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Storage study of prepared probiotic beverage by blending apple and orange juice

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Abstract
The probiotic beverage was prepared by blending apple and orange juice (60:40) of Total Soluble Solids (TSS) 13^0 Brix, inoculated using 10% mixed culture of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* (1:1) and fermented for 5 hrs was standardized. The physico-chemical characteristics of beverage were changed TSS (13 to 11.7 ^{0}Brix), pH (5.30 to 5.21), acidity (0.51 to 0.72%) and ascorbic acid (22.15 to 15 mg/100mL) in the fourth week of storage. The overall acceptability score of the sample was found to be continuously decreasing during a storage period of 4 weeks. The number of probiotic bacteria increased from an initial number of 2.8 x 10^9 to 4.5 x 10^9during second week of storage. However, viable counts of probiotic bacteria decreased after third and forth weeks of storage at refrigerated temperature. The microbial analysis of beverage was found to be free form yeast, mold and coliform bacteria. Finally, it can be concluded that probiotic beverage prepared by blending apple and orange juice was potential carrier of viable cells with good organoleptic characteristics in 4 weeks storage period.

Keywords: Apple and orange juice, probiotics, physico-chemical characteristics, microbial analysis

Introduction
Recently, consumer's awareness towards the relationship between food and health had led to an explosion of interest in healthy foods and beverages. The idea of health promoting foods is not new. Hippocrates wrote 2400 years ago *"Let food be the medicine and medicine be the food".* Now-a-day, healthy food means functional foods which exert beneficial effect on more specific body functions, in addition to the traditional nutritional effects. The well-known examples of functional foods are those containing or prepared with bioactive compounds like phytochemicals, dietary fibre, oligosaccharides and active friendly bacteria (probiotics) (Jankovic *et al*., 2010) [9].

Apple is the second most consumed fruit in the world (Drogoudi and Pantelidis, 2011) [6] and contains many phenolic compounds beneficial to human health (Wolfe *et al.,* 2003) [29]. It is reported that because of their high antioxidant capacity, phenolics offer protection from cancer, cardiovascular conditions and some age-related diseases (Knekt *et al.,* 1997; Kris-Etherton *et al.,* 2002; Ju *et al.,* 2012) [14, 15, 11].

Oranges are one of the most popular fruits around the world, they are a hybrid between pomelo (*Citrus maxima*) and mandarin (*Citrus reticulata*), which has been cultivated since ancient times. Citrus fruits and juice, as such, have long been valued for their wholesome nutritious and antioxidant properties and by virtue of their abundance in vitamins, antioxidants and minerals have many proven health benefits. Moreover, it is now well confirmed, in citrus fruits, the presence of biologically active non-nutrient compounds, such as phytochemical, antioxidants (Goulas and Manganaris, 2012) [8] and soluble or insoluble dietary fibres involved in reducing the risk for stroke (Kurl *et al.,* 2002) [16], chronic diseases like arthritis (Pattison *et al.,* 2004) [21], hypertension (Galati *et al.,* 1994) [7] and coronary heart diseases.

Materials and Methods
Preparation of apple and orange juice
Freshly harvested apple and orange fruits were procured from local market of Parbhani (Maharashtra). Apple and orange juice was prepared by using domestic mixer and blended in the proportion of 50:50, 60:40 and 70:30 respectively. Total soluble solids of the prepared beverage were maintained to 13^0Bx and stored at 4 ^{0}C.

Table 1: Apple and orange juice with variations in proportion

0	Orange
100	00
50	50
60	40
70	30

Probiotic Strains
Lactobacillus isolates, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were isolated and identified using phenotypic and genotypic methods in Department of Food and Industrial Microbiology, College of Food Technology, VNMKV, Parbhani.

