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Changes in Content of Tea Polyphenols in Tea Curd (Functional Food) Developed By Lactic Acid Bacteria (LAB) During Refrigerated Storage.

Sahoo Moumita[1]*, Sahoo Banishree [1], Dash Indira [1], Dethose Ajay[1], Jhonson Eldin[1], Rasu Jayabalan [1], Thangaraj P[2], Marimuthu S[2], Sasikumar AP[3], Joo-Won Suh[3], Seung Hwan Yang[3], and Kamila Goderska[4].

[1]Food Microbiology and Bioprocess Laboratory, Department of Life science, National Institute of Technology, Rourkela, Odisha- 769008, India.
[2]R & D Division, Parry Agro Industries Ltd.,Murugalli Bazaar, Valparai, Coimbatore (Dist), Tamil Nadu, India.
[3]Centre for Nutraceutical and Pharmaceutical Materials (8825), Myongji University (Science Campus), Yongin, Republic of Korea.
[4]Department of Fermentation and Biosynthesis, Faculty of Food Science and Nutrition, Poznan University of Life Sciences, Poland.

ABSTRACT

Tea curd is a functional food providing the health benefits of tea polyphenols and probiotics of curd. Biogenic amines, natural toxins produced by decarboxylation of amino acids, upon consumption in higher levels results in various pathophysiological condition seven leading to death. This work was aimed to study the effect of refrigerated storage on tea polyphenol contentsin tea curd produced by non-biogenic amine forming LAB. Among the probiotic bacteria isolated from different fermented foods in Rourkela, Odisha, India, two were found to be non-biogenic amine forming strains and were utilized for production of black tea curd (BTC) and green tea curd (GTC).The tea polyphenols showed variable stability during storage.There was decrease in contents of EGCG (72% and 74%) and ECG (76% and 74%) in BTC and GTC respectively after 1 day of refrigerated storage owing to conversion of these complex isomers of tea catechins to simpler forms.
Keywords: Functional food, green tea, black tea, biogenic amine, probiotics, tea curd

Corresponding author

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INTRODUCTION

Since ancient times, green tea has been considered as traditional Chinese medicine. Along with antioxidant and anti-cancer properties green and black tea also promote oral health and other physiological functions such as anti-hypertensive effect, controlling body weight, antibacterial and antiviral activity, anti-inflammatory activities, solar ultraviolet protection, increase in bone mineral density, anti-fibrotic as well as neuro-protective power. Increasing interest in its health benefits has led to the inclusion of green tea in various functional foods (1).All beneficial effects of tea have been attributed to the activity of the polyphenolic compounds having strong antioxidant properties, i.e. they protect our body from damage due to free radical-induced oxidative stress (2). Mainly three polyphenol groups are distinguished in tea: catechin, theaflavins and thearubigins (3). Seven catechins have been identified so far (−)-catechin (C), (+)-epicatechin (EC), (−)-epigallocatechin (EGC),(−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin 3-gallate (EGCG), (−)-gallocatechin-3-gallate (GCG) and (−)-catechin-3-gallate (CG) (4). Numerous epidemiological studies show that tea polyphenols mainly catechins decrease mortality from cardiovascular diseases and slow down the aging processes (5). Natural phenolics have been found to intervene at all stages of cancer development (6). EGCG is an extremely active compound with eight –OH groups determining its high antioxidant activity. The content of certain minerals and vitamins in tea increases its antioxidant potential (1). Tea catechins also have the novel characteristic of trapping reactive carbonyl species (RCS). Binding site for RCS trapping is the A ring of the catechins, whereas the preferred site for anti-oxidation is the B ring (7). Green tea is reported to contain a larger quantity of catechins than black tea (8). Green tea demonstrates a significant increase in plasma antioxidant capacity in humans which leads to a reduced oxidative damage in macromolecules such as DNA and lipids (9).Tea leaves are the only food product containing EGCG (10). Other compounds in tea include caffeine (3.5 %), an amino acid known as theanine (4%), lignan (6.5 %), organic acids (1.5 %), protein (15%), and chlorophyll (0.5%) (11). Caffeine mainly has effect on the CNS, stimulating alertness, facilitating association of ideas and decreasing fatigue (12). Tea catechins show high stability at pH<4 (13). The presence of tea does not significantly ($P < 0.05$) influence the characteristic microorganisms in yogurt. It has been reported that yogurt bacteria do not affect tea catechins when incubated together for 48 h. Green and black teas also do not show any effect on total lactic acid levels of the final products. In accordance to these findings, tea or tea catechins can be recommended to be added to yogurt for imparting beneficial effects on human health (14).

