Methodology
METHODOLOGY

The present study was undertaken with two major objectives:—

1) To determine the awareness among consumers regarding safety/quality of ready-to-eat (RTE) foods and
2) To determine the extent to which RTE foods sold in Bombay are microbiologically safe for consumption.

PROTOCOL OF THE STUDY

Part-I

The project was divided into two parts. Part-I consisted of two surveys conducted with different objectives as described below:

1) The Initial Survey: The primary purpose of this survey was to use the information obtained from it as a basis to determine the most commonly RTE foods and to select the same for microbiological analysis. Information sought through this survey was:

i) the frequency of eating out and the pattern of consumption,

ii) the particular types of RTE foods that are popular,

iii) the source of purchase,

iv) monthly expenses on RTE foods and

v) general awareness regarding safety/quality of RTE foods
The Second Survey was conducted simultaneously with the microbiological analysis of food samples. The main objective of this survey was to find out consumers' practices and knowledge/awareness regarding safety/quality of RTE foods. For this, a precoded questionnaire was constructed to obtain the following information.

A) Aspects included in knowledge/awareness among the consumers were:

i) knowledge regarding the types of food that generally spoil faster,

ii) knowledge about the modes of transmission of organisms and sources of contamination of food at various stages of production,

iii) awareness regarding food hygiene during production and distribution/sales,

iv) the particular RTE foods that they think generally carry food poisoning organisms and cause infection/poisoning,

v) awareness/knowledge and attitudes regarding the nutritional content of various RTE foods,

vi) awareness regarding their rights and responsibilities,

vii) awareness regarding the procedures for redressal and

viii) awareness regarding the various standards laid for the manufacturers involved in the production of food.

B. The aspects looked for the general practices followed while purchasing RTE foods were -
i) the frequency of consumption from different types of outlets,

ii) total amount spent on particular RTE food per month from different types of outlets,

iii) whether particular RTE food(s) are avoided and the reasons for the same,

iv) whether they are careful about the type of outlet and whether they avoid particular kinds of outlets,

v) while purchasing a RTE food do they -
   (a) see that the food is served hot (wherever appropriate),
   (b) try to find out when and where the food was made and
   (c) try to find out what ingredients went in preparation of the food.

vi) whether they consider the health and hygiene of food handlers,

vii) whether they look for any signs of staleness of the food,

viii) if served with a substandard RTE food, whether they would report it,

ix) whether they have had illness episodes due to consumption of RTE foods and whether they reported it,

x) whether they look for nutritional content of RTE foods and

xi) whether they avoid any specific RTE food(s) because of some nutritional reasons.
Microbiological analysis was done for the popular RTE food samples chosen as per the information obtained through the initial survey. The main objective was to find out the extent to which the RTE foods sold in the market are safe for consumption. For this, various samples were analysed for indicator organisms and some pathogenic organisms.

DESIGN OF STUDY

Part-I : Survey - I

The initial survey was conducted on 300 respondents. The purpose of the survey was to choose the most popular RTE foods for microbiological analysis. Pretesting of the questionnaire was done on 20 respondents to see if any modifications were required. The questionnaire is given in the Appendix-I. Three hundred families were selected on the basis of total monthly income using the following cut-off points.

i) Group-I : Those earning more than Rs.8,000/- per month.

ii) Group-II : Those earning Rs.2000/- to Rs.8000/- per month.

iii) Group-III: Those earning less than Rs.2000/- per month.

From each of these groups 100 families were selected for the survey. To obtain a representative sample, Bombay was divided into three main areas -
i) Group-I - Cuffe Parade, Nepeansea Road, Colaba, Peddar Road, Pali Hill, Malabar Hill and Juhu.

ii) Group-II - Dadar, Santacruz, Andheri and other suburban areas upto Borivli.

iii) Group-III - Slums and chawls in the suburban areas.

The respondents were primarily selected on the basis of their willingness to respond to the questionnaire.

Executives, businessmen, officers, college staff, students, housewives and other individuals were approached with a request to respond to the questionnaire.

Respondents were approached at their residence or their working places after prior appointment. They were first explained the purpose of the survey and were assured confidentiality of the information given. They were explained in detail how to answer the questionnaire and doubts, if any, were cleared. In case the language of the question/questionnaire was not clear, the question was explained in the local language and the respondent helped to fill up the form. They were given enough time to answer the questionnaire. On an average, the respondents required ten to twelve minutes to answer the questionnaire.

Survey - II

The second survey undertaken was an indepth one on consumer awareness regarding safety/quality of RTE foods. For this, a questionnaire was developed with the objective of
finding out people's attitudes, practices and knowledge/awareness regarding safety/quality of RTE foods. The objectives of the aspects looked for have been given in the protocol of the study. Most questions were of the closed-end type to make it easier for the respondent to answer. In the case of some of the closed-end type of questions, the question ended with an open-end format leaving some choice for the respondent as well as allowing the respondent to qualify the choices made or to elaborate upon answers given. For some aspects of knowledge, a five-point rating scale was used.

