of juice samples, considering the item and evaluation time, amounted to 32 samples. [9]

2-5- Physicochemical tests of fruit juice

Including the determination of total sugar, formaldehyde index, pH and Brix in fruit juice, which were conducted according to standard number 2685, the fruit juice testing method in the Urmia Food and Drug Control Laboratory.

1-2-5- Test for determining total sugar in fruit juice

In this method, sucrose, which is a disaccharide, is broken down into two molecules of glucose and fructose in the presence of acid and heat. The amount of reducing sugars produced is determined by the lane eynon method, which involves the conversion of divalent copper in Fehling's solutions to monovalent copper, ultimately resulting in the formation of a brick-red precipitate. The total percentage of sucrose and reducing sugars is reported as the total sugar percentage of the fruit juice.

2-2-5- Test for determining the formaldehyde index in fruit juice

The goal of determining the formalin index in various concentrates and fruit juices is to release the existing amino acids and titrate them with alkaline substances in the presence of neutral formalin. This index, which indicates the naturalness of fruit juice and concentrate, is used to determine the quality of various fruit juices and concentrates. The formalin index is the number of mL of 0.1 normal NaOH required to neutralize the amino acids present in 100 grams or 100 mL of the sample. In this method, the amino acids present in the fruit juice are titrated with alkaline substances in the presence of neutral formalin.

3-2-5- Test to determine the pH of fruit juice

In this method, by placing the calibrated pH meter electrode inside the sample, the pH of the sample is determined. Before starting the calibration, ensure that the electrode is in good condition. The calibration method of the pH meter device may vary depending on the manufacturer; however, the basic operation involves adjusting the device with a buffer solution close to the pH of the solution being tested. Store the pH 4 and 7

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buffers away from light and in the refrigerator. Each buffer solution container is usable for a maximum of 3 months after opening. Place the device's electrode in a 3 molar KCL solution after the test.

4-2-5-Brix Test (soluble solids in water) for fruit juice

Determination of Brix and refractive index in various fruit juice samples is carried out using a refractometer. Brix refers to the percentage of soluble solids in water. In this method, Brix (soluble solids in water) is determined based on grams of sucrose per 100 grams of sample using a refractometer.

2-6- Microbial tests for fruit juice

Including search and identify for lactic acid bacteria, acid-resistant bacteria, mold, and yeast, which was conducted according to standard number 3414, microbiology of fruit juice, vegetable juice, and their products in the Urmia Food and Drug Control Laboratory.

3 - Statistical Analysis

In this study, a completely randomized design was used for sample preparation. Then, for analyzing the data obtained from the sample tests, a two-way ANOVA without replication was used, and for comparison, the LSD analysis was conducted using Excel 2019 software. And in this study, the first type error level was $\alpha = 0.05$.

4- Results and Discussion

4-1- Viability of probiotic bacterium

The results showed that the viability of probiotic bacterium in the average samples of mango and orange juice was not significant. Additionally, based on the results over time, there is a significant difference in the average viability of the inoculated probiotic bacterium in the fruit juice samples during the storage period. Based on the results, the survival rate of probiotic bacterium during the 30 days of storage period decreased in all treatments of mango and orange juice examined. The low storage temperature of the samples, low pH, and high acidity in the samples prevent bacterial growth, thereby limiting their growth and ultimately reducing the microbial population over the storage period. Additionally, based on the obtained results, the viability of bacteria encapsulated with xanthan gum, soy protein isolate, and fructo-oligosaccharide was higher than that of free bacteria in fruit juice samples. In fact, due to the poly cationic and physical structure of the wall materials that create a protective layer around the bacteria, they strengthen the capsule walls and protect the bacteria. As a result, the resistance and viability of probiotic bacterium increase in the acidic conditions of the digestive system and fruit juice. Based on the results, no significant difference was observed in the average total sugar content of the samples over their storage period. The results of the total sugar content of the samples also showed that the mean between the mango juice samples was not significant. The results of the orange juice showed that there was a significant difference between the means of the samples (Table 1). However, according to the results, the difference between the orange juice samples Oc (Orange juice control sample) and O_8 (Orange juice eight sample) was not significant. As such, the highest total sugar content (0.001 ± 7.85) was related to the orange juice sample O_{15} (Orange juice fifteen sample). The reason for this behavior is likely due to the presence of xanthan gum alone in the capsule wall structure; in the O_2 (Orange juice two sample) sample, 75% xanthan gum, 25% soy protein isolate, and 1.5% fructo-oligosaccharide were used [14].

