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Development of Carrot Yoghurt with B Carotene, Gamma Amino Butyric Acid and Enhanced Antioxidant Properties

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ABSTRACT: The functional characteristics of carrot juice enriched yoghurt developed are reported in this study. Carrot juice at 10-20%, sugar (5 gm) was optimal for the yoghurt which was fermented using *Streptococcus thermophilus* and *Lactobacillus acidophilus*. Antioxidant activity was determined by DPPH and hydroxyl radical scavenging assays and Gamma amino butyric acid (GABA) by GABASE assay. EPS, β carotene, Riboflavin and Vitamin C were evaluated by standard procedures. Analytical results revealed GABA concentration of 6.04 mM/ gm, 5130.12 μ g, β carotene /100 gm yoghurt with notable (88.6%) antioxidant properties. EPS concentration was 4.40 mg, whereas Riboflavin and Vitamin C were 33.68 μ g and 67.31 μ g/100gm yoghurt. The yoghurt possessed excellent sensory properties, shelf life of 14 days, pH (4.43), syneresis (3ml) and titratable acidity (0.80 % lactic acid). Results of or study suggest that carrot yoghurt may offer a sustainable, affordable variation of yoghurt for the population, as a part of daily diet for nutritional adequacy.

KEYWORDS: Lactococcus lactis, Streptococcus thermophilus, carrot yoghurt, antioxidants, gamma amino butyric acid.

I. INTRODUCTION

Plant and animal foods and their various components are the primary vehicles that provide nourishment to human beings. Nutrition is vital for growth, development of humans and animals and in the prevention and treatment of disease. Unfortunately, an interplay of several factors lead to inadequacies in nutrition which translates to degenerative diseases. For instance cardiovascular and cerebrovascular disease, diabetes, osteoporosis and cancer are among the most common diseases affecting individuals, are all diet-affected. Increasingly in the diet/disease debate, the role that micronutrients play in promoting health and preventing non-communicable disease is receiving considerable attention. Nutrient deficiencies (Vitamin C, Vitamin B₂, and Calcium) are often common in people due to a number of factors such as their reduced food intake and a lack of variety in the foods they eat. Additionally, individuals often suffer from decreased immune function, which contributes to increased morbidity and mortality. Many significant age-related changes include the loss of cognitive function and deteriorating vision, all of which hinder good health and dietary habits especially in old age. Therefore, supplementation of micro-nutrients, antioxidants and probiotics is widely advocated to improve the immune response and prevent chronic disease. Yoghurt, a functional dairy food product, is an effective medium for delivering beneficial functional ingredients. As a low cost, culturally acceptable, nutrient dense food it assumes prominence for affording nutritional adequacy through diets thereby impacting nutritional levels of population. Yoghurt is also very suitable for the elderly for easily affording desired nutrition.

II. RELATED WORK

Carrot (*Daucus carota*) satisfies several conditions as pre-requisites for this purpose including availability and cost. Besides it possesses appreciable levels of carotenoids and dietary fibers. Carrot juice is rich in vitamin A, beta-carotene, minerals and it is easier to digest than raw or cooked vegetables. Carotenoids are important nutrients in fruit and vegetable based products to human health and nutrition because of their pro vitamin A function and antioxidant properties by Knockaert et al. [2012]. Carrots also contain high levels of glutamine which can be fermentatively converted to gamma amino butyric acid (GABA), a non-protein amino acid which is a major inhibitory



neurotransmitter [16]. GABA is involved in the regulation of cardiovascular functions, such as blood pressure and heart rate, and plays a role in the sensations of pain and anxiety by Mody et al. [1994]. GABA has been classified as an important bioactive in foods especially in fermented foods, GABA fortified foods is an emerging area of interest.

Thus far, few studies have investigated the possibility of yoghurt enriched with carrot juice as a means to expand the functionality of yoghurt especially Riboflavin, Vitamin C and GABA. Given the acceptability of yoghurt in all age groups, cultures and being particularly suitable for the aged, we attempted to explore whether a functional yoghurt supplemented with carrot juice could address the nutritional needs of the population. The development, characteristics and functional attributes of the carrot yoghurt is described in the present study.

