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Growth kinetics of Yeast for production of low alcoholic self carbonated beverage from carrot:amla juice

P. Sahota[1*], **G. Pandove**[1] **and T.S. Dhillon**[2]

[1] Department of Microbiology,[2] Department of Vegetable crops,
Punjab Agricultural University, Ludhiana-141 004, India
Email: gpandoveg@yahoo.co.in

Abstract. A technology to produce low alcoholic self carbonated beverage from carrot:amla juice is developed. The yeast culture Clavispora lusitaniae has the potential to produce less alcohol ($< 1\%$) and high bar pressure (1.5 bar) of carbon dioxide in fruit juice during fermentation. CO_2 produced adds tangy taste, body and sparkle to the beverage, additionally is effective against gram negative aerobic spoilage bacteria. Its fungistatic and bacteriostatic effect depends on the dissolution of gas in water phase of beverage. When crop is harvested, it often creates a glut-like situation, so to safeguard the interest of progressive horticulturists, the utilization of fruits for the production of low alcoholic self carbonated beverage is economical and an appropriate viable technology.

Results: The freshly prepared fermented carrot-amla beverage (1:1) has TSS 16.0°B, pH 3.5, acidity (% citric acid) 0.36%, brix acid ratio 44.44, alcohol 0.3% (w/v), $CO_2$0.9 bar and viable count 1.5×10^7 cfu/ml. Mean sensory scores of beverage did not differ significantly during storage at refrigerated temperature for three months.

Conclusion: This technology can minimize the post harvest losses, avoid fruit and vegetable glut in the market, and utilize astringent highly nutritive

fruit, minimize transportation cost and retain the nutrients in the fruits for three months. The carbonated beverage is simple, easy and can be prepared at small scale and pilot scale.
Key words: Growth kinetics, Low alcoholic, self carbonated, Yeast, growth curve

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1 Background

India stands second in the world for production of fruits and vegetables owing to the remarkable diversity of its geographical conditions. The country produces about 50 million tonnes of fruits per year but only 2% of this goes for processing, while over 25% is spoiled due to improper handling and storage results in quantitative and qualitatively losses (flavour, texture, nutritional value and safety) [1]. Consumers like carrot juice because of its high nutritive value, fiber, carbohydrates, vitamin A derived from its high α carotene (β ε-carotene), β-carotene content, colour, aromatic compounds and refreshing characteristics [2]. A major problem for processing carrot is color loss and requires double pasteurization [3]. Fruits like amla because of its high acidity and astringent taste, is not palatable for direct consumption, but its excellent nutritional and therapeutic values offer enormous potentiality for processing. Amla (*Emblica officinalis*) is a richest source of ascorbic acid, an antioxidant (600 mg/100g) which is said to be the second highest among all the fruits and a good source of choline, an effective free radical scavenger (256mg/100g). It contains 20 times as much as ascorbic acid as orange juice. Amla is exception among fruits as it contains substances which partially protect the ascorbic acid from destruction on heating or drying. As it is highly acidic so it protects its ascorbic acid. Blending of carrot juice with astringent, highly nutritious fruits like Amla can provide health beverages with medicinal and therapeutic values. The fermented beverage retains nutrients, and additionally CO_2 so produced is anti microbial and adds tangy taste, fizz and sparkle to the beverage. Carrot and Amla are available for short span of time in a year and result in seasonal glut. To make them available throughout the year in the form of beverage, the present study is pro-

posed with objective to develop a reliable, controllable, reproducible technology for the production of low alcoholic self carbonated beverage with shelf-life of three months.

Material and methods

Fruits and Vegetables: Carrot var. PC-34 was procured from the Department of Vegetable crops, PAU, Ludhiana. Amla var. chakaiya was obtained from Department of Horticulture, PAU, Ludhiana.

Extraction of juices: Healthy fruits and vegetables were washed with chlorinated water and peeled. Carrot juice was extracted aseptically and in hygienic conditions using Electronic juicer (INALSA), where as amla was passed through screw type extractor to extract juice. Extracted juice was filtered through muslin cloth.

Preparation of sugar solution: The granulated sucrose procured from local market of Ludhiana city, was boiled in equal water (500g/litre) for 5 minutes and then cooled to room temperature to prepare sugar solution.

Yeast culture : Yeast culture *Clavispora lusitaniae* was obtained from Department of Microbiology, PAU, Ludhiana, It has potential to produce less than 1% alcohol and 1.5 bar pressure of carbon dioxide.

