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ORIGINAL ARTICLE

Prevalence, Molecular Characterization and Antibiogram Assay of Human Pathogens Associated with an Indian Frozen Dessert Sold in Bangalore, India

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ABSTRACT

Kulfi is a traditional dairy-based frozen dessert sold in almost all the cities in India and relished by people of different age groups, mainly popular among the teenagers. The present study was undertaken to investigate the microbiological quality of kulfi sold in many parts of Bangalore, India. Twenty kulfi samples, comprising of seven different brands, were analyzed. Biochemical and molecular characterization revealed the presence of bacterial pathogens such as Streptococcus faecalis, Bacillus cereus, Bacillus subtilis, Bacillus coagulans, Staphylococcus epidermidis, Staphylococcus aureus, Staphylococcus citreus, Micrococcus luteus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis and psychrotolerant bacteria Yersinia pseudotuberculosis and Chryseobacterium gleum. Total viable counts of bacteria in the samples varied between 1.0-137 x 10^4 CFU/g. Presence of Lactobacillus sp. was also detected in few samples. Salmonella and Vibrio cholerae were not detected in any of the samples. Yeasts like Saccharomyces, Rhodotorula and Torulopsis were also encountered but no filamentous fungus was detected. Antibiogram assay indicated that ofloxacin and gentamicin were highly effective against gram positive bacteria, whereas, ciprofloxacin largely inhibited gram negative isolates. It is suggested that good quality water and pasteurized milk should be used for the preparation of kulfi inorder to avoid any foodborne pathogenic outbreaks in future.

Key Words: *Frozen desserts, contamination, kulfi, antibiogram, pathogens.*

INTRODUCTION

In India about 0.7% of the total milk produced is converted into frozen desserts like ice-cream and kulfi [1]. Kulfi is a traditional frozen dairy product of India, prepared by concentrating whole milk by boiling and adding sugars and dry fruits to the concentrated milk. It is then filled into aluminium or plastic cones and frozen in an earthen pot having a mixture of ice and salt [2]. Kulfis are mainly popular due to their easy affordability and wide availability in various flavors.

Though frozen dairy products have some nutritional significance, they lack therapeutic properties [3]. Recently, inferior microbial quality has become a serious drawback of market kulfi [2]. The various constituents used during its preparation include water, milk, sugars and dry fruits, which may act as sources of microbial contamination. Usually kulfis have shelf life of ten months when kept frozen but their quality deteriorates due to pathogenic contamination. Furthermore, improper processing, storage and distribution of kulfis may result in the introduction and survival of psychrotolerant bacteria in this food, which upon consumption, may lead to foodborne illnesses such as typhoid, sore throat etc [1].

Very few researches have been conducted on the quality surveillance of kulfis sold in India. A study conducted in Mumbai revealed the presence of various bacterial and fungal pathogens in 36 samples of kulfi [4]. Another investigation of 24 kulfi samples sold in Chennai indicated the occurrence of moderate to high psychrotrophic count of various *Pseudomonas* sp., especially in kulfis sold by road side local vendors [5].

The present study was conducted to investigate the microbiological quality of kulfis sold in Bangalore, identification of various pathogens associated with this frozen dessert and their antibiogram assay.

MATERIALS AND METHODS

The present study was conducted during the period from 11.06.2011 to 15.03.2012 at Genohelix Biolabs, Chamarajpet, Bangalore, India. All the media used during the course of the study were procured from Himedia Laboratories Pvt. Limited (A- 406, Bhaveshwar Plaza, Mumbai- 400086, India).

Collection of samples

Four overpopulated locations in Bangalore city, India, were chosen for the collection of kulfi samples. Preferred locations were Chamarajpet, Jayanagar, Banashankari and J.P. Nagar. Samples of kulfi were picked up from at least five shops in each area where the sale was maximum. All the samples were aseptically collected in sterile containers, stored at 4°C and analyzed within an hour of procurement.