LAB are generally recognized as safe (GRAS) and used to produce fermented foods and beverages, using various substrates, such as milk, vegetables, cereals, meat, etc (15,16,17).The application of certain LAB strains as probiotics, prebiotics and nutraceuticals have created new perspectives for research and consumption, attracting both food scientists and health professionals (18,19,20).Biogenic amines (BA) are toxic nitrogenous compounds formed by decarboxylation (removal of α-carboxyl group) of amino acids (21).The synthesis of BAs in bacteria is associated with the supply of energy and to help protect from acid stress (22,23). Recently, the genes of diverse strain dependent pathways producing BA were identified in LAB (24). The enzymes of these pathways are encoded by unstable plasmids (25,26). Some LAB strains can produce different amines simultaneously, suggesting that they might possess more than one amino acid decarboxylases (27,28,29). BA producing LAB are even reported to form part of the starters or adjunct cultures. Biogenic amines can be either monoamines including histamine (HIS), tyramine (TYR) and tryptamine (TRP), arising from histidine, tyrosine and tryptophan, respectively or diamines like putrescine (PUT) and cadaverine (CAD) produced from ornithine and lysine, respectively. Putrescine is a pioneer for the formation of polyamines, spermidine (SPD) and spermine (SPM) (30). Histamine and tyramine are mainly produced by LAB. Consumption of higher levels of biogenic amines will result in various patho-physiological effects such as hypotension or hypertension, cardiac palpitation, and even death (31). Secondary amines, such as putrescine and cadaverine, can also react with nitrite to form carcinogenic nitrosamines (21). Human sensitivity depends on the proper functioning of the detoxification systems, since biogenic amines are metabolized in the human gut by amine oxidases (32). Putrescine and cadaverine inhibit intestinal diamine oxidase and histamine-N-methyltransferase required for metabolizing histamine, leading to histamine toxicity (33). High levels of histamine in foods can have hazardous vasoactive effects (34) and allergy-like symptoms (35). High level of tyramine may cause migraine headaches and a sudden rise in blood pressure. Tyramine is the major mutagen precursor (36).Therefore; it is indispensable to use strains which do not produce BA, as starters or adjuncts (24).

This work was aimed to screen the LAB isolated from locally available curd and handia (homemade fermented rice) for biogenic amine formation using decarboxylase media to study the effect of refrigerated

storage on the content of tea polyphenols in GTC and BTC produced using selected non-biogenic amine forming LAB.

MATERIALS AND METHODS

Chemicals

All the amino acids (histidine, lysine, ornithine and tyrosine), *Lactobacillus* de Man-Rogosa-Sharpe (MRS) media and components of decarboxylase media were purchased from the Himedia, Mumbai, India. All the chemicals were of high analytical grade.

Isolation and Identification of Microbes

Probiotic microbes were isolated on MRS agar media from homemade and industry made curd (Omfed, Rourkela, Odisha) as well as from locally available Handia (Rourkela, Odisha), a homemade fermented rice. The samples were serially diluted separately to appropriate dilutions and then spread onto respective MRS agar plates. Then the various distinct colonies were isolated by streaking them individually onto separate MRS agar plates. The morphology of the isolated bacteria was studied by simple staining method using methylene blue and then observing under oil immersion microscope.

Screening For Non-Biogenic Amine Producers

The isolated microbes were repeatedly sub-cultured for 5 times in MRS broth. Then their ability to produce any biogenic amine was checked by growing them on specific modified decarboxylase media (37)(Table 1), containing different amino acids and that without any amino acid as control.

Table 1: List of components of the modified decarboxylase media

Components	Quantity (%)
Tryptone	0.500
Yeast extract	0.500
Meat extract	0.500
Sodium chloride	0.250
Glucose	0.050
Tween 80	0.100
$MgSO_4$	0.020
$MnSO_4$	0.005
$FeSO_4$	0.004
Ammonium citrate	0.200
Thiamine	0.001
K_2PO_4	0.200
$CaCO_3$	0.010
Pyridoxal-5-phosphate	0.005
Amino acid *	1.000
Bromocresol purple	0.006
Agar	2.000
pH	5.300

* Respective amino acids were added in the decarboxylase media while the control media lacks amino acid.

Acid Tolerance

The extent of acid tolerance of all the isolated bacteria was determined at pH ranging from 2 to 7.4. The active cultures were harvested by centrifugation for 10 min at 5000 rpm and 4°C. Pellets were washed once in phosphate buffered saline (PBS at pH 7.2). Then cell pellets were re-suspended in PBS having pH 2, 3, 4, 5.5 and 7.4 respectively and incubated at 37°C. Viable microorganisms were enumerated after 3 hr by spread plate method and 24 hr incubation at 37°C.

