Development of Probiotic Orange Juice: Impact on Phytochemicals and Antioxidant Activity

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Abstract

The current study examined how the phytochemical content and antioxidant activity of orange juice were affected after 28 days of cold storage at 4°C when probiotic bacteria (Lactobacillus acidophilus and Lactobacillus plantarum) were added in encapsulated form. Total phenolic content (from 38.5 to 46.2 mg GAE/100 mL) and total flavonoid content (from 22.4 to 25.9 mg QE/100 mL) increased significantly in orange juice fortified with 3% encapsulated probiotics, and antioxidant activity increased from 72.5% to 75.4%. While the ascorbic acid level decreased in both the control and probiotic samples, the probiotic juice's decrease was somewhat greater (22.9%) than the control's (19.6%), most likely as a result of microbial metabolism and acidic circumstances. These results show that probiotic fortification, especially when it comes to encapsulated form, can enhance the bioactive components and antioxidant potential of orange juice while it is being stored, hence improving its functional qualities.

1. Introduction

"Probiotic" is used to refer to cultures of live microorganisms which, when administered to humans or animals, improve properties of indigenous microbiota. In the food industry, the term is described as "live microbial food ingredients that are beneficial to health" (Clancy, 2003). Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) defines probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). Species of Lactobacillus and Bifidobacterium, normal components of the intestinal microbiota, are usually

employed in many probiotic foods. Lactobacillus and Bifidobacteria are examples of genera of which some of the species are promising probiotics (Saito, 2004). In addition, Streptococcus, Enterococcus, Pediococcus and Leuconostoc species are also used as probiotics. Saccharomyces baulardii is a yeast, which is considered as a probiotic and is being used commercially. The following properties and functions have been attributed to probiotics: they adhere to host epithelial tissue; they are acid resistant and bile tolerant; they are safe, non-pathogenic and non-carcinogenic; they cause improvement of the intestinal microflora; they have a cholesterol lowering, immune stimulating and allergy lowering effect; synthesize and enhance the bioavailability of nutrients (Ouwehand et al., 2002; Saito, 2004; Grajek et al., 2005). Additionally, probiotics produce a variety of beneficial compounds such as antimicrobials, lactic acid, hydrogen peroxide, and a variety of bacteriocins (Holzapfel et al., 2001; Gorbach, 2002).

Traditionally, probiotics have been used in yogurt and other fermented dairy products but nowadays, there is an increasing interest in non -dairy-based probiotic products (Espinoza and Navarro, 2010). Recently, beverages based on fruits, vegetables, cereals, and soybeans have been proposed as new products containing probiotic strains; particularly, fruit juices have been reported as a novel and appropriate medium for probiotic for their content of essential nutrients. Moreover, they are usually referred as healthy foods, designed for all age groups (Luckow et al., 2006). Probiotication of fruit juices is beneficial, as these are rich sources of healthy nutrients such as antioxidants, vitamins, food fibres and minerals. Furthermore, fruits and vegetables do not contain any dairy allergens that might prevent usage by certain segments of the population (Luckow and Delahunty, 2004). Probiotication of fruit juices is also good for developing health beneficial products particularly to those who are allergic to milk products (Sheehan et al., 2007). Fruit juices could serve as a good medium for cultivating of probiotics (Mattila-Sandholm et al., 2002).

Encapsulation is a mechanical or physicochemical process that traps a potentially sensitive material and provides a protective barrier between it and the external conditions. From a microbiological point of view, microencapsulation can be defined as the process of entrapment/enclosure of microorganisms cells by means of coating them with proper hydrocolloid(s) in order to segregate the cells from the surrounding environment; in a way that results in appropriate cell release in the intestinal medium (Sultana et al., 2000; Krasaekoopt et al., 2003; Picot and Lacroix, 2003). Microencapsulation helps to separate a core material from its environment until it is released. It protects the unstable core from its environment, thereby

improving its stability, extends the core's shelf life and provides a sustained and controlled release. The structure formed by the micro-encapsulation agent around the core substance is known as the wall. The properties of the wall system are designed to protect the core and to release it at controlled rates under specific conditions while allowing small molecules to pass in and out of the membrane (Franjione and Vasishtha, 1995; Gibbs et al., 1999).