Isolation and enumeration of microorganisms

Isolation and enumeration of microbes were performed using serial dilution and spread plate technique. One gram of kulfi sample was properly homogenized in an electric blender. One milliliter of the resultant homogenate was added to 9 mL of sterile 0.85% (w/v) saline in a test tube and serially diluted to obtain dilutions upto 10^{-5}. For bacterial isolation 0.1 mL of the appropriate dilution from each tube was aseptically pipetted out and plated onto different selective and differential media (Tryptic Soy agar, Mac Conkey agar, Cetrimide agar, Baird Parker's agar, Blood Glucose agar, Tributyrin agar, 10% Salt Milk agar, Eosin Methylene Blue agar, Plate Count agar, Lactobacillus de Mann Rogosa Sharpe agar, Skimmed Milk agar, Bile Esculin agar, Thiosulphate Citrate Bile salts Sucrose agar, Mannitol Salt agar, Polymyxin Egg Yolk Mannitol Bacitracin agar, Mannitol Yolk Red agar, Salmonella Shigella agar, Hichrome UTI agar and Mitis Salivarius Sucrose Bacitracin agar). All the bacterial plates were incubated under aerobic and anaerobic conditions (as per requirement) at 37°C for 24 to 48 h. The fungal isolation was done on Sabouraud Dextrose agar. The fungal plates were incubated at 27°C for 3 to 5 days. For microbial enumeration the plates were used to determine the number of colony forming units (CFU) per gram of kulfi sample.

Identification and biochemical characterization of the microbial isolates

Following incubation, the isolated colonies were pure cultured and Gram stained. Biochemical characterization of the isolated colonies was carried out using standard protocols [6]. Several biochemical tests performed were indole production, methyl red, Voges Proskauer, citrate utilization, starch hydrolysis, gelatin hydrolysis, lipid hydrolysis, urea hydrolysis, slide coagulase, tube coagulase, oxidase, motility, 6.5% NaCl test, 10% NaCl test, triple sugar iron test, ornithine-decarboxylase, sugar fermentation and haemolysis test. Bacterial identification was carried out according to Bergey's Manual of Determinative Bacteriology (9^{th} Edition). Identification of the fungal isolates was performed by lactophenol cotton blue staining and observation of macroscopic and microscopic characteristics. Identification of the yeast isolates was performed by Gram staining and observation of macroscopic and microscopic characteristics.

Molecular characterization of selected bacterial isolate

The selected bacterial isolate was cultured in Luria Bertuni broth and incubated at 37°C for 24 h in an orbital shaker at 150 rpm. Genomic DNA was extracted using Bacterial Genomic DNA Isolation Kit (Chromous Biotech Pvt. Ltd., Bangalore, India) according to the manufacturer instructions and visualized using 0.8% (w/v) agarose gel electrophoresis.

PCR amplification

The PCR amplification reactions were performed in a total volume of 25µL. Each reaction mixture contained the following solutions: 1.5µL genomic DNA, 1µL 10 pmol forward 16S rDNA primer (5'-AGAGTTTGATCCTGGCTCA-3'); 1µL of 10pmol reverse 16SrDNA primer (5'-ACGGCTACCTTGTTACGACT-3'); 1µL of 30 mM deoxyribonucleoside 5'-triphosphate (N= A,T,G,C) (dNTP's); 2.5µL of 10X PCR buffer and 1µL Taq polymerase (1U) (Chromous Biotech Pvt. Ltd., Bangalore, India) and water was added up to 25 µL. The thermal cycler (MJ Research PTC 200, USA) was programmed as follows: 2 min initial denaturation at 94°C, followed by 30 cycles that consisted of denaturation for 1 min at 94°C, annealing for 30 s at 57°C and extension at 74°C for 1 min and a final extension of 5 min at 74°C. The PCR amplified product was analyzed by 1.2% agarose gel electrophoresis using TAE buffer. The resulting DNA patterns were examined with UV

light under transilluminator, photographed and analyzed using gel documentation system (Herolabs, Germany).

