



Efficacy of Indian Probiotic Cultures

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Outline of presentation

- Probiotics - entry in India
- Status of probiotics at global level
- Need for Indigenous probiotics
- Role of potential biomarkers for screening and selection of potential strains
- Initiative for Developing Indigenous Probiotics of Indian origin at NDRI, Karnal (DBT)
- Desired Interventions from Indian Perspective
- Conclusion

Probiotics – the New Nutraceutical Stars enter in Indian Market



- An old concept, with a new attitude
- Probiotic based functional foods - a strong global market
- Japan, Europe - the major players
- Dairy based probiotic foods / drinks (> 50%)
- Probiotics gaining a foothold in India

Status of probiotic cultures at Global level

- *L. rhamnosus* GG (Valio)
- *L. casei* Shirota (Yakult)
- *L. plantarum* 299v (Probi AB)
- *L. johnsonii* La7 (Nestle)
- *L. reuteri* (BioGaia)
- *L. acidophilus* NCFM (Nestle)
- *L. casei* strain DN-114001 (Danisco)
- *B. animalis* DN 173010 (Danisco)
- *L. rhamnosus* 271 (Probi AB)
- *L. casei* (Chr Hansen)
- *L. acidophilus* La1
- VSL#3 (4 *Lactobacillus* spp.; 4 *Bifidobacterium* spp. and one streptococcus) (CD Pharma)

Efficacy / Prospects of probiotic strains

- Widely studied strains world wide
- Scientifically proven probiotics
- Clinical trials conducted for their efficacy
- However, results are debatable

- Several factors are known to influence this benefit

Timing of probiotic delivery

- Prophylactic, empiric or therapeutic

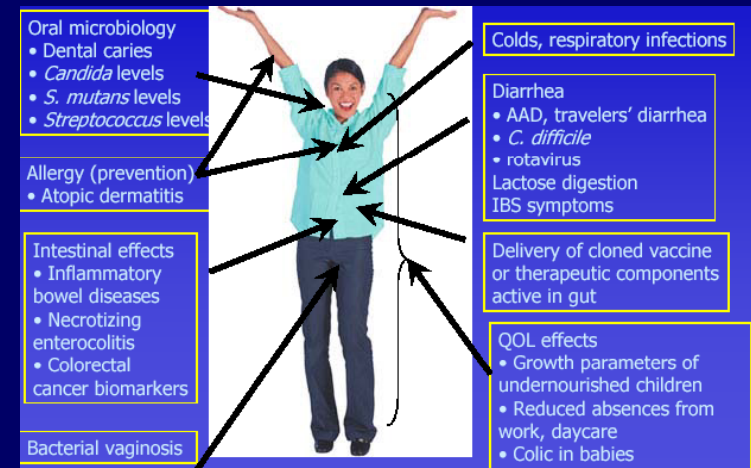
Specific strain of bacteria

- Various secreted components can modulate activity

Quantity of bacteria

Method of administration

Host factors



Major Issues for use in Indian population

- Data in Indian population - lacking ?
- Poor adhesion /colonization and short transit period in the Indian gut due to different gut ecology and food habits
- Conditioning effect - critical for acclimatization and optimal functionality
- Non-availability of established / Novel Indian probiotic strains with specific health functions to demonstrate their efficacy

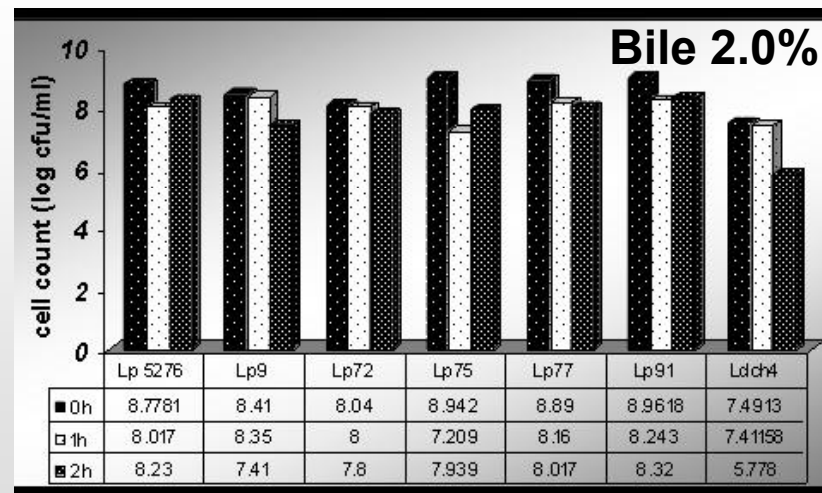
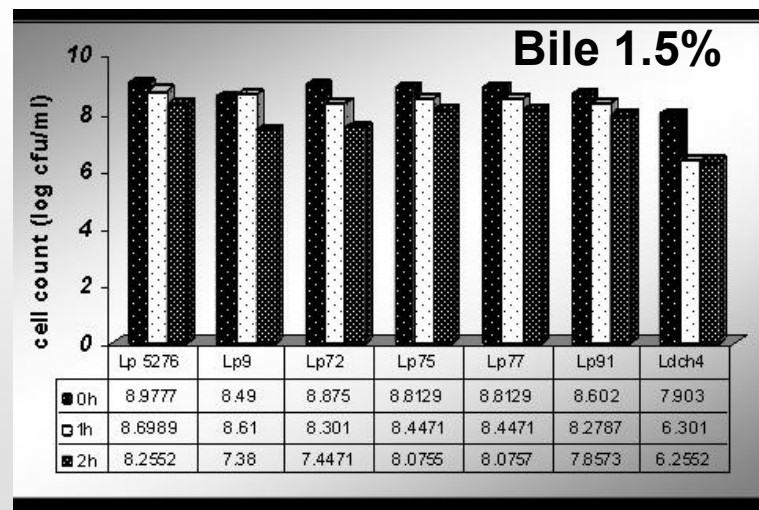
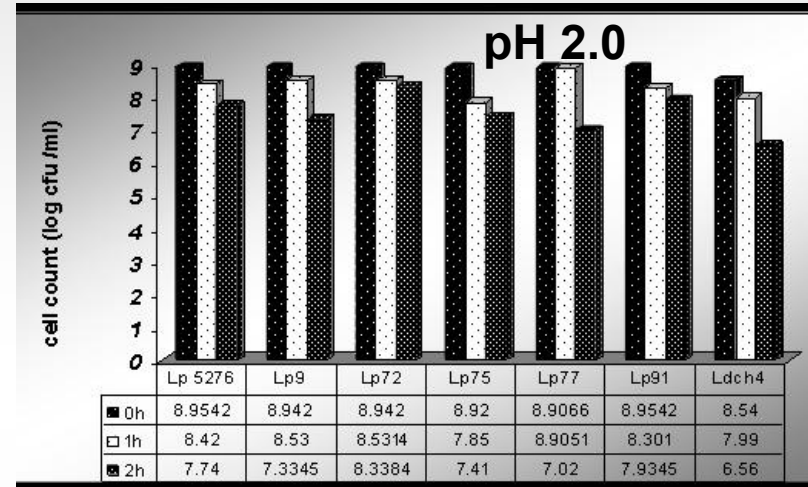
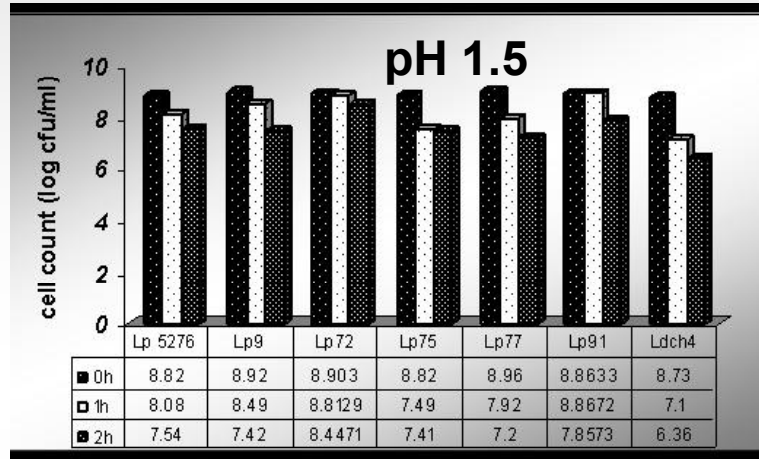
Why Need for Indigenous probiotics ?

