Chapter 3 Methods and Materials

Adolescence is an important phase in human lifespan. According to the World Health Organisation it is defined as 10-18 years of age which is characterised by physical, physiological, psychological and social changes. Hence, it is important to maintain health and nutrition of children in this phase of life. Various forms of undernutrition during adolescence not only affect growth and wellbeing of an adolescent but also affect health, productivity and quality of life as an adult.

School feeding programmes are seen as an effective strategy to improve nutritional status of school going children and adolescents. Mid Day Meal Programme of India is the World's largest school feeding programme Although, it has shown positive impact in terms of improvement in school enrolment, attendance, girl child enrolment as well as nutritional status, prevalence of undernutrition among school children and adolescents remains to be high.

Thus, there is a need to create awareness regarding nutrition, hygiene as well as importance of MDM in order to bring improvement in nutrition related outcomes of the programme with a focus on undernourished children and adolescents. Hence, the present study was carried out to evaluate the functioning of Mid Day Meal Programme in Rural Vadodara as well as to study impact of Nutrition Health Education on the nutritional status of undernourished children studying from 5^{th} to 8^{th} standard of Government primary schools of Rural Vadodara.

The methodology adopted in execution of this study is discussed in this chapter.

MEDICAL ETHICS AND APPROVALS

This study was approved by the Institutional Medical Ethics Committee (Approval no. IECHR/2014/9).

The following approvals were obtained for the study (Annexure 1 to 5):

1. Permission from Deputy Collector and Additional Commissioner Mid Day Meal Programme, Vadodara.
2. Permission from District Education Officer, Vadodara.
3. Permission from Principals of schools.

4. Permission from COO, Akshay Patra, Vadodara.
5. Consent from parents of school children.

Location of the Study: Rural Petrochemicals Area of Vadodara was selected for this study. This area is in the vicinity of the petrochemical companies situated in the outskirts of Vadodara. There are total 47 Government Primary schools in this area out of which 30 schools are co-ed and have both primary and upper primary sections. These 30 schools were purposively included in the sampling frame for further selection of sample.

The entire study was conducted in two phases:

Phase 1: Evaluation of Mid Day Meal Programme in Rural Vadodara

Phase 2: Impact of Nutrition Health Education on the Nutritional Status of Moderate and Severely Undernourished Upper Primary School Children of Rural Vadodara

PHASE 1: EVALUATION OF MID DAY MEAL PROGRAMME IN RURAL VADODARA

This phase was conducted to study the functioning of MDM in the Rural Vadodara. It was a cross-sectional phase which was further divided into three sub-phases:

Phase 1 A- Evaluation of MDM at school level (Academic Year 2017-18)

Phase 1 B- Evaluation of MDM at Centralised Kitchen

Phase 1 C- Nutrient Composition and Quality Attributes of MDM

Sample Selection and Data Collection

As mentioned earlier, rural petrochemicals area of Vadodara was selected for this study and out of 47 Government Primary schools in this area, 30 schools which were co-ed and had both primary and upper primary sections compromised the sampling frame.

Sample selection in Phase I A was done by cluster random sampling. Study design of this phase is shown in (Figure 3.1). The petrochemicals area was divided into three geographical clusters in such a way that each area had 10

co-ed Government primary schools with both primary and upper primary sections. Two schools from each area i.e. total 6 schools were randomly selected for this phase using random number tables. All the children studying in 5^{th} to 8^{th} standard were included in the study after obtaining written consent from their parents. Data on socio economic status, anthropometry and practices and perceptions were collected on all the children enrolled in the study. Information regarding school profile was also collected using a semi structured performa. (Annexure 6) Spot observations were carried out at the time of serving MDM once a month in all the schools for a period of six months.

These observations focused on execution of MDM and hygiene practices followed by children during the meal break. Further, random selection of one school from each geographical cluster was done to elicit information on 3 day diet recall. Data on three day diet recall was collected on a sub-sample of children selected from these three schools. Every fifth child from 5^{th}-8^{th} standard was included in the sample for dietary data analysis. These data were used for studying the dietary intakes of children and contribution of MDM to the daily diets of children.

Phase 1 B was evaluation of MDM at centralised kitchen. Observations were carried out at the centralised kitchen under this phase. It included observations of the process for a week and spot observations. The spot observations were carried out once a month for a period of six months. Observations were made on:

- Machinery and infrastructure facility
- Machinery and equipment
- Storage facility
- Sanitation and hygiene
- Food handling
- Cooking process
- Transportation of food.

The three schools enrolled for diet data analysis, were also included for nutrient and microbial analysis of MDM meals in Phase 1 C. Food samples of all the items on menu were aseptically collected from the kitchen in separate sterile

containers and stored in insulated box at a low temperature and sent for nutrient analysis. Food samples were collected from the centralised kitchen at the time of production for microbial analysis. Each item of the menu was sampled separately directly from the tiffin as soon as the food was put into the tiffin. These tiffins were then immediately closed with lids and labelled so as to ensure that correct tiffins are delivered to the school. The school was visited on the time of meal break and individual samples from the tiffins were collected. Samples were collected using sterile ladles and transferred into sterile airtight containers. These were immediately stored at low temperature in insulated boxes and sent for analysis. Sterile hand gloves, headgear, mask and apron were worn while collecting the sample. Hands were sanitized with 70% Iso Propyl Alcohol (70% v/v IPA) before collecting the sample. Thus, samples of all the items of 6 days menu in all the three selected schools as well as centralised kitchen were analysed for microbial parameters i.e. Total Plate Count, Coliforms, E.Coli, Yeast and Mould.

