ROLE OF LACTOBACILLUS (*L. FERMENTUM* AND *L. RHAMNOSUS*) FERMENTED SWEET WHEY ON REINFORCEMENT OF INTESTINAL EPITHELIAL FUNCTION

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
ANIMAL BIOCHEMISTRY
By

HARPREET KAUR
(M.Sc. Biochemistry)

ANIMAL BIOCHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL – 132 001 (HARYANA), INDIA

2021

Regn. No. : 15-P-AB-09

ROLE OF LACTOBACILLUS (*L. FERMENTUM* AND *L. RHAMNOSUS*) FERMENTED SWEET WHEY ON REINFORCEMENT OF INTESTINAL EPITHELIAL FUNCTION

By

HARPREET KAUR

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
ANIMAL BIOCHEMISTRY

Approved by

23/4/2021

(Dr. M.K. Tripathi)
EXTERNAL EXAMINER

(Dr. Rajeev Kapila)
MAJOR ADVISOR

Members of Advisory Committee

1. **Dr. Suman Kapila** __________
   Principal Scientist, Animal Biochemistry Division
2. **Dr. Sunita Meena** On leave
   Scientist, Animal Biochemistry Division
3. **Dr. Shilpa Vij** 27/4/2021
   Principal Scientist, Dairy Microbiology Division
4. **Dr. Shaik Abdul Hussain** 27/4/21
   Scientist, Dairy Technology
5. **Dr. A.K Singh** On leave
   Principal Scientist, Dairy Technology

DIVISION OF ANIMAL BIOCHEMISTRY
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL – 132 001 (HARYANA), INDIA

Dr. Rajeev Kapila, Ph.D.
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled, **"ROLE OF LACTOBACILLUS (*L. FERMENTUM* AND *L. RHAMNOSUS*) FERMENTED SWEET WHEY ON REINFORCEMENT OF INTESTINAL EPITHELIAL FUNCTION"** submitted by **Harpreet Kaur** in partial fulfilment of the requirement for award of the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL BIOCHEMISTRY** of the ICAR-National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

23/4/2021

Dated: 27-03-2021

Dr. Rajeev Kapila
MAJOR ADVISOR

ACKNOWLEDGEMENTS

I wish to express my deepest sense of gratitude to my respected guide Dr. Rajeev Kapila, Principal Scientist, Animal Biochemistry Division for his benevolence, sagacious guidance, persistent help, constant encouragement, care, and all kinds of painstaking help extended to me that led to successful completion of my research. Any success that I have or will receive in my journey through science will perpetually be dedicated, in part, to you. His unflinching courage and conviction will always inspire me. Personally, I feel above all his merits he is a great human being, who helped and supported me during my tough times and buttressed me to perform my work comfortably I have truly been very fortunate to have worked under his precious guidance.

I owe my sincere thanks to the members of my advisory committee Dr. Suman Kapila, Principal Scientist, Dr. Sunita Meena, Scientist, Animal Biochemistry Division, Dr. Shilpa Vij, Principal Scientist & Head, Dairy Microbiology Division, Dr. Shaik Abdul Hussain, Principal Scientist, Dairy Technology and my Director's Nominee, Dr. A.K Singh, Principal Scientist, Dairy Technology for their valuable suggestions and encouragement throughout the research period. Their critical evaluation along with prolific ideas and time to time suggestion helped me in the successful execution of my work. Financial assistance from Indian council of Agriculture Research (ICAR) and Indian Council of Medical Research (ICMR) are gratefully acknowledged.

I also wish to express my profound sense of gratitude and heartful thanks to Dr. R.R.B. Singh, Joint Director (Academic) of N.D.R.I. (Deemed University), for providing necessary facilities that helped me during my work.

I am very much thankful to Dr. Y.S. Rajput, Dr. S. K. Atreja & Dr. R.K. Sharma (Ex Heads), Animal Biochemistry Division, and Dr. Dheer Singh, Head, Animal Biochemistry Division for guidance that helped me during my work.

I consider it a great privilege to record my deepest sense of gratitude to Dr. T. K. Datta, Animal Biotechnology Division for providing free access to work in their lab throughout my research. I want to thanks them for their kindness.

I am thankful to my seniors Dr. Taruna Gupta, Dr. Syed Azmal Ali , Dr. Deepti Mittal, and Dr. Radha Yadav, for their encouragement, invaluable moral support and being there for me throughout. I am indebted towards their contributions. I wish to pay my special thanks to my loving friend Deepak, Deepti Mittal, Sonia Batan for their love, care, affection and support. My sincere thanks to them for their ready incorporation and willing help to overcome my miner and major difficulties to whatever extent they could.

I also wish to thanks my lab mates, Taruna, Swasti, Apurva, Shalaka for their help and support given to me. I am also thankful to my batchmates and friends: Deepti Mittal, Sonia Batan for their love, moral support and good memories shared with me and providing family, healthy and amusing atmosphere during my Ph.D. journey. I also do not forget to thank my lovely and friendly juniors always been there to shower their affection, care, and help.

Thanks to all faculty member and staff of Animal Biochemistry division for their co-operation and support, Baljit Ji, Raman ji, Ravikant sir and other staff for their help.

"Where emotions are involved, words cease to exist" and words become inadequate to express my profound sense of gratitude and love to my most admired parents (my strength) who brought me up to this stage and also the unforgettable support and affection showered on me. I also say thanks my other family members for their invaluable love, care, support and encouragement.

Not everyone is mentioned, but none is forgotten.

Harpreet Kaur

CONTENTS

Chapter No.	Title			Page No.
1	**INTRODUCTION**			**1-4**
2	**REVIEW OF LITERATURE**			**5-31**
	2.1	Fermented Dairy Products		5
		2.1.1	Dairy Whey and its application in human health	7
		2.1.2	Probiotic	12
		2.1.3	Fermented dairy product as probiotic vehicle	12
		2.1.4	Health benefits of probiotic functional food	14
			2.1.4.1 Obesity and Diabetes	15
			2.1.4.2 Lactose intolerance	15
			2.1.4.3 Allergies	16
			2.1.4.4 Oxidative stress	16
			2.1.4.5 Skin repair	17
			2.1.4.6 Hypercholesterolemia	17
			2.1.4.7 Gastrointestinal disorders	18
	2.2	Mode of action of probiotic fermented products		21
		2.2.1	Fermented product as a key player of immunomodulation	21
		2.2.2	Role fermented products in reinforcement of gut barrier integrity	24
		2.2.3	Intercellular junctional proteins	25
		2.2.4	Host antimicrobial peptides	26
		2.2.5	Modulation of pathogen recognition receptors (PRRs) signaling	27
			2.2.5.1 TLR-2 receptor	28
			2.2.5.2 TLR-4 receptor	29
	2.3	Cytokines release by intestinal epithelial cell		30
		2.3.1	IgA	31

3	**MATERIALS AND METHODS**			**32-73**
	3.1	Materials		32
		3.1.1	Chemicals	32
		3.1.2	Plastic ware	32
	3.2	Methods		33
		3.2.1	Bacterial strain and culture condition	33
		3.2.2	Purity checking of bacterial strains	33
		a.	Gram's staining	33
		b.	Nigrosin staining	33
	3.3	Preparation of sweet whey and its fermentation		34
	3.4	Chemical analysis of probiotic fermented whey		35
		3.4.1	pH and Titarable acidity	35
		3.4.2	Lactose content	35
		3.4.3	Total nitrogen content measurement	35
		3.4.4	Quantification of Protein Hydrolysis	36
	3.5	In-vivo study		37
		3.5.1	Animals	37
		3.5.2	Composition of basal diet	37
		3.5.3	Experimental design and grouping of animals	38
		A	Non-colitis groups	38
		B	DSS induced colitis model	38
		3.5.4	Collection of tissue sample	41
		3.5.5	Assessment of disease activity index (DAI)	42
	3.6	Evaluation of general health status		42
		3.6.1	Body weight and organ indices	42
		3.6.2	Hematological analysis of blood	42
	3.7	Examination of intestinal health		43
		3.7.1	Histology of intestine	43
		3.7.2	Immune responses in the gastrointestinal tract	43

			A.	Myeloperoxidase (MPO) activity	43
			B.	Measurement of IgA in intestinal fluid	44
			C.	C-reactive protein (CRP) level in intestinal fluid	45
			D.	Measurement of Interleukins in intestinal fluid	47
			E.	Expression of immune related genes	48
		3.7.3	Evaluation of intestinal barrier function		57
			A.	Transcriptional expression of epithelial junctional genes	57
			B.	Immunofluorescence assay	57
			C.	Intestinal Gut Permeability by FITC-Dextran	59
		3.7.4	Microbial count in fecal sample		60
	3.8	The mode of action of lactobacilli fermented whey on human cell line			60
		3.8.1	Epithelial cell line		60
		3.8.2	Inflammatory agents		60
		3.8.3	Maintenance and culturing of HT-29 cells		61
	3.9	Simulated gastrointestinal digestion			61
		3.9.1	Quantification of hydrolysed protein		63
	3.10	MTT assay			63
	3.11	Transcriptional expression of intestinal epithelial cells during *E. coli* challenge in presence of simulated digested probiotic fermented whey			64
	3.12	Protein quantification			65
	3.13	Nuclear translocation of NF-κβ in intestinal cells			66
		3.13.1	Western Blotting		67

	3.14	Statistical analysis		73
4	**RESULTS**			**74-84**
	4.1	Morphological features of bacterial cultures		74
	4.2	Growth of lactobacilli in whey media		75
		4.2.1	Growth and metabolites production by *Lactobacillus*	75
		4.2.2	pH and Titratable acidity	75
		4.2.3	Lan Eynon Method	75
		4.2.4	Protein percent	75
		4.2.5	Peptide concentration	75
	4.3	Assessment of probiotic fermented whey on general health status of weaning mice during non-colitis and DSS-induced colitis		76
		4.3.1	Change in body weight and organ weight in non-colitis	76
		4.3.2	Percent loss in body weight and organ weight in colitis	76
		4.3.3	Disease activity index	76
	4.4	Number of payer patches and colon health		77
	4.5	Hematological analysis of blood		77
	4.6	Histological examination of intestine and histological score of non-colitis and colitis animals		78
	4.7	Immune responses in the gastrointestinal tract		78
	4.8	Restoration of gut barrier functions		80
	4.9	Bacterial load in feces		81
	4.10	Morphological features of intestinal epithelial cells (HT-29 cells)		82
	4.11	MTT assay		82
	4.12	Impact of simulated digested probiotic fermented whey on expression of genes associated with epithelial barrier function during challenge with inflammatory agent (*E. coli*)		82
	4.13	Nuclear translocation of NF-κβ in intestinal cells		83

	4.14	Nuclear translocation of NF-κβ by western blotting	84
	4.15	Nuclear translocation of NF-κβ (p-65) in intestinal cells on challenge with inflammatory agents in the presence of probiotic simulated digested *L. rhamnosus* fermented whey	84
5	**DISCUSSION**		**85-93**
	5.1	Probiotic *Lactobacillus* fermented whey improves the general health status	85
	5.2	Probiotic *Lactobacillus* fermented whey maintain the immune response in gastrointestinal tract	87
	5.3	Probiotic *Lactobacillus* fermented whey improves the gut barrier function	89
	5.4	Probiotic *Lactobacillus* fermented whey augmented the modulation of genes associated with the epithelial barrier and immune function	91
	5.5	NF-κβ signalling in host intestinal epithelial cells by probiotic *Lactobacillus* during exclusion of *E. coli*	92
6	**SUMMARY AND CONCLUSION**		**94-97**
	6.1	The effect of lactobacilli fermented whey on protection of gut barrier integrity in colitis induced mice	94
	6.2	The mode of action of lactobacilli fermented whey on human intestinal epithelial barrier integrity	96
BIBLIOGRAPHY			i-xxx

LIST OF TABLES

Table No.	Title	Page No.
3.1	OPA working reagent	36
3.2	Proximate composition of semi-synthetic basal diet	38
3.3	Reaction mixture for cDNA preparation	52
3.4	Primer sequences for the amplification of genes	53
3.5	Reaction mixture for PCR	54
3.6	Program of gradient PCR for standardization of annealing temperature	55
3.7	Reaction mixture for qRT-PCR	55
3.8	Reaction program for the qRT-PCR	56
3.9	Preparation of stock for simulated digestion fluids	62
3.10	Sequences of primers used for gene amplification	64
3.11	Preparation of 12 % separating gel for SDS-PAGE	69
3.12	Preparation for 4.5 % stacking gel for SDS-PAGE	70
3.13	Composition of 1X running buffer (pH 8.3) for SDS-PAGE	70
3.14	Composition of 5X sample buffer for SDS-PAGE	70
4.1	Impact of probiotic fermented whey consumption on blood hematological parameters	77
4.2	Impact of probiotic fermented whey consumption during colitis induction on blood hematological parameters	79

LIST OF FIGURES

Figure No	Title	On/After Page No.
3.1	Flow chart for the preparation of whey	34
3.2	Experimental design for probiotic fermented whey consumption during colitis induction with dextran sulfate sodium (DSS) in mice for prophylactic evaluation	40
3.3	Overview of sample collection and parameter analysed under objective I	41
3.4	Assessment of expression of tight junctional and immune genes in intestinal epithelial cells during challenge with *E. coli* as inflammatory agent in presence of probiotic simulated digested fermented whey	65
4.1	Growth and metabolites utilised by *Lactobacillus* (*L. rhamnosus* and *L. fermentum*) during fermentation of whey	75
4.2	Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on body weight and organ indices	77
4.3	Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on body weight, organ indices and disease activity indices upon DSS challenge to induce colitis	77
4.4	Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on intestinal health	77
4.5	Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on intestinal health upon DSS challenge to induce colitis	79
4.6	Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on immune response in the gastrointestinal tract	79
4.7	Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on restoration of gut barrier functions	79

4.8	Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on immune response in the gastrointestinal tract after DSS challenge to induce colitis	79
4.9	Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on restoration of gut barrier functions upon DSS challenge to induce colitis.	81
4.10	HT-29 cell viability upon exposure to variable concentration of simulated digested probiotic fermented whey (*L. fermentum* and *L. rhamnosus*) and their supernatant	83
4.11	Transcriptional modulations in key genes associated with epithelial barrier function by simulated digested probiotic fermented whey (*L. rhamnosus* and *L. fermentum*)	83
4.12	Transcriptional modulations in key genes associated with epithelial barrier function by simulated digested probiotic fermented whey (*L. rhamnosus* and *L. fermentum*) on HT-29 cells during exclusion of *E. coli*	83
4.13	Influence of simulated digested fermented whey prepared from L. fermentum (MTCC: 5898) on nuclear translocation of NF-κβ p-65 subunit in intestinal epithelial cells (HT-29) during exclusion of *E. coli*	84
4.14	Influence of simulated digested fermented whey prepared from *L. rhamnosus* (MTCC:5897) on nuclear translocation of NF-κβ p-65 subunit in intestinal epithelial cells (HT-29) during exclusion of *E. coli*	84

LIST OF PLATES

Plate No.	Title	A/Page No.
4.1	Representing photographs of probiotics bacteria (*Lactobacillus rhamnosus* and *Lactobacillus fermentum*)	75
4.2	Representative images of *E. coli*	75
4.3	Representative images displaying cytoskeleton protein (Actin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey	81
4.4	Representative images displaying cytoskeleton protein (Claudin-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey	81
4.5	Representative images displaying cytoskeleton protein (ZO-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey	81
4.6	Representative images displaying cytoskeleton protein (Occludin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey	81
4.7	Representative images displaying cytoskeleton protein (Actin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis	81
4.8	Representative images displaying cytoskeleton protein (Claudin-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis	81
4.9	Representative images displaying cytoskeleton protein (ZO-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis	81

4.10	Representative images displaying cytoskeleton protein (Occludin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis	81
4.11	Representative images of HT-29 cells under phase contrast inverted microscope (40 X) on different days of culture A. 1st day B. 2nd day C. Confluent cells	83

ABBREVIATION

DSS	-	Dextran sodium sulfate
AD	-	Atopic Dermatitis
BSA	-	Bovine serum albumin
CFU	-	Colony Forming Unit
CLRs	-	C-type lectins receptors
cDNA	-	Complementary deoxyribonucleic acid
DAPI	-	4',6-diamino-2-phenylindole
DC	-	Dendritic cells
DEPC	-	Diethyl Pyrocarbonate
DMSO	-	Dimethyl Sulfoxide
DMEM	-	Dulbecco's Modified Eagle's Medium
EMB	-	Eosin Methylene Blue
E. coli	-	*Escherichia coli*
ECL	-	Enhanced Chemiluminisense
EDTA	-	Ethylene diamine tetra acetic acid
ELISA	-	Enzyme linked immuno sorbent assay
ERK	-	Extracellular Signal-Regulated Kinase
FBS	-	Fetal Bovine Serum
GIT	-	Gastrointestinal Tract
GALT	-	Gut Associated Lymphoid Tissue
HRP	-	Horseradish Peroxidase
IBD	-	Inflammatory Bowel Diseases
IEC	-	Intestinal Epithelial Cells
IL	-	Interleukin
IgA	-	Immunoglobulin A
ILF	-	Isolated Lymphoid Follicles
IFN-Ƴ	-	Interferon-gamma
JNK	-	Jun N-terminal kinase
LF	-	*Lactobacillus fermentum*
LR	-	*Lactobacillus rhamnosus*
LTA	-	Lipothechoic acid
LPS	-	Lipopolysaccride
LRR	-	Leucine Rich Repeat Domain
mRNA	-	Messenger Ribonucleic acid

MRS	-	De Man, Rogosa and Sharpe agar
MUB	-	Mucus Binding Protein
Map-A	-	Mucous Adhesion-Promoting Protein
MLN	-	Mesenteric Lymph Nodes
MCP-1	-	Monocyte chemotactic protein-1
MAPK	-	Mitogen Activated Protein Kinases
N.D.R.I.	-	National Dairy Research Institute
NF-κβ	-	Nuclear factor κB
NOD	-	Nucleotide-binding Oligomerization Domain
PAGE	-	Poly Acrylamide Gel Electrophoresis
PBS	-	Phosphate buffer saline
PCR	-	Polymerase Chain Reaction
PP	-	Peyer's patches
PG's	-	Peptidoglycans
PPARγ	-	Peroxisome proliferator activated receptor-γ
PRR	-	Pattern Recognition Receptors
PVDF	-	Polyvinylidene difluoride
qRT-PCR	-	Quantitative real-time polymerase chain reaction
RLRs	-	Rig-1-like receptors
ROS	-	Reactive oxygen species
SCFA	-	Short Chain Fatty Acid

ABSTRACT

The fermented foods provide essential nutritional components and bioactive molecules that have beneficial effects on several gastrointestinal disorders. Under present investigation potential protective effects of whey fermented with probiotic *Lactobacillus rhamnosus* MTCC-5897 and *L. fermentum* MTCC-5898 on reinforcement of intestinal epithelial barrier function were evaluated. In order to understand the protective role of probiotic *Lactobacillus* fermented whey, initially DSS-induced colitis mice were used as *in-vivo* model. The impact of consumption of *Lactobacillus* fermented whey was assessed on disease activity index, hematological and histological scores, release of cytokines and expression of other inflammatory markers as well tight junctional genes (occludin, claudin-1, ZO-1, actin) along with their cellular localization respectively. While intestinal epithelial cells (HT-29) were also used to elucidate their mode of action during exclusion of *E. coli* in presence of simulated digested fermented whey prepared with *Lactobacillus* (LR: *L. rhamnosus* MTCC 5897 and LF: *L. fermentum* MTCC 5898). Later, integrity of tight junctional genes was determined through qRT-PCR in HT-29 cells and mode of downstream signaling resulting in immunomodulation was also assessed through nuclear translocation of NF-κβ (p-65 subunit) using western blotting. Pre-consumption of whey fermented with probiotic *L. rhamnosus* and *L. fermentum* before colitis induction to mice significantly reduced ($p<0.01$) the disease activity index and improved ($p<0.05$) the hematological and histological scores. The considerably diminished levels ($p<0.01$) of pro-inflammatory markers (IL-4, TNF-α, CRP and MPO activity) and enhanced ($p<0.05$) presence of anti-inflammatory cytokine TGF-β with IgA in intestine on feeding lactobacilli fermented whey appeared to prevent inflammation even on colitis induction. Transcriptional modulations in pathogen recognition receptor (TLR-2/4) and tight junctional genes (*ZO-1, occludin, claudin-1*) along with localized distribution of junctional (claudin-1, occludin, ZO-1) and cytoskeleton (actin) protein improved immune homeostasis and intestinal barrier integrity. Besides, significant reduced ($p<0.05$) appearance of FITC-dextran marker in serum on consumption of lactobacilli fermented whey directly confirmed the healthy status of host gut. Ideal expression of epithelial barrier and immune genes in intestinal cells were found during 4 hr of incubation of intestinal epithelial cells (HT-29) with simulated digested probiotic *Lactobacillus* fermented whey as well as inflammatory agent (*E. coli*). Pre-exposure of intestinal cells with either of probiotic lactobacilli simulated digested whey followed by incubation with the inflammatory agent under exclusion assay enhanced the expression ($p<0.05$) of tight junctional genes (occludin, claudin-1, ZO-1) respectively. In case of hBD-2, antimicrobial peptide, mRNA expression did not show any major change in exclusion with *E. coli* with simulated digested fermented whey prepared with *L. rhamnosus* and *L. fermentum* respectively. However, simulated digestion of fermented whey prepared with probiotic *L. fermentum* significantly decreased NF-kβ mRNA expression during *E. coli* exclusion. Further western blotting showed that incubation of intestinal epithelial cells with inflammatory agent (*E. coli*) significantly translocated p-65 sub-unit of NF-kβ from cytoplasm to nucleus. Contrarily, simulated digestion of fermented whey prepared with *L. fermentum* and *L. rhamnosus* respectively attenuated the translocation of p-65 sub-unit into nucleus during exclusion assay. Thus this strategy of probiotic fermented whey consumption not only added value to the dairy by-product but also appeared to be useful as a suitable delivery vehicle for administration of probiotics. Moreover, presence of live probiotic bacteria in fermented whey appeared more effective in contracting the infection and pathogenesis induced by colitis.