- Better colonization / adhesion potential
- Longer transit time
- Health promoting functions of probiotics – highly strain and host specific
- High incidences of diarrhoeal diseases and other gastric disorders in Infants and immuno-compromised
- Probiotics as potential Immuno-modulators – Healthy guts
- **Indian probiotic strains might be better suited to Indian gut due to longer transit time, colonization / biofilm formations and conditioning effect**

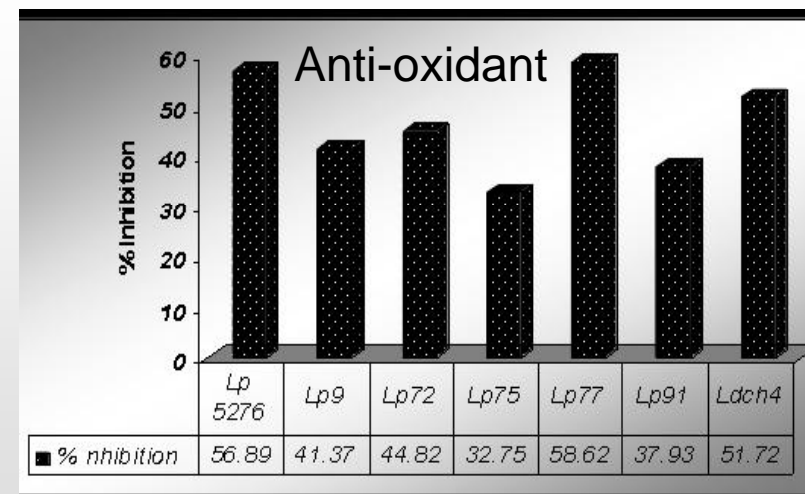
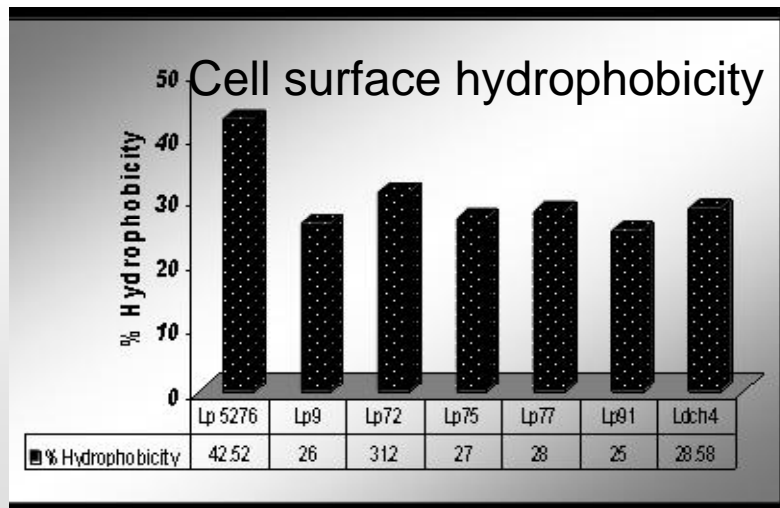
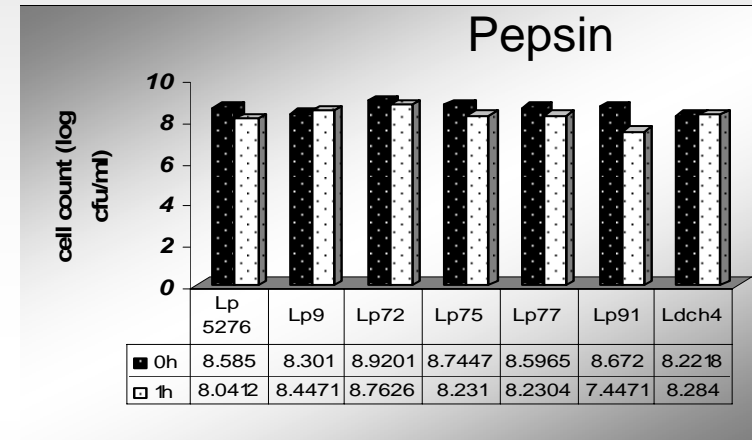
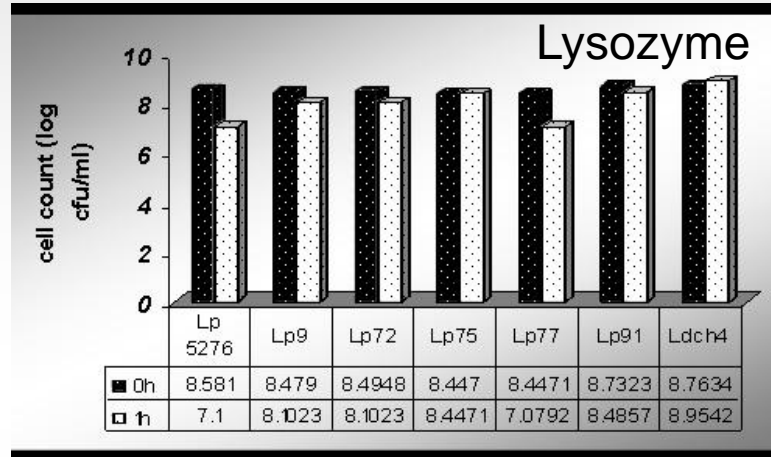
Initiative for Developing Indigenous Probiotics of Indian origin at NDRI, Karnal (DBT)

- More than one hundred cultures of indigenous Lactobacilli of human origin isolated and studied for their probiotic attributes and colonization potentials
- Tested for a battery of *in vitro* tests for culture short listing
- Selection of promising cultures based on strong colonization and *in vitro* probiotic attributes

