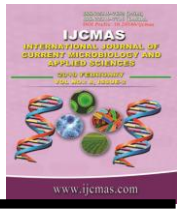
Int.J.Curr.Microbiol.App.Sci (2019) *8*(3): 2028-2034

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 03 (2019)**
Journal homepage: http://www.ijcmas.com



Original Research Article https://doi.org/10.20546/ijcmas.2019.803.241

Effect of Cold Storage on Viability of Probiotics in Non Dairy Probiotic Beverage Based on Carrot and Tomato Juice

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Keywords

L. acidophilus, *S. boulardii*, Temperature, Probiotic beverage, Cell viability

Article Info

Accepted:
15 February 2019
Available Online:
10 March 2019

A B S T R A C T

The carrot and tomato juice (70:30) with 18^0Bx inoculated using 10% mixed culture of *L. acidophilus* and *S. boulardii* (1:1) and fermented for 20 hours. The microbiological analysis showed that prepared beverage contained optimum level of cultures i.e. 8.5 $x10^9$ CFU/mL and yeast and mold $4.5x10^9$ CFU/mL was free from any traces coli-form bacteria. The loss of viability of probiotic cells that is *L. acidophilus* and *Sacchromyces boulardii* cultures were observed in beverage stored at refrigeration conditions for 28 days. It is studied that the viability of cultures decreased during storage, but the count was within the limits (10^7-10^9) that is *L. acidophilus* 4.7 x 10^7 and *S. boulardii* 4.5 x 10^7.

Introduction

Probiotic is the word means “for life” and it is generally used to name the bacteria associated with the beneficial effects for humans and animals. Probiotication is one of the methods to produce fermented functional foods. Addition of probiotics to food provides several health benefits including reduction in the level of serum cholesterol, improvement of gastrointestinal function, enhancement of immune system and reduction in risk of colon cancer (Burner and Donnel, 1998).

The development of probiotic beverages or products in food industry has gained importance for the last two decades. Considerable research and scientific findings on probiotic products have been well documented. The term probiotics was first used by Lilly and Stillwell in 1967, although this concept existed since ancient Greek times. Probiotics represent over 65 per cent of the functional food market (Agrawal, 2005). Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut. They are also called "friendly bacteria" or "good bacteria". Probiotics are

available to consumers mainly in the form of dietary supplements and foods. They can be used as complementary and alternative medicine (CAM) (Prado *et al.*, 2008).

World Health Organization and the Food and Agriculture Organization of the United Nations defined that probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host". The majority of products containing probiotics are dairy-based, which include yogurt and fermented milk beverage. In the last decade, there is an increasing interest in using nondairy ingredients as substrates for certain strains of lactic acid bacteria to deliver the physiological benefits of probiotics to wider group of consumers. A commercial probiotic product is considered as functional only if it contains 10^7 CFU/ml at the time of consumption (Charalampopoulos *et al.*, 2002).

Probiotic foods and beverages are manufactured by either method: (a) by adding the probiotic strains simultaneously with the standard cultures in the fermentation tank; (b) by adding the probiotic culture directly into nonfermented final products. Generally, species of *Lactobacillus* and *Bifidobacterium* are used in most of the probiotic applications. However, due to some drawbacks related to dairy products, there are emerging interests in using non-dairy ingredients as substrates for delivering the physiological benefits of probiotics to wider group of consumers (Prado *et al.*, 2008).

Carrots have also a unique combination of three flavonoids: kaempferol, quercetin and luteolin. They are also rich in other phenols, including chlorogenic, caffeic and p-hydroxybenzoic acids along with numerous cinnamic acid derivates. Among hydroxycinnamic acid and its derivates, chlorogenic acid represents 42.2% to 61.8% of total phenolic compounds detected in different carrot tissues. Carrot juice contains carbohydrates, dietary fiber, protein, fat, Vitamins A, C, B1, B2, B3, B6 and E. It also contains traditional antioxidants such as ascorbic acid, phytonutrient and beta-carotene (Gopalan *et al.*, 1996).

