The aim of the present research study was to detect the presence of food borne pathogens from selected dairy and non dairy foods, available in the market as well as processed in the laboratory. The study also included an assessment of the viability of probiotic bacteria especially *L. acidophilus* in yoghurt preparations during refrigerated storage. Further the study was also extended for production of bacteriocin by *L. lactis* and screening of nisin (a bacteriocin) producing gene in probiotic bacteria.

The present investigation was conducted at the Food Biotechnology Laboratory of the Post Graduate Department of Home Science, Sardar Patel University, Vallabhbh Vidyanagar during 2005-09.

The study was divided in to several parts:

1. Comparison of different Phenol-chloroform based methods for DNA isolation from *E. coli, S. aureus* and *S. typhi*
2. Molecular and bacteriological examination of milk from different milch animals with special reference to coliforms
3. Molecular and bacteriological detection of *E. coli, S. aureus* and *S. typhi* from milk samples
4. A comparison of methods for the detection of *E. coli* O157:H7 from artificially contaminated dairy products by PCR
5. Assessment of viability of probiotic bacteria and competitive growth of *L. acidophilus* in yoghurt during refrigerated storage using microbiological and molecular methods
6. Effect of pure nisin – a bacteriocin on the growth and survival of selected food pathogens
7. Bacteriocin nisin production by free and immobilized Lactococcus lactis
8. Screening for NisA promoter gene and RAPD profiling of potentially probiotic lactic acid bacteria

9. Molecular detection of food pathogens from retailed fast food samples by PCR

10. Molecular Detection of *E. coli*, *S. aureus* and *S. typhi* from local fast food and juice samples

11. Effect of enrichment media for PCR detection of *Listeria monocytogenes* and *S. aureus* from vegetable gravy
1. Comparison of different phenol-chloroform based methods for DNA isolation from *E. coli*, *S. aureus* and *S. typhi*

This part of the study deals with the methodology of the four different phenol chloroform based methods used for DNA extraction from the three bacterial strains namely *E. coli*, *S. typhi* and *S. aureus*.

**Bacterial culture**

*E. Coli* was obtained from the Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar. *Salmonella typhi* MTCC 531 and *Staphylococcus aureus* MTCC 740 were obtained from Microbial Type Culture Collection, Chandigarh. All bacterial strains were activated in Luria broth and then preserved in 50 % glycerol at -20°C for further use. These bacterial strains were used for DNA extraction.

**Method 1**

This method proposed by Wallace (1987) was followed for DNA extraction from *E. coli*, *S. typhi* and *S. aureus* in pure culture. *E. coli*, *S. typhi* and *S. aureus* were inoculated into 100 ml sterile Luria broth and incubated overnight (12 hours) at 37°C and 160 rpm. This incubated broth was used for DNA isolation for each individual organism.

2 ml of overnight culture was pelleted by centrifuging at 10,000 rpm for 15 min. The supernatant was discarded and to the pellets, 200 µl of proteinase K reaction buffer (10 mM Tris pH 7.8, 5 mM EDTA, 0.5 % SDS) was added. It was then incubated at 37°C for 1 hour. 200 µl of RNase A (50mg/ml) was added and incubated for 20 min at 37°C. After that, 20 µl of Proteinase K (100 mg /ml) was added and incubated for 1 hour at 37°C. Mixture was subsequently extracted one
after the other with equal volume of Phenol:chloroform:isoamyl alcohol (25:24:1) followed by Chloroform:isoamyl alcohol (24:1) and lastly by water saturated diethyl ether. Upper phase was removed and transferred to a new tube. From this, DNA was precipitated with 2 volumes of ethanol and thereafter washing with equal volume of 0.2 M NaCl (pH 8.0). The DNA pellet was dissolved in 100 µl sterile water and quantified by UV spectrophotometer at 260 and 280 nm. The flow chart is given below:
**Flow Chart for Method 1**

2 ml bacterial culture  
Centrifuged at 10,000 rpm for 15 min  
To the pellet, 200 μl proteinase K added  
Incubated at 37°C for 1 hr  
200 μl RNase A added and incubated for 20 min at 37°C  
Mixture extracted with equal volume of Phenol:Chloroform:isoamyl alcohol  
(25:24:1)  
Followed by equal volume of chloroform:isoamyl alcohol (24:1)  
Then, extracted with water saturated diethyl ether  
Upper phase was transferred to a new tube and DNA was precipitated using 2 volumes of ethanol and then washing with 0.2 M NaCl (pH 8.0)  
DNA pellet was dissolved in 100 μl sterile water  
Read at 260 and 280 nm and ratio calculated
Method 2

In the second method genomic DNA was isolated using a phenol-chloroform based DNA isolation method described by Saito and Miura (1963) in which 5 ml of overnight culture was used.

This method was performed according to Saito, H. & Miura, K. (1963). Briefly, 5 ml of overnight culture was harvested by centrifugation. The pellet was resuspended in 100 µl lysozyme solution [10 mg lysozyme in 100 ml buffer containing 0.15 M Nacl and 0.1 M EDTA (pH 8.3)]. 0.5 ml each of 1 % SDS, 0.1 M Nacl, 0.1 M tris HCL (pH 8.0) were added and incubated at 60°C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the supernatant was then further extracted with an equal volume of 5 M sodium perchlorate and centrifuged. After this, 0.1 ml of 3 M sodium acetate was added to the supernatant (aqueous phase) along with double the volume of absolute ethanol (double volume of the aqueous phase). DNA was pelleted by centrifugation, washed with 2 ml of 70 % ethanol and dried under vaccum. The DNA pellet was resuspended in 100 µl TE buffer [10 mM Tris HCL (pH 7.5), 0.1 mM EDTA]. DNA concentration was obtained by reading at 260 nm in a spectrophotometer.
Flow chart for Method 2

5 ml overnight culture centrifuged at 10,000 rpm for 15 min

Pellets suspended in 100 µl lysozyme solution

0.5 ml of 1 % SDS, 0.1 M NaCl, 0.1 M tris HCL (pH 8.0) added and incubated at 60°C

Extracted with equal volume of Phenol:chloroform:isoamyl alcohol (25:24:1)

Supernatant further extracted with equal volume of 5 M sodium perchlorate

Centrifuged at 8000 rpm for 20 min

0.1 ml of 3 M sodium acetate was added to the aqueous phase along with double volume of absolute ethanol

DNA was pelleted by centrifugation, washed with 2 ml of 70 % ethanol

Pellet dried under vaccume

DNA pellet was resuspended in 100 µl TE buffer (10 mM Tris HCl pH 7.5, 0.1 mM EDTA)

Read at 260 nm
Method 3

This method of DNA isolation was performed according to Hein et al. (2005).

For this, 1 ml of overnight culture was taken and centrifuged at 8,000 rpm for 12 min. To the pellet, 130 µl of digestion buffer [100 mM Tris HCL, 100 mM EDTA, 0.5 % SDS (pH 8.0)] and 200 µl of proteinase K was added and incubated for 3 hour at 20˚C. This was centrifuged at 5700 rpm for 15 min. Supernatant was discarded and the pellets were resuspended in 200 µl of TE buffer [10 mM Tris HCL, 1 mM EDTA (pH 7.5)], centrifuged at 5700 rpm for 5 min. and supernatant was discarded. The pellets were resuspended again in 200 µl of TE buffer and centrifuged at 5700 rpm for 5 min. Supernatant was removed, washed thrice with Phenol: Chloroform: Isoamyl alcohol (25:24:1) and resuspended in 200 µl of PCR buffer [50 mM KCL, 1.5 mM MgCl₂, 10 mM Tris HCL (pH 8.4)] and centrifuged at 5700 rpm for 5 min. To the pellet, 50 µl of PCR buffer and 5 µl of lysozyme were added. It was incubated at room temperature for 15 min. 2 µl of Proteinase k was added and then incubated at 60˚C in a water bath for 1 hour. It was then centrifuged at 10,000 rpm for 15 min and supernatant was collected. It was then quantified by UV spectrophotometer at 260 and 280 nm. Flow chart is as follows:
**Materials and Methods**

**Flow chart for method 3**

1 ml of overnight culture was taken

- centrifuged at 8,000 rpm for 12 min

To the pellet, 130 µl of digestion buffer and 200 µl of proteinase K added

- incubated for 3 hour at 20°C
- centrifuged at 5700 rpm for 15 min
- resuspended in 200 µl TE buffer (pH 7.5)

Pellet resuspended in 200 µl TE buffer

- centrifuged at 5700 rpm for 5 min

Supernatant was removed, pellet washed thrice with Phenol: Chloroform: Isoamyl alcohol (25:24:1)

- resuspended in 200 µl of
- centrifuged at 5700 rpm for 5 min

PCR buffer

To the pellet, 50 µl of PCR buffer and 5 µl of lysozyme were added

- incubated at R.T. for 15 min
- 2 µl of Proteinase k was added
- incubated at 60°C for 1 hr

- centrifuged at 10,000 rpm for 15 min and supernatant was collected

- Supernatant was quantified by UV spectrophotometer at 260 and 280 nm
Method 4

This method of DNA isolation was a modified method proposed by Sambrook et al., (1987).

For this, 2 ml of overnight grown culture was taken, centrifuged at 8000 rpm for 12 min. To the pellet, 260 µl of digestion buffer [100 mM Tris HCL, 100 mM EDTA, 0.5 % SDS (pH 8.0)] and 400 µl of proteinase K were added, incubated for 1 hour in water bath at 60˚C. After that, 20 µl of lysozyme was added, incubated for 5 min; 30 µl of RNase was added, incubated for 5 min and centrifuged at 7500 rpm for 25 min. To the supernatant, 450 µl of phenol, 500 µl of chloroform:isoamyl alcohol (24:1) and 50 µl Tris buffer [10 mM Tris HCL, 1 mM EDTA (pH 7.5)] was added, shaken well, centrifuged at 8000 rpm for 15 min, aqueous phase was taken out and again treated with 500 µl chloroform:isoamyl alcohol and centrifuged at 8000 rpm for 15 min. Aqueous phase was again collected and double the volume of ethanol or isopropyl alcohol (double the volume of the aqueous phase) was added, centrifuged at 7800 rpm for 10 min. The resulting pellet was dried and dissolved in TE buffer and DNA concentration was quantified using a UV spectrophotometer at 260 and 280 nm. Flow chart is given as follows:
2 ml of overnight grown culture was taken

- centrifuged at 8000 rpm for 12 min

To the pellet, 260 µl of digestion buffer and 400 µl of proteinase K added

- incubated for 1 hour in water bath at 60°C

20 µl of lysozyme was added, incubated for 5 min; 30 µl of RNase was added, incubated for 5 min

- centrifuged at 7500 rpm for 25 min

To the supernatant, 450 µl of phenol, 500 µl of chloroform:isoamyl alcohol (24:1) and 50 µl Tris buffer added

- centrifuged at 8000 rpm for 15 min

Aqueous phase treated with 500 µl of chloroform: isoamyl alcohol

- centrifuged at 8000 rpm for 15 min

Aqueous phase collected, double the volume of ethanol or isopropyl alcohol was added

- centrifuged at 7800 rpm for 10 min

Resulting pellet dried, dissolved in TE buffer

DNA concentration was quantified using a UV spectrophotometer at 260 and 280 nm and ratio calculated
2. Molecular and microbiological examination of milk from different milch animals with special reference to coliforms

The present study was planned to assess the quality of milk from different milch animals by detecting the presence of coliforms. Both bacteriological as well as molecular methods (like PCR) were compared for their efficacy (All chemicals and media used in this study were purchased from Titan Biotech, India).

Sample Collection
A total of twenty samples of raw milk from cow (5 samples), buffalo (5 samples) and goat (5 samples) were collected from local vendors and farmers of Vallabh Vidyanagar and Anand including AMUL brand pasteurized milk (5 samples) purchased from retail outlets in the month of April 2006. Raw milk samples were collected from individual cows, buffaloes or goats into sterile screw capped tubes and were directly transported to the laboratory and processed within 3 hours of collection. Cow milk samples were labeled C1 to C5, buffalo milk samples B1 to B5, goat milk samples G1 to G5 while pasteurized milk samples were labeled P1 to P5.

Parameters studied:
The study was carried out in two stages. Milk samples from different milch animals and the market samples were analyzed in the first stage by agar plate and sugar fermentation methods. In the second stage, confirmation of bacteria was carried out by Polymerase Chain reaction. The parameters studied were:

i. Total Plate count
ii. Violet Red Bile Agar (for coliforms)
iii. BGLB broth
iv. Yeast and mold count of milk samples
v. Gas production from lactose in LST (Lauryl tryptose) broth (Confirmation on EMB plates followed by Gram staining)
vi. Colonies from EMB plate - Brilliant Green Bile Broth (gas production)

vii. IMViC tests (Indole, Vogues Proskauer, Methyl Red, Citrate)

viii. DNA isolation and PCR assay

All the microbiological tests were performed according to Bacteriological Analytical Manual (1998).

**Bacterial culture**

*Escherichia coli* ATCC 11775 was obtained from American Type Culture Collection and was used in this study as reference strain (positive control). It was activated in Luria broth at 150 rpm for 24 hours at 37°C in environmental shaker. After that, activated culture was preserved at -20°C for further use. Whenever needed, *E. coli* culture was activated in Luria broth.

**Total Plate Count**

Nutrient agar (pH 7.4 ± 0.2) was used for total count. $10^{-1}$ and $10^{-2}$ dilutions of the all the milk samples were prepared. Then 0.1 ml of aliquote was taken from each dilution and spread on the solidified nutrient agar plates. These plates were incubated at $37^\circ$ C for 24 hours.

