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Characterization of microflora, antioxidant and antibacterial activities of Tepache – A fermented fruit beverage

[1]Deevena Jemima, [2]Sheila John, and [3]Sarah Jane Monica

[1,2]Department of Home Science, Women's Christian College, Chennai-600006 (India)
[3]Department of Food Science, M.O.P. Vaishnav College for Women (Autonomous) Chennai-600034 (India)

Abstract

Fruits and vegetables contain vitamins, minerals, organic acids, and phytonutrients. Consumption of fermented fruit-based beverages is increasing among consumers as they provide numerous health benefits. This research work aimed at studying the microbial composition, antioxidant and antibacterial activities of tepache prepared using whole pineapples and apples. The pH of the formulated beverage was 3.21 with a viscosity of 3.80cP. TPC and TFC were 100.38 ± 1.02 µg/mL GAE and 79.31 ± 0.89 µg/mL QE. The total energy and vitamin C content were 55.1 kcals and 14.76mg/100mL. Results of gram staining, biochemical tests, and sugar utilization profile showed the presence of gram-positive and catalase-negative lactic acid bacteria. The viable count was 4.2 x 10^9 CFU/mL. GC-MS analysis showed the presence of phenolic compounds, flavonoids, fatty acids, and esters of fatty acids. Tepache had good antioxidant activity and inhibitory action against *Escherichia coli, Micrococcus luteus,* and *Shigella flexneri*. This study signifies that fermented fruit beverages provide essential micronutrients and functional compounds possessing therapeutic properties beneficial to mankind. However, optimizing fermented fruit-based beverages in terms of physicochemical parameters, nutrient composition, microbial profile, and potential health benefits are required for them to serve as an alternate substitute for sugar-sweetened beverages.

Key words : fermentation, apple, pineapple, tepache, lactic acid bacteria.

Over the past few years, remarkable changes have been noticed in beverage consumption. Due to the adverse ill effects caused by the consumption of sugar-sweetened beverages (SSB), the percentage of consumers opting for such beverages has started to decline. Concurrently, the consumption rate of different varieties of tea, coffee, and traditional

[1]Res. Scholar, [2]Associate Professor, [3]Assistant Professor

beverages is gaining popularity among individuals of all age groups[18]. Fruits and vegetables represent an excellent source of dietary fibre, organic acids, phytonutrients, vitamins, and minerals. Processing of fruits and vegetables using traditional and modern techniques has led to the production of juices and beverages that contain essential micronutrients, non-nutritive components, and different probiotic strains[14].

Fermented refreshments include alcoholic and non-alcoholic beverages obtained from fruits and vegetables. Some of the traditional fruit-based beverages prepared in different countries include Gilaburu, Hardaliye, Jjudima, Koumiss, Shalgam, and Tepache[1,3]. Tepache is a traditional Mexican fermented refreshing beverage prepared with pineapple and brown sugar. Lactic acid bacteria (LAB) are facultative, gram-positive, non-sporulating micro-organisms that grow rapidly in all food substrates[22]. LAB isolated, characterized and identified from fermented fruit-based beverages maintain the gut microbiome, improves the digestibility of foods and alleviates the risk of developing lifestyle and gastric disorders[24].

Apples and pineapples are commonly consumed by individuals of all age groups. Apples are rich in dietary fibre, pectin, pectic oligosaccharides, polyphenols, and vitamins. Besides consuming apples in fresh form, apples are processed into different products such as fermented apple juice, puree, vinegar, cider, apple spirit, dehydrated and canned apples[17]. Pineapples are highly relished for their sweet taste and aroma. A considerable amount of proteolytic enzymes, ascorbic acid, fibre, and phytonutrients are present in pineapples. Pineapples are processed into various products such as jams, jellies, candies, vinegar, concentrated pineapple powders, wine, and probioticated pineapple juice[8].

According to the literature, tepache is either prepared with pulp, or peel or by combining both peel and pulp of pineapples. Currently, the role of fruit by-products in beverage fortification has kindled interest. Likewise, nutritionally enriched fermented fruit-based beverages can serve as a healthy alternative approach to treating and preventing metabolic diseases. Limited studies are available on the microbiological profile and health-promoting properties of tepache prepared by mixing different fruits. In this regard, the research work aimed at determining the microbial composition, antioxidant and antibacterial activities of tepache prepared using whole pineapples and apples.