Selection of promising cultures based on *in vitro* probiotic attributes

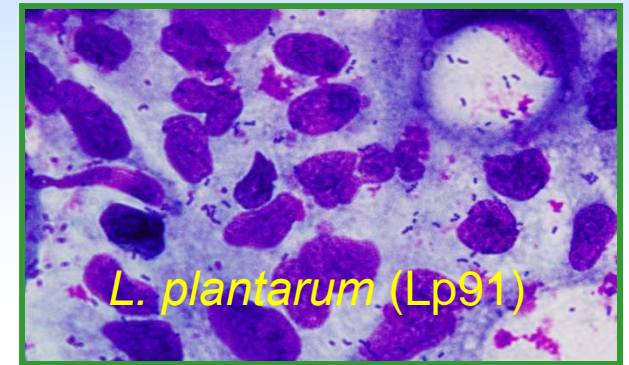
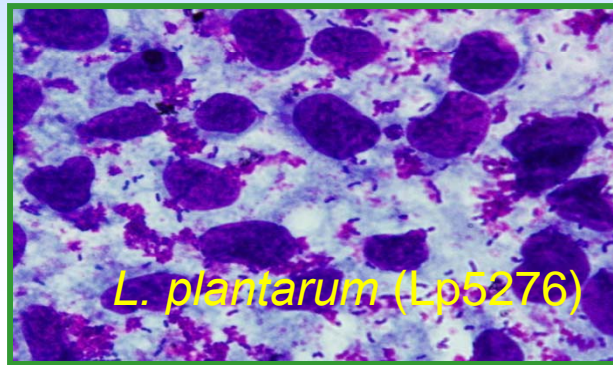
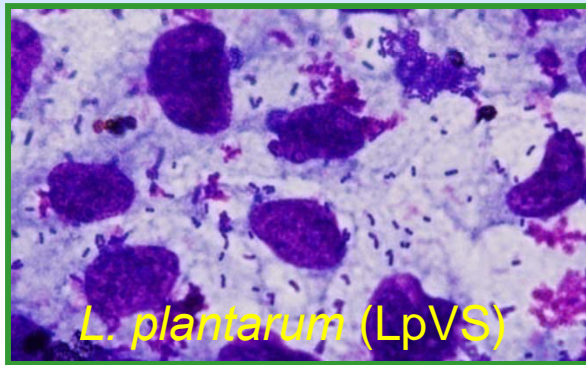


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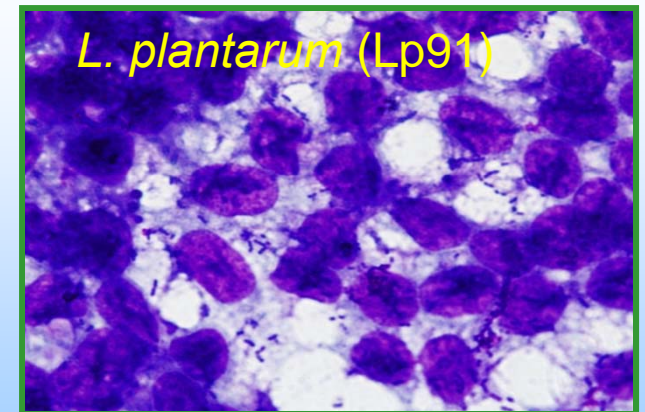
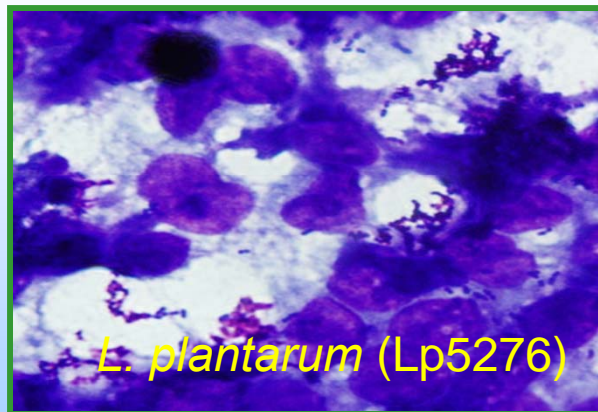
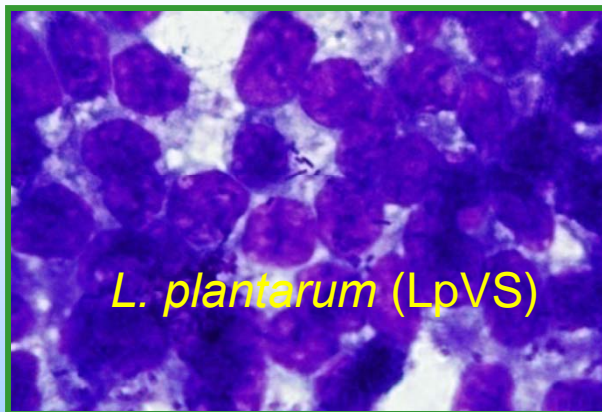


Adhesion of Lactobacilli (10^9 cfu/ml for 2 hrs) to human adenocarcinomal cell lines

Caco2 cell line:



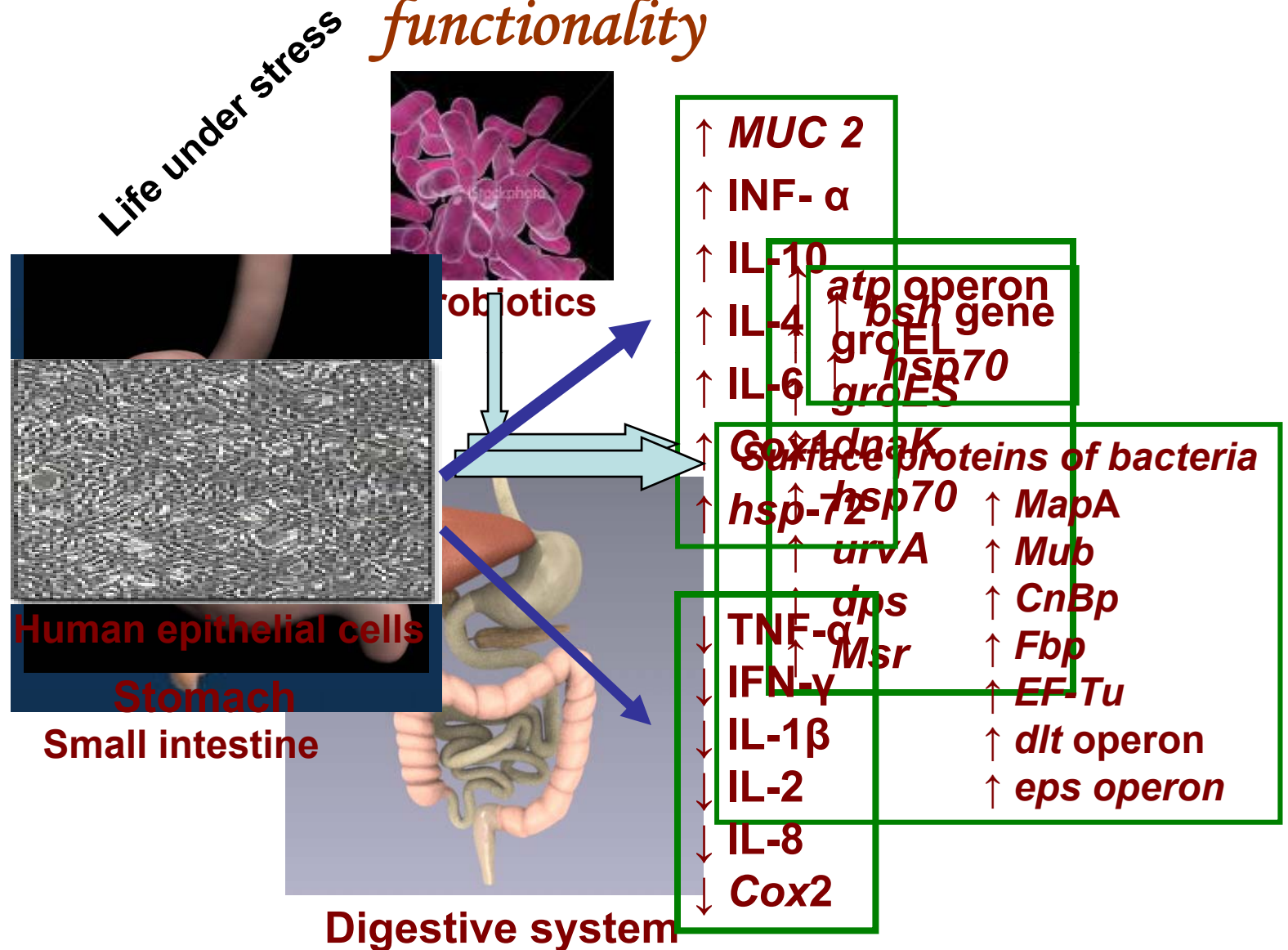
HT-29 cell line:



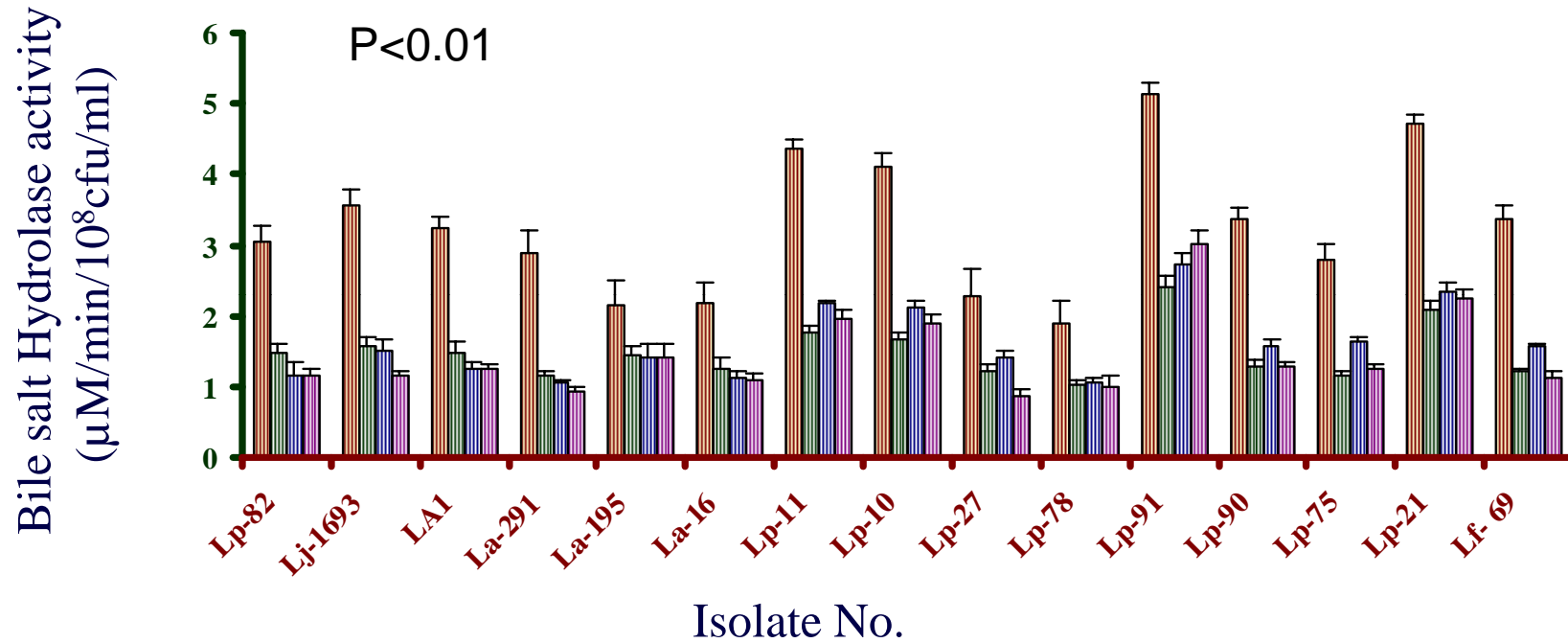
Role of Biomarkers for selection of probiotics

- Selection of a proven probiotic strain extremely crucial for application in clinical trials and product development
- Potential biomarkers need to be developed for screening and selection of probiotic strains

Expression of bacterial and host genes during transition in GIT of human that can be used as potential biomarkers for functionality



Quantification of BSH activity by modified ninhydrin assay



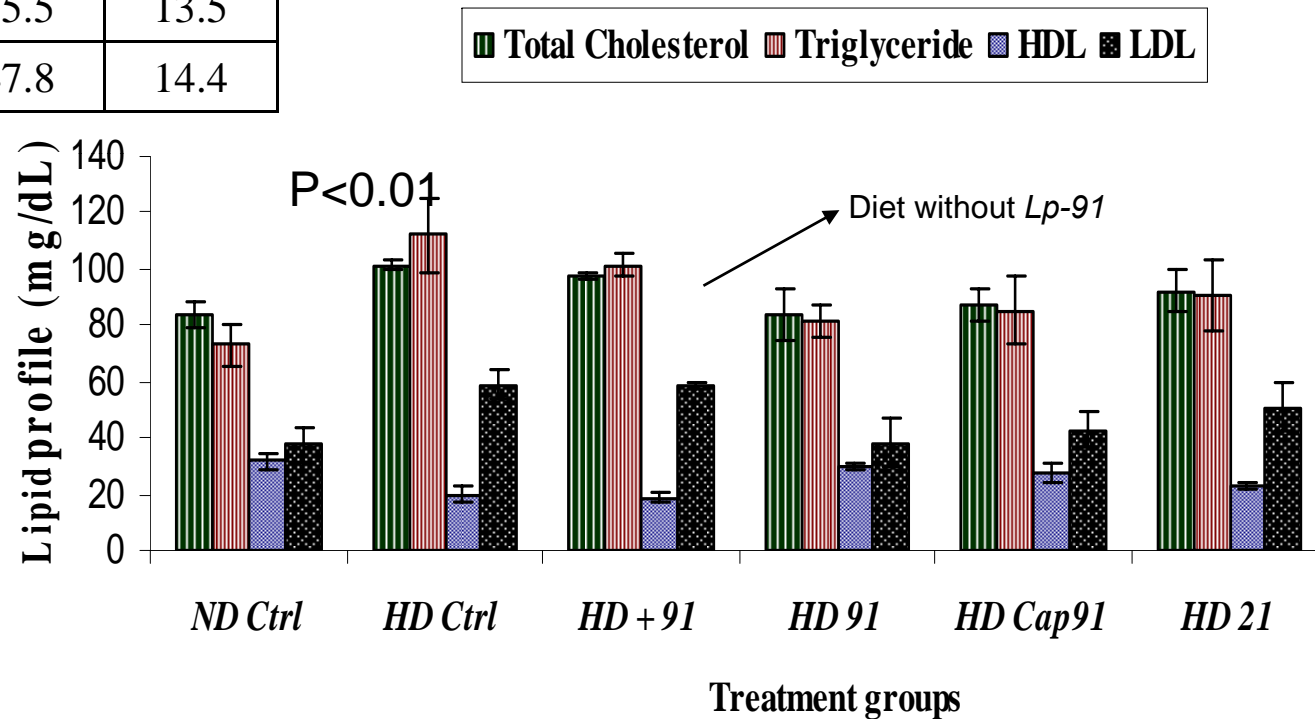
Bile salt hydrolase activity of lactobacilli cultures was quantified against different conjugated bile salts.

