



ISOLATION AND IDENTIFICATION OF BACTERIA IN ICE-CREAM SAMPLES MARKETING IN TIRUCHIRAPPALLI CITY, TAMILNADU, INDIA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author SGS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BK and MR managed the analyses of the study. Author MR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The microbiological quality of ice cream during retail marketing mainly depends on the contamination during the product handling as well as efficiency and sanitary conditions during frozen storage. Many food poisoning cases associated with the consumption of ice creams have been reported. We studied the bacteriological profile of ice cream sold at retail outlets in Tiruchirappalli, a city in Tamilnadu State, India. Ice cream samples were collected from ice cream shops. Random sampling method was followed to collect different flavours of ice creams from the shops. A total of 10 samples were collected from two different localities, in sterile containers and transported to the laboratory and were processed immediately. High level coli form count, presence of *Escherichia coli* and *Bacillus sp* indicated faecal contamination of ice creams thereby suggesting possible risk of infection involved in the consumption of such food. Presence of *Staphylococcus* an enterotoxin producer may cause serious health problems. Among the isolates from samples tested, only *Escherichia coli* and *Staphylococcus sp* were significant potential pathogens. However, presence of other organisms (*Bacillus*) could be attributed to unhygienic conditions during preparation, handling and serving of ice creams. Based on the biochemical characterization, the isolate 1 was identified as *Bacillus sp.*, the isolate 2 as *E. coli sp.*, the isolate 3 as *Staphylococcus sp.* The molecular characterization revealed that the *Bacillus sp.* was identified as *Bacillus subtilis*, this was further submitted to NCBI Genbank to get Accession number. The Accession number was MH071337.1. Health education of the vendors and strict implementation of hygienic standards may help to reduce the contamination rates. The results suggested negligence such as poor sanitation during the preparation/or storage of ice cream. These included the observed dirty premises, used utensils and the use of bare hands in preparing the products.

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1. INTRODUCTION

Ice cream is the major dairy product that dominates interest of large segments of the population. As a result, its production and consumption are rapidly increasing. The microbiological quality of ice cream during retail marketing mainly depends on the contamination during the product handling as well as efficiency and sanitary conditions during frozen storage [1].

Ice cream is a palatable, nutritive and healthy food. It is undoubtedly one of the most popular and favourite food products in many countries among children and adults especially during summer season. Several brands of ice cream with a variety of flavours are being marketed in country like India. Carbohydrate solutions give the sensation of sweetness and control the frozen and melting characteristics of ice cream. Ice cream mix properties depend primarily on lipid and protein quantities. The small quantities of emulsifier and hydrocolloid act as stabilizers of oil-in-water emulsions. For flavouring, natural flavours may be used such as real fruit, cocoa, coffee, ginger and vanilla. All these allow great variations in ice cream flavour, taste and way of structure formation [2].

Ice cream is a congealed dairy product made by milk fat (about 10-16%), sugar (9-12%), non-fat milk solids (about 9-12%), 0.20- 0.50% stabilizer and/or emulsifier, flavoring agents, coloring materials and thickeners Aaku EN, et al. [3]. It is a very good medium for microbial growth as it has high nutritive value with a long storage and neutral pH. As food, ice cream is a very nourishing product, with a great energy value due to its rich content in carbohydrates, lipids and proteins. It also contains vitamins (especially vitamin A and B) and mineral salts especially calcium and phosphorus salts. Ice cream is undoubtedly one of most popular and favorite food product among children and adults especially during summer season. Several brands of ice cream in variety of flavors have been marketed. Ice cream quality depends on the extrinsic factors like manufacturing procedure and also on intrinsic factors such as the ingredients used and their proportions Hankin and Hanna, [4].

Any of these may account for the various specific species of bacteria [5]. Ice cream is a nutritious food for human and also an excellent medium for the growth of many microorganisms some of which may cause diseases in human beings e.g. Cholera, typhoid, bacillary dysentery. Contaminated ice cream causes several outbreaks of gastrointestinal diseases in a

number of countries in Asia, Europe and North America [6,7,8].

Quality of ice cream depends on extrinsic factors that include manufacture procedure, as well as intrinsic factors that include the proportion of ingredients used. Primary sources of microbial contamination of ice cream include water and raw milk, whereas secondary sources include flavoring agents, utensils and handling. Possible sources of these microorganisms in ice cream have been reported to include raw materials used for the composition of ice cream-mix, such as milk and milk powder, cream, flavouring and colouring substances and sanitizer [9,10] and from contaminate air during processing [11].