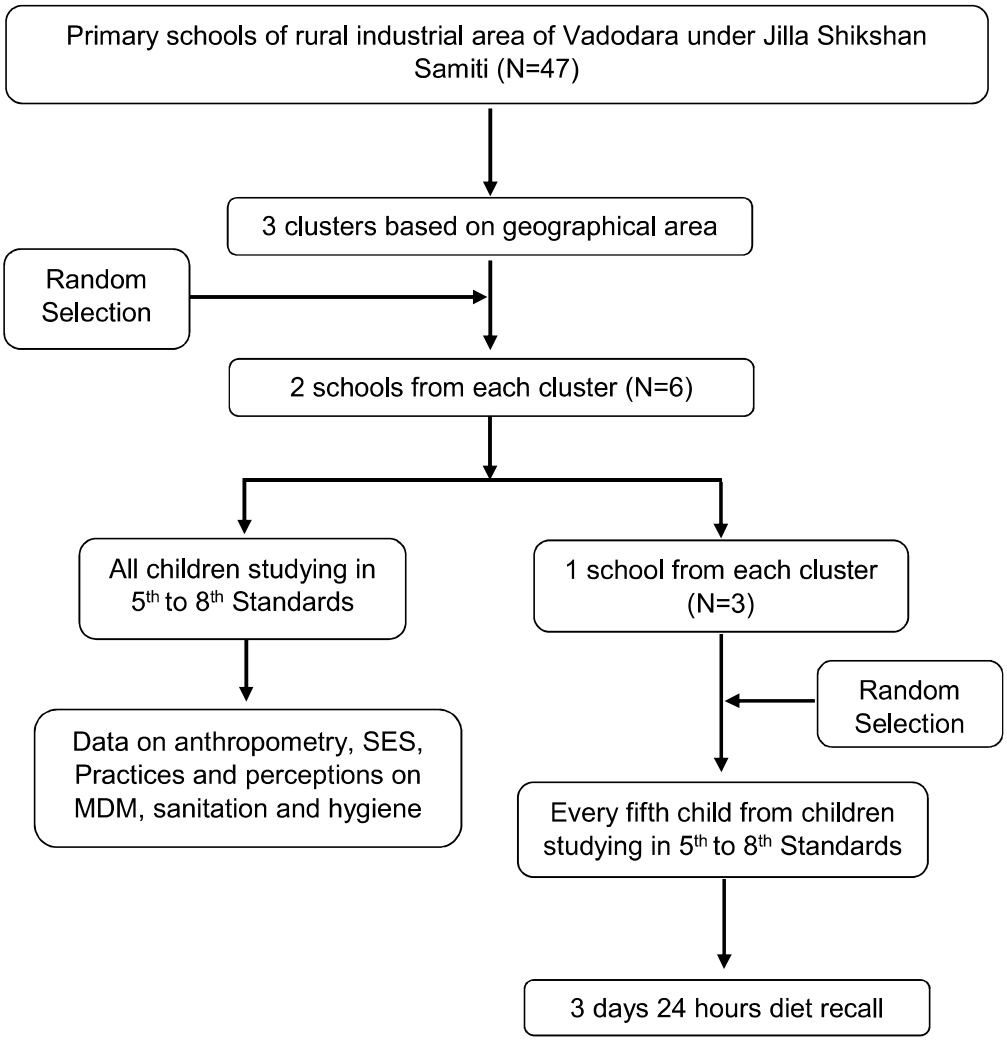
PHASE 2: IMPACT OF NUTRITION HEALTH EDUCATION ON THE NUTRITIONAL STATUS OF MODERATE AND SEVERELY UNDERNOURISHED UPPER PRIMARY SCHOOL CHILDREN OF RURAL VADODARA

Phase two was an experimental phase which was conducted to study the impact of giving NHE with using innovative and interesting yet easy to implement reinforcement sessions on nutritional status of children with low BMI for age (thin). It was divided into two phases.

Phase 2 A: Formative Research- screening of children using anthropometric measurements.
Phase 2 B: Impact evaluation of Nutrition Health Education

Hypothesis of this phase was, “NHE may improve the nutritional status in at least 25% of moderate and severely undernourished upper primary school children of rural industrial area of Vadodara.”

Figure 3.1 Study Design of Phase 1 A



Specific Objectives:

- To assess the magnitude of under nutrition in rural upper primary school children using WHO 2007 standards.
- To assess the prevalence of anemia amongst moderate and severely undernourished rural upper primary school children.
- To develop NHE material for moderate and severely undernourished upper primary school children.
- To assess the impact of NHE on, growth, Hb levels, practices and perceptions regarding MDM as well as sanitation and hygiene practices, MDM compliance, morbidity profile, cognitive development, physical work capacity and Dietary intake.

Sample Selection and Data Collection: A total of 6 schools from the 30 co-ed Government primary schools of rural petrochemicals area of Vadodara, having upper primary section were included in the sample using purposive sampling. This sample was mutually exclusive from the sample of Phase I. All the children studying in 5^{th} to 8^{th} standard were included in the screening. Their anthropometric measurements were taken for nutritional status assessment. Children were categorised into various categories of nutritional status according to BMI for age indicator using WHO 2007 growth reference standards for 5-19 years old children.

Children who were found to be thin (BAZ<-2SD- moderate and severe Undernutrition) were included in the sample after obtaining a written consent from their parents. Data on socio economic status, morbidity profile, physical work capacity, practices and perceptions as well as cognitive test was collected on all the children at baseline. A separate consent for drawing blood sample for performing haemoglobin estimation was obtained from the parents of children. Only those whose parents gave written consent were included for haemoglobin estimations and 24 diet recall. (Figure 3.2)

The selected six schools were further randomly divided into experimental and control group, with three schools in each group. Random numbers generated

in Microsoft Excel were used for randomisation. Nutrition Health Education intervention was given to the children studying experimental group for two months followed by a washout period of two months. Children in control group were kept under the standard care (Regular MDM + 60 mg weekly IFA) (Figure 3.3). Data on SES was collected at baseline. Other data was collected at the end of the intervention i.e. 2 months.