- ▨ GCA hydrolase activity.
- ▨ GDCA hydrolase activity
- ▨ TCA hydrolase activity.
- ▨ TDCA hydrolase activity.

Anti-Hypercholesterolemic effect of *Lp-91*

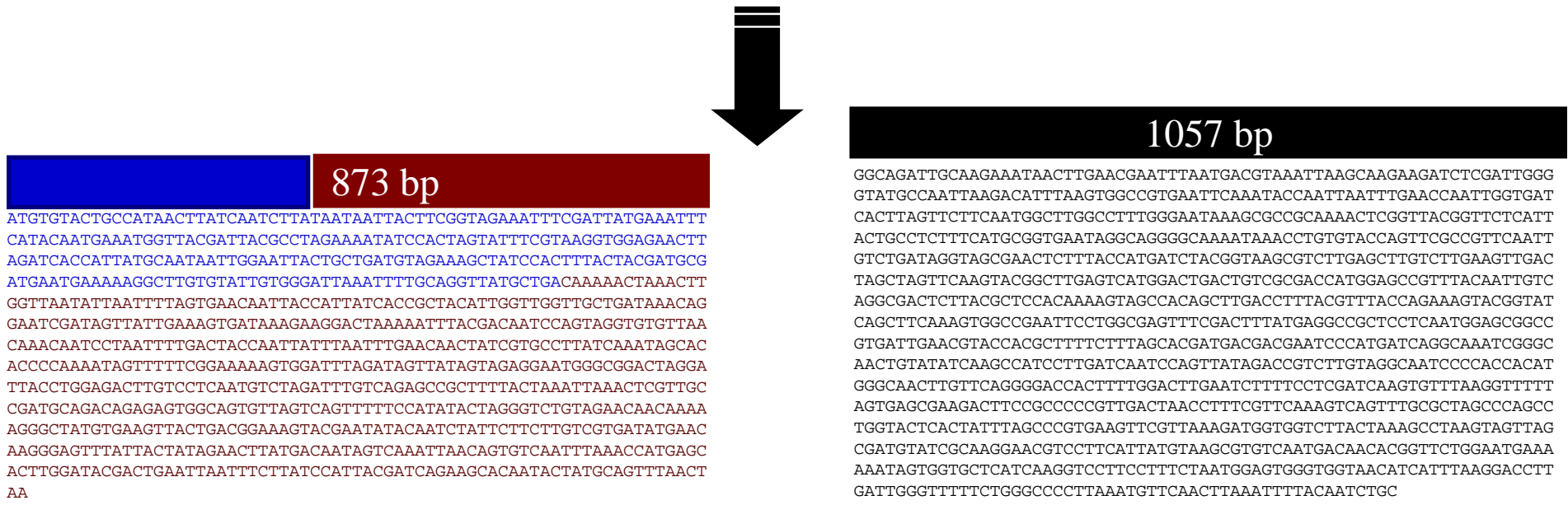
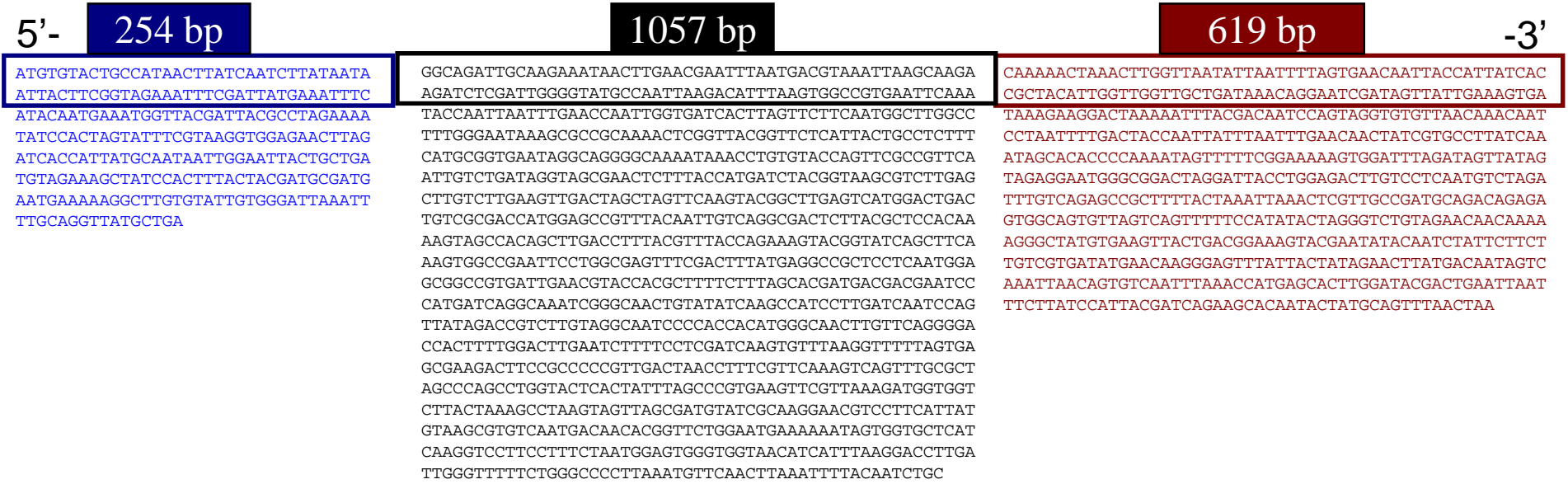
	Lp91	Lp21
TC ↓	17.2	9.1
TG ↓	27.3	18.9
LDL ↓	35.5	13.5
HDL ↑	47.8	14.4