**Violet Red Bile Agar count**

Violate Red Bile Agar (VRBA) (pH 7.4 ± 0.2) was used for coliform count. $10^{-1}$ and $10^{-2}$ dilutions of the all the milk samples were prepared. Then 0.1 ml of aliquote was taken from each dilution and spread on the solidified Violate Red Bile agar plates. Plates were incubated at $37^\circ$ C for 48 hours.

**Brilliant Green Bile Broth**

A loopful of positive growth on EMB plates were inoculated into Brilliant green Bile Broth (BGLB) and incubated at $37^\circ$C for 48 h. Gas production in BGLB was considered as positive reaction for the detection of coliforms.
Yeast and Mold Count of milk samples
Potato Dextrose Agar (PDA), pH 5.6 ± 0.2 was used for Yeast and Mold count. 10^{-1} and 10^{-2} dilutions of the all the milk samples were prepared. Then 0.1 ml of aliquote was taken from each dilution and spread on the solidified PDA plates. These plates were incubated at 25°C for 4 days.

Gas production from lactose
This test was performed to determine the presence of coliforms in the sample. The milk samples (0.1 ml) were inoculated in tubes containing LST (Lauryl tryptose broth) and incubated for 48 ± 2 h at 35°C. Gas production (displacement of medium from inner vial) or effervescence after gentle agitation was considered positive reaction. Positive tubes showing gas production were further confirmed for coliform presence. For this, contents were transferred from test tubes of LST broth and plated on Eosine methylene blue (EMB) agar plates. Gram staining of the colonies growing on EMB plates was carried out. All cultures appearing as Gram-negative, short rods were tested for the IMViC reactions and also re-inoculated back into LST to confirm gas production. The flow chart used is as follows:
Flow Chart for determination of Gas production from Lactose

Milk samples (0.1 ml)

Inoculated in tube of LST

Incubated at 35°C for 48 hrs

Positive reaction indicates effervescences on gentle agitation

Further confirmation

Collected milk samples were also transferred on EMB plates for further confirmation

Gram staining (of positive samples)

Gram – ve colonies taken for ImViC tests (Indole, Voges Proskauer, Methyl Red, Citrate)
IMViC tests:

The chemicals used for IMViC tests were purchased from Merck, Mumbai.

(i) Test for Indole production:

The diluted milk samples were inoculated in a tube of tryptone broth and incubated at 24 ± 2 hr at 35°C. Then, 0.2-0.3 ml of Kovac's reagent (Merck, Mumbai) was added into the tubes. Appearance of distinct red color in upper layer is considered a positive test.

(ii) Vogues Proskauer (VP)-reactive compounds:

The diluted milk samples were inoculated into a tube of MR-VP (Methyl Red Vogues- Proskauer) broth and incubated for 48 ± 2 h at 35°C. After transferring 1 ml content into another tube, 0.6 ml α-naphthol solution and 0.2 ml 40% KOH were added. Few crystals of creatine monohydrate were added. It was mixed well and allowed to stand for 2 hr. Development of eosin pink color was considered to be positive.

(iii) Methyl red-reactive compounds:

After VP test, MR-VP tubes were incubated for additional 48 ± 2 h at 35°C. After that, 5 drops of methyl red solution was added to each tube. Distinct red color was considered to be positive. Yellow colour was considered to be a negative reaction.

(iv) Citrate production:

To, prevent over production, milk samples were diluted with equal amount (1:1) of sterile distilled water. Diluted milk samples were inoculated into a tube of Koser's citrate broth and incubated for 96 h at 35°C. Development of distinct turbidity was considered to be positive reaction.
DNA isolation and PCR Assay

Milk samples were diluted and plated on Nutrient Agar plates. They were allowed to incubate at 37°C for 24 hours. Colonies which grew on nutrient agar plate were further inoculated into Luria broth for enrichment and incubated at 37°C with 160 rpm for 24 hours in orbital mechanical environment shaker. Cultures were confirmed as *E. coli* by growth on eosin methylene blue (EMB) agar and testing for the formation of indole. DNA isolation from these colonies was carried out according to Sambrook et al., (1987). All the chemicals used for PCR assay were purchased from Bangalore Genei, Bangalore.

Briefly, 1 ml of an overnight culture grown in LB broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1 mg lysozyme /ml in 0·15 M NaCl, 0·1 M EDTA, pH 8·0), followed by lysis using 1 % SDS, 0·1 M NaCl, 0·1 M Tris-HCl (pH 8·0) at 60°C for 20 minutes in a water bath. DNA was purified by extraction with an equal volume of phenol/ chloroform/isoamyl alcohol (25 : 24 : 1) in the presence of 5 M sodium perchlorate. A 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol were added and the nucleic acid was then pelleted by centrifugation, washed with double volume of 70 % ethanol. The DNA pellet was resuspended in 100 µl TE buffer (10 mM Tris/HCl, pH 7·5, 0·1 mM EDTA) and subjected to PCR amplification.

Primers used in this study were ECPAL-L 5’-GGCAATTGCGGCATGTTCTTCC-3’ and ECPAL-R 5’-CCGCGTGACCTTCTACGTTGAC-3’. The primer pair ECPAL-L and ECPAL-R is specific for all *Escherichia coli* and will amplify a control fragment of 280 bp from the exeC gene (PAL protein) (Kuhnert et al., 1995). DNA isolated from *Escherichia coli* ATCC 11775 was used as positive control.

2 µl of DNA template was added to 1.25 µl 10X buffer, 2 µl 25mM MgCl₂, 1 µl DNTPs mix, 1µl each of forward and reverse primer and 0.5 µl Taq DNA
Materials and Methods

Polymerase. Total volume made up to 50 µl by adding nuclease free sterile distilled water. PCR amplification was carried out using a PCR thermal cycler (Corbett Research, Australia). The temperature profile used was as follows: initial denaturation for 3 min at 94°C, followed by 35 cycles for 30 sec at 94°C (denaturation), 30 sec at 60°C (annealing) and 5 min at 72°C (extension). 10 µl of the cycled PCR reaction was analyzed on a 1.5% agarose gel containing 500 µg/ml ethidium bromide at 110 V for 2 h in TAE-buffer (100 ml: 5.4 g Tris, 0.4 g EDTA, 2.75 g Boric acid, pH 6.8) as gel running buffer. The photograph of gel was captured using α–Inotech Gel Documentation Unit.
3. Microbiological and molecular detection of *E. coli*, *S. typhi* and *S. aureus* from milk samples

This part of the study deals with the detection of food pathogens from the unbranded milk samples available in the local market. The milk samples were analyzed for the presence of *E. coli* (a gram negative bacterium), *S. typhi* (a gram negative pathogen) and *S. aureus* (a gram positive bacterium) from 10 different samples using PCR.

**Collection of milk samples**

A total of 10 milk samples were collected from local milk selling vendors of Anand and Vallabhb Vidyanagar. Of these, 5 were cow milk samples and the remaining 5 were buffalo milk samples. Raw milk was collected from individual cow & buffalo in sterile screw capped tubes and were directly transported to the laboratory. Samples were labeled from 1 to 10.

**Microbial analysis of milk samples**

All the samples were processed under aseptic conditions for microbial analysis. The milk samples were subjected to serial dilution. Before processing, personal & equipment hygiene was maintained by wearing clean apron and by cleaning hands with 95 % ethanol. Care was taken to prevent external contamination of the samples. Laminar surface was wiped with 95 % ethanol.

For microbial analysis of milk samples, following parameters were studied:

1. Total plate count - Nutrient agar
2. EMB plate count - Eosine Methylene Blue agar
3. VRBA plate count - Violet Red Bile agar
4. Yeast & mold count - Potato Dextrose agar

10 ml milk samples were suspended in 90 ml of sterile peptone water. Each sample was serially diluted upto $10^{-4}$ using sterile peptone water. For total plate count, 0.1 ml aliquote from the $10^{-4}$ tube was plated on Nutrient agar plates and
plates were incubated at 37°C overnight. Next day, colonies were counted and recorded accurately. Similarly, Violet red brilliant agar (VRBA) and Eosine methylene blue (EMB) agar were also used for conducting coliform and E. coli counts, respectively.

Potato Dextrose Agar (PDA), pH 3.5 ± 0.2 was used for yeast and mold count of food samples. $10^{-1}$ and $10^{-2}$ dilutions were prepared from the samples. Then 0.1 ml of aliquote taken from each dilution was spread on the solidified PDA plates. These plates were incubated at 25°C for 4-5 days.

**Preservation of colonies**

Bacterial colonies from N-agar plates were transferred to sterile 2 ml vials (Appendroff) under aseptic condition using a sterile wire loop. Colonies were suspended in 100 µl of sterile D/W, mixed properly for 5 to 10 sec, and centrifuged to get pellet. Colonies were preserved in 50 % glycerol. Prior to analysis, the culture was transferred to enrichment broth (Luria broth) for 18 hours for activation.

**PCR analysis**

For PCR analysis, boiling centrifugation method was used as described by Lampel *et al* (2000). For this, one ml aliquots of enrichment broths were centrifuged at 13,000 rpm for 3 minutes. The pellets were resuspended in 100 ml of sterile distilled water, heated to 95°C in a temperature controlled water bath for 10 min, cooled in ice and centrifuged at 13,000 rpm for 3 min. The resulting supernatants were directly used for PCR assay. 2 µl of supernatant was used for amplification in the final reaction mixture.

**Isolation of DNA from known bacterial strains of E. coli, S. typhi and S. aureus (Positive control)**

DNA was isolated from *E. coli*, *S. typhi* and *S. aureus* according to Sambrook *et al.* (1987) and this DNA was used as positive control in all PCR reactions.

About 2 ml of overnight grown culture was taken, centrifuged at 8000 rpm for 12 min. To the pellets, 260 µl of digestion buffer [100 mM Tris HCL, 100 mM EDTA,
Materials and Methods

0.5% SDS (pH 8.0)) and 400 µl of proteinase K was added, incubated for 1 hour in a water bath. After that, 20 µl of lysozyme was added, incubated for 5 min; 30 µl of RNase was added, incubated for 5 min and centrifuged at 7500 rpm for 25 min. To the supernatant, 450 µl of phenol, 500 µl of chloroform:isoamyl alcohol (24:1) and 50 µl Tris buffer [10 mM Tris HCL, 1 mM EDTA (pH 7.5)] was added, shaken well, centrifuged at 8000 rpm for 15 min, aqueous phase was taken out and again treated with chloroform:isoamyl alcohol, centrifuged at 8000 rpm for 15 min. Aqueous phase was again collected and double the volume of ethanol or isopropyl alcohol was added, centrifuged at 7800 rpm for 10 min. The resulting pellets were dried and dissolved in TE buffer. DNA concentration was quantified by UV spectrophotometer at 260 and 280 nm.

Master Mix preparation for PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X buffer</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTPs mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>DNA sample</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1 µl (0.3 µg/µl)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µl (0.3 µg/µl)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

Total reaction volume was made up to 50 µl by adding 42 µl sterile double distilled water.

Primers used:

E-Coli:

Primers:

EXPAL-L’5-GGCAATTGCAGATGTCTTCTCC-3’
EXPAL-R’5-CCGCGTGACCTTCTACGGTGCAC-3’ (Kuhnert et al., 1995)

PCR reaction was carried out in PCR tube in PCR thermal cycler (Corbett Research, Australia).
Materials and Methods

Reaction cycle:
Cycle 1: 3 min 94°C
Cycle 2 (40 cycles):
   30 sec 94°C
   30 sec 60°C
   1 min 72°C
Cycle 3: 72°C for 7 min

S. aureus:
The pair of primers detecting S. aureus consisted of
RDR327: 5' GCC GGT GGA GTA ACC TTT TAG GAG C3'
DG74: 5' AGG AGG TGA TCC AAC CGC A 3'.
The length of the resulting amplicon was 105 bp (Natar, Khodoud et al., 1999).

PCR cycle:
Cycle 1: 95°C for 5 min
Cycle 2 (40 cycles):
   95°C for 1 min,
   58°C for 1 min,
   72°C for 1 min,
Cycle 3: 72°C for 10 min

S. typhi:
For S. typhi, primers used were specific for a 389 bp fragment of invA gene and each reaction included 10 pmol of the
Forward primer Salm 3 (5'-GCTGCACGCGAAGCTGCAGGATT-3') and
Reverse primer Salm 4 (5'-TCCGCAGCGCGCGC-3').

Amplification of S. typhimurium targeting inv A consisted of:
Cycle 1: 95°C for 5 min;
Cycle 2: 40 cycles of
Materials and Methods

95°C for 90s,
58°C for 80s,
72°C for 2min.; and
Cycle 3: 72°C for 7min (Stevens and Jaykus, 2004).

The amplified DNA was detected after electrophoresis in 1.5 % Agarose gel containing 5 mg/ml ethidium bromide. The gel was visualized on a UV-light transilluminator (HaakeBuchler, Saddle Brook, NJ, USA). The photograph of gel was captured using α–Inotech Gel Documentation Unit.
4. A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially-contaminated dairy products using PCR

The present study was planned to evaluate various DNA extraction methods for detection of *E. coli* O157:H7 from artificially contaminated dairy products using PCR.

**Food Samples**
Foods (liquid skim milk, non fat dry skim milk powder, cheese) were purchased from local retail establishments.

**Bacterial culture**
Escherichia coli O157:H7 was obtained from the Bioscience Department, Sardar Patel University. It was maintained in Brain Heart Infusion (BHI) broth (Micro-master) at 37°C. Plate counts of log-phase cultures were confirmed by pourplating on Trypticase Soy Agar (TSA) (Titan Biotech) incubated at 37°C for 16 hrs.