Partial 16S rDNA sequencing and analysis of sequenced data

The partial 16S rDNA sequencing of the amplified product was performed at Chromous Biotech Pvt. Ltd., Bangalore, India. The 16S rDNA sequence was aligned manually with the available nucleotide sequences retrieved from the NCBI database by using BLASTN [7].

Antibiogram assay

The antibacterial susceptibility test against few selected bacterial pathogens was performed by agar disc diffusion [8]. Pure cultures of the bacterial isolates were inoculated in sterile Mueller Hinton broth tubes and incubated at 37°C overnight for preparation of the inocula. The bacterial cultures were swabbed on to the surface of sterile pre-solidified Mueller Hinton agar plates. Different standard antibiotic discs such as vancomycin, ofloxacin, gentamicin, clindamycin, ceftazidime, ciprofloxacin, tobramycin and cefotaxime were tested against gram positive and gram negative bacterial isolates. The bacterial plates were incubated at 37°C overnight and observed for the zones of inhibition. Diameters of the inhibitory zones were measured to the nearest millimeter using a millimeter scale.

Statistical analysis

All the antibiogram assays were conducted in triplicate and the data were analyzed using single factor analysis of variance (ANOVA). All the data are presented as mean ± standard deviation of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Kulfi is a very popular frozen dairy dessert from the Indian subcontinent. Although quite similar to ice-cream, it is denser and creamier. It is available in a variety of flavors and relished by people of all age groups. The basic ingredients of kulfi consist of ice, milk, water and sugars. Since water and raw milk generally harbor lot of potential pathogens, these may survive in the kulfis if inadequately pasteurized milk and/or poor quality water is used for the food processing. These pathogens, especially the psychrotolerant ones, may survive in the finished product and result in incidences of foodborne illnesses and food poisoning among the consumers.

In the present study the microbiological quality of 20 kulfi samples were analyzed. The kulfi samples were purchased from local street-side vendors, small establishments, retail outlets and supermarkets across four different locations in Bangalore. The 20 different kulfi samples belonged to 7 different brands: some local and some leading. All these samples were available in different flavors such as cardamom, almond, pistachio, saffron pistachio and chocolate. The kulfi samples were collected and transported to the research laboratory within icepacks, allowed to melt and the pH was recorded. The pH values of all the samples ranged from 6.7 to 7.5. This particular pH range might have provided a favorable environment for the survival and proliferation of majority of the pathogenic bacteria.

Enumeration of microorganisms

The bacterial enumeration of the kulfi isolates was carried out on plate count agar. Sample number 2, 15 and 16 revealed very high total viable counts ranging above 92 x 10^4 CFU/g. Sample number 1, 3, 4, 6, 7, 14, 17 and 18 demonstrated moderate counts ranging between 11-37 x 10^4 CFU/g, whereas, sample number 5, 8, 9, 10, 11, 12, 13, 19 and 20 showed low viable counts varying between 0-8 x 10^4 CFU/g. The significantly high load of bacteria as noted in 15% of the samples indicates the gross contamination of the kulfi samples either during processing, packaging, improper storage and/or distribution. A previous study conducted in Chennai revealed high psychrotrophic count in road side locally-vended samples (1.8 x 10^3 CFU/g) followed by that of samples from small scale producers (1.01 x 10^3 CFU/g) and branded samples from organized sector (3.5 x 10^2 CFU/g) [5]. In our study, the high bacterial counts associated with sample number 2, 15 and 16 may be attributed to the fact that these were collected from road side vendors selling kulfis which might have been prepared under dusty unhygienic conditions and from poor quality ingredients.

40% of the samples revealed moderate counts of bacteria which could be due to improper packaging, storage or distribution. On the other hand, the rest 45% of the kulfi samples showed

very low bacterial counts indicating the overall good bacteriological quality of these products as compared to others. It can also be correlated to the fact that some of these samples belonged to brands which were either obtained from retail outlets and/or supermarkets, wherein better hygienic standards, storage conditions and cleanliness are maintained. The significant results of bacterial enumeration have been presented in **Table 1**.

