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# Fermentation of vegetables juice by probiotic bacteria

**Roya Nosrati[1], Mahnaz Hashemiravan[2*], Maliheh Talebi[3]**

[1]*Food Science and Technology, Varamin –Pishva Branch, Islamic Azad University, Varamin,Iran, Department of Food Science and Technology, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran*

[3]*Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran*

**Key words:** Vegetables juice, probiotic, fermentation, *Lactobacillus casei*, *Lactobacillus plantarum*.

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**Abstract**

In this study, producing of fermentative functional drinks based on vegetables juice using lactic acid bacteria, including *Lactobacillus casei* and *Lactobacillus plantarum* was investigated. In this research, Microbial population, changes in pH, lactic acid and glucose were measured during fermentation for 24 h at 37$^0$C, and also viability of lactic acid bacteria, changes in pH and lactic acid were determined during 28 days storage at 4$^0$C. In order to produce probiotic vegetables juice, microbial suspension with an initial concentration of $10^8$ cfu/ml was produced. Various ratios of bacteria including (65% *Lactobacillus casei* + 35% *Lactobacillus plantarum*), (50% *Lactobacillus casei* + 50% *Lactobacillus plantarum*) and (35% *Lactobacillus casei* + 65% *Lactobacillus plantarum*) were added to the vegetables juice with concentrations of 2%, 3%, 4%. According to the obtained results, treatment Pc2 (35% *Lactobacillus casei* + 65% *Lactobacillus plantarum*) with 2% concentration, was recognized as the best treatment, because it had the maximum bacteria population, the minimum pH and the maximum amount of lactic acid. The results showed that, the amount of glucose reduced during the fermentation. Totally, the results of this study indicated that, mixed vegetables juice without any nutrient supplementation could be considered as a proper matrix for growth of lactic acid bacteria and functional beverage production.

* **Corresponding Author:** Mahnaz Hashemiravan ✉ m_hashemiravan@yahoo.com

## Introduction

Probiotics as nutritional supplements are defined as alive microorganisms which have useful effects by improving the intestinal microbial balance of the host (Fuller, 1989). Internationally, probiotics are defined as live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition (FAO/WHO, 2001). Most of probiotics belong to Bifid bacterium and Lactobacillus genus (Espinoza and Navarro, 2010; Vitali., 2012).Many health effects have been reported for probiotics such as anti-carcinogenic and anti-mutagenic effects (Mortazavian and Sohrabvandi, 2006; Pereira *et al*., 2011). Cholesterol reduction, reduction of blood ammonia levels (Prado *et al*., 2008, Saarela *et al*., 2000; Shah, 2001),stimulation of the immune system (Mazaheri Tehrani *et al*, 2010),diabetes prevention (Roble *et al*., 2010), treatment, and prevention ofrotavirus diarrhea (Peres *et al*., 2012), Restoration of the normal intestinal microflora after antibiotic therapy and increasing lactose tolerance (Prado *et al*., 2008). Probiotics are basically used in producing the fermented milk and other dairy products such as yogurt, ice cream and cheese (Saarela *et al*., 2006; Nualkaekul and Charalampopoulos, 2011). Despite fermentative dairy products, especially milk proteins are considered as good carrier substrates for probiotic microorganisms into the human digestive tract (Ritter *et al*., 2009),But, the two major disadvantages related to consumption of this products are lactose intolerant and cholesterol content (Espinoza and Navarro, 2010; Yoon *et al*., 2006). Therefore, development of non-dairy probiotic products such as fruits and vegetables juice is very important. Currently, somenon-dairy probiotic beverages have become commercial and its consumption is rising in the world (Moraru *et al*., 2007). Wide variety of fruits and vegetables and many strains of Lactobacillus have provided a great opportunity for the development and industrialization of non-dairy fermented beverages. Fruit and vegetable extracts are suitable for probiotics transfer due to having minerals, vitamins, dietary fiber and antioxidants (Moraru *et al*., 2007; Yoon *et al*., 2004). In this study, a mixture of vegetable juice as substrate of probiotic bacteria activity was investigated. The main characteristic of the vegetables used in this study is the existence of nutrients and antioxidant compounds such as vitamins, minerals, dietary fibers, lycopene, carotenoids and bio flavones. The objective of this study is to investigate desirability of mixed vegetable extracts a substrate for probiotic bacteria and the optimal time determination of the product storage and also, to determine the survival of probiotic bacteria in the product.

## Materials and methods

*Raw material*

The studied vegetables juice were mixture of, tomato, carrot, celery, spinach, lettuce, parsley and beet extracts. Tomatoes and carrots, were purchased from a local store, and after washing and peeling, were extracted by juicer (Parskhazar, Iran). Tomato extract was filtered by cloth filter. Tomato and carrot juice were frozen at -20°C prior to use (Pereira *et al*., 2011).