**DNA isolation methods**
Two methods were compared for their DNA extraction efficacy:

1. Solvent method
2. Concentration method

**DNA extraction from foods**
Prior to inoculation with *E. coli* O157:H7, 10 gm/10 ml of each food material was suspended in 90 ml sterile peptone water and from these serial dilutions were prepared. Dilutions were plated on Triptcase Soy Agar (TSA) with an overlay of Violet Red Bile Agar (VRB) (Titan Biotech) to confirm the absence of coliform contamination (Christen et al., 1993).
(1) Solvent method

Initial sample processing differed slightly according to commodity:

For liquid skim milk, a 10 ml sample was pre-treated with 1 ml of 25 % sodium citrate followed by vigorous shaking in an environmental shaker for 5 min.

Non fat dry milk (skimmed milk powder) (20 g) was suspended in 80 ml of 2 % sodium citrate, by vigorous shaking in an environmental shaker for 5 min.

In the case of cheese, 20 g were grated and blended for 2 min in 90 ml warm (40°C) 2 % sodium citrate with the use of blender prior to extraction according to standard methods (White et al., 1993).

For each food suspension, 10 ml portion was transferred to a centrifuge tube and inoculated with 1 ml serially diluted log phase *E. coli* O157:H7 (10^0-10^7 cfu/ 10 ml). These dilutions represent the final density in the food suspension immediately prior to DNA extraction.

The DNA was extracted using a modification of the method described by Drake *et al* (1996). Briefly, petroleum ether (2 ml) (Qualigens), 100 % ethanol (2 ml) and concentrated ammonium hydroxide (4 ml) (Qualigens) were added into 10 ml food portion in centrifuge tube, mixed, and the solution centrifuged at 8000 rpm for 10 min at room temperature in a centrifuge. The supernatant fluid was discarded and the pellet resuspended in 900 µl STET buffer [8 % sucrose (Qualigens), 0.5% Triton-X-100 (Qualigens), 50 mmol EDTA (Qualigens), 50 mmol Tris-HCl pH 8.0 (Qualigens)] and transferred to a 2 ml microcentrifuge tube. The tube was vortexed occasionally for 10 min. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed, and the tube centrifuged at 17 000 g for 10 min at room temperature. The supernatant fluid was transferred to a new tube and an equal volume of chloroform was added, mixed, and centrifuged as above. The clear aqueous phase was pipetted to a new tube and the DNA precipitated with 0.1 volume 3 M sodium acetate, pH 5.4, 1 volume cold isopropanol and 30 mg glycogen (Sigma).
The sample was centrifuged at 12,000 g for 30 min at room temperature to pellet the DNA. The air-dried pellet was resuspended in 15 µl nuclease-free water (Banglore Genei) and quantified at 260 nm using a Shimadzu UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan).

(2) Concentration method

All samples were pre-treated with sodium citrate, as described above, prior to inoculation with decimal dilutions of *E. coli* O157:H7 (10^0-10^7 cfu 10 ml^-1), as described in the solvent method.

Samples, i.e. liquid skim milk, nonfat dry skim milk powder and cheese, 10 ml of each pre-treated sample were also subjected to an initial solvent extraction (prior to bacterial concentration), in order to extract fat and protein, by gentle mixing with 2 ml 95 % ethanol and 4 ml petroleum ether. Samples were centrifuged at 8000 g for 10 min at room temperature. The bacteria-containing pellet was resuspended in 750-900 µl sterile 0.9 % NaCl. After this extraction, samples were used for DNA isolation.

Preparation of metal hydroxide

Metal hydroxide was prepared for its use in the concentration method. Metal hydroxide preparation was carried out according to Mckillip et al (2000). For preparation of titanous hydroxide, a 1.3 mmol/lit solution was prepared by the addition of 200 ml distilled water to 136.6 µl titanous chloride (Sigma). The solution was adjusted to pH 7 (± 0.2) by drop wise addition of 2 mol/lit ammonium hydroxide (Qualigens) with continuous stirring. The suspension was washed twice with 5 ml sterile 0.9 % NaCL (Qualigens). In the washing procedure, the hydroxide was mixed with sterile saline, allowed to settle for 10 min, centrifuged at 5000 rpm for 15 min, the clear top phase was decanted and the pellet was used for further study. The final volume of the washed titanous hydroxide was approximately 200 ml.
**Materials and Methods**

**DNA extraction**

For bacterial concentration, samples were centrifuged at 8000 g for 10 min at room temperature. The bacteria-containing pellet was resuspended in 750-900 µl sterile 0.9 % NaCl and transferred to a 2 ml microcentrifuge tube. An equal volume of titanous hydroxide suspension was added to the tube which was gently mixed to resuspend the pellet followed by shaking for 10 min in an elliptical motion to facilitate bacterial immobilization by the metal hydroxide. The tube was centrifuged at 1500 g for 5 min to pellet the bacteria-metal hydroxide complexes.

For subsequent DNA extraction, the pellet was resuspended in 600 µl guanidinium isothiocyanate (GITC) solution (containing 4 mol guanidinium isothiocyanate, 20 mmol sodium acetate, pH 5.2, and 0.1 mol dithiothreitol, all from Sigma) and 100 µl 2 % Triton X-100 (Sigma) and incubated at room temperature for 10 min with occasional shaking to solubilize the cells. The sample was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) (Qualigens), agitated briefly and centrifuged at 10000 rpm for 10 min. The DNA was precipitated from the aqueous layer, as described above, and quantified spectrophotometrically.

**DNA amplification**

DNA amplification was carried out by PCR. PCR primers targeted a 1.5 kb portion of the slt-II gene from *E. coli* O157:H7 as previously described by Meyer *et al* (1992).

2 µl of extracted DNA by both the methods was added to 1.25 µl 10 X buffer, 2 µl 25 mM MgCl₂, 1 µl DNTPs mix, 1 µl 20 pmol each of forward (5’-TGTAAGCTTAAAGCCGGACAGAGC-3’), reverse (5’-CCACGGATCCGGTTATGCCTC-3’) primer and 0.5 µl Taq DNA Polymerase. Total volume was made upto 50 µl by adding nuclease free sterile distilled water. PCR amplification was carried out using a PCR thermal cycler (Corbett
Research, Australia). The temperature profile used was as follows: initial denaturation for 3 min at 94°C, followed by 35 cycles for 30 sec at 94°C (denaturation), 30 sec at 54°C (annealing) and final extension for 7 min at 72°C (extension). 10 µl of the cycled PCR reaction was analyzed on 1.5 % agarose gel containing 500 ng/ml ethidium bromide at 110 V for 2h in TAE-buffer (1 Lit: 5.4 g Tris, 0.4 g EDTA, 2.75 g Boric acid). The photograph of gel was captured using α-Inotech Gel Documentation Unit. Controls included a negative control (without DNA) and a positive control (having template DNA) consisting of 1 µl DNA extracted from an overnight culture of E. coli O157:H7.
5. Assessment of viability of probiotic bacteria and competitive growth of *Lactobacillus acidophilus* in yoghurt during refrigerated storage

In the present study, survival of lactic acid bacteria was evaluated during refrigerated storage in yoghurt. Growth of *Lactobacillus acidophilus* was also monitored in the presence of other lactic acid bacteria during refrigerated storage of yoghurt for a period of 30 days. Both microbiological methods and PCR studies were carried out on the samples.

**Selection and Maintenance of culture**

*Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 0253, *Streptococcus thermophilus* NCDC 075, *Lactobacillus acidophilus* NCDC 013, *Lactobacillus plantarum* NCDC 020, *Lactobacillus fermentum* NCDC 214, *Lactobacillus paracasei spp. paracasei* NCDC 022 and *Lactobacillus casei* NCDC 297 were procured from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal, India.

Freshly prepared reconstituted skim milk (10 % skim milk powder) was autoclaved at 15 psi for 15 minutes, cooled to 42˚C and inoculated with loopful of respective individual cultures of *L. acidophilus* or *L. bulgaricus* or *S. thermophilus* or *L. Plantarum* or *L. fermentum* or *L. paracasei* or *L. casei* in test tubes of 50 ml capacity. These were incubated at 42˚C for 24 hrs. Tubes were then stored to 4˚C. Transfers were made once in two days and these were used as activated cultures.

**Control Yoghurt Preparation from Standardized Milk**

Control Yoghurt production was carried out according to Tamime and Robinson (1999). 100 ml standardized Amul milk (4.5 % fat, 8.5 % SNF) was taken in a beaker. Milk was heated to 45-50˚C. Before heating, 3 gm skim milk powder (SMP) was added to standardized milk to increase the solids not fat (SNF)
content and mixed properly. Heat treatment was given at 90°C for 5 minutes. The temperature was monitored constantly by a thermometer. It was then cooled to 45°C. Activated cultures of *L. bulgaricus* and *S. thermophilus* at 1 % w/v were added, mixed properly. It was then incubated at 42°C until acidity reached 0.9 % (8 hours). Product was prepared in three replicates and stored at 4°C in a refrigerator for 30 days.

**Experimental yoghurt preparation**

Experimental yoghurts were prepared to assess competitive growth of *L. acidophilus* in the presence of other lactic acid bacteria in the yoghurt product. The procedure was similar to that of control yoghurt preparation except for the inoculation time of *L. acidophilus* in the yoghurt product. Along with *L. bulgaricus* and *S. thermophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. paracasei* were also used for experimental yoghurt preparation. Three experimental yoghurt samples were prepared:

(A) Experimental I (Exp. A): In this yoghurt, *L. acidophilus* was inoculated with *L. bulgaricus* and *S. thermophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. paracasei*, each at 1 % w/v;

(B) Experimental II (Exp. B): In this yoghurt, *L. acidophilus* was inoculated 2 hours after the inoculation of *L. bulgaricus*, *S. thermophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. paracasei*, each at 1 % w/v;

(C) Experimental III (Exp. C): In this sample, *L. acidophilus* was inoculated 2 hours before the inoculation of *L. bulgaricus*, *S. thermophilus*, *L. plantarum*, *L. fermentum*, *L. casei* and *L. paracasei*, each at 1 % w/v.

All the products were prepared in three replicates and stored at 4°C for 30 days.

Parameters analysed were:

1. Microbial analysis of Control and Experimental yoghurt products (Total Lactobacillus Count, *L. acidophilus* count, Yeast and Mold count, Coliform count)
2. DNA isolation and PCR products confirmation of different lactobacilli from
Control and Experimental yoghurt samples

3. Proteolytic activity of Control and Experimental yoghurt products

4. Sensory evaluation of Control and Experimental yoghurt products

1. Microbial analysis of yoghurt samples

Microbial analysis conducted on yoghurt samples is presented in Table 1. Total lactic acid bacterial count was carried out on yoghurt samples on 0, 15 and 30 days of storage. Lactobacillus MRS agar (Hi-Media), pH 6.5 ± 0.2 was used and double layer pour plate method was performed. $10^{-1}$, $10^{-2}$,...$10^{-6}$ dilutions were prepared from the samples. 1 ml of diluted sample was mixed in MRS molten agar and it was then poured on previously solidified MRS agar plates. It was then incubated at 37°C for 48 hours.

$L.\ acidophilus$ MRS agar (Hi-media) media was used for counting of $L.\ acidophilus$. $L.\ acidophilus$ count was carried out on 0, 15 and 30th day of storage by using double layer pour plate method.

Potato Dextrose Agar (PDA) (Hi-Media), pH 3.5 ± 0.2 was used for yeast and mould count of yoghurt samples. $10^{-1}$ and $10^{-2}$ dilutions were prepared from the samples. Then 0.1 ml of aliquote taken from each dilution and spread on the solidified PDA plates. These plates were incubated at 25°C for 4-5 days. Yeast and mould count was performed at 0 day and on 30th day of storage.

For Coliform count of yoghurt samples, 3M™ Petrifilm™ Rapid Coliform Count (RCC) plates (Merk, Mumbai) were used. 0.1 ml of sample was spread on the plates and the plates were incubated at 42°C for 24 hours. Coliform count was carried out on 0 day and on the 30th day.
Table 1. Microbial analysis of Experimental and Control yoghurt Samples

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Microbial count</th>
<th>Media</th>
<th>Days of testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total lactic acid count</td>
<td>Lactobacillus MRS agar</td>
<td>0, 15 &amp; 30</td>
</tr>
<tr>
<td>2</td>
<td>L. acidophilus count</td>
<td>L. acidophilus MRS agar</td>
<td>0, 15 &amp; 30</td>
</tr>
<tr>
<td>3</td>
<td>Yeast &amp; mold count</td>
<td>Potato Dextrose Agar</td>
<td>0 &amp; 30</td>
</tr>
<tr>
<td>4</td>
<td>Coliform count</td>
<td>Petrifilm rapid coliform count</td>
<td>0 &amp; 30</td>
</tr>
</tbody>
</table>

- Double layer pour plate method was carried out for all counts except yeast and mold count.
2. DNA Isolation from Yoghurt samples

DNA isolation and PCR analysis was carried out from yoghurt samples to determine the presence of each individual inoculated lactic acid bacteria in the sample on 0 day and after a storage period of 30 days. DNA isolation from yoghurt samples was carried out on 0 day and on the 30th day according to the method described previously (Lick, Keller, Bockelmann, & Heller, 1998).

