CHAPTER – 3

MATERIALS & METHODS
PHASE I

In this phase, *in vitro* experiments were carried out to

- Evaluate the immunomodulatory activity of probiotic strains and selected the maximum, moderate and minimum bioactive strain

3.1 Procurement of different probiotic strains and maintenance

Following probiotic strains were procured from National Collection of Dairy Culture, Karnal.

- *Lactobacillus casei subsp. casei* 17 (LB 17)
- *Lactobacillus brevis* 403 (LB 403)
- *Lactobacillus delbrueckii* 405 (LB 405)
- *Bifidobacterium bifidium* BA3 233 (Bif 233)
- *Bifidobacterium bifidium* BD4 234 (Bif 234)
- *Bifidobacterium bifidium* BD1 235 (Bif 235)

The cultures so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. Bacterial cultures were grown and maintained for further use.

For *Lactobacillus spp*.

- Selective growth media - MRS
- Growth temperature - 37°C
- Growth conditions - Facultative anaerobes
- Growth time - 24 hrs
- pH - 6.8 ± 0.2
For *Bifidobacterium* spp.

Selective growth media - MRS

Growth temperature - 37°C

Growth conditions - Facultative anaerobes

Growth time - 24 to 72 hrs

pH - 6.8 ± 0.2

### 3.1.1 To study the growth pattern of probiotic strains

**Reagents:**

1. MRS Broth

**Procedure:**

All probiotic strains (*Lactobacillus casei subsp. casei* 17, *Lactobacillus brevis* 403, *Lactobacillus delbrueckii* 405, *Bifidobacterium bifidium* (BA3 233), *Bifidobacterium bifidium* (BD4 234), *Bifidobacterium bifidum* (BD1 235) were inoculated at the rate 1% in MRS broth and their growth pattern was monitored by measuring the absorbance at 600 nm.

### 3.2 *In vitro* evaluation of immunomodulatory activity for selection of probiotic strains

**3.2.1 Preparation of test samples**

The six strains i.e. LB 17, LB 403, LB 405, Bif 233, Bif 234 and Bif 235 after appropriate growth in MRS broth were collected and centrifuged at 4000 rpm at 4°C for 10 min. Supernatant
was discarded and pellet was washed twice with PBS (pH 7.4). Finally, cells were suspended in 10ml PBS, counted and were standardized as $1 \times 10^6$cells ml$^{-1}$ and $1 \times 10^9$cells ml$^{-1}$ for each strain.

### 3.2.2 Splenocyte isolation

Splenocytes were isolated from spleen by teasing the tissue. Cells were centrifuged ($400 \times$ g for 10 min at $4 \, ^\circ \, C$) and lysed by ACK lyses solution ($0.5M \, NH_4Cl$, $10mM \, KHCO_3$ and $0.1 \, mM$ disodium EDTA, pH 7.2). Splenocytes obtained were washed thrice in PBS, counted and adjusted ($2 \times 10^6$ cells/ml) in RPMI.

### 3.2.3 Incubation of splenocytes and test samples

The test samples (LB 17, LB 403, LB 405, Bif 233, Bif 234 and Bif 235) as $1 \times 10^6$cells ml$^{-1}$ and $1 \times 10^9$cells ml$^{-1}$ for each strain was incubated with $2 \times 10^6$ cells splenocytes/ml at $37^\circ C$ for 24 hrs in humidified CO$_2$ chamber.

Following techniques (details of techniques are given in chapter 4) were employed to evaluate the in vitro immunoactivity at two different concentrations i.e. $1 \times 10^6$cells ml$^{-1}$ and $1 \times 10^9$cells ml$^{-1}$ for each probiotic strain.

1. Nitroblue Tetrazolium Chloride (NBT) Reduction test (Hudson and Hay, 1989)
2. Inducible Nitric Oxide Synthase activity (Stuehr and Marletta, 1987)
3. Bactericidal activity (Raghuramulu et al., 1983)
**PHASE II**

In this phase,

- Selected probiotic strains showing maximum (LB 405), moderate (LB 403) and minimum activity (Bif 234) as well as their isolated DNAs were immunocharacterized *in vivo*.