Inoculum preparations: Carrot-Amla (1:1) juice was diluted in the ratio 1:2 with water, pasteurized at 82 $^\circ$ C for 15 secs, cooled and brix adjusted to 16 $^\circ$ B by adding sugar solution. A loopful of 24 hrs old yeast culture was transferred to juice in 250 ml Erlenmeyer flask and incubated at 20$\pm$5 $^\circ$ C for 24 hrs to achieve concentration 10^7–10^8 cfu/ml, which was used as starter culture.

Standardization of inoculum concentration : The inoculum concentration was optimized to achieve CO_2 level 0.9-1.5 bar and alcohol 0.4-1% level. The fermentation was carried out in one liter glass bottle by inoculating varying concentration of yeast culture (10^8 cells/ml) @ 0.2, 0.5, 0.8, 1.0, 1.5 % (v/v) in sterilized, diluted carrot-amla (1:1) juice, TSS (Total soluble solids) adjusted to 16 $^\circ$ B and incubated at 20$\pm$5 $^\circ$ C for 36 hrs. The bottles were analyzed for CO_2 (bar) and alcohol (w/v) at the end of fermentation.

Growth kinetics of yeast in beverage: Blended carrot-amla juice was diluted in the ratio 1:2 with water, pasteurized at 82 $^\circ$ C for 15 secs,cooled and brix adjusted to 16 $^\circ$ B by adding sugar solution. Inoculated with of 24 hrs old yeast culture and incubated at 20$\pm$5 $^\circ$ C for 24 hrs to achieve concentration 10^7-10^8 cells/ml, for use as starter culture

to inoculate large volume of juice to carry out growth kinetic study. Physico-chemical changes like pH, acidity (%), TSS ($^\circ$B), Brix acid ratio, alcohol content (% w/v), Carbon dioxide (bar), Total yeast count, Dry weight and Optical density was studied.
Dry weight: The dry weights were determined by centrifuging at 10,000 rpm for 20 mins. Cell biomass retained by the centrifuge was washed and recentrifuge with 10 ml of distilled water, dried at 60°C for 24 hours to constant weight.
Optical Density: The beverage was analyzed for growth in terms of increase in optical density at 600 nm on spectronic-20 (Baush and Lomb).
Enumeration: It was carried on GYE media.
Preparation of blended carrot amla beverage: The carrot–amla juices (1:1) were diluted in the ratio of 1:2 with water and after pasteurization at 82 $^\circ$ C for 15 secs, it was cooled, and brix was adjusted to 16 $^\circ$B followed by inoculation of *Clavispora lusitaniae* culture in standardized concentration. It was incubated for 36 hrs at 20$\pm$5 $^\circ$ C. The beverage was refrigerated for 24 hrs, siphoned, bottled and stored in refrigerated conditions.
Chemical Analysis of Juice: The pH was determined by using a pH meter, acidity expressed as Citric acid by titrating against standard NaOH (0.1N), total soluble solids was determined by using Erma hand refractometer (Unico). Alcohol (w/v) content and carbon dioxide was determined by AOAC [4] after slight modification.
Statistical analysis: Statistical analysis of the data was done by using GSTAT04 and CPCS1 software developed by Department of Math, Stat and Physics PAU,Ludhiana.

2 Results and discussion

Standardization of inoculum concentration: The fermentation of carrot-amla (1:1) juice, with TSS 16 $^\circ$B was carried out with varying concentration of culture *Clavispora lusitaniae*. It was observed that with increase in inoculum concentration from 0.2 to 1.5% (v/v), alcohol concentration also increased from 0.0 to 0.86% (w/v) (Fig. 2) and CO_2 increased from 0.0 to 3.2 bar (Fig. 2). Chaill et al., [5] observed that slight overpressure (0.3-0.6 atm) of CO_2slows yeast growth, though did not have any marked effect on fermentation rate. Gibbons and Westby [6] reported that higher inocula showed no advantages. Lower inocula resulted in lowered final yeast population and increased

fermentation time. Ghosh and Tyagi [7] have reported that the fermentation process was lowered as the inoculum concentration was increased could be due to lower rate of cell multiplication at higher inoculum levels. Strehaiano et al., [8] observed that the application of higher inoculum level, shortens the fermentation periods and it is economically non-viable.*Clavispora lusitaniae* at inoculum level of 0.5% (v/v) with 4.2×10^7 cfu/ml of inoculum produce 0.3 % ethanol (w/v) and 0.9 bar pressure of CO_2, which is desirable for production of low alcoholic self carbonated beverages (Table 1, Fig. 2).