Briefly, the yoghurt sample was properly homogenized and from the homogenate, 250 µl of yoghurt sample was taken. To it, 80 µl of sodium citrate, 150 µl of NaOH was added. It was centrifuged at 12,000 g for 2 minutes and then the upper layer of fat and the aqueous supernatant was discarded and the pellet was resuspended in 500 µl 5X SSC solution [Sodium chloride (Qualigens) /Sodium citrate solution c(NaCl) (Qualigens) = 0.75 mol /l & c(C$_6$H$_5$Na$_3$O$_7$) = 0.075 mol /l]. It was centrifuged for at least 2 minutes at 12,000g. The supernatant was discarded and resuspended the pellet in 500 µl 5 X SSC solution. It was then centrifuged for 2 minutes at 12,000 g and the supernatant was discarded. The pellet was resuspended in 500 µl of extraction/ lysis buffer [1 volume part of buffer solution A and 1 volume part of sucrose solution. Buffer solution A: c (Tris) =0.020 mol / l; c(Na$_2$EDTA) (Qualigens) =0.020 mol / l; c(NaCl) = 0.100 mol /l. pH was adjusted to 8.0 with HCl or NaOH. Sucrose solution: c(C$_{12}$H$_{22}$O$_{11}$) = 400g /l]. 50 µl of lysozyme solution (freshly prepared, 10mg/ ml in 0.25 M Tris HCl Buffer, pH 8.0) was added and incubated at 37°C for 1 hour. To it, 20 µl of RNase solution (5mg/ml in 50mM Na-acetate, pH 5) was added and incubated at room temperature for 15 minutes. 25 µl of SDS solution and 25 µl of protinase K solution ( 20 mg/ml, dissolved in sterile water) were added and incubated for 10 minutes at 60°C. 500 µl of phenol /chloroform /isoamyl alcohol (25:24:1) was added and mixed. Then it was centrifuged for 3 minutes at about 12,000 g. The upper aqueous phase was transferred to a new tube and 1 volume of chloroform/isoamyl alcohol (24:1) was added and mixed. Then it was centrifuged for 3 minutes at about 12,000 g. The upper phase was transferred to a new tube and 0.1 ml of sodium acetate solution and 1 ml of
isopropanol were added. It was kept for 30 minutes at room temperature and then centrifuged for 15 minutes at 12,000 g. The supernatant was discarded and washed the pellet carefully in at least 500 µl of ethanol solution. Then, it was centrifuged for 10 minutes at about 12,000 g. The supernatant was discarded; the pellet was dried and redissolved in 100 µl of TE buffer [10 mM Tris Cl (pH 8.0), 1 mM EDTA (pH 8.0)]. Isolated DNA samples were analyzed on 0.8 % agarose gel and observed in UV transilluminator for confirmation. DNA isolation was carried out on zero day of storage. It was performed after completion of incubation period, i.e. when acidity reached to 0.9 %.

**Polymerase Chain Reaction (PCR) from the isolated DNA**

The master mix preparation for PCR was prepared as follows: 1.25 µl 10 X buffer [10 mM Tris-HCL (pH 8.3), 50 mM KCl], 2 µl 25 mM MgCl2, 1 µl dNTPs mix, 2 µl DNA sample, 1 µl forward primer, 1 µl reverse primer, 1 µl (1 U) of Invitrogen Platinum Taq High Fidelity DNA Polymerase and the volume was made up to 50 µl by adding distilled water. All the chemicals used were of Invitrogen make. The primers used in the present study are given in Table 2.

Each PCR product was amplified according to the following conditions: 95°C for 3 min, followed by PCR cycles of denaturation at 94°C for 30 sec, annealing at temperatures specified in Table 3 for 60 sec and extension at 72°C for 1 min. The number of amplification cycles and the literature reference for each PCR are presented in Table 3. The PCR products were observed by using Agarose gel electrophoresis having 1.5 % agarose.

**3. Proteolytic Activity of Yoghurt samples**

Proteolytic activity of yoghurt samples was analyzed to observe the effect of storage of yoghurt samples on their proteolytic activity and also to observe the effect of competitive growth of probiotic lactic acid bacteria on proteolytic activity of yoghurt. Proteolytic activity of yoghurt samples was measured according to the method described by Abraham, De Antoni, & Anon, 1993. 100 ml of yoghurt was taken, stirred with a glass rod and the pH was set between 6.5-7.0 using 2 % EDTA (pH 12). It was then centrifuged at 5000 g for 15
minutes at 10°C. The resulting pellet was washed twice with 2 % EDTA and resuspended in sterile distilled water. For checking of proteolytic activity, milk agar plates were used. A small well (bore) was made in each plate with the help of a sterile borer. Then 0.1 ml of sample (pellet) was added in the well in each plate. The plates were incubated at 37°C for 24 hours and observed for the proteolytic zone (Composition of Milk agar: skim milk powder 1 gm %, agar 2 gm %, distilled water 100 ml, pH 6.8).

4. Sensory Evaluation
Sensory evaluation was carried out to assess the acceptability of all the prepared yoghurt samples on 0 day. Sensory evaluation and training of the panel members was carried out as mentioned by Karagul-YuCeer, Coggins, Wilson, & White, 1999. For the sensory evaluation of yoghurt samples, ten panelists were selected from research students & staff of Foods & Nutrition branch, Sardar Patel University. Panelists evaluated 20 gm portions of each yoghurt sample. Organoleptic studies included the colour, texture, flavor and overall acceptability. The scoring of samples was done using the modified Composite Scoring Test on a scale of 1 – 10 for each characteristic where 1 = poor quality and 10 = excellent quality.

Statistical analysis
Results are mean of three replications. Difference between variables were tested for significance using a one way analysis of variance procedure, Duncan’s t-test, using a level of significance p<0.05 (SPSS for windows 10.0).
Table 2. Primers used in this study for each organism

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Primer</th>
<th>Orientation</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus delbrueckii subsp. bulgaricus</em></td>
<td>Ldel-7</td>
<td>F</td>
<td>5'-ACA GAT GGA TGG AGA GCA GA-3'</td>
</tr>
<tr>
<td></td>
<td>Lac-2</td>
<td>R</td>
<td>5'-CCT CTT CGC TCG CCG CTA CT-3'</td>
</tr>
<tr>
<td></td>
<td>Lpla-3</td>
<td>F</td>
<td>5'-ATT CAT AGT CTA GTT GGA GGT-3'</td>
</tr>
<tr>
<td></td>
<td>Lpla-2</td>
<td>R</td>
<td>5'-CCT GAA CTG AGA GAA TTT GA-3'</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>Laci-I</td>
<td>F</td>
<td>5'-TGC AAA GTG GTA GCG TAA GC-3'</td>
</tr>
<tr>
<td></td>
<td>23–10C</td>
<td>R</td>
<td>5'-CCT TTC CCT CAC GGT ACT G-3'</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>Lactcase-F</td>
<td>F</td>
<td>5'-TGT CTC CAT CGA AGT TGA TC-3'</td>
</tr>
<tr>
<td></td>
<td>Lactcase-R</td>
<td>R</td>
<td>5'-AAC TTC TTC TCG AAG AGC TC-3'</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>Stp Th-F</td>
<td>F</td>
<td>5'-CAC TAT GCT CAG AAT ACA-3'</td>
</tr>
<tr>
<td></td>
<td>Stp Th-R</td>
<td>R</td>
<td>5'-CGA ACA GCA TTG ATG TTA-3'</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td>LF1</td>
<td>F</td>
<td>5'-AATACCGCATTACAACCTTTG-3'</td>
</tr>
<tr>
<td></td>
<td>LF2</td>
<td>R</td>
<td>5'-GGTTAAATACCGTCAACGT-3'</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em></td>
<td>W2</td>
<td>F</td>
<td>5'-CACCGAGATTCAACATGG-3'</td>
</tr>
<tr>
<td></td>
<td>Y2</td>
<td>R</td>
<td>5'-CCCCACTGCTGCCTCCCCTAGGAGT-3'</td>
</tr>
</tbody>
</table>

F: Forward; R: Reverse
### Table 3. Conditions for PCR amplification

<table>
<thead>
<tr>
<th>Species</th>
<th>Annealing temperature</th>
<th>No. of cycles</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>60°C</td>
<td>35</td>
<td>Song, Kato, Liu, Matsumiya, Kato, &amp; Watanabe, 2000</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>68°C</td>
<td>45</td>
<td>Song, Kato, Liu, Matsumiya, Kato, &amp; Watanabe, 2000</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>60°C</td>
<td>40</td>
<td>Drisko, Bischoff, Giles, Adelson, Rao, &amp; Mccallum, 2005</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td>58°C</td>
<td>35</td>
<td>Lick, Drescher, &amp; Heller, 2001</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em></td>
<td>50°C</td>
<td>35</td>
<td>Dickson, Riggio, &amp; Macpherson, 2005</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em></td>
<td>50°C</td>
<td>40</td>
<td>Desai, Shah, &amp; Powell, 2006</td>
</tr>
</tbody>
</table>
6. Effect of nisin on growth and survival of selected food pathogens

The present study was planned to evaluate the effect of the bacteriocin ‘nisin’ on selected food pathogens namely *S. aureus*, *L. monocytogenes* and *S. typhi* during yoghurt fermentation and storage. Effect of nisin was also evaluated on the growth of yoghurt starter cultures namely *L. bulgaricus* and *S. thermophilus*.

Bacteriological strains and growth conditions

A. Yoghurt starter cultures

*Lactobacillus delbrueckii* subsp. *bulgaricus* NCDC 09 and *Streptococcus thermophilus* NCDC 075 were procured from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal. Fresh or reconstituted skim milk (10 % skim milk powder) was autoclaved at 15 psi for 15 minutes, cooled to 42˚C and inoculated with a loopful of respective individual cultures of *L. bulgaricus* and *S. thermophilus*, in 50 ml capacity test tubes. These both were incubated at 42˚C. Tubes were then stored to 4˚C. Transfers were made once in two days and these were used as activated cultures.

B. Pathogens

*Salmonella typhi* MTCC 531 and *Staphylococcus aureus* MTCC 740 were obtained from the Microbial Type Culture Collection, Chandigarh. *Staphylococcus aureus* was stored on Baird-Parker agar plates (Titan Biotech, Bangalore) at 4˚C during the study. *S. typhi* and *E. Coli O157:H7* were grown in Luria broth (Titan Biotech) for 18-24 hours at 37˚C.

*Listeria monocytogenes* was obtained from MTCC (Microbial Type Culture Collection) Chandigarh. It was grown on a slant of Trypticase Soy Agar (TSA, Titan Biotech) and stored at 4˚C. Before each use, it was transferred to 10 ml of Trypticase Soy Broth [(TSB) (Bacto-Tryptone, 15 g; Bacto-soytone, 5 g; NaCl, 5
g; and distilled water, 1000 ml] (Titan Biotech) and incubated 16 to 18 hours at 37°C.

**Nisin**

Nisin (Nisaplin) was purchased from National Laboratories, Mumbai. Stock solution (37 x 10^3 IU/ml) was prepared by dissolving 0.1 g of nisin in 80 ml HCl (0.02 mol/ml) and holding at room temperature for 2 hours to complete dissolution. The volume was then made up to 100 ml with HCl (0.02 mol/l) and the solution was filter-sterilized by passage through a 0.22 μm Millipore membrane and stored at -20°C.

**Parameters studied:**

1. Effect of Nisin on yoghurt fermentation
2. Effect of Nisin on the growth of *S. aureus*, *S. typhi*, and *L. monocytogenes*
3. Effect of Nisin on the growth of *S. aureus*, *S. typhi*, and *L. monocytogenes* in Yoghurt

1. **Effect of Nisin on Yoghurt Fermentation**

As yoghurt starter cultures i.e. *L. bulgaricus* and *S. thermophilus* is reported to be sensitive to nisin (Kumar and Prasad, 1994a; Kumar and Prasad, 1994b; Vandenbergh, 1993), this preliminary experiment was carried out to determine the concentration of nisin which can be used without affecting significantly normal yoghurt processing and acid production.

Yoghurt trials were conducted as follows:

Nonfat dry milk (skimmed milk powder) was added to raw milk to adjust dry matter and fat contents to approximately 17 and 150 g/l, respectively. After thorough mixing, pasteurization was performed in a water bath at 80°C (internal temperature of milk) for 30 min. Of the pasteurized milk, 100 milliliters were dispensed in plastic cups and inoculated with the activated yoghurt starter (1ml *L. bulgaricus* and 1 ml *S. thermophilus* in 100 ml milk) to the level of 20 ml/l.
Nisin was added to the cups to a final concentration of 10, 50 or 100 IU/ml. A sample not containing nisin was used as control. The containers were incubated at 43°C for 24 hours and then transferred to the refrigerator (4-7°C).

**Parameters studied:**

The pH and acidity were measured every 4 hours up to 24 hours. pH measurements were done with a pH meter. Acidity was measured by titration with NaOH (0.1 N) in the presence of phenolphthalein indicator.
### Table 1. Yoghurt sample preparation with the addition of Nisin

<table>
<thead>
<tr>
<th>Nisin concentration (IU/ml)</th>
<th>Control</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
2. Effect of Nisin on *S. aureus*, *S. typhi* and *L. monocytogenes*

(A) Varying pH with constant nisin concentration

In the first part, fixed nisin concentration was used along with different pH values. Sample preparation is shown in Table 2.

Three series of test tubes (4 tubes in each series) containing 9 ml of reconstituted sterilized skimmed milk powder (10 %) were used. The tubes of each series were adjusted to different pH values (6.8, 5.5, 5.0 and 4.5) with 0.1 N HCL.

To the first series (test), nisin was added to a final concentration of 50 IU/ml along with 0.1 ml of overnight culture (16 to 18 h, $10^6$ cells/ml) of *L. monocytogenes* or *S. typhi* or *S. aureus*, respectively.

To the second series, only *S. aureus* or *S. typhi* or *L. monocytogenes* were added respectively to each tube to the same concentration. To enhance the activity of nisin on *S. aureus* and *S. typhi*, in the tubes having *S. aureus* and *S. typhi* cultures, 20mM EDTA was added as reported by Miller et al. (2001).