Preparation and fermentation of tepache:

Unblemished apples and pineapples were purchased from a local fruit market. The fruits were washed with tap water and dried at room temperature. Whole fruit including the peels was used for preparing tepache. The fruits were sliced using a sharp sterile knife. The top part of the fruits was discarded. Tepache was prepared according to the method described by Corona *et al.*[10] with slight modifications. 400 g of pineapple, 100 g of apple, 200 g of brown unrefined sugar, 10 cloves, and 2 cinnamon sticks were added to an airtight glass jar containing one litre of water. Since pineapple by itself acts as a good medium for the growth of *Lactobacillus* and

Bifidobacterium strains; no starter culture was used during the fermentation process. The contents in the glass jar were fermented under anaerobic conditions for 54 h at 22°C. The pH, viscosity, energy, and vitamin C content were estimated using standard procedures.

Characterization of microflora :

Initially, 1 mL of the fermented beverage was mixed with 9 mL of saline solution (0.85%) followed by the preparation of serial dilution. Later, this solution was streaked on MRS (deMan Rogosa and Sharpe) agar plates under aseptic conditions. The plates were incubated at 37°C for 48 h and observed for the growth of colonies. Characterization of isolates was done using the gram staining technique, biochemical tests, and sugar utilization profile.

Gram staining :

A loopful of the test culture was placed on a clean glass slide using an inoculation loop. The bacterial smear was heat fixed and crystal violet staining solution was added drop by drop. After 50 seconds, the smear was rinsed with distilled water followed by the addition of Gram's Iodine solution. The smear was allowed to rest for 45 seconds and was rinsed again. Later, 95% of ethyl alcohol (decolourizing agent) was added drop by drop and rinsed with distilled water. Finally, the smear was counterstained with safranin (counterstain dye) and was allowed to rest for 50-60 seconds. In the end, the slide was blot dried and observed under an oil immersion microscope for the presence of gram-negative and gram-positive bacteria[2].

Biochemical tests :

Indole test :

Briefly, 10 mL of nutrient broth was taken in a test tube, inoculated with the test culture, and incubated for 24 h at 37°C. After incubation, Kovac's reagent was added and observed for the formation of a cherry red ring.

Methyl red test :

For this test, 20 mL of Glucose Phosphate broth was taken in a test tube, inoculated with the test culture, and incubated for 24 h at 37°C. After incubation, methyl red reagent solution was added and observed for the development of pink colour.

Voges Proskauer test :

Initially, 20 mL of Glucose Phosphate broth was prepared and inoculated with the test culture. This broth was incubated for 24 h at 37°C. Barritt's A and Barritt's B reagents were added and observed for the presence of pink colour.

Citrate utilization test :

Simmon's citrate agar was prepared and 10mL of this agar solution was poured into a clean petri plate. After solidification, the culture was streaked on the agar plate, incubated for 24 hrs at 37°C, and observed for the formation of deep blue or green colour.

Catalase test :

A small amount of the test culture was spread on a sterile clean glass slide. Later, 3% hydrogen peroxide solution was added to the

slide and checked for the presence of effervescence.

Oxidase test :

An oxidase disc was placed on a clean glass slide and the test culture was swabbed on this glass slide using a sterile loop and checked for the formation of pink colour[16].

Sugar utilization profile :

The sugar utilization pattern of the test culture was determined using five different sugars viz glucose, fructose, sorbitol, mannitol, and lactose. These sugars were added to test tubes containing the basal media and inoculated with the test culture. All the test tubes were incubated for 48 hrs at 37°C in an orbital shaker. Acid production was confirmed through colour change[33].

Microbial analysis :

Plate count method was used to determine the colony units present in tepache. Man-Rogosa-Sharpe (MRS) agar was used and the colonies formed were counted after 48-72 hrs. Results are expressed as CFU\ml of tepache[6].

The extract required for further analysis was prepared by mixing 50mL of tepache with 100mL of methanol and reduced using a rotary vacuum evaporator at 45°C. The concentrated residues were stored at 4°C for further analysis.

Total phenol content (TPC) :

TPC was estimated using the Folin-Ciocalteu reagent method[30]. According to this method, 100µL of the test sample (1mg\mL) was mixed with 1 mL of Folin's reagent (1:10 diluted with distilled water). After 5 min, 1 mL of 20% Na_2CO_3 was added and the reaction mixture was incubated for 30 min. The absorbance was measured spectrophotometrically at 760 nm. TPC is expressed as µg\ml GAE.