Green and Black Tea Infusion

Green tea and black tea used in this study were manufactured from *Camellia sinensis* (L) *O. Kuntze* at Parry Agro Industries Limited, Valparai, Tamil Nadu, India. The tea infusions were prepared by boiling the green tea (2%) and black tea (2%) in milk separately for 5 minutes and filtered through the sterile cotton for preparing tea curd. The tea infusions were prepared in water for HPLC analysis as same as for tea curd.

Tea Curd Production

Both green and black tea curds were produced by the method described by Jaziri (14), under aseptic conditions by utilizing the screened biogenic amine non- producing LAB strains.

Effect of Storage On pH, Total Titratable Acidity, Total Microbial Count and Tea Polyphenols

Green tea and black tea curds produced after 6 hours of fermentation at 45°C were stored in refrigerator (Samsung, Model no. RT2534BACRR/TL/2013, India). Sampling was done after 1, 7, 14 and 21 days. Tea curd analyzed before storage was considered as 0 day.

pH Determination

The pH of the green and black tea curds were measured with an electronic pH meter (Sartorius model PB-11) after the specific time intervals under consideration.

Titratable Acidity Measurement

Total lactic acid content of the green and black tea curds were measured after the specific time intervals under consideration. 10 g of each curd and 10 ml of distilled water was taken into separate Erlenmeyer flasks. It was boiled to drive off CO_2 and 5 drops of 0.1% phenolphthalein was added to it after cooling. It was titrated with 0.1 N NaOH until a persistent pink colour was observed.

% of lactic acid = ml of NaOH × normality of NaOH × 9/weight of sample (g)

Total Microbial Count

Total viable count of*Lactobacillus* in green and black tea curds were determined by spread plate method using MRS agar with suitable dilution.

HPLC Analysis of Tea Polyphenols and Caffeine

Tea infusion was prepared in water and inoculated with microbial cultures. The analysis was carried out in Parry Agro Industries Limited, R&D Centre, Murugalli Bazaar, Valparai, Coimbatore (Dist), Tamil Nadu, India which is a NABL (National Accreditation Board for Testing and Calibration Laboratories) accredited laboratory for both microbiological and chemical testing as per IS/ISO/IEC 17025: 2005. Five ml of tea infusions were extracted with 20 ml of methanoland was passed through membrane filter (0.45 µM) into HPLC vials. A 10µl sample of filtrate was injected into a Shimadzu (Kyoto, Japan) HPLC system equipped with a diode array detector (SPD-M10Avp). Phenomenex Luna C-18(2) column (4.6 mm ID×25 cm, 5µm) was used for the analysis. The mobile phase was a mixture of 0.1% orthophosphoric acid (A) and acetonitrile (B). The gradient used was: 0-12 min, 15% B; 12-22 min, 25%, 22-30 min, 15% B. The flow rate and column temperature was maintained as 1.0 ml/min and 35°C, respectively. Detection of the tea polyphenols was carried out at 280 nm. The resolution peaks were then recorded on the HPLC chart according to the retention time of each compound. Quantification of tea polyphenols and caffeine was done from standard curves after the specific time intervals under consideration.

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RESULTS AND DISCUSSION

Isolation and Identification of Microbes

All total eight probiotic microbes were isolated from homemade curd, industry made curd and handia. Out of the four isolates from industry made curd as shown in Fig.1(a), three (OF1, OF3 and OF4) were found to be bacteria while OF2 was yeast. Only two bacteria (HM1 and HM2) were isolated from homemade curd as shown in Fig.1(b), while both the handia isolates shown in Fig.1(c) were yeasts (HN1 and HN2). Their basic morphology has been provided (Table 2a).