Cultures were incubated at 37°C for different periods of incubation as discussed below.

**Parameters studied:**

Growth of *L. monocytogenes* was monitored by plate count on Listeria selective agar plates (Merck, Mumbai) at 0, 4, 8, 24 and 48 hours. Growth of *S. aureus* was monitored on Baird-Parker agar plates (Merck, Mumbai) plated at 0, 4, 8, 24 and 48 hours. Growth of *S. typhi* was monitored on Salmonella differential agar plates (Merck, Mumbai) at 0, 4, 8, 24 and 48 hours.
### Table 2. Preparation of yoghurt samples with constant nisin concentration (50 IU/ml) with varying pH values

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>pH</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.8</td>
<td>5.5</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>L. monocytogenes</strong> (L.M.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^{st}) Series</td>
<td>Nisin + L.M.</td>
<td>Nisin + L.M.</td>
<td>Nisin + L.M.</td>
<td>Nisin + L.M.</td>
</tr>
<tr>
<td>2(^{nd}) Series</td>
<td>L.M.</td>
<td>L.M.</td>
<td>L.M.</td>
<td>L.M.</td>
</tr>
<tr>
<td><strong>S. aureus</strong> (S.A.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^{st}) Series</td>
<td>Nisin + S.A.</td>
<td>Nisin + S.A.</td>
<td>Nisin + S.A.</td>
<td>Nisin + S.A.</td>
</tr>
<tr>
<td>2(^{nd}) Series</td>
<td>S.A.</td>
<td>S.A.</td>
<td>S.A.</td>
<td>S.A.</td>
</tr>
<tr>
<td><strong>S. typhi</strong> (S.T.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^{st}) Series</td>
<td>Nisin + S.T.</td>
<td>Nisin + S.T.</td>
<td>Nisin + S.T.</td>
<td>Nisin + S.T.</td>
</tr>
<tr>
<td>2(^{nd}) Series</td>
<td>S.T.</td>
<td>S.T.</td>
<td>S.T.</td>
<td>S.T.</td>
</tr>
</tbody>
</table>

- Nisin concentration: 50 IU/ml
- Each test tube contained 10 ml of 10 % reconstituted skimmed milk powder
- Bacterial culture: 0.1 ml of overnight culture (16 to 18 h, 10 cells/ml) of *L. monocytogenes* or *S. typhi* or *S. aureus*
(B) Effect of varying pH and varying nisin concentration

In the second part, effect of varying pH with varying nisin concentration was studied.

Four series of test tubes (4 tubes in each series) containing 10 ml of luria broth for *S. aureus* and *S. typhi* was used while TSB for *L. monocytogenes* were used. Each series was adjusted to different pH values (6.8, 6.0, 5.5 and 5.0) with 0.1 N HCL. For each pH, four tubes of different nisin concentrations were used (10, 50, 100 and 200 IU/ml). This was prepared for each of the three pathogens studied.

Tubes were inoculated with 0.1 ml of overnight cultures (16 to 18 hours) of *S. aureus*, *S. typhi* and *L. monocytogenes* respectively incubated at 37°C for different periods of incubation. In the tubes having *S. aureus* and *S. typhi*, 20 mM EDTA was added. Negative and positive controls were prepared as described above. This is presented in Table 3.

**Parameters studied:**

Growth of the organisms was monitored by O.D. determinations at 600 nm at 0, 2, 4, 8, 24 and 48 hours.
Table 3. Sample preparation with varying pH and varying nisin concentration

<table>
<thead>
<tr>
<th>pH</th>
<th>S. typhi&lt;sup&gt;a&lt;/sup&gt;</th>
<th>S. aureus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L. monocytogenes&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nisin Concentration (IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>5.5</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>6.0</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>6.5</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup>: growth in luria broth, <sup>b</sup>: growth in Tripticase soy broth
3. Effect of Nisin on the growth of *S. aureus*, *S. typhi* and *L. monocytogenes* in Yoghurt

Two series of yoghurt cups (three tubes in each series) were prepared as described in experiment 1 (Effect of Nisin on yoghurt fermentation). In the first series; Nisin was added to them to a final concentration of 10 IU/ml. All cups were inoculated with 0.1 ml \((10^5 \text{ cfu/ml})\) of an overnight grown *S. aureus*, *S. typhi* and Listeria culture in separate cups. Second series was control (three tubes in the series), in which Nisin addition was not carried out. In the samples having added *S. aureus* and *S. typhi*, 20 mM EDTA was added along with nisin. Samples were incubated at 42°C for a period of 24 hours. It is presented in Table 2.
Table 2. Yoghurt sample preparation to study the effect of Nisin on *S. aureus*, *S. typhi* and *L. monocytogenes*

<table>
<thead>
<tr>
<th>Yoghurt Samples</th>
<th><em>S. aureus</em></th>
<th><em>S. typhi</em></th>
<th><em>L. monocytogenes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin (10 IU/ml)</td>
<td>+ nisin (control)</td>
<td>+ nisin (control)</td>
<td>+ nisin (control)</td>
</tr>
<tr>
<td>EDTA</td>
<td>20 mM</td>
<td>20 mM</td>
<td>20 mM</td>
</tr>
</tbody>
</table>

Incubation at 42°C until coagulation

+ nisin : addition of nisin
- nisin : nisin not added
Parameters studied:
They were then stored in the refrigerator (7°C) and sampled for plate count at 1, 2, 3, 10 and 15 days. *S. typhi* count was carried out on Salmonella Differential Agar Plate (Titan Biotech). *L. monocytogenes* count was carried out on Listeria Selective Agar Plate (Titan Biotech). *S. aureus* count was determined by surface plating of appropriate sample dilutions on Baird-Parker Agar Plates (Titan Biotech).
7. Bacteriocin nisin production by free and immobilized 

*Lactococcus lactis*

In the present study, bacteriocin production was carried out using *Lactococcus lactis*. Free bacterial cells and immobilized bacterial cells were evaluated for bacteriocin production.

**Bacterial strains**

*Lactococcus lactis* subsp. *lactis* ATCC 11454 was obtained from ATCC (American Type Culture Collection). The culture was activated in MRS broth at 30°C for 24 hours under stationary conditions. *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* were obtained from the Department of Biosciences, S.P. University, V.V. Nagar. These were streaked on nutrient agar slants, allowed to grow for 24 hrs at 37°C and stored in a refrigerator at 4°C. All the cultures were freshly activated before use. *M. luteus* was used as Nisin sensitive strain (Hilmi et al., 2006). *Bacillus subtilis* and *Staphylococcus aureus* were tested against Nisin produced by *Lactococcus lactis*.

**Bacteriocin production**

Two different media were used for the production of bacteriocin:

**Media 1 (MRS Broth)**

200 ml of MRS broth was prepared and autoclaved. 1 ml of 24 hr activated *Lactococcus lactis* inoculum was added and kept in an environmental shaker for 40 hrs at 30°C at 100 rpm for bacteriocin production. After fermentation, 1 ml broth was transferred into fresh MRS broth. A total of 5 consecutive transfers were given at the end of every 40 hrs and the supernatant of centrifuged culture broth of each transfer was used for agar diffusion assay for assessment of bacteriocin production.
**Materials and Methods**

**Media 2 (MRS Broth + 0.5 % sucrose)**

200 ml of MRS broth containing 1.0 g sucrose was prepared and autoclaved. 1 ml of activated *Lactococcus lactis* inoculum was added and kept in a shaker for 40 hrs at 30°C for 100 rpm for nisin production. After fermentation 1 ml broth was transferred into fresh MRS + 0.5 % sucrose medium. Continued for 5 consecutive transfers after every 40 hrs and the supernatant of the centrifuged culture filtrate of each transfer was used for agar diffusion assay.

**Immobilization of *Lactococcus lactis* by encapsulation:** Immobilization of *Lactococcus lactis* was carried out according to the method given by Ivanova et al (2002).

*Lactococcus lactis* was grown in 100 ml of MRS medium at 30°C for 24 hrs. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes and washed thoroughly with 0.85 % saline solution. The cells were resuspended in 3.5 ml peptone water (pH 6.8) (1g/lit). The cells were encapsulated by mixing one part culture concentrate with one part of 4 % sodium alginate solution. Uniform droplets were formed using sterile pipette which were collected in 500 ml of 4 % Barium chloride solution & stored overnight at 4°C. The beads were washed with sterile distilled water and transferred into 100 ml of sterile MRS broth and then kept for fermentation at 30°C, for about 40 hrs.

After 40 hrs beads were filtered (Whatman No.1) from the MRS broth under sterile conditions, washed with sterile saline solution and replaced in fresh media for a second cycle of fermentation. Here also 5 consecutive transfers were given and assessment of bacteriocin activity was carried out using agar diffusion assay.

**Purification of bacteriocin using ammonium sulphate**

The fermentation broth (approximately 150 ml) after the 5th transfer was centrifuged at 8000 rpm for 10 min. Supernatant was collected and slow addition of solid ammonium sulphate was done to obtain 60 % saturation (approx. 65 gm in 100 ml) with constant stirring at 4°C in a refrigerator. This mixture was again centrifuged at 8000 rpm for 20 min. Protein precipitate was dissolved in
phosphate buffer pH 7.2 and stored in a refrigerator at 4°C for assessing bacteriocin activity (Sample A); while the supernatant was again treated with solid ammonium sulphate for 80 % saturation. This protein precipitate was dissolved in phosphate buffer pH 7.2 and stored (Sample B) for bacteriocin activity.

Both the samples were used for assessing bacteriocin activity as it was considered that sample A would be a mixture of a variety of proteins while sample B would be a concentrate of bacteriocin.

**Agar diffusion assay for bacteriocin activity**

Agar diffusion assay was carried out to determine bacteriocin activity according to Wolf and Gibbson (1996).

Plates of 1.5 % nutrient agar containing 0.8 % (v/v) Tween 80 were prepared, solidified and overlaid with 1 % soft agar containing 100 µl of test bacterial suspension (Prepared by activating the bacterial strains *M. luteus*, *S. aureus* and *B. subtilis* in Luria broth, 10 ml broth of each culture was centrifuged to pellet bacterial cells, pellets were dissolved in 1 ml of sterile distilled water).

Boring was done on Nutrient agar plates using a sterile borer of diameter 7 mm. Stock nisin solution (1000 IU/ml) was prepared by adding 0.025 g of Nisin (Sigma) in 25 ml of 0.02 N HCl (Hi-media). Working standards (nisin concentrations of 10, 20, 30, 40 and 50 IU/ml) were prepared from stock solution. 100 µl of each concentration of working solution were added to each plate for each test micro-organism.

The supernatants of the two different media and the immobilized organism were neutralized using 4 N NaOH and then filtered using sterile 0.22 µm poresize bacteriological filters (Millipore). Appropriate quantity of standard nisin (100 µl) of each concentration and samples (100 µl) were added in the bore carefully. The plates were incubated at 37°C for 24 hrs to observe inhibition zones and their diameters were measured after 24 hrs of incubation. The bacteriocin activity of standard and samples were compared. Assay experiments were carried out in triplicates.
8. Screening for NisA promoter gene and RAPD profiling from Potentially Probiotic Lactic acid Bacteria

The present study was performed to detect the presence of NisA gene, which is the promoter gene in the Nisin gene cluster in lactic acid bacteria. Moreover, RAPD profiling of lactic acid bacteria was also carried out.

**Bacterial strains used**

*Lactobacillus acidophilus* NCDC 013, *Streptococcus thermophilus* NCDC 075, *Lactobacillus bulgaricus* NCDC 25, *Lactococcus lactis* subsp. *cremoris* NCDC 274, *Lactobacillus helveticus* NCDC 05, *Lactobacillus plantarum* NCDC 020 and *Lactobacillus casei* NCDC 297 were obtained from the National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal.

*Lactococcus lactis* subsp. *lactis* ATCC 11454 was procured from American Type Culture Collection Centre (ATCC).

*Lactococcus lactis* and *lactococcus cremoris* were activated in MRS (de Man Rogosa & Sharpe) broth at 30°C for 24 hours in stationary conditions while other bacterial strains were activated in MRS at 37°C in stationary conditions except for *L. plantarum* and *L. helveticus* which were kept in shaking conditions for 48 hours. Cells were collected by centrifugation and preserved in 50 % glycerol at 0°C. Sub-culturing was carried out according to further use.

**DNA isolation**

Two different methods were used for DNA extraction from the bacterial cultures.

(A.) Genomic DNA isolation from *L. acidophilus*, *L. bulgaricus*, *L. casei* and *S. thermophilus*
Genomic DNA from *L. acidophilus, L. bulgaricus, L. casei* and *S. thermophilus* was isolated according to the previously described method of Flamm & Hinrichs et al., 1984. Cells were pelleted out from 10 ml of overnight cultures and then washed in 2 ml of Sodium saline citrate (SSC) {0.75M NaCl and 0.075M Sodium citrate}. Pellets were resuspended in 1 ml 10 mM Tris HCl (pH 8) containing 20 % sucrose, lysozyme (2.5 mg/ml) & incubated at 37°C for 45 min. 9 ml of lysis buffer [10 mM Tris HCl (pH 8), 1 mM EDTA, 500 mg Proteinase k, 1 % SDS] was added & incubated for 30 min. at 37°C. Extraction was performed once with phenol:chloroform (25:24). DNA was precipitated by using equal volume of chilled ethanol.