Pretesting of the schedule

The survey schedule was pretested on 20 individuals before administering it to the actual study sample, for its accuracy and validity in fulfilling the set objectives. Required changes were made wherever it was felt necessary. The questionnaire is shown in Appendix-I.

Study population

The target population involved 500 individuals. They were chosen from different educational levels, ages, occupation, income and family background.

The respondents were primarily selected on the basis of their willingness to respond to the questionnaire. Only those who eat RTE foods and earn more than Rs.2,000/- per month were chosen. Different educational levels ranging from
non-graduates to professionals like doctors, engineers, scientists, and different occupations like clerical jobs, teaching, management, business, housewives and students were chosen. The age of the respondents ranged from 20-60 years.

Respondents were approached personally at their residence or their working places/colleges, after prior appointment. They were first explained the purpose of the survey and were assured confidentiality of the information given. They were explained in detail how to answer the questionnaire and doubts, if any, were cleared. They were given enough time to answer the questionnaire. Most of the respondents approached at their working premises could not respond to the questionnaire immediately and needed to be reminded and reapproached.

On an average, the respondents required 15-17 minutes to answer the questionnaire. Questionnaires returned blank or with less than 50% of the responses were determined invalid and excluded from the analysis. Out of 500 questionnaires, 394 were returned duly filled in (78.8%).

Part-II
Microbiological Analysis of the Selected Ready-To-Eat Foods
Sample selection

Selection of samples for microbiological analysis depended on the popularity of the food item which was derived from the analysis of survey (I) Protocol-I.
Classification of samples

A. **Vegetarian**
   1) Fried snacks.
   2) Steamed snacks.
   3) Baked snacks.
   4) Roasted snacks.

A. **Vegetarian**
   5) Other savouries.
   6) Milk-based sweets.
   7) Other sweets.
   8) Other RTE foods, e.g. Bhel puri, panipuri, etc.

A. **Non-vegetarian**
   1) Burgers.
   2) Patties/Cutlets.
   3) Pizzas.
   4) Samosas.

A. **Non-vegetarian**
   5) Lollypops.
   6) Rolls.
   7) Chops.
   8) Kababs.

The samples were picked from both urban and suburban shops and fast food counters on streets and railway stations within the city. A total of 25 vegetarian and 9 non-vegetarian food items were chosen. Three varieties of chutneys served with the RTE foods chosen, were also analysed separately bringing the total to 37 samples. The details of the samples analysed are given in Table-7.

Collection of samples

Standard procedures recommended by the FAO were followed for collection of samples. Totally 907 samples were collected from retail outlets. Packed samples were collected as such and open samples were collected in clean, dry, sterile, leakproof containers with wide mouth to contain 200 g
<table>
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<tr>
<th>CATEGORY</th>
<th>ITEM NO.</th>
<th>NAME OF SAMPLE</th>
<th>NO. OF SAMPLES</th>
<th>TYPES OF OUTLETS</th>
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<td>A. VEGETARIAN RTE FOODS</td>
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<td></td>
<td>20</td>
<td>KHOA</td>
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<td>18</td>
<td>Y Y</td>
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Note: Y - samples collected.
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<td>X. CHUTNEYS</td>
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<td>GREEN</td>
<td>37</td>
<td>66</td>
<td>69</td>
<td>Y</td>
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</table>

Note: * In case of Panipuri, puri, pani and Ragda were analysed separately.
** All the chutneys served with RTE foods were analysed separately.
Y Samples collected.
of sample. Liquid samples were collected in either disposable plastic bags or containers with screw-type caps. The samples were analysed within two hours of collection. Before removing the sample for analysis the outer surface of the packs was cleaned by washing or sponging with 70% alcohol. The packs were opened with a sterile cutting instrument and processed further.

**Processing of samples**

Standard FAO procedures were followed for processing of food samples. Approximately 200-250 grams of the food sample was homogenised by cutting/slicing/mashing and/or stirring with appropriate sterile instruments. Twenty five grams of such a sample was then blended with 225 ml of Buffered Peptone Water (BPW) in a sterile blender. This gave a dilution of $10^{-1}$. One ml of $10^{-1}$ dilution was then transferred to a test tube containing 9 ml of sterile BPW to get $10^{-2}$ dilution. Subsequent higher dilutions were made in a similar manner up to $10^{-7}$ for most food samples.

Quantitative microbiology included Total Viable Count (TVC) and total counts of *Staphylococcus aureus*, fecal coliform, fecal streptococci and sulphite-reducing Clostridia.

