1. **Title of the project:** “Evaluation of probiotics for anticarcinogenic effects”

2. **Significance and scope:**

   Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit to the host. A number of health and nutritional benefits of probiotic bacteria have been reported in the literature. These benefits include improving bowel habits and mineral absorption, modulating lipid metabolism and the immune system, and retarding the processes leading to cancer development. The potential mechanisms for induced anticarcinogenic activity of probiotics include antigenotoxicity, inhibition of colonic enzyme activity, control of potentially harmful bacteria, interaction with colonocytes, immune system modulation and production of physiologically active metabolites such as short chain fatty acids. The antigenotoxic properties of probiotic bacteria ingested with food can be promising in preventing the effect of food related mutagens and a potential measure for reducing colon cancer risk. The ability of probiotics to adhere to the intestinal mucosa prolongs their persistence in the intestine and thus, allows the bacteria to exert its beneficial effects. Probiotic bacteria are also able to enhance both non-specific and specific immune responses. Since microencapsulation techniques have been widely utilized to protect microorganisms, encapsulation of probiotic bacteria against adverse effect of acid and bile salts can be attempted in order to examine the survival behavior of microencapsulated probiotic bacteria. Some known potential probiotic bacteria will be taken up for exploring their protective attributes and viability studies with respect to prebiotics and microencapsulation technology.

3. **Current status of research:**

   i. **International**

   Wagar *et al.* (2009) tested immunomodulatory bioactivity of fermented soy beverage (SB) and dairy milk blend (MB) preparations on human intestinal epithelial cells (IEC) and determined the impact of freezing medium on culture survival prior to bioactivity analyses. Fermented SB and MB were
prepared using pure or mixed cultures of *Streptococcus thermophilus* ST5, *Bifidobacterium longum* R0175, and *Lactobacillus helveticus* R0052. Immunomodulatory bioactivity was assessed by testing selected SB and MB ferments on tumor necrosis factor alpha (TNFalpha)-treated IEC and measuring effects on Interleukin-8 (IL-8) production. Impact of timing of ferment administration relative to this pro-inflammatory challenge was investigated. The most pronounced reductions in IEC IL-8 production were observed when IEC were treated with either SB or MB ferment preparations prior to TNF alpha challenge. These results indicate that freezing-stable MB and SB ferments prepared with selected strains can modulate IEC IL-8 production in vitro, and suggest that yogurt-like fermented soy formulations could provide a functional food alternative to milk-based fermented products.

Raipulis *et al.* (2005) studied antigenotoxic activity of probiotic bacteria against furazolidone using the short-term bacterial assay SOS chromotest, with *Escherichia coli* PQ37 as the test organism. The supernatants from probiotic and furazolidone after co-incubation exhibited strong suppression on SOS induction produced by furazolidone on *E. coli* PQ 37 (stiA: lacZ). The highest genotoxicity inhibition was detected for *Bifidobacterium lactis* Bb-12 (92.0%) and for *Lactobacillus acidophilus* T20 (81.9%).

Medina *et al.* (2007) evaluated the ability of different strains of *Bifidobacterium longum* to induce cytokine production by peripheral blood mononuclear cells (PBMCs). Live cells of all *B. longum* strains greatly stimulated regulatory cytokine interleukin (IL)-10 and proinflammatory cytokine tumour necrosis factor (TNF)-α production. Strains of the same species also induced specific cytokine patterns, suggesting that they could drive immune responses in different directions. The probiotic strain *B. longum* W11 stimulated strongly the production of T helper 1 (Th1) cytokines while *B. longum* NCIMB 8809 and BIF53 induced low levels of Th1 cytokines and high levels of IL-10.

Makoto *et al.* (2006) screened lactic acid bacteria which had cellular or humoral immunomodulatory function from 51 strains isolated from Mongolian fermented milk by measuring the inductive activity of IL-12 and INF-.GAMMA. or
IgM, IgG and IgA, respectively. The probiotic properties of these 51 strains were studied by measuring the tolerance to stomach acid, bile and pancreatic fluid and the adherence to human enterocyte-like Caco-2 cells in in vitro tests. Results showed that the N-17 strain (Lactobacillus plantarum), which induced large quantities of IgA, strongly tolerated stomach acid, bile and pancreatic fluid and expressed strong adherence to human enterocyte-like Caco-2 cells.