III. METHODOLOGY

Chemicals and Media

All the chemicals and reagents used were of the highest analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Standard media components were purchased from Fisher Scientific (USA) or Sigma Aldrich (St. Louis, MO, USA) and Hi-media (Mumbai, India).

Microorganisms

Lactobacillus acidophilus ATCC 4312 and *Streptococcus thermophilus* (NDRI, Karnal) (starter culture) was used for yoghurt production. For microbial enumerations, tenfold serial dilutions of 1 gm yoghurt sample were prepared with 9 ml of 0.85% saline solution and 1 ml of the three highest dilutions was pour-plated in duplicate. Colonies counts of strains were enumerated on ST(*Streptococcus Thermophilus*) agar and MRS agar (deMann Rogosa Sharpe) under anaerobic conditions at 30 °C for 72 hours.

Preparation of Carrot Juice

Two hundred and fifty grams of good quality carrots (purchased from local market) were washed thoroughly under running water and scrubbed with a vegetable brush. Carrots were finely chopped or mashed. The puree was poured into the large measuring cup. The juice was strained, stored under refrigerated and used within 2 hrs.

Optimization of Carrot juice, Inoculum size and Sugar

Carrot juice, sugar and inoculums size was optimized as described by Salwa et al. [26]. 10%, 15% and 20% carrot juice was selected for further studies and inoculum size of 1% was optimized for yoghurt preparation, 5 gm of sugar was optimized for yoghurt preparation.

Preparation of Carrot Yoghurt

Plain and sugary yoghurt samples were produced both with and without sugar. Following overnight incubation at 37°C, both the cultures were centrifuged at 8000 rpm for 5 min at 4 °C with successive washing of pellets washed with 0.85% saline. Hundred ml of double toned milk was purchased and was heated to 80°C in a water bath for 10 minutes and cooled to room temperature. Different concentrations of carrot juice (CY 10%, CY 15%, CY 20%), both with and without sugar was then added into it. After incubation, the samples were cooled at room temperature for approximately 1 hr and all samples were refrigerated (4±1°C). The experimental yoghurts were stored for 14 days and analyzed after 1, 7 and 14 days of storage. For control, yoghurt (plain) marketed by local companies with valid FSSAI (Food Safety and Standards Authority of India) certification was used.

Sensory Evaluation, Storage and Viability of cultures

Sensory qualities of the carrot yoghurt along with control yoghurt were evaluated by a panel of judges by grading for color, smell, consistency, texture and overall acceptability score on a 9-point hedonic scale by Wichchukit et al. [34]. Carrot yoghurt samples were stored in sealed aseptic containers at 4 °C.

Analytical determination of yoghurt

Measurement of pH

The pH of yoghurt was determined using bench-top pH meter which calibrated with pH 7.0 and 4.0 standard buffers (Merck, Darmstadt, Germany). Twenty five ml of yoghurt was placed in a beaker, the probe was inserted and pH value was recorded by Joseph et al. [12]



Syneresis

The firmness of the yoghurt gel was measured as the susceptibility to syneresis based on the whey collected (amount of whey /100 gm of yoghurt) of freshly prepared (30 minute's refrigerated and ambient incubation) as well as after 14/21 days of refrigerated storage.