*Preparation of microorganisms and stock culture*

*Lactobacillus casei* PTCC1608 and Lactobacillus plantarum PTCC1058 were provided from Iranian Research Organization for Science and Technology. Their activation was taken in MRS broth (Merck, Germany) and was incubated at 37 ℃ for 24 hours under aerobic conditions. In order to produce sub-cultures from the bacteria, about 10 cc of the 24-hour culture were centrifuged at 3000 g for 5 minutes at 25 ℃ (Mokarram *et al*., 2010). Liquid medium was evacuated and sub-cultures were prepared by adding sterile glycerol (50%v/v) to the activated cultures. Sub-cultures were kept in sterile micro-tubes containing 8 cc of 24-hour culture suspension at -20 ℃ (Pereira *et al*., 2011). For more activating of bacteria, about 5 cc of 24-hour culture was added in to 95 cc MRS broth and was incubated under the same conditions (48-hour culture) (Mokarram *et al*., 2010; Mousavi *et al*., 2011).

*Pasteurization of vegetables extract*

In order to pasteurization of tomato juice and carrot juice was used of the water bath at 80 °C for 20 minutes (Kun *et al*., 2008).The mixed of vegetables extract including parsley, lettuce, spinach, celery and beet was purchased from Sanich Co. and was kept at 4 °C prior to use (Yoon *et al*., 2004). This mixed of vegatables extract was pasteurized for 5 min at 80°C (Mousavi *et al*., 2011).

*Inoculum preparation*

To determine the number of bacteria per ml was used of the 0.5 MacFarland standard suspension (Ashrafi, 2002).In order to achieve $10^8$ cfu/ml from each strain, 10 cc of 48-hour culture was centrifuged at 3000 g for 5 minutes at 25 ℃. Then, the upper floating liquid was separated and sterile peptone buffer 0.1% (Merck, Germany) was added to the mass of bacteria, shaked well and centrifuged under the same conditions again. This activity was repeated again to wash the bacteria completely (Mokarram *et al*., 2010). The cultures were diluted with sterile distilled water. Then was measured optical density of bacterial suspension by spectrophotometer at 625 nm. The optical density of bacterial suspension was corresponded with the optical density of 0.5 MacFarland (Ashrafi, 2002).

*Fermentation of vegatable juice*

The vegetables extract of San ich (with no preservatives) and pasteurized tomato juice and carrot juice were mixed with ratios of 44.5%, 33.5%, and 22% respectively and were prepared 2%, 3% and 4% concentrations by adding sterile distilled water. *Lactobacillus casei* and *Lactobacillus plantarum* with initial density of $10^8$cfu/ml and the ratios of 35%, 50% and 65% were inoculumed in to the pasteurized mixture of vegetables juice .Three samples of vegetables juice mixture with 2%, 3% and 4% concentrations were prepared as control. The samples were incubated at 37℃ for 24 hours. According to the report by Saxelin *et al.* (1999), the temperature of 37 to 40 ℃ is suitable for probiotic bacteria growth, especially *Lactobacillus casei*. After fermentation, the samples were stored at the refrigerator (4℃) for 4 weeks, for investigating considered factors.

The used codes in this study were as following: $C_1$p: 35% *Lactobacillus plantarum* + 65% Lactobacillus casei, $C_2$p: 50% *Lactobacillus plantarum* + 50% *Lactobacillus casei*, Pc: 65% *Lactobacillus plantarum* + 35% Lactobacillus casei. Non-hybrid codes of C2, C3 and C4 represented control samples with concentrations of 2, 3, and 4% of vegetables juice respectively.

The numbers 2, 3, 4 at the end of the cods refer to the concentrations of 2, 3, and 4% of vegetables juice respectively.

*Viable cell counts determination*

Viable cell counts were determined by serial dilution and standard plate method after fermentation. Dilutions of $10^{-8}$ and $10^{-9}$ were prepared from the fermented samples by using of sterile Ringer (Merck, Germany) solution. 1 ml of these dilutions, were plated in double plate and then, sterilized MRS agar (Merck, Germany) medium was poured on it (standard plate count method). The plates were incubated at 37℃ for 48 hours. Plates containing 20–350 colonies were measured and recorded as colony forming units (CFU) per mL of solution (Vinderola and Reinheimer, 2000). Also the viability of lactic acid cultures was determined during cold storage period at weekly intervals by using the mentioned method and expressed as cfu/ml. The number of lactic acid cultures was calculated with equation (1).