4-2- Physicochemical properties of fruit juice

4-2-1- Total sugar in fruit juice

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Based on the results, no significant difference was observed in the mean total sugar of the samples over their storage time. The results of the total sugar content of the samples also showed that the mean between the mango juice samples was not significant. While the results for orange juice showed that there was a significant difference between the sample means (Table 1). However, according to the results, the difference between the orange juice samples Oc (Orange juice control sample) and O8 (Orange juice eight sample) was not significant. The highest total sugar content (7.85 ± 0.001) was related to the O_{15} (Orange juice fifteen sample) orange juice sample. The reason for this behavior is likely due to the presence of xanthan gum alone in the capsule wall structure; in sample O_2 (Orange juice two sample), 75% xanthan gum, 25% soy protein isolate, and 1.5% fructo-oligosaccharide were used [32,33].

4-2-2- Index Formalin Fruit Juice

The formaldehyde index serves as an indicator for determining amino acids in fruit juices. The importance of the formalin index is that if amino acids are high during the production process, they combine with the sugars present in the juice, leading to non-enzymatic reactions such as the Maillard reaction, which ultimately darkens the color of the juice. The results over time indicated no significant difference between the average times during the storage period up to 30 days. Based on the results, there was a significant difference between the average formalin index of the mango juice samples and the orange juice samples. However, no significant difference was observed between the control mango juice sample with not encapsulated probiotic bacterium (Mc) and the mango juice containing (1.5×10^5 CFU/mL) encapsulated probiotic bacterium [M8 (Mango juice sample No. 8)]. According to the results presented in Table (1), the orange juice eight sample (O_8) with encapsulated bacteria had the highest formalin index, while the orange juice sample with free bacteria (Oc) had the lowest formalin index. The lowest formalin index in the juice with free bacteria is likely due to the greater tendency of non-encapsulated bacteria to consume sugars rather than amino acids, as the metabolism of sugars (especially fructose and glucose) occurs easily in them. Although their dependence on sugars is not the same, the results are consistent with the findings of Sabagh Pour Langaroudi et al., 2021, who reported that the formalin index in the juice sample with encapsulated bacteria was higher than that in the juice with free bacteria [17].

Table 1: Viability, Total Sugar and Formalin Index of probiotic Mango and Orange juice

Samples and Storage time	Viability (Log_{10}) CFU/mL	Total Sugar (%)	Formalin Index (%)
M_2	5.93±1.77[a]	13.12±0.00 [a]	4.06±0.00[c]
M_{15}	6.65±0.73[a]	13.17±0.00[a]	4.17±0.00[d]
M_8	6.88±0.57[a]	13.15±0.00[a]	3.82±0.00[b]
Mc	4.97±10.19[a]	13.04±0.00[a]	3.79±0.00[a]
O_2	5.65±2.51[a]	7.65±0.01[b]	5.06±0.00$_b$
O_{15}	5.59±2.47[a]	7.85±0.00[d]	5.17±0.00[c]
O_8	6.77±0.65[a]	7.71±0.00[c]	5.55±0.00[d]
O_C	4.86±10.68	7.62±0.01[a]	5.02±0.00[a]
Day 0	3.187±0.05[a]	10.48±8.15[a]	4.58±0.47[a]
Day 10	3.212±0.08[a]	10.39±8.48[a]	4.59±0.48[a]
Day 20	3.125±0.03[a]	10.39±8.47[a]	4.57±0.48[a]