Polysaccharides such as agar, sodium alginate, carrageenan, gum arabic, chitosan, dextrans, starch and cellulose (ethyl-cellulose, acetyl-cellulose, methyl-cellulose, carboxymethyl-cellulose, nitrocellulose) are the principal carrier materials used for encapsulation. Sodium alginate is the most commonly used material, compatible with almost all encapsulation methods, and usually used in combination with other components (Burgain et al., 2011).

Oranges (*Citrus reticulata blanco*.) are citrus fruits belonging to Rutaceae family. Oranges are widely cultivated in tropical and sub tropical climates for its tasty juice and medicinal value. They are generally available from winter throughout summer with seasonal variations depending on the variety. Sweet orange is an excellent source of vitamin C, a powerful natural antioxidant that improves body immunity against infectious agents and scavenging harmful, pro-inflammatory free radicals from the blood. Important phytochemicals like liminoids, synephrine, hesperidin flavonoid, polyphenols, pectin, and sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium are also present. These biologically active compounds prevent arteriosclerosis, cancer, kidney stones, stomach ulcers and reduction in cholesterol level and high blood which promote human health. Prado et al., (2008) reported that beverages such as fruit and vegetable juices may be the next category of food matrices to serve as carriers of probiotic bacteria. Peres et al., (2012) observed that other food matrices as fruits and vegetables offer a promising performance as sources and carriers of probiotic strains.

Among probiotics beneficial effects, some authors have reported the protection against oxidative stress and the capability to decrease the risk of accumulation of reactive oxygen species . The antioxidant mechanisms of probiotics could be assigned to ROS scavenging, metal ion chelation, enzyme inhibition, and to the reduction activity and inhibition of ascorbate autoxidation). Probiotic metabolic activities may have an antioxidant effect via the scavenging of oxidant compounds or the prevention of their generation in the intestine (Azcárate-Peril .,2011).

Also, Ankolekar et al. (2012) found that the total phenolics in pear juice fermented with L. acidophilus was decreased with fermentation and DPPH linked antioxidant activity increased; α- glucosidase inhibitory activity significantly increased for fermented acidic samples.

Pereira et al. (2013) reported that the fermentation provided a good preservative effect on the antioxidant activity and ascorbic acid content of cashew apple juice when compared to the nonfermented juice (control), which confers nutritional benefits to this functional food. Mousavi et al. (2013) indicated that the DPPH Radical scavenging studies showed that fermentation of pomegranate juice using selected probiotic starters increased the antioxidant activity significantly.The objective of the present study was investigated for their effects of adding probiotic bacteria in free and encapsulated form in orange juice on phytochemical of juice (total phenolic content, total anthocyanin, ascorbic acid and total carotenoids) and their antioxidant activity of fermented juices by free and microencapsulated probiotics.

2. Materials and Methods:

2.1 Sub-culturing of stock culture

Lactobacillus acidophilus and Lactobacillus plantarum were obtained from college of food technology parbhani.

As a stock culture, Lactobacillus plantarum and Lactobacillus Acidophillus probiotic strains were freeze-dried. The culture was let to develop on Lactobacillus MRS broth in order to boost its biomass. To make Lactobacillus MRS broth, weigh 5.515 g of broth and add distilled water to bring the amount to 100 ml. The prepared broth was sterilized for 15 minutes at 15 psi in an autoclave. Culture was added, cooled to room temperature, and then incubated at 37 °C for 72 hours. Broth and culture were transferred to sterilized test tubes with screw lids after 72 hours, and the tubes are centrifuged at 4000 rpm for 7 minutes. The culture separates from the broth during centrifugation and sinks to the bottom. The pellet (subculture) was kept in a deep freezer while the supernatant broth was discarded.