Ice-cream may become contaminated during production, transportation and/or storage with a number of microorganisms [12,13]. The contamination might cause during pasteurization period, during the addition of other ingredients or from crack plants, faulty packaging process and storage [14]. Primary contamination sources include water, raw milk and secondary contamination sources include flavoring agents, utensils and handling of the ice creams [15]. Post-pasteurization microbial contamination could be identified by the presence of coliform [16,17,18]. Bacteriological quality of ice cream reflects hygienic practice in production and is an indication of food safety. Hence, the present study was taken up to determine the bacteriological quality of ice cream marketed in Trichirappalli town, Tamilnadu and to assess the potential of this frozen product to pose risk to public health issues.

2. MATERIALS AND METHODS

2.1 Study Area

Trichirappalli is a city in the Indian state of Tamil Nadu and the administrative headquarters of Trichirappalli District. It is the fourth largest municipal corporation in Tamil Nadu and also the fourth largest urban agglomeration in the state. It is located almost at the geographic centre of the state. Trichirappalli is geographically located at 10.8050°N 78.6856°E.

2.2 Sample Collection

A total of 10 samples of industrially produced ice-cream were purchased from Ice cream shops and retail outlets within Trichirappalli city. The samples were

collected in a sterile container and brought to the laboratory immediately for proper analysis.

2.3 Preparation of Serial Dilution

1 ml of each ice-cream sample was pipetted out aseptically and transferred into a sterile test-tube containing 9 ml of peptone water to give a dilution of 1:10, from this dilution, a serial dilution of up to 10^{-3} was carried.

2.4 Bacteriological Examination of Samples Collected

2.4.1 Primary isolation of bacteria from samples

Using the pour plate technique, the prepared samples of concentration range 10^{-1} and 10^{-2} were used for inoculation. 1ml of each of this dilution was inoculated on nutrient agar in duplicate. It was rocked and allowed to solidify, inverted to prevent condensation of moisture on the surface of the agar and were incubated at 37°C for 24 hours. At the end of incubation, the number of colonies was counted with the aid of a colony counter. Average of duplicate plates were counted and recorded as the numbers of colony forming unit (cfu/ ml) of each ice-cream sample. The bacteria plate counts per ml were recorded.

2.5 Purification and Storage of Isolates

Bacterial colonies that appeared on the primary isolation plates were subcultured onto fresh nutrient agar plates to obtain pure cultures of the different isolates. The final cultures containing discrete colonies were transferred onto slants made with test tubes containing nutrient agar. The slants were stored in the refrigerator at 37°C for further studies outlined below.

2.6 Characterization and Identification of Isolates

The bacteria isolates from different samples were grouped on the basis of colonial morphology. The criteria used, where the size of the colony, color, surface, edge, slope and elevation as described by Bergy's manual of systematic bacteriology.

2.7 Microscopic Examination of the Isolates

Smears of each of the different bacterial isolates was made with a clean- grease free slide and air dried. These were directly observed under the microscope using the oil immersion objective (x100). The

bacterial isolates were stained using Gram's Method of staining to study the morphological appearance of the cell as well as their Gram reactions (Whether they are Gram positive or Gram negative) and cell arrangement. Gram positive cells retained the purple color of the initial dye while the Gram negative cells retained the pinkish- red color of the counter stains.

2.8 Biochemical Tests

Biochemical tests were performed to confirm the identity of the isolated bacteria 24 hours old cultures were used to perform the biochemical test.

2.9 Microphotograph of Bacteria

The isolated bacterial species were identified based on the biochemical characterization. The isolate 1 was identified as *Bacillus* sp, the isolate 2 as *E. coli*, the isolate 3 as *Staphylococcus* sp. These bacteria were micro photographed using photo microscope.

2.10 Molecular Characterization

The above studied efficient isolate 1 was identified as *Bacillus* species. This was further characterized by 16S rRNA molecular characterization technique.

2.11 Preparation of Genomic DNA and PCR Amplification of the 16s rRNA Gene

The genomic DNA was isolated from the isolate according to the procedure described by Marmur et al. [19] and the small subunit of rRNA gene was amplified using the two primers 16S1 (5'-GAGTTTGATCCTGGCTCA-3') & 16S2 (5'-CGGCTACCTTGTTACGACTT-3'), which are complementary to the conserved regions at the 5' - and 3' ends of the 16S rRNA gene corresponding to positions 9-27 and 1477-1498 of the *Escherichia coli* 16S rRNA gene [20].