सारांश

किण्वित खाद्य पदार्थ आवश्यक पोषण घटक और जैव सक्रिय अणु प्रदान करते हैं, जो कई जठरांत्र संबंधी विकारों पर लाभकारी प्रभाव डालते हैं। वर्तमान जांच में दो स्वदेशी प्रोबायोटिक लैक्टोबैसिलस (LR: *L. rhamnosus* MTCC 9897 और LF: *L. fermentum* MTCC 5898) के साथ किण्वित मट्ठा की संभावित सुरक्षात्मक प्रभावों के तहत आंतों के उपकला अवरोध के सुदृढीकरण पर मूल्यांकन किया गया था। प्रोबायोटिक लैक्टोबैसिलस किण्वित मट्ठा की सुरक्षात्मक भूमिका को समझने के लिए, शुरू में इन-विवो मॉडल के रूप में डीएसएस-प्रेरित कोलाइटिस चूहों का उपयोग किया गया था। लैक्टोबैसिलस किण्वित मट्ठा की खपत के प्रभाव का मूल्यांकन रोग गतिविधि सूचकांक, हेमटोलॉजिकल और हिस्टोलॉजिकल स्कोर, साइटोकिन्स की रिहाई, अन्य दाहक मार्कर जंक्शनल जीन (ZO-1, occludin, claudin-1, actin) और सेलुलर स्थानीयकरण की क्रमशः अभिव्यक्ति की गयी। जबकि, आंतों के उपकला कोशिकाओं (एचटी -29) का उपयोग, *E. coli* की उपस्थिति में लैक्टोबैसिलस (LR: *L. rhamnosus* MTCC 5897 और LF: *L. fermentum* MTCC 5898) के साथ तैयार की गई किण्वित मट्ठा की कार्रवाई की विधि में देखने के लिए किया। बाद में, एचटी -29 कोशिकाओं में क्यूआरटी-पीसीआर के माध्यम से जंक्शन जीनों की अखंडता और डाउनस्ट्रीम सिग्नलिंग का मूल्यांकन NF-κβ (पी-65 सबयूनिट) के नाभिक स्थानान्तरण द्वारा पश्चिमी सोख्ता के माध्यम से किया गया था। *L. rhamnosus* और *L. fermentum* के साथ मट्ठा की पूर्व खपत ने रोग गतिविधि सूचकांक (पी<0.01), हेमेटोलॉजिकल और हिस्टोलॉजिकल स्कोर (पी <0.05) को चूहों में काफी हद तक कम कर दिया। लैक्टोबैसिली किण्वित मट्ठा द्वारा साइटोकिन्स मार्कर (IL-4, TNF-α, CRP and MPO) की अभिव्यक्ति स्तर (पी <0.01) में कमी और विरोधी दाहक साइटोकाइन (TGF-β) के स्राव में काफी (पी <0.05) वृद्धि दिखाई दी, जो कोलाइटिस प्रेरण सूजन को रोकता है। रोगजनक मान्यता रिसेप्टर (TLR-2/4) और जंक्शन जीन (ZO-1, occludin, claudin-1) में ट्रांसक्रिप्शनल मॉड्यूलेशन के साथ-साथ जंक्शनों के स्थानीयकृत वितरण (ZO-1, occludin, claudin-1) और साइटोस्केलेटन (actin) प्रोटीन ने प्रतिरक्षा होमियोस्टेसिस और आंतों की अखंडता में सुधार किया। इसके अलावा, लैक्टोबैसिली किण्वित मट्ठा की खपत पर सीरम में एफआईटीसी-डेक्सट्रान मार्कर की कमी ने मेजबान आंत की स्वस्थ स्थिति की पुष्टि की। आंतों की कोशिकाओं में प्रोबायोटिक लैक्टोबैसिली सिम्युलेटेड डाइजेस्टेड ने बहिष्करण परख के तहत, पूर्व-प्रसार के बाद *E. coli* के ऊष्मायन ने तंग जंक्शन जीनों (ZO-1, occludin, claudin-1) की अभिव्यक्ति (पी <0.05) को बढ़ाया। HBD-2, रोगाणुरोधी पेप्टाइड के मामले में, mRNA अभिव्यक्ति में *E. coli* के साथ बहिष्करण में कोई बड़ा परिवर्तन नहीं दिखा। हालांकि, *E. coli* अपवर्जन के दौरान प्रोबायोटिक *L. fermentum* के साथ तैयार किण्वित मट्ठा सिम्युलेटेड डाइजेस्टेड ने NF-kβ mRNA अभिव्यक्ति को काफी कम कर दिया। पश्चिमी सोख्ता में, *E. coli* की उपस्थिति में साइटोसोल से नाभिक तक NF-kβ (पी-65 उप-इकाई) में महत्वपूर्ण स्थानान्तरण देखा गया। इसके विपरीत, क्रमशः *L. rhamnosus* और *L. fermentum* के साथ तैयार किए गए किण्वित मट्ठा सिम्युलेटेड डाइजेस्टेड ने बहिष्करण परख के दौरान पी-65 उप-इकाई के नाभिक स्थानान्तरण में कम कर दिया। इस प्रकार प्रोबायोटिक किण्वित मट्ठा खपत ना केवल डेयरी उप-उत्पाद के लिए मूल्य को जोड़ा, बल्कि प्रोबायोटिक्स के लिए भी एक उपयुक्त वितरण वाहन के रूप में उपयोगी साबित हुआ। इसके अलावा, किण्वित मट्ठा में जीवित प्रोबायोटिक जीवाणुओं की उपस्थिति कोलाइटिस से प्रेरित संक्रमण और रोगजनन से निपटने में अधिक प्रभावी दिखाई दी।

CHAPTER –1

Introduction

INTRODUCTION

Whey is a by-product of casein and cheese produced in dairy industry. A majority of the whey produced globally is sweet whey. Traditionally, whey is a troublesome waste product of cheese factories and a serious cause of environmental pollution. It can be converted to different products, such as condensed whey, dry whey, dry modified whey, whey protein concentrate, whey isolates, and dried lactose through various technologies and processes. However, technologies involved in separation of whey proteins and its storage require high-energy processes that add to the cost and maintenance of cheese plants. Moreover, anaerobic systems used to treat whey as organic wastewater in the cheese industry also result in the production of methane (CH_4) gas. Nowadays, whey considered as valuable co-product due to its high biological value and nutritional applications. Sweet whey also contains appreciable amount of lactose which can be fermented by lactic acid bacteria. This lactose component makes the whey fit for the preparation of fermented beverages. Whey protein is the excellent source of protein having high nutritional and functional properties beside these it is also used as food ingredient due to its beneficial physical properties i.e high solubility, water absorption, gelatinization and emulsifying capacities. Whey proteins (β-lactoglobulin, α-lactoalbumin, immunoglobulin, serum albumin and lactoferrin) also have anti-inflammatory, anti-carcinogenic and immunomodulatory properties (Madureira *et al.* 2007). Recently goat whey also showed immunomodulatory effects in colitis model by inhibiting the NF-κβ master inflammatory regulator which helps in the maintenance of barrier integrity and promotion in gut health (Araujo *et al.* 2017).

Human gastrointestinal tract (GIT) has the largest area (200-400 m^2) colonised with diverse variety of microbes. Altered microbiota with aberration towards normal microflora is postulated to be central predisposing factor in disease pathogenicity (Xavier and Podolsky, 2007). Inflammatory bowel disease (IBD) is an idiopathic recurrent illness of gastrointestinal tract (GIT) consisting of ulcerative colitis and Crohn’s disease usually affecting people of all ages (Carvalho and Cotter, 2017). IBD is multifactorial disease and its etiology is still

poorly understood and likely to be correlated with stress, diet, environmental factors and microbiota dysbiosis which lead to loss of epithelial barrier integrity due to overzealous immune response. Thereby, it is critically important to develop the approaches to prevent the occurrence of IBD. Intestinal epithelium is critical barrier complemented by well evolve innate immune system which is populated by cells such as neutrophils, dendritic cells (DC), macrophages, innate immune lymphoid cells (ILC) and antimicrobial peptide (β-defensin) that poised to defend against pathogenic intrusions and curtail immune response to maintain the state of hypo-responsiveness to commensal microbiota (Khor *et al.* 2011). Cytokines and chemokines are soluble protein mediators which are critical for cellular communication and support intestinal mucosal homeostasis but can also be key driver of inflammation and inflammation associated damage (Peterson and Artis, 2014; Neurath, 2014). Under physiological conditions, intestinal epithelial lining involved in the maintenance of the tight junction proteins and intercellular structure consist of transmembrane proteins (Zonula-occluden: ZO-1, Occludin and Claudin) by regulating the level of pro-inflammatory and anti-inflammatory cytokines required for intestinal homeostasis (Al-Sadi *et al.* 2009). Impaired tight junction proteins and increase in epithelial permeability were established for mucosal inflammation in IBD patients. DSS-induced colitis model is one of the widely accepted model for ulcerative colitis because it resemble closely with human IBD model in order to study gastrointestinal physiology (Chassaing *et al.* 2014).

Dietary strategies such as probiotic bacteria fermented dairy products have been reported for beneficial effect on human health by improving intestinal microbial balance and various health attributes such as anti-carcinogenic, anti-obesity, immune modulator, antimicrobial and many others. Fermented milk products possess components that are produced during fermentation such as peptide, exopolysaccharide etc. that contribute to immunomodulation with the activation of innate immunity via toll like receptor (TLRs) which further lead to effector adaptive immune response (Vinderola *et al.* 2006 a). However, TLRs dysregulation mainly TLR-4 in intestinal epithelial cells (IEC) might be one cause of immune imbalance and excessive inflammation in IBD (Cario, 2010). TLR signaling proceeds through PAMPs (pathogen associated molecular pattern

recognition) to downstream complex intercellular cascade activation which finally outcomes in downstream signaling activation with the release of pro-inflammatory response (Kordjazy *et al.* 2018). In IBD patients, different pro-inflammatory cytokines are released in excess which further lead to instrumental progression of disease. In gut mucosa, activation of TLR-2 and suppression of TLR-4 are considered as anti-inflammatory activity (Bermudez-Brito *et al.* 2012, a). Usage of probiotic fermented milk products have been shown to regulate the impaired TLR expression and therefore attenuated the inflammatory process (Santiago-López *et al.* 2018). TLR-2 modulation by probiotics regulated the pro-inflammatory response via increasing several negative regulators to suppress TLR-4 expression. Intestine remains to be protected from exaggerated inflammatory response with the activation of barrier repair and epithelial maintenance pathways (Kamdar *et al.* 2013). Thus epithelial restitution was regulated by balancing pro and anti-inflammatory cytokines and defensive immune response using probiotic bacteria have also been implicated in modulation of epithelial permeability for the barrier function (Bauché and Marie, 2017). Recently effect of fermented whey with a probiotic bacterium on gut immune system has been investigated concisely as an interesting alternative for development of a new food additive for pig production (Liao and Nyachoti, 2017).

Two indigenous probiotic strains, *Lactobacillus rhamnosus* (MTCC 5897) and *Lactobacillus fermentum* (MTCC 5898), isolated in our laboratory has played a critical role in the regulation of immunomodulatory activities by modulating the expression of different cytokines (IL6, IL8, TNF-α and IL23) and Toll-like receptors (TLR-2,-4 and -9) along with regulation of nuclear translocation of NF-κB in intestinal cells (Gupta *et al.* 2021: In Press). Both bacterial strains were previously well characterised as probiotic due to their acid tolerance, bile tolerance and adherence properties (Sharma *et al.* 2014 a,b). They also maintained Th1/Th2 immune homeostasis, anti-oxidative status and prevented translocation of pathogenic *E. coli* in aging mice (Sharma *et al.* 2014 a,b). Feeding of *L. rhamnosus* (MTCC 5897) fermented milk to mothers during the suckling period and to their offspring after weaning improved the ovalbumin-induced allergic symptoms in mice (Saliganti *et al.* 2015). In the intestinal cell model, these *Lactobacillus* strains also enhanced the gut barrier integrity in a

strain specific manner during exclusion and competition with *E. coli* (Bhat *et al.* 2019; Bhat *et al.* 2020). Thus keeping in view the foregoing discussion, it is hypothesized that the above protective results of these laboratory strains may be translated again using dairy whey which will not only add value to the dairy by-product but also appear useful as a suitable delivery vehicle for administration of probiotics. Hence, present work was designed with the following objectives using *in vivo* and *in vitro* experimental models

1. **To assess the effect of lactobacilli fermented whey on protection of gut barrier integrity in colitis induced mice.**
2. **To elucidate the mode of action of lactobacilli fermented whey on human intestinal epithelial barrier integrity.**

CHAPTER –2

Review of Literature

Functional foods are those foods that provide health benefits beyond the provision of essential nutrients (e.g., vitamins and minerals), when they are consumed at efficacious levels as part of a varied diet on a regular basis. Functional foods are important as they are considered as one of the fastest-growing segments of the food industry.

Functional foods can also be defined as nutritive items that, in addition to facilitating nutrients and energy, constructively modify one or more targeted functions in the body, by augmenting a definite physiological response and/or by reducing the risk of disease (Nicoletti, 2012).

The functional foods of plant or animal origin according to their action can be categorized as:

1. Vitamins and minerals fortification (e.g., vit C, folic acid, calcium, iron)
2. Cholesterol reduction (e.g., omega-3 fatty acids, phytosterols).
3. Dietary fiber (e.g. nondigestible carbohydrates and lignin).
4. Probiotics, prebiotics, and synbiotics (e.g., yoghourt, kefir, fruits, vegetables).
5. Phytochemicals (e.g., phenolic compounds, carotenoids, lycopene) (Lagouri *et al*. 2019)

2.1 Fermented dairy products

Milk is the richest source of nutrients coupled with bioactive functional properties that helps in maintenance of growth and nourishment of infants. Milk comprehends omega-3 fatty acids, phytosterols, isoflavins, conjugated linoleic acid, minerals and vitamins, so has great importance in the development of functional foods. . *In-vitro* and *in-vivo* studies have shown that fermented dairy products (fermented milk, curd, yogurt, cheese, koumiss, paneer and kefir) assisted in the maintenance of normal mucosal homeostasis and modulate the immune response in a positive fashion which provides protection against various metabolic and pathogen mediated diseases by anti-oxidative, anti-microbial, anti-

fungal, anti-inflammatory, anti-diabetic and anti-atherosclerotic activities. The linkage between fermentation, nutrition and health is important and has been popularized with the best known fermented dairy foods such as fermented milk, yogurt and cheese, etc. (Bourlioux *et al.* 1988 and Yoda *et al*. 2014).

Yoghurt: Probiotic (*L. rhamnosus* GG) and prebiotic (dietary fiber) yoghurt was not simply a food gradient but also its consumption help in improvement of inflammatory bowel syndrome (IBS) and maintenance of fecal microflora in patients with reduction in putrefactive bacteria *Clostridium difficile* and *E. coli*. Thus probiotic yogurt was related with alleviation of IBS symptoms like constipation, abdominal pain, bowel movement frequency and flatulence after 6 weeks of consumption (Lee *et al.* 2013).

Cheese: Cheeses are fermented dairy products which have a strong potential for delivering beneficial microorganisms into the human intestine, due to their specific chemical and physical characteristics. Cheeses have higher pH levels, lower titratable acidity, higher buffering capacity, more solid consistency, relatively higher fat content, higher nutrient availability and lower oxygen content than yogurts. These qualities protect probiotic bacteria during storage and passage through the gastrointestinal tract (Karimi *et al*. 2011 and Ong *et al.* 2006). Whey is also a by-product of cheese industry produced in millions of tons having proteins which represent approximately 20% of total protein content in bovine milk. Whey could also be used as media for beneficial bacteria due to the presence of sufficient amount of lactose as source of energy (Koller *et al.* 2012).

Koumiss: Koumiss is fermented dairy beverage of ancient origin known by several names koumiss, kumiss, kumis, kymis, kymmyz. It is traditionally made milk beverage of the horse milk originated from the nomads of Central Asia. Extensively formed in Russia, Turkic, Bulgar, Kazakhstan in Western Asia predominantly for its health promoting potential. Mangolia considered it as a national drink and called as Airagand distilled koumiss called as Araka. Koumiss has mild alcoholic and sour taste. Koumiss and kefir are almost related as both are prepared from yeast and lactic acid fermentation except it is manufactured from liquid starter in contrast to kefir grains (Ørskov *et al.* 1995).

Kefir: Kefir ‘fermented dairy drink’ originated from Northern Caucasus, Mongolian, Tibetan mountains thousands of years back and is different and less

popular than other fermented milk products such as yogurt, cheese. It is manufactured through traditional fermentation of milk based on starter-kefir grains that contain various bacteria and yeast which live in symbiosis that influence organoleptic and sensory characteristics of drink.

2.1.1 Dairy Whey and its application in human health

Types of dairy whey and its composition

Initially, whey was defined as a by-product of casein and cheese produced in dairy industry and least commonly used around the world, usually disposed as waste causing high environmental pollution. Nowadays, whey considered as valuable co-product due to its high biological value and nutritional applications. The worldwide production of whey has been reported 165 million tons; among which cheese whey contributes the maximum percent (95%). In addition to this, Chhana and paneer is the major source of whey in India (Ha and Zamel, 2003).

Whey represents about 85-95% of the milk volume and retains 55% of milk nutrients. The most abundant of these nutrients are lactose (4.5-5% w/v), soluble proteins (0.6-0.8% w/v), lipids (0.4-0.5% w/v) and mineral salts (8-10% w/v of dried extract). Whey constitutes more than half of total milk solids, lactose, and water-soluble vitamins, including whey protein (20% of total protein) containing essential amino acid such as lysine, isoleucine, threonine and tryptophan in excess to FAO standard (Boumba *et al.* 2001). The main whey protein are beta-lactoglobulin (BLG) (58%) and alpha-lactalbumin (13%), while immunoglobulin, serum albumin and protease peptone present in lesser amount. Whey salts include NaCl and KCl (more than 50%), calcium salts (primarily phosphate) and others. Besides those, whey also contains lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds like urea and uric acid, B group vitamins and so on. There are two main types of whey; acid and sweet. Acid whey has a pH less than 5 and sweet whey has a pH above 5 (6-7 mainly). The type of the whey produced depends on the procedure used for casein precipitation. Acid whey is formed as the pH is lowered and the colloidal calcium phosphate is solubilised, the casein micelle structure of milk is disrupted and the casein proteins aggregate releasing calcium phosphate into the whey. Acid whey has higher ash and lower protein contents than sweet whey. Thus, their use in

alimentation is more limited than that of sweet whey, because of their acidic flavour and high saline content (Pescuma *et al.* 2008). On the basis of method of cheese manufacture, composition of whey depends. Sweet whey is a product derived from manufacturing pressed cheese or of rennet casein i.e. cheddar and mozzarella cheese. The mineral composition of sweet and acid whey is determined by the method of curd formation, either acid precipitation or rennet coagulation. Sweet whey is produced when rennin cleaves κ-casein on the surface of the casein micelle, destabilizing the casein complex, releasing casein-macropeptide into the whey, the remaining hydrophobic α, β paracasein flocculates together in the presence of calcium to form the cheese curd. It is very biodegradable (~99%) with high organic content (~ 70 g COD/l) and low alkalinity content. To produce 1 kg of cheese about 9 kg of whey is generated (Yadav *et al.* 2014) and because of its low concentration of milk constituents (6-7 % dry matter), whey has commonly been considered a waste product (Tsutsumi and Tsutsumi, 2014). About 50% of worldwide cheese-whey production is treated and transformed into various food products. About 45% of this amount is used directly in liquid form, 30% as powdered cheese-whey, 15% as lactose and its byproducts and the rest is as cheese-whey-protein concentrates. Many researches is still being conducted with whey to find new whey products (Mollea *et al.* 2013).

Whey protein is the excellent source of protein having high nutritional and functional properties beside these it is also used as food ingredient due to its beneficial physical properties i.e high solubility, water absorption, gelatinization and emulsifying capacities. Many non-expensive techniques such as ultrafiltration, microfiltration and spray drying have been developed to concentrate the whey protein selectively, so that it can be commercialized in food and pharmaceutical industries. Whey protein are present in different forms like whey protein concentrate (WPC), whey protein hydrolysate (WPH), whey protein isolate (WPI). These are taken as protein supplements because of their rapid digestion and also involved in increasing skeletal muscle protein synthesis. Both human and bovine whey proteome have been reported which provided information in relation to use of healthy dairy products for infants (Murakami *et al.* 1998; Tay and Gam 2011; Bian *et al.* 2014). Initially, 211 proteins were

identified and quantified by using iTRAQ among which 133 were found to be differentially expressed (Yang *et al.* 2013). Recently, using iTRAQ a total 584 proteins were identified and quantified from which 424 were differentially expressed in human and bovine colostrum and mature milk. Differentially expressed proteins also involved in many immune cascades (Yang *et al.* 2017). Thus, analysis of large number of proteins increase our knowledge regarding bovine and human whey protein. Importance of whey proteome is well documented in immune homeostasis and barrier integrity. On the basis of gene ontology (GO) functional annotation whey protein have various biological function. Previously, quantified bovine milk whey protein were analyzed by GO annotation and were found to be related with cellular process, intercellular organelles and molecular function (Reinhardt *et al*. 2012). In recent study, it was explained that bovine milk whey involved in immune response (34.8%), cell communication (17.4%), extracellular part (55.7%) and cytoplasm (23.5%) (Liao *et al.* 2011). Whey is not only a source of nutrient but also modulate many physiological functions in the body. This is especially true for intestine tract because of continuous interaction of intestine with dietary antigens.

Application of whey on human health

Whey and whey protein induces immune tolerance to antigens or towards diseases which in-turn leads to barrier integrity. The large number of health effects associated with consumption of whey or whey protein have been well established for example diminished inflammatory gene expression and protection against diarrhea induced by DSS (Sprong *et al.* 2010). Whey peptide extract also played major role in gut microbiota modulation. These beneficial effects also be mediated by whey protein extract which leads to the enhancement of mucosal innate immunity during early life (Pérez-Cano *et al.* 2007). Fermented foods are also known to possess various nutritional and therapeutic properties. In this regard, lactic acid bacteria (LAB) played a major role towards the positive health effects of fermented whey and related products. It is also important to note that besides probiotic LAB, possess other nonbacterial components that are produced during fermentation, such as peptides and exopolysaccharides, which can contribute to immunomodulation.

Whey not only contains essential nutrients but also a number of natural bioactive substances such as α-lactalbumin, lactoferrin, lactoperoxidase, lysozyme, EGF and TGF that have many beneficial effects on animal health (Gupta *et al.* 2011). Milk-derived whey protein concentrate rich in TGF-β has been developed by the food industry for infant and children's nutrition which has been proposed to stabilize epithelial barrier function and protect against inflammatory barrier impairment (Hering *et al.* 2011). Furthermore in reporter gene assays, TGF-β1 in WPC increased the activity of the claudin-4 promoter and protected against IFN-γ induced barrier impairment in HT-29/B6 cells in a dose dependent manner (Hering *et al.* 2011). Similarly the WPC supplementation has protective effects on intestinal integrity in which WPC reduced the expression of intestinal pro-inflammatory cytokines and increased the expressions of TJ proteins such as occludin and ZO-1 in LPS challenged piglets (Xiao *et al.* 2016). Whey also exerted beneficial effects on a wide variety of gastrointestinal disorders such as inflammatory bowel disease and necrotizing enterocolitis in animal models (Xaio *et al.* 2016). Araújo *et al.* (2017) indicated the increased expression of proteins such as mucins, occludin proteins and cytokine signaling suppressors upon treatment by whey.

The anti-inflammatory effects of goat whey was studied in a mouse intestine colitis induction by 2,4-dinitrobenzenesulfonic acid (DNBS) that resembled human Inflammatory bowel disease (IBD). Goat whey improved the symptoms of intestinal inflammation namely by decreasing the disease activity index, colonic weight/length, and leukocyte infiltration. Also goat whey increased the expression of proteins such as mucins, occludin proteins and cytokine signaling suppressors (Araújo *et al.* 2017). Bovine WPE (whey protein extract) reduced apoptosis of human blood neutrophils and exerts a dose-dependent priming effect on these cells which on subsequent stimulation increased the functions of adherence, chemotaxis, phagocytosis, oxidative burst, and degranulation. WPE increased not only neutrophil chemotaxis but also the surface expression of CD11b, a mediator of neutrophil adhesion to endothelial cells (Rusu *et al.* 2010). The bioactive forms of proteins and growth factors present in low-heat-treated WPC may induce greater proliferation and an enhanced immune response. Lactoferrin and TGF-β2 were retained at greater

levels in low-heat-treated WPC than in standard WPC in vitro condition (Nguyen *et al.* 2016). G-WPC (Glycated Whey Protein Concentrate) prepared via a non-enzymatic Maillard reaction had no cytotoxicity on RAW264.7 macrophage cells and caused increased expression of various cytokines (i.e., TNF-α, IL-1β and IL-6 mRNA) which showed ability to enhance phagocytosis (Chun *et al.* 2016).

Modified WPC can also suppress the mitogen-stimulated secretion of interferon, as well as the surface expression of interleukin-2 receptor, when added to T and B lymphocyte cultures (Cross *et al.* 1999). Neutrophils incubated with whey protein extract through α-LA, β-LG, stimulated the accumulation of IL-1Ra in excess of IL-1β through NF-kB and MAPK dependent mechanism (Rusu *et al.* 2010). Additionally, bovine lactoferrin function in inducing apoptosis on human gastric adenocarcinoma cells SGC-7901 by the inhibition of Akt activation (Xu *et al.* 2010). Lactoferrin (LF) and lactoperoxidase (LP) in response to mitogenic stimulation, inhibited the proliferation and interferon gamma (IFN-y) production of ovine blood lymphocytes and had no effect on LPS induced ovine blood lymphocyte proliferation, production of IL-1β, TNF-α by ovine bronchoalveolar lavage (BAL) macrophages and on bovine natural killer (NK) cell activity (Wong *et al.* 1996). Likewise, low heat treated whey protein concentrate from acid whey played a major role in cytokine response and proliferation in intestinal epithelial cells (IEC) as compared to low heat treated sweet whey (Nguyan *et al.* 2016). Bioactive peptides present in whey protein concentrate (WPC) enhanced immune response specifically at mucosal site by increasing the CD8αα, intestinal epithelial lymphocytes (IEL) and natural killer (NK) cells by lactoferrin which enhances innate immunity in early life (Perez-Cano *et al.* 2007).

Araujo *et al.* (2017) proved that goat whey inhibited NF-κB p65 and p38 MAPK signaling pathways which consequently down-regulated the gene expression of various proinflammatory markers TNF-α, IL-1β, IL-6 in colonic tissue of mice with DNBS induced colitis. Rusu *et al.* (2010) reported that WPE induced a time-dependent phosphorylation of IκBα (inhibitor of NF-κB) in neutrophils, so that it was removed from NF-κB and finally NF-κB gets activated which leads to activation of immune response.

2.1.2 Probiotic

The word probiotic was coined by Kollath which means “for life” and according to Lilly and Stillwell, probiotics are substances produced by microorganism which endorse the growth of other microorganisms. Utmost accepted definition of probiotics states that these are live microorganism, when administered in adequate amounts confer beneficial effects on the host (FAO/WHO 2001). Daily consumption of 10^8 -10^9 cfu/100g or 100ml of food containing probiotic assist dietary and microbial balance by regulating mucosal and systemic immunity (Begum *et al.* 2017). In Japan, probiotics are consumed as frozen culture tablets whereas in Europe due to biasness against medication, probiotics are only consumed through their inclusion in food (Kolacek *et al.* 2017). Dairy product plays a predominant role as probiotic carrier. Probiotic foods confer large number of health effects, for example improved lactose intolerance, prevention of inflammatory disorder, dysbiosis, diabetic, osteoporosis, carcinogenesis, *Helicobacter pylori* infections and various scientific publications demonstrating their effectiveness in gastrointestinal tract disorders as well. In addition to this, other fermented products like kimchi, kefir, and cheese also being continuously used for their nutritional value due to the presence of the probiotics.

2.1.3 Fermented dairy product as probiotic vehicle

Among total milk produced, only 7% of it is utilized in preparation of fermented milk products in India (Aneja *et al.* 1990). Three most common fermented dairy products are Indian dahi, shrikhand (sweetened concentrated curd), and lassi (stirred curd). Fermented dairy products are also regarded as ideal for the delivery of probiotic bacteria in human gut. These products provide a suitable environment in which their growth is promoted. Fermentation carried out with probiotic organism combines the benefits of both bacteria and fermented products by increasing bioactive peptides as shown in Table 2.1 (Heller, 2001). Dairy products such as cream, cheese, yogurt, *Acidophilus-bifidus* milk, koumiss, kefir and fermented beverages have long been considered as best matrix for the probiotic microbes with increased content of lactic, butyric, citric and acetic acids that not only enhance the organoleptic properties but also bio-functional efficacy of products (Homayouni *et al.* 2012). Rezac *et al.* (2018) surveyed many

fermented foods and observed that mostly they contained 10^{5-7} lactic acid bacteria per ml or gram, although there was considerable variation based on geographical region and sampling time. In general, cultured dairy products consistently contained higher levels of bacteria up to 10^9/ml or g. According to Karimi *et al.* (2012), recommendations for the minimum viable counts of each probiotic strain in gram or millilitre of probiotic products vary when it comes to providing health benefits related to probiotic organisms.