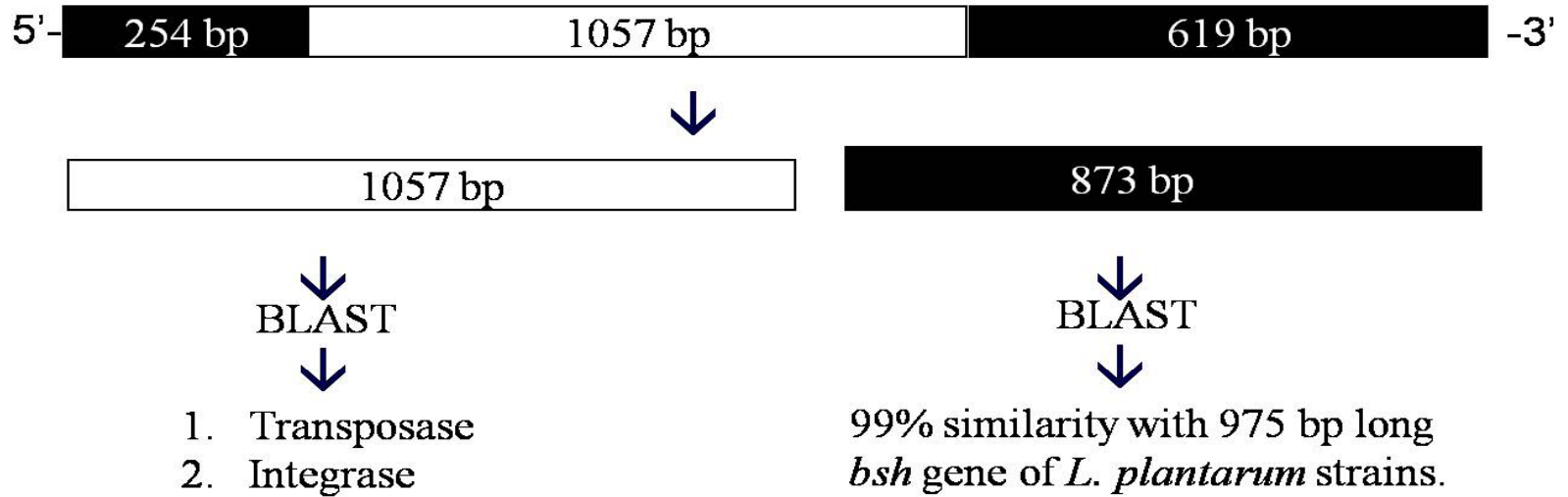
Duration of treatment:- 21 days



ND, Normal Diet; HD, Hypercholesterolemic diet; HD+91, Hypercholesterolemic diet without *Lp-91*; HD91, Hypercholesterolemic diet containing noncapsulated *Lp-91*; HDCap91, Hypercholesterolemic diet mixed with Microencapsulated *Lp-91*; HD21, Hypercholesterolemic diet mixed with *Lp-21* and HD Shirota, Hypercholesterolemic diet mixed with *L. casei* (Shirota).

Transposon mediated insertional inactivation of *bsh* gene of *L. plantarum* 77 strain.





Cell surface proteins as probiotic markers for colonization

- **Mucus Binding Protein (MBP)**
- **Fibronectin Binding Protein (FnBP)**
- **Collagen Binding Protein (CBP)**
- **EF-Tu**
- **LTA**

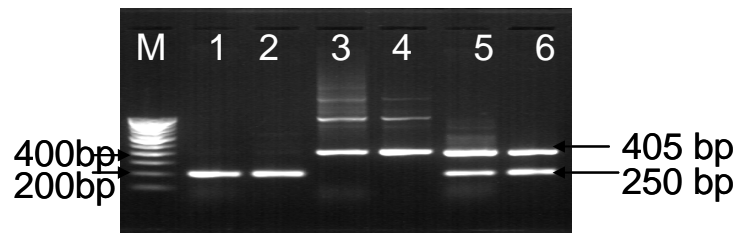


Fig. Multiplex PCR with primer pairs LBLMA1 /R-161 and MubD1_F87/ MubD1_R0.5

Lanes: M-100 bp marker;

1-2- Genus specific PCR product of Lp9,Lp91

3-4- 'Mub' specific PCR product with primer MubD1_F87/ MubD1_R0.5 of Lp9,Lp91

5-6- Multiplex PCR with both primer for Lp9,Lp91 .

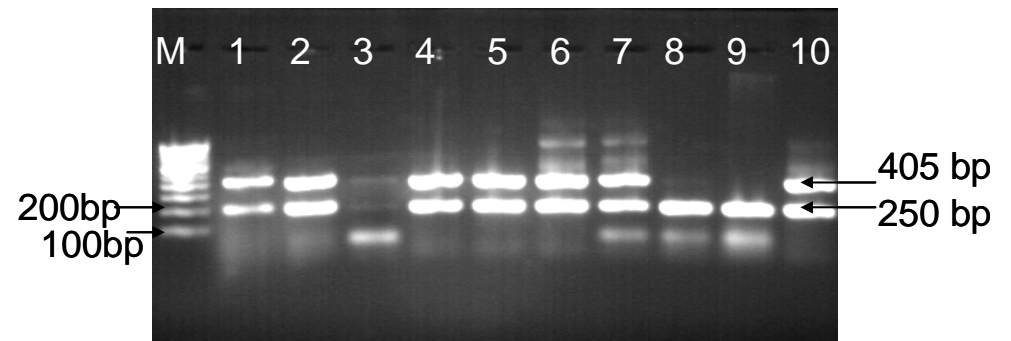


Fig. Multiplex PCR for *L. plantarum* with primer pairs LBLMA1 /R-161 and MubD1_F87/ MubD1_R0.5

Lanes: M-100 bp DNA ladder; 1-Lp10; 2-Lp20; 3-Lp75; 4-Lp76; 5-Lp77; 6-Lp5276; 7-Lps2; 8-Lp44; 9-Lp45; 10-Lp68

PCR Assays

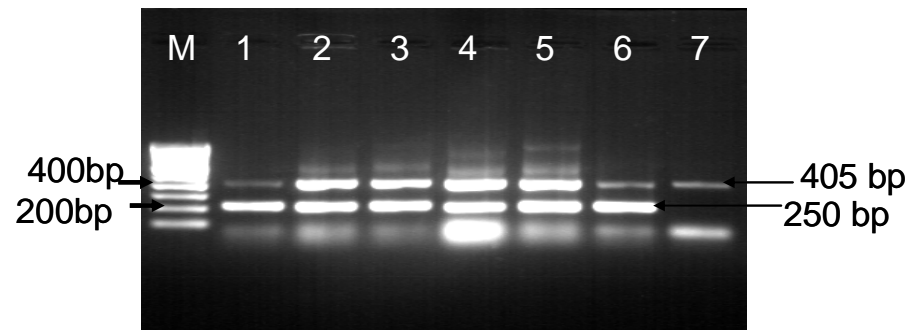


Fig. Multiplex PCR for *L. casei* , *L. acidophilus* and *Bifidobacterium bifidum* with primer pairs LBLMA1 /R-161 and MubD1_F87/ MubD1_R0.5