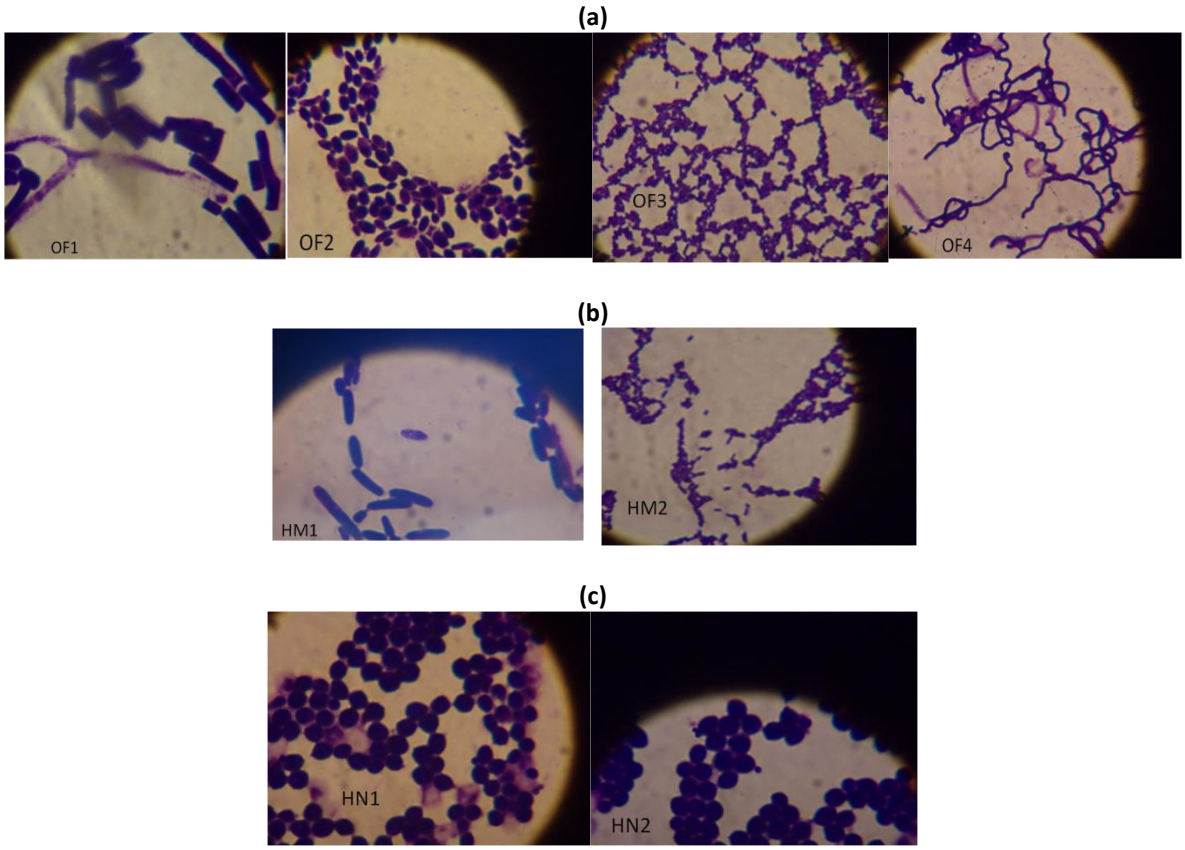

Fig.1: Isolates from (a) omfed curd (OF1, OF2, OF3, OF4); (b) homemade curd (HM, HM2) and (c) handia (HN1, HN2)

Table 2: (a) Morphology and biogenic amine production potential of isolated microbes, (b) acid tolerance of the selected bacteria

(a)

Name of the strain	Morphology	Tyrosine (Tyramine)	Ornithine (Putrescine)	Lysine (Cadaverine)	Histidine (Histamine)	Selection
OF1	Long rod [b]	Yes	Yes	No	Yes	No
OF2	Oval Shape [y]	Yes	No	No	No	No
OF3	Long rod [b]	No	No	No	No	Yes
OF4	Long rod [b]	No	No	No	No	Yes
HM1	Long rod [b]	Yes	Yes	Yes	Yes	No
HM2	Long rod [b]	No	No	No	No	Yes
HN1	Oval Shape [y]	Yes	No	No	No	No
HN2	Oval Shape [y]	No	No	No	No	No

* [b] shows that the isolated organism is bacteria while [y] shows that it is an yeast