NUTRITION HEALTH EDUCATION

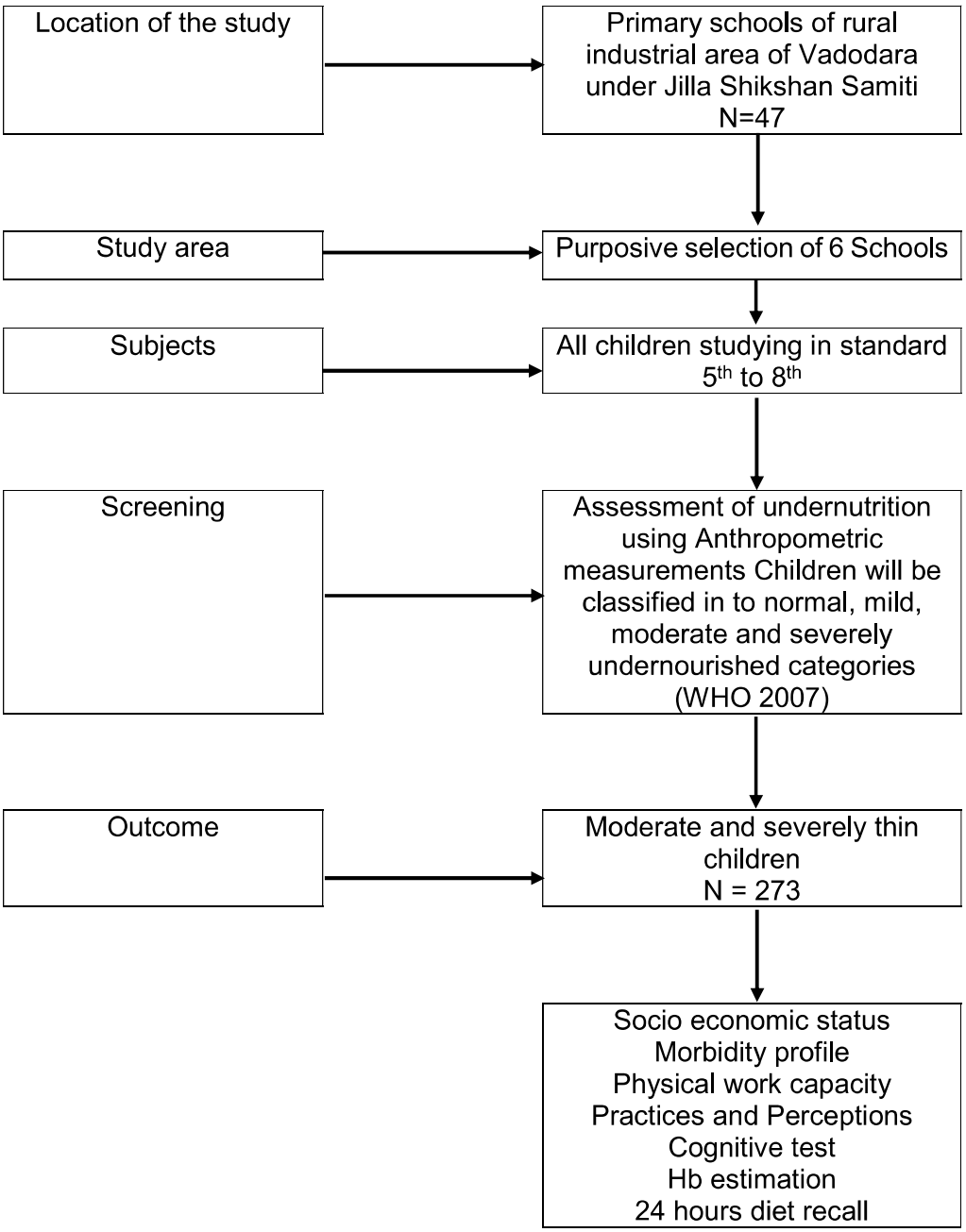
Nutrition Health Education was given to all the children studying in 5^{th} -8^{th} standard in the experimental group school for 60 days. Nutrition Health Education strategy was planned as an easy to implement intervention. It was a two months intervention: one main session followed by reinforcement sessions every fifteen days. Reinforcement sessions included, *Sanedo* (a form of Gujarati folk song), game and skit. In addition to these various posters containing NHE messages placed at strategic locations in the school. NHE was given to all children but data was collected only for the moderate and severely undernourished children.

The main NHE session was a talk on health and nutrition using a PowerPoint presentation. This talk and presentation was in the local language i.e. Gujarati. These interactive NHE sessions were conducted separately for each class so as to make the communication easy to understand for each age group. The sessions were followed by a small question answer interaction.

Key concepts covered in the NHE are as follows:

- Balanced Diet
    - Importance of balanced diet
    - Consumption of commonly consumed fruits and vegetables
    - Role and function micronutrients- iron, Vitamin A, iodine and zinc
    - Importance of IFA tablets
    - Use of iodized salt
- Mid Day Meal Programme
    - Objectives and functioning of the Mid Day Meal programme
    - Importance of Mid Day Meal
- Sanitation and hygiene

Figure 3.2 Study Plan for the Formative Research



- Importance of sanitation and hygiene
- Hand-washing techniques

The schools had televisions for e-learning. These televisions were used to impart the NHE.

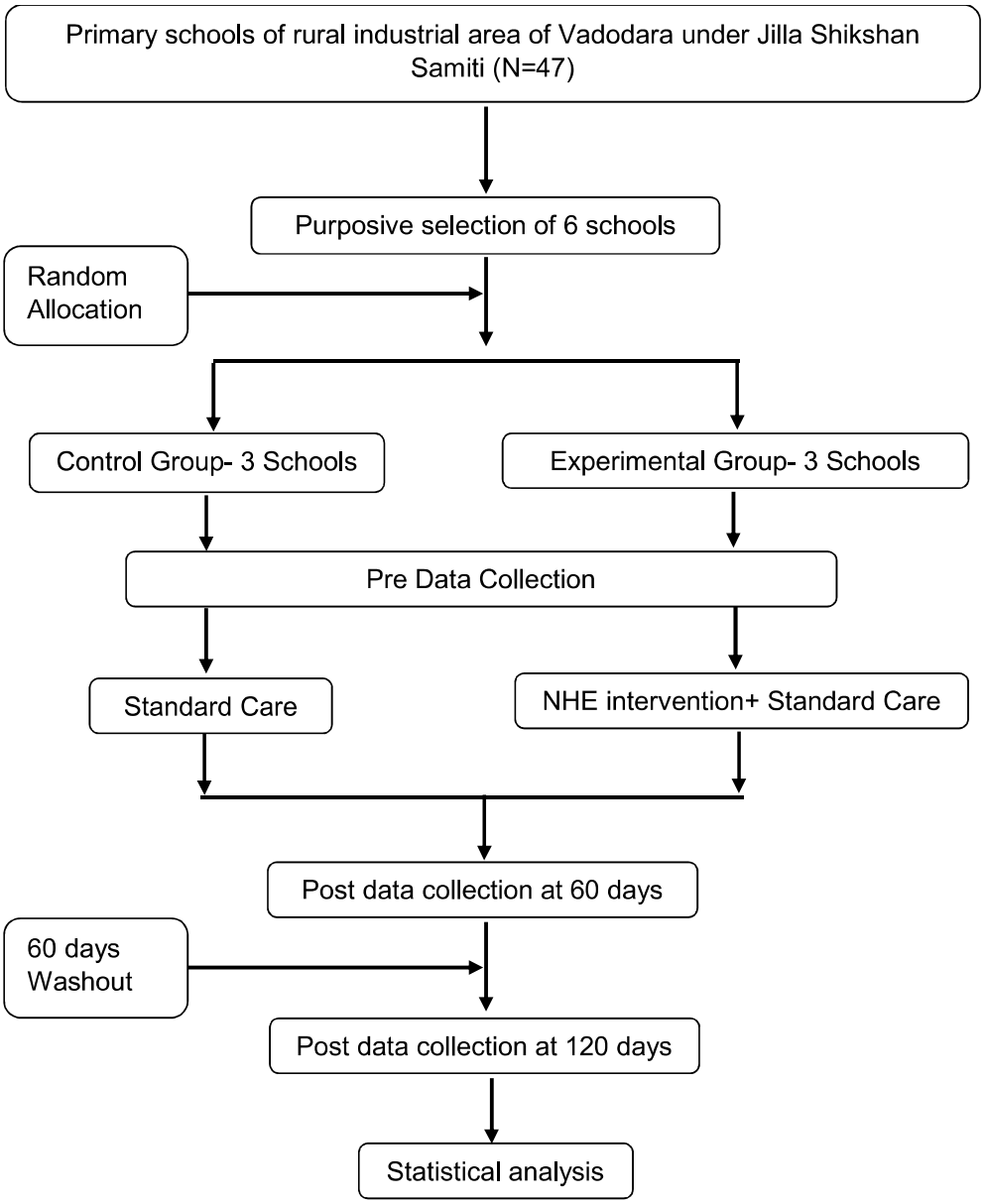
Second session was at fifteenth day. A folk song form '*Sanedo'* is very popular among communities in Gujarat. This was adopted for making a song for reinforcement of key messages. Lyrics was written to convey the key messages which were covered in the main session and was set to the tune of *Sanedo.* This song was titled as *'Tandurasti no Sanedo'.* Copyright for literary work was obtained for this song from the Copyright Office of Government of India (Copyright No: 13386/2016-CO/L). It was recorded with the help of The Faculty of Performing Arts of The Maharaja Sayajirao University of Baroda. Children were gathered in the assembly area of the school, where this song was played. A group of girls also danced to the song. This was also followed by an interaction and question answers with students for reinforcing the messages.