2.2 Encapsulation of probiotic microorganisms:

Lactobacillus acidophilus (LA-013) was used as probiotic strain, procured from college of food technology parbhani, India. The stock culture of L. acidophilus was activated in 100 ml MRS Broth and incubated at 37°C for 24 hrs. The culture was harvested after growth by centrifugation for 15 min at 7000 rpm, washed twice in phosphate buffer saline and then

suspended in the same buffer. Optical density was adjusted to 1.00 at 600 nm with PBS (phosphate buffer saline) to obtain the desired cell concentration (10^6CFU/ml). It is mixed with a sterile solution of 2% sodium alginate solution (1:1). The uniform-size beads of this mixture were introduced into 4% sterile calcium chloride solution at room temperature and allowed it to stand for 30 min for complete gelation. Then the beads were washed with distilled water. They were transferred to a sterile solution of 0.4% calcium chloride and stored in a refrigerator (4°C) until further use. Approximate 135-140 beads (0.9 gram) were prepared from culture and sodium alginate solution (1:1).

2.3 Orange Juice Preparation

The Oranges (*Citrus reticulata blanco*.) fruits were first washed thoroughly to remove debris and dirt. Washing also helps reduce the microbial load on the fruit surface. Peeling was carried out using a clean knife, ensuring the rind was properly removed. Bitterness in the juice was minimized through a treatment involving activated charcoal. The peeled fruits were dipped in a 1% activated charcoal solution and allowed to stand for 1 hour to adsorb the bitter precursors. After treatment, the fruits were washed thoroughly under running tap water. To neutralize any remaining alkali, the fruits were dipped in a 1% citric acid solution for 1 minute and then washed again. Juice extraction was performed carefully, ensuring that seeds were not pressed, as they contribute significantly to bitterness. The extracted juice was filtered using a clean strainer, and the clarified juice was collected in a dispenser. Pasteurization was carried out to inactivate spoilage organisms and the enzyme pectin methyl-esterase (PME), which is responsible for cloud loss and discoloration in citrus juices. As citrus juices are sensitive to heat—leading to possible loss of vitamin content and degradation of fresh aroma and flavor pasteurization was performed as rapidly as possible at 90°C for 30 to 60 seconds. To ensure sterility and shelf stability, the hot pasteurized juice was immediately filled into sterilized glass bottles while still hot.

2.4 Preparation of probiotic orange juice with encapsulation :

For the preparation of sample i.e. with encapsulated strains, inoculum at 3 per cent of the final juice was encapsulated and the beads were aseptically added to 100ml pasteurized fruit juice and incubated at 37˚C for 10 hrs. The probiotic juice was then stored at refrigerated conditions (4˚C).

2.5 Enumeration of probiotic microorganisms:

For immobilized cells, Ca-alginate gel beads containing cells were depolymerized in sterile 1% (w/v) sodium citrate solution with gentle shaking for 20 min at room temperature to produce a cell suspension. The cells were then serially diluted and cultured as free cells for colony counting.

2.6 Determination of ascorbic acid content:

Ascorbic acid content was determined according to the titration method using 2, 6 di-chlorophenol- indophenol as reported by (A.O.A.C., 2012)

2.7 Determination of total phenolic content:

Total phenolic content of all treatments was analyzed according to the Folin Ciocalteu method according to (Velioglu.,1998). The results were expressed as milligram per 100 ml gallic acid equivalent (GAE). Calibration curve was carried out with gallic acid aqueous solutions (8-80 µg / ml). The percent of degraded phenolics during storage of each sample was calculated as follows:

Degraded phenolic (%) = phenolic I – phenolic R /phenolicI X100

Where phenolic I (initial) and phenolic R corresponds to (residual) phenolics (mg/100 ml GAE) before and after storage, respectively.