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Day 30	3.212±0.03[a]	10.39±8.48[a]	4.57±0.47[a]

M2: Mango juice sample 2, MC: Mango juice Control sample, O2: Orange juice sample 2, O_C: Orange juice Control sample, M8: Mango juice sample 8, M15: Mango juice sample 15, O8: Orange juice sample 8, O15: Orange juice sample 15

Table 2: pH, Acidity and Brix of probiotic Mango and Orange juice

Samples and Storage time	pH	Acidity(%)	Brix (%)
M_2	3.175±0.02 [a]	0.292±0.00 [a]	13.2±0.06 [a]
M_{15}	3.125±0.04 [a]	0.297±0.00 [a]	12.95±0.01 [a]
M_8	3.15±0.12 [a]	0.315.±0.00 [a]	13.00±0.04 [a]
Mc	3.15±0.01 [a]	0.25±0.00 [a]	13.17±0.01 [a]
O_2	2.95±0.01 [a]	0.48±0.00 [a]	11.97±0.02 [a]
O_{15}	3.35±0.01 [a]	0.47±0.00 [a]	11.85±0.01 [a]
O_8	3.15±0.01 [a]	0.497±0.00 [a]	11.82±0.06 [a]
O_C	3.425±0.02 [a]	0.385±0.00 [a]	11.9±0.06 [a]
Day 0	3.187±0.05 [a]	0.406±0.01 [a]	12.63±0.45 [a]
Day 10	3.212±0.08 [a]	0.382±0.00 [a]	12.52±0.37 [a]
Day 20	3.125±0.03 [a]	0.363±0.01 [a]	12.40±0.47 [a]
Day 30	3.212±0.03 [a]	0.341±0.00 [a]	12.37±0.44 [a]

M2: Mango juice sample 2, MC: Mango juice Control sample, O2: Orange juice sample 2, O_C: Orange juice Control sample, M8: Mango juice sample 8, M15: Mango juice sample 15, O8: Orange juice sample 8, O15: Orange juice sample 15

3-2-4- pH

According to the results of the statistical analysis, there was no significant difference in the acidity and pH levels of various samples of mango and orange juice containing probiotic bacterium. Additionally, the acidity and pH levels of the fortified fruit juice samples over a storage period of up to 30 days were also not significant. [28]

4-2-4- Brix (soluble solids)

The results of the statistical analysis, which showed the minimum significant difference for the mean of the samples for the Brix of orange and mango juice samples in Table (2), indicated no significant difference between the various fruit juice samples. Additionally, according to the time results, which showed the minimum significant difference over the storage time of the samples, no significant difference was observed between the mean times of the samples. [29]

4-2-5- Sensory evaluation of fruit juice

The sensory evaluation of mango and orange juice samples containing encapsulated and free probiotic bacterium was conducted by 15 evaluators on the first, tenth, twentieth, and thirtieth day's post-production. The results indicate that no significant difference was observed between the mean samples of orange and mango juice studied. [31]

Table 3: Organoleptic evaluation of probiotic Mango and Orange juice

Samples and Storage time	Organoleptic evaluation
M_2	2.25±0.25 [a]
M_{15}	1. 5±0.33 [a]
M_8	2.00±0.66 [a]

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Mc	1.25±0.25 [a]
O_2	2.25±0.25 [a]
O_{15}	2.00±0.66 [a]
O_8	1.75±0.91 [a]
O_C	1.5±0.33 [a]
Day 0	1.37±0.26 [a]
Day 10	1.62±0.55 [a]
Day 20	2.00±0.57 [a]
Day 30	2.25±0.25 [a]

M2: Mango juice sample 2, MC: Mango juice Control sample, O2: Orange juice sample 2, O_C: Orange juice Control sample, M8: Mango juice sample 8, M15: Mango juice sample 15, O8: Orange juice sample 8, O15: Orange juice sample 15