'Passing the parcel' game was chosen for the second reinforcement session. This game format made it possible to involve all the children in a class at the same time. Since it required all the children to sit throughout the game, it was easy to manage the children and avoid chaos. Chits were prepared with questions on the key messages of the main NHE session. Children were made to sit in a big circle and music was played. A parcel was passed while music played and the child who had the parcel when music stopped was asked to pick up a chit and answer the question. The child was given clues to improve the thought process for the possible answer to the question. All the children attempting to answer the questions were encouraged and cheered to ensure a positive feeling. If other children wanted to add on to the answer given by that child, they were given opportunity to share their thoughts. It was followed by explanation by the researcher.

Children were involved in making drawings on key messages given to them in the NHE.

A small skit in Gujarati was prepared for the last reinforcement session. A group of volunteers were trained to play that skit. All the children from classes 5th to

Figure 3.3 Experimental Plan for Impact Evaluation of NHE



Standard care: Regular MDM + 60 mg weekly IFA supplementation (under the ongoing NIPI in the schools)

Primary outcome: Reduction in the prevalence of undernutrition among the upper primary school children

Secondary outcome: Improvement in other parameters such as MDM consumption and sanitation and hygiene practices.

8th were gathered in the assembly area and the skit was performed in front of them. This was also followed by an interactive session of ten minutes where students as well as teachers shared their thoughts on the key messages.

Posters were developed on nutrition, sanitation and hygiene as well as importance of MDM and were displayed at strategic locations in the school. Posters related to hand washing and personal hygiene were displayed near the hand washing stations. Those related to MDM were displayed in the areas where students are served MDM. Rest of the posters were placed at key places such as corridors where they would catch children's attention. These posters were displayed for the whole intervention period.

The NHE session using PowerPoint presentation the main NHE strategy conducted after pre data collection. Reinforcement sessions were conducted after every 15 days. *'Sanedo'* was used at the 15th day as the first reinforcement strategy. Modified passing the parcel game was used as a reinforcement strategy at the 30th day. Children were involved in making drawings on key messages in the 3rd reinforcement session at 45th day. Last session of reinforcement was done on 60th day. A skit was performed by children in the 4th reinforcement strategy. (Table 3.1)

Methods for Data Collection:

Socio Economic Status

Data on Socio Economic Status of children were collected using a pre-tested semi-structured performa. Name, Age and sex of the child were recorded in the performa. Information regarding family background of the children such as religion, caste, type of family, total number of family members, total family income education and occupation of parents was elicited from the children. Date of birth (for calculating age), religion and caste were verified with the school records. (Annexure 7)

Anthropometry:

Anthropometry, measurement of human body, is one of the approaches used for assessment of nutritional status. Anthropometric measurements assess the

Table 3.1 NHE Strategy

Number of days	PowerPoint presentation	Posters	Sanedo	Game	Drawings	Skit
Baseline	√	√	-	-	-	-
15	-	√	√	-	-	-
30	-	√	-	√	-	-
45	-	√	-	-	√	-
60	-	√	-	-	-	√

size, shape, and proportions of the human body. Weight and height are among the commonly used anthropometric measurements in development programmes.

Weight: A calibrated platform weighing balance of SECA was used for weighing the children. The weighing balance was validated for calibration was placed on a hard flat surface. Children were asked to empty their pockets, remove footwear and heavy clothing such as sweaters and jackets. Children were asked to stand erect on the scale, looking straight ahead, without touching anything. Weighing balance was checked for zero before every reading. Weight was measured with an accuracy of 100 gm.

Height: A flexible, non-stretchable fiberglass tape was used for measuring height of the children. Measuring capacity of the tape was 150 cm with an accuracy of 1mm. The tape was fixed vertically on a smooth wall perpendicular to the ground. It was made sure that the ground was smooth and there was no skirting on the wall. The tape was fixed leaving 100cms from the ground making the total measuring capacity of the tape as 250cms. Children were asked to remove their footwear and headgear (if they were wearing any). They were asked to stand erect with the shoulder, hips and heels touching the wall, heels together and looking straight ahead. The head was held comfortably erect, arms hanging loosely by the sides. A thin smooth scale was held on the top of their head in the centre, crushing the hair at the right angles to the tape. Height was read from the lower edge of the ruler to the nearest 0.1 cm.

Age: Date of birth was recorded from the school register as a secondary source. Age was calculated in complete years and months from the date of birth to date of anthropometric measurements.

Body Mass Index: Body Mass Index (BMI) or Quetelet's index is among the most well-known indicator of several indexes and ratio that can be derived from anthropometric measurements. (CDC 2009) It uses the variables weight and height to measure body fat stores. (Bruce 2003) It is the ratio of weight (in kg) to recumbent length or standing height^2 (in m^2).

Anthropometric Indices: Nutritional status of the children was assessed using anthropometric indices namely BMI for age, height for age and weight for age (for children below 10 years of age). WHO 2007 growth reference standards for the age group of 5-19 years were used to calculate the prevalence of malnutrition in the sample.