Lanes: M-100 bp marker; 1- *L. casei* J7; 2- *L. casei* J9; 3- *L. casei* J13; 4- *L. casei* 17; 5- *L. casei* S3; 6- LA1 (standard); 7- BB12 (*Bifidobacterium bifidum*)

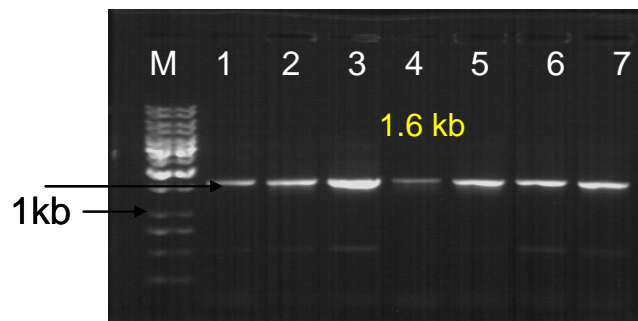


Fig. 'Mub' PCR with primer pair mubN_F0.93 / MubN_R1.65
Lanes: M-250 bp DNA ladder; 1- *L. brevis* (standard); 2-Lp9; 3-Lp91; 4-Lp72; 5-Lp-77; 6-Lp10; 7-Lp20

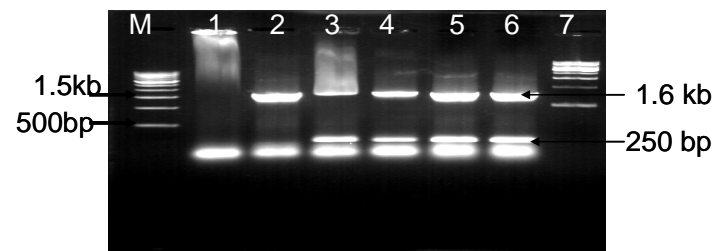


Fig. Multiplex PCR for Lp9 with primer pairs LBLMA1/R-161 and MubN_F0.93/ MubN_R165
Lanes: M- Supermix DNA ladder; 1-2-(1:0.5 ratio of Genus and 'Mub' primers); 3-4- (1:1 ratio of Genus and 'Mub' primers); 5-6- (0.5:1 ratio of Genus and 'Mub' primers); 7- 1kb DNA ladder (Chromous)

PCR Assays

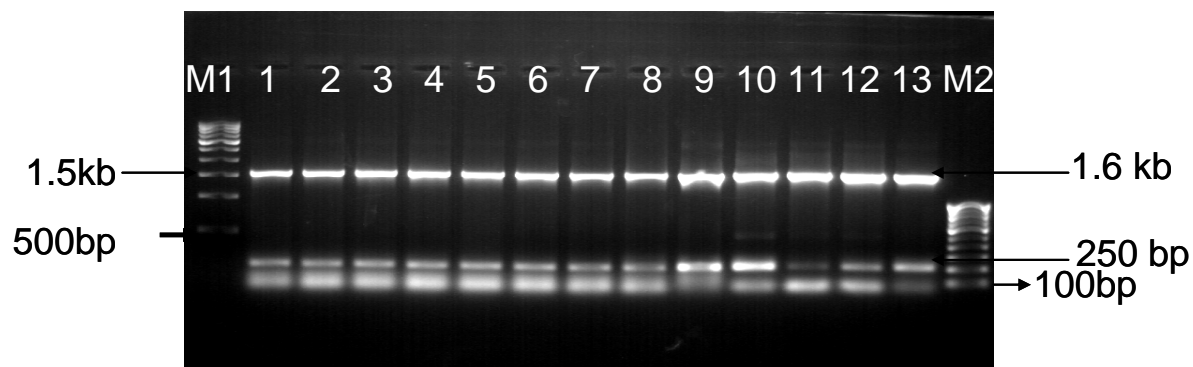
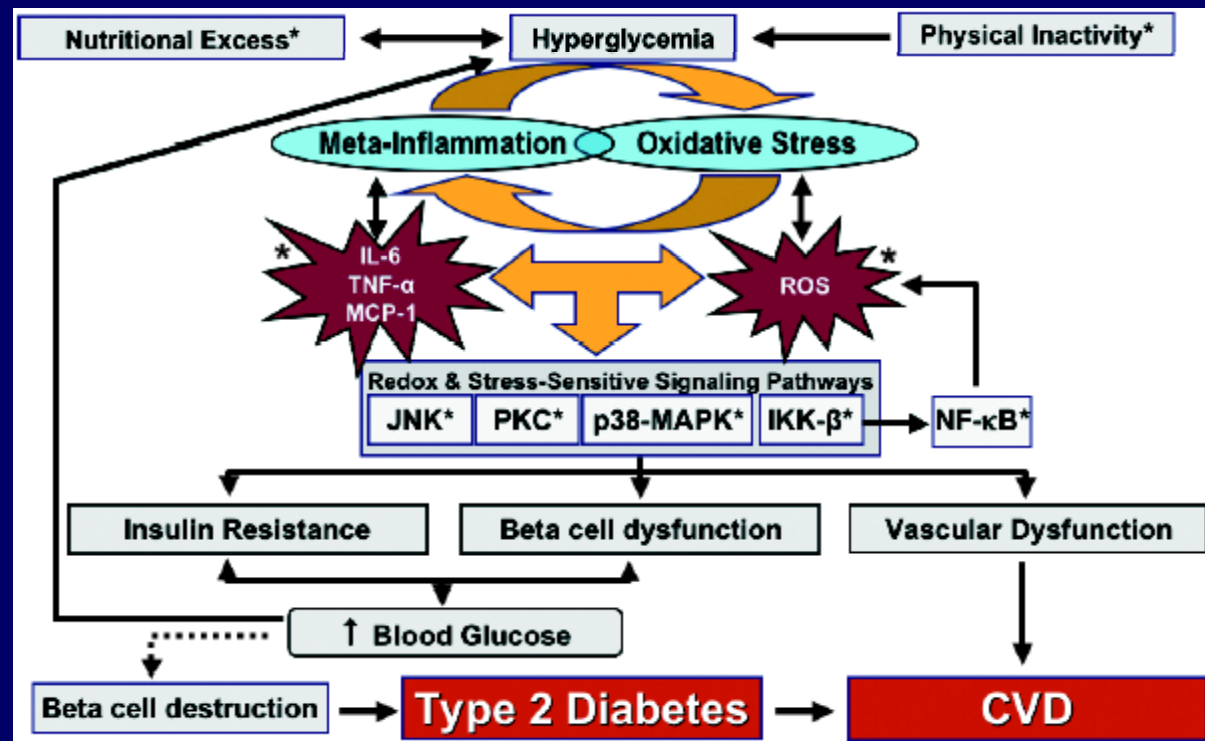


Fig. Multiplex PCR for *L. plantarum* isolates with primer pairs LBLMA1/ R-161 and MubN_F0.93/ MubN_R165
Lanes: M1-500 bp DNA ladder; 1-*L. brevis* (standard); 2-Lp5276 (standard); 3-Lp9; 4-Lp10; 5-Lp20; 6-Lp21; 7-Lp72; 8-Lp75; 9-Lp76; 10-Lp77; 11-Lp90; 12-Lp91; 13-Lp-122; 14-Lp130; M2-100 bp DNA ladder

Potential Biomarkers for Selection of Probiotics against specific diseases

- **Probiotics – Highly strain and host specific**
(Specific health promoting functions)
- Inflammatory diseases, IBD, Crohn's disease, Ulcerative colitis, constipation etc.
- Gastro-intestinal disease
- Cancers – colon, bladder etc.
- Autoimmune diseases – Diabetes, Rheumatoid arthritis
- Allergies – rhinitis
- Respiratory tract diseases – pneumonia etc.
- Liver diseases
- Hypertension, obesity etc.