Equation 1 $$\mathbf{N = \frac{\Sigma C}{V(n1+0/1n2)d}}$$

ΣC: total colonies in plates of two successive dilutions, V: volume of inoculated dilution in each plate as ml, $n_1$: number of counted plate in the first dilution (thicker), $n_2$: number of counted plate in the second dilution(thinner),d: dilution index based on the first of counted dilution (thicker) (Iran National Standard No. 5272).

*pH determination*

pH was measured with pH meter (SAT-2002, Iran) (Iran National Standard No. 2685).

*Lactic acid determination*

Total acidity, expressed as lactic acid(mg/100cc), was measured by titration with titrazol 0.1N NaOH (Merck, Germany) to pH 8.2 (Iran National Standard No. 2685).

*Glucose sugar measurement*

Quantitative analysis of glucose sugar before and after fermentation in vegetables juice, was carried out by High Performance Liquid Chromatography (Agilent-1200, U.S.A) equipped by auto sampler(G1329) with injection volume of 20 µl. separation was conducted by $NH_2$ column (25cm×4mm), at 30°C. UV-Vis detector (G1314)at 193 nm was used to identify glucose sugar. Acetonitrile: water (75:25) was employed as mobile phase at a flow rate of 1.25 ml/min (Shaw and Wilson, 1983). Glucose sugar content was expressed as mg/ml.

*Statistical analysis*

All experiments were carried out in triplicate. The results are expressed as mean±S.D (standard deviation). Mean analysis was carried out using Duncan's multiple range tests at 95% level. Variance analysis was conducted by SPSS software (version 20) and the graphs were drawn using Excel 2007 software.

**Results**

*Changes in pH and lactic acid*

Variations mean of pH and lactic acid after 24 hours fermentation and during 4 weeks maintenance in refrigerator at $4^0$C have been shown in Table (1).

**Table 1.** Variations mean of pH and lactic acid in various concentrations of vegetable juice and various ratios of *Lactobacillus casei* and *Lactobacillus plantarum* during 24 hours fermentation at 37 $^0$C and 4 weeks storage at $4^0$C± standard deviation.