Total flavonoid content (TFC) :

TFC was estimated using the Aluminium Chloride method[7]. Initially, 500µL of the test sample was mixed with 0.5 mL of 5% $NaNO_2$. Later, this solution was incubated for 15 min by adding 0.5 mL of 10% $AlCl_3$ and 1 mL of 1M NaOH. The absorbance was measured spectrophotometrically at 510 nm. TFC is expressed as µg\mL QE.

Antioxidant activity :

DPPH assay :

The ability of tepache to scavenge DPPH (1, 1 diphenyl -1-picrylhydrazyl), a stable free radical was determined using the method given by Blois[5]. In brief, 1 mL of 0.1 mM DPPH solution was added to different concentrations of the test sample (50-300µL). After 30 min of reaction in dark, the absorbance was measured spectrophotometrically at 517 nm. Results are expressed as % inhibition of DPPH free radical.

FRAP assay :

Different concentrations of the test sample (50-300µL) were mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% $K_3[Fe(CN)_6]$. The test tubes containing

the reaction mixture were incubated in a water bath at 50°C for 20 min. Later, 1 mL of 10% TCA was added. Finally, 500µL of 0.1% freshly prepared $FeCl_3$ was added and the absorbance was measured spectrophotometrically at 700nm. Results are expressed as absorbance values measured at 700 nm.

Phosphomolybdenum reduction assay :

Various concentrations of the test sample (20-120µL) were mixed with 1 mL of reagent solution that contained 0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The test tubes were covered with an aluminium foil sheet and incubated at 95°C for 90 min. The absorbance was measured spectrophotometrically at 695 nm. Results are expressed as absorbance values measured at 695 nm.

Antibacterial activity :

The antibacterial activity was determined using the agar well diffusion method. Bacterial strains used in the study were Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Shigella flexneri, Micrococcus luteus and Klebsiella pneumoniae. In brief, 20mL of freshly prepared nutrient agar was poured into the petri plates and was allowed to solidify. The bacterial strain was streaked on the surface of the media using a sterile cotton swab. Wells were punched using a sterile cork borer. Different concentrations of the test sample were loaded into the wells using a microphipette. The petri plates were incubated for 24 h. Results are expressed in terms of zone inhibition in millimeters.

Chromatographic profiling :

The volatile compounds were identified using gas chromatography-mass spectrometry (Agilent technologies 6890 N JEOL GC Mate II GC-MS model). The analytes were separated on an HP-5 column (30 m x 0.25 mm with 0.25 µm film thickness). The temperature was maintained at 50°C initially after which the temperature was steadily increased to 280°C at a rate of 10°C\min. Pure helium (99.9999%) was used as the carrier gas at a constant flow rate of 1 mL\min. The following MS conditions were used: ionization voltage of 70 eV, ion source temperature of 250°C, interface temperature of 250°C, and mass range of 50-600 mass units. The injection was performed by split mode (10:1). The compounds were identified using the standard mass spectral database of the National Institute Science and Technology Library[4].

The morphological and biochemical characterization of microflora is presented in Table-1. Gram staining showed the presence of gram-positive cocci that appeared in clusters. Results of biochemical tests confirmed the presence of lactic bacteria since a positive reaction was observed only for the methyl red test. Sugar utilization tests indicated that sugars viz glucose, fructose, and lactose were fermented by the isolates which was evident through colour change and acid production. The viable count of bacteria was found to be 4.2×10^9 CFU\mL of tepache.

The electron-donating potential was determined using 2, 2' -diphenyl-1- picryl hydrazyl radical (DPPH) free radical which is based on the ability to scavenge DPPH free radical. A gradual decrease in the absorbance

value indicates the free radical scavenging activity of the reaction mixture. FRAP and phosphomolybdenum assay were used to measure the reducing power of the reaction mixture. FRAP assay is based on the ability to reduce Fe^{3+} complex to Fe^{2+} form while phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) complex at an acidic pH. An increase in the absorbance value indicates the reducing power of the reaction mixture. Results of free radical scavenging activity and reducing power assay highlight that tepache had good antioxidant activity (Table 2). The IC_{50} value for DPPH assay was 198.51µg/mL.