Kinetics of Yeast Clavispora lusitaniae in beverage: Growth kinetics of Yeast *Clavispora lusitaniae* was perfomed in carrot-amla (1:1) juice to understand the growth and metabolic activities of yeast throughout fermentative phase. The growth curves (Fig. 3 (a), (b) of optical density, viable cell count (log_{10} no.of cells per ml), and Cell biomass which showed normal patterns with first a short lag period of 4 hrs followed by exponential growth up to 72 hrs as indicated by sharp increase in optical density, viable cell count ($\log_{10}$ no.of cells per ml) and cell biomass from 0.25–0.61 (Table 2), 7.42-10.56 (Table 2), 0.002–0.45 (Table 3) respectively. Stationary phase was shortened and no increase in cell biomass was observed. Viable cell count started decreasing from 96 hrs to 120 hrs showing death phase, decrease in yield of growth and acceleration of yeast death, this could be due to inadequate supply of nitrogenous substances, vitamins, concentration of dissolved oxygen, accumulation of toxic metabolites and feed back inhibition. Similar observations were also reported [9]. Giovanelli et al., [10] also studied kinetics of grape juice fermentation under aerobic and anaerobic condition and found that under anaerobic condition sugars were exhausted within 200 hrs and number of cells reached to $1x10^8$ cell/ml. The studies depicts that the amount of ethanol (1% w/v) and CO_2 (1.5 bar) was produced during the exponential phase cell (Fig. 4). Results showed that unutilized sugar (76.25%) was left in fermentable substrate. It is inferred that yeast *Clavispora lusitaniae* produces less alcohol (1% w/v) and more carbon dioxide (1.5 bar) which gives sparkle, tangy taste to beverage. Fermentation is partially ceased because of carbon dioxide bar pressure in juice. Low alcoholic self carbonated beverage is sweet in taste because of partial fermentation and availability of residual sugar. Physico-chemical changes like decrease in pH 3.5 to 3.3, Brix acid ratio 44.44 to 27.72 and increase in acidity 0.36 to 0.44 were also observed (Table 5, Fig. 5).

Shelf life studies: Shelf-life of low alcoholic self carbonated carrot-amla (1:1) blended beverage stored at refrigerated temperature was studied and evaluated fortnightly for organoleptic, chemical and microbiological qualities . The results in Table 6 shows that only small amount of alcohol produce during storage period of three months (1%). It is due to the non alcoholic nature of fermentation by yeast *Clavispora lusitaniae* and also due to very less utilization of sugar as evident from small reduction of TSS from 16-11^{0}B. The CO_2 increased from 0.9-1.5 bar. The yeast count did not increase much during storage. This may be due to the combined effect of high initial concentration ($1.5x10^7$), partially anaerobic condition, high CO_2 pressure and low temperature.It is also reported that elevated CO_2 inhibit glycolysis, Kreb cycle intermediates and enzymes such as succinate dehydrogenase activity, leading to the accumulation of only succinate,there by reducing the formation of citrate/isocitrate and 2-ketoglutarate and Brix acid ratio (BAR) from 44.44 to 25.00. The pH of the beverage decreased from 3.5 to 3.3 and acidity increased from 0.36 to 0.44. The decrease in pH and increase in acidity could be attributed due production of CO_2 that form weak acid on dissolution. These results are in accordance with Kitabatake *et al*., [11] who reported decreased in pH while increase in acidity of traditional non-alcoholic beverages. Ilamaran and Amutha [12] reported gradual decrease in BAR content of carbonated banana beverage during storage. Sahota and Sunil [13] also prepared non alcoholic naturally carbonated beverage from three plum varieties satluj purple, kala amritsari and alucha after optimizing the fermentation condition. Sensory data revealed good mean sensory scores for all sensory attributes during storage period. The fermentated beverage retains nutrients, and additionally CO_2 so produced is anti microbial and adds tangy taste, fizz and sparkle to the beverage with out the addition of any preservative.