Preparation of Starter Culture
Starter culture was prepared with the help of method described by Mousavi *et al.*, (2010) [19] with slight modifications. *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* was cultivated separately in the MRS broth for 48 h at 37 ^{0}C. To obtain the biomass, 10 mL of the separately cultivated MRS broths were mixed in equal proportion (1:1) and centrifuged at 4000 rpm for 10 min. The obtained biomass was washed with sterile saline solution twice to remove the residual MRS media. Thus, inoculum was prepared. It was then introduced into pasteurized blend of apple and orange juice (100 mL) for making it 10% concentration of probiotics. The inoculated juice was then incubated at 37 ^{0}C for 24 hr and was treated as starter culture for preparation of final beverage.

Preparation of probiotic blend of apple and orange juice
The starter culture (10 mL) was then added to the pasteurized blend of apple and orange juice (100 mL) to obtain 10% inoculation. It was allowed to ferment in incubator at 37 ^{0}C for 5 hr. After incubation, the beverage was kept at refrigeration temperature for future use.

Physico-chemical analysis of probiotic blend of apple and orange juice
The physico-chemical properties of blended apple and orange juice were determined: These were as follows;

Total soluble solids (T.S.S.), acidity and pH
Total soluble solids were measured immediately after extraction using hand refractometer (ERMA make).Acidity, expressed as per cent lactic acid, was determined by titration against 0.1N NaOH using phenolphthalein as an end point indicator. The pH value was obtained by using a digital pH meter (ELICO LI612) after standardizing it with buffers of pH 4.0 and 9.0 (Ranganna, 1991) [22].

Ascorbic Acid (vitamin C)
Ascorbic acid contents of samples were determined according to the titration method using 2, 6-dichlorophenol indophenols (Ranganna, 1991) [22].

Sensory evaluation of probiotic blend of apple and orange juice
Prepared probiotic blend of apple and orange juice was evaluated for organoleptic characteristics like color, flavor, taste, and overall acceptability by a panel of semi trained judges, comprised of postgraduate students and academic staff members of College of Food Technology, V.N.M.K.V., Parbhani. Samples were scored based on a nine point hedonic scale. Judges were asked to rate the product on 9 point Hedonic scale with corresponding descriptive terms ranging from 9 'like extremely' to 'dislike extremely'(Meilgaard *et al.*, 1999) [18].

Microbial analysis of probiotic blended apple and orange juice
The viable count of mixed culture was determined by the standard plate count method using Man-Rogosa-Sharpe agar (MRS agar) and the results were expressed as CFU/mL juice. The yeast and mold count of beverage was determined using potato dextrose agar medium. The coli-form and basically *E. coli* are the indicator microbes of water contamination by feces. The coli-form gives red pink color colonies on the MacConkey agar. Plates were incubated at 37 ^{0}C for 48-72 hr (Chris *et al*., 2006) [4].

Statistical Analysis
The data obtained was analyzed statistically by Completely Randomized Design (CRD) as per the procedure given by Panse and Sukhatme (1967). The analysis of variance revealed at significance of $P< 0.05$ level, S.E. and C.D. at 5% level is mentioned wherever required.

Results and Discussion
A- 50:50(apple juice + orange juice),
B- 60:40(apple juice + orange juice),
C- 70:30(apple juice + orange juice).

Amongst all the blends, blend B of apple and orange juice (60:40) was found to be most acceptable. Similar results reported by Biswas *et al*., (2016) [2] recorded for sensory attributes of *Aloe Vera* and Pineapple Juice Blended Beverage. Tiwari *et al*., (2015) [28] recorded the observation on preparation and storage of blended RTS beverage from bael and *Aloe vera*.

Physico-chemical properties of standardized probiotic blend of apple and orange juice sample
Physico-chemical parameters play a significant role in the quality of the juice and also survival of probiotic cultures. The probiotic blend of apple and orange juice were analyzed quantitatively for their chemical compositions. The data of various parameters such as total soluble solids, pH and percent acidity are presented in Table 2.