BMI for age: BMI is an anthropometric indicator used for adults. The relationship among weight, height, and fat in children and adolescents depends on their stage of development and sex as these are the phase of life cycle of growth. (Cashin and Oot, 2018) Hence, BMI is recommended to be used for children and adolescents using the same formula as for adults, but it is compared with age and sex specific z-scores or percentiles of reference standards. BMI for age (BAZ) is an indicator of short term nutritional status. A low BMI for age reflects acute undernutrition. It can be a result of rapid weight loss in children due to insufficient quality or quantity of food, severe and/or repeated infections, or a combination of these two. A low BMI for age is known as thinness where as high BMI for age shows overweight or obesity.

Height for age: Stunting is defined as a low height-for-age for children. Stunting in an indicator of long term undernutrition i.e. chronic undernutrition. Short-term changes in the nutritional status cannot be reflected in height for age. It is associated with chronic insufficient food intake, frequent infections and sustained incorrect feeding practices. Children with z-scores < -2.00 SD are said to be stunted and those < -3.00 SD severely stunted.

Morbidity profile:

A pre-tested structured questionnaire was used to collect information regarding common morbidities such as cough, cold, fever, headache, stomach ache, constipation, vomiting, diarrhoea, tooth ache, loss of appetite experienced by children with the reference period of 15 days as a part of morbidity profile. Morbidity profile was obtained to study relationship to undernutrition and haemoglobin levels. (Annexure 9)

Nutrient intake:

Information on dietary intake of children was elicited using the 24 hours dietary recall method in order to study quality and quantity of their diet. Standardised locally used utensils, standard cups and spoons as well as food models were used as aids for taking recall. Nutrient content of raw foods for calculations were taken from Indian Food Composition Tables (NIN 2017). Nutrient content given on the nutrition label for packaged foods were used for nutrient calculations. The calculations were made for macronutrients like energy, protein, fat and micronutrients like iron and calcium and compared with the Recommended Nutrient intakes for Indians (2020).

A three day diet recall in phase one and one day diet recall at three points (pre intervention, post intervention and washout) was collected to study the daily nutrient intake among children and nutrient intake through MDM. (Annexure 12)

Hemoglobin estimation:

A trained lab technician was hired to collect children's blood samples. Approximately 2ml blood was withdrawn as an intravenous blood sample for estimating the hemoglobin of the children. Disposable syringes were used. On the same day, the blood samples were sent for analysis to LAB CORE, Vadodara. Hemoglobin estimation was done using a fully automatic *Abbot Five Part Haematolgy Analyzer* which works on Multi Angle Polarized Scatter Separation Method (MAPSS). Hemoglobin estimation was done only on those children whose parents gave written consent for giving blood sample. Anemia was classified by using WHO, 2011 criteria (Table 3.2).

Physical work capacity:

Step test was used for measurement of this parameter. Step test was performed on all the moderate and severely under-nourished children to measure their physical work capacity. To perform this test, a 20 cm high step was selected in each school for the children. The numbers of steps were counted for 3 minutes.

Table 3.2 Anemia Classification (Hb, g/dl)

Age (Y)	Non anemic	Mild	Moderate	Severe
9-11 years	≥11.5	11-11.4	8-10.9	<8
≥12	≥12	11-11.9	8-10.9	<8

Source: WHO, 2011

Practices and Perceptions:

A pre-tested open ended questionnaire was used to collect information pertaining to practices and perceptions regarding consumption of MDM, IFA supplementation in school, sanitation and hygiene practices (Annexure 11).

Cognitive development:

Information on the cognitive development was collected using a combination of two cognitive test known as RAVEN's Progressive Matrices and Clerical test. A pretested perfoma was used as a self-administered tool. The children were given necessary instructions and explanation regarding the tool before they took the test. (Annexure 13)

RAVEN's Progressive Matrices: It is a non-verbal group test also known as Raven's Matrices which is typical used in educational settings. It helps in measuring abstract reasoning of a person. It can be used for a wide age groups range, i.e. from children above 5 years of age to elderly.

The principle of this test is to present a series of drawings (objects, animals, plants…) organized according to a certain pattern (AAAA, ABAB…). One drawing is missing in the series, symbolized by a "?". The series is followed by 4 drawings among which the child must choose one to make the first series complete. This sub-test measures inductive reasoning. It requires that the child induces the underlying rules organizing the series. This process requires also planning abilities because the child has to 'try' (visualize mentally) one answer, check what would be the series if the answer was a certain one; then conclude whether his answer is correct or not; and if not try another solution, etc.

Clerical Test: This test involves simple mathematical calculations and finding words or alphabets from a given paragraph. This test helps assess the simple grasping powers and analytical skills of the children.

Spot observations:

Spot Observations were made in the schools to observe the execution of MDM in school. Information regarding, serving of MDM, sanitation and hygiene practices followed by children in the school, consumption of MDM, role of teachers, left over and plate waste management was collected using a semi structured observation check list. Observations were made in both phase I and phase II.

Spot observations were also conducted at the centralised kitchen of TAPF once a month over a period of six months. A semi structured observation guide was used for the spot observations (Annexure 14) In addition to this, detailed observations of functioning of the kitchen were carried out for a week.

In phase II, spot observations were conducted to understand if there were any changes in the hand-washing practices as well as MDM consumption among children post intervention (Annexure 10)

Nutrient Analysis

Nutrient composition analysis of food samples collected from the centralised kitchen was done at an NABL accredited laboratory- Hemshell Services, Vadodara. Food samples were analysed for moisture content, carbohydrates, fat, protein, total ash, calcium and iron content.

Determination of moisture (loss on drying)

Apparatus: Hot Air Oven- maintained at 130ºC ± 3ºC

Procedure:

- In cooled and weighed dish (provided with cover) previously heated to 130ºC ± 3ºC, accurately weigh 2 g well mixed test portion.
- Uncover Test portion, Place the dish in oven maintained at 130ºC ± 3ºC for 1 hour. (1 hour drying period begins when oven temperature is actually 130ºC.)
- Cover dish while still in oven, transfer to desiccator, and weigh soon after reaching room temperature.

Calculation:

$$\text{Moisture, percent by mass} = \frac{100\ (M1-M2)}{(M1-M)}$$

Where; M - weight of empty dish

M1- weight of Moisture dish with material before drying.

M2- weight of moisture dish after drying.

(AOAC 20th Edition 925.10)

Determination of ash content

Apparatus: Muffle Furnace- maintained at 550ºC

Procedure:

- Weigh 3-5 g well mixed test portion into shallow, relatively broad ashing dish (Crucible) that has been ignited, cooled in desiccator and weighing soon after reaching room temperature.
- Ignite in furnace at 550ºC (dull Red) until light Grey Ash results, or to constant weight.
- Cool in desiccator and weigh soon after reaching room temperature.
- Calculate Total ash content using following formula.