Tomato (*Solanum lycopersicum* Mill.) belonging to the family Solanaceae and is the most important warm season fruit vegetable both nutritionally and economically grown throughout the world. It is one of the most important "protective foods because of its special nutritive value and its widespread production (Kavya, 2013). Among the processed tomatoes, juices may also be considered as health-promoting beverages (Naga *et al.*, 2016).

Since, in addition to being delicious and nutritious, the carrot and tomato juice may be an excellent medium for the supplementation of existing nutraceutical components with probiotic culture.

Thus, pertaining to the above discussion, in response to the demand from increasingly health conscious consumers for nutritive value and medicinal properties of carrot and tomato therefore for developing probiotic carrot and tomato beverage all steps and protocol are given in this research.

Materials and Methods

Preparation of carrot and tomato juice

Freshly harvested carrot and tomato fruits were procured from local market of Parbhani (Maharashtra). Carrot and Tomato juice was prepared by blanching of carrot and Tomato at 60^0C for 20 min. Then blend the juice in ratio of 70:30 of carrot and tomato juice. Its soluble solids was maintained to 18^0Bx, add stabilizer xanthan gum (0.2%) and stored at 4^0C before use.

Probiotic strains

Probiotic isolates, *Lactobacillus acidophilus and Sachharomyces boulardii* were identified using phenotypic and genotypic methods in Department of Food and Industrial Microbiology, College of Food Technology, VNMKV, Parbhani. Stock solution was prepared by adding sterile glycerol (50% v/v) to the activated culture. The glycerol stock culture was stored at -20 ^{0}C in sterile screw cap tubes.

Preparation of starter culture

The starter culture was prepared with the help of method described by Thakur M and Sharma (2017), with slight modifications. *L. acidophilus and S. boulardii* was cultivated separately in the MRS broth and Potato Dextrose Broth for 24-h at 37^{0}C. To obtain the biomass, 10 mL of the separately cultivated MRS broths (5ml) and Potato dextrose broth (5ml) 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 and Potato dextrose media. Thus, inoculum was prepared.

It was then introduced into pasteurized carrot and tomato juice blend (100 mL) for making it 10% concentration of probiotics. The inoculated juice was then incubated at 37^{0}C for 24 h and was treated as starter culture for preparation of final beverage.

Preparation of probiotic beverage

Above prepared starter culture (10mL) was then added to the pasteurized (at 78^0 C for 30 min) carrot and tomato juice blend (100 mL) to obtain 10% inoculation. It was allowed to ferment in incubator at 37^{0}C for 20 h. After incubation, the beverage was kept at refrigeration temperature for future use.

Sensory analysis of probiotic beverage

The sensory evaluation of carrot and tomato based probiotic beverage was carried out by 10 semi-trained panel members comprised of postgraduate students and academic staff members of the faculty who had some previous experience in sensory evaluation. The panel members were requested in measuring the terms identifying sensory characteristics and in use of the score. Judgment were made through rating products on a 9 points Hedonic Scale with corresponding descriptive terms ranging from 9 “like extremely” to 1 “dislike extremely” with respect to different quality attributes such as colour, flavour, taste, aroma, mouthfeel and overall acceptability.

Statistical analysis

All processing equipments and analysis of samples were run in triplicate. Analysis of variance was calculated using standard ANOVA procedure. The data obtained for various treatments was recorded and statistically analyzed by complete randomized design (CRD) to find out the level of significance as per the method proposed by Panse and Sukhatme (1957). The analysis of variance revealed at significance at $P< 0.05$ level. The standard error (SE) and critical difference (CD) at 5 % level were mentioned where required.

Microbial analysis of probiotic beverage

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 37^0C for 48-72 hours (Chris *et al.,* 2006).