Table-1: Characterization of microflora present in Tepache

Test	Result	Inference
Gram staining		
Gram Staining	Gram-positive cocci	Presence of purple colour spherical shaped bacteria in clusters
	Biochemical tests	
Indole test	Negative	Absence of colour change
Methyl red test	Positive	Presence of bright red colour
Voges Proskauer test	Negative	Absence of colour change
Simmon's citrate utilization	Negative	Absence of colour change
Catalase test	Negative	Absence of formation of effervescence
Oxidase test	Negative	Absence of purple colour formation
Sugar utilization profile		
Glucose	Positive	Colour change from red to orange
Fructose	Positive	Colour change from red to orange
Sorbitol	Negative	Absence of colour change
Mannitol	Negative	Absence of colour change
Lactose	Positive	Colour change from red to orange

Table-2. Antioxidant activity

DPPH assay		FRAP assay		Phosphomolybdenum assay	
Concentration µg/mL	% Inhibition	Concentration µg/mL	Absorbance @ 700 nm	Concentration µg/mL	Absorbance @ 695 nm
50	27.74 ± 4.72	50	0.07 ± 0.01	20	0.66 ± 0.02
100	29.15 ± 3.88	100	0.12 ± 0.02	40	0.73 ± 0.03
150	36.79 ± 3.38	150	0.19 ± 0.01	60	0.80 ± 0.01
200	50.51 ± 3.80	200	0.28 ± 0.03	80	0.84 ± 0.04
250	55.23 ± 1.66	250	0.42 ± 0.02	100	0.85 ± 0.05
300	65.93 ± 5.33	300	0.68 ± 0.05	120	0.86 ± 0.07

Alkaloids, saponins, tannins, terpenoids, and phenolic compounds present in fruits impede the growth of pathogenic microorganisms. Tepache inhibited the growth of Escherichia coli, Micrococcus luteus, and Shigella flexneri which increased with an increase in concentration. Maximum inhibition was observed in Escherichia coli with a zone of inhibition of 26 at 625µg/mL.

Table-3. Antibacterial activity

Bacterial strains	Standard (Tetracycline)	Zone of inhibition (mm)			
		250 µg	375 µg	500 µg	625 µg
Escherichia coli	13	16	20	22	26
Micrococcus luteus	16	10	13	18	20
Shigella flexneri	19	13	20	22	27
Bacillus subtilis	18	-	-	-	-
Staphylococcus aureus	15	-	-	-	-
Klebsiella pneumoniae	18	-	-	-	-

Fig. 1. GC-MS chromatogram of Tepache

Table-4. Compounds identified in tepache using GC-MS

Retention time	Name	Structure	Molecular weight	Molecular formula
13.1	Benzoic acid, 3-(1-methylethyl)-		164.201	$C_{10}H_{12}O_2$
13.9	8-endo-Methyl-7-exo-phenylbicyclo(4.2.0)oct-1(2)-ene		198.98	$C_{15}H_{18}$
14.7	Cyclohexanol, 4-(1,1-dimethylethyl)-1-phenyl-, trans-		232.367	$C_{16}H_{24}O$
15.95	Palmitic acid, methyl ester		270.457	$C_{17}H_{34}O_2$
16.6	Cetylic acid		256.43	$C_{16}H_{32}O_2$
17.73	Octadecanoic acid, methyl ester		298.511	$C_{19}H_{38}O_2$
18.38	Nitrofurazone		280.279	$C_{17}H_{12}O_4$
19.27	Bicyclo (3.2.0)hept-2-en-6-ol,exo-6,7-diphenyl-5-methoxy-		292.372	$C_{20}H_{20}O_2$
20.15	Coumarine,3(2,4-dinitrophenyl)-		312.237	$C_{15}H_8N_2O_6$
21.5	2H-Naphtalen-1-one,3,4-dihydro-6-methoxy-2-(4-methoxybenzylideno)-		294.35	$C_{19}H_{18}O_3$
23.32	But-2-enediamide,N,N-bis(4-methoxyphenyl)-		326.352	$C_{18}H_{18}N_2O_4$