**Genomic DNA isolation from *L. lactis, L. cremoris, L. helveticus* and *L. plantarum***

Genomic DNA isolation from *L. lactis, L. cremoris, L. helveticus* and *L. plantarum* was extracted by the method described by Gavilan et al., 1992. Briefly, Bacterial strains were grown in MRS broth at 37°C for 24 hours. Cells were harvested in early stationary phase, resuspended in TS buffer (50mM Tris Hydrochloride [pH 8], 0.25mM sucrose) containing lysozyme (10µg/ml) and incubated at 37°C for 1 hr. 0.1 % (v/v) of 20 % Sodium Dodecyl Sulphate (SDS) was added. Extractions were performed with phenol-chloroform (25:24), then extracted once with chloroform. DNA was precipitated with equal volume of isopropanol. Pellet were dried and dissolved in TE buffer (10mM Tris HCl, 1mM EDTA [pH8] ), containing RNase (2µg/ml).

The presence of DNA was checked using Agarose gel electrophoresis. The purity of DNA was checked by measuring the Optical Density at 260 and 280 nm and taking the ratio of the absorbance values. (If A260 : A280 was equal to 1.8 then DNA was pure while <1.8 indicated contamination of RNA and >1.8 indicated presence of protein. The concentration of DNA, was measured by checking O.D. at 260 nm (1 O.D. corresponds to 50µg/ml).
Screening for *NisA* gene

*NisA*, precursor of Nisin gene was screened from 8 Lactic acid bacterial DNA through PCR.

Oligonucleotide primers used for PCR and for sequencing were 5’-CGCGAGCATAATAAACGGCT-3’ and 5’-GGATAGTATCCATGTCTGAAC-3’, which were complementary to regions 80 bp upstream and 29 bp downstream of the coding regions of the NisA and nisZ genes, respectively (Mulders et al., 1991).

Master mix prepared for PCR analysis was as follows: 1.25µl 10X buffer (incomplete), 2 µl 25mM MgCl2, 1 µl DNTP s mix, 2 µl DNA sample(100ng), 1µl forward primer, 1µl reverse primer, 0.5µl Taq Polymerase. Total volume was made upto 50 µl by adding nuclease free sterile distilled water. The thermal profile consisted of initial denaturation at 93°C for 3 min, followed by 40 repeats of 93°C for 1 min, 54°C for 1 min., 72°C for 2 min and final extension was carried out at 72°C for 5 min.

The amplified product was checked by agarose gel electrophoresis in 1.5 % agarose gel.

**DNA elution from gel and sequencing of DNA**

DNA band of interest was excised from the agarose gel using a sharp sterile blade. Larger pieces were crushed into smaller pieces. 2.5 volumes of sodium iodide solution were added and the gel was incubated in a water bath at 50-55°C for 2 to 3 min to solubilize it. To the solubilized gel, 15 µl of glass solution (Merck, Mumbai) was added to samples containing 5 µl or less DNA. The contents were mixed thoroughly and spun at 12000g for 30 sec. Supernatant was discarded and the pellet containing DNA was taken and wash buffer (Merck, Mumbai) was added to remove glass solution, vortexed and spun at 12000g for 30 sec. The supernatant was discarded again. Tubes were incubated at 37°C in the water bath for 10 min. For elution of DNA, the pellet was resuspended in 1x TE buffer, vortexed and incubated at 45°C for 5 min. This step was repeated thrice, all the
fractions were pooled of glass solution. The sequencing of DNA was carried out by Sanger method (Dideoxy chain termination) at Banglore Genei, India. The primers used for sequencing were
5’-CGCGAGCATAATAAACGGCT-3’ and
5’-GGATAGTATCCATGTCTGAAC-3’ (Mulders et al., 1991).

Statistical analysis
(i) For RAPD Profiling:
NTSYS pc 2.0 was used for Jaccard’s co-efficients of similarity (Jaccard, 1908), UPGMA (un-weighed pair group method using arithmetic averages) and SAHN clustering. Phylogenetic tree was also prepared by NTSYSpc 2.0. Nei’s Unbiased Measures of Genetic Identity (Nei, 1978) and Genetic distance was found by using POPGENE Version 3.1.
(ii) For NisA gene detection:
BLASTN programme was used to obtain the homology. From this homology, the phylogenetic tree was obtained.

RAPD analysis
Random Amplified Polymorphic DNA (RAPD) of all 8 lactic acid bacterial DNA was carried out using 10 different primers (MWG Biotech, Germany). The primers used for RAPD-PCR with their sequences are given in Table No. 1. Master Mix prepared for RAPD analysis was as follows:
1.25 µl 10 X buffer
2 µl 25 mM MgCl2,
1 µl DNTP s mix,
2 µl DNA sample (100ng),
1 µl primer,
0.5 µl Taq Polymerase.
Total volume was made upto 50 µl by adding nuclease free sterile distilled water.
## Table No.1 Primers used in RAPD profiling

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence from 5' to 3'</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1254</td>
<td>CCGCAGCCAA</td>
<td>Zapparoli et al., 1999</td>
</tr>
<tr>
<td>OPA 02</td>
<td>TGGGCGTCAA</td>
<td>Reenen &amp; Dicks et al., 1996</td>
</tr>
<tr>
<td>M13</td>
<td>GAGGGTGCCGGTTCT</td>
<td>Zapparoli et al., 1999</td>
</tr>
<tr>
<td>CRA 23</td>
<td>GCGATCCCCCA</td>
<td>Daud &amp; Khaled et al., 1997</td>
</tr>
<tr>
<td>OPL 07</td>
<td>AGGGCGGAAC</td>
<td>Dicks et al., 1995</td>
</tr>
<tr>
<td>P1</td>
<td>GCGGCGTCGTATTACATGC</td>
<td>Cocconcelli &amp; Parisi et al., 1997</td>
</tr>
<tr>
<td>P4</td>
<td>ATCTACGCAATTCACCCTAAC</td>
<td>Rebecchi &amp; Crivori et al., 1998</td>
</tr>
<tr>
<td>OPL 16</td>
<td>AGGTGGCAGGG</td>
<td>Dicks et al., 1995</td>
</tr>
<tr>
<td>CRA 25</td>
<td>AACGCCGCAAC</td>
<td>Daud &amp; Neilan et al., 1997</td>
</tr>
<tr>
<td>OPL 5</td>
<td>ACGCAGGGCAC</td>
<td>Reenen et al., 1996</td>
</tr>
</tbody>
</table>
Materials and Methods

Table 2. Thermal profile used for RAPD analysis

* Different Temperatures used for different primers:

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>94°C for 3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 2</td>
<td>94°C for 1 min.</td>
</tr>
<tr>
<td></td>
<td>34°C* for 1 min.</td>
</tr>
<tr>
<td></td>
<td>72°C for 2 min.</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>72°C for 5 min.</td>
</tr>
</tbody>
</table>

34°C: For primers CRA 23, OPA 02, OPA 07, 1254;
32°C: For primers CRA 25, OPL 05, OPL 16;
52°C: For primer M13;
61°C: For primer P4;
57°C: For primer P1.
The amplified product of all these primers were loaded in 1.5 % agarose gel against marker ladder for comparison of base pairs and bands were observed in UV transilluminator. The photograph of gel was captured using α–INOTECH Gel Documentation Unit at BRD School of Biosciences, S.P. University, V.V.Nagar.
9. Molecular Detection of Food Pathogens from Retailed Fast Food Samples by PCR

The present study was carried out to detect food pathogens especially *E. coli*, *Salmonella typhi* and *Staphylococcus aureus* from retailed fast food samples namely Samosa, Paubhaji and Vadapau which are sold by local vendors.

**Procurement and Maintenance of bacterial strains**

*E. Coli* was obtained from the Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar. *Salmonella typhi* MTCC 531 and *Staphylococcus aureus* MTCC 740 were obtained from Microbial Type Culture Collection, Chandigarh. *Staphylococcus aureus* was stored on Brain Heart Infusion (BHI) agar plates at 4°C during the study period. The other two bacterial strains were activated in Luria broth and then preserved in 50 % glycerol at -20°C for further use. These bacterial strains were used as positive control strains for DNA isolation and PCR amplification.

**Procurement of food samples**

A total of 30 different fast food samples were collected from local vendors. Sampling was performed during an outbreak of typhoid, diarrhoea and dysentery in the local areas during the month of August - September. Ten samples each of Samosa, Paubhaji and Vadapau were collected. Samosa is made up of wheat flour, potatoes, spices and is fried in oil. Paubhaji is made up of potato and tomato gravy, vegetables (onion, red pepper), spices, salt prepared in oil eaten with bun pieces. Vadapau consist of a mashed vegetable preparation mainly potatoes stuffed between bun pieces. Approximately 250g of each food type was collected and placed in sterile beakers covered with aluminum foil and transported to the laboratory.
Processing of samples and preparation of artificially contaminated sample (Positive control)

Sterile beakers were filled with 10.0 g of the food sample and 90 ml of sterile peptone water. Samples were homogenized thoroughly with the help of a blender and incubated at 37°C for 18 hours as an enrichment step to maximize the number of bacterial cells. Prior to enrichment, 1 ml of the suspension was plated on nutrient agar plate to confirm the initial bacterial load.

Potato gravy was used as positive control for each organism; the organisms studied being *E. coli*, *S. typhi* and *S. aureus*. For this, 10 gm potatoes were boiled, peeled and crushed. 90 ml sterile peptone water was added to it and autoclaved. Overnight active cultures of each individual organism were added separately to each preparation to achieve a final concentration of $1 \times 10^6$ CFU/g of the prepared gravy after incubation. The samples were incubated at 37°C for 12 hours at 160 rpm. Concentration of *E. coli*, *S. typhi* and *S. aureus* was checked by plating the sample on EMB (Eosine Methylene Blue) agar, McConkey agar and Brain Heart Infusion (BHI) agar plates, respectively.

Two-step centrifugation method was evaluated to concentrate bacterial cells prior to DNA amplification, using potato gravy as control. Briefly, the two-step method incorporated one centrifugation step (4000 rpm for 15 min at 4°C) to remove large food particulates and a second centrifugation step (11000 rpm for 10 min at 4°C) to concentrate the bacterial cells in the supernatant that was recovered after the first centrifugation. Cells recovered in the precipitate and supernatant of each centrifugation were enumerated by pour plating on Nutrient agar plate. For this, cells were suspended in 0.1 ml of sterile distilled water. This was then added to molten agar, which was then poured on to Nutrient agar.

From the results of plating, Percent recovery (or loss) was calculated as previously described by Lucore et al., 2000; and Stevens and Jaykus, 2004.

$$\text{Percent recovery} = \left( \frac{\text{total population in recovered pellet or recovered supernatant after centrifugation} \times 100}{\text{total population in sample before centrifugation}} \right)$$

$$\text{percent loss} = \left( \frac{\text{total population in discarded supernatant or discarded pellet after centrifugation} \times 100}{\text{total population in sample before centrifugation}} \right)$$
The parameters studied were:

1. Standard Plate count
2. Coliform count
3. Yeast and mold count
4. DNA isolation and PCR analysis

**Standard Plate Count (SPC)**
The method of standard plate count was followed according to Andrews, 1992 to determine the total colony count of the samples. A series of dilutions of the samples \(10^{-1}, 10^{-2}, 10^{-3}, \text{ and } 10^{-4}\) was made and 0.1 ml aliquote was plated on nutrient agar plates in petri dishes. After incubation at 37°C for 48 hours, the colonies were counted.

**Coliform count**
Coliform count was carried out according to the previously described method of Colins et al., 1995. A series of dilutions of the samples \(10^{-1}, 10^{-2}, 10^{-3} \text{ and } 10^{-4}\) was made and 0.1 ml aliquote was plated on violet red bile lactone agar. The plates were incubated at 30°C for 24 hours.

**Yeast and Mold Count**
The yeast and mold count method was carried out by the previously described method of Andrews, 1992 to enumerate the yeast and mold of the samples. Potato Dextrose agar medium was employed, in which organisms other than yeast and mold were inhibited using acidified media. A series of dilutions of the samples \(10^{-1}, 10^{-2}, 10^{-3}, \text{ and } 10^{-4}\) was made and 0.1 ml aliquote was plated on PDA plates. After incubation at 25°C for 5 days, the colonies were counted.

**Isolation of DNA**
DNA extraction was carried out from the colonies which grew on nutrient agar plates. Colonies were picked up from N-agar plates and were transferred to 100 ml sterile Luria broth for enrichment. Enrichment was carried out at 37°C at 150 rpm for 12 hours. DNA extraction was performed from this broth. DNA extraction was carried out using the phenol-chloroform method (Sambrook et al., 1997).
Lysozyme used in the study was purchased from Sigma, whereas NaCl, EDTA, Phenol, chloroform, isoamyl alcohol, Tris HCl were purchased from Hi-Media.

Briefly, 1 ml of LB broth was harvested by centrifugation. The pellet was resuspended in lysozyme solution (1 mg lysozyme /ml in 0.15 M NaCl, 0.1 M EDTA, pH 8.0), followed by lysis using 1 % SDS, 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0) at 60°C. DNA was purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) in the presence of 5 M sodium perchlorate. A 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol were added and the nucleic acid was then pelleted by centrifugation, washed with 70 % ethanol. The DNA pellet was resuspended in TE buffer (10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA) and subjected to PCR amplification.