5- Conclusion

The inoculation of the probiotic bacterium *Lactobacillus acidophilus* with different ratios of soy protein isolate, xanthan gum, and fructo-oligosaccharide into two fruit juices, mango and orange, was conducted. Based on the results, there is a significant difference in the average viability of the inoculated probiotic bacterium in the fruit juice samples over the storage period. According to the results, the viability of the bacteria decreased over 30 days of storage in all tested mango and orange juice treatments. The low storage temperature of the samples, low pH, and high acidity in the samples hindered bacterial growth, limiting their growth and ultimately reducing the microbial population over the storage period. The use of a three-component capsule wall of xanthan gum, soy protein isolate, and fructo-oligosaccharide for encapsulating the bacteria resulted in higher viability of probiotic bacterium in the fruit juices. Additionally, the stability of the physicochemical properties of the probiotic fruit juice during one month of storage at 4 ◦C can lead to consumer acceptance of the produced product as a functional beverage.

6. References

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چکیده

هدف از این پژوهش، ارزیابی زنده‌مانی باکتری *لاکتوباسیلوس اسیدوفیلوس* PTCC 1643 میکروکپسوله با ایزوله پروتئین سویا، صمغ زانتان و فروکتوالیگوساکارید به عنوان مواد دیواره به روش خشک کردن انجمادی پس از تلقیح به دو آب میوه انبه و پرتقال و بررسی ویژگی‌های فیزیکوشیمیایی آب میوه‌ها در دوره نگهداری می‌باشد. آزمون‌های بررسی زنده‌مانی باکتری پروبیوتیک، تعیین اسیدیته، pH، بریکس، قند کل، اندیس فرمالین، ویژگی‌های میکروبی و ارزیابی حسی آب میوه، انجام گردید. ارزیابی نتایج با آنالیز واریانس دو طرفه[1] ANOVA بدون تکرار و برای مقایسه تحلیل LSD[2] با استفاده از نرم افزار Excel 2019 نمونه‌ها نشان داد که زنده مانی باکتری‌ها در نمونه‌های مختلف آب میوه انبه و پرتقال، دارای تفاوت معنی-دار نبود. همچنین بر اساس نتایج زمان، اختلاف معنی‌داری در میانگین زنده‌مانی باکتری پروبیوتیک تلقیح شده در نمونه آب میوه، طی زمان نگهداری وجود دارد. دمای پایین نگهداری نمونه‌ها، pH پایین و اسیدیته بالا در نمونه‌ها مانع رشد باکتری‌ها شده، به طوریکه رشد آن‌ها را محدود می‌کند و در نهایت جمعیت میکروبی در طول زمان نگهداری کاهش می‌یابد. همچنین بر اساس نتایج حاصل شده، میزان زنده‌مانی باکتری درون پوشانی شده با صمغ زانتان، ایزوله پروتئین سویا و فروکتوالیگوساکارید بیشتر از نمونه‌های آب میوه با باکتری آزاد بود. در واقع به علت ساختار چند کاتیونی و فیزیکی مواد دیواره که لایه پوششی اطراف باکتری ایجاد می‌کنند موجب استحکام دیواره میکروکپسول‌ها و محافظت از باکتری می‌گردند. بر اساس نتایج نمونه و زمان، اختلاف معنی داری در ویژگی‌های فیزیکوشیمیایی آب میوه ها شامل اسیدیته، pH، قند کل، اندیس فرمالین میانگین نمونه‌ها در طی زمان ماندگاری آن‌ها و نوع آب میوه مشاهده نگردید. از نظر ارزیابی حسی آب میوه‌ها در طی زمان و بسته به نوع آب میوه، نتایج بین میانگین نمونه‌های آب پرتقال و آب انبه مورد مطالعه تفاوت معنی‌داری مشاهده نشد.

1-Two-way ANOVA
2- Least significant difference

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