2.12 16s rRNA Gene Sequencing

The purified PCR product, approximately 1.5Kb in length, was sequenced using the primers 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 (5'-CGGCTACCTTGTTACGACTT-3'), the same which were already used for PCR amplification. Sequencing of the purified PCR product was carried out using 5 pmol of a given sequencing primer and 8 µl ready-reaction mix from either the Big Dye Terminator sequencing kit (Perkin Elmer). The obtained sequence was involved for Blast analysis for the closest match and the same were submitted in NCBI GenBank for getting accession number.

3. RESULTS AND DISCUSSION

The results obtained in this study represent the current status of microbiological quality of ice cream being sold in Trichirappalli town. All the analyzed ice cream samples (n = 5), three samples showed contamination of notable bacterial species (*Bacillus*, *E. coli* and *Staphylococcus*) which indicates fecal contamination. According to Indian Food Safety Standards and Regulations [21], Total Mesophylic

Aerobic Bacterial Count (Table 1) of ice cream should not exceed 2,50,000 cfu/g. In the present study, it was observed that the Total Mesophylic Aerobic Bacterial Count for all the branded ice creams was 3×10^4 to 7×10^4 cfu/g.

Morphological and cultural characteristics of the organisms isolated from ice cream samples were tabulated in Table 2. Table 3 shows the biochemical characteristics of the isolated organisms.

Table 1. Total number of mesophylic aerobic bacterial count from ice-cream samples

S. No.	Name of the ice cream	Totalplate count/gm
1	Aavin	3.0×10^4 cfu /g
2.	Amul	6.3×10^4 cfu /g
3.	Arun	4.5×10^4 cfu /g
4.	Kwality wall's	6.7×10^4 cfu /g
5.	Cornetto	7.0×10^4 cfu /g

Table 2. Morphological and cultural characteristics of the organisms isolated from ice cream samples

S. No	Name of the organisms	Gram's staining	Motility	Nutrient agar
1.	<i>E. coli</i>	Negative	Motile	Large transparent
2.	<i>Staphylococcus</i>	Positive	Nonmotile	Golden yellow
3.	<i>Bacillus</i>	Positive	Motile	White opaque



Fig. 1. *Bacillus* sp.



Fig. 2. *E. coli*

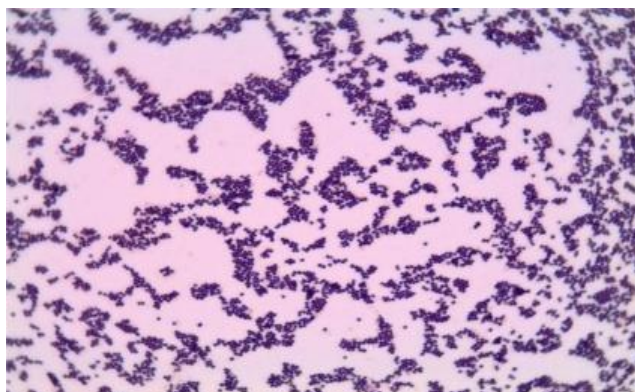


Fig. 3. *Staphylococcus* sp

The molecular characterization revealed that the *Bacillus* sp, was identified as *Bacillus subtilis*, this was further submitted to NCBI Genbank to get Accession number. The Accession number was MH071337.1.

***Bacillus subtilis* strain MK 16S ribosomal RNA gene, partial sequence**

GenBank: MH071337.1

[FASTA Graphics](#)

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 ACCESSION MH071337 VERSION MH071337.1
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 ORGANISM [Bacillus subtilis](#) Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; *Bacillus*.
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 AUTHORS Susan Suganya, G. and Kiruthika Devi, B.
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Table 3. Biochemical characteristics of the isolated organisms

S. No	Biochemical test	<i>E. coli</i>	<i>Staphylococcus sp</i>	<i>Bacillus sp</i>
1	Indole production	Positive	Negative	Negative
2	Methyl red	Positive	Negative	Negative
3	Vogues proskeur	Negative	Negative	Positive
4	Citrate utilization t	Negative	Positive	Positive
5	Glucose	Acid & gas	Acid	Acid
6	Lactose	Acid & gas	Acid	Acid
7	Mannitol	-	Acid	Acid
8	Sucrose	Acid	Acid	Acid
9	Urease	Negative	Negative	Positive
10	Catalase	Positive	Positive	Positive
11	Oxidase	Negative	Negative	Positive
12	H ₂ S	Negative	Positive	Positive
13	Gelatin	Negative	Positive	Positive