3.3 Isolation of DNA from probiotic strains (Atashpaz *et al.*, 2010)

Reagents:

1. Lysis Buffer (2% CTAB ,100mMTris HCl, 1.4 M NaCl, 1% PVP, 20mM disodium salt of ethylenediaminetetraacetic acid (Na$_2$EDTA),0.2% LiCl, pH:8.0)
2. Chlorform: Isoamyl solution (Hi-media)
3. Isopropanol (Merck)
4. 70 % Ethanol (Merck)
5. TE Buffer (10mM Tris HCl, 1mM EDTA, pH : 8.0)

Procedure:

1. An overnight culture (1.5 ml of MRS broth and 1.5% glycine) containing 1 x 10$^9$ cells ml$^{-1}$ was pelleted at 8000 rpm (microcentrifuge) at 25°C for 5 minutes and resuspended in 800µL Lysis buffer. The samples were incubated for 2h at 65°C with gentle shaking after every 10 min.
2. The sample was centrifuged at 10,000 rpm for 5 min at 4°C. The supernatant was transferred into a new tube and an equal volume of a Chlorform: Isoamyl (24:1 vol/vol) was added. Then, the tube was gently flipped several times.
3. The sample was again centrifuged at 12000 rpm for 8 min at 4°C. The upper phase was then transferred into a new tube.
4. In order to increase the yield of extracted DNA, 100 µL of 5M sodium acetate was added into the tube and mixed by gentle flipping.
5. Equivalent volume of isopropanol (-20°C) was added to precipitate the DNA and washed with 70% ethanol to remove residual contamination.

6. The supernatant was discarded and pellet was dried at room temperature. The pellet was dissolved in 20-30 µL of TE.

7. The concentration and purity of DNA were analyzed spectrophotometrically by measuring \( \text{OD}_{260}/\text{OD}_{280} \). Only the DNA with \( \text{OD}_{260}/\text{OD}_{280} \) ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1% agarose gel (100V for 20-40 min).

3.4 *In vivo* immuno-characterization of selected probiotics as well as their DNAs

Selected probiotics having maximum (LB 405), moderate (LB 403) and minimum activity (Bif 234) along with their isolated DNA (DNA LB 405, DNA LB 403 and DNA Bif 234) were tested for *in vivo* immunomodulatory potential in male swiss albino mice (18-22gm).

3.4.1. Animals

The animals were divided into respective groups each of minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CP-CSEA-2010-40). Experiments were carried out as per the guidelines of committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

3.4.2. Experimental design and groups of animals

Group I: Untreated Control (kept on normal diet)

Group II: Positive control (25mgkg\(^{-1}\) Levamisole i.p for 17 consecutive days)
Group III: Immunized control (immunization with SRBC and were fed on normal diet)

Group IV: Bif 234 (10^9 cells day^{-1} mouse^{-1} as oral dose for 17 consecutive days)

Group V: DNA Bif 234 (75µg mL^{-1} mouse^{-1} three injections in left tibialis anterior muscle after 6 days)

Group VI: LB 403 (10^9 cells day^{-1} mouse^{-1} as oral dose 17 consecutive days)

Group VII: DNA LB 403 (75µg mL^{-1} mouse^{-1} three injections in left tibialis anterior muscle after 6 days)

Group VIII: LB 405 (10^9 cells day^{-1} mouse^{-1} as oral dose 17 consecutive days)

Group IX: DNA LB 405 (75µg mL^{-1} mouse^{-1} three injections in left tibialis anterior muscle after 6 days)

All groups were treated with respective doses as given above. On day 4 of treatment the animals were immunized with a single dose of SRBC (Sheep red blood cells). The blood was withdrawn from retro orbital plexus on day 0, 8 and 13 to assess the humoral immune response. On day 15th the SRBC’s were given in the left hind foot pad and the lateral paw received only normal saline for cell mediated DTH reaction. The cell mediated immune response was assessed by measuring the footpad thickness of each animal from day 15th to day 18th (The technique for DTH is given in chapter 4).

The animals were sacrificed on day 18th, the spleen was excised and splenocytes were separated as described in section 3.2.2. The separated splenocytes were then assessed for macrophage function (NBT, iNOS and bactericidal activity is given in chapter 4).