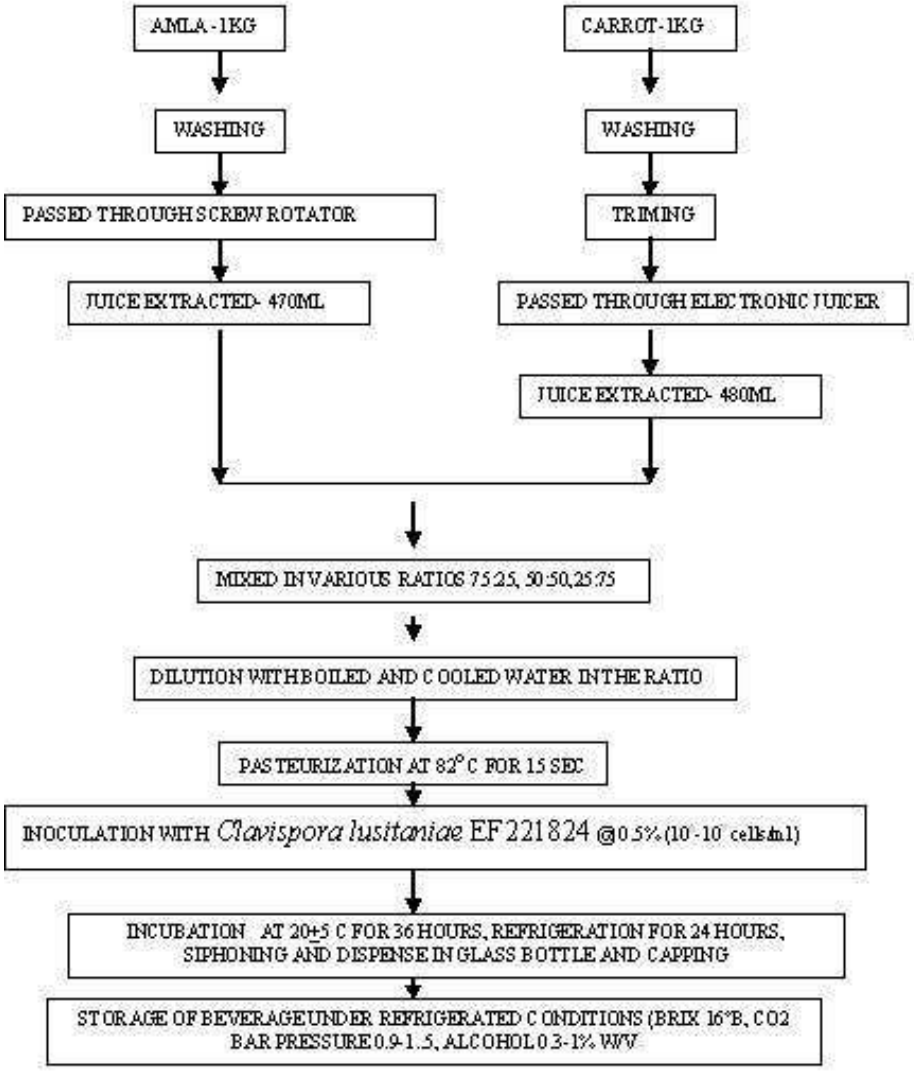


Figure 1: Flow diagram for preparation of low alcoholic self carbonated beverage from blended carrot-amla juices.

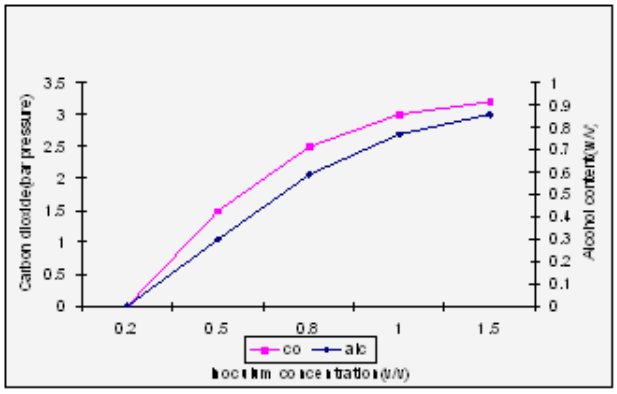


Figure 2: Effect of inoculum concentration on alcohol content % (w/v) and carbon dioxide (bar pressure) in beverage.

Table 1: Effect of inoculum concentration on Yeast growth and alcohol content in the beverage.