| Forth week | | Third week | | Second week | | First week | | First day | | |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | Lactic acid mg/100cc | pH | Lactic acid mg/100cc | pH | Lactic acid mg/100cc | pH | Lactic acid mg/100cc | pH | Lactic acid mg/100cc | treatments |
| 4/66±0/0$^{a}$ | 270±0/0$^{e}$ | 4/66±0/0$^{a}$ | 270±0/0$^{b}$ | 4/66±0/0$^{a}$ | 270±0/0$^{b}$ | 4/65±0/02$^{ab}$ | 270±0/0$^{a}$ | 4/65±0/02$^{abc}$ | 270±0/0ab | C4 |
| 4/16±0/1$^{b}$ | 426±34/1$^{abcd}$ | 4/2$^{b}$±0/16$^{b}$ | 402±68/1$^{a}$ | 4/3$^{a}$±0/14$^{bc}$ | 363±67/5$^{a}$ | 4/53±0/1$^{bc}$ | 294±22/6$^{a}$ | 4/60±0/0$^{bcd}$ | 279±15/$^{a}$a | C1p4 |
| 4/1$^{a}$±0/12$^{b}$ | 423±32/4$^{abcd}$ | 4/2$^{a}$±0/15$^{b}$ | 399±66/3$^{a}$ | 4/40±0/15$^{b}$ | 354±58/$^{a}$a | 4/55±0/1$^{bc}$ | 291±18/7$^{a}$ | 4/60±0/04$^{bcd}$ | 279±15/$^{a}$a | C2p4 |
| 4/14±0/1$^{b}$ | 426±34/1$^{abcd}$ | 4/2$^{b}$±0/171$^{b}$ | 402±68/1$^{a}$ | 4/37±0/17$^{bcd}$ | 369±76/$^{a}$a | 4/5$^{b}$±0/12$^{bc}$ | 297±27/0$^{a}$ | 4/59±0/0$^{bcd}$ | 285±2$^{a}$/0a | Pc4 |
| 4/69±0/01$^{a}$ | 234±9/0$^{e}$ | 4/68±0/0$^{a}$ | 234±9/0$^{b}$ | 4/6$^{a}$±0/01$^{a}$ | 234±9/0$^{b}$ | 4/68±0/01$^{ab}$ | 234±9/0$^{b}$ | 4/68±0/01$^{ab}$ | 234±9/0b | C3 |
| 4/13±0/1$^{a}$$^{b}$ | 375±57/$^{a}$$^{cd}$ | 4/1$^{b}$±0/1$^{b}$ | 363±36/$^{a}$a | 4/2$^{b}$±0/1$^{cd}$ | 354±37/$^{a}$$^{a}$ | 4/39±0/05$^{c}$ | 303±13/7$^{a}$ | 4/5$^{b}$±0/10$^{cd}$ | 279±15/$^{a}$a | C1p3 |
| 4/15±0/13$^{b}$ | 366±41/$^{b}$d | 4/18±0/1$^{b}$ | 351±46/$^{a}$a | 4/21±0/08$^{cd}$ | 354±37/$^{a}$$^{a}$ | 4/4$^{b}$±0/1$^{c}$ | 297±15/$^{a}$a | 4/55±0/05$^{cd}$ | 276±10/$^{a}$a | C2p3 |
| 4/1$^{b}$±0/17$^{b}$ | 384±57/$^{b}$bcd | 4/15±0/13$^{b}$ | 366±41/$^{a}$a | 4/20±0/07$^{d}$ | 354±37/$^{a}$$^{a}$ | 4/39±0/12$^{c}$ | 303±13/7$^{a}$ | 4/5$^{b}$±0/1$^{a}$$^{d}$ | 282±20/$^{a}$a | Pc3 |
| 4/73±0/03$^{a}$ | 144±32/4$^{f}$ | 4/7$^{b}$±0/02$^{a}$ | 144±32/4$^{c}$ | 4/73±0/03$^{a}$ | 144±32/4 $^{c}$ | 4/73±0/03$^{a}$ | 144±32/4$^{c}$ | 4/73±0/03$^{a}$ | 144±32/$^{a}$c | C2 |
| 3/81±0/01$^{c}$ | 444±20/$^{a}$ab | 3/86±0/04$^{c}$ | 414±18/0$^{a}$ | 3/9$^{b}$±0/06$^{e}$ | 36384/$^{a}$ $^{a}$ | ۰۰/$^{a}$±0/08$^{d}$ | 306±15/$^{a}$a | 4/1$^{a}$±0/07$^{e}$ | 258±20/$^{a}$ab | C1p2 |
| 3/8$^{a}$±0/03$^{c}$ | 435±31/6$^{abc}$ | 3/87±0/05$^{c}$ | 414±18/0$^{a}$ | 3/9$^{b}$±0/06$^{e}$ | 381±41/$^{a}$a | 4/0$^{b}$±0/08$^{d}$ | 294±20/$^{a}$a | 4/17±0/0$^{b}$e | 255±2$^{a}$/0ab | C2p2 |
| 3/7$^{a}$±0/01$^{c}$ | 474±22/6$^{a}$ | 3/84±0/04$^{c}$ | 429±22/6$^{a}$ | 3/89±0/0$^{a}$e | 390±2$^{a}$/0$^{a}$ | 3/99±0/08$^{d}$ | 306±15/$^{a}$a | 4/1$^{a}$±0/06$^{e}$ | 261±23/8ab | Pc2 |

Values in a same column and with the same letter do not have significant difference (p> 0.05).

According to the (Table 1) initial pH of vegetables juice with 4, 3 and 2% concentrations before fermentation were 4.65±0.02, 4.683±0.01 and 4.73±0.03 respectively that, during fermentation and 4 weeks storageat $4^0$C, the amount of pH gradually decreased and lactic acid increased as well. The results showed that effect of time on pH and lactic acid was significant (p<0.01).

After 24 hours fermentation, and one week storage at 4 °C, no dramatic reduction occurred in pH (increase in lactic acid) of the samples with 4% concentration and this difference was not significant (p>0.05). but, after the second, third and fourth weeks, sample Pc4 had the maximum amount of lactic acid and the minimum amount of pH, and there was a significant difference in pH andlactic acid betweenPc4 and control (C4) samples (p<0.05). The maximum acidity and minimum pH after 24 hours fermentation and during 4 weeks storage at $4^0$C related to treatments Pc3 and Pc2, between 3% and 2% concentrations respectively that had no significant difference with

the other ratios of bacteria in these concentrations (p>0.05). The results showed that, various ratios of the probiotic bacteria did not cause significant difference in pH reduction and increase of lactic acid (p>0.05) but, various concentrations of vegetables juice caused a significant difference in pH reduction and lactic acid increasing (p<0.01). Generally treatments containing 2% of vegetable juices, had the highest level of lactic acid and the lowest pH. Graph (1) and (2) show that, the minimum pH and maximum lactic acid related to the treatment Pc2 (35% Lactobacillus casei +65%Lactobacillus plantarum) with 2% concentration in 28th day of maintenance at $4^0$C.

*Changes inbacterial growthafterfermentation andduring storage*

The number of bacteria was investigated after 24 hours fermentation at 37 $^0$C and during 4 weeks storage at $4^0$C (Table 2).