Fermented dairy products continue to be the primary delivery vehicle for food and beverage products featuring beneficial or probiotic microbes that help in maintenance of health (Figure 2.1). Firstly fermented dairy products such as yogurt are generally understood to contain live microbes because unlike many other fermented foods, dairy products are typically not pasteurized or heat treated at the end of production, which ensures microbial viability (Marco *et al.* 2017). Moreover microbes have been well studied for their growth and survival in dairy products. Davidson *et al.* (2000) evaluated the viability of probiotic strains in low-fat yogurt using *Streptococcus salivarius* ssp. Thermophiles, *L. delbrueckii* ssp. Bulgaricus, *B. longum* and *L. acidophilus*, and verified that culture bacteria did not decrease during frozen storage without altering its sensory characteristics. Further, the yogurt matrix appeared good vehicle for microbes due to its composition, which includes milk proteins, fat and lactose, as well as other compounds. Moreover, its frozen state contributes to its efficiency. Additionally, yogurt should have relatively high pH values 5.5 to 6.5, in order to favor an increased survival of lactic cultures during storage. The lower acidity also results in increased consumer acceptance, especially among consumers who prefer milder products (Cruz *et al.* 2009). Similarly, *Saccharomyces boulardii*, had the ability to grow in bio-yogurt and reached maximum counts exceeding 10^7 cfu/g. The number of yeast populations was substantially higher in the fruit-based yogurt, mainly due to the presence of sucrose and fructose derived from the fruit. Despite the inability of *S. boulardii* to utilize lactose, the yeast species utilized available organic acids, galactose and glucose derived from bacterial metabolism of the milk sugar lactose present in the dairy products (Lorens-Hattingh and Viljoen, 2001). Similarly fermented milks supplemented with lemon and orange fibers increased the counts of *L. acidophilus* and during

cold storage compared to the control set (Sendra *et al*. 2008). Likewise cheeses are fermented dairy products which have a strong potential for delivering beneficial microorganisms into the human intestine, owing to their specific chemical and physical characteristics. Cheeses have higher pH levels, lower titratable acidity, higher buffering capacity, more solid consistency, relatively higher fat content, higher nutrient availability and lower oxygen content than yogurts. These qualities protect probiotic bacteria during storage and passage through the gastrointestinal tract (Karimi *et al*. 2011 and Ong *et al*. 2006). Moreover, fermentation allows for the retention and optimization of microbial viability and productivity, and it simultaneously preserves probiotic properties (Ross *et al.* 2002; Stanton *et al*. 2003). Studies conducted on animal and human models have been focused on the effects of dairy product on cytokine production, phagocytic activity, antibody production, T-cell function and Natural Killer (NK) cell activity (Nagao *et al*. 2000: Forssten *et al.* 2011). Whey could also be used as media for beneficial bacteria due to the presence of sufficient amount of lactose as source of energy (Koller *et al.* 2012). In addition, fermented whey has the potential to cause an increase in the RBC and lymphocytes in cases of anaemia, is of great importance in the defense of the body against infections. It is therefore conceivable that the consumption of fermented whey by apparently healthy individuals might boost the cells of the immune system and may also play a role in cases of allergic reactions (Adebolu TT *et al.* 2016).

2.1.4 Health benefits of probiotic functional food

Probiotics are animate microbial food add-ons that offer many health benefits, as they provide assistance in sustaining excellent stability and maintainence of the composition of the intestinal microbiota and uplift the resistance against infection by pathogens. Probiotics can be deliberately considered as prospective functional foods, as they provide health benefits much more than the conventional nutritional foods. The need of probiotic functional foods is quickly and gradually on the rise for the reason that of increased consciousness of the public about the influence of food on health. Probiotics are now evolving as a promising key category of food supplement around the globe. There are nowadays abundant of evidences backed up with high-quality, scientific clinical data to support that probiotic contribution could undeniably be

successful in different types of diarrheal diseases, the modulation of immune function, the inhibition of colon cancer, and other chronic gastrointestinal inflammatory disorders. The potential efficiency of probiotics in treating or preventing neurological diseases is developing as a subject of great interest. In the topical years, considerable interest has been committed to ascertain the functions and therapeutic effects of probiotics in an extensive range of neurological conditions (Begum *et al.* 2017).

2.1.4.1 Obesity and Diabetes

Diabetes is a global health problem in the world. Probiotic food has anti-diabetic property. Wang *et al.* (2015 a,b) reported that the intake of *L. paracasei*, *L. rhamnosus* or *Bifidobacterium animalis* subsp. Lactis individually reduced conditions like hyper-insulinemia, hyperglycemia, and glucose intolerance considerably and thus presenting a promising preventive alternative to treat metabolic syndrome. Ostadrahimi *et al.* (2015) demonstrated that consumption of probiotic-fermented milk (kefir) in diabetic patients in comparison with conventional fermented milk decreased the fasting blood glucose and HbA1C levels. Similarly, oral administration of milk fermented in 8-week-old male rats with *L. casei* strain AP was the most effective against various diabetic parameters and similar in effect to that of the commercially available antidiabetic drug metformin (Widodo *et al.* 2019). Another study displayed that consumption of probiotic fermented milk containing *L. rhamnosus* (MTCC 5897), *L. fermentum* (MTCC 5898) and *L. rhamnosus* (MTCC 5957) had anti-diabetic effect individually and in combination through controlling glucose metabolism, inflammation status, serum lipid profile in diabetic rats as compared to control group (Yadav *et al.* 2018).

2.1.4.2 Lactose intolerance

Lactose intolerance may occur as a disease in countries with generally lactose tolerant individuals. In lactose in- tolerant children lactose may be toxic and damage the intestinal mucosa giving rise to symptoms similar to those in coeliac disease. In view of the widespread occurrence of lactose intolerance, it is of interest to reduce the concentration of lactose in milk (Dahlqvis *et al.* 1977). Fermented milk appears to be tolerated in lactose intolerant subjects because of the lower concentrations of lactose compared with low fat milk. It is also possible

that lactase-containing microorganisms originating from the fermented milk continue to be active in the intestinal tract and participate in hydrolysis of ingested lactose (Garza *et al.* 1979). Healthy volunteers, females and males, ranging from 18 to 50 year from Sweden consumed fermented milk products, especially yogurt and acidophilus. None of the subjects complained about gastrointestinal discomfort after ingesting yogurt or acidophilus milk, therefore considered as an alternative in formulating diets for lactose intolerant subjects (Alm *et al.* 1982). Similarly, intake of probiotic showed reduction in duration of diarrhoea (Agustina *et al.* 2012), inflammatory responses and encouraged the growth of microorganisms in digestive tract.

2.1.4.3 Allergies

Probiotic fermented products intake alleviates symptoms of food based allergies in susceptible individuals. A meta-analysis performed on supplementation with different combination of lactobacilli in atopic dermatitis in normal and allergic risk groups displayed positive impacts (Panduru *et al*. 2015). Intake of probiotic *L. gasseri* KS-13, *B. bifidum* G9-1, and *B. longum* MM-2 in placebo control study revealed anti-allergic effect in young adults suffering from seasonal allergies through modulation of immune response (Dennis-Wall *et al.* 2017). Liu *et al.* (2017b) demonstrated that probiotic *B. infantis* CGMCC313-2 administration inhibited the secretion of allergen-induced IgE antibody as well secretions of Th2 cytokines and further attenuated allergic inflammation in allergen-induced airway inflammation and β-lactoglobulin induced food allergies in a mouse model. Likewise, Saliganti *et al*. (2015) demonstrated the anti-allergic effect of probiotic *L. rhamnosus* (MTCC 5897) against ovalbumin allergies in weanling mice.

2.1.4.4 Oxidative stress

Bioactive peptide produced by probiotics act as a natural antioxidant and reduces oxidative stress. Probiotic *B. animalis* 01 in aging mice displayed increased anti-oxidative enzymes function and controlled oxidation of lipid by reducing MDA content, lipofuscin level and monoamine oxidases activity (Shen *et al*. 2011). Wang *et al*. (2017) demonstrated anti-oxidative effect of *Bacillus amyloliquefaciens* SC06 in intestinal porcine epithelial cells through reduction of reactive oxidative species and controlling Nrf-2 and Keap-1 pathways. Das and

Goyal, (2015) isolated *L. plantarum* DM5 from fermented beverages which showed anti-oxidative effect through reduction in hydroxyl radical, which prevented auto-oxidation of ascorbate and synthesis of γ-amino butyric acid. In another study, Kullisaar *et al*. (2003) revealed that consumption of fermented goats milk improved anti-atherogenicity in healthy human subjects, it prolonged resistance of the lipoprotein fraction to oxidation, lowered levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and glutathione redox ratio, and enhanced total antioxidative activity in 21 days of period.

2.1.4.5 Skin repair

Use of probiotics as nutricosmetics in global market is also evolving rapidly due to changes in lifestyle, aging population and consumer awareness. In a placebo-control study, supplementation of probiotic *L. paracasei* NCC 2461 in female groups for 2 months decreased the incident of skin sensitivity and enhanced skin barrier functions (Guéniche *et al*. 2014). Weill *et al*. (2013) demonstrated that cell wall component, Lipoteichoic acid (LTA), isolated from *L. rhamnosus* GG displayed immunomodulatory effect against UV radiation and inhibited development of skin tumor. Khmaladze *et al*. (2019) compared the effect of probiotic *L. reuteri* DSM and its cell lysate on general skin health. They found that probiotic bacteria provided better protection against UVR-B mediated inflammatory pathway, inhibited photoaging through its anti-inflammatory, anti-microbial effect besides augmented skin barrier related gene. Miyazawa *et al*. (2018) demonstrated that oral feeding of *L. rhamnosus* GG improved skin health through maintaining moisture content and preventing water loss from skin.

2.1.4.6 Hypercholesterolemia

Cardiovascular Diseases (CVD) are currently a leading cause of death globally and is still flourishing, spreading and has become prevalent over the whole world. The WHO has predicted that up to 40% of all deaths will be related to CVD by 2030, affecting approximately 23.6 million people around the world. Indeed, the risk of heart attack is three times higher in patients with hypercholesterolemia than those who have normal blood lipid contents (Davis *et al*. 1990). To prevent hypercholesterolemia considerable interest has instigated in dietary and pharmacological interventions to prevent cholesterol absorption as well. Dairy products fermented with the lactic acid bacteria can be anticipated to

result in a lowering of the circulating cholesterol concentrations, thus diminishing the risk of CVD in the population. These bacteria must be bile resistant and have the ability to deconjugate bile acids and bind cholesterol. If these criteria are fulfilled, fermented dairy products can be viewed as functional foods in the lowering of elevated cholesterol concentrations, and hence, in the prevention of CVD (St-Onge *et al*. 2000).

2.1.4.7 Gastrointestinal disorders

Fermented food supplemented with probiotics *Lactobacillus, Bifidobacterium* species as well as designer probiotics are the most widely used to cure inflammatory bowel diseases condition by modulating intestinal flora and gut immune response (Amer *et al*. 2018). Probiotics mainly balance the gut intestinal flora and reduce the chances of colonisation by pathogenic bacteria which lead to improvement of gut health. Chen *et al*. (2013), evaluated the administration of specific dose of *L. acidophilus* in colitis mice which relieved the symptoms of Ulcerative colitis (UC) by modifying the gut microbiota composition of distal colon. Use of broad-spectrum antibiotics treatments lead to the development of antibiotics associated diarrhea. Fermented milk containing probiotic *Bifidobacterium bifidum* YIT 10347 affects gastric and lower abdominal symptoms after eating with the production of gastro-protective mucin in functional dyspepsia individuals (Gomi *et al*. 2015). A study by Guyonnet *et al*. 2009 showed that the daily consumption of probiotic food containing the specific *B. lactis* DN-173 010 strain is able to improve GI well-being and digestive symptoms in adult women reporting minor digestive disorders. Effect of fermented products on different type of diseases as discussed following in Table 2.2.

Table 2.2: **Effect of fermented products on different type of diseases (**Kaur *et al.* 2020**)**

Fermented dairy food	Microorganism	Disorder	Mode of action
Fermented milk	*Bifidobacterium breve*, *B. bifidum*, and *L. acidophilus* YIT0168	Ulcerative colitis	Preventive effects in ulcerative colitis relapse, reduced *B. vulgatus* and luminal butyrate with concomitant reduction in albumin in murine model
	L. paracasei CNCM I 1518	Cirrhosis	Decreased proinflammatory mediators, oxidative damage and improve gut dysbiosis in rats
	L. rhamnosus MTCC 5897 and *L. rhamnosus* MTCC 5957	Hypercholestremia	Increased antioxidative activities and decreased lipid peroxidation in wistar rats
Fermented oat gruel in fruit drink,capsules	*Lactobacillus plantarum* 299v (DSM9843)	Irritable bowel syndrome, Clostridium difficile infection	Sufficient dose (10^8 cfu/g)stimulates mucosal immune system and increases defense action to limit the dissemination of pathogen in immune-competent mice model
Camel milk (Shubat drink)	0.1% direct vat set (DVS) yogurt culture	Diarrhea	Anti-microbial agents in camel milk showed antiviral activity against diarrhea causing viruses in rats
Koumiss	*L. helveticus* NS8	Ulcerative colitis	Protective against colitis and associated with immunomodulatory activity in-vitro condition LcZhang modulate immune response, with increase in sIgA local immunity effect mainly to the release of various cytokine involved in systemic immunity

Kefir	*Lactobacillus lactis* subs., Leuconostoc subs., Streptococcus thermophilus, *Lactobacillus* subs., and yeast of kefir *Lactobacillus helveticus* R389 or L89	Inflammatory bowel disease (IBD) Breast cancer	Ameliorate the colitis induced symptoms in dose dependent manner, relives from disease induced diarrhea and macroscopic damages caused in mucosal wall Increased IL-10 concentration and decreased levels of IL-6.
Yogurt	*Lactobacillus acidophilus* + *Bifidobacterium animalis* subsp lactis	Improves microbiota	Beneficial effect on stool frequency, defecation condition and stool consistency in adult women with constipation constipated women after 1 and 2 wk of consumption
Tofu	*Lactobacillus plantarum* K68	Ulcerative colitis	Decreased the production of pro-inflammatory TNF-a IL-1b, IL-6 and colonic mRNA expression levels of TNFa, cyclooxygenase-2, (COX-2), forkhead box P3 (Foxp3), suppressors of cytokine signalling 3 (SOCS3) and TLR4 was reduced
Fermented milk	*Lactobacillus acidophilus* B, *Lactobacillus bulgaricus* Lb, *Lactobacillus bulgaricus* 448, *Bifidobacterium longum* B6, *Streptococcus thermophilus* MC	Anti-allergic effect	The results showed that the ratios of IFNγ to IL-4 of both spontaneous and ovalbumin-stimulated secretions in splenocytes from mice receiving BAL had increased

2.2 Mode of action of probiotic fermented products

2.2.1 Fermented product as a key player of immunomodulation

Inflammation is a response of immune system to different kind of harmful stimuli, such as pathogens, damaged cells and toxin compounds. It is a common cause of various chronic diseases like cardiovascular, diabetes, arthritis, inflammatory bowel diseases and cancer (Chen *et al.* 2018). Lactic Acid Bacteria (LAB) have shown immunomodulatory and anti-inflammatory activities. There is ability of several LAB strains to enhance both innate and adaptive immunity, in animal models, after the ingestion of fermented milks. The effects of LAB are very strain-dependent and many lactobacilli act on payer's patches to stimulate IgA production, phagocytosis process and possess anti-inflammatory and anti-allergic activities by reducing the production of cytokines and immunoglobulin E (IgE) (Chapat *et al.* 2004; Clancy *et al.* 2003).

Under *In-vivo* trials

Probiotic fermented products applications and immunomodulatory effects are studied in various animal and clinical models. In mice model, animals fed with novel fermented whey protein-based product Malleable Protein Matrix (MPM) exhibited strong reduction of the ear inflammation with no side effects, as compared to hydrocortisone, were observed. The MPM seemed to reduce neutrophil extravasation in tissue as evidenced by blood polymorphonuclear cells and ear myeloperoxidase content (Beaulieu *et al.* 2007). Zheng *et al.* (2014) revealed that feeding of *B. breve* for 9-days before induction of DSS-colitis in mice prevents inflammation through modulation of T-cell composition by decreasing Th_1 and increasing Th_2 and T-reg associated cytokines in the distal colon. Oral intake of probiotic *L. casei* CRL 431 before challenge with *Salmonella typhimurium* displayed protective effect through downregulation of TNF-α and upregulation of IFN-ϒ, IL-6 and IL-10 production in the lamina propria of the small intestine of mice (Castillo *et al.* 2011). The fermented whey dairy beverage treatment displayed a significant effect on animal survival (70%), translocation of the pathogen to the liver (2 out of 10), histopathology (fewer lesions), and inflammation protective effect against *Salmonella Typhimurium* infection in the murine model (Cordeiro *et al.* 2019). Bacterial fermented Nipa

vinegar were shown to have a positive effect on inflammation and lipid metabolism due to the active ingredients present in Nipa vinegar, particularly the polyphenols, which enhanced the anti-infammatory, hypolipidaemic effect and weight loss in obese mice relative to those of synthetic acetic acid vinegar (Beh *et al.* 2017). de LeBlanc *et al.* (2008) evaluated the influence of milk fermented by *L. casei DN-114001, L. delbrueckii subsp. bulgaricus or S. thermophilus* on the immune response in BALB/c mice for 98 consecutive days. The results demonstrated that the oral administration of fermented milks increased IgA+ TNF-α, interferon (IFN)-γ, IL-12 and IL-10- producing cells in most intestinal samples. The cell population values of CD4 and CD8+ were also increased in the lamina propria of the intestine. However, no modifications were observed on mucosal sites distant from the gut. Solids (including bacteria) and liquid supernatant obtained from kefir were also reported to induce cytokine production *in vivo* by peritoneal macrophages and adherent cells isolated from peyer's patches (Vinderola *et al.* 2006 a). In a study performed in Japan by Nagao *et al.* (2000), *L. casei* Shirota-fermented milks were administered orally over 3 weeks to healthy subjects. The results showed that the consumption of fermented milks with these bacteria increased the level of NK cell activity, which is beneficial for people with low activity in these cells, and thus prevents disease, as assessed in peripheral blood mononuclear cells (PBMC).

In-vitro trials

In vitro models based on the use of cell lines or specific cells of the immune system are particularly attractive. These models may be used to measure the induction of nonspecific and specific immune responses by measuring parameters such as the production of cytokines and mediators by antigen- presenting cells and cellular markers for different cell populations. Cell-free concentrated whey from *B. breve* C50–fermented milk (BbC50sn) induced a specific interleukin-10 (IL-10)-rich cytokine profile and prolonged DC survival via a TLR-2 pathway. The anti-apoptotic BbC50sn signal surpasses the proapoptotic effect of lipopolysaccharide on DCs (Granier *et al*. 2013). Laffineur *et al.* (1996) evaluated the immunomodulatory activity of culture supernatants that were obtained from milk fermented by different LAB (*L. helveticus, L. casei* ssp. *Casei* 31, *L. casei* subsp. *rhamnosus* 4008, *L. acidophilus* 41, *L. delbrueckii* subsp.

bulgaricus 1208 and *Streptococcus thermophilus* 052) exhibited and the results showed that only the culture supernatants from *L. helveticus* strains were able to promote lymphocyte proliferation, but they did not modulate the cytotoxic activity of NK cells the proliferative response and cytotoxic activity in human PBMC. In addition, the impact of fermented milks' supernatant by *L. helveticus* R389 at pH 6 controlled the expression of calcineurin and the reinforcement of the epithelial barrier, as determined by evaluating parameters such as transcellular Ca2+ and E-cadherin expression. The results showed that the supernatant of fermented milks enhanced the gut mucosal immunity by improving the mechanisms that reinforce the epithelial and nonspecific barriers and gut function at the sites of infection, with improved expression of the enzyme calcineurin, an important signal in the network that activates the gut immune system (Vinderola *et al*. 2007).

The intestinal epithelial barrier is the main defense mechanism against infection and inflammation. The disruption of its integrity is one of the primary cause of many intestinal disorders. Bacterial surface layers are supramolecular cell envelop structures that are abundant in gram positive and gram negative bacteria (Liu *et al*. 2020). As the outermost structure of the cells, the surface layer lattice is generally considered to be the first bacterial component that have direct interaction with the intestinal epithelium. Surface layer proteins (SLPs) can protect the intestinal barrier by affecting F-actin distribution and modulating the tight junctional proteins at the mRNA and protein level (Prado *et al.* 2017). Previous studies identified diets rich in fruits, vegetables or whole grains as critical modulators of the gut microorganisms (Tuohy *et al.* 2012) based on their content in fibers, phenolic compounds and prebiotics. Moreover, diets containing fermentable fibers and resistant starches result in increased gut fermentation and SCFA (Shot Chain Fatty Acid) production. In turn, increase in SCFA production would also increase protection of the epithelium through strengthening the barrier as mediated by increased TJ protein production and TEER, as well as decreased permeability and bacterial translocation. (Guzman *et al.* 2013). In brief, initially probiotic fermented products stimulate host cells for production of antimicrobial peptides such as bacteriocins or beta-defensins (α and β- defensins) and antagonize the pathogens. They also form lactic acid and acetic acid that has strong antimicrobial activity against various group of gram-

negative bacteria. Probiotic fermented product released components prevent the pathogenic colonization by blocking mucosal adherence site or depriving the nutrients. this mechanism is termed as competitive exclusion. Probiotics microbe present in fermented product able to regulate immune response by interacting with dendritic cells (DC), monocytes/macrophages and lymphocytes in-turn increases the IgA response. Thus components produced from dairy fermented probiotic bacteria influence the epithelial barrier integrity and enhances tight junctional protein expression via modulating the TLRs. Thus, overall intestinal barrier defense consists of tight junctional adhesion complex, mucosal layer, antimicrobial peptide, secretory IgA, TLRs, cytokines that play major role in gut-immune homeostasis (McGuckin *et al.* 2009).

2.2.2 Role fermented products in reinforcement of gut barrier integrity

Fermentation carried out with probiotic organism combines the positive images of both bacteria and fermented products (Heller, 2001). Moreover, the consumption of probiotic bacteria via fermented milk is an ideal way to re-establish the balance of intestinal microbiota (Lourens-Hattingh and Viljoen, 2001). After consumption of probiotic-fermented milk products, some micro-organisms (e.g. *Bifidobacterium longum*) become part of the human intestinal microbiota whereas others such as *Lactobacillus casei* indirectly exert their effects in a transient manner as they pass through by remodelling or influencing the existing microbial community (Gogineni *et al.* 2013). The broth obtained by *L. acidophilus* fermentation in the presence of enriched extracts from *E. sativa* seeds, have a high concentration of glucoerucin, induced a significant decrease in the CXCL8 expression in Caco-2 cells following EHEC infection and reduced epithelial disruption caused by EHEC (Bonvicini *et al*. 2020). *Lactobacillus casei* BL23 and milk work synergistically to prevent damage to epithelial barrier integrity induced by pro-inflammatory cytokines (Zhai *et al.* 2019). Likewise, consumption of *L. plantarum* Lp91 in the form of fermented dairy food could increase the functioning of intestinal barrier in normal health as well as enteric infection conditions. The mechanism of protection against infection was revealed by the modulation of key regulatory receptors TLR-2, TLR-4 and by upregulating the expression of the tight junction genes along with secretory components; mucin-2, β-defensin-2 and cathalicidin (Rokana *et al*. 2016). The *L. paracasei* 01 fermented milk beverage reduced macromolecule permeability and strengthened

intestinal epithelial integrity caused by dextran sodium sulfate-induced damage. Besides, fermented milk beverage prepared with live *L. paracasei* 01 in the cell medium increased intestinal epithelial cell (Caco-2) growth, decreased lipopolysaccharide/tumor necrosis factor-α (TNF-α)/interferon-γ (IFN-γ)-induced Caco-2 cell death and chemokine CCL-20 production (Chen *et al.* 2016).

2.2.3. Intercellular junctional proteins

The tight-junction (TJ) is a major cellular component for maintenance of tissue integrity and barrier function. It has a complex molecular composition that forms the continuous intercellular barrier against external agents in the intestine. The integral membrane components of TJ include claudins, occludins and zona-occludin that maintains the epithelial barrier integrity (Ulluwishewa *et al.* 2011). Epithelial barrier function is an important feature that helps to preserve epithelial integrity and active transport function. When impaired, a passive loss of water and solutes leads to leak-flux diarrhea and the unwanted invasion of noxious luminal antigens perpetuates mucosal inflammatory processes. Thus, the terms "gut barrier" and "intestinal permeability" are often used interchangeably, although they refer to different functional aspects of the mucosa. Epithelial cells comprise the largest amount of surface area and are sealed at paracellular interfaces by tight junctions (Ewaschuk *et al.* 2008). Inflammatory bowel disease (IBD) is often associated with altered tight junction barrier function that allows for the passage of microbial antigens into underlying immune tissues. Increased intestinal epithelial permeability, also known as "leaky gut," is associated with a variety of gastrointestinal disorders, including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), celiac disease, and the early stages of colon cancer development (Bischoff *et al.* 2014). In IBD, altered permeability increases the translocation of proinflammatory stimuli into the lamina propria (LP), triggering inflammatory cytokine-mediated changes to the tight junctions that result in permeability changes (Clayburgh *et al.* 2004). TJ are not static barriers but highly dynamic structures that are constantly being remodeled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria. TJ is comprised of over 50 proteins that helps in the maintenance of epithelial integrity. In a strain and dose-dependent manner,

numerous probiotic strains have been shown to directly alter tight junction protein expression in both *in vivo* and *in vitro* models (Zhou *et al.* 2010).