Qualitative microbiology included detection of the presence of species of *Salmonella*, *Shigella*, Coagulase positive *Staphylococci* and *Yersinia enterocolitica.* Commercially available dehydrated media (Hi-media) were used.
for the analysis and were prepared as per manufacturer's instructions. Stains, indicators and various reagents were prepared as per standard procedures (FAO, 1979; Cheesbrough, 1985 and standard APHA technique, 1966).

Preliminary Bacteriological Procedures

Quantitative estimations

1) Total Viable Count (TVC)

Standard pour plate technique was employed for the estimation of TVC. One ml from each of the three chosen dilutions was dispensed on a sterile petridish in duplicates to which molten Plate Count Agar (PCA), cooled to 45 - 50°C was poured and mixed thoroughly by rotating the plates. These plates were incubated at 37°C for 24-48 hours and colonies were counted on a colony counter. Average of plates showing countable colonies (plates with 30-300 colonies) was used for calculating the TVC.

2) Fecal Coliform Count

Violet Red Bile Agar (VRBA) was used for rapid identification and enumeration of fecal coliforms following pour plate technique (Anonymous, 1962). One ml inoculum from each of the three chosen serial dilutions was dispensed into a sterile petri plate in which molten VRBA (45-50°C) was poured. Plates were incubated at 44°C for 24-48 hours. Typical coliform colonies showing deep red-purple colonies with zone of bile precipitate were counted (Weling, 1978).
3) **Fecal Streptococcal Count**

The method followed as prescribed in the FAO manual (1979) Bile Esculin Azide Agar (BEAA) was used for enumeration of fecal streptococci. An inoculum of 0.1 ml was spread on plates of BEAA with an 'L'-shaped glass rod. The plates were incubated at 44°C for 24-48 hours. Typical pinpointed black colonies were identified as fecal streptococci.

4) **Staphylococcus aureus** count

The method used for enumerating Staphylococci was as per USP XXI (1985). The procedure remained same as has been described for the fecal streptococcal count. After inoculation on Vogel-Johnson Agar (VJA) plates they were incubated at 37°C for 24-48 hours. Typical black colonies with yellow halo were counted as *Staphylococcus aureus*. Coagulase test was performed as per FAO manual (1979) for confirmation with a slight modification, i.e. in place of rehydrated rabbit plasma, fresh rabbit plasma was used.

5) **Clostridial Count**

Sodium Polymyxin Sulphadiazene Agar (SPS Agar) was used for enumeration of *Clostridium* species (Angelotti et al., 1961). A quantity of 0.1 ml from each of the three chosen serial dilutions was incorporated in Veillon's tube, 4-5 ml molten SPS agar (45-50°C) was poured over it and shaken. After setting, another layer of SPS agar was poured over it and incubated at 44°C for 24-48 hours. Typical black cotton like colonies were counted as Clostridia.
Qualitative estimations

This was aimed at detecting the possible presence of a few of the common bacterial pathogens in the samples of RTE foods.

Various differential and selective media (Hi-media, Bombay) were used employing spread plate technique. Inoculum (0.1 ml) from lowest dilution ($10^{-1}$) was inoculated into the appropriate media and spread uniformly with the help of a sterile 'L'-shaped glass rod.

1) Detection of Salmonella

The method as described in FAO manual (1979) was followed with minor modifications in the media used, i.e., in place of Brilliant Green Phenol Red Agar and Bismuth Sulfite Agar, Salmonella Shigella Agar (SSA) and Brilliant Green Agar (BGA) were used. Ten grams of sample was added to 20 ml of BPW and incubated at 37°C for 24 hours for pre-enrichment. The inoculum was added in Selenite Cystine Broth (SCB) and incubated at 44°C for 18 hours for enrichment. After incubation, inocula was streaked into BGA and SSA plates simultaneously and incubated at 37°C for 24 hours. Pink colour colonies on BGA and pink-red colonies with black centres on SSA were further confirmed as Salmonella. Several biochemical tests were performed as stated in the FAO manual (1979).
Biochemical Tests

i) TSI Agar : Red slope and yellow butt with bubbles or cracks.

ii) Urease : Negative.

iii) Motility : Positive.

iv) Indole : Negative.

v) VP reaction : Negative.

vi) Carbohydrate fermentation : Mannitol and glucose positive.

For carbohydrate fermentation tests, ready-made Hi-media discs were used for the respective carbohydrate fermentation tests. The procedures followed was as per the instructions given by the manufacturer on individual packs. Discs of lactose, mannitol, glucose, sucrose, oxidase and citrate were used for testing.

The details of the procedure of other biochemical tests is given in the Appendix-II.

Serotyping

Salmonella cultures were sent to Central Research Institute (CRI), Kasauli for serotyping.