**Table 2.** The mean of the bacteria survival as cfu/ml in fermented vegetables juice with various concentrations of vegetables juice and various ratios of *Lactobacillus casei* and *Lactobacillus plantarum* during 24 hours fermentation at 37 $^0$C and 4 weeks storage at $4^0$C ±standard deviation.

| survival (cfu/ml) | | | | | |
|---|---|---|---|---|---|
| Fourth week | Third week | Second week | First week | First day | |
| | | | | | treatment |
| 1/900 ×$10^{10}$±0/793×$10^{10a}$ | 2/466×$10^{10}$±0/709×$10^{10a}$ | 2/700×$10^{10}$±1/442×$10^{10a}$ | 5/536×$10^{9}$±6/681×$10^{9c}$ | 2/276×$10^{8}$±1/602×$10^{8b}$ | $C_1p4$ |
| 1/263×$10^{10}$±0/866×$10^{10a}$ | 2/126×$10^{10}$±1/572×$10^{10a}$ | 2/626×$10^{10}$±1/960×$10^{10a}$ | 4/383×$10^{9}$±5/097×$10^{9c}$ | 2/103×$10^{8}$±1/474×$10^{8b}$ | $C_2p4$ |
| 2/076×$10^{10}$±1/637×$10^{10a}$ | 2/833×$10^{10}$±1/553×$10^{10a}$ | 3/266×$10^{10}$±1/761×$10^{10a}$ | 1/790×$10^{10}$±2/697×$10^{10bc}$ | 5/433×$10^{8}$±3/780×$10^{8b}$ | Pc4 |
| 1/986×$10^{10}$±0/987×$10^{10a}$ | 2/500×$10^{10}$±1/126×$10^{10a}$ | 3/200×$10^{10}$±0/871×$10^{10a}$ | 2/433×$10^{10}$±0/850×$10^{10abc}$ | 2/566×$10^{9}$±1/858×$10^{9b}$ | $C_1p3$ |
| 1/433×$10^{10}$±0/714×$10^{10a}$ | 2/153×$10^{10}$±1/920×$10^{10a}$ | 2/800×$10^{10}$±0/400×$10^{10a}$ | 8/476×$10^{9}$±13/456×$10^{9bc}$ | 4/473×$10^{8}$±3/491×$10^{8b}$ | $C_2p3$ |
| 2/873×$10^{10}$±0/325×$10^{10a}$ | 3/466×$10^{10}$±0/351×$10^{10a}$ | 4/066×$10^{10}$±0/450×$10^{10a}$ | 3/206×$10^{10}$±0/816×$10^{10ab}$ | 5/296×$10^{9}$±4/303×$10^{9b}$ | Pc3 |
| 2/536×$10^{10}$±0/545×$10^{10a}$ | 2/220×$10^{10}$±1/611×$10^{10a}$ | 3/866×$10^{10}$±1/171×$10^{10a}$ | 2/933×$10^{10}$±1/258×$10^{10abc}$ | 4/006×$10^{9}$±0/943×$10^{9b}$ | $C_1p2$ |
| 2/003×$10^{10}$±1/547×$10^{10a}$ | 2/986×$10^{10}$±1/766×$10^{10a}$ | 3/116×$10^{10}$±0/076×$10^{10a}$ | 2/733×$10^{10}$±1/159×$10^{10abc}$ | 2/533×$10^{9}$±0/404×$10^{9b}$ | $C_2p2$ |
| 3/100×$10^{10}$±1/000×$10^{10a}$ | 4/033×$10^{10}$±1/677×$10^{10a}$ | 4/433×$10^{10}$±1/767×$10^{10a}$ | 4/300×$10^{10}$±0/984×$10^{10a}$ | 2/720×$10^{10}$±0/713×$10^{10a}$ | Pc2 |

Values in a same column and with the same letter do not have significant difference (p> 0.05).

The results in table 2 present that the bacteria population increased from an initial number of $10^8$ cfu/ml after 24 hours fermentation at 37 $^0$C. The maximum bacteria growth was observed in treatment Pc2 by 2.720×$10^{10}$± 0.713×$10^{10}$cfu/ml after 24 hours fermentation which had significant difference with other ratios of bacteria in 3% and 4% concentrations of vegetables juice (p<0.05). There was no significant difference in bacteria population between different ratios of bacteria in 3% and 4% concentrations of vegetables juice during this time(p>0.05). After one week storage at 4 $^0$C, the bacteria population increased in all treatments and the maximum mean of bacteria growth related to the treatment Pc2 by 4.300×$10^{10}$±0.984×$10^{10}$cfu/ml which was significantly higher than treatment $C_1p4$ , $C_2p4$ and Pc4 in 4% concentration and treatment $C_2p3$ in 3% concentration (p<0.05). After 2 weeks maintenance of vegetables juice at $4^0$C, bacteria population increased in all samples and the maximum bacteria population was observed in treatment Pc2 by 4.433×$10^{10}$± 1.767×$10^{10}$ cfu/ml(Fig 3),but, no significant difference was seen among other the treatments (p>0.05). Bacteria counting assay after 3 and 4 weeks storage at $4^0$C showed that, viable cell count decreased in all treatments and no significant difference was observed between treatments (p>0.05). The maximum amount of viability after 4 weeks storage at $4^0$C related to treatment Pc2 by 3.100×$10^{10}$±1.000×$10^{10}$ cfu/ml. Totally, the results showed that, the effect of vegetables juice concentration and time (p<0.01) and the effect of bacteria ratios (p<0.05) were significant on number of lactic acid cultures.