LGG (*L. rhamnosus* GG) fermented milk also regulated intestinal epithelial homeostasis and potentially prevented intestinal inflammatory diseases through activation of EGFR (epidermal growth factor receptor) by LGG-derived proteins in DSS induced colitis mice model (Yoda *et al.* 2014). Visser *et al.* (2009) reported that milk protein components improved intestinal barrier function by altering the expressions and functions of claudin. Enteroinvasive *E. coli* (EIEC) infection lead to the disruption and disorganization of the actin cytoskeleton but these effects were reversed by incubating the epithelial cells with *L. plantarum*, which increased density of actin filaments at the junctional regions as tight junctional proteins have been closely associated with the cytoskeleton (Qin *et al.* 2009). The *L. paracasei* 01-fermented milk beverage provided intestinal protection effects by strengthening the epithelial barrier function via increasing TEER and decreasing the level of proinflammatory cytokine *in-vitro* (Chen *et al.* 2015). Furthermore, the immunoprotective effect of probiotic Dahi containing *L. acidophilus* LaVK2 and *Bifidobacterium bifidum* BbVK3 on DSS-induced ulcerative colitis in mice was demonstrated by significantly reducing TNFα, IL-6, and IFN-γ cytokines (Jadhav *et al.* 2013). Additionally, administration of VSL#3 probiotics decreased TNFα and IL-6 and increased IL-10 serum levels in a DSS-induced colitis model of inflammation (Dai *et al.* 2013).

2.2.4 Host antimicrobial peptides

The intestinal epithelium plays an active role by secreting a vast array of antimicrobial peptides and proteins (AMPs) into the lumen that contribute to the multilayered defense against luminal microorganisms (Muniz *et al.* 2012). Besides their bactericidal property it also play a major link between innate and adaptive immune system. However, the largest amounts of AMPs are produced by enterocytes lining the gastrointestinal tract and by Paneth cells in the small intestine. Expression of some Paneth cell antimicrobials varies along the small intestine, with the highest amounts produced in the ileum (Karlsson *et al.* 2008). They have also been shown to be overexpressed in the inflamed colon in IBD (Wehkamp *et al.* 2002 and Cunliffe *et al.* 2001). β-Defensins are synthesized by various epithelial cells such as in the skin, respiratory tract, and gastrointestinal

tract (O'neil *et al.* 2008). Whereas expression of human β-defensin-1 may be constitutive while β-defensin-2 is induced by bacterial infection (Takahashi *et al.* 2001 and Krisanaprakornkit *et al.* 2000).

Several studies have shown the antagonistic effect of probiotic against various pathogenic microorganisms through the release of AMPs as a defensive response from the epithelial surface (Bermudez-Brito *et al.* 2012 b). Schlee *et al.* (2008) showed that β-defensins expression or secretion was significantly up-regulated in Caco-2 cells upon stimulation by several lactobacilli species and VSL#3. Similary, Caco-2 cells differentially regulated the increase in HBD-2 expression after LAB treatment in TNF-α induced inflammatory condition (Kanmani and Kim, 2020). Consumption of *L. reuterii* probiotic bacteria for 14 days significantly enhanced the β-defensin-2 expression via NF-κB signaling cascade in wistar rats (Kusumaningsih *et al.* 2016). Piglets orally administered with different probiotic combinations displayed the increased expression of β-defensin-2 in the duodenum with the coadminstartion of RJGP16 and B1 (Deng *et al.* 2013). Mondel *et al.* 2009 showed that under *in vitro* (Caco-2) and *in vivo* (human) milieu probiotic *E. coli* mixture enhanced the synthesis of antimicrobial human β-defensin-2.

2.2.5 Modulation of pathogen recognition receptors (PRRs) signaling

Pattern recognition receptors (PRRs) have the ability to relay "danger signals" to the host in order to intercede an early innate immune response. Enterocytes express PRRs i.e TLRs (Toll like receptor). The activation of PRRs include multiple signaling pathways such as nuclear factor (NF-κB) and mitogen activated protein kinases (MAPKs) which lead to the induction of proinflammatory and anti-microbial responses (Llewellyn and Foey, 2017). Saliganti *et al.* (2016) reported that the supplementation of probiotic (*L. rhamnosus*) fermented milk either to the mothers during the suckling period (BD-PS) exclusively or subsequently to their offspring during the post weaning (BDPS+PW) period effectively controlled the abnormal deviations in immune response by increased expression of TLR-2 and decreased expression of inflammatory markers (COX-2 and MCP-1) in the intestine of mice during suckling-weaning transition.

2.2.5.1 TLR-2 receptor

Effect of fermented dairy products on toll like receptor signaling is studied in various *in-vitro* or *in-vivo* models (Chen *et al.* 2012). TLR-2 play a major role in immunomodulation after it was recognized by various microbial components such as lipopeptides/lipoproteins from various pathogens, peptidoglycans and lipoteichoic acid (LTA) from bacterial surface and variable number of dietary components (Kamdar *et al.* 2013). Sensing of probiotics by dendritic cells (DC), epithelial cells and macrophages is governed by the TLR receptor which work as pattern recognition receptor. TLR-2 forms heterodimer with TLR-1 and TLR-6 which is the initial event in cascade of events leading to significant innate immune activation that leads to activation of adaptive immunity to pathogens. TLR-2 heterodimers generally inititate a MyD-88 dependent intracellular signaling pathway which induces the translocation of NF-kB to modulate gene transcription and consequent inflammatory cytokine production (Oliviera *et al.* 2012). TLR-2 -/- deficient DCs are not able to produce T-reg cells development and IL-10 secretions (Round *et al.* 2011). Likewise, probiotic *L. plantarum* BFE 1685 and *L. rhamnosus* 33 GG treatment also found responsible for the immunomodulatory effect through TLR-2 on interaction with intestinal epithelial cells HT-29 (Pinto *et al.* 2009). Probiotic supernatant containing bioactive components act via TLR-2 and NOD-2 pathways in HEK293 cells also showed immunomodulatory effect (Harb *et al.* 2013). Jiang *et al.* (2012) revealed that *L. acidophilus* induced TLR-2 signalling played vital role in regulation of immune signaling in intestinal cells (Caco-2). These numerous studies depicted that TLR-2 signaling is necessary by probiotic bacteria to show its anti-inflammatory effect. Moreover, Dahi and milk demonstrated a significant increase in TLR-2 as compared to negative control group in *Salmonella* challenged mice (Rokana *et al.* 2016). It also noted from previous studies (Ulluwishewa *et al.* 2011) that epithelial resistance has been enhanced by activation of TLR-2 using probiotic bacteria and their fermented biogenic components. The activation of TLR-2 receptor was observed by intact and mildly hydrolysed whey proteins only and not by casein hydrolysates in TLR reporter cell lines (Kiewiet *et al.* 2017). Rakoff-Nahoum and Comstock, (2014) indicated that elimination of TLR-2 increased the susceptibility to DSS-induced colitis as, the severe mortality was seen in MyD88-

/- animals as the result of defective signaling of multiple TLRs induced by various commensal-derived products. TLR-2-deficient animals have also been shown to develop more severe colitis after DSS administration than their wild-type counterparts as a result of a dysregulated interaction with intestinal microflora and altered epithelial permeability characteristics (Albert *et al.* 2011).

2.2.5.2 TLR-4 receptor

TLR-4 has been shown to be involved in defense against pathogens as well as establishing commensal colonization and maintaining tolerance to commensal bacteria.

TLR-4 expression and sensitivity to its ligand and lipopolysaccharide (LPS), are increased in the setting of intestinal injury associated with crohn disease and ulcerative colitis and the presence of different proinflammatory cytokines (IFN-γ and TNF-α). Lee *et al.* (2009) displayed that probiotic strain *L. suntoryeus* inhibits expression of inflammatory mediator such as Cox-2, TNF-α, IL-1β and IL-6 through down-regulation of TLR-4 pathways in 2,4,6-trinitrobenzene sulfonic acid-induced colitis mice. The two probiotic strains (*Lactobacillus and Bifiidobacterium*) protected against colonic inflammation by decreasing inflammatory factor TNF-α via TLR-4 signaling inhibition in colonic tissue of colitis mice (Yang *et al.* 2013). Upon epithelium disruption, TLR-4 activation stimulates inflammatory cytokine and chemokine expression with recruitment of innate and adaptive immune cells to restrict the bacterial invasion. For instance, increased TLR-4 signaling leads to enhanced IL-6 and IL-12 expression and neutrophil recruitment, in enteric *Toxoplasma gondii* infection and in dextran-sodium sulfate (DSS) colitis. Chiu *et al.* (2013) revealed that lactobacilli bacteria can generate tolerance against LPS induced TLR-4 pathways through induction of anti-inflammatory cytokine (IL-10) and other factors like PPAR-α regulated by NOD pathways in HepG2 cells. The intestinal flora has been shown to be affected by the TLR-4 signaling. On the other hand, regulation of the microbiota by TLR-4 appears to be attributable to alterations in gastrointestinal motility, which may assist in clearance of pathogens and maintenance of commensal populations, goblet cells differentiation and antimicrobial peptides expression. The above findings demonstrates that defective TLR-4 signaling may cause a shift in intestinal microbiota and

activation of inflammatory signaling pathways that leads to the development of intestinal diseases.

2.3. Cytokines release by intestinal epithelial cell

The intestinal epithelial cells are the first line of interaction with microorganisms (Galdeano and Perdigon, 2006). The activation of these cells leads to production of various immunomodulatory molecules such as cytokines. Probiotic lactobacilli can elicit innate and adaptive immune response in host via binding to pattern recognition receptor (PRR) including TLR and NOD expressed on immune cells and intestinal epithelium (Wells, 2011). The immunomodulatory activity of cell free supernatant derived from *L. helveticus* LH-2-fermented milk was assessed in murine macrophages (RAW 264.7 cell line). The results suggested that IL-6, TNF-α and IL-1β production was significantly higher in macrophages that were stimulated with cell-free supernatant in comparison with LPS-stimulated macrophages (Tellez *et al.* 2010). Martinez *et al.* (2015) suggested that ingestion of probiotic products by children may result in improved innate immunity through a significant increase in natural killer cells and other specific immune factors that may improve their health status. The interaction between probiotic strains and enterocytes is important for the controlled production of cytokines and chemokines secreted by epithelial cells in order to modulate immune response. Probiotic organisms can modulate the *in-vitro* expression of pro and anti-inflammatory molecules in a strain-dependent manner. *L. paracasei* CBA L74 fermented milk preparations for infant formula had a strong anti-inflammatory activity *in vitro* and protects against colitis or enteric pathogens *in-vivo*. So, *L. paracasei* CBA L74 fermented formula can provide immune benefits to formula-fed infants and also play a major role in intestinal epithelial barrier integrity (Zagato *et al.* 2014). Traditionally fermented mare's milk koumiss samples and isolated strain of *L. helveticus,* NS8 showed immunomodulatory effects and enhanced the intestinal epithelial barrier integrity *in-vivo* and *ex-vivo* conditions. Furthermore, NS8 was also able to diminish the proinflammatory cytokine of lipopolysaccharide (LPS) in mouse macrophage cell line RAW264.7 by inducing higher level of IL-10 (Rong *et al.* 2015). The administration of probiotic fermented milk as a dietary supplement during the re-nutrition process in a murine immunodeficiency model by malnutrition found to

be a good adjuvant diet to improve the gut and systemic immune response for the protection against *Salmonella* infection (Galdeano *et al.* 2011). Feeding of probiotic dahi for four months increased the phagocytosis and cytokine production in macrophages and enhanced *in-vitro* proliferative capacity of lymphocytes (Kaushal and Kansal, 2011). Meyer, (2007) evaluated the effects of daily consumption over 4 weeks of a commercial fermented dairy beverage on cytokine production in the peripheral blood of healthy young women. The results showed that the consumption of the fermented product increased the production of proinflammatory cytokines, IL-1β and TNF-α in addition to IFN-γ.

2.3.1 IgA

The specific immune response involves the presence of lymphocytes (T and B cells), which provides a protective mechanism against a possible re-infection. The cells of the innate immune system play a crucial part in the initiation and subsequent direction of the adaptive immune responses. In this regard, IgA production by B cells, which helps to maintain intestinal humoral immunity by binding to antigens by limiting their access to the epithelium (Delcenserie *et al.* 2008). Consumption of kefir, a stirred beverage made from milk fermented with a complex mixture of bacteria including lactobacilli and lactococci, has been reported to increase levels of serum anti-cholera toxin IgA antibody and IgA secreting cells in gut associated lymphoid tissue of old rats (Thoreux and Schmucker, 2001). Solids (including bacteria) and liquid supernatant obtained from kefir were reportedly able to induce cytokine production *in vivo* by peritoneal macrophages and adherent cells isolated from Peyer's patches (Vinderola *et al.* 2006 a). Furthermore, kefir consumption not only increased the phagocytic activity and modulated the immune response in mice, but it also increased the IgA level in the small intestine and the production of cytokine IL-4, IL-10 and IL-6 (Vinderola *et al.* 2006 b). Sharma *et al.* (2014) reported the remarkable increase in pathogen-specific IgA and IgG1 antibodies by *Lactobacillus rhamnosus* fermented milk which attenuated the spreading of *E. coli* either by presenting them to complement system and immune cells for destruction.

CHAPTER –3

Materials & Methods

3.1 Materials

3.1.1 Chemicals

Dextran sodium sulphate (DSS), RNA later (Sigma), FITC-Dextran (fluorescein isothiocyanate-dextran), formalin, RPMI (Roswell Park Memorial Institute Medium), antibiotics (penicillin, streptomycin, ampicillin), sodium bicarbonate, dimethyl sulfoxide (DMSO), bovine serum albumin, diethylpyrocarbonate (DEPC), Tri Reagent, chloroform, 2-propanol, ethidium bromide (EtBr), agarose, primers, DAPI (4',6-diamino-2-phenylindole), anti-ACTB (β-actin), HRP-conjugated goat anti-rabbit IgG, X-ray film (Kodak) and X-ray developer & fixer were procured from Sigma-Aldrich, St Louis, USA. De Man, Rogosa and Sharpe agar (MRS Agar), MRS Broth, Gram's stain, and nigrosin stain were purchased from Hi-media Pvt. Ltd, Mumbai, India. Fetal bovine serum (FBS), nuclease-free water, gel loading dye (6X), cDNA synthesis kit and Maxima Sybr qPCR master mix (2X) with ROX were supplied by Thermo Scientific, USA. Cytoplasmic and nuclear protein extracting reagents were purchased from Infobio Pvt. Ltd, New Delhi, India and polyvinylidene difluoride (PVDF) membrane was procured from Millipore, MA, USA. Protein molecular weight markers and Clarity TM Western ECL Substrate were obtained from Bio-Rad Pvt. Ltd. California, USA Qualitative filter papers (Grade-1) and chromatography paper (3 mm) were procured from Whatman Asia Pacific Pvt. Ltd., Mumbai, India. All other chemicals used in this study were of analytical grade and purchased from Sisco research laboratories (SRL) Pvt. Ltd., Mumbai, India.

3.1.2 Plastic ware

For *in vitro* experiments, cell culture flask (25 cm^2), plates and plastic dishes were purchased from Nest biotechnology Pvt Ltd., China. Other plastic wares were obtained from Tarsons Products Pvt. Ltd., Kolkata, India, Genetix Asia Pvt. Ltd., New Delhi, India, Thermo Fisher Scientific Pvt. Ltd., Massachusetts, USA.

3.2 Methods

3.2.1 Bacterial strain and culture condition

Two potential probiotics *Lactobacillus rhamnosus* (MTCC: 5897) and *Lactobacillus fermentum* (MTCC: 5898) were previously isolated from household curd and 10-month-old infant feces respectively in our laboratory. Both cultures were already characterized for their probiotic attributes such as bile tolerance, acid tolerance and adherence to intestinal epithelial cells previously and also identified by 16 S RNA sequencing (Sharma *et al.* 2014). The cultures were maintained in freeze-dried form and as well as in MRS broth supplemented with 20% glycerol at -80°C. For experiments, cultures were activated in MRS broth with 1% inoculum from stock and further sub-cultured twice in MRS broth for 18 hr at 37°C. Cultures were stored at 4°C between the transfers. Microscopic morphological identification and purity of lactobacilli culture was checked every time before the initiation of experiment by Gram staining.

3.2.2 Purity checking of bacterial strains

Gram Staining and Nigrosin Staining were used to confirm the morphology of probiotic culture.

a. Gram's staining

Initially, the bacterial smear was prepared by a loop of full bacterial suspension and then they were fixed by mild heating. Bacterial smear was primary stained with crystal violet (2%) for 1 min and the extra strain was removed with water and Gram iodine as a mordant which was applied for 1 min to trap the violet colour. After washing, decolourizer acetone or 95% ethyl alcohol was applied to extract violet colour complex. In last, the bacterial smear was stained with safranin for one minute. Then slides were air-dried and visualized in oil immersion under the microscope at 100X.

b. Nigrosin staining

In nigrosin or acid staining, one loop of bacterial culture and Nigrosin strain was used to make a smear. The slide was air-dried and visualized in oil immersion under the microscope.

3.3 Preparation of sweet whey and its fermentation

Buffalo milk was obtained from experimental dairy, National Dairy Research Institute, Karnal. The milk was pasteurized at 72°C with no holding and cooled to 30°C and then enzyme rennet (1mg/100L) was added to the milk. Continuous and consistent stirring was carried out till clear whey separated out. When coagulation was completed, stirring was stopped and curd was allowed to settle down for 5 min. The whey was then drained out into a clean sterile vessel using a double lined muslin cloth. The whey so obtained was kept under refrigeration temperature till use. Before fermentation, whey was reconstituted with 4% whey protein concentrate (Davisco, foods International. Inc.) followed by pasteurization at 80°C for 30 min (Pescuma *et al.* 2008). Probiotic fermented whey (PFW) was prepared by inoculating above supplemented whey with 2% of activated bacterial culture (LR: MTCC 5897 and LF: MTCC 5898) respectively followed by incubation for 12 hr at 37°C to obtain a final bacterial count of 1×10^9 colony-forming units (cfu/ml). Probiotic fermented whey supernatant (LRWS and LFWS) of respective lactobacilli was also separated by centrifugation at 7000 rpm for 15 min at 4°C. The number of bacteria in the PFW was determined by plate counting on MRS agar plates after aerobic incubation at 37°C for 12 hr.

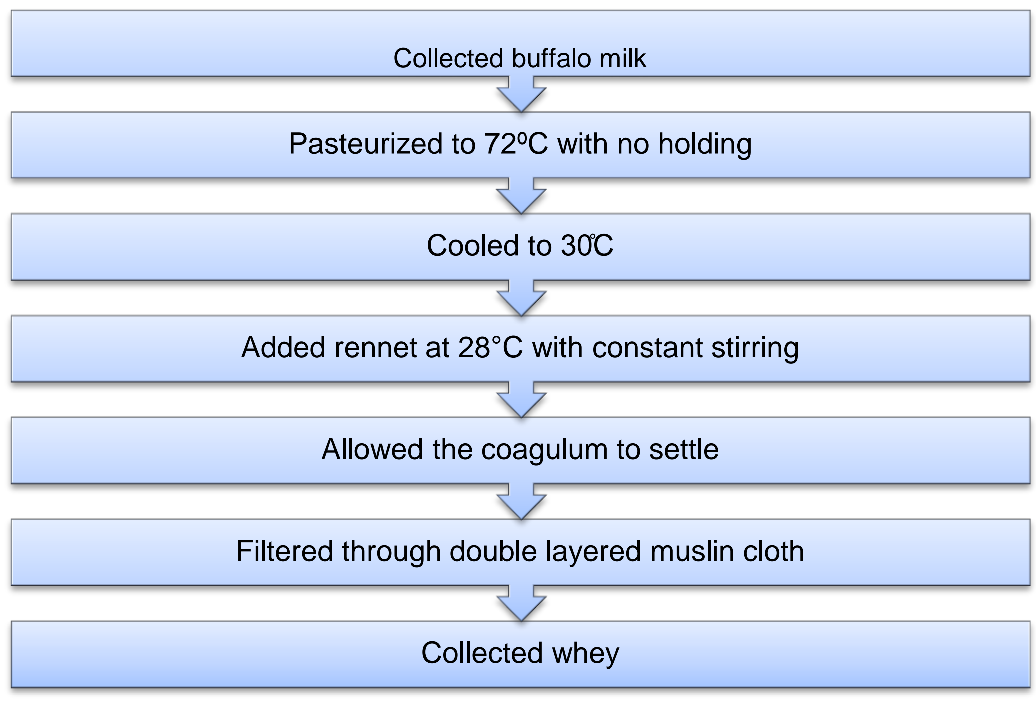

Figure 3.1: Flow chart for the preparation of whey

3.4 Chemical analysis of probiotic fermented whey

3.4.1 pH and Titarable acidity

The pH measurement was done through pH meter (Systronics, pH meter 361) and titarable acidity of sample was determined by the method recommended by the AOAC (1990).

3.4.2 Lactose content

Lactose content in the product was determined by Lane Eynon method as described in IS: SP part –IX (1981).

3.4.3 Total nitrogen content measurement

Reagents

Kjeldahl catalyst mixture: Dissolved 3.5 g of K_2SO_4 (A.R. grade) and 0.105 g $CuSO_4$ in distilled water (1litre).

Sulphuric acid (98%)

NaOH (40%): Dissolved in 40 g of NaOH in 100 ml of distilled water.

NaOH (15%): Dissolved in 15 g of NaOH in 100 ml of distilled water.

Boric acid (2%): Dissolved in 20 g of Boric acid in 1000 ml of distilled water.

Indicator solutions: Dissolve 0.25 g of methylene blue and 0.375 g of methylene red in 300 ml of 95% ethanol.

Procedure

Total nitrogen in fermented whey samples was estimated by micro Kjeldahl method. A weighed quantity of the sample (about 5 ml) was digested in digestion tubes with 20-30 ml concentrated H_2SO_4 in the presence of small quantity (2-3 g) of digestion mixture (sodium sulphate and copper sulphate in ratio of 10:1); till the solution became colourless. After digestion, the contents were cooled and volume was made to 100 ml. 10 ml of aliquot was distilled in Kjeldahl distillation apparatus (KELPLUS Nitrogen Analyzer) after adding 10-15 ml of 40% NaOH solution to make the content alkaline. About 60-75 ml of distillate (light green colour) was collected into an Erlenmeyer flask containing 10 ml of 2% boric acid solution with mixed Tashiro's indicator (10 ml 0.2%

bromocresol green and 20 ml 0.1% methyl red indicator). The distillate was then titrated against standard N/100 H_2SO_4 solution and the end point was recorded when the colour changed to slight pinkish. Volume of N/100 H_2SO_4 solution used in titration was recorded. The crude protein content in the fermented whey sample was calculated by multiplying the nitrogen content with the factor 6.25.

3.4.4 Quantification of protein hydrolysis

The extent of hydrolysis of fermented whey proteins were achieved in terms of number of free N-terminal amino acids. It was determined with OPA (O–Phthaldialdehyde) method as per Nielsen and Jensen, (2001) with slight modification.

Reagents

Borax (0.1 M): To prepare 100 ml of borax solution, 3.81 g of sodium tetra borate was dissolved in distilled water and the volume was made to 100 ml and stored at room temperature.

SDS Solution (20%): Dissolved 20 g of SDS in distilled water, volume made to 100 ml and stored at room temperature.

O–Phthaldialdehyde (OPA) solution: Dissolved 400 mg of O–Phthaldialdehyde in 10 ml of methanol and stored at 4°C in dark.

OPA working reagent: The OPA working reagent was made by mixing of following reagents, as shown in table-3.1 and diluting to a final volume to 50 ml with distilled water. The OPA reagent was always prepared fresh before use and kept in dark. OPA working reagent was prepared by mixing the following chemicals as shown by table-3.1

Table: 3.1 OPA working reagent

Sl. No	Chemicals	Volume (ml)
1	0.1M Borax	25
2	20% SDS	2.5
3	OPA solution	1.0
4	β-Mercaptoethanol	0.1

L–Leucine standard solution (1 mg/ml): Dissolved 10 mg of L-Leucine in distilled water and final volume was made upto 10 ml in volumetric flask.

Procedure

Different volumes of L-Leucine standard solutions containing (15, 22.5, 30, 37.5, 45, 52.5, 60, 67.5, 75 µg of Leucine) were taken in glass test tubes. To it 3 ml of OPA was mixed and hold for 2 min in dark. For blank and samples 10 µl of each distilled water and hydrolysates were mixed with 3 ml of OPA reagent respectively. The absorbance was read exactly after 2 min. L-Leucine was used as positive control and distilled water as a negative control. The linearity of the assay was assessed by standard curve prepared by plotting amount of L–Leucine against absorbance.