Calculation:

$$\text{Total Ash, percent by mass (on dry basis)} = \frac{10000\ (M2-M)}{(M1-M)(100-W)}$$

Where; M - weight of empty dry crucible

M1- weight of crucible with material before ashing

M2- weight of crucible after ashing

W- Percent moisture content of sample

(AOAC 20th Edition 923.03)

Determination of carbohydrate content

Carbohydrate content was determined by difference using following formula.

Carbohydrate, percent by mass= Total solids - (Protein + Fat + Ash)

Where; Total solids = 100 - Moisture content

(AOAC 20th Edition 986.25)

Determination of fat content

Reagents:

- Ethyl Alcohol
- Dilute HCl (25+11)
- Petroleum Ether (boiling point<60ºC)
- Diethyl Ether

Apparatus:

- Water bath
- Mojonnier Fat extraction apparatus
- Glass Container (Flask- Beaker)
- Hot air oven

Procedure:

- Place 2g test portion in 50ml beaker, Add 2 ml Alcohol and stir to moisten all particles to prevent lumping on addition of Acid.
- Add 10 ml HCl (25+11), mix well. Set baker in waterbath held at 70-80ºC, and stir at frequent intervals during 30-40 minutes.
- Add 10 ml Alcohol and cool.
- Transfer mixture to Mojonnier fat extraction apparatus.
- Rinse beaker into extraction tube with 25 ml diethyl ether, added in 3 portions; stopper flask (with Glass, cork, neoprene or other synthetic rubber stopper not affected by solvents) and shake vigorously 1 min.
- Add 25 ml Redistilled Petroleum ether and again shake vigorously 1 min.
- Let stand until upper liquid is practically clear or centrifuge 10 min at 600rpm.

- Draw as much as possible of ether- Fat solution into weighed 125 ml beaker-flask containing porcelain cheeps or broken glass. Before weighing beaker dry it and similar flask in oven at 100ºC; then keep it in Desiccator and allow it to cool and weigh.
- Re-extract liquid remaining in tube twice, each time with only 15 ml of each ether. Shake well on addition of each ether. Draw off clear ether solution into same flask as before and wash tip of extraction tube and rubber cork with few ml of mixture of the 2 ether in equal volumes, free from suspended water.
- Evaporate ether slowly on steam water bath; then dry fat in oven at 100ºC to constant weight.
- Remove flask from oven, keep it in Desiccator and allow it to cool and weigh.
- Correct this weight by Blank determination on reagent used.
- Report as percent fat by acid hydrolysis.

Calculation:

$$\text{Fat, percent by mass} = \frac{\text{Weight of Extracted Fat} \times 100}{\text{Weight of Sample}}$$

(AOAC 20th Edition 922.06)

Determination of protein content

(Block Digestion method using Copper catalyst and steam Distillation into Boric Acid)

Apparatus:

- Protein Digestion unit with Heating Block
- Protein Distillation unit
- Digestion Tubes
- Titration Flask & Burette
- Fume Exhaust manifold

Reagents:

- Concentrated H_2SO_4 (95-98%) as Digestion Acid
- Catalyst Mixture: Potassium Sulphate & Copper sulphate in the Ratio of 5:1 (5gm of Potassium sulphate + 1gm of Copper sulphate)
- Sodium Hydroxide solution: 40% (w/w) make up 400gm NaOH as 1 Ltr by using distilled water in a standard flask.
- Boric Acid: 4%. Make up 40gm H_3BO_3 as 1Ltr by using distilled water in a standard Flask. And add 3ml mixed indicator (Methyl Red & Bromocresol green) solution will be light Orange colour.
- Mixed Indicator : (Methyl Red & Bromocresol Green)
- Dissolve 0.2 g Methyl Red and dilute to 100 ml in 95% ethanol. Dissolve 0.1 g Bromocresol Green and dilute to 50 ml in 95% ethanol. Mix 1 part of Methyl Red solution with 5 parts of Bromocresol green solution.
- Hydrochloric Acid standard solution : 0.1N HCL

Principle: The material is digested in H_2SO_4 to convert the Protein N to $(NH_4)_2SO_4$ at a boiling point elevated by the additional K_2SO_4 with a Cu catalyst to enhance the reaction rate, Ammonia is liberated by Alkaline steam distillation and qualified titrimetrically with standardized acid, aluminium block heated increase the efficiency of the digestion.

The digestion must be contain residual H_2SO_4 to retain the NH_3, water is added manually or automatically to the digest to avoid mixing concentrated alkali with concentrated Acid and to prevent the digest from solidify, concentrated NaOH is added to Neutralize the Acid and make the digest basic, and the liberated NH_3 is distilled into a Boric acid solution and titrated with a stronger standardized acid, HCl, to a colorimetric endpoint. The same endpoint detection system must be used for the standardization of the HCl and for the analyte.

The analyte is referred to as “crude” protein because the method determines N, a component of all Protein. The Amount of protein in most materials is calculated by multiplying % N by 6.25.

Procedure:

- Turn on Block digester and set temperature profile for heat to 420ºC. Start the programme and allow it to gain temperature.
- Weigh 0.1-3.0gm of Test portion (Test portion depends on protein content) into Digestion tube. Add weighed Digestion mixture Approx. 3 gm into each Tube.
- Add 10ml of concentrated H_2SO_4 into each tube.
- Keep it all sample and Blank tubes on heating block for Digestion. Let reaction subside in Fume hood in exhaust system.
- After completion of Digestion process switch off the digestion system.
- Remove rack of tubes with exhaust still in place, and put in the stand to cool for 10-20 minutes cooling can be increased by using commercial Air blower or by placing in hood.
- Wearing Gloves and eye protection, predilute digests manually before distilling. Carefully add a few ml of deionized water to each tube. If spattering occurs, the tubes are too hot. Let cool for few more minutes. Add water to each tube to a total volume of approximately 20-30ml. This is a convenient stopping point. (If distillation unit equipped with steam addition for equilibration is used, the manual dilution steps can be omitted. About 20-30ml water is then automatically added during the distillation cycle.
- Start distillation unit. Keep tube in distillation unit for steam distillation.
- Run the programme or manually add 40ml 40% Alkali, 10ml distilled water into digestion tube and 25ml of 4% Boric Acid mixed with indicator into graduated Flask for titration. Then start the process.
- Allow to running distilled water into the system for generating steam and running tap water for condensing purpose. Steam distil until ≥150 ml distillate is collected.
- Remove receiving Flask containing violet end point. Titrate it with 0.1N HCl solution until the solution goes back to pink.
- Record millilitre of HCl and calculate the Nitrogen content and Protein content using following formula.

