MATERIALS AND METHODS
3. MATERIALS AND METHODS

The present investigation was undertaken to standardize a method for preparation of whey-based tropical fruit beverages containing probiotic organisms \(L. \textit{rhamnosus}\) (LBKV3) bearing a MTCC Accession No. 5463. The beverage thus prepared was analyzed for its compositional, microbiological and quality attributes and acceptability. Studies were also conducted to ascertain its in vitro antimicrobial properties followed by effect of feeding of such beverage on the gastrointestinal microbiota of the randomly selected children. A brief account of materials used and the methodologies adapted for execution of the investigation to establish the objectives are as hereunder:

3.1. MATERIALS

3.1.1. Procurement and standardization of whey:

Pooled cow milk was obtained from the local milk vendors of Udgir town. It was used for \textit{Paneer} preparation by traditional method in the laboratory of Department of Dairy Science, Shivaji College, Udgir Distt. Latur. Whey obtained was standardized to 4% total solids (0.5% fat and 3.5% SNF) and stored under
refrigerated conditions till its utilization for preparation of whey-based tropical fruit juices containing probiotic organisms. *Paneer* whey was selected in the present investigation due to its easy availability and vast demand for *Paneer* from the local users like the restaurants. Similarly, the *Paneer* can be prepared in small quantities by local milk producers. One additional benefit of using *Paneer* whey is the fact that unlike the cheese whey, *Paneer* whey does not contain any residual rennet activity.

### 3.1.2. Procurement and processing of figs:

Fresh ripened figs were obtained from the fig orchard of a local farmer's field. After proper blenching, figs were subjected to drying in open sunlight and then it was converted into pulp by using domestic mixer-cum-grinder. The average composition of fig pulp thus prepared in the laboratory was protein (3.3%), Carbohydrates (48%) and fat (1.5%). The fig pulp was stored under refrigerated conditions till its use.
3.1.3. Procurement and composition of Alfonso pulp:

Commercially available preserved Alfonzo mango pulp was obtained from local market of Udgir town. The average composition of the pulp was protein (0.82%), carbohydrates (17%) and fat (0.27%). Mango pulp was stored under refrigerated conditions till its use.

3.1.4. Selection and maintenance of probiotic culture:

The pure culture of *L. rhamnosus* (LBKV3) bearing a MTCC Accession No. 5463 isolated by Khedkar *et al.*, 1988 and deposited at Institute of Microbial Technology, Chandigarh was used in this study. Pure culture of this proven probiotic organism was obtained from Anand Agriculture University, Anand. This culture was selected as the test culture to study its suitability in preparation of whey-based tropical fruit beverages and its therapeutic benefits in children in the present investigation. The culture was maintained in sterile screw-cap glass tubes containing MRS broth. Before being used for the study at least three subculturings were performed to get the desired activity in the culture.
3.1.5. **Sugar:**

Fine crystalline sugar of commercial grade was obtained from the local market of Udgir.

3.1.6. **Stabilizer:**

Stabilizer, namely carboxy methyl cellulose (CMC) (LOBA Chemie, Bombay) at 0.1% level was tried in whey beverages.

3.1.7. **Preservatives:**

Sorbic acid (LR grade, SD-fine Chem Ltd., Mumbai) and sodium benzoate (LR grade SD-fine Chem Ltd., Mumbai) were used as preservatives in beverage.

3.1.8. **Selection of children for feeding trial of the beverage:**

The most acceptable whey-based tropical fruit beverage containing $10^5$ cfu/100 ml of probiotic organisms was tested for its effect on faecal microbiota of randomly selected children. Twenty one healthy children of 2-5 years, not taking any antibiotic treatment, were randomly selected for this trial. Parent of these children were informed about the details of feeding trial, sampling of the faecal matter and duration of the feeding trial.
3.1.9. Packaging material:

The beverage samples during study were packaged in clean and sterilized glass bottles. Sanitized (soaking in 100 ppm chlorine solution for 15 min) LDPE pouches (capacity 100 ml) and stored at refrigerated temperature (7±1°C).

3.2. METHODS

3.2.1. Preparation of whey-based tropical fruit beverage containing probiotic organisms:

The whey-based tropical fruit beverage containing 10⁵ cfu of probiotics per single serving/dose of probiotic organisms viz. *L. rhamnosus*-LBKV3 was prepared. Flow diagram of the said method as depicted in Fig. 3.1.:
FIG. 3.1: FLOW DIAGRAM FOR PREPARATION OF WHEY-BASED TROPICAL FRUIT BEVERAGE CONTAINING PROBIOTICS

1. RECEIVING FRESH PANEER WHEY
2. FILTRATION/CLARIFICATION/STANDARDIZATION (4% TS)
3. NEUTRALIZATION OF WHEY WITH CaCO₃
4. ADDITION OF CANE SUGAR @10%
5. HEAT TREATMENT (80°C/15 MIN.)
6. COOLING TO 37°C
7. FORTIFICATION WITH TROPICAL FRUIT PULP (5, 10& 15%) AND ADDITION OF 0.1% BEVERAGE STABILIZERS & PRESERVATIVES
8. GRINDING IN MIXER-CUM-GRINDER
9. INOCULATION WITH 2% L. rhamnosus-LBKV3
10. COOLING TO 5°C
11. WHEY-BASED TROPICAL FRUIT BEVERAGE CONTAINING PROBIOTICS PREPARATION
3.2.2. Analysis of the whey-based tropical fruit beverage:

The whey-based tropical fruit beverage containing three different levels of the fruit pulp was analysed for its composition, sensory characteristics, viable counts of the probiotic organisms and acceptability based on 9-point Hedonic scale.