*Changes in glucose content*

The amount of glucose sugar in 2, 3 and 4% concentrations of vegetable juice was measured after 24 hours fermentation at 37 $^0$C by HPLC (Table 3). The initial value of glucose in 2%, 3% and 4% concentrations were 40.4, 55.2 and 60.1 mg/ml respectively.

**Table 3.** Mean of glucose sugar (mg/ml) in fermented vegetables juice with various concentrations and different ratios of *Lactobacillus casei* and *Lactobacillus plantarum* during 24 hours fermentation at 37 $^0$C ±standard deviation.

| Glucose sugar mg/ml | Treatment |
|---|---|
| 60/100±0/000$^a$ | C4 |
| 42/866±1/628$^d$ | $C_1$p4 |
| 45/600±1/868$^c$ | $C_2$p4 |
| 39/266±1/069$^e$ | Pc4 |
| 55/200±0/000$^b$ | C3 |
| 38/333±1/115$^e$ | $C_1$p3 |
| 42/833±1/150$^d$ | $C_2$p3 |
| 33/600±2/722$^f$ | Pc3 |
| 40/400±0/000$^e$ | C2 |
| 19/400±0/655$^g$ | $C_1$p2 |
| 21/266±1/778$^g$ | $C_2$p2 |
| 18/500±0/984$^h$ | Pc2 |

Values in a same column and with the same letter do not have significant difference (p> 0.05).

According to the graph 4, the amount of glucose sugar of control treatments had no change after 24 hours fermentation at 37 $^0$C, but this factor dropped in other treatments($p<0.05$). The highest level of sugar reduction related to treatment Pc2 that had significant difference with other samples ($p<0.05$). According to the Table 3, the lowest level of remained sugar in 4% concentration of vegetables juice related to the sample Pc4 by 39.26± 1.06 mg/ml which had significant difference with $C_2P4$ and $C_1p4$($p<0.05$). The minimum remained sugar in treatments with 3% concentration belonged to treatment Pc3 by 33.600±2.722 mg/ml which had significant difference with other samples in this concentration ($p<0.05$). The results presented that, the effect of vegetables juice concentration was significant on glucose consumption ($p<0.01$).

**Discussion**

*changes in pH and lactic acid during fermentation and cold storage*

The results of this study, showed that, bacteria growth in vegetables juice with various concentrations has led to pH reduction and increase in lactic acid. The main reason of this case has been attributed to the sugars consumption and production of organic acid by lactic acid cultures (Moraru *et al.*, 2007). A negative correlation there was between pH and lactic acid, during fermentation at 37 $^0$C and 4 weeks of cold storage at 4 $^0$C, it means that, by pH reduction, lactic acid increased(fig.5)

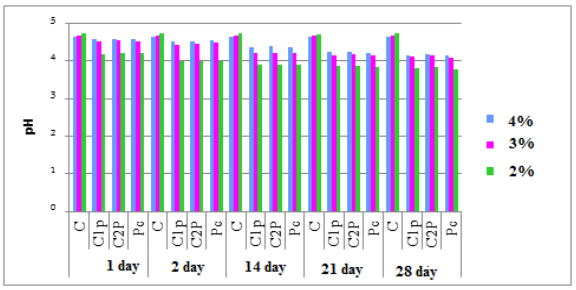


**Fig. 1.** Effect of vegetables juice concentration and various ratios of bacteria in different days of cold storage on pH.

Sample of Pc2 (35% Lactobacillus casei + 65% *Lactobacillus plantarum*) with 2% brix had minimum pH and maximum rate of lactic acid after fermentation and during 4 weeks cold storage. This results are in agreement with those presented by Yun *et al.* (2005) who studied fermentative beet juice using *Lactobacillus casei*, *Lactobacillus plantarum*, Lactobacillus acidophilus and Lactobacillus delbrueckii (30°C for 72 h). They reported that, *Lactobacillus plantarum* decreased pH more than Lactobacillus casei and also, *Lactobacillus plantarum*, reduced the pH from an initial value of 6.3 to 4.2 after 24 hours fermentation while, *Lactobacillus casei* decreased this value from 6.3 to 5.