**PCR analysis**

Isolated DNA samples were taken for PCR amplification using a PCR thermal cycler (Corbett Research, Australia). Primers used are given in Table 1. For positive control, DNA isolated from pure strains of *E. coli*, *S. aureus* and *S. typhi* were used. Reaction volume of PCR consisted of 10X buffer-1.25 µl, 25 mM MgCl₂-2 µl, 1 µl -DNTP s mix, 2 µl -DNA sample (100 ng), forward and reverse primer 1µl each, 0.5µl -Taq Polymerase. Total volume was made upto 100 µl by adding nuclease free sterile distilled water.
**Table 1:** Primers used for the detection of *E. coli*, *S. typhi* and *S. aureus*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. Coli</em></td>
<td>ECPAL-L 5'-GGCAATTGCGGCATGTCTTCTCC-3'</td>
<td>Kuhnert and Frey, 1995</td>
</tr>
<tr>
<td></td>
<td>ECPAL-R 5'-CCGCGTGACCTTCTACGATGCAC-3'</td>
<td></td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>Salm3 5'-GCTGCGCGCGAACGGCGAAG-3'</td>
<td>Stevens and Jaykus, 2004</td>
</tr>
<tr>
<td></td>
<td>Salm4 5'-TCCCGGCAGAGTTCCATT-3'</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>DG74 5'-AGG AGG TGA TCC AAC CGC A-3',</td>
<td>Matar et al., 1999</td>
</tr>
<tr>
<td></td>
<td>RDR327 5' GCC GGT GGA GTA ACC TTT TAG GAG C-3'</td>
<td></td>
</tr>
</tbody>
</table>
The temperature profile used for *E. coli* was as follows:

Cycle 1 (initial denaturation): 94°C for 3 min,
Cycle 2 (35 cycles):
  30 sec at 94°C (denaturation),
  30 sec at 60°C (annealing) and
  5 min at 72°C (extension)
Cycle 3 (Final extension): 72°C for 7 min.

The temperature profile used for *S. aureus* was as follows:

Cycle 1 (initial denaturation): 95°C for 5 min,
Cycle 2 (40 cycles):
  1 min at 95°C (denaturation),
  1 min at 58°C (annealing) and
  72°C for 1 min
Cycle 3 (Final extension): 10 min at 72°C.

The temperature profile used for *S. typhi* was as follows:

Cycle 1 (initial denaturation): 5 min at 95°C,
Cycle 2 (40 cycles):
  95°C for 90 sec (denaturation),
  58°C for 80 sec (annealing) and
  72°C for 2 min (extension).
Cycle 3 (Final extension): 7 min at 72°C.

10 µl of the cycled PCR reaction was analyzed on 1.5 % agarose gel containing 500 µg/ml ethidium bromide at 110 V for 2 h in TAE-buffer (100 ml: 5.4 g Tris, 0.4 g EDTA, 2.75 g Boric acid). The photograph of gel was captured using α – Inotech Gel Documentation Unit, at Department of Biosciences, Sardar Patel University.
Bacterial identification

DNA isolated from one of the samples of ‘Paubhaji’ could not get amplified by the primers for *E. coli*, *S. aureus* and *S. typhi*. Hence, isolated DNA from this sample was sent for commercial bacterial identification services (Banglore Genie, India). Using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using high-fidelity PCR Polymerase (Banglore Genie, India). The PCR product was cloned in plasmid DNA and plasmid DNA was bi-directionally sequenced using the forward, reverse and an internal primer (Banglore Genie, India). Sequence data was aligned and analyzed for finding the closest homologs for the microbe in the Genebank database.
10. Molecular Detection of *E. coli*, *S. aureus* and *S. typhi* from ‘Panipuri water’ and sugarcane juice samples by PCR

This part of the study deals with the detection of food pathogens from ‘Panipuri water’ and sugarcane juice samples available in the local market. These food samples were analyzed for the presence of *E. coli*, *S. typhi* and *S. aureus* from 20 different samples; 10 each of water preparation of panipuri and sugar cane juice respectively using PCR.

Collection of material
A total 20 samples were collected; ten samples each of water preparation of ‘Pani Puri’ and sugar cane juice, respectively. Samples were collected in sterile screw capped tubes from the local street vendors of V.V. Nagar. All the samples were directly transported to the laboratory under aseptic conditions. Labeling of samples was done from 1 to 10.

Maintenance of aseptic condition
All the samples were processed under aseptic conditions for microbial analysis. Food samples were subjected to serial dilution. Before processing, personal & equipment hygiene was maintained by wearing clean apron and by cleaning hands with 95 % ethanol. External contamination of the samples was prevented. Laminar surface was wiped with 95 % ethanol.

Preliminary Microbial analysis of samples
Prior to PCR analysis, preliminary microbial analysis was performed. Following parameters were analyzed:

1. Total plate count
2. *E. coli* count
3. Coliform count

4. Yeast and mold count

**Coliform and *E. coli* count**

10 ml food samples were suspended in 90 ml of sterile peptone water. Each sample was serially diluted up to $10^{-6}$. For total plate count, 0.1 ml aliquote from each dilution was plated on Nutrient agar plates and plates were incubated at 37°C overnight. Next day, colonies were counted and recorded accurately according to the dilution of the samples. In this way, violet red brilliant agar (VRBA) and Eosine methylene blue (EMB) agar were used for coliform and *E. coli* count.

**Yeast and Mold count**

Potato Dextrose Agar (PDA), pH 3.5 ± 0.2 was used for yeast and mold count of food samples. Dilutions were prepared from the samples. Then 0.1 ml of aliquote was taken from each dilution and spread on the solidified PDA plates. These plates were incubated at 25°C for 4-5 days.

**Preservation of colonies**

Bacterial colonies from N-agar plates were transferred to 2 ml vials (appendroff tubes) under aseptic conditions using a sterile wire loop. Colonies were suspended in 100 µl of sterile D/W, mixed properly for 5 to 10 sec, centrifuged to pellet out and colonies were preserved in 50 % glycerol. Prior to PCR analysis, the culture was transferred to enrichment broth for 18 hours for incubation.

**PCR analysis**

For PCR analysis, boiling centrifugation method was used as described by Soumet et al (1994). In this, one ml aliquots of enrichment broths were centrifuged at 13,000 rpm for 3 minutes. The pellets were resuspended in 100 ml of sterile distilled water, heated to 95°C in a temperature controlled water bath for 10 min, cooled in ice and centrifuged at 13,000 rpm for 3 min. The resulting supernatants were directly used for PCR assay. 2 µl of supernatant was used for amplification in the final reaction mixture.
Materials and Methods

Isolation of DNA from bacterial strains of *E. coli*, *S. typhi* and *S. aureus*

DNA was isolated from *E. coli*, *S. typhi* and *S. aureus* according to Sambrook et al., (1987) and these DNA samples were used as positive control in all PCR reactions.

In detail, 2 ml of overnight grown culture was taken, centrifuged at 8000 rpm for 12 min. To the pellets, 260 µl of digestion buffer [100 mM Tris HCL, 100 mM EDTA, 0.5 % SDS (pH 8.0)] and 400 µl of proteinase K was added, incubated at 60°C for 1 hour in water bath. After this, 20 µl of lysozyme was added, incubated for 5 min; 30 µl of RNase was added, incubated for 5 min and centrifuged at 7500 rpm for 25 min. To the supernatant, 450 µl of phenol, 500 µl of chloroform:isoamyl alcohol (24:1) and 50 µl Tris buffer [10 mM Tris HCL, 1 mM EDTA (pH 7.5)] added, shaken well, centrifuged at 8000 rpm for 15 min, aqueous phase was taken out and again treated with chloroform:isoamyl alcohol, centrifuged at 8000 rpm for 15 min. Aqueous phase was again collected and double the volume of ethanol or isopropyl alcohol was added, centrifuged at 7800 rpm for 10 min. The resulting pellets were dissolved in TE buffer and DNA concentration was quantified by UV spectrophotometer at 260 and 280 nm.

Master Mix preparation for PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X buffer</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>25 mM MgCl2</td>
<td>2 µl</td>
</tr>
<tr>
<td>dNTPs mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>DNA sample</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1 µl (0.3 µg/µl)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1 µl (0.3 µg/µl)</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

Total reaction volume was made up to 50 µl by adding 42 µl sterile double distilled water.
Primers used:

**E-Coli:**

Primers:-
EXPAL-L’5-GGCAATTGCAGCATGTTCTTCC-3’
EXPAL-R’5-CCGCGTGACCTTCTACGGTCGAC-3’ (Kuhnert et al., 1995)

PCR reaction was carried out in a PCR tube in PCR thermal cycler (Corbett Research, Australia).

Reaction cycle:
Cycle 1:- 3 min 94°C
Cycle 2 (40 cycles):-
30 sec 94°C
30 sec 60°C
1 min 72°C
Cycle 3 :- 72°C for 7 min

**S. aureus:**

The pair of primers detecting S. aureus consisted of
RDR327: 5’ GCC GGT GGA GTA ACC TTT TAG GAG C3’
DG74: 5’ AGG AGG TGA TCC AAC CGC A 3’.

PCR cycle:
Cycle 1: 95°C for 5 min
Cycle 2 (40 cycles):
95°C for 1 min,
58°C for 1 min,
72°C for 1 min,
Cycle 3: 72°C for 10 min

**S. typhi:**

For S. typhi, primers used were specific for a 389 bp fragment of invA gene and each reaction included 10 pmol of the

Forward primer Salm 3 (5’-GCTGCGCGCAACGGCGAAG-3’) and
Materials and Methods

Reverse primer Salm 4 (5'-TCCGGCAAGGTTCCCATT-3').

Amplification of *S. typhimurium* targeting *inv A* consisted of:

Cycle 1: 95°C for 5 min;
Cycle 2: 40 cycles of
95°C for 90s,
58°C for 80s,
72°C for 2min.; and
Cycle 3: 72°C for 7min (Stevens and Jaykus, 2004).

The amplified DNA was detected after electrophoresis in 1.5 % Agarose gel containing 5 mg/ml ethidium bromide. The gel was visualized on a UV-light transilluminator (HaakeBuchler, Saddle Brook, NJ, USA). The photograph of gel was captured using α –Inotech Gel Documentation Unit.
11. Effect of enrichment media for PCR detection of *Lysteria monocytogenes* and *S. aureus* from vegetable gravy

The present study was conducted (1) to develop a PCR-based method that can detect *L. monocytogenes* and *S. aureus* in enriched food suspension, (2) to determine whether the type of enrichment medium has an influence on the sensitivity and on the time required for detection of organisms using PCR based method.

**Bacterial strain and culture conditions**

*Listeria monocytogenes* MTCC 657 and *Staphylococcus aureus* subspp. *aureus* MTCC 740 strain were used for artificial contamination of vegetable gravy. *Listeria monocytogenes* was maintained on tryptic soy yeast extract agar (TSAYE, Fluka) plates with weekly sub-cultivation. Tryptic soy yeast extract broth was inoculated from these plates and incubated at 37°C for 18-20 hours for preparing inocula for artificial contamination of food samples. *Staphylococcus aureus* was maintained in Brain Heart Infusion (BHI) agar (Hi media) plates at 4°C.

**Enrichment Media**

Half Fraser (HF) (Merck, Mumbai), buffered peptone water (BPW) (Merck, Mumbai), Triptic Soy Yeast Extract Broth (TSBYE) (Merck, Mumbai) and universal pre-enrichment broth (UPB) (Difco, USA) were used as enrichment broths for *L. monocytogenes*. Listeria selective agar plates (Titan Biotech, Delhi) plate was used as selective medium for Listeria isolation.
Preparation of vegetable gravy

Vegetable gravy prepared in the laboratory was used as the model food system for the growth of *L. monocytogenes* and *S. aureus*. Potato, tomato and other ingredients were obtained from the local vegetable market. 500 gm potatoes were boiled, peeled and diced. The other ingredients consisted of: 1 gm each of cooked, diced vegetables - carrots, beans, peas and cauliflower; 0.5 gm chopped onions; 0.5 gm ghee; 4 green chopped chillies; 250 gm chopped tomato; 1 table spoon red chilli powder; 1 table spoon ginger garlic paste; pinch of salt to taste. Ghee was heated in a pan, then onion, green chillies, ginger garlic paste and tomatoes were added, sautéed over medium heat for 2-3 minutes. After that, red chilli powder, mixed vegetables and potatoes were added. It was cooked for 15 minutes. This gravy preparation was used for inoculation of *L. monocytogenes* and *S. aureus*.

Inoculation of *L. monocytogenes* and enrichment

Enrichment of food sample was tested in four enrichment broths (Half Fraser, Buffered Peptone Water, Tryptic Soy Yeast Extract Broth and Universal Pre-enrichment Broth) for *L. monocytogenes*. $10^{-1}$……$10^{-4}$ dilutions of an 18-20 hour culture of *L. monocytogenes* were made in sterile Buffered peptone water. 1 ml of buffered peptone water (pH 7.2 ± 0.1) (BPW) containing varying counts of *L. monocytogenes* was added to 200 ml of each of the broths (HF, BPW, TSBYE and UPB).

