5. SUMMARY

Presently, the attitude of people, especially those from urban areas, towards food is rapidly changing, and as such there has been a growing demand for probiotic fermented milk products, in particular. A prerequisite for the development of a probiotic dairy product is the introduction of probiotic cultures such as *Bifidobacterium* spp. with proven beneficial characteristics. These organisms should have high cell concentration and should be resistant to gastric acids and bile in order to remain active in the intestines and should possess antimicrobial properties against human pathogens. In fact, the recent advances in the area of probiotic research show a greater promise in new product development of functional foods based on milk. It is in this direction that new and innovative type of yoghurts and yoghurt products are available in the market as a result of phenomenal increase in the per capita consumption of these products during the last decade. Although the food market in the advanced countries such as the United States of America, Japan and Australia has been heavily loaded with the functional foods like probiotic fermented milk products, the availability of this kind of products in the Indian food market is scanty. Indeed, a perusal of literature on probiotic fermented dairy products clearly reveals that the information in this area of research is very limited. The present investigation is, therefore, particularly concerned with the development of probiotic yoghurt with the following objectives of

- isolating and characterizing potential strains of *Bifidobacterium* spp. from their natural environments for use as probiotic cultures,
• studying the antibacterial activity against human intestinal pathogens and human gut barrier properties such as acid and bile tolerance of *Bifidobacterium* spp. in addition to their capability of utilizing milk sugar, lactose,

• finding the suitability of *Bifidobacterium* spp. for their use in yoghurt, a major fermented dairy product, by studying their interactions with standard yoghurt starter cultures in terms of acid production,

• optimizing the process for the preparation of different varieties of probiotic yoghurts such as plain set yoghurt, flavoured set yoghurt, and flavoured stirred yoghurt,

• assessing the physico-chemical, microbiological changes and organoleptic evaluation of probiotic yoghurts during storage at refrigerated temperature,

• establishing the impact of whey protein concentrate supplementation on the viability of *Bifidobacterium* spp. and physicochemical and sensory attributes of probiotic yoghurts, and

• evaluating the beneficial effects of probiotic yoghurt in terms of animal body weight, reduction in counts of intestinal coliforms, and change in serum cholesterol involving albino rats as biological models.

Initially to isolate and characterize the potential strains of *Bifidobacterium* spp. from their natural environments, twenty-five faecal samples from breast-fed infants were collected and pour-plated using a selective culture medium, NPNL, under anoxic conditions in an anaerobic chamber. The isolates were characterized and identified as strains of *Bifidobacterium* spp. based on their morphology, biochemical reactions,
fructose-6-phosphate phosphoketolase activity, and sugar fermentation patterns following the protocols described in Bergey’s Manual of Systemic Bacteriology.

Three well-characterized isolates, i.e. two strains of *Bifidobacterium bifidum* (BB-F1 and BB-F2) and one of *Bifidobacterium longum* (BB-F3), and four standard strains of *Bifidobacterium bifidum* (NCDC-233, NCDC-229-A, NCDC-255, and DSM-20456) and two strains of *Bifidobacterium longum* (1941 and BB-536), and one strain of *Bifidobacterium animalis* (BB-12), procured from other laboratories were evaluated for their probiotic attributes like antibacterial activity, pH and bile tolerance.

All the strains of *Bifidobacterium* spp. included in the present investigation were effective against the common human intestinal pathogenic bacteria such as *E. coli*, *Salmonella typhi*, *Shigella dysenteriae* and *Yersinia enterocolitica*. While pH 1.0 was inhibitory to all strains of *Bifidobacterium* spp., incubation at pH 3.0 even for 3 h did not affect the growth in terms of viable counts. However, exposure of the strains to pH 2.0 moderately inhibited the bacterial growth. To find out bile sensitivity of the probiotic bacterial strains, the time required was recorded for the organism to reach an OD₆₀₀ of 0.3 in MRS agar medium containing oxgall. Even with 0.3% bile in the medium, the time delay in reaching the specified growth was significant with the strains of *Bifidobacterium* spp. isolated from infant faecal samples, indicating that these strains are more bile-tolerant. In addition, the lactose utilization efficiency of these strains was assessed by their β-galactosidase activity. The strains obtained from other laboratories seem to be efficient in utilising lactose than those isolated from the infant faecal samples.
Based on the overall performance of these attributes, four standard strains of *Bifidobacterium bifidum* and two strains of *Bifidobacterium bifidum* isolated in the present study were selected for evaluating their biocompatibility in terms of titratable acidity, pH, viability, and proteolytic activity, when grown in association with the traditional starter cultures used for the preparation of yoghurt. Based on the optimal growth capabilities in associative growth trials, two potential strains *Bifidobacterium bifidum* DSM 20456 and NCDC 229-A were chosen for the standardization of probiotic yoghurts.