Fermentation is a process whereby micro-organisms act on food substrates and break the complex molecules into simpler units. Fermentation improves the physicochemical properties, nutrient profile, and sensorial parameters of food products[11]. Fermented fruit beverages contain different probiotic strains and are high in antioxidant compounds. There are different ways of fermenting foods and beverages such as a) fermentation by microflora that is naturally present in the raw materials b) fermentation by adding suitable starter cultures to the raw materials c) fermentation by heat treated materials by starter cultures[31]. The present study was designed to study the microbial composition, antioxidant and antibacterial activities of tepache prepared using whole pineapples and apples. The initial and final pH value of tepache was 4.42 and 3.21 with a viscosity of 3.80cP. This decrease noted in pH after fermentation might be due to the production of organic acids namely lactic and acetic acid. The pH values observed in this study are in agreement with those reported by Moreno Terrazas[22]. Tepache is a traditional fermented beverage that is commonly consumed by Mexicans. Beneficial bacterial strains such as *Lactobacillus lactis*, *L. paracasei*, *L. pentosus*, and *L. plantarum* have been isolated from tepache. These bacterial strains maintain the gut microflora, modulate the immune system and contribute to many other therapeutic properties[2,28]. In this study, results of gram staining, biochemical tests, and sugar utilization profile confirmed the presence of gram-positive catalase-negative lactic acid bacteria. The viable bacterial count was 4.2×10^9 CFU/mL. The total energy and vitamin C content were 55.1 kcals and 14.7mg/100mL.

Beyond the nutritional and physicochemical advantages, current studies highlight the beneficial aspects of fermented fruit and vegetable based-beverages. Koh *et al.*[9] in their study documented that the fermentation of pumpkin beverages with *L. mali* exhibited a hypoglycaemic effect. The beverage had the potential to significantly inhibit the action of alpha-glucosidase. Yan *et al.* (2019) stated that fermentation of blueberry pomace with *L. rhamnosus*, *L. plantarum-1*, and *L. plantarum -2* had excellent antihyperlipidemic properties. Beverages obtained by fermenting blueberries, blackberries, and pears were reported to possess anti-inflammatory activity along with the ability to maintain the integrity of intestinal barriers[13,15,34]. Results of the present study proved that tepache had good antioxidant and antibacterial activities.

Antioxidants exert their action upon free radicals through two elemental mechanisms. The first mechanism of action is the chain-breaking mechanism where antioxidants donate electrons to scavenge free radicals. The second mechanism is quenching certain chain-initiating catalysts by antioxidants[21]. Results of free radical scavenging activity and reducing power assay highlight that tepache had good antioxidant activity. Phenolic compounds act as natural antioxidant compounds. Results of preliminary qualitative analysis of phytochemicals confirmed the presence of alkaloids, phenols, flavonoids, glycosides, steroids, terpenoids, and saponins in tepache. The total phenol content and total flavonoid content of tepache were 100.38 ± 1.02 µg/mL GAE and 79.31 ± 0.89 µg/mL QE. The findings of this study are on par with the existing body of literature stating that phenols and flavonoids contribute to

to be an effective method used to improve the

Universally, fermentation is visualized

of fatty acids.

fermented fruit-based beverages.

protective and therapeutic benefits of such

required to corroborate and authenticate the

randomized controlled clinical trials are

fermented beverages, scientific evidence from

promoting properties of different non-dairy

model studies support the physiological health-

Through findings of in vitro and animal-based

which was followed by *Shigella flexneri*.

with a zone of inhibition of 26 at 625µg/mL

inhibition was observed in *Escherichia coli*

in a concentration-dependent manner. Maximum

lutea, and *Shigella flexneri* which increased

growth of *Escherichia coli*, *Micrococcus*

cinnamon worked synergistically to inhibit the

present in pineapple, apples, cloves, and

teapache. Different bioactive compounds

enhancing the flavor, aroma, and taste of

contributed to antibacterial activity apart from

cloves and cinnamon could have also

Previous and recent research findings have

confirmed the antimicrobial property of

cinnamon and cloves[22,26]. The addition of

and cloves were used while preparing teapache.

sensorial parameters. In this study, cinnamon

increase the shelf life and enhance various

added to fermented foods and beverages to

juniper berries, and red chillies are commonly

micro-organisms. Spices such as cloves, garlic,

dysbiosis by inhibiting the growth of pathogenic

of phenolic acids in preventing intestinal

activity. Cueva et al.[12] documented the role

Dietary polyphenols exhibit antimicrobial

of fatty acids.

compounds, flavonoids, fatty acids, and esters

analysis also showed the presence of phenolic

antioxidant activity[33]. Furthermore, GC-MS

chemical, physical and nutritional properties of different foods and beverages. The research work aimed at the characterization of microflora and determining tepache's antioxidant and antibacterial activities. This nutritionally enriched fermented beverage made using the whole pineapple and apple proved to be a good source of phytonutrients. Further, more studies are needed to authenticate the protective effects of fermented fruit-based beverages.

Conflict of Interest: The authors declare no conflict of interest.

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