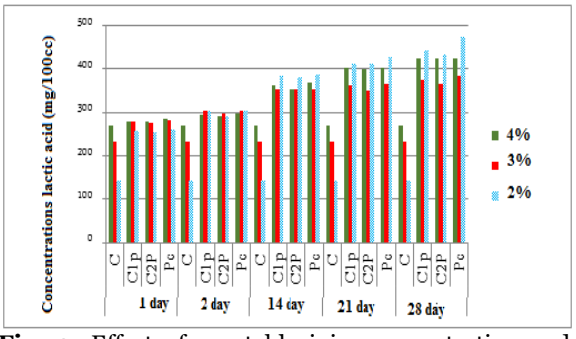


**Fig. 2.** Effect of vegetable juice concentration and various ratios of bacteria in different days of cold storage on lactic acid.

The results of present study showed that, treatments with lower concentration (2%) had the maximum reduction in pH and increase in acidity. Saw *et al.* (2011) also studied the production of tropical juices using *Lactobacillus casei*, *Lactobacillus acidophilus*

and *Lactobacillus delbrueckii* subsp. *bulgaricus*, and they reported that, pH in juices with lower level of brix had more reduction than juices with higher level of brix which is in agreement with the results of present study.

The pH of the sample Pc2 reached from an initial value of4.14 to the minimum value (3.78) after 28 days of cold storage at 4 $^0$C. Priera *et al.* (2011) in an investigation showed that, inoculating *Lactobacillus casei* to Cashew apple juice, dropped pH from an initial value of 4.28 to 3.79 at the end of 42 days of cold storage at 4$^0$C. Also, Guo *et al.* (2009) reported that, *Lactobacillus casei* after 24 hours fermentation, reduced pH of fermented milk to 5.59 and after 28 days storage in refrigerator at 4 $^0$C, pH reached 4.6.It could be concluded that, the pH level of probiotic products depend on probiotic species and base of product which may be based on water or milk. For example, it has reported that, the products based on water, have reduced pH higher and faster during the storage period (Pereira *et al.*, 2011).

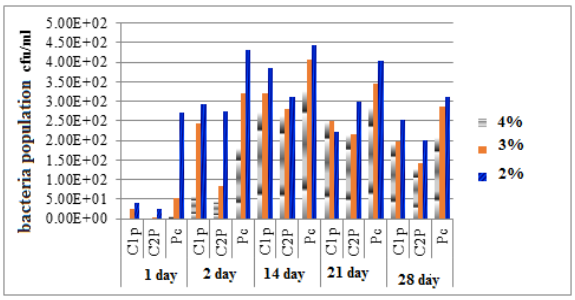


**Fig. 3.** Effect of vegetables juice concentration and various ratios of bacteria in different days of cold storage on the bacteria population.

*Changes in bacteria population during fermentation and cold storage*

According to the results of bacterial growth in the vegetable juice, it was observed that, treatment Pc2 (35% *Lactobacillus casei* + 65% *Lactobacillus plantarum*) with 2% brix, had the highest growth during fermentation and the maximum viable cell counts during 4 weeks cold storage at 4$^0$ C. During 14$^{th}$ day of cold storage at 4$^0$ C, bacteria population increased in all of the treatments, and it was decreased during the third and fourth weeks of cold storage at 4$^0$ C in all treatments. The main reason for decrease of viability of probiotic cells have been attributed to pH reduction of the environment and accumulation of organic acid as a consequent of bacteria growth and fermentation (Yoon *et al.*, 2004). Periera *et al.* (2011) found that, *Lactobacillus casei* grew during cold storage period, and viable cell counts reached from an initial value of 7.48 log cfu/ml to more than 8 log cfu/ml during 42 days storage at 4$^0$C. Also in present study, bacteria population increased during two weeks of cold storage.

Probably, the reason of increase in probiotic cells population in samples with 2% concentration was the inhibitory effect of high concentrations of soluble solids. Saw *et al.* (2011) reported that, by dropping the amount of soluble solids, the rate of bacteria growth increased. They also presented that, high level of brix prevent of probiotic cells growth which is in agreement with the results of this study.

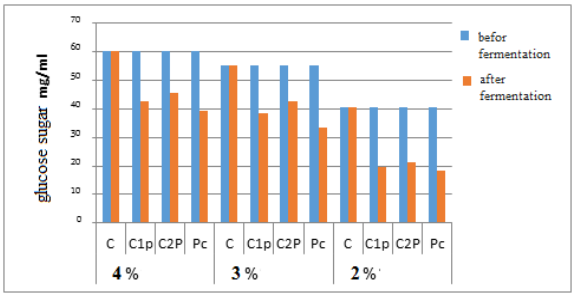


**Fig. 4.** Effect of vegetables juice concentrations and various ratios of bacteria on glucose sugar during fermentation.