Survival of the microencapsulated probiotics, Lactobacillus acidophilus 547, Bifidobacterium bifidum ATCC 1994, and Lactobacillus casei 01, in stirred yoghurt from UHT- and conventionally treated milk during low temperature storage was investigated by Krasaekoopt et al. (2004).

Ewaschuk et al. (2006) assessed the ability of probiotic bacteria to exert anticarcinogenic effects through the production of conjugated linoleic acids. Incubation of probiotic bacteria (VSL3 Lactobacillus acidophilus, L. bulgaricus, L. casei, L. plantarum, Bifidobacterium breve, . infantis, B. longum, and Streptococcus thermophilus) in presence of linoleic acid yielded conjugated linoleic acid as measured by gas chromatography.

Apostolou et al. (2001) tested the ability to adhere to human Intestinal mucus for lactic acid bacteria of clinical blood culture, human fecal and dairy origin. The blood culture isolates were found to adhere better than the dairy strains. Of the Lactobacillus rhamnosus strains (nine clinical, 10 fecal and three dairy), blood culture isolates adhered better than the fecal strains.

Ouwehand et al. (2001) assessed the potential of new probiotic strains i.e. Lactobacillus brevis PEL1, L. reuteri ING1, L. rhamnosus VTT E-800 and L. rhamnosus LC-705 for their adhesion properties using the human intestinal mucus model. Three of the tested strains showed significant adhesion properties in the mucus model, while L. brevis PEL1 had intermediate adhesion and L. rhamnosus LC-705 adhered poorly.

Lian (2002) examined the survival of microencapsulated and free cells of bifidobacteria in simulated gastric juice (pH 2.0 and 3.0) and bile solution (0.5% and 2.0%) and found that microencapsulated bifidobacteria exhibited a lower
population reduction than free cells during exposure to simulated gastric environment and bile solution.

Cenci (2007) studied the inhibitory effect of 21 bacilli strains, previously characterized by tDNA-PCR, on four genotoxins, in vitro using the short-term assay SOS-Chromotest. All strains had a high inhibitory activity against 4-nitroquinoline-1-oxide and N-methyl-N-ø-nitro-nitrosoguanidine (direct agents), whereas the inhibitory activity was high or moderate against 2-amino-3,4-dimethylimidazo[4,5-f] quinoline and aflatoxin B1 (indirect agents).

ii. National

Jamuna and Jeevaratnam (2004) isolated lactic acid bacteria from traditional fermented foods (appam batler and pickles) and screened them for bacteriocin production. Two bacilli, LABB and LABP (one from each source) producing bacteriocin were characterized. LABB was found to be thermobacterium growing at 45°C while LABP was streptobacterium growing at 15°C. LABB was identified as *Lactobacillus acidophilus* while LABP as *Lactobacillus casei*. Their bacteriocin showed broad inhibitory spectrum against the indicator organism tested.

Bhatia and Rani (2008) evaluated the health effects of orally delivered probiotics (live and dead *S. thermophillus*) in swiss albino mice with major focus on its effect on immune response. Immune response was assessed by various methods such as NBT reduction by splenocytes, inducible nitric oxide synthase expression, phagocytosis, DTH and ELISA were the tests employed to assess the cell mediated and humoral immune responses. They observed that even the dead bacteria could modulate the immune response.

Deshpande and Dhakephalkar (2006) isolated *Lactobacilli* from faeces of healthy chickens. The cultures were screened for probiotic properties which included tolerance to low pH, bile salts, digestive enzymes, antagonistic activity towards pathogenic bacteria, enzyme production, aggregation and coaggregation properties and cell surface hydrophobicity. Three strains were found to retain almost 60% viability after exposure to low pH, bile salts (0.3%) and digestive
enzymes. These strains exhibited high cell surface hydrophobicity and auto-aggregating nature in addition to the production of protease.