Calculation:

$$\text{Kjeldahl Nitrogen, \%} = \frac{(V_S - V_B) \times N \times 14.01}{W \times 10}$$

$$\text{Crude Protein, \%} = \text{\% Kjeldahl N} \times F$$

Where;

V_S = volume in ml of standardized acid used to titrate a Test;

V_B = volume in ml of standardized acid used to titrate a blank

N = Normality of standard HCl

W= weigh in g of test portion or standard

F = Factor to convert N to protein

F factors are 5.70 for wheat, 6.38 for dairy products and 6.25 for other Feed materials.

(AOAC 20th Edition 2001.11)

Determination of iron in foods

Principle: Organic matter in the sample is destroyed by ashing and the resulting ash is dissolved in hydrochloric acid and diluted to a known volume with water.

Whole of the iron present in the aliquot of ash solution is reduced with hydroxylamine hydrochloride and the Fe(II) is determined spectrophotometically as its coloured complex with, α-α-dipyridyl, the solution being buffered with acetate buffer solution. Absorption of the resulting complex is read at 510 nm.

Reagents:

- Magnesium Nitrate solution: (50% w/v) Dissolved 50 g of $Mg(NO_3)_2.6H_2O$ in water and dilute to 100 ml with water.
- Concentrated Hydrochloric acid
- Hydroxylamine Hydrochloride solution: (10% w/v) Dissolve 10 g H_2N OH.HCL in water and dilute to 100 ml.

- Acetate buffer solution: Dissolve 8.3 g of anhydrous NaOAC (previously dried at 100ºC) in water, add 12 ml of Glacial Acetic acid and dilute to 100 ml.
- Alpha,alpha-dipyridyl solution: (0.1% w/v) Dissolve 0.1 g of Alpha,alpha-dipyridyl in water and dilute to 100 ml. Keep this reagent in cool and dark place.
- Iron standard solution: (0.01mg Fe/ml) Dissolve 0.3512 g $Fe(NH_4)_2(SO_4)_2.6H_20$ in water, add 2 drops of concentrated HCL and dilute to 100 ml. Dilute 5 ml of solution(f) to 250ml.
- Orthophenanthroline solution: (0.1% w/v) Dissolve 0.1 g of O-Phenanthroline in 80 ml of water at 80ºC, cool and dilute to 100 ml with water. Keep in cool and dark place.

Apparatus: Spectrophotometer / colorimeter to read absorption at 510nm.

Preparation of Sample:

- Weigh accurately, a suitable quantity of well homogenized sample, into a cleaned and tared silica dish. If sample contains more water, dry on a water bath. Char the sample (in the dish) on law flame of a burner till all the volatile matter escapes and smoking ceases.
- Transfer the dish to a cold muffle furnace and raise the temperature slowly to 450ºC. Continue ashing at 450ºC till practically carbon-free ash is obtained (If carbon is present in ash even after 4-5hrs of ashing, remove the dish from furnace, cool and moisten the ash with 1 ml of magnesium Nitrate solution, dry on water bath/ hot plate and ash in furnace at 450ºC.)
- After the ash is carbon free remove the dish from furnace and cool.
- Add 5 ml of concentrated HCL letting acid rinse the upper portion of the dish and evaporate to dryness on a water bath.
- Dissolve residue by adding exactly 2.0 ml of concentrated HCL.
- Heat for 5 min on steam bath with watch glass covering the dish. Rinse the watch glass with water, Filter into a 100 ml volumetric flask, and cool and dilute tom volume.

Procedure:

- Pipette 10 ml aliquot of ash solution into 25 ml volumetric flask, and Add 1 ml hydroxylamine Hydrochloride solution.
- After 5 min, add 5 ml of Buffer solution and 1 ml Orthophenanathroline solution or 2 ml of dipyridyl solution and dilute to volume.
- Determine absorbance of solution at 510 nm.
- From Absorbance reading, determine Fe content present in Aliquot of Ash solution taken by referring to standard curve.

Preparation of Standard Curve:

- Pipette 0.0, 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 ml of Fe standard solution (F-II) into a series of 25 ml volumetric flasks and add to each of them exactly 0.2 ml of concentrated HCL.
- Dilute each of them to exactly 10 ml with water, and then add reagents in the same way as for the sample,
- Plot the quantity of Fe (in mg) against the absorbance.

Calculation:

$$\text{Iron content of sample (mg Fe/100 gm sample)} = \frac{\text{Quantity of Fe in aliquot of ash solution (from calibration curve)}}{\text{Aliquot of Ash solution taken For determination}} \times \frac{\text{Total volume of Ash solution}}{\text{Wt. of the sample taken for ashing}} \times 100$$

(FSSAI Manual Method for Analysis of Metals & AOAC 20th Edition 944.02)

Determination of calcium

Reagents:

- Hydrochloric acid - 0.1N
- Sodium Acetate solution - 20%
- Sodium Oxalate solution - Saturated
- Dilute sulfuric acid - one volume of concentrated sulfuric acid diluted with 4 volume of water.

- Standard Bromocresol Green Indicator solution- Prepared by dissolving 0.1 g of Bromocresol green in 100 ml of rectified spirit.
- Standard Potassium Permanganate solution - 0.05 N
- Acetic Acid

Procedure:

- Weigh accurately about 25 g of the composite sample in a previously weighed 50 to 100 ml crucible.
- Heat the dish at 100ºC until water is expelled, and continue heating slowly until the swelling stops.
- Place the dish in a closed Muffle furnace at 525ºC ± 20ºC and leave until white ash is obtained.
- Treat the ash with 25 ml or more of 0.1N hydrochloric acid and heat to in incipient boiling on a asbestos plate till all the soluble constituents have completely dissolved.
- Cool and filter through the Whatman No.1 Filter paper into a 250 ml graduated flask.
- Take a 50 to 100 ml of aliquot of the solution obtained in a 400 ml beaker and add 8 to 10 drops of bromocresol green indicator solution and then the Sodium Acetate solution till the colour of the solution is distinctly blue.
- Adjust the pH of the solution at 4.4 to 4.6 adding Acetic acid drop by drop until the colour changes to distinct Green.
- Filter the solution and bring it to boil. While still Hot, add the saturated Sodium Oxalate solution drop wise as a long as any precipitate forms, and then Add an Excess, and keep on boiling water bath one hour.
- Allow to stand for 3 hours or longer, preferably overnight.
- Decant the colour solution through a quantitative filter paper.
- Pour 15 to 20 ml of Hot water on precipitate and again decant the clear solution.
- Dissolve any precipitate remaining on a filter paper by washing with a Hot dilute hydrochloric acid into the original beaker; wash the filter paper thoroughly with Hot water.

