Integrated Pre-clinical Safety and Efficacy Analysis of Potential Probiotic *Limosilactobacillus fermentum* NCDC 400: A Computationally Guided *In vitro* and *In vivo* Investigation

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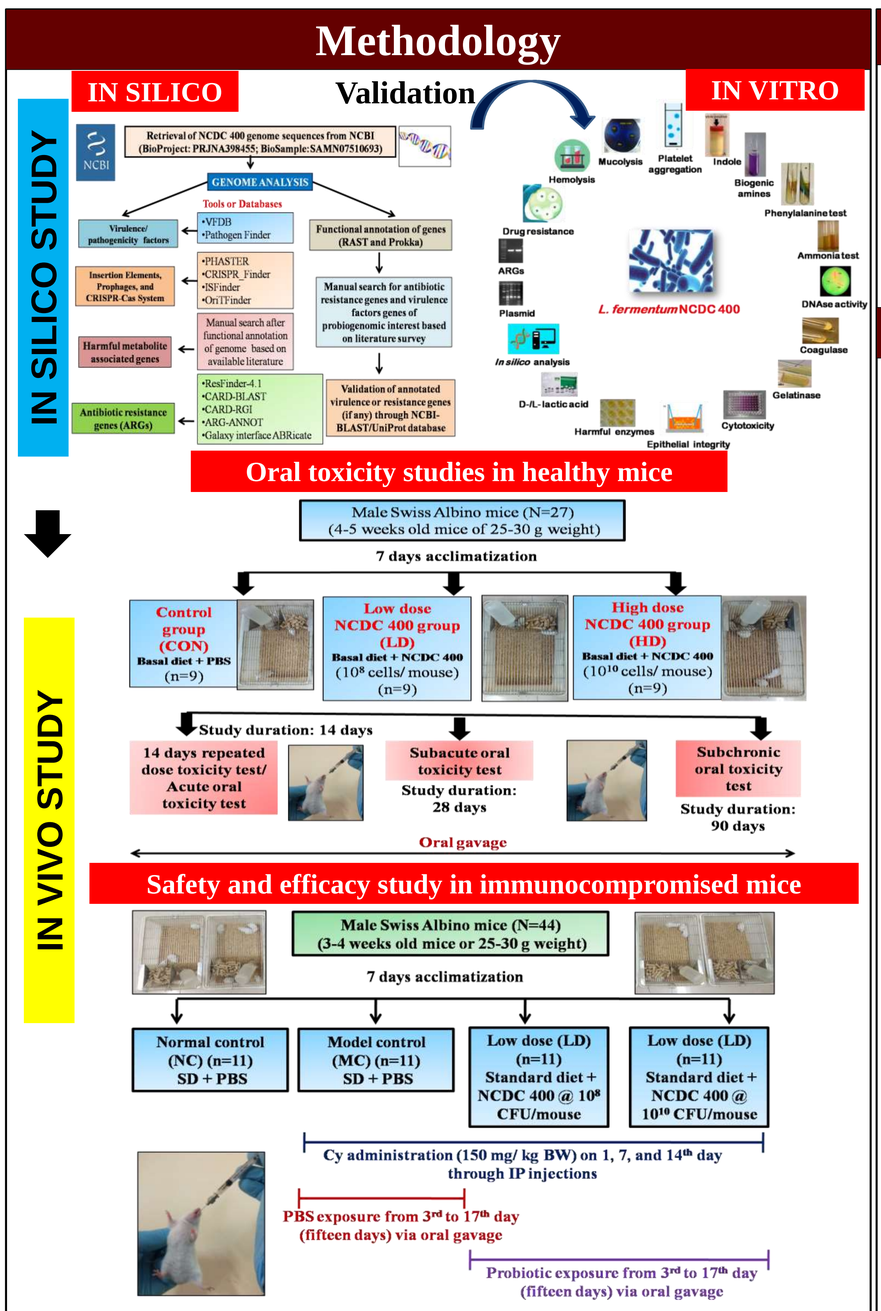
Introduction

Probiotics are amply studied and applied dietary supplements. Nevertheless, the emerging clinical evidence on probiotics-mediated potential risks, especially among immunocompromised individuals, necessitates careful and in-depth safety studies before clinical translation. In addition, the preponderance of probiotics in fostering various theoretical risks, such as production of harmful metabolites and enzymes, virulence factors, drug resistance etc. have been increasingly reported. Hence, it is paramount to appraise the benefit-to-risk ratio of any potential probiotic strain before their human administration. In congruence with ICMR/ DBT guidelines, the present study was undertaken to evaluate the pre-clinical safety and efficacy of potential probiotic *Limosilactobacillus fermentum* NCDC 400, an in-house industrially important and potential probiotic strain, using suitable *in silico* approaches and *in vivo* models.