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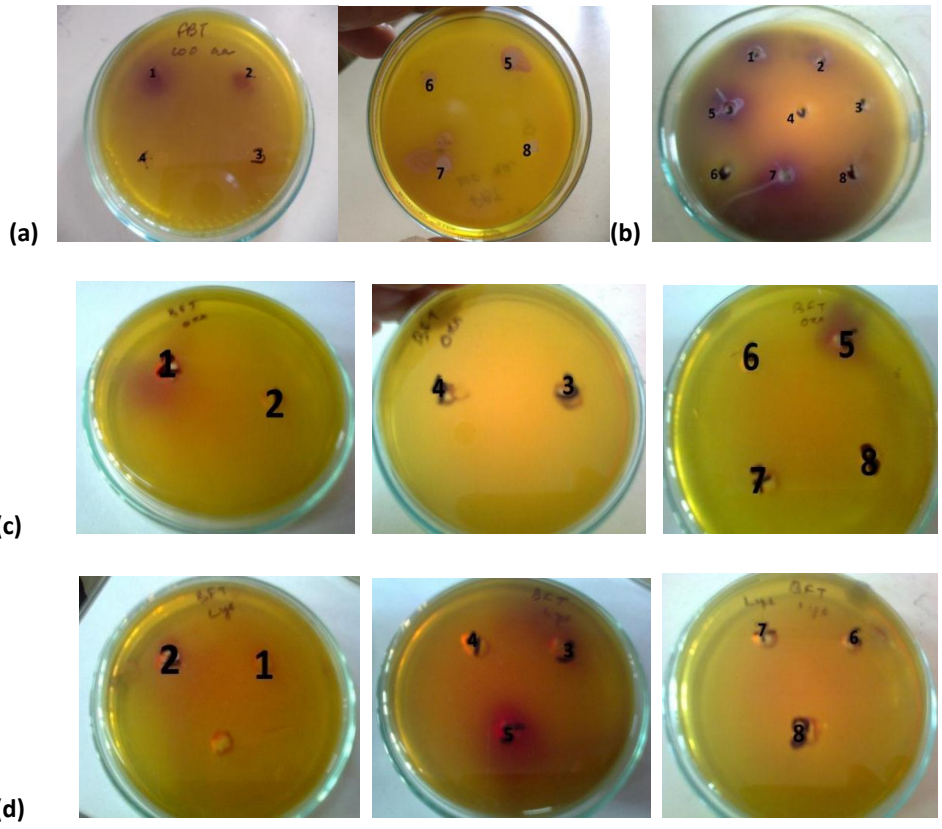
(b)

Bacteria	pH 5.5	pH 4	pH 3	pH 2
OF3	Growth	Growth	Growth	No Growth
OF4	Growth	No Growth	No Growth	No Growth
HM2	Growth	Growth	Growth	No Growth

Screening For Non-Biogenic Amine Producer

Screening for the production of biogenic amines was done using decarboxylase media with respective amino acids and control media lacking amino acids. Production of amines by microorganisms will change the color from yellow to purple due to bromocresol purple. Color production should be less in control media with respect to the media with amino acids. Among the isolated bacteria, three strains (OF3, OF4 and HM2) did not show color formation in all the amino acids (lysine, tyrosine, histidine and ornithine) tested as well as in control.The microbial strains which were not producing biogenic amines in decarboxylase media were selected for further studies and for the production of tea curd.

Itis clearly evident from Fig. 2(a) that, 1 (OF1), 2 (OF2), 5 (HM1) and 7(HN1) were producing biogenic amines even though the control medium is lacking amino acids by utilizing the protein sources like tryptone, yeast extract and beef extract of the medium itself. While 3 (OF3), 4 (OF4), 6 (HM2) and 8 (HN2) were non-producers of biogenic amines. In accordance to Fig. 2(b), 3 (OF3), 4 (OF4), 6 (HM2) and 8 (HN2) were non-producers of tyramine from tyrosine, while rest all gave positive result by showing purple zones. As evident from Fig. 2(c), 2 (OF2), 3 (OF3), 4 (OF4), 6 (HM2), 7 (HN1) and 8 (HN2) were non- producers of putrescine from ornithine, while 1 (OF1) and 5 (HM1) were showing purple zones due to decarboxylase activity. As clearly visible in Fig. 2(d), only 5 (HM1) formed a purple zone, while all others were non- producers of cadaverine from lysine as they lack decarboxylase activity. In accordance to Fig. 2(e), only 1 (OF1) and 5 (HM1) were producing histamine from histidine, while the other three isolates 2 (OF3), 3 (OF4) and 4 (HM2) were showing negative result due to absence of purple zone. Table 2(a) summarizes biogenic amine production by the isolated microbes.



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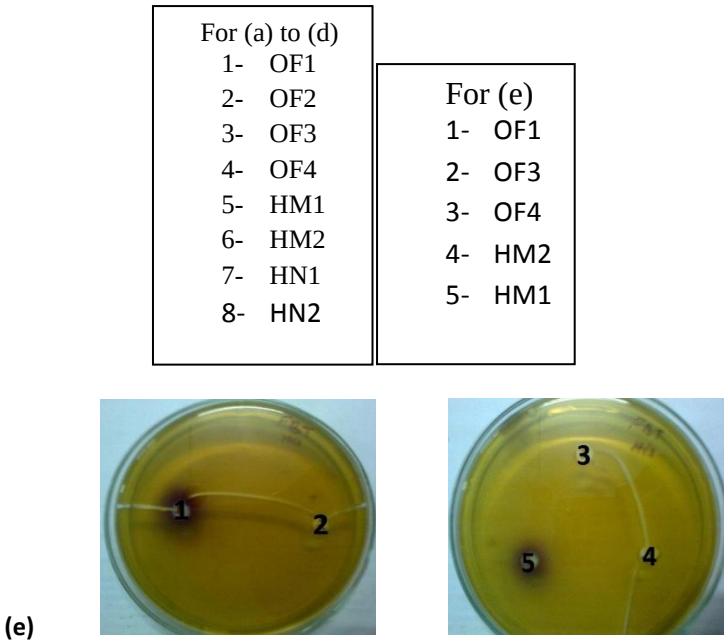