Titrateable acidity

The titrateable acidity was determined by titrating 15 ml of the yogurt with 0.1 M sodium hydroxide until the yoghurt reached a pH value of 8.2, corresponding to the end point of the phenolphthalein by Joseph et al. [13]. The spent NaOH volume was then recorded and the acid percentage of the yoghurt was calculated using the formula:

$$\text{Titrateable acidity} = \frac{\text{Titre value} \times M \times 90 \times 100}{\text{Volume of sample} \times 1000}$$

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Scavenging assay

For DPPH scavenging assay, 250 µl carrot yoghurt extracts were mixed with DPPH (3 ml of 60 mmol/L in ethanol). 250 µl of water serves as control. The mixture was allowed to stand at room temperature for about 1 hr. Constant OD was recorded at 517 nm in a spectrophotometer by Shetty et al. [28]. The scavenging activity was calculated by:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

Hydroxyl radical scavenging activity

Hydroxyl-radical scavenging assay was used to assess the hydroxyl radical scavenging ability of the carrot yoghurt extract. The reaction mixture comprised of FeCl₃ (100µM), EDTA (104µM), H₂O₂ (1mM) and 2-deoxy ribose (2.8mM) were mixed with or without extracts at the concentration of 9 µM, the volume was made up to 1ml with phosphate buffer (20 mM, pH 7.4). The absorbance was read at 535 nm. The control, lacking antioxidant or extract was considered as 100% deoxyribose oxidation. The percentage of hydroxyl radical scavenging activity of the extracts was determined accordingly in comparison with control by Ningappa et al. [21]

Extraction and Quantification of Exopolysaccharide

Exopolysaccharides was extracted from yoghurt according to method described by Uemura et al. [33]. Precipitation of Casein was done by adjusting the pH to 4.6 with 2M NaOH solution and then removing it by centrifugation (10,000 g, 4°C for 20 minutes). Whey fraction was neutralized by additional NaOH solution. Insoluble protein was removed by centrifugation (4°C, 10,000 rpm, and 20 minutes).

For quantification, 5% phenol solution was dissolved in distilled water. Sample(s) (400 µl) were dispensed into glass tube and 400 µl of prepared phenol solution was added in it. 400 µl of 5% phenol solution in water was added in 400 µl of distilled water. Concentrated sulphuric acid (2 ml) was added thereafter into the solution tube. Samples were allowed to stand for 10 minutes, stirred and let stand for 10 minutes at 30°C, absorbance measured at 540 nm spectrophotometrically along with the control.

Evaluation of functional properties of the Carrot Yoghurt

Estimation of Ascorbic Acid

The method of redox titration was carried out using iodine solution (British Pharmacopeia, 2003 pp. 163-164). The reaction proceeded with the oxidation of ascorbic acid by iodine to dehydroascorbic acid and conversion of iodine to iodide. To 10 ml aliquots of carrot yoghurt extracts in 250 ml conical flasks, 4-5 drops of 1 % starch indicator was added. Titration was performed with 0.05 M iodine solution till the end point (blue-black color, due to starch-iodine complex) was reached.

Estimation of Riboflavin (Vitamin B₂)

Riboflavin was determined calorimetrically, briefly, 0.8 ml of the sample was added to 0.2 ml of 1 M sodium hydroxide. To 0.4 ml of the resulting solution, 1 ml of 0.1 M potassium phosphate buffer (pH 6) was added for neutralization. Absorbance at 444 nm was measured using a spectrophotometer (Shimadzu). Concentration of riboflavin in the food samples was estimated with riboflavin (SRL, Mumbai, India) as standard.



Qualitative analysis of γ -amino butyric acid (GABA)

GABA in carrot yoghurt was presumptively detected using Thin-Layer Chromatography (TLC) with some modifications by Cho et al. [7]. Silica gel (30-40%) in distilled water was layered on clean glass plate using TLC applicator. Spotting was performed using 2 μ l of GABA standard (GNC, USA) along with 20 μ l of the food extract on the silica plate. The elution solvent for TLC analysis was n-butanol, acetic acid, water in the ratio 4:1:1 which was allowed to run in a sealed glass chamber for 40 minutes. The TLC plate was stained with ninhydrin solution and was incubated at 110°C in a hot air oven for development of red-violet spots.