3.5 *In-vivo* study

3.5.1 Animals

Male albino weanling mice (4 weeks old) weighing 16-19 g were obtained from the small animal house of ICAR-National Dairy Research Institute, Karnal, Haryana, India, to conduct the experiments. Prior to the experiment, all animals were acclimatized in polypropylene cages under controlled conditions of temperature (24 ± 1 °C), humidity (55 ± 5%), and light (12 hr light/dark) for a week and given semi-synthetic basal diet and water *ad libitum*. Care and handling procedure for animals were approved by the Institutional Animal Ethics Committee (ICAR-NDRI: Approval no. 41-IAEC-18-60; dated: January 27, 2018).

3.5.2 Composition of basal diet

Weanling mice were fed with semi-synthetic basal diet (BD) and water *ad libitum*. The proximate composition of semi-synthetic basal diet is shown in table 3.2 having 11·5 % starch, 70 % bengal gram (black chickpea) flour, 6·5 % soyabean oil, 2·1 % mineral mixture, 1 % vitamin mixture and 8.9 % cellulose.

Salt mixture (AOAC, 2005) required for 10 kg diet (500 g) contained $CaCO_3$, 180.7 g; $CoCl_2.6H_2O$, 0.0115; $CuSO_4.5H_2O$, 0.238 g; $FeSO_4.7H_2O$, 13.5 g; KH_2PO_4, 194.5; KI, 0.04 g; $MgSO_4.7H_2O$, 49.09 g; $MnSO_4.H_2O$, 2.005 g; NaCl, 59.65 g; and $ZnSO_4,7H_2O$, 0.274 g. Vitamin mixture (100 g) comprised of biotin, 4 mg; folic acid, 20 mg; vitamin B_{12}, 0.3 mg; menadione, 50 mg; para aminobenzoic acid, 1 g; meso-inositol, 1 g; thiamine, 50 mg; riboflavin, 80 mg;

pyridoxine, 50 mg; calcium pentothenate, 0.4 g and starch, 97.34 g. Vitamin A (2×10^5 IU), vitamin E (10^3 IU) and vitamin D (2×10^4 IU) were incorporated in diet through oil/ fat.

Table: 3.2 Proximate composition of semi-synthetic basal diet

S.No.	Particulars	Percentage (%)
1	Crude Protein	12.55
2	Crude Fiber	6.77
3	Oil	7.12
4	Carbohydrates	67.98
5	Acid Insoluble Ash	0.11
6	Moisture	5.58

3.5.3 Experimental design and grouping of animals

A. Non-colitis groups

Under this experimental study animals were randomly distributed into six groups with each group containing eight animals (Fig. 3.2). Non-colitis negative control group (1: NC), mice were fed with dough prepared from the basal diet (5g/animal/day) in water and supplied with drinking water *adlibitum*. Whey fed vehicle control group (2: WC) received dough prepared with sweet whey (@ 2 ml whey/animal/day). Probiotic fermented whey (PFW) groups (3: LRFW and 4: LFFW) received dough prepared with respective lactobacilli fermented whey (@ 2ml/animal/day having 2.0×10^9 cfu). Similarly, dough prepared with bacterial free fermented whey (5: LRWS and 6: LFWS) of respective probiotic strains (*L. rhamnosus* and *L. fermentum*) was fed to animal groups.

B. DSS induced colitis model

Under this study animals were randomly distributed into six groups having eight animals in each group (Fig. 3.2). Among the six groups: Colitis induced control group (1: DSS) included mice fed with basal diet but drinking water was replaced with 5% DSS (DSS, MW-500, Sisco Laboratories, Pvt. Ltd, India) from 23rd day onwards. Whey fed second control group (2: W+DSS) in which colitis was induced, mice were fed with dough prepared from BD and sweet whey (@

2ml/mice/day) instead of water to obtain consistency and administered 5% DSS *adlibitum* from 23rd day onwards. In experimental groups, dough supplemented with sweet whey was replaced with dough prepared with probiotic fermented whey (@ 2.0×10^{9} cfu/mice/day) containing respective *lactobacilli* (3: LRFW+DSS and 4: LFFW+DSS). Similarly, remaining two groups of animals (5: LRWS+DSS; 6: LFWS+DSS) were fed with dough prepared with respective fermented whey supernatants (@ 2 ml/mice/day). Clinical signs (diarrhea, hemoccult) and body weight (BW) of all the animals were recorded daily during the colitis induction period. At the end of experiment (29th day) animals were sacrificed by cervical dislocation in order to assess the preventive effect of fermented whey and its supernatant on DSS-induced colitic mice in contrast to healthy control and whey treated animals.

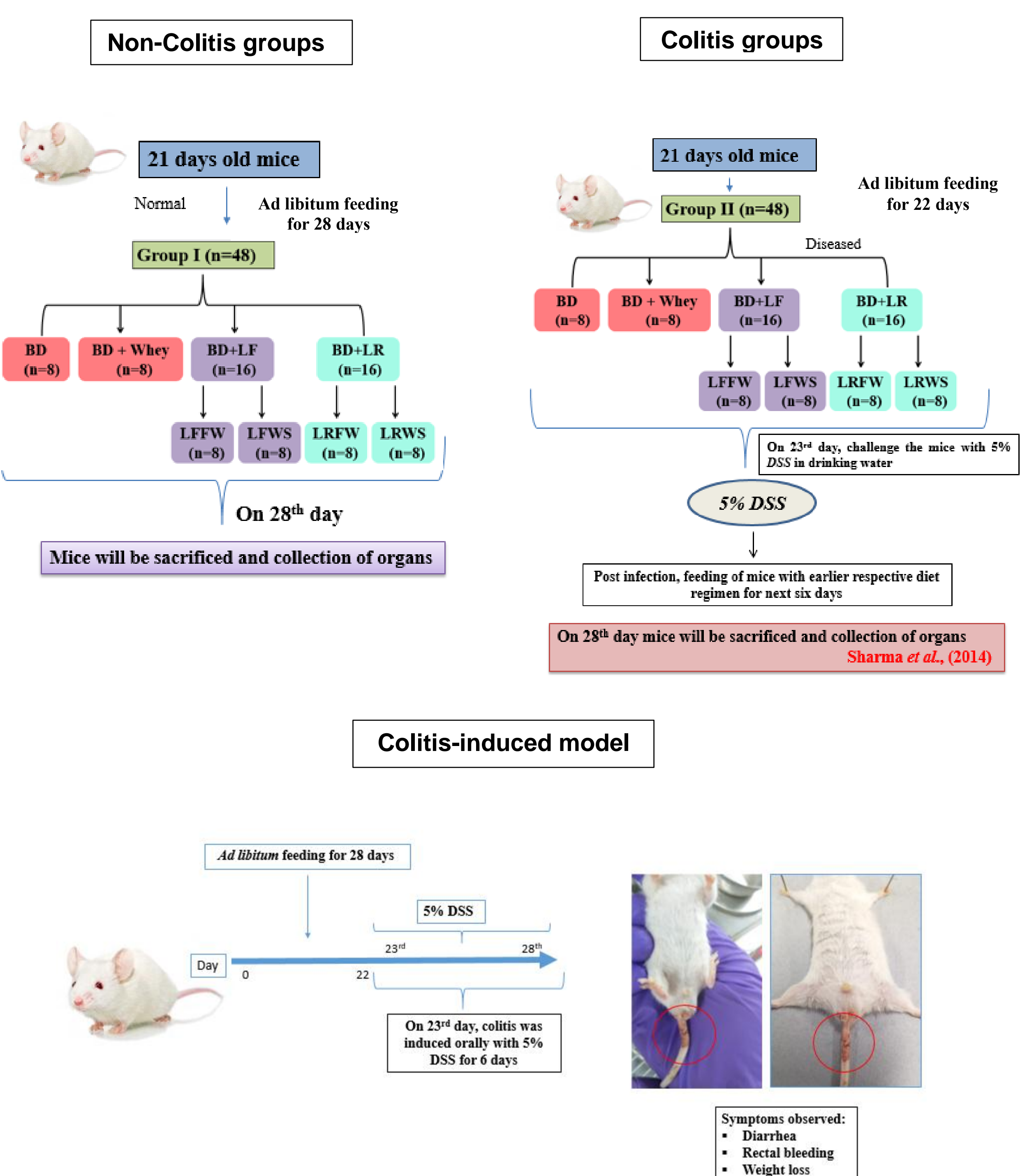

Figure 3.2: Experimental design for probiotic fermented whey consumption during colitis induction with dextran sulfate sodium (DSS) in mice for prophylactic evaluation

3.5.4 Collection of tissue sample

At the end of feeding period, before sacrifice of the animals fasting blood glucose was estimated from tail vain by glucometer (AccuSure ™ Soul, New Delhi, India). After that animals were euthanized with diethyl ether over dose and tissue samples were aseptically collected for further analysis. The intestine of experimental mice were carefully removed from gastrodeudenal to ileocaecal junction and measured their weight and length. Its contents were flushed out with 2.5 ml phosphate buffer saline (PBS, pH 7.4) and simultaneously teased with sterile needle. Subsequently it was centrifuged at 2000 g for 30 min to collect the supernatant which was stored at -80°C until analysed. Afterwards, one part of intestinal tissues was fixed in formalin for hematoxylin & eosin (H & E) staining, and immunohistochemistry. Other part of intestinal sample was stored at -20°C for conducting RT-qPCR and determining MPO activity. Blood samples of experimental animals were collected by cardiac puncture to carry out hematological assessment and determination of intestinal damage by fluorescein isothiocyanate (FITC)-dextran permeability test.

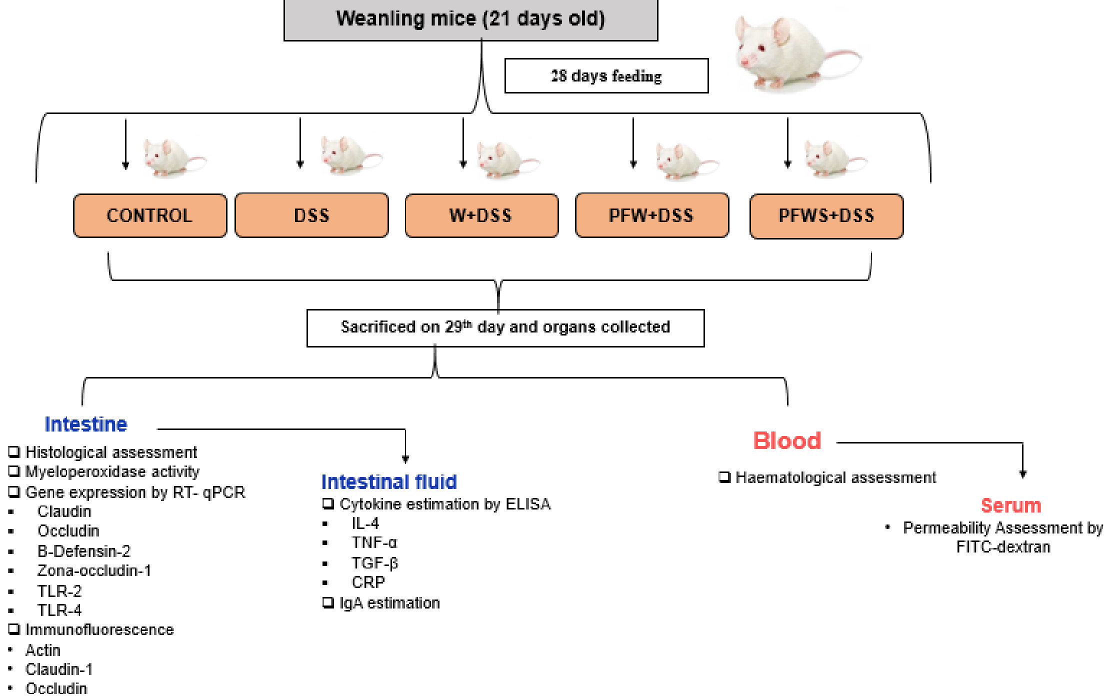

Figure 3.3: Overview of sample collection and parameters analysed under objective I

3.5.5 Assessment of disease activity index (DAI)

Intestinal disease activity was assessed based on weight loss, stool consistency, and gross rectal bleeding scored daily for 6 days during induction of colitis. The overall disease severity was assessed with a clinical scoring system: stool consistency (0, normal; 2, loose stool; 4, watery diarrhea); bloody stools (0, normal; 2, slight bleeding; 4, gross bleeding); and body weight loss (0, none; 1, decreased 1%-5%; 2, decreased 5-10%; 3, decreased 11%-15%; 4, decreased >15%) (Cooper *et al.* 1993). These values were assessed for each animal, and the sum of the 3 values constituted the DAI.

3.6 Evaluation of general health status

3.6.1 Body weight and organ indices

After acclimatization period all the animals attained the weight approximately 19-20 g. Successively, weight of each mouse was recorded on daily basis. After that average weight of all mice in respective groups was compared. On conclusion of experiment, animals were sacrificed and organs (Spleen/Liver/Kidney) were collected to determine their weight index which was calculated by dividing organ weight by total body weight.

$$\textbf{Organ index} = \frac{\textbf{Weight of organ}}{\textbf{Total body weight}}$$

3.6.2 Hematological analysis of blood

NH sodium Heparin VACUETTE® (Greiner bio-one) tubes coated with spray dried lithium, ammonium or sodium heparin were used for collection of blood. The hematological analysis of blood was carried out with automated blood analyzer machine (MS4Se-Melet Schloesing Laboratories India) for determination of Hb, RBC count, WBC count, MCV, MCHC, MCH.

Fasting blood glucose level was also measured just before sacrificing the animals after overnight fasting by procuring blood from the tail of the animals. AccuSure™ soul (Microgene Diagnostic Systems Pvt. Ltd.) blood Glucose Monitoring system was used to measure the blood glucose level.

3.7 Examination of intestinal health

3.7.1 Histology of intestine

Colon samples of 1cm were fixed in buffered formalin at room temperature. The fixed tissue was then embedded in paraffin and the 3µm sections of the tissues were cut with Senior Rotary Microtome (Radical, RMT-30, Ambala, India). The slides having tissue sections were deparaffinized by heating on burner flame and then were placed in xylene solution for 3-4 minutes. Then the slides were hydrated by passing them through the decreasing alcohol concentrations. After proper hydration of tissue sections, slides were stained with hematoxylin solution for 3-5 minutes and then washed in running tap water. Later, slides were dipped in and out of 0.5% hydrochloric acid and washed with tap water for 1-2 minutes followed by their immersion in ammonia water and rinsed in 95% alcohol. Finally, slides were dipped in eosin dye for 30-60 seconds. Then the slides were dehydrated by dipping in increasing grades of alcohol from 75 to 95% by giving soak time of 60 seconds in each alcohol gradient. Then slides were cleared by dipping them in xylene for one minute and slides mounted on DPX with a coverslip. Histological scores were assigned (Suwal *et al.* 2018) for the destruction of the crypt structure (0-4: basal one-third damage, basal two-third damage, only surface epithelium intact, entire crypt, and entire epithelium lost), the depth of the lesions (0-3: none, mucosal, submucosal, transmural) and the degree of inflammatory cell infiltration (0-3: none, slight, moderate, severe). Sum of the above scores (destruction in the crypt structure, depth of the lesions, and degree of inflammatory cell infiltration) of each mouse was recorded as the histopathological damage score.

3.7.2 Immune responses in the gastrointestinal tract

A. Myeloperoxidase (MPO) activity

MPO is a critical enzyme in generation of lethal free radicals, characteristic of neutrophil invovlved in pathogen evading machinery.

Reagents

Potassium phosphate Buffer (PPB, 50mM, pH, 6.0): PPB was made by adjusting pH of 50mM KH_2PO_4 with 50 mM K_2HPO_4 to 6.0.

Cetyl trimethyl ammonium bromide (CTAB, 0.5%)

O-dianisidine dihydrocholride (ODD, 0.167 mg/ml)

Hydrogen Peroxide (H_2O_2, 1%): Concentration of H_2O_2 was determined spectrophotometrically by using a fixed volume of H_2O_2 in phosphate buffer. The absorbance was recorded at 240 nm and the concentration of H_2O_2 was calculated with Beer-Lambert law using 43.6 M^{-1} cm^{-1} as the molar extinction coefficient H_2O_2.

Procedure

MPO activity was measured according to the method described by Rodriguez-Palacios *et al*. (2015). Briefly, the colon tissue (50 mg/mL) was suspended in potassium phosphate buffer (50 mM, pH 6.0) having 0.5% CTAB and sonicated for 10sec followed by freezing thawing repeatedly for 3 cycles. Later tissue suspension was centrifuged at 12,500 rpm at 4ºC for 5 min. MPO activity in supernatant (0.1 ml) was measured by incubating it at 28°C with 0.5% of o-dianisidine dihydrochloride and 1% H_2O_2 as substrates at 460 nm (UV-visible double beam spectrophotometer) in every 30 sec interval for 5 min. One unit of enzyme is defined as that amount which converted 1μM of substrate into product/mg of tissue/min considering extinction coefficient of o-dianisidine as 1.13 x 10^4/M.cm at 28°C.

B. Measurement of IgA in intestinal fluid

Total IgA was estimated by sandwich ELISA kit procured from Koma biotech, Seoul, Korea.

Reagents

50 mM Bicarbonate buffer (pH 9.6): Na_2CO_3 (530 mg) and $NaHCO_3$ (420 mg) were dissolved in 100 ml water each and pH of $NaHCO_3$ was adjusted with Na_2CO_3 to 9.6.

Coating antibody: Coating antibody was prepared by diluting 1:100 in coating buffer

IgA standard: Reconstitute 1 vial of standard protein in 0.325 ml sterile water to a concentration of 10,000 ng/ml. Then dilute in assay diluent at 1:2 serial dilutions from concentration 125 ng/ml to 1.95 ng/ml.

Detection antibody: Detection antibody was prepared by diluting 1:4000 in assay diluent

Phosphate-Buffered Saline (PBS) (10 mM, pH 7.4).

Assay diluent: 1% Bovine serum albumin in PBS (prepared fresh).

Wash Buffer: PBST: 0.05% (v/v) Tween 20 in PBS.

Blocking Buffer: 1% Bovine serum albumin in PBS (Prepared fresh).

TMB substrate: TMB solution: substrate solution (1:2).

Stop solution (2N H_2SO_4): Prepared by slowly adding 5.61 ml of stock H_2SO_4 to 94.36 ml of distilled water.

Procedure

Total IgA was estimated in intestinal fluid by sandwich quantitative ELISA kit procured from Koma biotech, Seoul, Korea. Briefly, the wells of 96 well plates (Hi-media, Mumbai, India) were coated overnight (4°C) with 100 µl of coating IgA antibody (diluted 1:100 in bicarbonate buffer). Plates were washed three times with wash buffer (PBS/T) and free binding sites were blocked with 200 µl of blocking buffer at room temperature for 1 hr. After washing four times with PBS/T, 100 µl of serially diluted standard IgA (1.9-125 ng/ml) was added in duplicate to appropriate wells. The intestinal fluid samples (1:1000 times diluted) were also added to the respective wells and incubated at room temperature for 1 h. Thereafter, plates were washed again with PBS/T thrice, followed by the addition of 100 µl of horseradish peroxidase (HRP) conjugated anti-mouse IgA detection antibody (1:10000). Then plates were incubated at 37°C for 1 h and washed four times to remove unbound antibodies. Lastly, 100 µl TMB (3,3,5,5-tetramethyl diamine benzidine containing 0.03% H_2O_2), was added to the plate and incubated for 15-30 min at room temperature in dark to develop color. The reaction was stopped with 2N H_2SO_4 (50 µl/well). Plates were read at 450 nm on microplate reader (BioTek Instruments, Winooski, Vermont, USA).

C. C-reactive protein (CRP) level in intestinal fluid

CRP is a classic circulatory marker of inflammatory. The plasma level of this protein was estimated by using pre-antigen coated ELISA kit (Boster

Immunoleader, Pleasanton, California, USA) in a sandwich ELISA format according to manufacturer's protocol.

Reagents

Standard Diluent (1X)

Lyophilized standard: Serially diluted in standard diluent (1X) over a range of concentration (312.5-2000 pg/ml)

Anti-Mouse CRP Pre-coated 96-well strip microplate

Assay Diluent (1X)

Mouse CRP Biotinylated antibody (100x): Diluted the stock concentration 100 times in assay diluent to prepare working concentration (1X)

Avidin-Biotin-Peroxidase Complex (100x): Diluted the stock concentration 100 times in assay diluent (1X) to prepare working concentration (1X)

Wash solution (1X)

TMB substrate (1X)

Stop solution (1X)

Procedure

The sealed pre-coated CRP (Boster Immunoleader, Pleasanton, California, USA) was carefully opened and allowed to attain room temperature. Serially diluted standard protein was then coated over appropriate wells (100 µl/well) while wells with standard diluent only served as control. Intestinal fluid samples (undiluted: 100 µl/well) were added in appropriate wells and plate was sealed with plate sealer and incubated for 2 h at 37°C. Then plate was washed thrice with wash solution to remove unbound protein. Subsequently, 100 µl of 1X Biotinylated Anti-Mouse CRP antibody was added and incubated at 37°C for 90 min. After incubation, the wells were aspirated and rinsed with wash solution thrice (1 min) and snapped on blotting paper to remove any residual buffer. Later, 100 µl of Avidin-Biotin-Peroxidase complex was added in each well and incubated for 30 min at 37°C. The wells were again washed five times with wash solution and 90 µl of TMB was added to each well and incubated for 30 min at room temperature. Finally, the reaction was stopped with 100 µl of stop solution.

After that plates were read at microplate reader (BioTek Instruments, Winooski, Vermont, USA) at 450 nm.

D. Measurement of Interleukins in intestinal fluid

Immunomodulatory effect of probiotic fermented whey in experimental animals was determined by measuring the secretions of interleukins such as Tumor necrosis factor-alpha (TNF-α), Interleukin-4 (IL-4) and Tumor Growth Factor-βeta (TGF-β).

Reagents

Coating Buffer (10X): Coating buffer was diluted to 1X with deionized water.

Capture Antibody (250X): Respective anti-mouse capture antibodies specific to TNF-α, IL-4 and TGF-β were diluted to 1X in coating buffer.

Phosphate-Buffered Saline (PBS) (10 mM, pH 7.4)

Wash buffer (PBS/T): 0.05% (V/V) Tween 20 in PBS

Assay Diluent: Assay Diluent A (5X) was diluted to 1X in PBS.

Standard Reconstitution: The lyophilized standards were reconstituted by adding 0.2 ml of 1X assay diluent to make a final standard stock solution. Working concentrations of standard proteins for TNF-α (7.8 to 500 pg/ml), IL-4 (7.8 to 500 pg/ml), TGF-β (8 to 1000 pg/ml) were prepared in assay diluent by serial dilution.

Detection Antibody (250X): Biotin conjugated anti-mouse TNF-α, IL-4 and TGF-β, were diluted to 1X in assay diluent.

Detection Enzyme (250X): Avidin-HRP conjugate was diluted to 1X with respective assay diluents.

Substrate Solution: 1X TMB Substrate.

Stop Solution (2N H_2SO_4): Prepared by slowly adding 5.61 ml of stock H_2SO_4 to 94.39 ml of distilled water.

Procedure

The release of inflammatory mediators from intestinal epithelial cells was detected in intestinal fluid by sandwich ELISA following manufacturer's instruction. Briefly, 96-well plates were coated with 100 μl of capture

antibody/well and plates were incubated at 4°C overnight. The next day, unbound capture antibodies were aspirated and plates were washed with wash buffer 3-4 times. Afterward, free binding sites of wells were coated with 250 µl of blocking reagent (assay diluent) and incubated at room temperature for 1 hr. Then plates were aspirated and washed 4 times with wash buffer. Serial dilutions of standards of respective cytokines were made in assay diluent to make the standard curve. Serially diluted standards and undiluted intestinal fluid (100 µl) were added to appropriate wells and incubated at room temperature for 2 hr. Thereafter, the unbound samples were removed by washing thrice with PBST. Subsequently, addition of respective detection antibody (100 µl/well) against TNF-α, IL-4 and TGF-β were added in wells. The plates were again incubated for 1 hr at room temperature. Then wells were aspirated and washed 4 times with PBST. Later, 1X Avidin-HRP conjugated detection enzyme (100 µl/well) was added and plates were incubated for 30 min at room temperature. Subsequently, plates were aspirated and washed 5 times by soaking 30 sec to 1 min. Finally, TMB substrate (100 µl/well) was added and plates were incubated for 15-20 min in dark. The reaction was stopped with 2N H_2SO_4 (100 µl/well) and absorbance was taken at 450 nm.

E. Expression of immune related genes

Gene expression by RT-qPCR

Isolation of total RNA

Total RNA was isolated from the intestinal tissue by the single-step RNA isolation method of Livak and Schmittgen (2001) using TRI^{TM} Reagent**.**

Reagents

Di Ethyl Pyro Carbonate (DEPC, 0.1%): One ml of DEPC was diluted to 1 L with double distilled water and stirred overnight.

RNAse free plastic ware: Plastic wares (tips, microcentrifuge tubes) were made RNase free by soaking in DEPC (0.1%) for 24 hr at room temperature. Then the plastic wares were removed and dried at 37°C followed by autoclaving. The RNase free plastic wares were stored in a clean RNase free working area.

TRI Reagent (Sigma)

DNAse and RNAse free Chloroform

DNAse and RNAse free Iso-propanol

Nuclease free water

Ethanol (75%): Diluted 75 ml of commercially available absolute ethanol to 100 ml with DEPC treated or nuclease-free water.