Fig.2: Decarboxylase activity (a) in absence; and presence of amino acids (b) tyrosine; (c) ornithine; (d) lysine; (e) histidine.

OF3, OF4 and HM2 were selected for further acid and bile tolerance studies before being used for tea curd production, because they were unable to produce any of the biogenic amines due to lack of decarboxylase activity. Only bacterial strains which were not producing biogenic amines with the tested amino acids were selected and yeast strain (HN2) was omitted.

Acid Tolerance

Selection of candidate probiotics for consumption is based on their ability to withstand low pH, necessary to ensure that they will survive gastric passage *en route* to the intestines in a sufficient concentration (38). Microbial tolerance to acidic pH was tested using PBS with different pH ranging from 5.5 to 2.0. Strains selected (OF3 and HM2) for fermenting tea curd were shown to be resistant to pH from 5.5 to 3.0 but not for pH 2.0 (Table 2b).

Tea Curd Production

LAB ferment milk and produce lactic acid thereby lowering the pH of the milk and this increased acidity causes the milk protein casein to clump together and precipitate out of the remaining liquid which contains only whey proteins, this process is known as curdling or coagulation. The coagulated casein assumes a solid or gel like structure known as curd or yogurt. Green and black tea curds were prepared using the tea (2%) infusions prepared in milk. Tea infusions in milk were fermented at 45°C for 6 hours using the screened acid tolerant biogenic amine non- producers OF3 and HM2. After 6 hours of fermentation, the curd was stored in small screw capped bottles (Fig. 3a) under aseptic conditions in a refrigerator.

Effect of Refrigerated Storage On pH, Titratable Acidity And Total Microbial Count In Tea Curd

Sampling was done at 0, 1, 7, 14 and 21 days of refrigerated storage, in such a way that only one tube was taken at a time for analysis to prevent microbial contamination. GTC and BTC were found to be contaminated by fungus after 21 and 14 days of refrigerated storage respectively. Samples were tested for pH, titratable acidity, total microbial count and quantity of tea polyphenols.Table 3(a) and Table 3(b) show the effect of refrigerated storage on pH, total acidity and total microbial count of GTC and BTC respectively. In both the cases there was a gradual decrease in pH from 0^{th} day to 14^{th} day and then there was a slight increase on 21^{st} day. The decrease in pH is owed to increase in lactic acid content due to fermentation by starter cultures. Lactic acid fermentation implies to an anaerobic biological process occurring in some

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bacteriawhere cellular energy and the metabolite lactate are produced by catabolism of lactose, glucose, fructose and sucrose. The most commercially important lactic acid-fermenting bacteria are *Lactobacillus* commonlyutilized in the production of yogurt/ curd.The slight increase in pH observed after 14th day of refrigerated storage might be due to the fungal contamination. Titratable acidity was measured in terms of content of total lactic acid.In both the cases there was a gradual increase till the 7th day followed by a fall on 14th day and then again it increased on 21st day.In both the cases there was a twofold decrease in bacterial load on 7th day and then the curds showed fungal contamination from 14th day in case of black tea curd and 21st day in case of green tea curd. The decreased number of bacteria after 7 days of refrigerated storagewas caused by acid shock (low pH), which influenced the multiplication of bacteria.

Table 3: Summary of effect of storage on pH, titratable acidity and total microbial count of (a) GTC and (b) BTC

(a)

Refrigerated Storage (day)	pH	Titratable acidity (g lactate / L)	No. of bacteria (CFU / ml)
0	4.10	1.26	2.27×10^6
1	4.04	1.35	1.84×10^6
7	4.00	1.71	1.36×10^3
14	3.80	1.17	1.11×10^3
21	4.10	1.62	0.40×10^2

(b)

Refrigerated Storage (day)	pH	Titratable acidity (g lactate / L)	No. of bacteria (CFU / ml)
0	4.10	1.35	2.30×10^6
1	4.00	1.44	2.00×10^6
7	3.60	1.80	1.61×10^3
14	3.50	1.26	1.10×10^3
21	4.05	1.53	0.69×10^2