3.4.3 Immunization (Alsever and Ainslie, 1941)
Sheep blood was collected in Alsever's solution in the ratio 1:2 and was centrifuged at 400 \times g for 10 min at 4 °C. The erythrocyte pellet obtained was washed and suspended in PBS (0.1 M, pH 7.2) for further use. All mice were antigenically challenged intraperitoneally with a single dose (100µl ml\(^{-1}\) of \(1 \times 10^7\) cells/ml) of sheep red blood cells (SRBC).

3.4.4. Humoral Immune Response

To assess the humoral immune response, blood was withdrawn from retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8th and 13th (post immunization). The serum was separated and assayed by direct haemagglutination for the anti-SRBC antibody titer. The technique of **direct haemagglutination is given in chapter 4.**
PHASE III

In this phase, experiments were carried out to compare the mechanism of immunopotentiation of best selected strain DNA with its pure culture (LB405) and to study the biotherapeutic efficacy.

- The development of anti BSA antibodies
- Expression of Th-1(IFN-gamma) and Th-2 (IL-4) mediated cytokines
- The biotherapeutic potential was evaluated as antidiabetic, anticholesteramic and immunorestorative potential in immune suppressed host

3.5. Anti-BSA antibodies in sera by Indirect ELISA (Haghighi et al., 2005).

Selected probiotic having maximum immunoactivity (LB 405) along with their isolated DNA (DNA LB 405) was tested for development of antibodies against BSA by Indirect ELISA.

3.5.1. Animals

The male swiss albino mice (18-22gm) were divided into respective groups each having minimum six animals, housed individually in the departmental animal house and was exposed to 12 hr cycle of light and dark.

3.5.2. Experimental design and groups of animals

Group I: Untreated Control (kept on normal diet)

Group II: Immunized control (immunization with BSA and were fed on normal diet)

Group III: LB 405 (10^9 cells day^{-1} mouse^{-1} as oral dose for 17 consecutive days)
Group IV: **DNA LB 405** (75µg mL$^{-1}$ mouse$^{-1}$ three injections in left tibialis anterior muscle after 6 days)

Group V: **LB 405 + immunized**

Group VI: **DNA LB 405 + immunized**

### 3.5.3. Immunization

All mice were antigenically challenged intraperitoneally with three doses (0.25 ml PBS containing 100µg BSA) of Bovine Serum Albumin. The blood was withdrawn from retro orbital plexus on 0$^{th}$, 8$^{th}$ and 13$^{th}$ day to assess the antibody.

Technique is given in chapter 4.

### 3.6 Effect of most potent DNA LB 405 and LB 405 on cytokine expression

Selected probiotic having maximum immunoactivity (LB 405) along with its isolated DNA (DNA LB 405) was tested for expression i.e. Th-1(IFN-gamma) and Th-2 (IL-4) mediated cytokines by Indirect ELISA.

#### 3.6.1. Animals

The male swiss albino mice (18-22gm) were divided into respective groups each having minimum six animals, housed individually in the departmental animal house and was exposed to 12 hr cycle of light and dark.

#### 3.6.2. Experimental design and groups of animals

**Group I:** Untreated Control (kept on normal diet)

**Group II:** Positive Control (25 mg kg$^{-1}$, Levamisole i.p. for 17 consecutive days)

**Group III:** Immunized control (immunization with SRBC and were fed on normal diet)
Group IV: LB 405 \((10^9 \text{cells day}^{-1} \text{mouse}^{-1} \text{as oral dose for 17 consecutive days})\)

Group V: DNA LB 405 \((75 \mu\text{g mL}^{-1} \text{mouse}^{-1} \text{three injections in left tibialis anterior muscle after 6 days})\)

3.6.3 Immunization (Alsever and Ainslie, 1941)

Sheep blood was collected in Alsever’s solution in the ratio 1:2 and was centrifuged at 400 \(\times\) g for 10 min at 4 °C. The erythrocyte pellet obtained was washed and suspended in PBS \((0.1 \text{M, pH 7.2})\) for further use. All mice were antigenically challenged intraperitoneally with a single dose \((100\mu\text{l ml}^{-1}\text{of }1 \times 10^7 \text{cells/ml})\) of sheep red blood cells (SRBC).

Technique is given in chapter 4.