Mishra and Prasad (2005) undertook an in vitro study to check the various characteristics such as acid and bile tolerance, adhesion and cell surface hydrophobicity, antimicrobial effect on common pathogens and cholesterol reduction by seven *Lactobacillus casei* strains. All the strains were able to resist low pH; NCDC 63 and VT strains were able to tolerate 1% and 2% bile concentrations. The ability to antagonize common pathogens was observed in all strains but this activity was attributed to production of organic acids and no specific compound caused the inhibitory effect. NCDC 17 showed maximum reduction in cholesterol level after 48 h incubation with buffalo plasma as the source of cholesterol.

References


4. Deshpande M and Dhakephalkar PK. 2006. *In vitro* assessment of probiotic potential of *Lactobacilli* isolated from poultry. Paper presented in 47th annual conference of Association of Microbiologists of India, held at Department of Biotechnology, and Bioinformatics Centre, Barkatullah University, Bhopal from Dec 6-8,2006


4. Research Objectives (50 words)
1. To study known probiotic bacteria for antigenotoxicity and immunomodulatory effects.
2. To study adhesion property of probiotic bacteria
3. To study the effect of probiotics on fecal enzyme(s) involved in carcinogenicity
4. To study viability of probiotic strains using prebiotics and micro-encapsulation.

5. Methodology describing work elements
1. Screening of probiotic bacteria for protective attributes will be done according to following protocol:
   (I) Antigenotoxicity of probiotics
   The antigenotoxicity of probiotic bacteria against genotoxins such as 4-nitroquinoline-1-oxide (4-NQO) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) will be determined by using following short term methods:
   (a) Cell preparation and genotoxin-cell co incubation
   The cultures will be grown overnight in MRS broth, washed and resuspended in saline. Genotoxins will be added and co-incubation was maintained under agitation. The supernatant will be recovered by centrifugation and filtered by membrane filter. The resultant filtrate will be used for determining residual genotoxic activity.
   (b) Ames test
   The mutagenicity will be estimated by measuring the extent of reverse mutation of S. typhimurium his- TA 100 strain. An overnight culture of the test strain will be added to lactobacilli-genotoxin supernatant. The mixture will be mixed with soft agar and poured on minimal agar plate containing histidine. Bacterial revertants will be counted after incubation.
   (c) SOS chromotest
The genotoxicity will be evaluated by SOS response in E.coli PQ37 strain. PQ37 strain will be incubated overnight in Luria broth plus ampicillin and then transferred into same medium and incubated for 2 h. The fractions of culture will be mixed with sample containing genotoxin or supernatant of genotoxin treated culture and incubated. The induction of SOS DNA repair system will be evaluated by induction of β- galactosidase.

(d) **Viability evaluation**

Cell viability after co-incubation with genotoxins will be tested by plate count.

(II) **To study the Immunomodulatory properties of probiotic bacteria**

The immunological properties of probiotic bacteria have raised a lot of interest in recent years due to their immune- stimulating properties. Several strains of probiotic bacteria have been reported to display stimulatory properties on cells of the innate immune system in vitro, including macrophages and NK cells. For immune cell stimulation, bacterial cells will be grown till stationary phase, washed and resuspended in phosphate buffered saline. Peripheral Blood Mononuclear Cells (PBMCs) will be isolated from peripheral blood of healthy donors and these cells will be induced for cytokine production by using probiotics.

(2) **Adhesion properties of probiotic bacteria:**

(1) **Adhesion assay**

**Adhesion of probiotic bacteria on caco-2 cells**

(a) **Caco-2 cell culture:**

The Caco-2 human colon adenocarcinoma cell line will be cultured in Dulbecco’s modified Eagle’s medium (DMEM) with L-glutamine, fetal calf serum Fetaclone II and gentamycin sulphate. The incubation will be carried out in 10 per cent CO₂ atmosphere.

(b) **Preparation of Probiotic bacteria and Caco-2 for adhesion assay:**

For adhesion assay, the Caco-2 cells will be seeded in 24-well standard tissue culture plates. The cells will be maintained for 2 weeks for full
differentiation. The number of cells will be determined by trypsinisation of the monolayer and counting by using a haemocytometer.