Identification of microorganisms

Based on the Gram's staining, the bacterial isolates from the kulfi samples were categorized into gram positive rods in chains with endospores, gram positive cocci in short chains, tetrads, pairs and clusters, gram positive rods scattered with endospores, gram negative rods scattered, gram negative rods in pairs and scattered, gram positive cocci in clusters, gram positive serpentine like rods in chains and gram positive cocci in chains. The conventional biochemical characterization of these kulfi isolates indicated the presence of several pathogens such as *Streptococcus faecalis*, *Bacillus cereus*, *B. subtilis*, *B. coagulans*, *Staphylococcus epidermidis*, *S. aureus*, *S. citreus*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa, Proteus mirabilis* and psychrotolerant bacterium *Yersinia pseudotuberculosis*. Some of these bacteria might have originated from the raw milk and processing water, while others constitute the normal microflora of human and animal skin. *Yersinia pseudotuberculosis* is a gram negative psychrotolerant bacterium that causes pseudotuberculosis disease in animals and occasionally in humans through the foodborne route [9]. The pathological implications of these microorganisms on public health have been thoroughly described [10, 11].

With the advancement of technology in molecular study, primers have been developed by researchers to specifically target the 16S rDNA gene sequence of the microorganisms [12]. The molecular characterization of a selected β-haemolytic bacterial isolate number O was carried out after PCR amplification of the 16S rDNA sequence of the genomic DNA using the universal forward and reverse primers. The 1500 bp long PCR-amplified product was then partially sequenced. The BLASTN search results using 16S rDNA sequenced data as the query sequence revealed that isolate number O was very closely related to *Chryseobacterium gleum* strain CCUG 14555 with GenBank accession no. AM232812.1. The homology value of BLASTN search showed 99% sequence similarity with the registered 16S rDNA gene sequence in NCBI data base. *Chryseobacterium gleum* (previously included in the *Flavobacterium* IIb species) is a gram negative aerobic bacillus that produces a distinct yellow to orange pigment [13]. Chryseobacteria are widely distributed in nature and found primarily in soil and water [14]. They are known to cause nosocomial infections. This bacterium has also been found to produce β-lactamase that possesses the property of hydrolyzing most β-lactam antibiotics, including carbapenems [15]. This and other psychrotolerant bacteria in the kulfi samples might have originated from the raw milk or water used during the product preparation and thus would have survived even in the freezing temperatures of kulfi [16]. The beta hemolytic nature of some of these psychrotrophs may emphasize their potential to colonize the upper respiratory tract and cause severe sore throat, pharyngitis, tonsillitis and other difficult to treat infections. The occurrence of psychrotrophic *Pseudomonas fluorescens*, *P. aeruginosa* and *P. putrifaciens* in kulfi had been previously reported [5].

Presence of *Lactobacillus* sp. was also detected in few samples. The prevalence of lactobacilli in the kulfi samples may be attributed to the use of milk during the manufacturing process. Spores of lactic acid bacteria may withstand the pasteurization process and survive even at the freezing temperatures of kulfi. *Salmonella* and *Vibrio cholerae* were not detected in any of the samples. In another study, the presence of coagulase positive *S. aureus* and *E. coli* were noted, which were found to survive in kulfi even after 40 days. These bacteria might have come either from the zoonotic sources, milk handlers or from the water contaminated with faecal coliforms [17]. A high count of these bacteria in food may result in staphylococcal intoxication or diarrhea. Similarly, bacteriological assessment of *matka* kulfi in Bikaner city revealed the presence of *E. coli* and *Staphylococcus* sp. in all the 25 samples analyzed. Faecal streptococci count of all the samples was more than 1.8×10^3 per ml, where as psychrophilic count in most of the samples was in the range of 10^4 to $>10^5$ per ml [18]. Our findings are in agreement with a previous report on kulfi analysis carried out in Mumbai, which showed the prevalence of various microbial contaminants such as *Staphylococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Micrococcus luteus*, *Klebsiella aerogenes*, *Enterobacter aerogenes*, *Proteus* sp., *Escherichia coli* and *Shigella* sp.