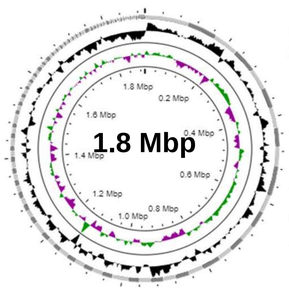
Methodology



Assays performed: General health status, hematology, serum biochemistry, histopathology, cytokines and Igs ELISA assays, oxidative markers, spleenocytes proliferation assay, genotoxicity, bacterial translocation assay, and gut microbiome profiling

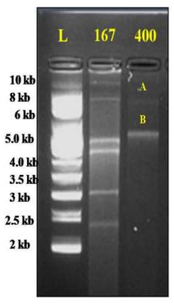
Results

CIRCULAR GENOME MAP



- **56 tRNA** genes and **15 rRNA** genes
- 423 GO terms : Cellular components
- 1,466 GO terms: Molecular functions

PLASMID PROFILING



NCDC 400 had two plasmids which do not code for virulence and antibiotic résistance genes

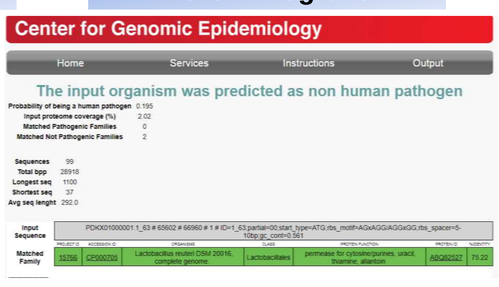
Antibiotic Resistance Genes (ARGs)

Sl. No.	Accession	Name of gene identified in genome	E-value	Identity	Score
1	3004853	16S rRNA mutation conferring resistance to capreomycin	0	80	953
2	3003436	16S rRNA mutation conferring resistance to kanamycin	0	80	953
3	3003437	16S rRNA mutation conferring resistance to viomycin	0	80	953
4	3003480	16S rRNA mutation conferring resistance to streptomycin	0	80	953
5	3003481	16S rRNA mutation conferring resistance to amikacin	0	80	953

VanT gene

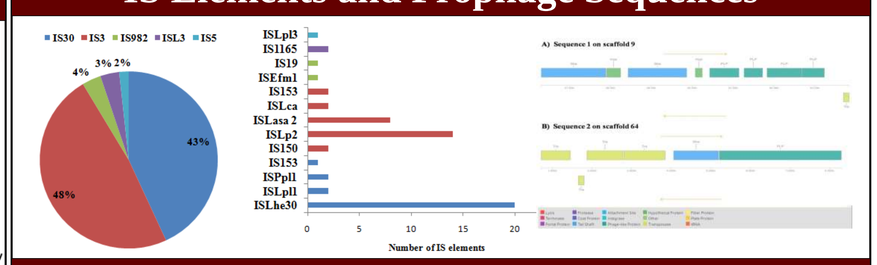
Intrinsic resistance

No OriT regions



No horizontal gene transfer

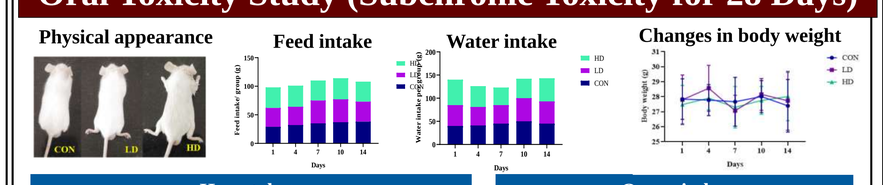
IS Elements and Prophage Sequences



In -vitro Tests

Test	Results	Test	Results
Hemolysis/ DNase/Urease	Absent	Coagulase	Absent
Mucin degradation		Platelet aggregation	
Ammonia production		Indole production	
Cytotoxicity to Caco-2 cell		Biogenic amines	
Gelatinase / harmful intestinal enzymes		Phenylalanine degradation test	