Procedure

To analyze the mRNA expression, total RNA was extracted by Trizol Reagent (Sigma) from mouse intestinal tissue following manufacturer's instructions. Samples have already been suspended in one ml of Trizol Reagent so 200 µl chloroform was added to the TRI reagent containing tissue homogenate and kept at room temperature for 5 min after vigorous shaking for 15 sec. The milky solution so formed was centrifuged at 12,000 g for 15 min at 4°C. The cell lysate separates into three phases: lower red colour organic phase contains protein and a colourless upper aqueous phase containing RNA with DNA at the interphase. The upper aqueous layer (200 µl) was carefully transferred to an RNase-free fresh microcentrifuge tube without disturbing the lower organic and inter-phase. Chilled isopropanol (500 µl) was added to the separated upper aqueous layer and the contents were mixed gently and kept in ice for 10 min to precipitate the RNA. The sample was centrifuged at 12,000 g for 10 min at 4°C and the supernatant was discarded without disturbing the RNA pellet. The pellet was washed with 1 ml of 75% ethanol by centrifuging at 7500 g for 5 min at 4°C. Later, ethanol was decanted and the tube was left open in a laminar flow hood for about 5 min to partially dry the pellet. The RNA pellet was then dissolved in 20 µl of nuclease-free water.

Assessment of the quantity and quality of RNA

The isolated RNA was checked for its quantity and quality. Total RNA was quantified by microvolume spectrophotometer (Take 3, BioTek Instruments, Vermont, USA) using 2 µl RNA solution against a nuclease-free water blank. The purity of the sample was decided based on the optical density ratio of A260/A280

nm. Samples having ratios ranged between 1.85-1.95 were considered acceptable and hence used for further analysis. The concentration of RNA in these samples were determined using the following formula

Concentration of RNA (ng/µl) = OD at 260 nm x Dilution factor x 40

Evaluation of RNA integrity

The quality of isolated RNA was assessed by 1.5% agarose gel electrophoresis using the protocol of Livak and Schmittgen (2001).

Reagents

Agarose (1.5%): 0.45 g of agarose was added in 30 ml of 1X TBE buffer

Tris Borate EDTA buffer (TBE,10X, pH 8.3): Tris borate EDTA buffer was prepared by dissolving 10 g of Tris base, 0.74 g of EDTA and 7.5 g of boric acid in 60 ml of distilled water and the pH was adjusted to 8.3 with 1.0 N NaOH. The volume was made to 100 ml with distilled water and filtered the solution with the Whatman filter for storage at 4°C. TBE solution was diluted 10 times with distilled water before use.

Ethidium bromide (10 mg/ml): Dissolved 10 mg of ethidium bromide in 1 ml of distilled water and stored at room temperature (RT). The working concentration of 0.5 µg/ml was made by dissolving 1.5 µl ethidium bromide stock solution in 30 ml of 1X TBE buffer.

Gel loading dye (6X): Diluted 1:5 with RNA sample.

Procedure

Agarose (0.45 g) was added to 30 ml of 1X TBE buffer and dissolved by melting the agarose until a clear transparent solution appeared. It was allowed to cool and then 1 µl ethidium bromide (0.5 µg/ml) was added to it. The melted solution was then poured into a casting tray containing a comb (5.2 cm long, 4 mm wide) and was allowed to harden resulting into air bubbles free matrix. The gel solidified within 20-30 min and was ready to use. The gel was submerged in a horizontal electrophoresis tank (Genei, Bangalore) containing 350 ml of 1X TBE buffer. The comb was removed carefully and 5 µl of isolated RNA samples were mixed with 1 µl of 6X DNA loading dye and dispensed carefully into the

wells. Immediately after loading the sample, the system was connected with an electric power supply unit (Atto Model - AE 8750). Electrophoresis was carried out at a constant voltage of 80 V at room temperature. The gel was constantly monitored and the migration was judged by observing the movement of the dye present in the loading buffer. When the dye front reaches the bottom of the gel (~60 minutes), the electric current was switched off. The gel was visualized and imaged using Alpha Imager gel documentation system. The integrity and quality of the sample were checked by the appearance of ribosomal RNA (rRNA) bands with the intensity of the upper band (28S) was about double the intensity of the lower band (18S).

Preparation of cDNA using reverse transcriptase

The cDNA synthesis was carried out using the "RevertAid^{TM} First-strand cDNA synthesis kit".

Reagents

Total isolated RNA

RevertAid^{TM} First-strand cDNA synthesis kit

Procedure

cDNA synthesis was performed by adding total RNA (1 µg), random hexamer (0.5 µg/ µl) and oligo-dT (0.2 µg/ µl) primers to DEPC treated water and incubating at 65°C for 5 min in a thermocycler (GenePro, Bioer). Then the reaction mixture was chilled on ice and spun down. RNAase inhibitor (20 U/µl), reaction buffer, dNTPs (10 mM) and reverse transcriptase (200 U/µl) as shown in table 3.3 were then added to it and mixture was incubated for 10 min at 25°C followed by 42°C for 30 minutes. The reaction was terminated by heating at 95°C for 3 min in a thermocycler. The prepared cDNA was stored at -20°C until further use.

A negative RT control and negative template control reactions were also run to check any contamination of genomic DNA or RNA in reagents respectively. In the negative RT control reaction, all components except RT enzyme were used. On the other hand, reagent contamination for RNA template

was checked by negative template control (NTC) where all reagents except RNA template were added in the reaction mixture. Final confirmation of successful cDNA synthesis was done by checking the amplification product of a housekeeping gene such as GAPDH using endpoint PCR.

Table 3.3. Reaction mixture for cDNA preparation

S. No.	Components of RT Reaction	Volume
1.	RNA	1.0 µg
2.	Random Hexamer (0.5 µg/ µl)	0.5 µl
3.	Oligo dT primer (0.2 µg/ µl)	0.5 µl
4.	dNTP Mix (10 mM)	2.0 µl
5.	RNase Inhibitor (RI) (20 U/µl)	1.0 µl
6.	Reverse Transcriptase (RT) (200 U/µl)	1.0 µl
7.	Reaction buffer (5X)	4.0 µl
8.	Total volume (Made by DEPC treated water)	20 µl

Optimization of conditions for Polymerase chain reaction (PCR)

Primer designing

Primer designing is an important step for the amplification of the desired DNA product. Primers were designed through the NCBI primer designing tool. This tool provided a different combination of target-specific primers based upon sequences present in the NCBI nucleotide database. Primers sequences were selected based on specific properties as mention below:

- Primer length should be between 18-25 base pairs.
- Melting temperatures (Tm) should be the same (55-60°C) for both forward and reverse primers.
- The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.
- GC Clamp: The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3'

end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

- Formation of secondary structure like a hairpin, self-dimer, cross-dimer which all forms due to inter or intra molecular interaction should be avoided.
- Primers with long runs of a single base like AAAAAAA, CCCCC should generally be avoided as they can misprime.

Software for checking primer integrity

Primer stat and BLAST software were used to evaluate potential PCR primers for the expression of specific genes. This software provided a report describing the properties of each primer which included GC content, secondary structure formation (hairpin, self-annealing) and single/dinucleotide run, etc. BLAST searches were performed against other genomes to determine the specificity of the primers. After appropriate evaluation, the primers were custom synthesized from Sigma Pvt. Ltd., India. The primer sequence and predicted product size are given below in table 3.4.

Table: 3.4. Primer sequences for the amplification of genes

Target gene	Primer sequence 5'-3'	Amplicon size (bp)
Tight junctional genes		
GAPDH	F 5'GCCTCGTCCCGTAGACAAAA3' R 5'CAATCTCCACTTTGCCACTGC3'	140
Claudin-1	F 5'CCCTTCAGCAGAGCAAGGTT3' R 5'TAGGGCAACCAAGTGCCTTT3'	123
Occludin	F 5'GACTCGGCTCTCACGGAAAC3' R 5'TAGCTCGGGCATTTCTGGTG3'	124
ZO-1	F 5'CCTAAGACCTGTAACCATCT 3' R 5' CTGATAGATATCTGGCTCCT 3'	82

Immune related genes		
hBD-2	F 5'GCCCTTTCTACCAGCCATGAG3' R 5'GACACAGTACCCTCCATTGGT3'	151
TLR-2	F 5'AAGAGGAAGCCCAAGAAAGC3' R 5'CGATGGAATCGATGATGTTG3'	199
TLR-4	F 5' CTGCCAGAGACATTGCAGGA3' R 5'CTGCCAGAGACATTGCAGGA3'	201

Standardization of annealing temperature for PCR

Polymerase chain reaction (PCR) was standardized using specific primers. The cDNA was amplified using the reaction mixture as shown in table 3.5 by adding cDNA, both forward and reverse primers along with PCR master mix in total volume of 25 µl. cDNA control was made by replacing cDNA with nuclease free water. The reaction mixture was subjected to gradient PCR for standardisation of annealing temperature using the program as shown in table 3.6.The PCR product was assessed in 1.5 % agarose gel electrophoresis along with 100 bp ladder.

Table 3.5. Reaction mixture for PCR

S. No.	Component	Volume	Concentration
1.	cDNA (50 ng/µl)	1 µl	2 ng
2.	Forward Primer (10 µM)	1.0 µl	0.4 µM
3.	Reverse Primer (10 µM)	1.0 µl	0.4 µM
4.	PCR Master mix (2X)	12.5 µl	1 X
5.	Nuclease free water	9.5 µl	Upto 25 µl

Table 3.6. Program of gradient PCR for standardisation of annealing temperature

Stages	Programme Name	Steps	Temperature	Time
Stage 1	Initial denaturation		95°C	5 min
Stage 2	Amplification (35 cycles)	Denaturation	95^{0}C	30 sec
		Annealing	55 ^{0}C- 60^{0}C	30 sec
		Extension	72^{0}C	45 sec
Stage 3	Final extension		72 ^{0}C	5 min
Stage 4	Cooling		4 ^{0}C	Pause/infinite

Relative expression of genes by RT-qPCR

cDNA was subjected to relative quantification by qRT-PCR using specific primers. qRT-PCR was performed using SYBR Green in a total volume of 10 µl reaction as shown in table 3.7. For convenience, q-PCR master mix containing below-listed components except cDNA was prepared for the simultaneous analysis of all samples. The content of the q-PCR mix was dispensed carefully into each well of the PCR plate while keeping on ice. Later, 1 µl of the cDNA sample (25 ng) was then added to the each well and contents were mixed properly and finally PCR plate was placed onto the real-time PCR machine (7500 Fast-Real Time PCR, Applied Biosystems, Foster City, California, USA) to run the q-PCR program as shown in table 3.8.

Table 3.7. Reaction mixture for qRT-PCR

S. No.	Components	Volume	Concentration
1.	cDNA (1:1)	1.0 µl	25 ng
2.	Roche SYBR Green I Master (2X)	5.0 µl	1X
3.	Forward primer	0.5 µl	10 µM
4.	Reverse primer	0.5 µl	10 µM
5.	PCR grade water	3.0 µl	Upto 10.0 µl

Melting curve analysis was performed at a temperature range of 60-95°C. Fluorescence was measured once every cycle after the extension step using filters for SYBR Green (at 492 nm excitation and 530 nm emission).

Analysis of relative target gene expressions

Generation of quantitative data by real-time PCR is based on the number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the Quantification cycle). The relative expression ratio of the target gene was tested for significance as per method given by Pfaffl and Hageleit (2001).

Table 3.8. Reaction program for the qRT-PCR

Stages	Programme Name	Steps	Temperature	Time
Stage 1	Pre-incubation / Holding stage	Step 1	50ºC	20 sec
		Step 2	95°C	10 sec
Stage 2	Amplification (40 cycles)	Denaturation	95ºC	15 sec
		Annealing and Extension	60ºC	45 sec
Stage 3	Melting curve	Step 1	95ºC	15 sec
		Step 2	60ºC	1 min
		Step 3	95ºC	30 Sec
		Step 4	60ºC	15 sec
Stage 4	Cooling		4ºC	Pause/infinite

$$\Delta C_P\ (ES) = C_P\ (HKGS) - C_P\ (TGS)$$

$$\Delta C_P\ (C) = C_P\ (HKGC) - C_P\ (TGC)$$

$$R = (2^{\Delta C_P\ (ES)}) / (2^{\Delta C_P\ (C)})$$

Where,

C_P	–	Crossing point
C_P (TGS)	–	Crossing point of the target gene in the sample
C_P (HKGS)	–	Crossing point of housekeeping gene in the sample
C_P (TGC)	–	Crossing point of the target gene in calibrator
C_P (HKGC)	–	Crossing point of the housekeeping gene in calibrator
ΔC_P	–	Difference between the crossing points
ΔC_P (ES)	–	Difference between the crossing points in the experimental samples
$\Delta C_P^{(C)}$	–	Difference between the crossing points in calibrator
R	–	Relative expression of the target gene

3.7.3 Evaluation of intestinal barrier function

A. Transcriptional expression of epithelial junctional genes

The effect of fermented whey and its supernatant on epithelial barrier function in different animal groups was evaluated by expression of related genes at mRNA level by using RT-qPCR. The expression level of important genes such as Claudin-1, Occludin, Zonula-occludin 1 (ZO-1) as shown above in table 3.4 which are involved in the maintenance of intestinal barrier functions were assessed.

B. Immunofluorescence assay

Reagents

Phosphate-Buffered Saline (PBS) (10 mM, pH 7.4)

Xylene (Thomas Baker)

Alcohol grades: 100% (absolute alcohol), 95% (v/v), 85%, and 70%.

Citrate buffer (10mM sodium citrate, pH 6.0): Prepared by dissolving tri-sodium citrate 2.94 g in 1000 ml of distilled water. pH adjusted with 10mM citric acid to 6.0 and stored at 4°C.

PBS with Triton X-100 (0.05% v/v PBST, Sigma): Sections were permeabilised by PBST. Dissolved 25 µl of Triton X-100 in 50 ml of PBS.

Bovine serum albumin (BSA, 2% w/v, Sigma): Dissolved 1 gm in 50 mL of PBS.

Primary antibody Claudin-1 (0.2 mg/ml Pierce Thermofischer Scientific): Anti-mouse claudin-1 polyclonal primary antibody raised in rabbit was used at dilution 1:400 in PBS: BSA (2:1) mixture.

Primary antibody Occludin (*6 µg/ml Invitrogen Thermofischer Scientific*): Anti-mouse occludin polyclonal primary antibody raised in rabbit was used at dilution 1:50 in PBS: BSA (2:1) mixture.

Secondary antibody (FITC conjugated secondary antibody: 1.5 mg/ml, Pierce, Thermo Fischer): FITC labelled anti-rabbit secondary antibody raised in goat was diluted in PBS (1: 200).

FITC conjugated phalloidin (0.1 mg/ml Sigma): 0.1 mg of *FITC phalloidin* was dissolved in 1 ml of DMSO (cell culture grade, Himedia), aliquoted and stored at -20ºC. The final concentration of 0.1mg/ml was prepared in PBS on the day of use for actin staining.

4',6-diamidino-2-phenylindole (DAPI, 500 µg/ml): Dissolved 500 µg of DAPI in 1 ml PBS.15 µl of this prepared solution was added in 2 ml of 1% BSA before use.

Procedure

Immune-histochemical staining was used for localization of proteins associated with tight junctions and cellular cytoskeleton. Unstained histological slides were subjected to de-paraffinization by xylene and rehydration in decreasing gradient of ethanol sections. The slides were washed thrice with PBS followed by boiling in 10 mM boiling citrate buffer (pH 6.0) for 15-20 min to unmask the antigens followed by cellular permeabilisation with 0.05% triton-X-100 in phosphate buffer for 20 min. To reduce non-specific binding, intestinal sections were blocked with 2% bovine serum albumin (BSA) followed by overnight incubation with primary antibodies (Thermo fisher Scientific, Massachusetts, USA) raised in rabbit against claudin-1 (0.2 mg/mL; 1: 400 dilution) and occludin (6ug/ml, 1:50 dilution) at 4°C. Subsequently, slides were

washed with PBS and incubated with FITC (fluorescein isothiocyanate) labelled anti-rabbit IgG secondary antibody raised in goat (1.5 mg/ml; 1:200 dilution) for 1 h. Finally tissue sections were treated with DAPI (500 ug/ml) for 5 min and washed five times with PBS to reduce the background noise. Intestinal morphology and tight junctional protein fluorescence was seen under fluorescent microscope (Olympus optics BX60 microscope; Olympus, Tokyo, Japan).

To delineate the effect of probiotic fermented whey under inflammatory conditions on skeletal filaments, f-actin staining was done using FITC labelled phalloidin. Phalloidin is a toxin obtained from the *Amanita phalloides* that binds actin filaments specifically and thus is often used to study its distribution in the cells. The fixed tissue slides were processed for actin staining by using FITC-phalloidin. The slides were de-paraffinization by xylene and rehydration in decreasing gradient of ethanol followed by boiling in citrate and permeabilised with 0.05 % triton-X-100. Again slides were washed and stained with FITC conjugated phallodin (0.1 mg/ml, 1:100, Sigma-Aldrich) and lastly stained with DAPI (500 ug/mL). To remove any traces of non-specific fluorescence, profuse washing was done and slides were examined under Olympus BX60 (Olympus) to visualize distribution of F-actin filaments in epithelial tissue of villi.

C. Intestinal gut permeability by FITC-Dextran

Reagents

FITC-dextran: 60 mg/Kg body weight

Phosphate-Buffered Saline (PBS) (10 mM, pH 7.4)

Procedure

Intestinal permeability is often used to check the gut inflammation by enteral administration of non-digestible markers that do not cross the mucosal barrier. FITC-Dextran 4000 is often employed to assess the *in vivo* disturbance of the gut barrier integrity in rodents (Volynets *et al.* 2016). Thus the same tracer molecule was used under present investigation to assess the intestinal permeability of murine model according to instruction previously described by Wang *et al.* (2015). Briefly, mice distributed in different groups as described in section 3.5.3 and figure 3.2 were given with FITC labeled dextran (60mg/100g of body weight) using oral gavage after fasting for 6-7 hours. Finally blood was

collected after 4 hours of FITC-dextran intubation by cardiac puncture of mice and serum was prepared. Serum was diluted by a ratio of 1:10 with PBS and then 100 μl of diluted serum was added in black 96-well microtiter plates (SPL Life Sciences, Korea). After that FITC-dextran concentration was measured in serum with Multimode Plate Reader (TECAN, infinite M200 Pro, Austria) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Standard curve of FITC-dextran (5-400 ng) in non-treated serum (diluted 1:10 v/v with PBS) was also prepared to determine the level of FITC-dextran released in the blood due to leakage in intestine.

3.7.4 Microbial count in fecal sample

Feces were collected in sterile tube before sacrificing animals on 29th day and feces were processed within 1 hr of collection for fecal bacterial count enumeration. Feces were homogenized in sterile PBS (pH 7.4) than diluted serially followed by plating on MRS agar and EMB agar of appropriate dilution (10^5). Plates were incubated at 37°C for 24-48 hr for morphological assessment of colonies.

3.8 The mode of action of lactobacilli fermented whey on human cell line

3.8.1 Epithelial cell line

HT-29 cells used in this study were a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells. When these cells were cultured under specific conditions, they polarised so that their phenotype, morphology, and functions resemble the enterocytes lining in the small intestine. For this study, HT-29 cells undergone 15-20 passages, were obtained from RCB, Delhi (India).

3.8.2 Inflammatory agent

Under present investigation, live *E. coli* (ATCC 14849) was procured from the the National collection of Dairy cultures (NCDC), National Dairy Research Institute, Karnal, Haryana, India. The culture was grown and maintained 37ºC for 18 hr in nutrient broth (Himedia Laboratories, Mumbai, India) stored at 4ºC until further use. Before the experiments, the purity of *E. coli* culture was confirmed on eosin methylene blue (EMB) agar where metallic green colonies appeared after 18-24 hr of incubation at 37ºC. Bacterial cell number was determined again

by plate count method on EMB agar to obtain desired cell number (1-2×10^8 cfu/ml) by dilution and adjusting optical density to 1.3 at 600 nm.

3.8.3 Maintenance and culturing of HT-29 cells

Reagents

Foetal bovine serum (FBS): 10% FBS was used for the preparation of RPMI media. Before use, fetal bovine serum was heat-inactivated at 56°C for 30 min, aliquoted and stored at -20°C until used.

Modified Eagle's Medium (RPMI): RPMI media was prepared by dissolving 16.4 g of RPMI powder, 2.10 g of sodium bicarbonate, sodium pyruvate 110.1 mg, HEPES 5.96 g, penicillin 61 mg, streptomycin 100 mg and 10% FBS in 1 litre of autoclaved water. Then the pH of the media was adjusted between 7.3-7.4 using 1N NaOH. Finally, prepared media was filtered through 0.22 μm autofill assembly and incubated at 37°C overnight to ensure its sterility. It was further stored at 4°C until used.

Procedure

HT-29 cells were routinely maintained in 25 cm^2 flask containing RPMI media. The cells were grown at 37°C in a humidified incubator perfused with 5% CO_2. The media in the flasks containing culture cells was usually replaced with new media every day by complete aspiration of old media. When cells reached 80-90% confluency during 3 days, they were harvested by trypsinizing the cells from the surface of the flask and centrifuged at 1000 rpm for 5 min. The obtained pellet was re-suspended in fresh media for sub-culturing (1: 2).

3.9 Simulated gastrointestinal digestion

Reagents

Enzymes: Alpha amylase from porcine pancreas (EC 3.2.1.1 ≥5 units/mg solid), Pepsin from porcine gastric mucosa (EC 3.4.23.1; ≥2,500 units/mg of solid), pancreatin from porcine pancreas EC 232-468-9; 4×USP) and bile salts (160 mM).

Electrolyte stock solution: KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ (H_2O), $(NH_4)_2CO_3$, $CaCl_2$

Milli-Q water was used for the preparation of all solutions

Procedure

Simulated digestion of probiotic fermented whey was carried out using stock solutions as shown in table: 3.9. Five milliliter of fermented whey samples were dissolved in 5 ml of simulated saliva fluid (SSF) for 2 min. Then, the mix was diluted at a ratio of 50:50 (v: v) in simulated gastric fluid (SGF) containing pepsin from porcine gastric mucosa.

After two hours of gastric digestion, digestion in intestinal phase was carried out by mixing the gastric phase with the same volume of simulated intestinal fluid (SIF) containing pancreatin from porcine pancreas (100 U trypsin activity/ml of final mixture, Sigma-Aldrich) and porcine bile extract (160 mM). All simulated fluids were incubated at 37ºC before use. Digestions were performed at 37ºC in a shaking water bath (Model: BS-11, Lab companion, Jeio Tech Inc., U.S.A.) at 100 rpm. Lastly, the enzymes were inactivated by heating at 95°C for 15 minutes, followed by cooling to room temperature according to Tagliazuchhi *et al.* (2016). Samples were kept at -20ºC until analysis.

Table 3.9: Preparation of stock for simulated digestion fluids

Constituent	Stock conc.		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
	g/l	mol/l	Vol. of stock	Conc. in SSF (mmol/l)	Vol. of stock (ml)	Conc. in SGF (mmol/l)	Vol. of stock (ml)	Conc. in SGF (mmol/l)
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
$CaCl_2(H2O)_2$	44.1	0.3		1.5 (0.075*)		0.15 (0.075*)		0.6 (0.3*)

*In brackets is the corresponding Ca^{+2} concentration in the final digestion mixture

3.9.1 Quantification of hydrolysed protein

Quantification of hydrolysed protein in simulated digested fermented whey prepared from *L. rhamnosus* and *L. fermentum* was carried out using OPA (O-phthaldehyde) method as per Nielsen and Jensen, (2001) as described previously in section 3.4.4.

3.10 MTT assay

Under the present investigation, this assay was used to determine the safety of simulated digested sample of probiotic fermented whey (LF: 5898 and LR: 5897) respectively at different amounts (1ng-100µg) on human epithelial HT-29 cell before onset of various experiments on junctional integrity assessment.

Reagents

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide), Sigma, USA Solution: 5 mg of MTT was dissolved in 1 ml of PBS.

Phosphate-Buffered Saline (PBS) (10 mM, pH 7.4).

DMSO (Dimethyl sulphoxide, Himedia Laboratories Pvt. Ltd. Mumbai).

Procedure:

The intestinal HT-29 cells were cultured in 25 cm^2 cell culture flask using filter sterilized RPMI and were incubated in a CO_2 incubator at 37°C in a humidified incubator perfused with 5% CO_2 till confluency. For MTT assay, cell were seeded (1×10^5 cells/well) in 96 well plates and were allowed for the overnight attachment at 37°C before exposure to simulated digested samples. After attachment, adherent cells were treated separately with 50 µl of simulated digested samples having different concentrations (5 ng-500µg) of hydrolysates per well prepared from simulated digested probiotic fermented whey (LF: 5898 and LR: 5897) suspended in 150 µl of antibiotic free RPMI media for 4 hr at 37°C. Subsequently, the cells were washed with PBS and 10 µl of MTT (5 mg/ml) along with 90µl of RPMI per well was added to achieve a final concentration of 0.5 mg/ml. The cells were further incubated for 4 h at 37°C. After completion of incubation, half of the solution from the wells were removed and 100 µl of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. The solution was properly mixed by avoiding bubble formation and read at absorbance 570 nm and background noise was read at 660 nm on microplate reader (BioTek Instruments, Winooski, Vermont, USA).