Inoculum concentration (v/v)	Initial Yeast count (cfu/ml)	Final Yeast count (cfu/ml)	CO_2 (Bar pressure)	Percent alcohol (w/v)
0.2	$5.0x10^4$	$1.0x10^6$	0.0	0.00
0.5	$5.0x10^5$	$4.2x10^7$	0.9	0.30
0.8	$8.0x10^5$	$7.0x10^7$	2.5	0.59
1.0	$1.0x10^6$	$9.0x10^7$	3.0	0.77
1.5	$1.5x10^6$	$1.2x10^7$	3.2	0.86

Growth conditions

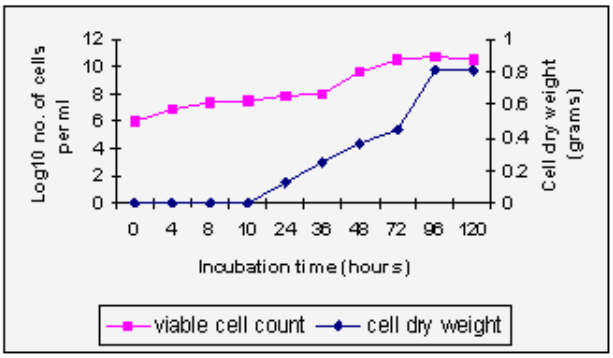
Yeast culture–*Clavispora lusitaniae*

Incubation temperature–20±5°C

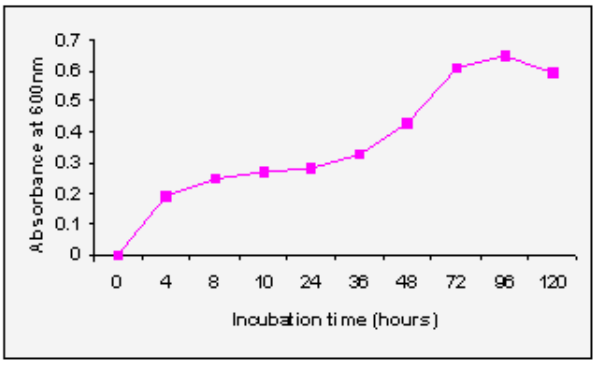
Incubation period–36h

Brix of Juice–16°B

pH of Juice–3.5



(a) Growth of yeast in beverage in terms of viable cell count and cell dry weight



(b) Growth of yeast in beverage in terms of optical density

Figure 3:

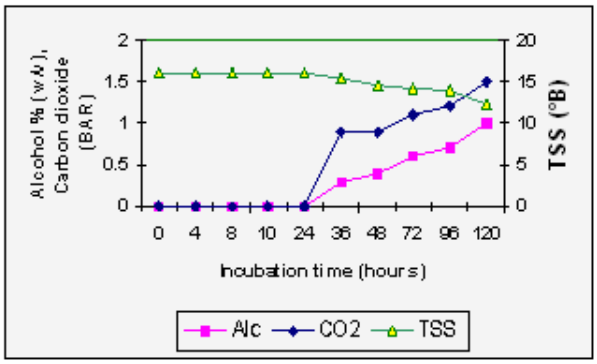


Figure 4: Association between Alcohol, Carbon dioxide and TSS during growth of *Clavispora lusitaniae* in Beverage.

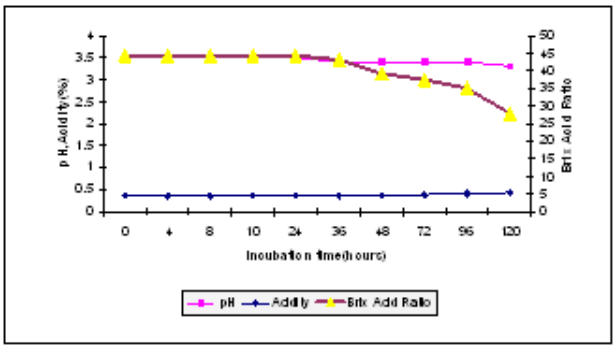


Figure 5: Physico-chemical changes during growth of *Clavispora lusitaniae in beverage*.

Table 2: Growth of Yeast in beverage in terms of optical density and cell count.

Incubation Time (hrs)	Optical density at 600 nm	$\log_{10}$ no. of cell per ml
0	0.00	6.0
4	0.19	6.9
8	0.25	7.42
10	0.27	7.46
24	0.28	7.90
36	0.33	8.04
48	0.43	9.61
72	0.61	10.56
96	0.65	10.76
120	0.59	10.48

Growth conditions.