Development of DM2 and CVD through oxidative-inflammatory cascade



Evaluation of the evidence - Preclinical Studies

- **Cell lines (*In vitro*)**
 - Too simplistic to mimic human systems
 - Important to screen strain characteristics
 - Important to understand mechanisms
- **Small experimental animal models (*In vivo*)**
 - Cannot be used for proof of efficacy
 - Excellent for safety

Evaluation of the evidence - Clinical Studies

- **Human target population RDBPCT :
cornerstone of efficacy in human
studies**
- **Phase I, II and III**
- **Preventive / therapeutic**
- **Critical Data analysis**
- **Post market surveillance studies**

Identification of probiotics – a pre-requisite

- Physiological function – highly strain specific
- Identification at strain level – very important
- False claims / labeling – spurious products
- Molecular based identification – more reliable at genus, species and strain level
 - 16s rRNA sequencing
 - House keeping genes (rpo, tuf etc.)

Lb. casei (13), *Lb. paracasei* (4), *Lb.fermentum* (25), *Lb. plantarum* (26), *Lb. delbrueckii* (2), *Lb.rhamnosus*(2), *Lb.reuteri*(4) and *Lb. acidophilus* (2)

*Nucleotide sequence of unique RAPD band of *Lb casei* with primer OPBB-02*

- **>OPBB-02**
- CACTGGCTGGGAAGGCACCGAGTTGTATGTTTCAGCTAGTTCCGG
AAGGCAAATTTGAAGGTGACACGTTAAATCCGTATTTTCTGATCA
AAACCGCGGATGAAGCGTTCAGCTTGTGGTCACCGACTGACTGT
GACATTCTCGCTGAAGATTGGCAACTTGTGACGCATGATAGCAT
TCGATTTTAGCGGCAAAACGGTTGTTGTGACTGGTGCGGCATCG
GGAATCGGCGCGGCTCAGGCAGCGGCGTTTACAGCAGCTGGTG
CACGGGTTATTGGCGTCGATCTTCAGCCGATGACTGGGCTAGCC
GTCACCATTCAGGCAGATGTCAGTAAGGCCGCGACAGCAGAAA
ACATCGTTCGTGACTATGCGCCCGACATTGTCTGCAACACGGCG
GGCATTCTGGATGGTTATCAAGACGTGCGCCGCCACTGATCTGGC
AACCTGGCAGCACATTCTTGATGTTGATCTCACCAGCCAGT
- **>probe1**
- **5'-TGGCAGCACATTCTTGATGTTGATCTCACCAG -3'**

Probiotics : Few Questions which remain unresolved

Selection of probiotic culture – what should be the criteria to define the most ideal probiotic strain for a specific disease

- Monostrain vs multistrain ?
- Will cell free extracts work ?
- Quantity and quality of probiotic needed for desired effect ?
- How best to assess the activity / viability ?
- Which Probiotics remain viable in GI tract ?
- How long do they remain in the gut to be effective?
- The best target site in pathogenesis of a particular disease for interventions
- Cell lines / cell culture and Animal models
- Lack of correlation (*In vitro* versus *in vivo* assays) e.g. *in vitro* evaluation of adhesion using cell lines do not account for complex and changing mucus lining
- Probiotic safety ?
- Clinical trials on human subjects for validating health benefits

Points to ponder

- **Probiotic definition or selection criteria should include:**
 - Ability to generate an immune response
 - *In vitro* assays to show that probiotics activate immune cells
- **Does *in vitro* screening based on acid and bile predict *in vivo* survival capacity (other stresses like oxidative, nutrient limitation, antimicrobial components etc. might influence?)**
- **Selection of probiotics on the basis of Adhesion potential using *in vitro* assays is being debated ? Can not be extrapolated to GIT (*in vivo*) due to**
 - Host defense systems
 - Competition for nutrients and space with commensals
 - Mucosal shedding
 - Peristaltic flow that continuously washes GIT
- **Hence, extrapolation of these findings to clinical applications requires caution**

Current problems with “probiotics” in India

- Extravagant claims without research
 - Specific species and strain effects
- Lack of good manufacturing practices
 - Quality assurance
 - Label vs content
 - Viability of bacterial species
- Validated biomarkers for assessing function and activity
- Identification needs to be done using molecular tools
 - 16S rRNA
 - FISH
- No specific guidelines currently
 - In India

Desired Interventions from Indian Perspective

- A comprehensive database on Indian probiotic cultures, their diversity and novel physiological functions for human health, well being and disease mitigation need to be generated as a National priority
- Indian gut – a rich habitat of diversified microbiota (gut ecology - food habits and regional and anthropological differences) – can be explored by metagenomic approaches
- High probability of biodiversity amongst probiotic strains from different gut niches at Genomics and Proteomics level
- Initiatives for mining the complete genome of promising Indian strains and the functional genes associated with novel physiological function
- Search for Novel probiotic strains (Lactobacilli and Bifidobacteria) with specific physiological functions in the gut using comparative genomics, Transcriptomics, Proteomics approaches etc.

Contd...

- Identification of potential Biomarkers for evaluating functionality of probiotics because greatest challenge concerns functionality of probiotics to strengthen health claims probiotics confer on host health
- Development of consortia of proven strains for ethnic dairy based product development (Dahi / Lassi) for boosting mucosal immunity
- Biosafety evaluation of selected probiotics and appropriate validation of health claims through clinical trials in target human subjects
- Efforts should be made to conduct clinical trials in Indian population for comprehensive evaluation of the efficacy of the Indian probiotic cultures against diarrhoeal as well as life style diseases vis a vis established western cultures to prove their efficacy

Time for a paradigm shift

Supply viable beneficial bacteria or a substrate which enhances a specific beneficial bacteria instead of trying to eliminate the pathogen ?

“Bioecological control”

Conclusion

- **“Thy Food Shall Be Thy Remedy”**
Hippocrates
- **Disease can be:**
 - Prevented
 - Mitigated
 - Treated
- **Appropriate Choice of :**
 - Clinical proven probiotics
- **We need to continue rigorous evaluation of assumptions and hypothesis to discover novel probiotics**

Acknowledgement

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