Based on the biochemical characterization, the isolate 1 was identified as *Bacillus sp*, the isolate 2 as *E. coli*, the isolate 3 as *Staphylococcus sp*

The results obtained in this study represent the current status of microbiological quality of ice cream being sold in Trichirappalli town. In the present study, it was observed that the Total Mesophilic Aerobic Bacterial Count for all the branded ice creams was 3.0×10^4 to 7.0×10^4 cfu /g. The bacteria counts in all the products fell within acceptable limits. Ice creams, a dairy product with high nutritional value and the presence of easily useable fats, carbohydrate and protein is an ideal environment for spoilage by microorganisms. A study was conducted regarding bacteriological quality of local made open scoop ice creams sold by street hawkers indifferent areas of Jalandhar city, Punjab which showed heavy contamination of bacteria ranging from 0.1×10^9 to 10.2×10^9 cfu/g [22]. The microbial counts in ice cream samples may have resulted from inadequate processing, such as initial improper cooling of the hot ice cream mix, which may lead to multiplication of microorganisms present in ice cream immediately after pasteurization [23]. It is also indicated that high microbial count occurrence in virtually sterile mix may occur when cooled slowly at a temperature conducive to spore germination [24].

All the 5 samples of ice-cream examined showed positive for one type of bacteria or the other. Three different types of bacteria were isolated from the samples of ice-cream examined. The presence of heavy contamination of notable bacteria (*Bacillus*, *E. coli* and *Staphylococcus*) which indicates faecal contamination.

The presence of coli form organisms is taken as an indication that other pathogenic organisms may also present in the sample [25]. During the present study, among the ten samples eight of the samples showed presence of coli form bacteria. The maximum coli form count was 7.0×10^4 cfu /g in the ice cream sample and minimum count was 3.0×10^4 cfu /g in the branded samples. Coli forms being non-spore formers should be susceptible to pasteurization. Their post pasteurization presence in ice creams indicates faulty heat process during preparation. Also, the other reasons for contamination may either come from water, lack of personal hygiene of the ice cream manufacturer, utensils used for ice cream. It was observed that coli form bacteria were present in all samples. Similar, findings were also reported by

Anuranjini et al. [26] also reported the incidence of high coli form count in ice creams marketed in Mangalore town.

Further, the isolation and confirmation of bacteria was done by Gram's staining and biochemical tests based on the Bergey's manual of bacterial identification. It was observed that about 30% samples showed presence of *E. coli* with maximum *E. coli* count (6.7×10^4 cfu /g) in ice cream sample. A study conducted by Ahmed and Shakoori [27] reported 640-683 *E. coli* colonies per 100 ml of drinking water. Such water can be the major source of contamination when used for preparation of ice creams. Once the ice cream become contaminated, freezing temperature later could not make the product safer [28].

About 20% ice cream samples showed presence of *Staphylococcus* species most of them were purchased from ice cream shops in the city. The maximum occurrence of these organisms was in ice cream samples is 7.0×10^4 cfu /g. It is assumed that the possible sources of this organism in ice cream hands, skin and clothing of handlers where it is commonly found; [29]. There are other sources like coughing, talking and sneezing which produce droplets. These droplets could settle on ice cream during transportation, storage and retailing [23].

Moreover, *Bacillus* species (4.5×10^4 cfu /g) also isolated from some ice cream samples. It was observed that about 40% samples showed presence of *Bacillus* species. Consumption of ice cream contaminated with entero pathogenic bacteria such as *Bacillus* has been the cause of several disease outbreaks [30].

Contaminated ice creams could be responsible for food poisoning in masses. Therefore, the presence of possible pathogenic organisms in the analyzed ice cream samples should be viewed with concern by the consumers. Not only bacteria but some viruses and fungal species are also responsible for spreading diseases. It is needed to continuously monitor the food products. Sanitary education through training and workshops to producers, distributors and retailers during processing and sale of the product can be the possible remedy to avoid such problems.