Probiotic bacteria from broth culture will be obtained by centrifugation and washed once with PBS buffer and once with the buffer used in the assay. The exact number of probiotic bacteria used in the assay will be determined by plate counting on MRS agar.

(c) Adhesion assay:

The adhesion assay will be carried out in 24-well standard tissue culture plates. After washing the Caco-2 monolayer twice with PBS, bacterial suspension will be added to each well and incubated for 30 minutes in atmosphere with 10 % CO$_2$, at 37°C. The unattached bacteria will be removed by washing. In order to enumerate the attached bacteria, each well will be treated with Triton X-100. Mixture of lysed Caco-2 cells and bacteria will be plated on MRS agar and will be enumerated after 48 hours.

(3) Viability studies on probiotic strains using prebiotics and microencapsulation technology:

a. Microencapsulation of probiotics will be done in different edible food carriers such as alginate and/or carrageenan. The viability studies will be carried out on these microencapsulated probiotics.

b. Prebiotics aids the survival and prolong the survival of probiotic bacteria. So different prebiotics will be screened which support maximum survival of probiotic bacteria. For this: The probiotic isolates will be cultured in reconstituted skim milk containing different concentrations of prebiotics such as oligosaccharides. The viability will be determined by storage at 4 °C for different intervals of time.

c. The prebiotic and probiotic (synbiotic) with maximum survivability will be converted into effective formulation.

(4) Effect of probiotics on fecal enzymes involved in carcinogenicity:
Colon cancer in rats/mice will be induced by using 1-2 dimethylhydrazine (DMH) as a carcinogenic agent. The large intestine will be removed and fecal enzyme like glucuronidasse/azoreductase/nitroreductase will be determined in following groups:

1. Control : No inoculation to Mice
2. Bacteria administered to Mice
3. Carcinogenic agent administered to Mice
4. Carcinogenic agent + Bacteria administered to Mice
5. Carcinogenic agent + prebiotic+ Bacteria administered to Mice
6. Carcinogenic agent + synbiotic+ Bacteria administered to Mice

in rats/mice models in which colon cancer would be induced by using 1-2 dimethylhydrazine (DMH) or DLD-1 human colon cancer cells as a carcinogenic agents.

### 6. Time-Activity Chart

<table>
<thead>
<tr>
<th>Time</th>
<th>Proposed work</th>
</tr>
</thead>
<tbody>
<tr>
<td>First month</td>
<td>Procurement/Ordering of material for experiments</td>
</tr>
<tr>
<td>Next 4 months</td>
<td>Antigenotoxicity of probiotics</td>
</tr>
<tr>
<td>Next 2 month</td>
<td>Adhesion properties of probiotic bacteria</td>
</tr>
<tr>
<td>Approx. 4 months</td>
<td>To study the Immunomodulatory properties of probiotic bacteria</td>
</tr>
<tr>
<td>Approx. 4 month</td>
<td>Viability studies on probiotic strains using prebiotics and microencapsulation technology:</td>
</tr>
<tr>
<td>Approx. 6 months</td>
<td>Effect of probiotic bacteria on fecal enzymes involved in carcinogenicity</td>
</tr>
<tr>
<td>Next 3 months</td>
<td>Analysis of data, preparation of final report</td>
</tr>
</tbody>
</table>
Expected Output (150 words)

It will give an insight into the anticarcinogenic and immunomodulatory potential of probiotic bacteria. Effective formulation of synbiotic will be produced which can be used for further evaluating for use in human clinical trials for proposed beneficial effects. It will scientifically validate the proposed effects of probiotic bacteria under in vitro and in vivo experiments. Further, continuation of work may be done by studying and isolating bioactive molecules contributing specifically to proposed effects in this project study. This work have the potential to provide mechanistic insights into the role of probiotic bacteria (which are mostly part of gut microflora) in colon carcinogenicity, a disease that is responsible for significant morbidity in the adult population, and may ultimately lead to novel approaches for prevention and treatment of this disorder.