Surprisingly, the percentage of yeasts and molds was low. Among the fungi, *Aspergillus* sp., *Penicillium* sp., *Mucor* sp., *Rhizopus* sp., *Absidia* sp., *Saccharomyces* sp. and *Rhodotorula* sp. were encountered in kulfi samples previously [4]. Similarly, yeasts like *Saccharomyces*, *Rhodotorula* and *Torulopsis* were also encountered in the present study but no filamentous fungus was detected. The yeast forms might have originated from the milk and/or sugar used during the product preparation. The presence of yeast counts may also indicate the post-processing or aerial contamination of the products [19]. The detailed report of microbial identification has been elucidated in **Table 1**.

Table-1: Enumeration and identification of microorganisms isolated from different brands of kulfi

Sl. No.	Types of Kulfi	Total Viable Count (x 10^4 CFU/g)	Microorganisms detected
1	Brand 1 Chocolate flavored	14	A, D-1, E, M
2	Brand 1 Almond flavored	TNTC*	B, E
3	Brand 1 Cardamom flavored	20	A, E, F
4	Brand 2 Cardamom flavored	37	A, B, D-2, M
5	Brand 2 Almond flavored	1	A
6	Brand 2 Pistachio flavored	11	A, E, J
7	Brand 2 Chocolate flavored	12	A, I-7, L
8	Brand 3 Almond flavored	-	-
9	Brand 3 Chocolate flavored	8	A, B, J, L, M, yeast
10	Brand 4 Saffron Pistachio flavored	4	A, D-2, E, I-7, yeast
11	Brand 4 Almond flavored	5	F, G, Y-1
12	Brand 5 Chocolate flavored	4	A, B, I-7, M
13	Brand 5 Pistachio flavored	6	I-1
14	Brand 6 Chocolate flavored	37	B, D-1, L, M
15	Brand 6 Pistachio flavored	100	D-2, E, I-1, I-7, M
16	Brand 6 Almond flavored	92	B, D-1, L, M
17	Brand 6 Cardamom flavored	17	D-1, M, yeast
18	Brand 6 Saffron Pistachio flavored	23	I-2, O
19	Brand 7 Chocolate flavored	3	B, M, Y-2, yeast
20	Brand 7 Almond flavored	2	B

Keys: *, Too numerous to count; A, *Streptococcus faecalis*; B, *Bacillus cereus*; D-1, *Bacillus subtilis*; D-2, *Klebsiella pneumoniae*; E, *Escherichia coli*; F, *Staphylococcus epidermidis*; G, *Yersinia pseudotuberculosis*; I-1, *Pseudomonas aeruginosa*; I-2, *Proteus mirabilis*; I-7, *Bacillus coagulans*; J, *Staphylococcus aureus*; L, *Lactobacillus* sp.; M, *Micrococcus luteus*; O, *Chryseobacterium gleum*; Y-1, *Staphylococcus citreus*; Y-2, *Staphylococcus citreus*