Yeast culture–*Clavispora lusitaniae*

Incubation temperature–20±5°C.

Inoculum concentration–0.5% (v/v)

Table 3: Growth of yeast *Clavispora lusitaniae* in beverage in terms of cell biomass.

Incubation Time (h)	Cell dry weight (grams)
0	0
4	0
8	0.002
10	0.003
24	0.130
36	0.248
48	0.363
72	0.450
96	0.809
120	0.809

Growth condition

Yeast culture–*Clavispora lusitaniae*

Incubation Temperature–20±5°C

Inoculum concentration–0.5% (v/v)

Table 4: Production of alcohol and $C0_2$during growth of yeast in beverage.

Incubation Time (h)	Alcohol (w/v)	CO_2 (Bar pressure)
0	0.00	0.00
4	0.00	0.00
8	0.00	0.00
10	0.00	0.00
24	0.00	0.00
36	0.30	0.90
48	0.40	0.90
72	0.60	1.10
96	0.70	1.20
120	1.00	1.50

Growth conditions

Yeast culture–*Clavispora lusitaniae*

Incubation temperature–$20 \pm 5^{\circ}$ C

Inoculum concentration–0.5%

Table 5: Effect of Yeast growth on physico-chemical characteristics of beverage.

Incubation Time (h)	pH	Acidity (%)	TSS (^{0}B)	Brix acid ratio
0	3.5	0.36	16.0	44.44
4	3.5	0.36	16.0	44.44
8	3.5	0.36	16.0	44.44
10	3.5	0.36	16.0	44.44
24	3.5	0.36	16.0	44.44
36	3.4	0.36	15.5	43.05
48	3.4	0.37	14.5	39.19
72	3.4	0.38	14.2	37.37
96	3.4	0.40	14.0	35.00
120	3.3	0.44	12.2	27.72

Growth conditions

Yeast culture–*Clavispora lusitaniae*

Incubation temperature–20±5 ˚ C

Inoculums concentration–0.5% (v/v)

Table 6: 180

Effect of storage on low alcoholic self carbonated carrot:amla (1:1) blended beverage.

Carrot: Amla (50:50)	Fresh	15	30	45	60	75	90	F-ratio	CD at 5%
TSS (°B)	16.0	16.0	15.85	15.10	14.25	13.20	11.00	321.96	0.35
pH	3.50	3.50	3.40	3.40	3.40	3.30	3.30	-	NS
Acidity (%)	0.36	0.36	0.37	0.41	0.42	0.43	0.44	-	NS
Brix acid ratio	44.44	44.44	42.89	36.82	33.92	30.70	25.00	875.43	0.8488
Alcohol (w/v)	0.30	0.40	0.60	0.70	0.70	0.80	1.00	-	NS
CO_2	0.90	0.90	0.90	1.20	1.20	1.20	1.50	-	NS
Total plate count (cfu/ml)	1.5×10^7	3.5×10^7	4.0×10^7	4.5×10^7	6.5×10^7	8.0×10^8	9.5×10^8	17.27	0.35×10^9

NS-Non-significant

Table 7: Effect of storage on blended low alcoholic self carbonated carrot: amla (1:1) beverage.

Carrot: Amla (50:50)	Fresh	15d	30d	45d	60d	75d	90d	F-ratio	CD at 5%
Color	8.0± 0.50	8.2± 0.45	8.1± 022	8.1± 022	7.9± 0.22	7.8± 0.45	7.8± 0.45	1.17	NS
Appearance	8.1± 0.22	8.0± 0.50	8.0± 0.50	8.1± 0.22	7.9± 0.22	8.0± 0.50	7.9± 0.22	1.16	NS
Texture	7.8± 0.84	7.7± 0.45	7.9± 0.22	7.8± 0.45	7.8± 045	7.7± 045	7.7± 0.45	0.10	NS
Taste	8.2± 0.84	8.1± 0.22	8.1± 0.22	7.9± 0.22	7.7± 0.45	7.8± 0.45	7.9± 0.22	0.88	NS
Aroma	7.8± 0.84	7.7± 0.45	7.6± 0.55	7.8± 0.45	7.8± 0.45	7.9± 0.22	7.9± 0.22	0.24	NS
Overall Acceptability	7.8± 0.84	7.7± 0.45	7.9± 0.22	7.8± 0.45	7.8± 0.45	7.9± 0.22	7.6± 0.55	0.24	NS

NS-Non-significant

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