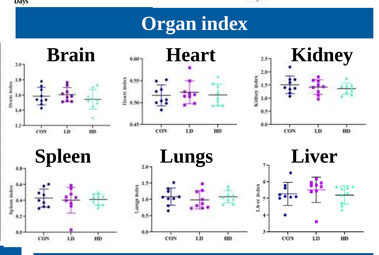
Oral Toxicity Study (Subchronic Toxicity for 28 Days)



Hematology

Parameters	Unit	CON	LD	HD
RBC	10^6/ µL	8.51 ± 0.53	8.34 ± 0.06	8.73 ± 0.37
Hb	g/ dL	12.4 ± 0.65	12.56 ± 0.03	12.80 ± 0.17
HCT	%	44.76 ± 3.19	45.60 ± 0.20	45.4 ± 1.70
MCV	fl	52.80 ± 0.60	54.66 ± 0.66	54.30 ± 0.87
MCH	pg	14.56 ± 0.17	15.00 ± 0.10	15.23 ± 0.43
MCHC	%	27.73 ± 0.58	27.5 ± 0.20	28.06 ± 1.01
MPV	fl	7.63 ± 0.08	7.73 ± 0.23	7.86 ± 0.17
WBC	10^3/µL	10.97 ± 0.78	12.5 ± 0.26	11.34 ± 0.87

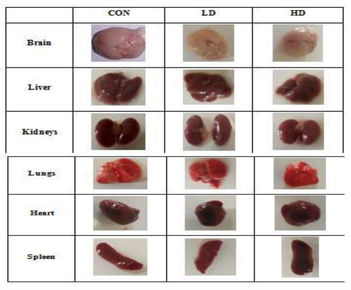
Organ index



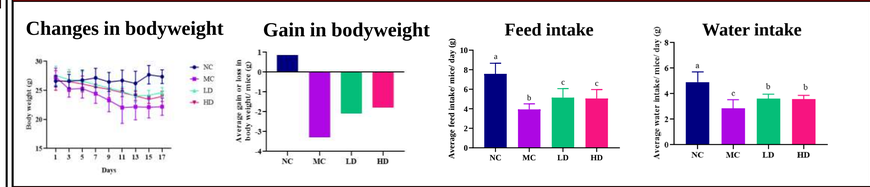
Clinical biochemistry

Parameters	Unit	CON	LD	HD
ALT/ SGPT	Unit/ L	37.53 ± 5.16	39.82 ± 1.28	37.50 ± 5.85
AST/ SGOT	Unit/ L	54.12 ± 0.71	57.77 ± 3.59	58.09 ± 3.43
Urea	mg/ dL	54.64 ± 3.35	52.36 ± 4.40	54.08 ± 2.99
Creatinine	mg/ dL	0.49 ± 0.19	0.54 ± 0.21	0.47 ± 0.11
LDH	Unit/ L	271.35 ± 15.66	269.32 ± 13.47	284.23 ± 15.13
Total protein	g/ dL	7.13 ± 0.03	7.11 ± 0.16	7.17 ± 0.17
Calcium	mg/ dL	10.24 ± 0.73	10.98 ± 0.14	11.17 ± 0.32
Phosphorous	mg/ dL	13.79 ± 0.29	13.36 ± 0.50	12.94 ± 0.23
Triglycerides	mg/ dL	94.88 ± 7.80	97.43 ± 6.79	92.77 ± 3.02
Total cholesterol	mg/ dL	108.66 ± 3.06	113.60 ± 5.22	119.5 ± 2.21
LDL-cholesterol	mg/ dL	31.50 ± 2.53	32.67 ± 3.34	35.59 ± 2.22
HDL-cholesterol	mg/ dL	60.24 ± 4.47	63. 90 ± 3.85	60.86 ± 2.53
VLDL-cholesterol	mg/ dL	18.67 ± 1.56	19.48 ± 1.35	18.55 ± 0.60

Gross necropsy



Safety and Efficacy Analysis in Cyclophosphamide Induced Immunocompromised Mice Model