Antibiogram assay

Antibiogram assay of the kulfi isolates indicated that ofloxacin and gentamicin were highly effective against the gram positive bacteria, while vancomycin and clindamycin were moderately effective. The significant results of the antibiogram assay have been outlined in **Table 2**. Ofloxacin showed the highest inhibition against *B. coagulans* (24 ± 0.05 mm), whereas, gentamicin was most effective against *S. citreus* (26 ± 0.05 mm). On the other hand, both vancomycin and clindamycin exhibited maximum inhibition against *S. citreus* (24 ± 0.08 mm and 40 ± 0.24 mm, respectively). In case of the gram negative bacteria ciprofloxacin largely inhibited majority of the isolates, with maximum inhibition against *Yersinia pseudotuberculosis* (40 ± 0.20 mm). Tobramycin and gentamicin were moderately effective and demonstrated maximum zones against *Chryseobacterium gleum* (20 mm). Cefotaxime greatly inhibited *Klebsiella pneumoniae* (22 ± 0.16 mm) and *Proteus mirabilis* (22 ± 0.15 mm), while ceftazidime was moderately effective against *Proteus mirabilis* (16 mm). Most of the isolates exhibited resistance to ceftazidime. Species of *Chryseobacterium* such as *C. indologenes* which is most similar to *C. gleum* with a 16S rRNA gene sequence similarity of 98-99%, is resistant to many antibiotics, as reported earlier [20, 21].

Table-2: Results of antibiogram assay showing inhibitory zones (in mm) against the bacterial isolates

Gram positive bacteria					
Isolates	Vancomycin	Ofloxacin	Gentamicin	Clindamycin	Ceftazidime
A	20 ± 0.00*	18 ± 0.00	20 ± 0.00	22 ± 0.03	-
B	19 ± 0.03	23 ± 0.03	20 ±0.09	25 ± 0.01	14 ± 0.02
D-1	19 ± 0.03	22 ± 0.06	20 ± 0.02	09 ± 0.00	-
F	19 ± 0.05	20 ± 0.00	23 ± 0.04	25 ± 0.20	-
I-7	21 ± 0.02	24 ± 0.05	21 ± 0.16	10 ± 0.00	-
J	19 ± 0.03	20 ± 0.00	19 ± 0.06	18 ± 0.01	-
M	18 ± 0.05	22 ± 0.05	21 ± 0.03	24 ± 0.03	-
Y-1	24 ± 0.08	16 ± 0.18	26 ± 0.05	40 ± 0.24	-
Y-2	18 ± 0.11	12 ± 0.09	20 ± 0.01	23 ± 0.12	10 ± 0.00
Gram negative bacteria					
	Ciprofloxacin	Tobramycin	Gentamicin	Cefotaxime	Ceftazidime
D-2	24 ± 0.02	15 ± 0.05	12 ± 0.00	22 ± 0.16	15 ± 0.04
E	19 ± 0.05	13 ± 0.06	13 ± 0.05	20 ± 0.08	15 ± 0.05
G	40 ± 0.20	12 ± 0.00	16 ± 0.08	-	-
I-1	32 ± 0.50	17 ± 0.01	12 ± 0.00	09 ± 0.05	-
I-2	22 ± 0.01	11 ± 0.02	11 ± 0.02	22 ± 0.15	16 ± 0.00
O	28 ± 0.03	20 ± 0.00	20 ± 0.01	19 ± 0.05	-

Keys: A, *Streptococcus faecalis*; B, *Bacillus cereus*; D-1, *Bacillus subtilis*; D-2, *Klebsiella pneumoniae*; E, *Escherichia coli*; F, *Staphylococcus epidermidis*; G, *Yersinia pseudotuberculosis*; I-1, *Pseudomonas aeruginosa*; I-2, *Proteus mirabilis*; I-7, *Bacillus coagulans*; J, *Staphylococcus aureus*; L, *Lactobacillus* sp.; M, *Micrococcus luteus*; O, *Chryseobacterium gleum*; Y-1, *Staphylococcus citreus*; Y-2, *Staphylococcus citreus*; *, values are mean ± S.D. (n=3)

CONCLUSION

Kulfi is a very popular frozen dessert sold in India. The use of raw milk and poor quality water for its preparation may result in microbial contamination. The present study was an attempt to assess the microbiological quality of kulfi sold in Bangalore. Our results revealed the occurrence of various pathogenic bacteria including some psychrotolerant β-haemolytic forms. Consumption of such contaminated kulfis can eventually act as a health hazard causing various infections among the consumers. It is suggested that periodical surveillance of the quality of kulfi be practised in order to prevent any outbreak in future.

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