3.11 Transcriptional expression of intestinal epithelial cells during *E. coli* challenge in presence of simulated digested probiotic fermented whey

HT-29 cells were seeded in a 6-well plate with a density of $1X10^5$ cells/well in a humidified CO_2 incubator. Then confluent HT-29 cells were initially stimulated with simulated digested probiotic fermented whey sample followed by challenge with *E. coli* as inflammatory agent as shown in figure 3.4. Finally, the cells were harvested in 1 ml TRI reagent for isolation of total RNA followed by cDNA synthesis. Thereafter expression of the following immune genes was assessed by qRT-PCR using primer sequence (Table 3.10) as mentioned previously in section 3.7.2 (E). Cells without any post *E. coli* challenge were considered as control respective of simulated digested whey (SDW), simulated digested whey fermented with *L. rhamnosus* and *L. fermentum* (SDFW and SDRW) throughout this study.

Table 3.10: Sequences of primers used for gene amplification

Gene	Sense/antisense Primer	Amplicon Length (bp)
Genes related to junctional integrity		
Occludin	5'AGAACAGAGAAAGATCACTATGAGACA3' 5'CTTTGTTGATCTGAAGTGATAGGTGGA3'	114
Claudin-1	5'GCACATACCTTCATGTGGCTCAG3' 5'TGGAACAGAGCACAAACATGTCA3'	92
Zona-occluden	5'TGATGGTGTCCTACCTAATTCAACTCA3' 5'GAACGCCAGCTACAAATATTCCAACA3'	140
GAPDH	5'GCACCGTCAAGGCTGAGAAC3' 5'TGGTGAAGACGCCAGTGGA3'	138
Genes related to immune signalling		
TLR-2	5'AGCACTGGACAATGCCACAT'3 3'ACCATTGCGGTCACAAGACA'5	113
TLR-4	5'CAAGAACCTGGACCTGAGCTT'3 3'AAAAGGCTCCCAGGGCTAAA'5	200
NF-κβ	5'ATGTGGGACCAGCAAAGGTT'3 3'CACCATGTCCTTGGGTCCAG'5	134

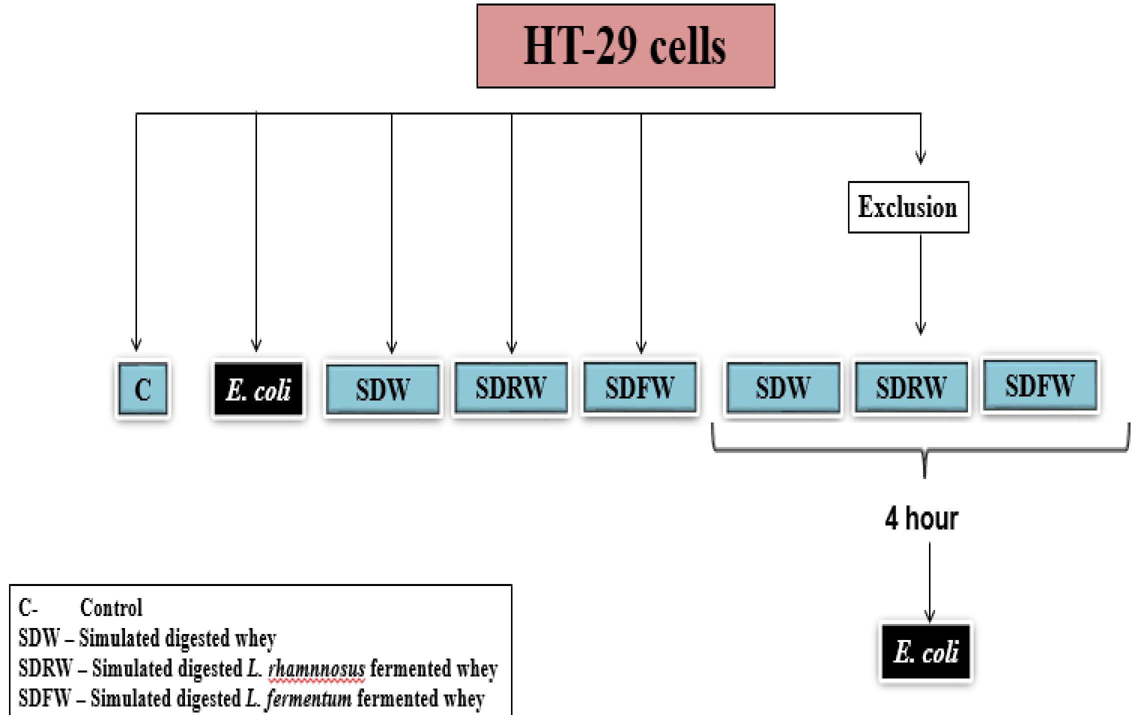

Figure 3.4: Assessment of expression of tight junctional and immune genes in intestinal epithelial cells during challenge with *E. coli* as inflammatory agent in presence of probiotic simulated digested fermented whey

3.12 Protein quantification

To measure protein in various sample method of Lowry *et al.* (1951) was used.

Reagents

Solution A1 –Sodium hydroxide solution (0.8%): 2 g of sodium hydroxide was dissolved in 200 mL of distilled water and the volume was made up to 250 mL in the volumetric flask.

Solution A2 –Sodium carbonate (4%): 8 g of sodium carbonate was dissolved in 150 mL of distilled water and the volume made up to 200 mL in the volumetric flask.

Solution B1- Sodium potassium tartrate solution (2%): 1 g of sodium potassium tartrate was dissolved in 40 mL of distilled water and the volume was made to 50 mL in the volumetric flask.

Solution B2–Copper sulphate pentahydrate (1%): 500 mg of copper sulphate pentahydrate was dissolved in 40 mL of distilled water and the volume was made to 50 mL in the volumetric flask.

Lowry's Reagent: Lowry's reagent was prepared fresh just before use by mixing Solution A1, A2, B1, and B2 in a ratio of 5: 5: 1: 1 respectively.

Folin and Ciocalteu's Phenol Reagent (2N): Commercial available 2.0 N Folin and Ciocalteu's phenol reagent were mixed with an equal volume of distilled water. The reagent was always prepared just before use.

Bovine Serum Albumin (BSA, 1 mg/ml): Dissolved 100 mg of BSA in 75 ml of distilled water and the volume was made up to the mark in 100 ml volumetric flask.

Procedure

Different aliquots (10-100 ml) of standard BSA solution (1 mg/ml) and samples (10 µl) were added in clean tubes. The volume of each test tube was made to 0.1 ml with distilled water. To these, 1.0 ml of freshly prepared Lowry's reagent was added and allowed to stand at room temperature for 10 min. Then 0.1 ml of freshly diluted Folin and Ciocalteu's phenol reagent was added to each tube, mixed well immediately and kept at 37°C for 45 min in dark. The absorbance was read at 660 nm in Specord 200 double beam spectrophotometer (Analytik Jena, Japan) after adjusting the blank to zero. The standard curve was prepared from the known concentration of BSA and the amount of protein in samples was determined by extrapolating against the standard curve.

3.13 Nuclear translocation of NF-κβ in intestinal cells

NF-κβ is a pivotal mediator of pro-inflammatory gene induction which regulate innate and adaptive immune responses. In intestinal cells, expression and secretion of interleukins on stimulation with probiotic lactobacilli fermented whey and inflammatory agents could be linked to NF-κβ activation. HT-29 cells were grown either on 18 mm coverslip in 35 mm Petri plates or in 6-well plates to achieve confluency and then treated with *L. rhamnosus* fermented whey (50 ug), *L. fermentum* fermented whey (50 ug), live *E. coli* (10^8 cfu/ml) respectively for 4 hr before conducting nuclear translocation of NF-κβ. A similar experiment

was also conducted during exclusion of inflammatory agents (*E. coli*) by either of the probiotic strains fermented whey.

3.13.1 Western Blotting

Initially, cytoplasmic and nuclear fractions of HT-29 cells obtained after treatment with probiotics strains fermented whey or inflammatory agents as described above were electrophoresed according to the protocol of Laemmli, (1970) and then blotted on PVDF membrane for western blotting analysis.

a. Preparation of cell lysate

Reagents

PBS/Phosphatase Inhibitor Solution (1X)

Complete Hypotonic Buffer (1X)

Complete Nuclear Extraction Buffer (1X)

Nuclear Extraction Dithiotheritol (DTT: 10mM)

NP-40 (10%)

Procedure

After treatment to HT-29 cells as described above, cells were washed thrice with PBS and finally harvested in 1 ml PBS followed by centrifugation at 300 g for 5 min to make cell pellet. Then the supernatant was discarded and 5 ml of PBS/ Phosphatase Inhibitor Solution (1X) was added to the cell pellet and centrifuged at 300 g for 5 min at 4°C. PBS/ Phosphatase Inhibitor Solution (1X) wash was repeated for one more time. After discarding the supernatant 500 µl ice cold 1X complete hypotonic buffer. Mix gently by pipetting and cells were incubated on ice for 15 min to allow cells to swell followed by the addition of 100 µl NP-40 (10%). Subsequently, cytoplasmic proteins were extracted in the supernatant by centrifugation at 14,000 g for 5 min and stored at -20°C. To the residual precipitate, 50 µl of nuclear protein extract containing nuclear extraction buffer (1X) was added and kept for 15 min while mixing it properly by vortexing under chilled conditions. Finally, the supernatant containing nuclear extract was obtained by centrifugation at 14,000 g at 4°C for 10 min and stored at -20°C. The

supernatants were analysed for protein content using the Lowry method as described above in section 3.15.

b. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Reagents

Acrylamide: Bis-Acrylamide solution (30%): Prepared by dissolving 29 g of acrylamide and 1 g of N' N' methylene-bis acrylamide in 100 ml of distilled water and filtered with Whatman no. 1 filter paper before storage at 4°C in the dark.

Stacking gel buffer (Tris-HCl, 0.5 M pH 6.8): 6.05 g of Tris was dissolved in 100 ml of distilled water and pH was adjusted to 6.8 with 6 N HCL and stored at 4°C.

Separating gel buffer (Tris-HCl, 1.5 M, pH 8.8): 18.15 g of Tris base was dissolved in 100 ml of distilled water and pH was adjusted to 8.8 with 6 N HCL and stored at 4°C.

SDS (10%): The reagent was prepared by dissolving 10 g of SDS in 100 ml of Milli Q water and stored at room temperature.

Ammonium persulphate (APS: 10%): Dissolved 100 mg of APS in 1 ml of distilled water.

Ethylenediamine (TEMED): Used as such commercial available form Sigma

Procedure

Preparation of separating and stacking gels

All the glass plates of electrophoresis apparatus were wiped using 70% ethanol. Glass plates were fitted in casting frame and leakage was checked with water. The separating gel was prepared in glass beaker by using different component as described above in table 3.8. APS and TEMED were added just before polymerization. The solution was mixed well by pipette. The solution was poured smoothly by the side of the cassette from the top by avoiding entrapment of any air bubble in the gel. Immediately monomer solution was overlaid with distilled water to make top of the gel to horizontal. Gel was allowed to polymerize for 30-45 min, without any disturbance. The stacking gel was prepared in glass beaker as described in table 3.9. Water was discarded before pouring the

stacking gel solution on the top of separating gel. A well forming comb (1 mm) was inserted into stacking gel and allowed to polymerize without any disturbance for 30-45 min. After polymerization, comb was removed straight up slowly and gently without affecting wells. Finally, well was rinsed with distilled water.

Sample preparation and loading

Cell lysate as described above were removed from -20°C and volume corresponding to 30 µg of protein was taken in eppendorf. Then equal volume of loading dye (Table 3.11) was added in tubes and sample was vortexed for 10 sec followed by boiling at 100°C for 5 min. Then the samples were cooled at RT for 15 min followed by centrifugation at 1000 g for 1 min. Then samples were loaded into the wells of stacking gel. Pre-stained molecular weight marker was added in one well to check movement and transfer efficiency after blotting. Gel was run at 60 Volts through the stacking part and voltage was increased up to 90 Volts after the proteins migrate through the separating gel. The migration was allowed to continue until the blue dye front reached at the end of the glass plates, but did not migrate off the gel.

Table 3.11. Preparation of 12 % separating gel for SDS-PAGE

S.No.	Chemicals	Volume
1.	Acrylamide / Bis (30% stock)	6 ml
2.	Tris-HCl (1.5 M,pH 8.8)	3.75 ml
3.	SDS (10%)	150 µl
4.	Deionized Water	5 ml
5.	Ammonium per sulfate (10%) Freshly prepared	75 µl
6.	TEMED	10 µl
7.	Total volume	15 ml

Table 3.12. Preparation for 4.5 % stacking gel for SDS-PAGE

S.No.	Chemicals	Volume
1.	Acrylamide / Bis (30% stock)	0.6 ml
2.	Tris-HCL (0.5 M,pH 6.8)	1.0 ml
3.	SDS (10%)	40 μl
4.	Deionized Water	2.4 ml
5.	Ammonium per sulfate (10%)	20 μl
6.	TEMED	5.0 μl
7.	Total volume	4 ml

Table 3.13. Composition of 1X running buffer (pH 8.3) for SDS-PAGE

S.No.	Chemicals	Volume
1.	Tris base	6.06 g / L
2.	Glycine	14.415g / L
3.	SDS (1%)	1g /L

Table 3.14. Composition of 5X sample buffer for SDS-PAGE

S.No.	Chemicals	Volume
1.	Tris-HCl, (1 M, pH 6.8)	1.75 ml
2.	SDS (25%)	2 ml
3.	β-mercapto ethanol	1.25 ml
4.	Glycerol	4.5 ml
5.	Bromophenol Blue (0.25 %)	0.5 ml
6.	Total volume	10 ml

c. Blotting on PVDF membrane

Reagents

Blotting Buffer: Prepared by dissolving 12.12 g of Tris (50 mM), 57.66 g of Glycine (384 mM), 0.2 g of SDS and 400 ml of methanol (20%). Made final volume 2000 ml with deionized water.

Washing Buffer: Also known as Tris Buffered Saline with Tween-20 (TBST) buffer. It was prepared by dissolving 4.84 g of Tris (20 mM), 17.53 g of NaCl (150 mM) and 2 ml of Tween-20 (0.1%) in 900 ml of deionized water. the pH of the solution was adjusted to 7.6 with the help of 6 M HCl and the final volume was adjusted to 2000 ml with distilled water.

Blocking Reagent (5%): 5 g of skim milk (non-fat milk)/ Bovine serum albumin was dissolved in 100 ml of TBST.

Primary antibody: NF-κβ P-65 antibody is a mouse monoclonal IgG_1 antibody (1:200) (Santa Cruz, California, USA).

Secondary antibody: Anti-mouse IgG produced in goat conjugated with Horseradish peroxidase used as Secondary antibody (1: 500) (Invitrogen).

Clarity Western ECL substrate

Procedure

Gel with separated proteins obtained after SDS-PAGE was used for western blotting after removing it from the electrophoresis apparatus using the following steps.

Equilibration of gel and activation of membrane

The gel was rinsed in water and the staking part of the gel was removed. Then separating gel was equilibrated in transfer buffer for 15 min. PVDF membrane was used to transfer proteins from the gel (SDS-PAGE) and this membrane was further used to probe with the antibody for identification and quantification of proteins associated with the NF-κβ pathway. For this process, the PVDF membrane was cut according to the size of the gel. Due to its hydrophobic nature, it was dipped in methanol for 30 sec to activate the positive charge of the membrane. Immediately the membrane was transferred into a

glass tank containing distilled water for 5 min, to remove excess of methanol. Initially, the membrane will float on water and move in a zig-zag manner if positive charges were activated properly. Subsequently, the membrane was equilibrated in transfer buffer for 5 min.

Assembly of the transblot apparatus and running the blot

The transfer sandwich was assembled under buffer taken in a rectangular clean tray to minimize trapping of air bubbles. A stack of about eight number of Whatman filter papers cut in equal dimension as of the foam pad along with two number of foam pads was immersed in transfer buffer to remove air bubbles. The transfer sandwich was arranged in the following order from bottom to top (cathode to anode):

- Black portion support on the bottom
- One Foam pad
- 3 MM Whatman filter paper (4 No.)
- Separating gel
- PVDF membrane: The membrane was also reversed to have glossy surface down facing the gel and cut edge at the bottom would come at the right-hand side matching the cut corner of the gel
- 3 MM Whatman filter paper (4 No.)
- One foam pad
- White plastic support on top

The transfer sandwich was closed properly and placed in a transfer tank filled with chilled transfer or blot buffer. Then protein transfer was carried out at 45 volts for 3 hr. Protein transfer efficiency on the membrane was assessed via the transfer of pre-stained marker on the membrane.

Blocking

The membrane was washed in distilled water and the nonspecific sides of the membrane were blocked by incubating the membrane overnight in 5% skim milk (non-fat dried milk) prepared in TBST buffer at 4°C.

Primary antibody incubation

The next day, the membrane was removed from 4°C and the blocking solution was aspirated completely. The membrane was washed three times for each 5 min with TBST buffer. Then the membrane was incubated with primary antibody (NF-κβ mouse monoclonal IgG, 1:200 dilution) prepared in TBST buffer supplemented with 2% BSA at room temperature for 4 hr. Later, the membrane was removed and washed 3 times with TBST buffer for 5 min with gentle agitation.

Secondary antibody incubation

In this step, the membrane was incubated with secondary antibody (Anti-mouse IgG-conjugated peroxidase produced in goat, 1:500) prepared in TBST supplemented with 2% BSA for 2 hr at room temperature. Then the membrane was removed and washed 3 times in TBST buffer for 5 times with gentle agitation.

Immunoblot detection by Enhanced chemiluminescent (ECL)

After the last washing, the membrane was removed and kept on plastic sheet. Enhanced chemiluminescent reagent (ECL) was added on the membrane by taking luminal and peroxidase reagent in equal ratio. The mixture was protected from light by wrapping the vial with aluminium foil and used immediately. Then the membrane was kept in a ziplock and image was developed through the X-ray imaging system.

3.15 Statistical analysis

Data were analysed using GraphPad Prism (Version 5.01) software. Experimental results are presented as mean ± SEM (standard error mean). Data were subjected to analysis of variance (ANOVA) and the Tukey test was used to separate the means ($p<0.05$), which were considered statistically significant.

CHAPTER –4

Results

RESULTS

Two laboratory isolates of probiotic cultures, *Lactobacillus rhamnosus* (LR**:** MTCC 5897) and *Lactobacillus fermentum* (LF**:** MTCC 5898) fermented whey preventive effect was evaluated on intestinal epithelial barrier integrity *in vivo* in mice under colitis induced condition and *in vitro* to study its mechanism of action. Their basic probiotic attributes which mainly includes bile tolerance, acid tolerance and adhesion of probiotic to intestinal cells were already established in our laboratory. Both of these indigenous probiotic cultures also showed anti-immunosenesense characteristics during *in-vivo* trials on aged mice (Sharma *et al*. 2014) and helped in alleviating the allergic responses in weanling mice (Saliganti *et al*. 2015). Hence present study was designed to gain further insight in the maintenance of gut-immune homeostasis by *Lactobacillus* fermented whey on intestinal epithelial barrier integrity *in vivo* in mice under inflammatory conditions and *in vitro* to study its mechanism of action. The animals were fed with probiotic *Lactobacillus* (*Lactobacillus rhamnosus and Lactobacillus fermentum)* fermented whey followed by the induction of colitis by DSS. Further, studied the preventive effects by assessing the expression of tight junctional, defensin, pattern recognition receptor (PRR) and level of interleukins in intestine. Later changes in expression of genes in intestinal cells and translocation of NF-κβ from their cytoplasm to nucleus further validated the immunomodulatory responses of probiotic fermented whey.

4.1 Morphological features of bacterial cultures

The morphological features of both probiotic strains are shown in plate 4.1 by Gram's staining, negative staining and colony morphology. LR appeared as extended rods which occurred in long chains, while LF appeared as short rods present as single or in pair. On MRS agar, LR form small, translucent, round colonies, whereas LF form large, translucent, circular and rough colonies. Another bacteria, *Escherichia coli* (ATCC 14948) was used to induce inflammatory milieu *in vitro*. Morphological features of *E. coli* are shown in plate 4.2 by Gram's staining, negative staining and colony morphology. *E. coli*

appeared as small rod shaped bacteria which form small chains having characteristic green metallic sheen on EMB agar.

4.2 Growth of *Lactobacillus* in whey media

4.2.1 Growth and metabolites production by *Lactobacillus*

Lactobacillus fermentum (LF: MTCC 5898) and *Lactobacillus rhamnosus* (LR: MTCC 5897) were able to grow in whey supplemented media and their growth kinetics was standardized at 37ºC in 12 h of incubation as represented in the figure 4.1 (A). The LR and LF both were able to obtain a population of 9.3 log cfu/ml and 8.7 log cfu/ml respectively, when grown in supplemented sweet whey for 12 h and subsequently attained a stationary growth phase.

4.2.2 pH and Titratable acidity

Lactobacillus fermentum (LF: MTCC 5898) and *Lactobacillus rhamnosus* (LR: MTCC 5897) fermented whey showed constant decrease in pH (6.5-5.8; LR and 6.4-4.5; LF) with the simultaneous increase in titratable acidity (0.2-0.4; LR and 0.2-0.3; LF) during 12 h of incubation at 37ºC as depicted in (Fig. 4.1B& C).

4.2.3 Lan Eynon Method

In accordance to the growth behavior, the amount of lactose consumed in LRFW and LFFW was estimated by Lan Eynon method as shown in figure 4.1. Lactose content varies from 5.5%-4.6% in LRFW and from 5.0%-4.5% in LFFW in 12 h of incubation period (Fig. 4.1D).

4.2.4 Protein percent

Regarding protein percent, a decrease was observed in whey fermented by both the cultures LR and LF as estimated by Kjeldahl method showed variation from 4.7-3.1% and 4.8%-4.1% respectively (Fig. 4.1E).

4.2.5 Peptide concentration

The LRFW showed the more proteolytic activity as compared to LFFW; the concentration of amino acid increased rapidly in LRFW from 0.9 mg/mL at 0 h reaching maximum 2.6 mg/mL at 12 h (Fig. 4.1F). While, LFFW exhibited 0.6 mg/mL at 0 h reaching maximum 1.72 mg/mLin 12 h of fermentation period.