$$R_f = \frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}}$$

Quantitative Estimation of GABA

Carrot yoghurt samples were centrifuged at 15,000 rpm for 10 minutes and supernatant (2 μ l) was added to 800 μ l of methanol, and incubated at 25°C in a water bath for 10 minutes. The reaction mixture was dried in incubator overnight and 1ml of 70mM LaCl₃ was added. The 1ml assay system contains 550 μ l of supernatant, 200 μ l of 0.5 M K⁺ pyrophosphate buffer (pH 8.6), 150 μ l of 4mM NADP⁺, 50 μ l of 2.5 units GABASE per ml, and 50 μ l of 20 mM α -ketoglutarate. The initial absorbance was read at 340 nm before adding α -ketoglutarate, and the final absorbance was read after 60 minutes. The difference in the A₃₄₀ value was used to calculate GABA content in yoghurt against GABA standard. [7].

Determination of β -Carotene in prepared Yoghurt

One gram portion of carrot yoghurt was mixed with 10 ml of acetone. The mixture was thoroughly homogenized in a Philips blender for 5 minutes and sodium sulphate was added (pinch) to the mixture. The solution was transferred to a separating funnel and petroleum ether was added. Upper layer (petroleum ether) of the two layers which formed contains beta-carotene, while lower layer acetone dissolves polar compounds. The O.D of the upper layer of petroleum ether was recorded spectrophotometrically at 450 nm.

The β -carotene content was calculated by: -

$$\text{Carotene} = \frac{\text{OD} \times 13.9 \times 10^4 \times \text{volume of aqueous layer}}{\text{Weight of sample} \times 560 \times 1000}$$

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD) from three independent parallel experiments. P<0.05 was considered as statistically significant when compared to relevant controls. The mixture was allowed to stand at room temperature for about 1 hr. Constant OD was recorded at 517 nm. The scavenging activity was calculated by:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}}$$

IV. EXPERIMENTAL RESULTS

Bacterial culture stability in Yoghurt

The yoghurt stored overnight at 4°C (approx. 24 hrs) exhibited a remarkable increase in the viable counts as compared to the counts after 7 and 14 days. In refrigerated yoghurt, the total population of organisms in live and active culture yoghurt must be 10⁸cfu/g at the time of manufacture and in frozen yoghurt it must be at least 10⁷cfu/g at the time of manufacture. In the present study, viability of the cultures (Fig 1) was retained and conformed to the specifications for a period of 14 days in line with previous reports. [5]

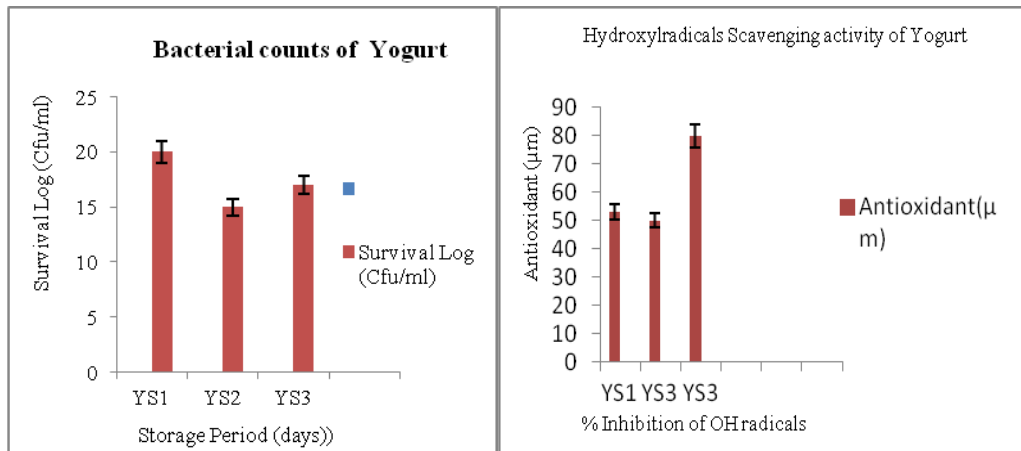


Fig.1 shows Survival of *L.acidophilus* in carrot yoghurt. Over a period of at 4⁰ C. YSi, YS 2 and YS3 represent the various proportions of carrot juice (CY 10%, CY 15%, CY 20%) used for formulating the yoghurt, whereas , Fig 2 is an Free radical scavenging activities of carrot joghurt. YS1, YS2 and YS3 represent the different proportions carrot juice used for formulating the yoghurt.