3.7 Therapeutic potential of most potent DNA of \textit{Lactobacillus delbrueckii}\n
LB 405 (DNA LB 405) and LB 405

The therapeutic potential of bioactive DNA of \textit{Lactobacillus delbrueckii} 405 (DNA LB 405) was assessed as antidiabetic, anicholesterolamc and immunorestorative agents in swiss albino mice.

3.7.1 Antidiabetic potential

3.7.1.1. Induction of experimental diabetes (Ozbek \textit{et al.}, 2004)

Reagents

1. Alloxan monohydrate

Procedure:

Experimental diabetes was induced by intraperitoneal (i.p.) administration of alloxan monohydrate in mice which had been subjected to overnight fasting. Total dose of alloxan \((150 \text{mg/kg b.wt})\) was administered in three injections at intervals of 24 h \((50 \text{mg/kg b.wt each time})\).
After 72 h, animals showing blood glucose level above 200 mg/dl (diabetic) were selected for study.

3.7.1.2. Collection of blood and determination of blood glucose

Blood of control and experimental mice was collected from orbital sinus puncture using a heparinized capillary glass tube. The blood samples so collected were analyzed for blood glucose levels by glucose estimation kit (Bayer health care LLC, Ireland). Blood glucose levels were estimated according to the method of Dunn & McLetchie (1943).

3.7.1.3. Experimental design

Diabetic swiss albino mice of either sex were divided into six groups (n = 6).

Group I: **Untreated control** i.e. mice fed basal feed.

Group II: **Drug control** (Glyburide, 10 mg/kg b.wt)

Group III: **LB 405** i.e. Hyperglycemic mice dosed with LB 405 $10^9$ cells day$^{-1}$ mouse$^{-1}$ as oral dose

Group IV: **DNA LB 405** i.e. Hyperglycemic mice dosed with DNA of LB 405 in left tibialis anterior muscle (Millan et al., 1998) at the rate of 75 µg mL$^{-1}$/mouse

Group V: **LB 405 + Drug** i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and LB 405 ($10^9$ cells day$^{-1}$ mouse$^{-1}$).

Group VI: **DNA LB 405 + Drug** i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and DNA of LB 405 (75 µg mL$^{-1}$/mouse$^{-1}$).

Experiment involved the acute and sub-acute study. The **acute study** involved the estimation of blood glucose levels at 0, 2, 4, 6, and 24 h after administration of LB 405, DNA LB 405, drug and combinations with all the doses given at one time.

The **sub-acute study** involved the repeated administration of drug for 28 days at a prefixed time and blood glucose levels were estimated on days 7, 14, 21, and 28. The data were represented as mean blood glucose level and standard error mean (SEM).

3.7.1.4. Oral glucose tolerance test (OGTT) in normal and diabetic mice

Non-diabetic and diabetic mice were divided into six groups (n = 6)
Group I: Untreated control i.e. mice fed basal feed.

Group II: Drug control (Glyburide, 10 mg/kg b.wt)

Group III: LB 405 i.e. Hyperglycemic mice dosed with LB 405 (10^9 cells day^{-1} mouse^{-1}) as oral dose

Group IV: DNA LB 405 i.e. Hyperglycemic mice dosed with DNA of LB 405 in left tibialis anterior muscle (Millan et al., 1998) at the rate of 75µg mL^{-1}/mouse

Group V: LB 405 + Drug i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and LB 405 (10^9 cells day^{-1} mouse^{-1}).

Group VI: DNA LB 405 + Drug i.e. Hyperglycemic mice dosed with glyburide (10 mg/kg b.wt) and DNA of LB 405 (75µg mL^{-1} mouse^{-1}).

Prior to commencement of experiment, the animals were fasted overnight. After 30 min of doses administration, animals were loaded with D-glucose solution (2.5 mg/kg) and blood glucose levels were monitored at 0, 30, 60, and 120 min after glucose loading.

3.7.2. Anticholesteremic activity

3.7.2.1. Induction of experimental Cholesteremia

Reagents:

1. Cholesterol

Procedure:

Cholesterolemia in mice was induced by feeding them on 1 % cholesterol mixed in normal diet. The total dose of 200mg/kg b.wt. was given over a period of 7 days. After seven days animals showing serum cholesterol level above 120 mg/dl were selected for the further experiment. The day microbial diet started was considered as day 0.

3.7.2.2. Experimental animal design

Group I: Untreated control i.e. mice fed basal feed.