Also higher population of lactic acid cultures in Pc2 was due to faster growth of *Lactobacillus plantarum* that this bacterium there was more than *Lactobacillus caseiin* this treatment. Yun *et al.* (2004) reported that, the population of four lactic cultures (*Lactobacillus casei, Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus delbrueckii*) with initial inoculation (> $10^5$ cfu/ml) reached more than $10^8$ cfu/ml after 48 hours incubation of tomato juice at 30 $^0$C. For example viable cell counts of *Lactobacillus plantarum* and *Lactobacillus casei* were 2×10$^9$cfu/ml and 9×$10^8$ cfu/ml respectively that indicated faster growth of *Lactobacillus plantarum* compared to the

other cultures. They reported thatviable cell counts of *Lactobacillus casei, Lactobacillus acidophilus , Lactobacillus delbrueckii* and *Lactobacillus plantarum* were $1.7\times10^8$, $1.4\times10^9$, $8.1\times10^8$ and $3.4\times10^6$ cfu/ml respectively, after 4 weeks of cold storage at $4^0$C. It is important to have a significant number of viable lactic acid bacteria in the product for maximum health benefits (Shah, 2001) the results of present research indicated that the viable cell counts were higher than $10^{10}$ cfu/ml after 4 weeks of cold storage at $4^0$C. Consequently, fermented vegetables juice could be considered as a probiotic beverage without any nutrient supplementation.

*Sugar variations during the fermentation*

According to the assay of sugar reduction, it was found that, the amount of glucose decreased in all treatments after 24 hours fermentation. This was due to the growth of probiotic cells and organic acids production. In many researches, glucose has been introduced as the most important carbohydrate source for probiotic Lactobacilluses (Mousavi *et al*., 2011; Helland *et al*., 2004).

The results indicated that, Pc2 (35% *Lactobacillus casei* + 65% *Lactobacillus plantarum*) with 2% brix had the maximum reduction of glucose content after 24 hours fermentation. Yun *et al.* (2004) reported that, *Lactobacillus plantarum* reduced sugar content faster than the other strains during tomato juice fermentation (30 °C for 72 h). This bacterium decreased the sugar content from an initial value of 32.4 mg/ml to 25.2, 21 and 19.3 mg/ml after 24, 48 and 72 hours fermentation respectively. These authorsin other study on fermentative cabbage juice by *Lactobacillus plantarum, Lactobacillus delbrueckii* and *Lactobacillus casei* (30 °C for 72 h)presented that, *Lactobacillus plantarum*, consumed sugar content more than other cultures (Yun *et al*., 2006), which are in agreement with the results of this study.

Muraro *et al.* (2007) investigated the production of fermentative celery juice and beet juice with and without pulp by Bifidobacterium BB12.They reported that, fermentative sugar content in celery juice with pulp decreased 57.11% and viable cell counts increased 18.65% and reached $1.2\times10^8$ cfu/ml after 48 hours fermentation. In cloudy beet extract, fermentative sugar content reduced with less slope than cloudy celery extract but, increase of the bacteria number was the same as in the celery extract. They also reported that, by decreasing the fermentative sugar content, increased treatable acidity.

Also Buruleanu*et al.* (2009), evaluated effect of inulin prebiotic on quality of lactic acid produced by fermentation of carrot and beet extract, and they reported that, the amount of glucose reducedby inoculation of Bifidobacterium BB12after 48 hours fermentation at $37^0$C, and in contrast the amounts of lactic acid and acetic acid increased in these extracts, that this reports are correspond with the obtained results of present research.

**Conclusion**

According to the results, *Lactobacillus casei* and *Lactobacillus plantarum* grew and survived in the vegetables juice without any nutrient supplementation during 4 weeks storage at 4 $^0$C. This fermented vegetables juice had adequate number of probiotic bacteria for maximum health benefits. Also results showed that, sample Pc2 (35% *Lactobacillus casei* + 65% *Lactobacillus plantarum*) with 2% brix had the maximum growth during the fermentation and maximum viability during the storage period. It was due to *Lactobacillus plantarum* grew faster than Lactobacillus casei during the fermentation, and level of brix in Pc2 was lower than the others. Also, the minimum pH and maximum acidity was observed in this sample. pH reduction was due to the production of organic acids as a result of carbohydrates consumption by probiotic cells in the vegetables juice. Glucose sugar reduced during fermentation which was because of the bacteria growth and production organic acids.

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