Table 2: Physico-chemical properties of probiotic beverage

Properties	Values
TSS (^{0}Brix)	13.00
Acidity (%)	0.51
pH	5.30
Ascorbic acid (mg/100mL)	22.15

TSS of the extracted juice was adjusted to13^0 Brix before inoculation of strains. From Table 2, it is observed that during 5 hr incubation the free strains reduced the TSS to 12.4 ^{0}Brix along with reduction in pH of the juices leading to increase in acidity. Martin-Diana *et al.*, (2003) [17] reported that adding probiotic starter culture caused decrease in pH value of the beverage at the same time titratable acidity was found to be increased. These results are also similar with the findings of Salwa and Diekmann, (2000) [23] for pH as there is an inverse relationship between acidity and pH.
Percent acidity of the sample was 0.51.The prepared probiotic juices showed a decline in ascorbic acid content which may be attributed to treatments and processing conditions before and after juice extraction. The ascorbic content of the sample decreased to 22.15 mg/100ml.

Sensory evaluation of blended juice sample during storage
The sensory evaluation of the prepared probiotic samples was performed at weekly intervals for a period of 4 weeks to examine the acceptance and the scores are presented in table 3.

Table 3: Sensory evaluation of beverage during storage

Sample B (60:40)	Probiotic beverage during storage			
Time in Weeks	Color	Flavor	Taste	Overall acceptability
0	8.6	8.4	8.0	8.7
1	8.5	7.9	7.8	8.5
2	8.3	7.4	7.5	7.7
3	8.2	7.0	7.1	7.4
4	8.1	6.6	6.6	7.0
Mean	8.3	7.4	7.4	7.8
SE ±	0.914	0.1033	0.1015	0.0639
CD @ 5 %	0.1293	0.3109	0.3057	0.1925

*Each value is the average of 3 determinations.

As per the sensory scores of prepared probiotic juice, it was found that there was not much difference in the sensory characteristics when the samples were consumed just after incubation. However, the data presented in table 3, showed that the overall acceptability score of all the sample was found to be continuously decreasing during a storage period of 4 weeks. This may be attributed to the unfavourable deterioration reactions during storage. Similar results were reported by King *et al.*, (2007) [12].

Effect of storage on physico-chemical properties of probiotic beverage at refrigerated storage
The sample B (60:40) containing strains had shown maximum consumer acceptability than other samples. Hence, storage study was conducted for Sample B for period of 4 weeks. The quality parameters selected for investigation include TSS, pH, percent acidity and ascorbic acid content and the obtained results are presented in Table 4.

Table 4: Physico-chemical changes in probiotic beverage during storage

Time in weeks	TSS (^{0}Brix)	pH	% Acidity (Lactic acid)	Ascorbic acid
0	13.0	5.30	0.51	22.15
1	12.3	5.27	0.55	21.05
2	12.1	5.26	0.62	19.00
3	12.0	5.21	0.70	16.00
4	11.7	5.21	0.72	15.00
Mean	12.22	5.25	0.62	18.64
SE ±	0.0914	0.0822	0.0091	0.0495
CD @ 5%	0.2752	0.2474	0.0257	0.1489

*Each value is the average of 3 determinations.

It could be revealed the TSS concentration found to be declined from an initial value of 13 to 11.7 ^{0}B during a storage period of 4 weeks. The changes in pH during refrigerated storage was found to be 5.30, 5.27, 5.26, 5.21 and 5.21 on the day of preparation, first week, second week, third week and forth week after production respectively. A LAB cultures may have utilized carbohydrates and produced small amount of organic acid thus lowering the pH of the product during storage. The changes in pH and growth of probiotic cultures are much related to each other.
Shukla *et al*., (2013) [23] also reported a decline in pH of probiotic beverage from whey and pineapple juice after 28 days period of storage. In a study by Ding and Shah (2008) [5], it was concluded that probiotic strains reduced the pH of the juice during storage regardless of whether they are in free or encapsulated form. It was also observed that the titratable acidity of sample increased during storage which may be attributed to increase in acids because of breaking down of sugars to acids by LAB cultures. The acidity values of the beverage significantly increased from 0.51% on the day of preparation to 0.72 percent in the fourth week of storage. These results are in agreement with those reported by Tangular and Erten (2012) [25].
The Jeney-Nagymate and Fodor (2008) [10] reported that the stability of ascorbic acid decreases with increase in temperature and pH. This destruction by oxidation is a serious problem in that a considerable quantity of the vitamin C contents of food is lost during processing, storage and preparation. Ajibola *et al*., (2009) [1] also concluded that ascorbic acid decreases gradually during storage especially at temperature above 0˚C.