Results and Discussion

Sensory evaluation of probiotic beverage

The probiotic beverage were used to sensory analyzed because the overall acceptability of the developed probiotic beverage is to be checked by different sensory evaluation panel and find out which is more delicious and tasty out of 6 prepared samples having different fermentation periods 12, 16, 20, 24, 28 and 32 hrs respectively.

The data in the Table 1 shows that, no significant effect was found among various treatments for appearance, color and consistency. The sample MIT_3 was most preferred in terms of taste and flavour while MIT_1 was least preferred. The MIT_3 sample was preferred because of higher metabolic activity of probiotics in enhanced fermentation period, as said above. Its mean scores for taste, flavour and overall acceptability were 8, 8 and 8, respectively, which were significantly higher ($p<0.005$) than other samples. The values of taste and flavor for MIT_1 sample were 6.5 and 6.8 respectively. The overall acceptability values for all samples varied from 6.5 (MIT_1) to 8 (MIT_6). As observed from it, the most preferred sample by panelists was MIT_3.

Microbial analysis of probiotic carrot and tomato beverage

The growth of undesirable organisms will spoil the product and may lead to food borne diseases affecting the healthy lives. Therefore, performing microbial analysis is mandatory in probiotic based products to assess their safety. The data related to microbiological analysis of probiotic beverage is tabulated in Table 2.

In the present work, the count of beneficial bacteria was detected as 8.5 $x10^9$ CFU/mL and 4.5$x10^9$ CFU/mL of yeast and mold beverage. This count was in range for a product to be called as probiotic (shah N.P 2001).

On the other hand, coli-form count was also determined. And they were not detected in the sample, which showed that the product was free of any pathogenic microbes and safe for consumption.

Effect of storage on viability of probiotics

It is imperative from a health view-point that probiotic strains selected for commercial use retain their viability and functional activity throughout the shelf-life of the delivery product. Therefore, the viability of the lactic cultures is the most important factor during refrigerated or ambient storage which is dependent on the level of oxygen in products, oxygen permeation of the package, fermentation time, and storage temperature. The changes in the counts of probiotic microorganisms during the storage are presented in Table 3.

From the Table 3, it is observed that the probiotic cultures were capable of surviving in the product at 4^oC for 28 days as shown above. The initial microbial population of *L. acidophillus* (TPC) was 8.5 $x10^9$ CFU/mL and Yeast and Mold that is, *Sachharomyces boulardii* was 4.5$x10^9$ just after fermentation. The viable count reduced over the storage period. At the end of 28 days, the microbial count (TPC) reduced to 4.7 $x10^7$ CFU/mL and 4.5$x10^7$ of *saccharomyces boulardii* was detected in sample stored at refrigerated temperature (4^oC). Temperature, which enhances the mortality effect of organic acids, is one of the most important factors on the viability of probiotics. The cell wall of lactic acid bacteria consists of saturated, unsaturated

and cyclic carbon chains, which will vary depending on parameters like temperature, pH, NaCl concentration and medium content. Linoleic and oleic synthesis will occur at acidic situations. These acids will absorb hydrogen in an acidic environment increasing the permeability of proton in membranes, and therefore, leading to viability increase when confronted with hostile conditions throughout acidic situation during storage at refrigeration temperature (Sheehan *et al.,* 2007).

From the standpoint of consumer's health benefits, the selected probiotic cultures must maintain their viability and functionality during the product storage period (Sheehan *et al.,* 2007). For the maximum health benefits, the minimum number of probiotic organisms in a food product should be 10^6 CFU/g (Shah, 2001). In the present investigation, the microbial count was detected higher than this limit for this sample and count was significantly higher in sample throughout the storage period.

The viability losses or the reduction of probiotics' count may be due to the decrease of pH values, post process acid production (Wang *et al.,* 2002), sensitivity to oxygen (Frank *et al.,* 1988) and metabolites such as hydrogen peroxide and ethanol and to bacteriocins produced by lactic acid bacteria (Medina and Jordano, 1994).