2) Detection of Coagulase positive Staphylococci

Standard APHA technique (1966) was followed for qualitative detection of Staphylococcal spp. An inoculum of 0.1 ml from 10^-1 dilution was spread on plate of mannitol salt agar with the help of sterilised 'L' shaped glass rod and incubated at 37°C for 24-48 hours. Bright yellow colonies were picked up and transferred to nutrient broth and
incubated at 37°C for 24-48 hours for further confirmation using following criteria.

i) Hemolysis Test

Inoculum was streaked onto blood agar plates and incubated at 37°C for 24 hours.

ii) Coagulase Test

Standard procedure recommended by FAO was followed with minor modifications (in place of rehydrated rabbit plasma, fresh rabbit plasma was used). A test tube containing 5 ml of Brain Heart Infusion (BHI) broth was inoculated and incubated at 37°C for 24 hours. From the resulting growth 0.1 ml was transferred to 0.3 ml of fresh rabbit plasma in small coagulation tubes and incubated at 35°-37°C. Clotting was looked for every half an hour up to 6 hours (FAO manual, 1979).

iii) Mannitol Broth Fermentation Test

The inoculum (0.1 ml) from nutrient broth was dispensed onto the tubes containing mannitol broth and incubated at 37°C for 24 hours. Production of acid was noted.

iv) Catalase Test: Positive (details given in Appendix-II).

Epidemiological Typing

It was done on the basis of differences in the susceptibility of isolates towards discs of six different
antibiotics, viz., Amicacin, Carbenicillin, Cefotaxime, Tobramycin, Cefsulodin and Netilmicyn (Oxoid). It was performed by disc diffusion method as described by Cruickshank et al. (1975), using nutrient agar medium.

Briefly, a swab-full of the nutrient broth culture was plated uniformly on a nutrient agar plate. It was allowed to dry and then six antibiotic discs were gently seeded onto the surface of the agar keeping a gap of about 2 cm between the two discs. A control strain of Staphylococcus (S.aureus ATCC 25923) was used for comparison. All plates were incubated at 37°C for 24 hours. Interpretation was done by referring to the zone size interpretation chart (Bauer et al., 1966). The zone of inhibition was measured by a scale in millimeters both horizontally and vertically and average of both was matched with the given zone size categorised as Sensitive (S), Resistant (R) and Intermediate (I) and were grouped accordingly. The concentration of all antibiotics in discs was 30 μg except for Carbenicillin which was used in a concentration of 100 μg.

3) Detection of Shigella

FAO standard technique (1979) was followed for detection and confirmation of Shigella in the RTE food samples. SSA and Xylose Lysine Deoxycholate (XLD) were used for the detection of Shigella. A quantity of 0.1 ml of 10^-1 dilution of the inoculum was spread on each plate using an 'L' shaped glass rod (sterile). The plates were incubated at
37°C for 24 hours. On SSA, pink colonies and on XLD agar, red colonies were confirmed biochemically for Shigella.

**Biochemical Tests**

Confirmation was done by proceeding with the following biochemical tests:

1. **TSI** : Red slant, yellow butt, no gas, no H₂S.
2. **Urease** : No red color (-ve).
3. **Motility** : Non-motile.
4. **KCN** : No growth.
5. **Indole** : Positive or negative.
6. **VP** : Negative.
7. **Carbohydrate** : No gas.
8. **Citrate** : No growth.

The details of the procedure for biochemical tests is given in Appendix-II.

**Serological Identification**

This could not be performed because of practical difficulties.

**Detection of Yersinia**

Yersinia Selective Agar Base (Hi-media) was used to prepare a selective agar medium for cultivation of *Yersinia enterococitica* to which Yersinia Selective Supplement was added as per the instructions given in Hi-media Product information. It was then poured into petridishes. A quantity of 0.1 ml of $10^{-1}$ dilution of the inoculum was
spread on each plate, using an 'L' shaped sterile glass rod. The plates were incubated at 32°C for 18-48 hrs. Typical Yersinia colonies showed transluscent growth with dark pink centre and bile precipitate.

Serotyping

Yersinia cultures were sent to University of Lund, Department of Medical Microbiology, Malmö General Hospital, Sweden, for serotyping.

Examination of primary culture plates, microscopic examination and motility examination is given in the Appendix-II.

ANALYSIS OF DATA

Part-I

Survey-I

Frequency distribution of the survey data was done and means of expenditures according to three income categories were calculated.

Survey-II

Frequency distribution was taken for all the responses. Knowledge scores were given to the responses to questions on safety aspects, nutritional aspects and total knowledge. The practice and the knowledge scores were then correlated with demographic data like age, sex, educational qualification, occupation, income, marital status and type of family. For this Pearson's correlation and Chi-square were applied using
SPSS. Student's 't' test was also applied wherever applicable.

**Part-II**

To analyse the results of microbiological quality, frequency distribution of the counts was taken. Analysis of variance was done to determine the effect of kind of sample, effect of place and effect of packaging on the quality of RTE foods.