Viability of probiotic LAB cultures in probiotic beverage during refrigerated storage
The probiotic beverage should contain strains sufficient number of viable microbial cells. The viability of probiotics during storage is of paramount importance because for a probiotic food to confer health benefit and the number of cells should be > 107CFU/mL or g at the time of consumption. The viable cell counts were enumerated and shown in Table 5.

Table 5: Probiotic cell viability in beverage

Times in weeks	Viability (CFU/mL) of probiotic LAB cultures
0	$2.8x10^9$
1	$2.9x10^9$
2	$4.5x10^9$
3	$2.4x10^9$
4	$1.3x10^9$

The number of probiotic bacterias increased from an initial number of 2.8 x 10^9 to 4.5 x 10^9during second week of storage. However, viable counts of probiotic bacteria decreased after third and forth weeks of storage at refrigerated. Although the viable count decreased, it was still above 10^8CFU/mL which was higher than the therapeutic minimum dose. Similar results are reported by Teanpaisan *et al*., (2015) [26].
These results are also in good accordance with Ding and Shah (2008) [5] who reported that free probiotic bacteria rapidly lost their viability in the apple juice within the four-week period. Results indicated that both orange juice and apple juice were both too acidic for probiotic growth there for there is decline in viability.

Microbial analysis of probiotic beverage during storage
The accepted probiotic juice sample was subjected to microbial studies for total plate count, yeast and mould count and *Coliform* growth during the storage period as per method adopted by Cappuccino and Sherman, (1996) [3]. The results recorded are presented in Table 6.

Table 6: Microbial analysis of probiotic beverage

Time in Weeks	Total Plate Count (cfu/ml)x10^8	Yeast & Mould Count (cfu/ml)x10^3	Coliform Count
1	$2.7x10^8$	ND	ND
2	$3.8x10^8$	$1.4X10^3$	ND
3	$4.9x10^8$	$1.1X10^3$	ND
4	$4.7x10^8$	$1.0X10^3$	ND

Steinkraus (1996) [24] reported that yeast counts were strongly correlated with LAB count. Co-metabolism between yeast and LAB and *Bifidobacterium bifidum* may exist, where the bacteria provide the acid environment, which selects the growth of yeast, that in turn; provide vitamins and other growth factors to the bacteria. The progressive decrease in yeast and mould count might be due to resultant increase in acidity during storage.

The results from table 6 also revealed that the probiotic beverage was free from *Coliform* throughout the storage period of 4 weeks at refrigerator temperature as result of good hygienic and sanitary conditions, during the preparation of probiotic beverage.

Conclusion

The blend of apple and orange juice was inoculated with probiotic cultures (10%) of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* (1:1) and fermented for 5 hr. Results showed that the chemical parameters were in sufficient amount for providing nutrition and bioactive components to consumers with good organoleptic characteristics during 4 weeks storage period. Microbiological analysis found that the beverage contained the desired level of probiotic cultures (109CFU/mL) which is helpful for maintaining the health of gastro intestinal tract. Further, the prepared beverage did not contain any traces of yeasts and molds and also coli-form bacteria, thus indicating that beverage is containing only health benefitting bacteria.

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