Group II: Positive control i.e. hypercholesteremic mice.
Group III: **Drug control** i.e. hypercholesteremic mice treated with Atorvastatin (10 mg/kg, b.wt.)

Group IV: **LB 405** i.e. Hypercholesteremic mice dosed with LB 405 ($10^9$ cells day$^{-1}$ mouse$^{-1}$) as oral dose

Group V: **DNA LB 405** i.e. Hypercholesteremic mice dosed with DNA of LB 405 in left tibialis anterior muscle (Millan et al., 1998) after 6 days at the rate of 75µg mL$^{-1}$ mouse$^{-1}$.

Group VI: **LB 405 + Drug** i.e. Hypercholesteremic mice dosed with Atorvastatin (100mg/kg, b.wt) and LB 405 ($10^9$ cells day$^{-1}$ mouse$^{-1}$).

Group VII: **DNA LB 405 + Drug** i.e. Hypercholesteremic mice dosed with Atorvastatin (100mg/kg,b.wt) and DNA of LB 405 (75µg mL$^{-1}$ mouse$^{-1}$).

Animals received respective doses consecutively for 15 days. Blood samples were obtained from retro-orbital plexus on 16th day. Serum was separated by centrifugation at 3000 rpm for 15 minutes.

**3.7.2.3. Estimation of total serum cholesterol (Wybenga, et al., 1970)**

Blood cholesterol level of animals was checked by using commercial diagnostic reagent kit manufactured by Span Diagnostic Ltd. India.

**Reagents:**

The kit contained three reagents:

1. Cholesterol Reagent- I
2. Working Cholesterol Reagent (200ng)-II
3. Precipitating Reagent- III

**Procedure:**

Briefly, to the 3ml of cholesterol reagent-I added 150µL of serum sample, 150 µL of reagent II and 150 µL of reagent III (T). For the standard, 3ml of cholesterol reagent-I was taken and to it 150 µL of reagent-II was added and for blank 3 ml of reagent-I was taken. Then, mixed well separately and kept the test tubes immediately in the boiling water exactly for 90 sec. Cooled
immediately to room temperature and the absorbance of standard and test (lavender color complex) was noted against blank with spectrophotometer at 540nm.

The concentration of cholesterol in mg/dl of the test samples was calculated as:

\[
\text{\% Cholesterol (mg/dl)} = \frac{\text{O.D. of Test (T)}}{\text{O.D. of Standard (S)}} \times 200
\]

3.7.3. Immunorestorative potential in immune suppressed host

3.7.3.1. Induction of Immunosuppression (Rania, 2004)

Reagents:

1. Hydrocortisone

Procedure:

Swiss albino mice (25-27gm) were made immunosuppressed by giving them hydrocortisone (HC) obtained from Wyeth Lederie Limited on day 1\textsuperscript{st} and 4\textsuperscript{th} (i.p.) i.e. 5 mg/kg b.wt. and after that they were divided into following groups and were given respective doses.

3.7.3.2. Experimental design

Group I: Untreated control i.e. mice fed basal feed.

Group II: Only Hydrocortisone (5 mg/kg b.wt.)

Group III: LB 405 (10\textsuperscript{9} cells day\textsuperscript{-1} mouse\textsuperscript{-1}) i.e. mice which were immunosuppressed were given LB 405

Group VI: DNA LB 405 (75\mu g mL\textsuperscript{-1} mouse\textsuperscript{-1}) i.e. mice which were immunosuppressed were given DNA of LB 405

After making the animals immune suppressed the animals were treated with respective doses for 17 consecutive days. On day 4\textsuperscript{th} of treatment all the groups were immunized with a single dose of SRBC as mentioned in section 3.4.3. and blood was collected from retero orbital plexus on day 0, 8\textsuperscript{th} and 13\textsuperscript{th} for humoral immune response by direct haemagglutination as mentioned in chapter 4. All SRBC primed groups were challenged intradermally on day 15 with SRBC and
footpad thickness was measured at 0, 24, 48 and 72 h to assess delayed type hypersensitivity response (measure of cell mediated immune response) as mentioned in chapter 4. The animals were sacrificed on day 18th, their spleen was excised and assayed for NBT reduction test, iNOS and bactericidal activity as mentioned in chapter 4.