Employing *Bifidobacterium bifidum* DSM 20456 and NCDC 229-A as probiotic cultures, three different probiotic yoghurts such as plain set yoghurt, flavoured set yoghurt and flavoured stirred yoghurt were prepared using the standardized milk containing 4% fat and 12% solids-not-fat following the standard protocols involving minor modifications. In making different probiotic yoghurts containing the starter cultures, *Streptococcus salarius* ssp. *thermophilus* NCDC 074 and *Lactobacillus delbrueckii* ssp. *bulgaricus* NCDC 009, obtained from NCDC, National Dairy Research Institute, Karnal, Haryana were routinely used. All the yoghurt samples packed in 100 ml polyethylene cups were stored at refrigerated temperature (4°C) for further analysis. The samples were periodically tested at 0, 3, 7, 14, 21, 28, and 35 days of storage for changes in physico-chemical properties like titratable acidity, pH, viability of *Bifidobacterium* spp., and for overall acceptability of the product adopting a 9-point Hedonic scale by a panel of five well-trained judges.

Interestingly pH in both flavoured set, and flavoured stirred yoghurts prepared in the presence or absence of selected *B. bifidum* strains marginally increased when compared with that of plain set yoghurt. The titratable acidity
in all three types of yoghurts either remained constant or decreased marginally. Furthermore, the viable counts of *B. bifidum* strains gradually declined with time of product storage at 4°C. In all, the probiotic yoghurts prepared in the present study were found to be acceptable until 3 weeks.

For testing the efficiency of whey protein concentrate (WPC) supplementation in improving viability of the selected probiotic organisms (*Bifidobacterium bifidum DSM 20456*) physico-chemical characteristics and sensory attributes, the probiotic yoghurts were prepared by adding 0.5 and 1.0% WPC to yoghurt milk. These yoghurt samples were analyzed after storing at refrigerated temperature at 0, 3, 7, 14, 21, 28, and 35 days for improvement in the viability of *Bifidobacterium spp.* and for the changes in the titratable acidity, pH and overall acceptability of the product. The probiotic yoghurt prepared with 0.5% WPC supplementation using *B. bifidum* DSM 20456 as a probiotic culture in combination with traditional yoghurt cultures was found to be superior in retaining the optimal levels of *B. bifidum* population even after 4 weeks of storage, with insignificant changes in pH and titratable acidity and with an overall acceptability of the probiotic yoghurts.

The nutritional impact of the standardized probiotic yoghurt having *B. bifidum* DSM 20456 as probiotic culture in combination with traditional yoghurt cultures was studied in a biological system involving male albino rats of the same age group by following a feeding schedule lasting for 45 days. Three groups, viz., control group with normal diet, a second group fed with yoghurt-mixed feed and a third group with probiotic yoghurt-mixed feed, were included in the study. The body weight, faecal bifidobacterial counts, intestinal coliform counts and serum cholesterol levels in albino rats were recorded at 0,
15, 30 and 45 days of feeding regime. Significant differences in body weight gain in albino rats fed with yoghurt alone or probiotic yoghurt were observed in comparison with control the group. There was an appreciable increase in the counts of bifidobacteria in faecal samples fed with probiotic yoghurt while a significant reduction in intestinal coliform counts was found in rats fed with probiotic yoghurt than those kept on feed with or without the ordinary yoghurt. With respect to the serum cholesterol levels in rats, no significant difference could be observed between the three treatment groups during a 6-week period of study.

The present investigation clearly indicates that either \textit{B. bifidum} DSM 20456 or NCDC 229-A could be an effective culture in the preparation of probiotic yoghurts involving Indian strains, \textit{Streptococcus salvarius} ssp. \textit{thermophilus} NCDC 074 and \textit{Lactobacillus delbrueckii} ssp. \textit{bulgaricus} NCDC 009 as traditional yoghurt cultures. Furthermore, the present results suggest that both strains 229-A and DSM 20456 are the most promising for their use as dietary adjuncts in fermented milk products like yoghurt based on their overall performance including antimicrobial activity against pathogenic enteric bacteria. Also, it is evident that ingredients like WPC up to a 0.5% supplementation in making the probiotic yoghurt will be of greater help in maintaining the required levels of probiotic organisms in the product during a 4-week period of storage, with little or no adverse effect on the physico-chemical and sensory characteristics of the product. The probiotic bacterium (strain DSM 20456) exhibited greater capability of colonizing the rat intestine besides reducing the populations of intestinal pathogenic coliforms. The reduction in serum cholesterol observed in albino rats was, however, not
appreciable, and needs further rigorous experimentation to establish the impact of probiotics on regulatory mechanism of serum proteins.

To sum up, following are the salient findings of the present investigation.

• Ten strains of *Bifidobacterium* spp. were isolated from 25 faecal samples, and three strains of *B. bifidum* were observed to be suitable for use as probiotic organisms.

• Employing two probiotic cultures (DSM 20456, NCDC 229-A), three different types of yoghurts viz. plain set, flavoured set, flavoured stirred were prepared, and assessed for their consumer acceptability. Both the phisico-chemical and sensory attributes of probiotic yoghurts prepared were proved to be beneficial.

• Though feeding a probiotic yoghurt to albino rats significantly contributed for an increase in body weight followed by a reduction in coliform counts and a gradual increase in bifidobacterial counts, there was no significant change in serum cholesterol.