(a)

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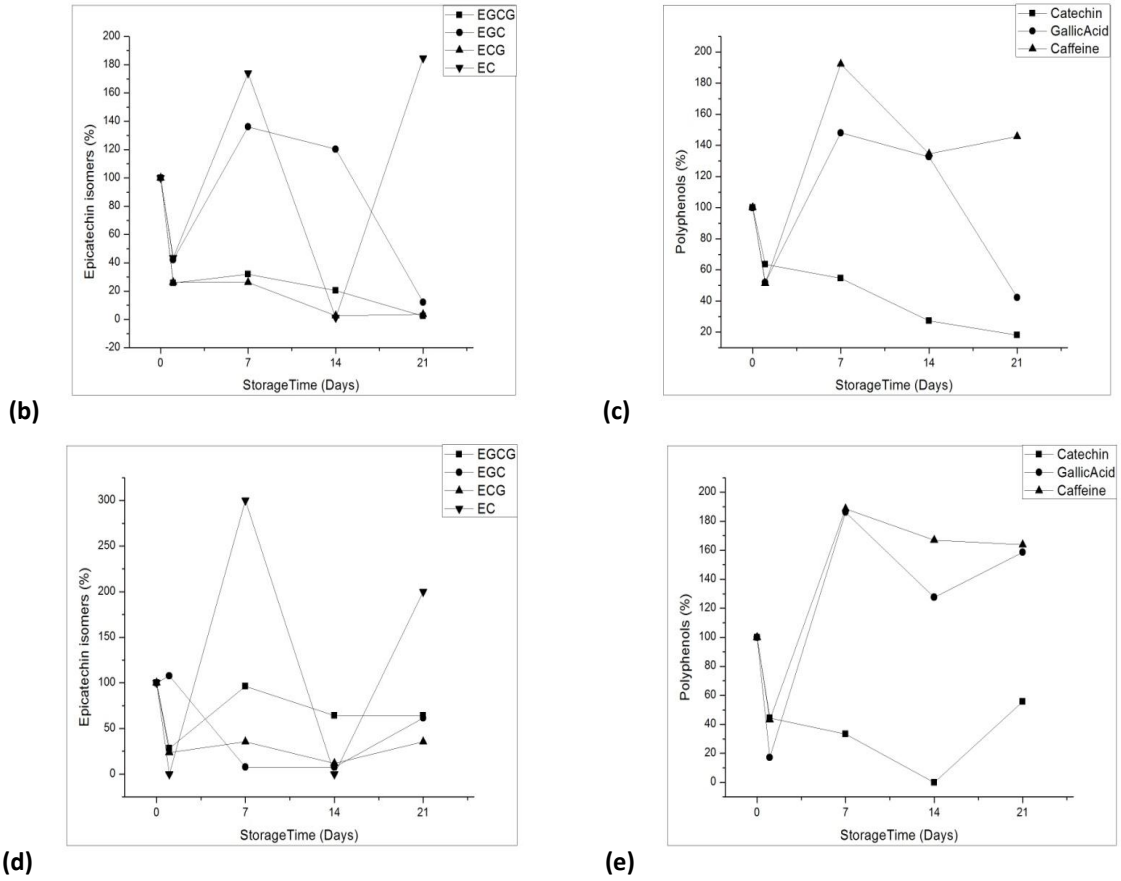

Fig 3: (a) Black tea curd (BTC) and green tea curd (GTC). (b) Effect of storage on levels of epicatechin isomers in GTC. (c) Effect of storage on levels of polyphenols in GTC. (d) Effect of storage on levels of epicatechin isomers in BTC. (e) Effect of storage on levels of polyphenols in BTC.