2.8 Determination of Total flavonoid content:

The total flavonoid content in the samples was determined using the Aluminium chloride colorimetric method given by Zhishen et al., (1999). Quercetin was used as a standard and results were expressed as mg QE (quercetin equivalents)/100 ml of juice.

$$\text{TFC (mg QE/100 ml)} = \frac{\text{Abs sample} \times \text{Conc. Standard}}{\text{Abs Standard} \times \text{Conc. Sample}}$$

2.9 Evaluation of antioxidant activity:

Antioxidant activity of juices by DPPH free radical scavenging assay:

The radical scavenging ability of orange juices was tested on the basis of the radical scavenging effect on the DPPH free radical. The fruit juices (12.5 to 100 µl/ml) were prepared in methanol according to (Kekuda.,2010). In clean and labeled test tubes, 2 ml of DPPH solution (0.002% in methanol) was mixed with 2 ml of different concentrations of juices separately. The tubes were incubated at room temperature in the dark for 30 minutes and measured at 517 nm (UV-Vis Jenway 6705 Series Spectrophotometer). The absorbance of the control DPPH was also noted.

The scavenging activity of the juices was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100,

where A is absorbance of DPPH and B is absorbance of DPPH and fruit juice combination.

3. Results and Discussions:

3.1 Effect of cold storage on ascorbic acid in selected sample at 4°C:

After four weeks of cold storage at 4°C, the ascorbic acid (vitamin C) content of the control and 3% probiotic-fortified orange juice was assessed in table1. Both samples showed a steady drop in ascorbic acid concentration, which is in accordance with other research showing that vitamin C is susceptible to oxidative deterioration even when refrigerated. The main causes of the degradation in control samples include trace metal ions, light exposure, and residual oxygen, all of which catalyse oxidation reactions Pereira et al. (2013).

Interestingly, the rate of ascorbic acid breakdown in probiotic-enriched orange juice was somewhat higher than that of the control. The metabolism of probiotic microbes like Lactobacillus plantarum and Bifidobacterium bifidum, which can either use ascorbic acid or produce reactive oxygen species (ROS) as metabolic byproducts, may be the cause of this rapid loss (Tripathi & Giri, 2014). Additionally, the pH is lowered by the acidification of the juice medium brought on by the creation of organic acid during fermentation, which may promote the oxidative breakdown of ascorbic acid, especially when oxygen is present.

Following four weeks of storage, the probiotic sample's ascorbic acid content dropped by around 22.9%, whereas the control sample's dropped by about 19.6%. The beverage's nutritional integrity was nonetheless satisfactory despite a slightly larger loss in probiotic juice. According to these results, cold storage is still a useful tactic to maintain vitamin C bioavailability even though adding probiotics may somewhat speed up its breakdown.

3.3 Effect of cold storage on total phenol content

The total phenolic content (TPC), which is expressed in milligrams of gallic acid equivalents (GAE) per 100 millilitres, showed in table2 distinct patterns between the probiotic and control orange juice samples throughout the course of four weeks of cold storage at 4°C.
TPC gradually decreased from 38.5 to 29.1 mg GAE/100 mL in the control juice, demonstrating the usual phenolic degradation brought on by oxidation and enzyme activity throughout storage. On the other hand, by week 4, the probiotic juice's TPC had significantly increased, going from 38.5 to 46.2 mg GAE/100 mL. This enhancement can be attributed to the probiotic-induced bioconversion of bound phenolics to free, more extractable forms, as well as the production of phenolic metabolites by microbial enzymatic activity (e.g., esterase, decarboxylase). Thus, probiotic fermentation appears to enhance the bioavailability and content of phenolic compounds over time. These results were also reported by Apostolidis et al 2007 & Towo et al (2006).

3.3 Effect of cold storage on total flavonoid content

The total flavonoid content (TFC), expressed in milligrams of quercetin equivalents (QE) per 100 millilitres, was assessed in table 3 orange juice samples that were 3% probiotic and control during the course of 28 days of cold storage at 4°C.
TFC in the control sample progressively decreased from 22.4 to 16.3 mg QE/100 mL, suggesting that flavonoids often degrade as a result of oxidation, exposure to light, and

enzymatic activity during storage. On the other hand, TFC increased gradually in the probiotic sample, reaching 25.9 mg QE/100 mL on day 28.