3.2.2.1. Fat content:

Whey and beverages were analyzed for fat as per the procedure given in ISI handbook (1981).

3.2.2.2. Protein content:

Protein content was estimated by using Kjel-Plus machine, Chennai. The procedure followed by Menefee and Overman (1940) as well as instructions supplied by Kjel-plus analyzer was used for the estimation of protein.

3.2.2.3. Ash content:

Ash content in the whey and beverages was determined as per the method prescribed in AOAC (1980).
3.2.2.4. Total solids content:

Total solids of whey as well as beverages were estimated by gravimetric method using Mojonnier Milk Tester as per the procedure described in Laboratory Manual (1959).

3.2.2.5. pH:

The pH of fresh whey and beverages were determined using digital pH meter (Systronic Digital pH meter, Model 335, India).

3.2.2.6. Titratable acidity:

The titratable acidity (expressed as % L.A.) of fresh whey as well as beverage samples was determined as per the procedure mentioned in ISI Handbook (1981).

3.2.2.7. Specific gravity:

The specific gravity of beverages were estimated at 20°C by using a standard specific gravity bottle of 25 ml capacity, taking distilled water as the standard liquid.
3.2.2.8. Viscosity:

Viscosity of beverages was measured by using viscometer at 20°C, with probe rotating at a speed of 500 rpm (level 10) and observations were taken after 25 sec.

3.2.3. Enumeration of viable counts:

A 10 g sample of freshly prepared beverage was drawn after mixing the contents thoroughly. It was transferred aseptically to 90 ml of sterile 0.1 percent peptone and mixed well. Further dilutions were prepared using 9 ml buffer blanks. The MRS agar was used for the enumeration of culture organism (L. rhamnosus-LBKV3) in the product.

3.2.4. Sensory evaluation of the whey-based fruit beverages containing probiotics:

Fresh samples of whey-based tropical fruit juices prepared and stored in plastic bowls (100 ml) were coded in order to obscure their identity. These were presented to a panel of seven judges to establish the overall acceptability of the product. The nine-point Hedonic Scale was used for judging the product. The
contents of Table 3.1 depicts the scorecard. Prior to actual judging, the judges were informed about the quality attributes of the product and practically acquainted with the same.

TABLE 3.1: NINE-POINT HEDONIC SCALE FOR EVALUATION OF ACCEPTABILITY OF THE WHEY-BASED TROPICAL FRUIT BEVERAGE CONTAINING PROBIOTICS

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Score</th>
<th>Score description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>Liked Extremely</td>
</tr>
<tr>
<td>2.</td>
<td>8</td>
<td>Liked very much</td>
</tr>
<tr>
<td>3.</td>
<td>7</td>
<td>Liked moderately</td>
</tr>
<tr>
<td>4.</td>
<td>6</td>
<td>Liked slightly</td>
</tr>
<tr>
<td>5.</td>
<td>5</td>
<td>Neither liked nor disliked</td>
</tr>
<tr>
<td>6.</td>
<td>4</td>
<td>Disliked slightly</td>
</tr>
<tr>
<td>7.</td>
<td>3</td>
<td>Disliked moderately</td>
</tr>
<tr>
<td>8.</td>
<td>2</td>
<td>Disliked very much</td>
</tr>
<tr>
<td>9.</td>
<td>1</td>
<td>Disliked extremely</td>
</tr>
</tbody>
</table>

3.2.5. Feeding trials:

The twenty-one randomly selected children were grouped into three groups each comprising of seven children. Faecal matter of all the children was analyzed for its microbial counts prior to commencement of the
feeding trial. Group A was receiving 100 ml of whey-based fig beverage containing $10^5$ cfu per 100 ml of the beverage, group B was receiving 100 ml of whey-based mango beverage containing $10^5$ cfu of probiotic organisms per 100 ml of the beverage and group C (control group) was receiving 100 ml of whey-based mixed fig and mango beverage containing no any probiotic organisms. Feeding trial was conducted continuously for a period of 21 days.

3.2.6. *In vitro* antimicrobial properties of the whey-based tropical fruit beverage:

To study the *in vitro* antimicrobial activity, the cell-free culture filtrates (CFC) of the test beverages and the control beverage without probiotics was obtained. The cup-well assay method was used to know the antimicrobial properties of the products. *Escherichia coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and enterotoxigenic *Staphylococcus aureus* were the selected test organisms to study the antibacterial activity of the CFC filtrate of the product.
3.2.7. Microbiological analysis of faecal matter of the children:

It comprised of enumerating the putrefactive and friendly microbiota present in the faecal matter of the children before commencing the feeding trial, at an interval of 7 days during the trial and 7 days after terminating the feeding trial of 21 days. By using the selective media i.e. DeMan Rogosa & Sharpe (MRS agar) for probiotic strain and MacConkey’s agar for coliforms, Nutrient agar for standard plate count, Staphylococcus agar for *Staph. aureus* and Pseudomonas agar for *Ps. aeruginosa*. All of the dehydrated media were obtained manufactured by Himedia and were used to enumerate the viable counts of the organisms. The media were prepared a fresh and utilized before a period of one month of its preparation.

3.2.8. Statistical analyses:

The data obtained during investigation was subjected to appropriate statistical analyses, as per the procedures mentioned by Steel and Torrie (1960). In general the Split-plot design with one main and one sub-plot was used for effect of feeding the beverage on GIT microbiota of the children.