Analytical Determination of the sample

Changes in pH during yoghurt storage

The acidity of control yoghurt increased during the refrigerated storage after 14 days, this may be due to the fact that the yoghurt starter culture *S. thermophilus* is active even at refrigerated temperature and still can produce small amounts of lactic acid by fermentation of lactose which results in noticeable pH decrease by Deva et al. [8].Changes in pH of the carrot yoghurt remained almost stable even for up to 14 days (Table 3).



Figure 3. Carrot yoghurt formulated with 20% carrot juice. Sensory and other parameters were found optimal at this concentration of carrot juice.

Titratble acidity

The titratble acidity (0.80 % lactic acid) observed was higher in carrot yoghurt, than that (0.76 %) obtained in control yoghurt (Table 3).

Syneresis

The Syneresis in control was 15 ml while in carrot yoghurt (Table 3) it was 3 ml which suggested that the firmness of the yogurt was maintained till 14th day. However increase in values was not observed. It has been suggested that EPS production is one of the most important properties of dairy strains of *S. thermophilus*. The EPS not only act as in-situ produced natural bio-thickeners improving the viscosity, but also modifies the texture, mouth feel besides preventing syneresis in yoghurt. Therefore a positive role in modulating syneresis in the carrot yoghurt may be attributed to EPS.

Sample	pH	Syneresis (ml)	pH	Syneresis (ml)	pH	Syneresis (ml)	pH	Syneresis (ml)
	0 Day	0 Day	1 Day	1 Day	7 Days	7 Days	14 Days	14 Days
Control Yoghurt	4.03±0.03	1±0.01	4.00±0.05	3±0.03	3.90±0.02	8±0.07	3.80±0.01	15±0.02
Sample Yoghurt	4.31±0.03	0±0.02	4.31±0.07	2±0.02	4.32±0.03	3±0.06	4.33±0.06	3±0.01

Table 3. Changes in pH and Syneresis (ml) of control yogurt and carrot yogurt with different time interval. Data are represented as mean±SD (n=3)

Estimation of Vitamin C and Riboflavin

Vitamin C and Riboflavin content in carrot yoghurt and that of control revealed slight increase in the concentration of ascorbic acid in the test sample and a considerable improvement in riboflavin content from the control. Storage of the samples at 4°C did not significantly ($p>0.05$) alter the amount of Vitamin C and Riboflavin (Table 5).

Storage Period (hours)	Vitamin C content (serving size of 100 grams)		Vitamin B ₂ content (serving size of 100 grams)		β-Carotene content (serving size of 100 grams)	
	Control yoghurt (mg)	sample yogurt (mg)	Control yoghurt (mg)	Sample yogurt (mg)	Control yogurt (μg)	Sample yogurt (μg)
RDA	90-120 mg/day		1.2-1.6 mg/day		390-18000 μg/day	
0	110.43	267.61	15.55	33.68	1120.56	5130.12
24	109.56	266.80	15.12	33.33	1120.43	5128.34
48	109.23	266.32	14.78	32.67	1119.32	5128.23

Table 5. Vitamin C, Vitamin B₂ and Beta-carotene content of control and carrot yogurt over storage time.

Estimation of Beta-Carotene

The β-carotene content in carrot yoghurt was notable (Table 5). High concentrations of carotenoids, especially β-carotene in carrots make important dietary sources for prevention of cancers, free radical scavengers, anti-mutagenic and immune-enhancers [17]. Therefore, it may enhance longevity and decrease the onset of chronic diseases in humans.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect

Carrot yoghurt exhibited high DPPH radical scavenging activity of 88.6% (Table 4). Higher yoghurt antioxidant activity is beneficial in two respects, firstly to delay the oxidation process of lipids in yoghurt that are responsible for the formation of off-flavors and undesirable chemical compounds by Kedare et al. [14] and secondly, to increase dietary antioxidants which are crucial in preventing the progressive impairment of pancreatic beta-cell function due to oxidative stress [17].