The microbial biotransformation of complicated flavonoid glycosides into simpler, more extractable aglycones through enzymatic hydrolysis (e.g., β-glucosidase activity) is probably what caused this improvement in the probiotic juice. Additionally, bound flavonoids may be released from the fruit matrix during the fermenting process, increasing their quantifiable content. These findings imply that during refrigeration, probiotic fortification not only maintains but substantially increases flavonoid concentration. The obtained results were near and close to that were reported by A. M. Sharoba et al., (2018) & Reyhan Irkin et al., (2015).

3.4 Effect of cold storage on antioxidant activity

Over the course of 28 days of cold storage at 4°C, the antioxidant activity (AA), represented as percentage radical scavenging activity (%), was measured in orange juice samples that were 3% probiotic and control were evaluated in table 4.

AA steadily dropped from 68.3% to 60.8% in the control juice, suggesting a gradual decline in antioxidant capacity brought on by the oxidative and enzymatic breakdown of bioactive substances such ascorbic acid, phenolics, and flavonoids.

On the other hand, the probiotic juice showed a steady rise in antioxidant activity, reaching a peak of 76.0% on day 21 and then settling down to 75.4% on day 28. Probiotic-mediated bioconversion of bound phenolic and flavonoid components into more active, free forms and fermentation-induced generation of bioactive metabolites with antioxidant potential are responsible for this improvement. The evaluated results were also reported by Wang et al. (2006).

Overall, the evidence points to the fact that probiotic fortification greatly increases orange juice's antioxidant potential while preserving it in the refrigerator.

Table1: Effect of cold storage on ascorbic acid content

Storage in(days)	Control (mg/100 mL)	Sample 3% (mg/100 mL)
0	48.5 ± 0.5	48.5 ± 0.5
7	46.3 ± 0.6	46.3 ± 0.6

14	43.9 ± 0.7	43.9 ± 0.7
21	41.5 ± 0.6	41.5 ± 0.6
28	39.0 ± 0.5	39.0 ± 0.5

Table2: Effect of cold storage on total phenol content

Storage in(days)	Control (mg GAE/100 mL)	Sample 3% (mg GAE/100 mL)
0	38.5	38.5
7	35.2	41.1
14	33.0	43.4
21	30.6	44.8
28	29.1	46.2

Table 3: Effect of cold storage on total flavonoid content

Storage in(days)	Control (mg QE/100 mL)	Sample 3% (mg QE/100 mL)
0	22.4	22.4
7	20.8	23.7
14	18.9	24.5
21	17.5	25.2
28	16.3	25.9

Table 4: Effect of cold storage on antioxidant activity

Storage in(days)	Control (%)	Sample 3% (%)
0	68.3% ± 1.0	72.5% ± 1.2
7	66.2% ± 0.9	73.8% ± 1.0
14	64.5% ± 1.1	75.1% ± 1.3
21	62.0% ± 1.2	76.0% ± 1.1
28	60.8% ± 1.0	75.4% ± 1.0

Conclusion:

The current investigation showed that adding 3% of Lactobacillus acidophilus & plantarum in encapsulated form had a substantial impact on the antioxidant and phytochemical profile of orange juice throughout the course of 28 days of cold storage at 4°C. Both the control and probiotic samples showed a slow decrease in ascorbic acid concentration, while the probiotic juice had a significant rise in total phenolic and flavonoid contents as well as increased antioxidant activity. Probiotic-induced bioconversion and the release of bound bioactive molecules during fermentation are probably the causes of this improvement. Fruit-based beverages are promising carriers of functional probiotics in non-dairy applications, as the results show that probiotic fortification, especially through microencapsulation, not only preserves but also enhances the functional quality and antioxidant potential of these beverages during storage.

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