Enrichment broths (200 ml) inoculated with 1 ml of peptone water (containing different dilutions of *L. monocytogenes*) was added to homogenised vegetable gravy (25 gm). It was then incubated at 30°C for 24 hours. The flow chart is shown as follows:
Overnight (18-20 hrs) culture of *L. monocytogenes* in BPW

Dilutions

\[ \times 10^1 \quad \times 10^2 \quad \times 10^3 \quad \times 10^4 \]

Dilutions inoculated into broths

HF, BPW, TSBYE, UPB

HF, BPW, TSBYE, UPB

HF, BPW, TSBYE, UPB

HF, BPW, TSBYE, UPB

200 ml of above + 25 gm Vegetable gravy

200 ml of above + 25 gm Vegetable gravy

200 ml of above + 25 gm Vegetable gravy

200 ml of above + 25 gm Vegetable gravy

Incubated at 30˚C for 24 hrs
The number of *L. monocytogenes* cells in the inoculum was determined by plating the mixture on Tryptic Soy Yeast Extract Broth (TSAYE) agar. Plates were incubated at 37°C for 48 hours. Three detection procedures were applied to each sample. The flow chart is as follows:
Food sample (25 gm) + Enrichment Broth (200 ml)

Artificial contamination with *L. monocytogenes* 

\(10^1\)-\(10^4\) cfu), mixed for 3 min

Incubation for 24 hrs at 30°C

Procedure A

Procedure B

Procedure C

Three different procedures were carried out to evaluate the effect of subculturing, different media and incubation time on the detection of organisms by PCR

**Procedure A:** Detection of *L. monocytogenes* using PCR after 24 hrs incubation

**Procedure B:** Detection of *L. monocytogenes* using PCR after 48 hrs incubation without subculturing

**Procedure C:** Detection of *L. monocytogenes* using PCR after 48 hrs incubation with subculturing
Legend used in this study:

1) EB: Enrichment Broth (4 in number) - HF, BPW, TSBYE and UPB

2) EB,A: detection of *L. monocytogenes* with PCR after **24 hrs** incubation from enrichment broth

3) EB,B: detection of *L. monocytogenes* with PCR after **48 hrs** incubation from enrichment broth **without subculturing**

4) EB,C: detection of *L. monocytogenes* with PCR after **48 hrs** incubation from enrichment broth **with subculturing**

5) CO: detection of *L. monocytogenes* with PCR from **suspect colonies on Listeria selective agar plates**

6) CO,A: detection of *L. monocytogenes* with PCR **after 24 hrs** incubation from suspect colonies on Listeria selective agar plates

7) CO,B: detection of *L. monocytogenes* with PCR from suspect colonies on Listeria selective agar plates **after 48 hrs** incubation of enrichment broth **without subculturing**

8) CO,C: detection of *L. monocytogenes* with PCR from suspect colonies on Listeria selective agar plates **after 48 hrs** incubation of enrichment broth **with subculturing**
Procedure A (detection of *L. monocytogenes* using PCR after 24 hrs incubation):

Two ml of incubated enrichment broth were used for PCR detection (PCR\textsubscript{EB, A}) of *L. monocytogenes*. Enrichment broth was also streaked on Listeria selective agar plates (Titan Biotech, Delhi). The plates were incubated at 37°C for 24 hours. Upto five suspected listeria colonies were picked up from Listeria selective agar plates for identification using PCR (PCR\textsubscript{CO, A}). Flow chart is as follows:
Procedure A

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *L. monocytogenes* (10\(^1\)-10\(^4\) cfu), mixed for 3 min,

Incubation for 24 hrs at 30˚C

Taken for PCR

Taken for plating

**PCR\(_{EB,A}\)**

Listeria selective agar plates

Incubation at 37˚C for 24 hrs

**PCR\(_{CO,A}\)**
**Procedure B:** The 24 hour enrichment broth was re-incubated for an additional 24 h at 30°C. Two ml of 48 hour enrichment broth was used for PCR detection (PCR$_{EB}$, B) of *L. monocytogenes*. 48 hour enrichment broth was also streaked on Listeria selective agar plates. The plates were incubated at 37°C for 24 hrs. Upto five suspect listeria colonies were picked up from Listeria selective agar plates for identification using PCR (PCR$_{CO}$, B). Flow chart is as follows:
Procedure B

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *L. monocytogenes* (10^1^-10^4 cfu), mixed for 3 min

Incubation for 24 hrs at 30°C

Taken for PCR  
Taken for plating

PCR_{EB,B}  
Listeria selective agar plates

Incubation at 37°C for additional 24 hrs

PCR_{CO,B}
Procedure C: One ml aliquot from the 24 hour enrichment broth was subcultured to a 9 ml portion of enrichment broth and reincubated for an additional 24 hours at 30˚C. After that, 2 ml of enrichment broth was used for PCR detection (PCR\(_{EB,c}\)) of *L. monocytogenes*. Enrichment broth was also streaked on Listeria selective agar plates. Plates were incubated at 37˚C for 24 hour. Upto five suspected listeria colonies were picked from Listeria selective agar plates for identification using PCR (PCR\(_{CO,c}\)). Flow chart is as follows:
**Procedure C**

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *L. monocytogenes* 

(10^1-10^4 cfu), mixed for 3 min,

incubation for 24 hrs at 30°C

Enrichment broth having food sample

From this, 1 ml was transferred

1 ml culture transferred in 9 ml Enrichment Broth

Incubation for 24 hrs at 30°C

used for PCR 

Listeria selective agar plates

Incubation at 37°C for 24 hrs 

Used for plating 

**PCR**

**EB,C**

**CO,C**
Inoculation of *S. aureus* and enrichment

Four enrichment broths Trypton soy broth (TSB), Baird staphylococcus enrichment Broth (BSEB), Staphylococcus Aureus enrichment broth (SAEB) and Luria broth (LB) were tested for *S. aureus*.

1 ml of buffered peptone water (pH 7.2 ± 0.1) (BP) containing varying counts of *S. aureus* was added to each of the broths (TSB, BSEB, SAEB and LB). 10⁻¹ dilutions of an 18-20 hour culture of *S. aureus* were made in sterile BP. Enrichment broths (200 ml) inoculated with 10 cfu, 10² cfu, 10³ cfu and 10⁴ cfu of *S. aureus* were added to homogenised vegetable gravy (25 gm). Homogenization was carried out with the help of a blender for 5 min. It was then incubated at 30°C for 24 hours. The number of *S. aureus* cells in an inoculum was determined by plating decimal dilutions of *S. aureus* culture suspension in BP on Baird Parker agar plates. Plates were incubated at 37°C for 48 hours. Aliquot of 25 gm of food sample was always checked for the naturally present *S. aureus* (without inoculation). All enrichment broths were incubated at 30°C for 24 h. Three detection procedures were applied to each sample. The flow chart is as follows:
Materials and Methods

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *S. aureus* (10^1-10^4 cfu), mixed for 3 min

Incubation for 24 hrs at 30°C

Procedure A

Procedure B

Procedure C

Three different procedures were carried out to evaluate the effect of subculturing, different media and incubation time on the detection of organism by PCR

**Procedure A:** detection of *S. aureus* using PCR after 24 hrs incubation

**Procedure B:** detection of *S. aureus* using PCR after 48 hrs incubation without subculturing

**Procedure C:** detection of *S. aureus* using PCR after 48 hrs incubation with subculturing
**Procedure A:** Two ml of incubated enrichment broth were used for PCR detection ($\text{PCR}_{\text{EB},A}$) of *S. aureus*. Enrichment broth was also streaked on Baird Parker agar plates (Titan Biotech, Delhi). The plates were incubated at 37°C for 24 hours. Upto five suspected staphylococcus colonies were picked up from Baird Parker agar plates for identification using PCR ($\text{PCR}_{\text{CO},A}$). Flow chart is as follows:
Procedure A

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *S. aureus* 

\((10^1 - 10^4 \text{ cfu})\), mixed for 3 min

Incubation for 24 hrs at 30˚C

Enrichment broth having food sample

used for PCR

Used for plating

\(\text{PCR}_{\text{EB,A}}\)

\(\text{Baird Parker agar plates}\)

Incubation at 37˚C for 24 hrs

\(\text{PCR}_{\text{CO,A}}\)
Procedure B: The 24 hour enrichment broth was re-incubated for an additional 24 h at 30°C. Two ml of 48 hour enrichment broth was used for PCR detection ($\text{PCR}_{\text{EB}, \ B}$) of *S. aureus*. Also, 48 hour enrichment broth was streaked on Baird Parker agar plates. The plates were incubated at 37°C for 24 h. Up to five suspected staphylococcal colonies were picked up from Baird Parker agar plates for identification using PCR ($\text{PCR}_{\text{CO}, \ B}$). Flow chart is as follows:
Procedure B

Food sample (25 gm) + Enrichment Broth (25 ml)

Artificial contamination with *S. aureus* 

$(10^1-10^4 \text{ cfu})$, mixed for 3 min

Incubation for 24 hrs at 30°C

Enrichment Broth having food sample

Incubation for 24 hrs at 30°C

Used for plating

used for PCR

PCR$_{EB,B}$ Baird Parker agar plates

Incubation at 37°C for additional 24 hrs

PCR$_{CO,B}$
Procedure C: One ml aliquot from the 24 hour enrichment broth was subcultured to a 9 ml portion of enrichment broth and reincubated for an additional 24 hours at 30˚C. After this, 2 ml of enrichment broth was used for PCR detection ($\text{PCR}_{\text{EB, C}}$) of \textit{S. aureus}. Enrichment broth was also streaked on Baird Parker agar plates. Plates were incubated at 37˚C for 24 hour. Up to five suspected staphylococcal colonies were picked up from Baird Parker agar plates for identification using PCR ($\text{PCR}_{\text{CO, C}}$). Flow chart is as follows:
**Procedure C**

Food sample (25 gm) + Enrichment Broth (25 ml)

- Artificial contamination with *S. aureus* (10^1-10^4 cfu), mixed for 3 min
- Incubation for 24 hrs at 30°C
- From this, 1 ml was transferred
- 1 ml culture in 9 ml Enrichment Broth
- Incubation for 24 hrs at 30°C
- Used for PCR
- Used for plating
- **PCR**
- **Baird Parker agar plates**
- Incubation at 37°C for additional 24 hrs
- **PCR**
Materials and Methods

**PCR template preparation**

PCR templates were prepared from 24 hour enrichment and 48 hour enrichment broths and from upto 5 suspected listeria (on Listeria selective agar plates) or *S. aureus* colonies (Baird-Parker agar plates). PCR template was prepared from both the organisms using boiling method according to Ran He et al. (2005).

Overnight-grown culture from enrichment broth was harvested by centrifugation at 10,000 rpm for 15 min. The pellet was washed once with double-distilled water and most of the supernatant was removed. Then the pellet was vortexed after adding 100 µl sterile distilled water and placed in a boiling water bath with a closed cap. Boiling for 2 min was followed by 1 min centrifugation at 10,000 rpm. The supernatant was saved and stored at -20˚C in the freezer until PCR was performed.

**PCR detection of *L. monocytogenes***

A reaction mixture of 50 µl was constituted which contained 5 µl of PCR buffer 10x (Tris-HCl 100 mmol l⁻¹, KCl 500 mmol l⁻¹, Triton X-100 1 %) (Banglore Genei), 3 mmol l⁻¹ MgCl₂ (Bangalore Genei), 150 µmol l⁻¹ of each dNTP (dATP, dTTP, dCTP, dGTP) (Banglore Genei), 1 µmol l⁻¹ of each primer (LL5: AAC CTA TCC AGG TGC TC , LL4: CGC CAC ACT TGA GAT AT) (Primers were selected according to Thomas et al., 1991) (MWG Biotech, Germany), 0.2 µl Tween 20 (0.05 g ml⁻¹) (Banglore Genei), 0.5 U Taq DNA Polymerase (Invitrogen) and 1 µl of cell lysate. Total reaction volume was made upto 50 µl by adding nuclease free sterile distilled water.

PCR assays were performed in a DNA thermal cycler (Corbett Research, Australia). Template DNA was initially denatured at 95˚C for 5 min followed by 40 cycles of 1 min denaturation at 94˚C, 1 min primer annealing at 60˚C and 2 min extension at 72˚C. After a final 8 min extension step at 72˚C, tubes were held at
4°C. Control samples were included in each PCR. The positive control was standard containing approximately $10^7$ cells ml$^{-1}$ \textit{(L. monocytogenes MTCC 657)}. In negative control, 1 μl of sterile distilled water was used instead of bacterial cell lysate.

**PCR detection of \textit{S. aureus}**

A reaction mixture of 50 μl contained 5 μl of PCR buffer 10x (Tris-HCl 100 mmol l$^{-1}$, KCl 500 mmol l$^{-1}$, Triton X-100 1 %) (Banglore Genei), 3 mmol l$^{-1}$ MgCl$_2$ (Banglore Genei), 150 μmol l$^{-1}$ of each dNTP (dATP, dTTP, dCTP, dGTP) (Bangalore Genei), 1 μmol l$^{-1}$ of each primer (Forward RDR327: 5' GCC GGT GGA GTA ACC TTT TAG GAG C3' and Reverse DG74: 5' AGG AGG TGA TCC AAC CGC A 3’) (MWG Biotech, Germany), 0.2 μl Tween 20 (0.05 g ml$^{-1}$) (Banglore Genei), 0.5 U Taq DNA Polymerase (Invitrogen) and 1 μl of cell lysate.

Total reaction volume was made upto 50 μl by adding nuclease free sterile distilled water. The length of the resulting amplicon was 105 bp. (Natar, Khodoud et al., 1999).

PCR cycle for \textit{S. aureus} consisted of

- **Cycle 1:** 95°C for 5 min
- **Cycle 2:** 95°C for 1 min,
  - 58°C for 1 min,
  - 72°C for 1 min, (40 cycles)
- **Cycle 3:** 72°C for 10 min

**Detection of amplification products**

A portion (8 μl) of PCR product was analysed by electrophoresis in 1.5 % agarose gel containing ethidium bromide (0.5 μg ml$^{-1}$) in Tris-acetate-EDTA buffer 0.5 X at 14 V cm$^{-1}$. The gels were visualised in UV-light and documented by Gel Doc (Bio-Rad, USA). A known molecular weight marker 100 bp DNA Ladder (Banglore Genei) was analysed along with samples.
**PCR results interpretation**

Presence of DNA amplification product of 520 bp was taken as a positive result for the presence of *L. monocytogenes* in the food sample. Similarly, the Presence of a DNA amplification product of 105 bp was taken as a positive result for the presence of *S. aureus*.