Sample	Titrateable Acidity (%)	EPS (g/100 gm)	% DPPH Radical Scavenging Activity
Control yogurt	0.76±0.07	0.979±0.04	43.3% ±1.5
Sample yogurt	0.80±0.05	4.40±0.03	88.6%±1.7

Table 4. Comparison of Titrateable acidity, EPS Content and DPPH radical scavenging activity of commercial and carrot yogurt. N.D: Not detected. Data are represented as mean±SD (n=3)



Hydroxyl radical scavenging activity

Hydroxyl radicals are known to be the most reactive of all the reduce forms of dioxygen and are thought to initiate cell damage in vivo. The yoghurt EPS scavenges the hydroxyl radicals up to the 79% at 10 μ M concentration (Fig 2) whereas standard at 400 μ M scavenged 52-55%.

Quantification of Exopolysaccharide

The carrot yoghurt produced reasonable quantities of EPS (4.4 mg/100 gm) and was significantly higher ($p < 0.05$) in comparison to control yoghurt (Table 4). EPS is an important constituent for good texture, consistency and stability of the yoghurt for its texture promoting abilities and high water binding capacity

Qualitative and quantitative determination of GABA in carrot yoghurt (20%)

The identification of GABA was qualitatively assessed by TLC on the basis of Retention Factor ($R_f = 0.63$) with authentic GABA samples followed by quantization using GABASE assay. The GABA concentration (mM) in carrot yogurt was found to be 6.08 mM/gm. Yoghurt with high concentration of GABA produced by *S.thermophilus* was reported by Chen et al. [6]. The *S.thermophilus* strain used in this study possessed the ability to produce GABA as observed in small quantities. However, GABA production by *L.acidophilus* was reasonable accounting for the high levels of GABA observed in the carrot yoghurt (Table 6). The functional effects of GABA for treatment of sleeplessness, depression, and autonomic disorders, chronic alcohol related symptoms and stimulation of immune cells has been extensively reported [22] and [23]. Therefore, GABA enriched yoghurt may deliver therapeutic benefits for relieving stress and sleeplessness for the aged.

After 13 th day of Storage at 4°C	SAMPLES			
	Control	CY 10%	CY 15%	CY 20%
Attributes				
Smell (9)	5.1±0.03	6.1±0.03	6.0±0.03	7.4±0.02
Consistency (9)	5.9±0.02	6.0±0.02	7.0±0.01	7.2±0.05
Texture (9)	6.0±0.06	5.9±0.06	6.0±0.02	7.1±0.04
Color and appearance (9)	5.2±0.03	6.0±0.03	6.2±0.06	7.4±0.07
Overall Acceptability (9)	5.7±0.04	6.2±0.04	6.3±0.05	7.2±0.06

VI. CONCLUSION

Innovations in nutrition research and education can provide the basis for solutions to larger health-related issues, allowing individuals to live healthier, more productive lives. Simple affordable innovations in enhancing nutritional status of diets in this regard may help enable an acceptable dietary option for general masses.

Table 2. Sensory evaluation of control yoghurt and carrot yoghurt after 13th day of storage at 4 °C. Data represented as mean \pm S.D (n=3).

Nutrient deficiencies such as Vitamin C, Vitamin B2, and β carotene) are common in people due to a number of factors such as their reduced food intake, lack of variety in the foods consumed in addition to socio-economic factors. The present study indicates that incorporation of carrot juice in yoghurt (produced by using *S. thermophilus* and *L. acidophilus*) imparts high nutraceutical benefits in terms of Vitamin C, Riboflavin, β -carotene and GABA. The developed carrot yoghurt is appropriate for inclusion as a part of regular diet, given its sensorial acceptability and shelf stability. However, clinical trials are recommended prior to its commercialization.



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