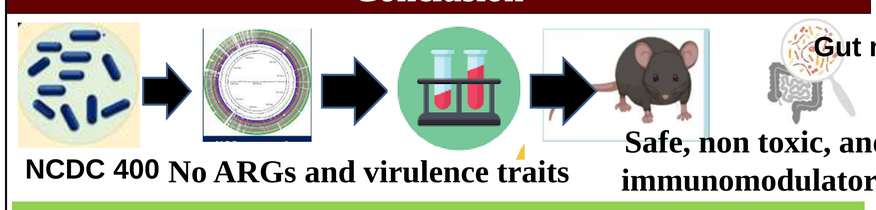
Hematology

Parameters	Unit	NC	MC	LD	HD
RBC	10^{12}/L	$8.73^a \pm 0.12$	$6.90^b \pm 0.05$	$7.39^b \pm 0.14$	$6.76^b \pm 0.25$
Hb	g/dL	$14.03^a \pm 0.20$	$11.83^b \pm 0.28$	$13.16^b \pm 0.37$	$12.90^b \pm 0.15$
HCT	%	$41.46^a \pm 1.4$	$29.7^b \pm 0.66$	$30.16^b \pm 0.64$	$30.3^b \pm 0.35$
MCV	fL	$46.46^a \pm 1.77$	$42.83^a \pm 0.66$	$41.5^a \pm 1.57$	$43.2^a \pm 0.36$
MCH	pg	$18.46^a \pm 0.31$	$17.73^a \pm 1.10$	$17.06^a \pm 0.61$	$17.5^a \pm 0.28$
PLT	10^9/L	$597.33^a \pm 8.22$	$234.00^b \pm 15.50$	$278.33^b \pm 11.83$	$250.00^b \pm 10.56$
PCT	%	$0.35^a \pm 0.02$	$0.33^a \pm 0.01$	$0.32^a \pm 0.011$	$0.37^a \pm 0.04$
WBC	10^9/L	$7.13^a \pm 0.28$	$1.36^b \pm 0.31$	$2.03^b \pm 0.47$	$2.46^b \pm 0.20$
LYM	10^9/L	$3.66^a \pm 0.13$	$0.43^b \pm 0.03$	$0.91^b \pm 0.26$	$1.70^c \pm 0.32$
MON	10^9/L	$0.43^a \pm 0.03$	$0.13^b \pm 0.03$	$0.18^b \pm 0.03$	$0.21^b \pm 0.10$
GRAN	10^9/L	$3.2^a \pm 0.30$	$0.73^b \pm 0.14$	$0.90^b \pm 0.25$	$0.63^b \pm 0.31$

Clinical Biochemistry

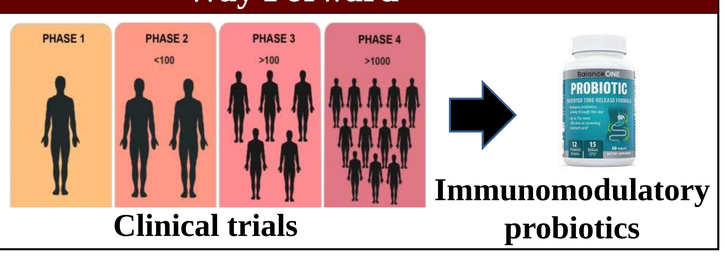
Parameters	Unit	NC	MC	LD	HD
ALT/ SGPT	Unit/ L	$50.05^a \pm 4.97$	$111.16^c \pm 2.53$	$88.46^b \pm 4.54$	$80.89^b \pm 1.53$
AST/ SGOT	Unit/ L	$54.12^a \pm 1.26$	$116.90^c \pm 7.46$	$92.53^b \pm 1.74$	$90.53^b \pm 4.03$
Creatinine	mg/ dL	$0.38^a \pm 0.01$	$0.90^a \pm 0.03$	$0.50^a \pm 0.10$	$0.41^a \pm 0.06$
LDH	Unit/ L	$217.33^a \pm 8.35$	$755.00^c \pm 6.92$	$711.00^c \pm 10.13$	$693.00^b \pm 3.51$
Total protein	g/ dL	$7.45^c \pm 0.16$	$4.99^a \pm 0.44$	$6.22^b \pm 0.03$	$6.18^b \pm 0.09$
Calcium	mg/ dL	$10.87^a \pm 0.16$	$11.96^a \pm 0.55$	$10.35^a \pm 0.78$	$10.03^a \pm 0.92$
Triglycerides	mg/ dL	$85.15^a \pm 0.90$	$117.00^c \pm 3.51$	$98.66^b \pm 1.22$	$96.59^b \pm 3.05$
cholesterol	mg/ dL	$160.33^a \pm 8.16$	$202.3^b \pm 3.13$	$173.90^a \pm 6.99$	$183.11^{ab} \pm 4.81$
LDL	mg/ dL	$65.91^b \pm 8.12$	$112.13^a \pm 3.12$	$97.75^a \pm 6.11$	$100.85^a \pm 5.83$
HDL	mg/ dL	$75.72^b \pm 1.64$	$66.83^a \pm 1.45$	$63.11^a \pm 2.19$	$62.94^a \pm 2.11$
VLDL	mg/ dL	$17.03^a \pm 0.18$	$23.4^c \pm 0.70$	$19.73^b \pm 0.24$	$19.31^b \pm 0.61$