- Then Re precipitate, while boiling hot, by the addition of Sodium Acetate and little of Saturated Sodium Oxalate solution.
- Allow to stand for 3 Hours or more as before, filter through the same filter paper, and wash with Hot water until it is chloride free.
- Perforate the apex of filter cone, wash the precipitate with 50ml of hot water into the beaker used for precipitation.
- Add roughly 10 ml of dilute sulfuric acid, heat to 90ºC and titrate with the standard potassium permanganate solution.

Calculation: Calculate as percent calcium, one millilitre of 0.05 N Potassium permanganate solution is equivalent of 0.001 g of calcium.

(Indian standard IS 2860: 1964)

Microbial Analysis: Food samples collected were analysed for Total Plate Count, coliforms, E. Coli, yeast and mould to look into the food safety of the meals.

Total Plate Count: Food samples were analysed for Total Plate Count using colony count at 30ºC by the pour plate technique (ISO 4833-1:2013). It is a method appropriate for food products for human consumption.

In this method, liquid test sample or initial suspension in a specified quantity is dispensed into an empty petri dish and mixed with a specified molten agar culture medium to form a poured plate. Other plates are prepared under the same conditions using decimal dilutions of the test sample or of the initial suspension. The plates are incubated under aerobic conditions at 30°C for 72 hours. The number of microorganisms per gram or per millilitre of the test sample is calculated from the number of colonies obtained in the plates containing fewer than 300 colonies.

Coliforms: It is a measure of sanitary quality of food items. Colony count technique was used for coliforms count present in the food samples using the ISO 4832: 2006 method.

Test samples were prepared as per SOP based on ISO 6887. A weighed quantity of sample was inoculated into 90 ml peptone salt solution to make initial

dilution and it was homogenized well. This initial dilution was further used for 4 subsequent serial dilutions. A specified molten agar culture was mixed with the dilutions to form pour plates. The plates were incubated at 30ºC-37 ºC for 24 hrs± 2 hrs. Colonies were counted on each plate post incubation. The number of microorganisms per gram or per millilitre of the test sample is calculated from the number of colonies obtained in the plates containing fewer than 150 colonies.

E.coli: It is used as an indicator organism in food or water for water faecal contamination. Horizontal method for the detection and enumeration of presumptive E.coli- most probable number technique (ISO 7251:2005) was used as a method of test for E.coli.

A liquid selective enrichment medium is inoculated with a specified quantity of the initial suspension of the test sample.

The tube was incubated at 37 °C for up to 48 hours and was examined for gas production after 24 and 48 hours. If the tube had given rise to opacity, cloudiness or gaseous emission, it was subcultured to a tube containing a liquid selective medium (EC broth) which was incubated at 44 °C for up to 48 hours and examined for gas production after 24 and 48 hours. If a rise to gaseous emission was observed, it was subcultured to a tube containing indole-free peptone water. This tube was incubated at 44 °C to 8 hours. This tube was examined for indole production resulting from the degradation of tryptophan in the peptone constituent. Tubes showing opacity, cloudiness or gas production in the liquid selective enrichment medium and whose subcultures have produced gas in the EC broth and indole in the peptone water at 44 °C are considered to contain presumptive Escherichia coll. The results are given as the "presence" or "absence" of presumptive Escherichia coll in x g or x ml of product.

Enumeration method: Three tubes of double-strength and three tubes of single-strength liquid selective enrichment medium were inoculated with a specified quantity of the initial suspension. Three tubes of single-strength liquid enrichment medium were inoculated with a specified quantity of the initial suspension. Then, under the same conditions, another three tubes of the

single-strength medium were inoculated with a specified quantity of decimal dilutions of the initial suspension. The tubes of double- and single-strength medium were incubated at 37 °C for up to 48 hours. These tubes were examined for gas production after 24 and 48 hours. Each tube of double-strength medium that had given rise to opacity, cloudiness or gaseous emission, and each tube of single-strength medium that had given rise to gaseous emission, was subcultured to a tube containing a liquid selective medium (EC broth). These tubes were incubated at 44 °C for up to 48 hours and were examined for gas production after 24 and 48 hours. Each tube of medium that had given rise to gaseous emission was subcultured to a tube containing indole-free peptone water. These tubes were incubated at 44 °C for 48 hours and examined for indole production resulting from the degradation of tryptophan in the peptone constituent. The most probable number of presumptive E.coli was determined by means of the MPN table, according to the number of tubes of single- and double-strength medium whose subcultures produced gas in the EC broth and indole in the peptone water at 44 °C.

Yeast and Mould: Yeast and mould are also used as microbiological indicators of food safety. Method used for their count was enumeration of yeast and mould count (ISO 21527-1:2008). A weighed quantity of sample was added to 90 ml of diluent. Serial decimal dilutions were prepared. Each dilution (1 ml) was transferred to agar medium and carefully spreaded as quickly as possible over the surface of the agar plate, trying not to touch the sides of the dish using the spreader. The plates were allowed to dry with their lids on for about 15 min at laboratory temperature for the inoculum to be absorbed into the agar. Then, the plates were inverted the plates and incubated for 5 days then re-incubate at 25 °C ± 1°C. After the incubation period, colonies on each plate and the plates containing fewer than 150 colonies were counted. The number of yeast and mould per gram sample were counted separately.

Various methods and tools used for data collection are summarized in Table 3.3.

Table 3.3 Various Methods and Tools Used in Data Collection

Indicators	Methods
Anthropometric Measurments	Standard method For Weight And Height measurements
Socio Economic Status	Pretested Semi Structured Questionnaire
Practices and Perception	
Dietary Pattern	24 hour Dietary Recall method
Morbidity Profile (15 days reference period)	Pre-Tested Open Ended Questionnaire
Haemoglobin	Abbott 5 Part Haematology Autoanalyser
Physical Work Capacity	Step Test
Cognition Score	Raven & Clerical test
Spot observation	Observation check list
Nutrient Analysis:	
Moisture	AOAC 20th Edition 986.25
Carbohydrate	AOAC 20th Edition 986.2
Fat	AOAC 20th Edition 922.06
Protein	AOAC 20th Edition 2001.11
Iron	AOAC 20th Edition 944.02
Calcium	Indian standard IS 2860: 1964
Microbial Analysis:	
Total Plate Count	ISO 4833-1:2013
Coliforms	ISO 4832: 2006
E.Coli	ISO 7251:2005
Yeast and Mould	ISO 21527-1:2008