When probiotic cells are present in low pH environments (<4.5), increased energy is required to maintain the intracellular pH, resulting in a lack of ATP for other critical functions and thereby causing cell death (Nualkaekul *et al.,* 2011). In addition, because probiotics are devoid of the electron transport chain and/ or catalase enzyme; the presence of oxygen can cause formation and accumulation of toxic metabolites in cells, which can lead to cell death by oxidative damage (Boza Mende˙z *et al.,* 2012; Talwalkar and Kailasapathy, 2004). The continuous exposure to oxygen under acidic conditions during storage is the main reason for the reduction in probiotic counts (Sheehan *et al.,* 2007).

The viable cell population of *L. plantarum* and *L. delbrueckii* remained at an acceptable level (10^6 CFU/mL) after one week of cold storage, but their microbial population decreased below the minimum accepted after 2 weeks (Yoon *et al.,* 2005).

Table.1 Organoleptic evaluation of probiotic carrot and tomato beverage (on 9 point hedonic scale)

S. No.	Samples	Appearance	Color	Taste	Flavor	Consistency	Overall Acceptability
1	**MIT_1**	7.5	7.8	6.5	6.8	7.6	6.5
2	**MIT_2**	7.6	7.8	6.9	7	7.7	6.8
3	**MIT_3**	**8**	**8**	**8**	**8**	**8**	**8**
4	**MIT_4**	7.8	7.8	7.4	7.5	7.8	7.3
5	**MIT_5**	7.9	7.9	7.5	7.6	7.9	7.4
6	**MIT_6**	7.6	7.8	7	7.2	7.6	7
	F-value			41.650	28.650		41.200
	SE±	NS	NS	0.0816	0.0816	NS	0.0816
	CD at 5%			0.2512	0.2512		0.2512

- Each value is an average of ten determinations

Table.2 Microbial analysis of probiotic beverage

S. No.	Parameters	Observations
1	Total plate count (CFU/mL)	8.5×10^9
2	Yeast and mold count (CFU/mL)	4.5×10^9
3	Coli-form count (MPN/mL)	ND

Table.3 Effect of storage during 4 weeks on viability of probiotic cultures in probiotic carrot and tomato beverage at 4^0C

Sr. No.	Parameters (CFU/mL)	Storage Period				
		$0^{\#}$ Day	7 Days	14 Days	21 Days	28 Days
1.	*L. acidophilllus*	8.5×10^9	2.5×10^9	5.3×10^8	9.3×10^7	4.7×10^7
2.	*S. boulardii*	4.5×10^9	8.3×10^8	1.2×10^8	8.9×10^7	4.5×10^7

-Each value is an average of three determinations
$^{\#}$ - Just after fermentation/incubation
ND - not detected

In conclusion, the pasteurized carrot and tomato juice (70:30) was inoculated with probiotic cultures (10%) of *L. acidophilus* and *S. boulardii* (1:1) and fermented for 20 hrs) was found to be most appropriate to improve the quality of beverage. Then, the prepared beverage was analyzed for microbiological characteristics. It was then stored in refrigeration temperature. The loss of viability of probiotic cells that is *L. acidophilus* and *Sacchromyces boulardii* cultures were observed in beverage stored at refrigeration conditions for 28 days. Microbiological analysis found that the beverage contained the desired level of probiotic cultures (10^9CFU/mL) which is helpful for maintaining the health of gastro intestinal tract. It is found that the viability of cultures decreased during storage, but the count was within the limits (10^7-10^9) that is *L. acidophilus* 4.7×10^7 and *S. boulardii* 4.5×10^7. Further, the prepared beverage didn't contain any traces of coli-form bacteria, thus indicating that beverage is containing only health benefitting bacteria.

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How to cite this article:

Shaikh Uzma, A., H.W. Deshpande and Kulkarni, D.B. 2019. Effect of Cold Storage on Viability of Probiotics in Non Dairy Probiotic Beverage Based on Carrot and Tomato Juice. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 2028-2034. doi: https://doi.org/10.20546/ijcmas.2019.803.241