Conclusion

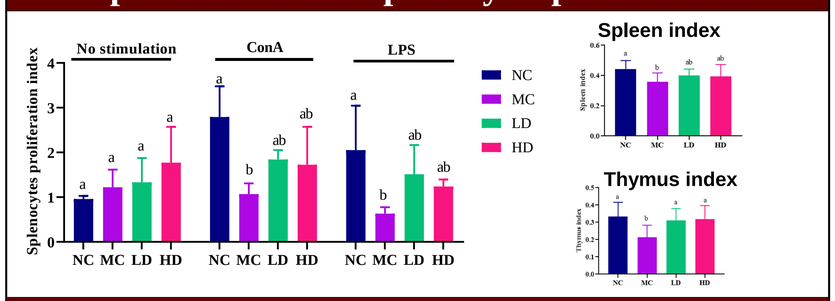


(No Observable Effect Level (NOEL): 10^{10} CFU/mouse/day)

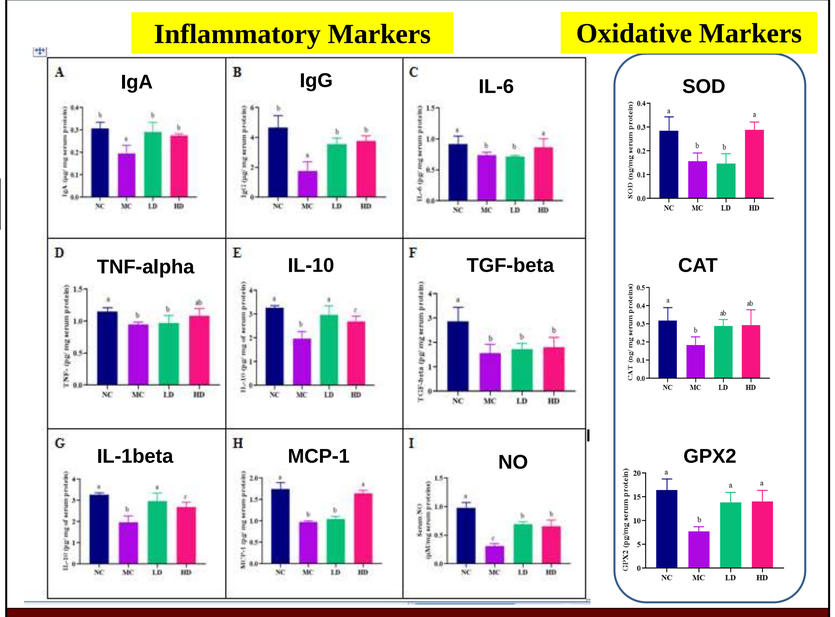
Way Forward



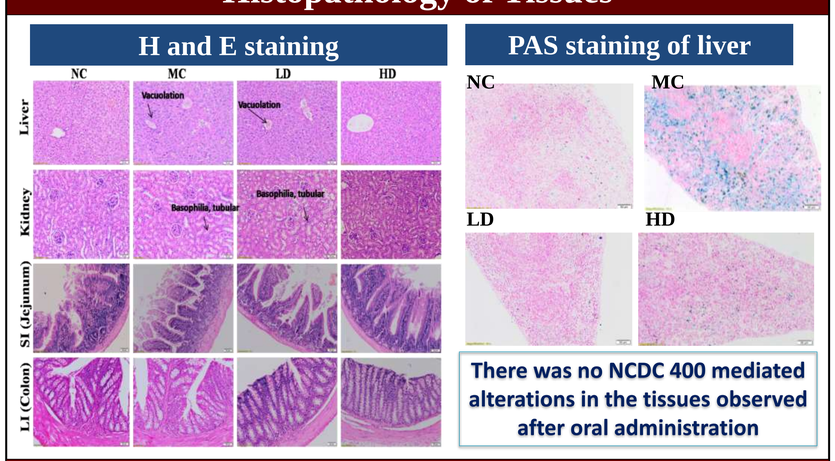
Spleen Index and Splenocytes proliferation