Effect of Refrigerated Storage on Tea Polyphenol Contents in Tea Curd

While Fig. 3(b) and Fig. 3(c) depict the effect of refrigerated storage on contents of epicatechin isomers and tea polyphenols respectivelyin GTC, Fig. 3(d) and Fig. 3(e) depict the effect of refrigerated storage on contents of epicatechin isomers and tea polyphenols respectivelyinBTC.Tea polyphenols in the present study were showing varying stability in GTC and BTC during refrigerated storage.There was decrease in contents of EGCG (72% and 74%) and ECG (76% and 74%) in BTC and GTCrespectively after 1 day of refrigerated storage. Then there was a slight increase in their concentration on 7th day of storage as compared to that on 1st day.Their concentrations again gradually decrease upto the 21st day. While EGC content increases on 1st dayand decreases thereafter in case of BTC whereas in case of GTC its concentration first decreases (58%) and then increases (20%) till 14th day and then again decreases (88%) on 21st day. EC content increases (100% and 85%) in case of BTC and GTC respectively on 21st day. There was gradual decrease (44% and 82%) in catechin concentration in BTC and GTC respectively. There was increase (33%) in concentration of gallic acid till 14th day after a decrease (48%) on 1st day and then it decreases (58%) on 21st day in case of GTC while it increases (59%) till 21st day after a decrease (83%) on 1st day in case of BTC.Caffeine concentration decreases (49% and 57%) on 1st day in GTC and BTC respectively followed by increase till 21st day (46% and 63%) in case of GTC and BTC respectively.There is great variation in tea polyphenol contents which may be due to three possible reasons; first the different components of tea have varying stability. But, since most of the tea polyphenols are highly stable at acidic pH (39) hence, this variation is considered to be brought about by the microbial enzymes. Biotransformation of EGCG to EGC and ECG to EC by enzymes excreted by microorganisms in curd could be the reason for the increased concentration of EGC and EC, even greater than the initial concentration, as observed on 7th day. Secondly, the complex polyphenolic compounds like epicatechin epimers show conversion or degradation to simpler ones.Finally, the epicatechin isomers adsorbed by the acid-sensitive microbial cells are released when they lose viability (40).Effective concentration of polyphenols

available in tea curd cannot be correlated with previous works since studies related to bioavailability of tea polyphenols in human volunteers are lacking in the literature. Although there are records of many research works on the bioavailability and efficiency of tea polyphenols against various diseases in animal models and different cell lines, it cannot be directly correlated with human systems since there are several influencing factors. Moreover, the efficiency of polyphenols in tea curd cannot be directly compared with other studies due to the presence of polyphenols in fat medium. Hence, our future works will focus on the study of effectiveness of tea polyphenols available in tea curd.

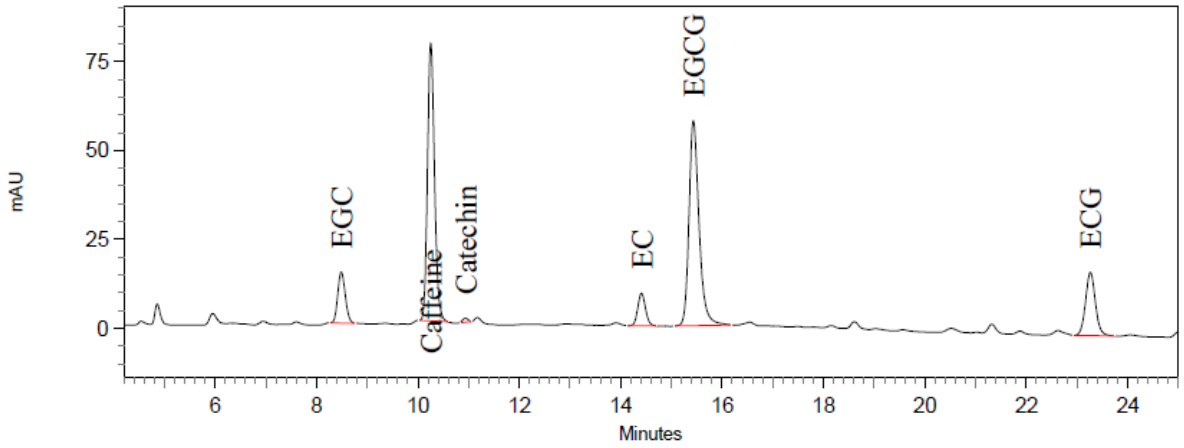

Fig 4: The liquid chromatogram of all the tea polyphenols

Fig. 4 shows the liquid chromatogram of all the tea polyphenols while table 4 shows the retention time of each tea polyphenol and the area occupied by each peak in the chromatogram.

Table 4: The retention time of each tea polyphenol and the area occupied by each peak in the chromatogram

Name	Retention Time	Area
Gallic acid	3.904	120713
EGC	8.491	147220
Caffeine	10.251	749813
Catechin	10.944	7921
EC	14.411	96314
EGCG	15.435	794741
ECG	23.264	240513
Totals		2157235

CONCLUSION

From the present study two strains were identified as biogenic amine non-producers hence can be used as starters or adjuncts during preparation of tea curd. Identification of the strains is under process. The tea polyphenols were found to have varying degrees of stability. This is generally due to three possible reasons, biotransformation of EGCG and ECG by microbial enzymes, degradation of complex epicatechin isomersinto their simpler forms and release of the epicatechin isomers from acid-sensitive microbial cells. Further studies on bioavailability and potential effectiveness of the tea polyphenols in tea curd are inevitable.

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