The presence of *Staphylococcus* sp. in industrially produced ice-cream could be from where it is commonly found, hands, skin and clothing of handlers. *Staphylococcus* sp. has been known to cause Staphylococcal food poisoning due to ingestion of improperly stored food in which *Staphylococcus* has grown. Coughing, talking, sneezing produces droplets which could settle on icecream during transportation,

storage and retailing. The presence of possible pathogenic organisms in the analyzed ice-cream should be viewed with concern by the consumers, producing company and the Government since food poisoning by *Bacillus* sp and *E. coli* sp is possible through consumption of contaminated icecream. Bryan [31] reported that proper handling of ice-cream is very important both during manufacturing and storage to prevent outbreak of food borne diseases. Source of contamination could be as a result of contamination from various sources notable handlers during preparation especially under unhygienic condition, also from retailers, most of them do not store ice-cream at the appropriate storage temperature. Even the refrigerated samples are not always maintained between 40°C and 60°C due to unstable power outage. Prescott et al. [32] reported that refrigeration at 50°C retards bacterial growth. High temperature therefore favours and encourages bacterial growth which eventually causes spoilage.

The results suggested negligence such as poor sanitation during the preparation/or storage of ice cream. These included the observed dirty premises, used utensils and the use of bare hands in preparing the products. Even the raw milk could be a possible source of contamination. Analysis of raw milk samples in different checkpoints of milk chain system showed that the samples were heavily contaminated by both coli forms and general bacterial load. In fact fecal infection is one of most important difficulties in production of ice cream and these bacteria's are considered as one of important agents of diarrhoea in children.

Although the hygienic qualities of ice-creams were improved in the past years, the relatively high unsatisfactory rates of soft ice-cream were observed. One main reason is that the preparation of ice-cream involves handling at both factory and retail level. At retail shops, improper storage temperature and prolonged storage time affect the hygienic quality of ice-cream mix. The problem may be aggravated by contamination from vending machines which had not been properly cleansed and disinfected [33].

The results suggested negligence such as poor sanitation during the preparation/or storage of ice cream. These included the observed dirty premises, used utensils and the use of bare hands in preparing the products.

4. RECOMMENDATIONS -ADVICE TO TRADE

Key steps in production must be observed by the trade. These include - (a) Obtain ingredients, such as

milk, cream and ice-cream mix, from licensed and reputable sources; (b) Store ingredients at proper temperature (frozen items: -18°C or below; chilled items: $0-4^{\circ}\text{C}$); (c) Maintain the ice-cream mix ($< 7^{\circ}\text{C}$) and ice-cream products ($<-18^{\circ}\text{C}$) at adequate temperatures from manufacturing factory to retail outlets; (d) Discard the defrosted products and do not re-freeze any melted ice-cream for sale; (e) Drain off and discard the leftover of ice-cream daily; (f) Maintain all equipment and utensils in clean and good condition; and (g) Observe hygienic practices during all preparation and handling processes.

4.1 Advice to Public

Consumers are advised to pay attention on the following –

(a) Buy ice-cream from reputable shops; (b) Do not buy and consume any hard ice-cream which is out of expiry date or not in a good quality; (c) Store ice-cream products in freezer after purchasing; (d) Observe the hygienic conditions of the retail shops including common area, equipment, utensils and food handling; (e) Consume ice-cream immediately; and (f) Do not eat too much ice-cream and take a balanced diet.

5. CONCLUSION

As per the Bureau of Indian standards (BIS) regulations, the total bacterial counts in the ice creams should not exceed $25 \times 10^4/\text{g}$ and coli form count should not be more than 100/g. The ice creams screened in the present study were within the BIS limits for coli form and total aerobic bacterial counts, thereby indicating no high levels of contamination and higher risk of infection. High level coli form count, presence of *E. coli* and *Bacillus* indicated faecal contamination of ice creams thereby suggesting possible risk of infection involved in the consumption of such food. Presence of *Staphylococcus* an enterotoxin producer may cause serious health problems.

Among the isolates from samples tested, only *E. coli* and *Staphylococcus* were significant potential pathogens. However, presence of other organisms (*Bacillus* sp) could be attributed to unhygienic conditions during preparation, handling and serving of ice creams. Ice creams may also get contaminated if the ice cream preparation machines are exposed to dust and flies. Health education of the vendors and strict implementation of hygienic standards may help to reduce the contamination rates. The results suggested negligence such as poor sanitation during the preparation/or storage of ice cream. These

included the observed dirty premises, used utensils and the use of bare hands in preparing the products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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