Immunomodulatory and Anti-oxidant Potency

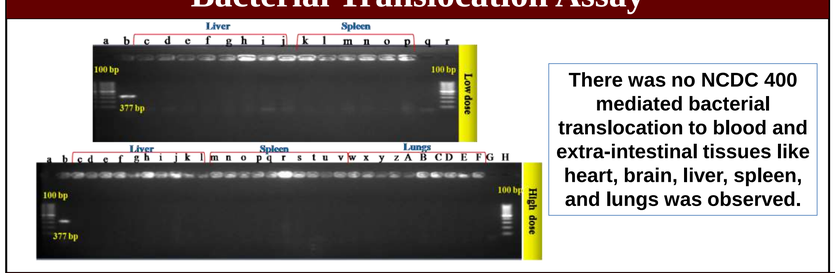


Histopathology of Tissues



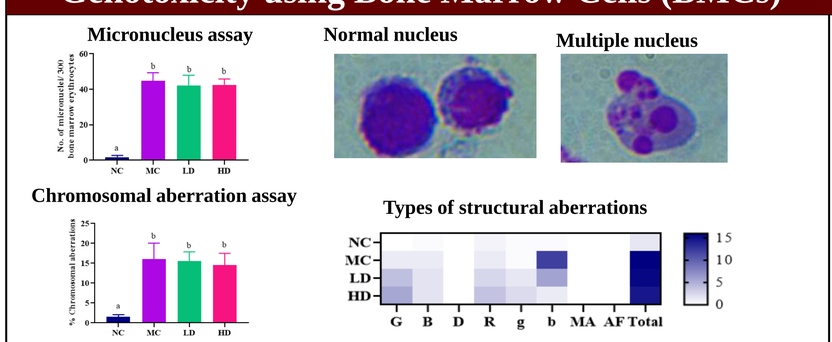
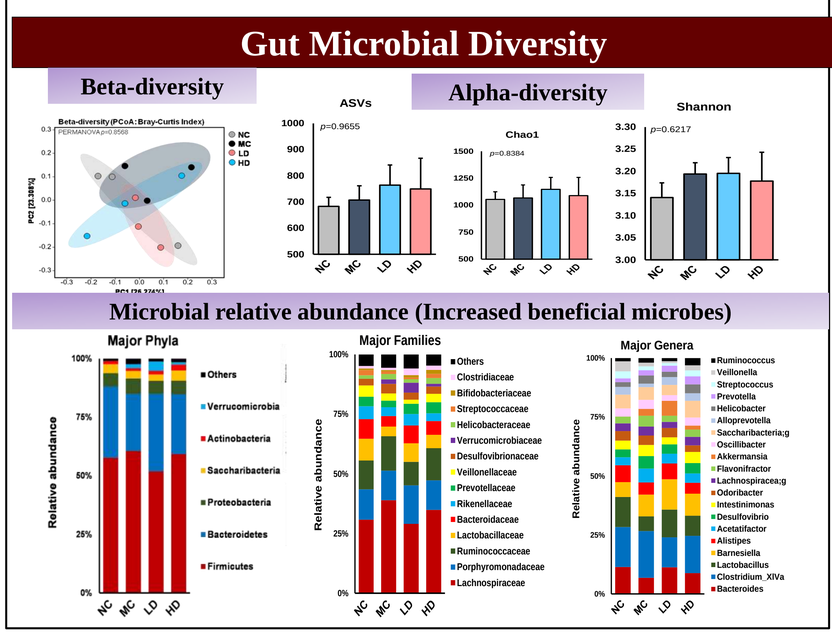
There was no NCDC 400 mediated alterations in the tissues observed after oral administration

Bacterial Translocation Assay



There was no NCDC 400 mediated bacterial translocation to blood and extra-intestinal tissues like heart, brain, liver, spleen, and lungs was observed.

Genotoxicity using Bone Marrow Cells (BMCs)



Gut Microbial Diversity