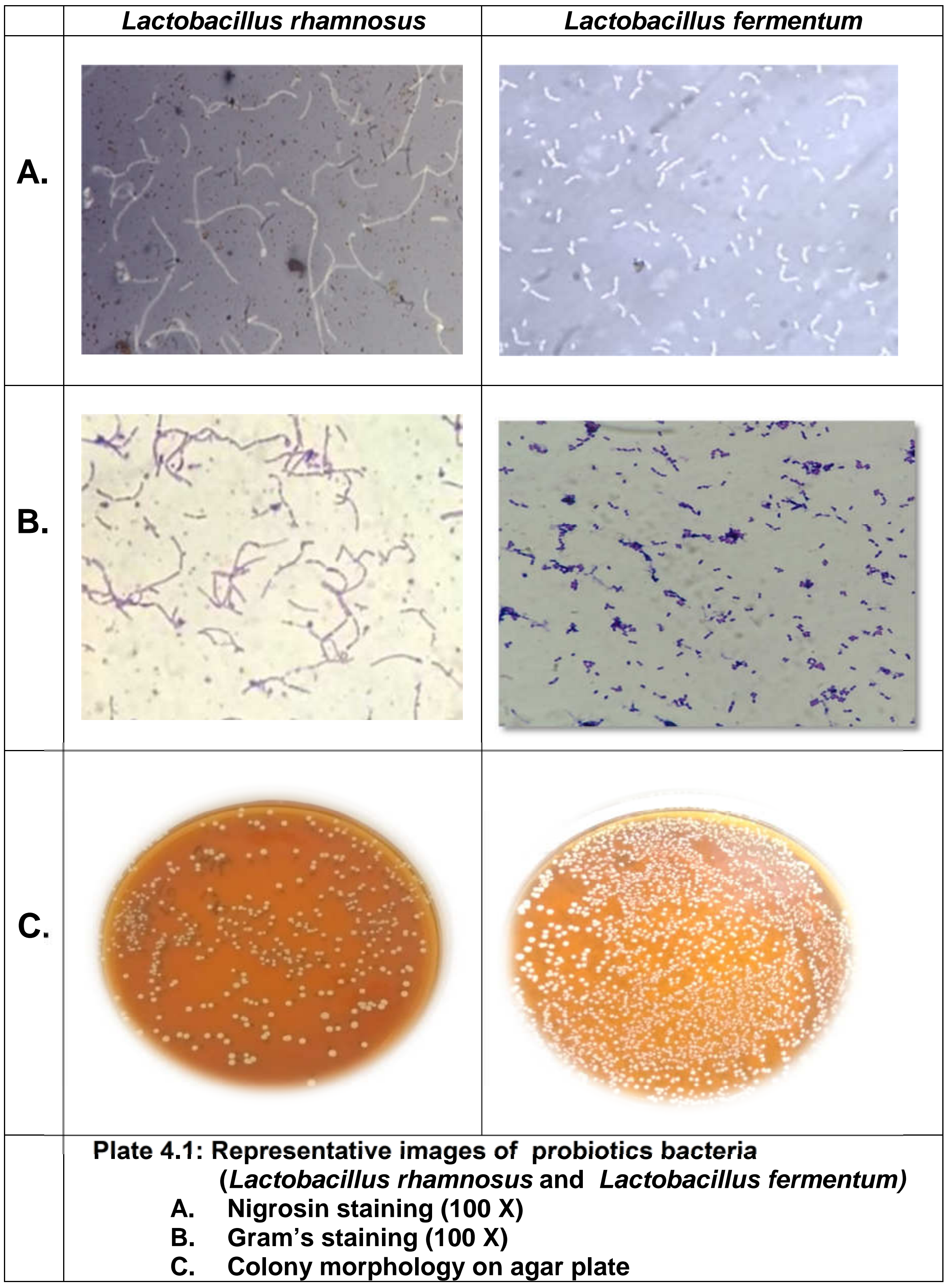

Plate 4.1: Representative images of probiotics bacteria (*Lactobacillus rhamnosus* and *Lactobacillus fermentum*)

- **A. Nigrosin staining (100 X)**
- **B. Gram's staining (100 X)**
- **C. Colony morphology on agar plate**

A

B

C

Plate 4.2: Representative images of *E. coli*

A. **Nigrosin (negative) staining (100 X)**

B. **Gram's staining (100 X)**

C. **Colony morphology on agar plate**

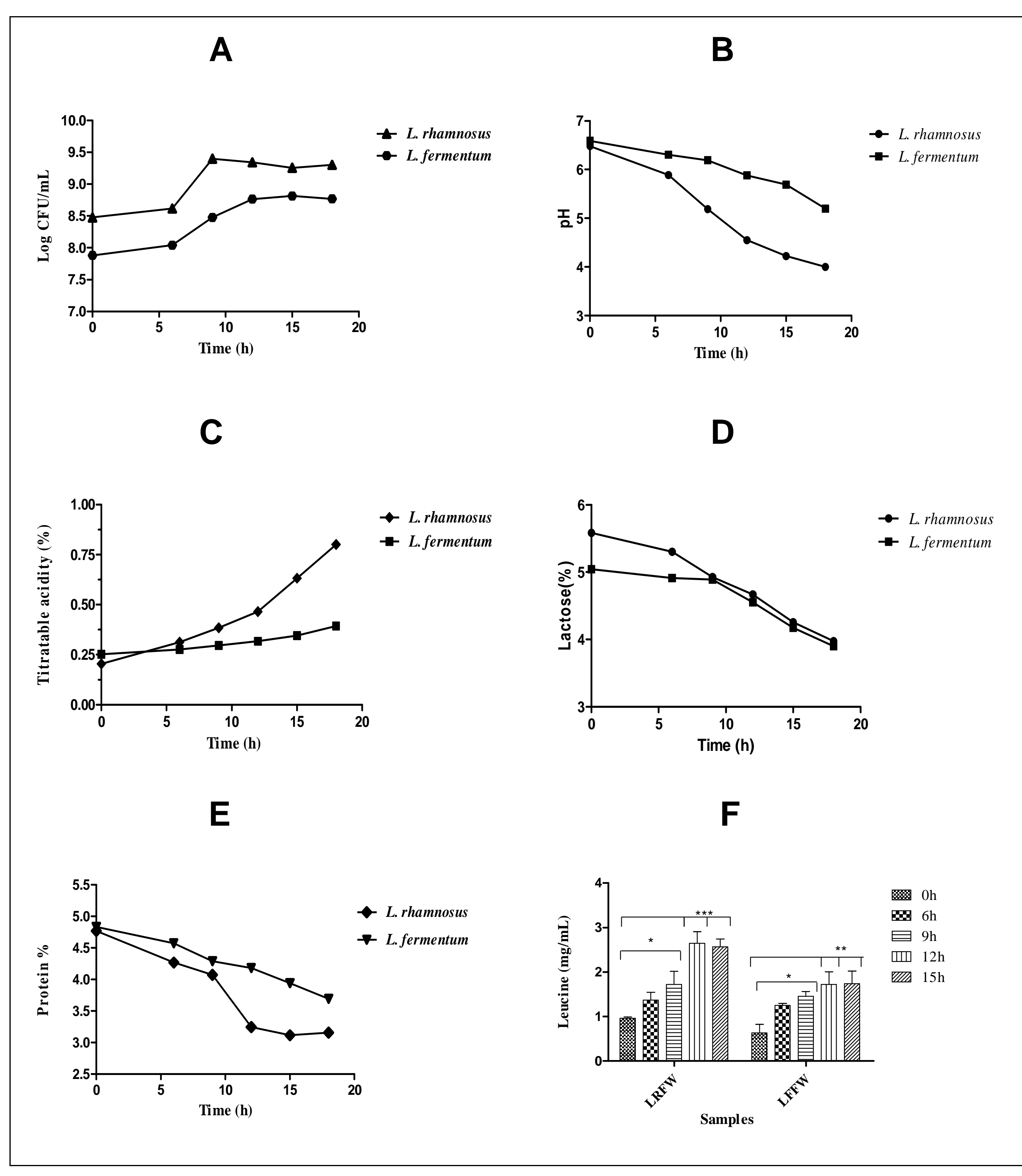

Figure 4.1 Growth and metabolites utilised by *Lactobacillus (L. rhamnosus and L. fermentum)* during fermentation of whey

4.3 Assessment of probiotic fermented whey on general health status of weaning mice during non-colitis and DSS-induced colitis

4.3.1 Change in body weight and organ weight in non-colitis

After 28 days of intervention all groups exhibited no health issues in any of the experimental group throughout the period. Mice in this study fed with basal diet, LRFW, LFFW and NFW exhibited significant increase ($p<0.001$) in body weight as compared to the control group. Similar, increased trend in body weight was followed by the LRFS and LFFS than control group at the end of feeding period (Fig. 4.2A). No change in organ indices and glucose were found in all the feeding groups (Fig. 4.2 B-E).

4.3.2 Percent loss in body weight and organ weight in colitis

The Swiss albino mice during their feeding periods under colitis induction are as shown in figure 3.2. After colitis induction, DSS-colitis animals showed significantly ($p<0.01$) higher loss in percent body weight than animal groups fed with whey, probiotic fermented whey and its supernatant respectively (Fig. 4.3A). The results obtained showed that there was no major change in liver, kidney index (Fig. 4.3C &D) and glucose (Fig. 4.3B) in the mice respective treatment groups as compared to control. Further, the higher spleen index ($p<0.01$) and its larger size observed in DSS-colitis group indicated systemic inflammatory response than control on colitis induction (Fig. 4.3E). Mice pre-fed with probiotic fermented whey significantly reduced ($p<0.05$) the spleen index in contrast to probiotic fermented whey supernatant fed group where insignificant changes were observed than DSS induced colitis animals.

4.3.3 Disease activity index

Disease activity index (DAI) was characterized as an amalgamation of loss in body weight, stool consistency and rectal bleeding which were scored to analyze the anti-inflammatory potential of probiotic fermented whey and its supernatant. Simultaneously, DSS and W+DSS fed-groups displayed significantly higher ($p<0.01$) disease activity indices (8.1 ± 0.98 and 5.3 ± 0.80) after six days of colitis induction as compared to animals fed with probiotic fermented whey (DAI: 2.1 ± 0.06) and its supernatant (DAI : 2.8 ± 0.54) respectively (Fig. 4.3F).

4.4 Number of payer patches and colon health

Non-colitis

Peyer's patches play an important role in immune surveillance of materials within your digestive system. Immune surveillance refers to the process by which your immune system recognizes and destroys potential pathogens. Animals of healthy non-colitis grousp as showed by normal payer patches number with the absence of inflammation (Fig. 4.4A). Whereas the colon weight and length remain normal in all feeding groups (Fig. 4.4B-C).

Colitis

DSS fed animals in colitis induced group showed higher ($p<0.01$) number of payer patches in intestine, whereas they remained similar to healthy control animals when colitis was induced after feeding whey, LRFW, LFFW, LRWS and LFWS to respective animal groups (Fig. 4.5A). DSS induced intestinal inflammation significantly ($p<0.01$) reduced the colon weight and length also. But, whey, LRFW, LFFW, LRWS and LFWS ($p<0.01$) fed animals prevented the shortening and diminution ($p<0.01$) in weight of colon even after colitis induction and brought the parameters close to normal healthy control (Fig. 4.5B-C).

4.5 Hematological analysis of blood

Non colitis

The effect of probiotic fermented whey feeding on health status of animals was also determined by estimating different blood components. At the end of experimental feeding, all the animals were sacrificed and blood was taken in heparinized tubes for analysis by blood analyzer machine. Table 4.1 shows the results of hematological parameters such as hemoglobin (Hb), white blood cells (WBCs), red blood cell count (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH), where no significant differences between the control and probiotic treated groups were observed.

Colitis

Impact of DSS administration on induction of colitis like symptoms by stimulating inflammatory response were also evidenced by significantly increased neutrophils ($p<0.05$) and lymphocyte ($p<0.01$) cell count with reduced

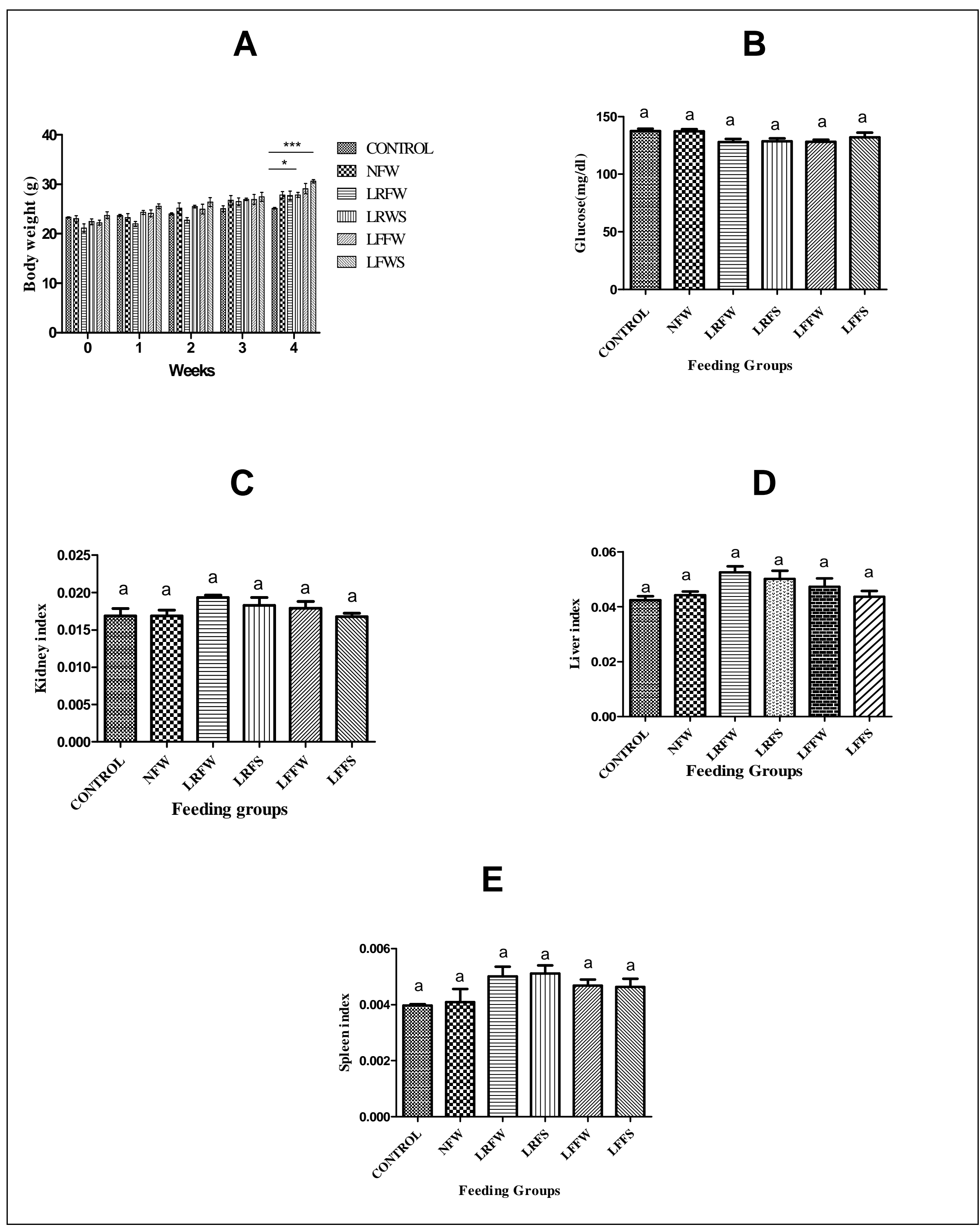

Figure 4.2 Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on body weight and organ indices

Values are expressed as mean ± SD (n = 5). Superscript letters indicate significant differences at $p<0.05$

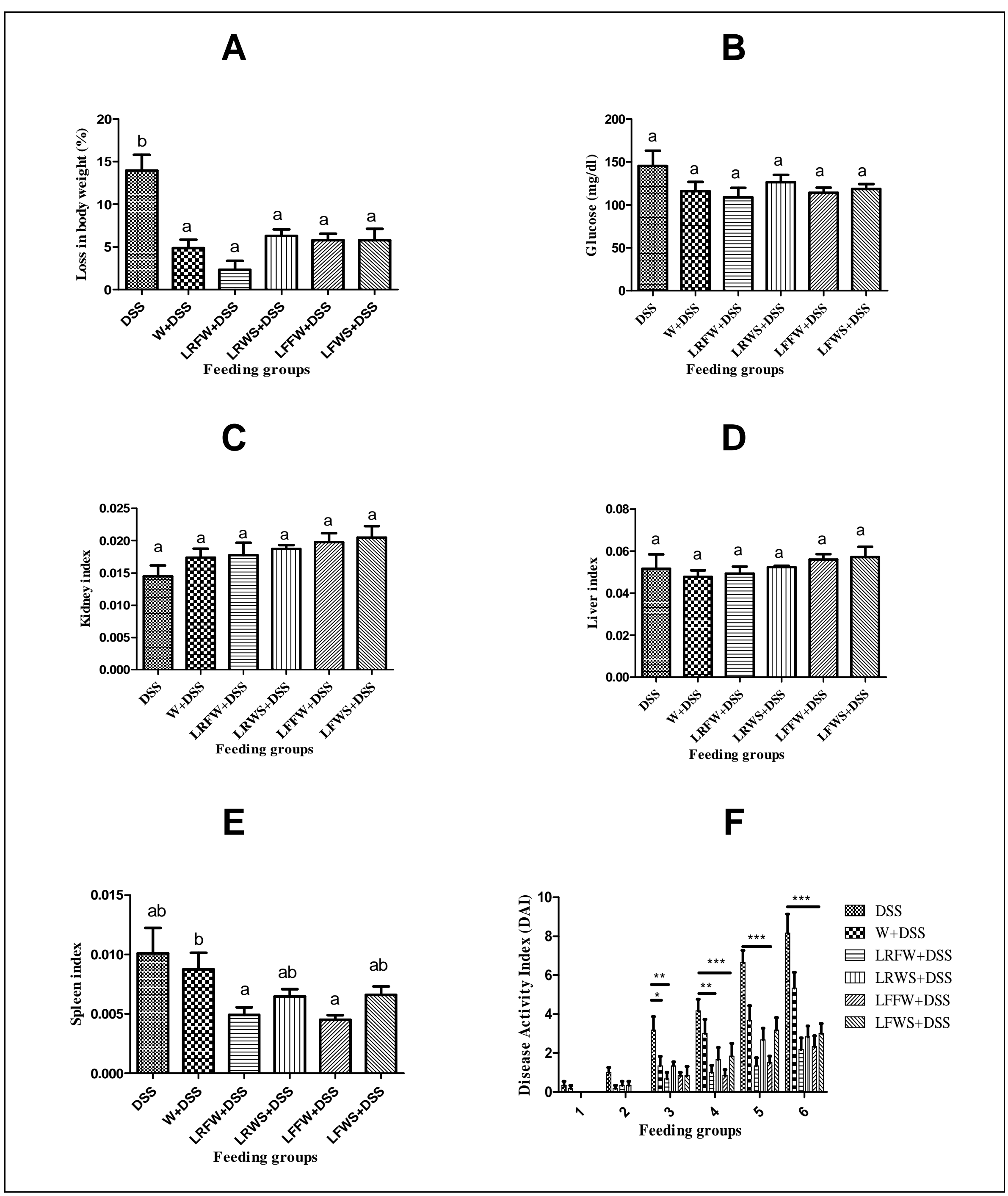

Figure 4.3: Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on body weight, organ indices and disease activity indices upon DSS challenge to induce colitis

Values are expressed as mean ± SD (n = 5). Superscript letters indicate significant differences at $p<0.05$

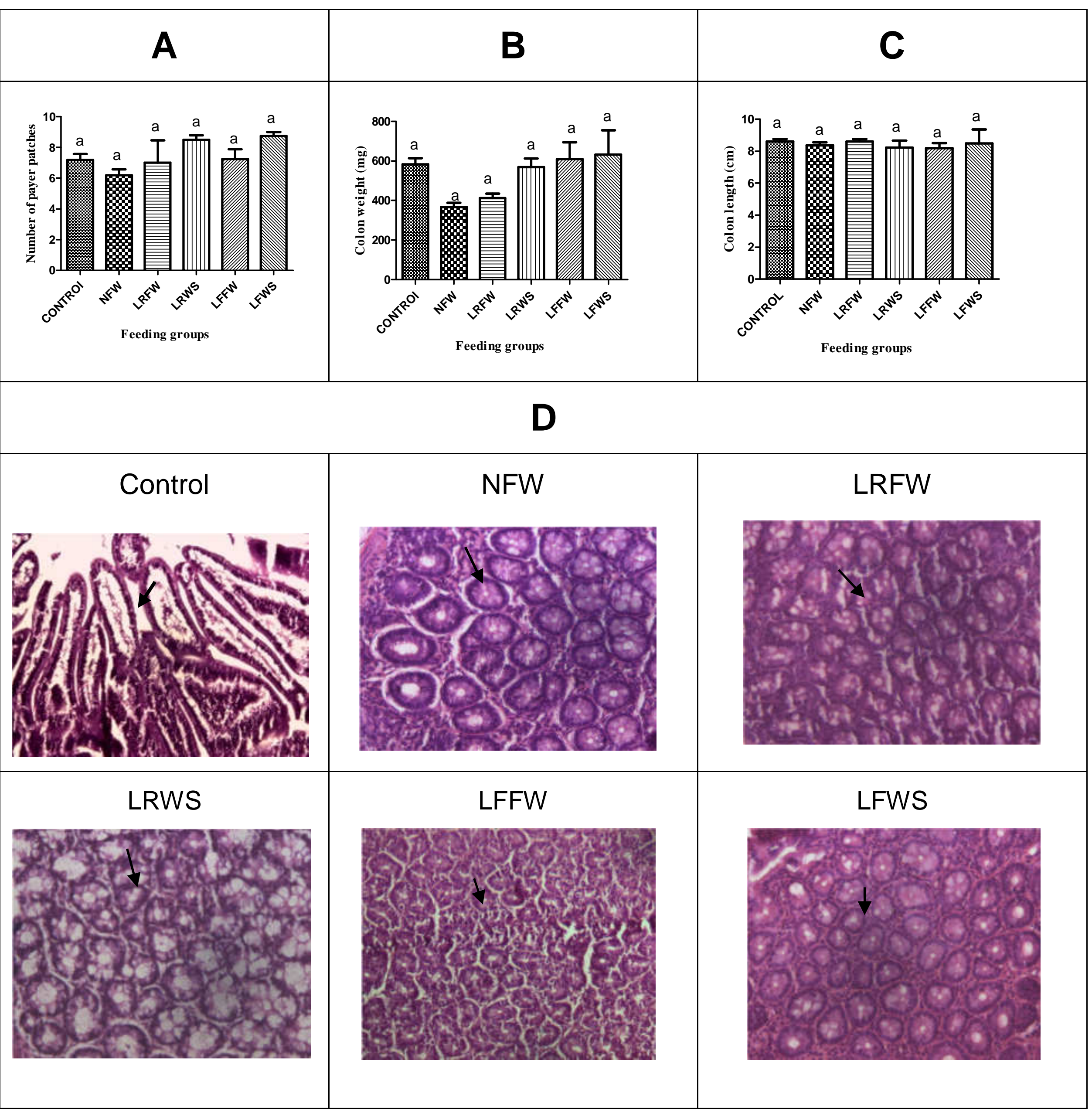

Figure 4.4: Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on intestinal health

A: Number of payer patches
B. Colon weight
C. Colon length
D. Histological examination of colon after H &E staining (at 400X)

Values are expressed as mean ± SD (n = 5). Superscript letters indicate significant differences at $p<0.05$

Table 4.1: Impact of probiotic fermented whey consumption during on blood hematological parameters.

Hematological parameters	Control	NFW	LRFW	LRFS	LFFW	LFFS
Hb (g/dl)	12.7 ± 1.04[a]	13.4 ± 0.55[a]	16 ± 1.19[a]	14.2 ± 2.42[a]	12.7 ± 0.72[a]	12.7 ± 0.45[a]
WBC $10^{3/mm3}$	9.0 ± 1.02[a]	11.3 ± 5.09[a]	14.41 ± 2.93[a]	13.2 ± 3.65[a]	10.6 ± 2.55 [a]	11.2 ± 2.96 [a]
RBC $10^{6/mm3}$	8.4 ± 0.69 [a]	8.8 ± 0.44 [a]	8.6 ± 0.33 [a]	8.59 ± 0.34 [a]	9.5 ± 0.52 [a]	8.7 ± 1.1 [a]
MCHC (g/dl)	36.8 ±1.0 [a]	37.7 ±0.86 [a]	36.6± 0.90 [a]	37.7 ± 1.6 [a]	38.5 ± 0.81 [a]	37.4 ± 0.93 [a]
MCV (FI)	55.6 ± 0.50 [a]	56.4 ± 1.3 [a]	55.5 ± 0.32 [a]	57.2 ± 2.26 [a]	49.9 ± 3.6 [a]	60.76 ± 2.4 [a]
MCH (pg)	15.08 ± 0.21 [a]	15.10 ± 0.30 [a]	15.14 ± 0.35 [a]	14.72 ± 0.47 [a]	16.68 ± 0.94 [a]	16.6 ± 5.42 [a]

Values are mean ± SD of five animals. Values with different alphabet are significantly different at $p<0.05$

($p<0.01$) hemoglobin content which appeared to reverse upon consumption of LRFW and LFFW (Table 4.2).

4.6 Histological examination of intestine and histological score of non-colitis and colitis animals

Non-colitis

Histopathology is the study of biological tissues at microscopic level to evaluate the disease associated with that tissue and to observe it at very fine detail. The results of histopathological examination of intestinal tissues after probiotic fermented whey and its supernatant supplementation are shown in (Fig. 4.4D). Animals of non-coltis groups as showed by normal colons with full organ preservation and absence of inflammation in villi.

Colitis

While Histological abrasions of colon in DSS and W+DSS group fed mice were recorded with severe grade of inflammation as observed by loss in colon architecture, erosion of epithelium by infiltration of neutrophils, damaged lamina propria with marked increase in thickness of muscular layer than healthy control animals. Conversely, feeding animals with LRFW and LFFW significantly lowered ($p<0.01$) histological score where mice exhibited almost intact colon histology by preserving crypt architecture due to reduced neutrophil infiltration. Similarly, mice fed with LRWS and LFWS showed mild to moderate grade of inflammation in histopathology with the non-significant decrease in histological score as compared to DSS control group (Fig. 4.5 D& E).

4.7 Immune responses in the gastrointestinal tract

Non-colitis

MPO activity

Pre-feeding of LRFW and LFFW prior to colitis induction were also confirmed by non-significant change in MPO activity inflammatory bio-markers on (Fig. 4.6A)

Release of inflammatory markers

Effect of feeding NFW and different fermented whey cultures of lactobacilli (*L. fermentum* MTCC 5898 and *L. rhamnosus* MTCC 5897) and their supernatant on release of cytokines depicted by the set of graphs in figure 4.6B.

In NFW fed group there was no significant variation in the proinflammatory cytokines (IL-4 and TNF-α) than control group. Similarly, LFFW and LRFW and their supernatant also showed no significant variation in the proinflammatory cytokines IL-4 and TNF-α release. Likewise, in CRP level there was no statistically significant differences observed between the experimental and control group (Fig. 4.6B). However, TGF-β brings the non-significant increase in LFFW and LRFS group while the remaining experimental groups showed no major change as compared to control group. Moreover, change in total IgA level in intestinal fluid as shown in figure 4.6A. No statistically significant differences in IgA level were observed among the experimental groups over the 28 days study duration.

Estimation of gene expression of immune related genes

The LRFS and LFFS groups displayed significant increase in β-defensin-2 as compared to control and NFW group. However, there was no significant difference observed in LRFW and LFFW fed groups in transcript level of β-defensin-2 (Fig. 4.7B). On the other hand, gene expression of TLRs after lactobacilli fermented whey (LR and LF) and their supernatant were depicted in figure. 4.7B. Non-significant change in TLR-4 expression was observed in all of experimental groups. While, statistically significant variation was observed in LRFS fed group among all the experimental groups in transcript level of TLR-2 than control and NFW groups after 28 days feeding period.

Colitis

MPO activity

The results of reduction in inflammatory bio-markers on pre-feeding of LRFW and LFFW prior to colitis induction were also confirmed by significantly reduced MPO activity (Fig. 4.8A) as a direct measure of neutrophils infiltration in intestinal tissue as shown previously through histopathological examination.

Release of inflammatory markers

Animals exposed to DSS also stimulated inflammatory response by significantly enhancing ($p<0.01$) pro-inflammatory cytokines such as IL-4 and TNF-α along with C reactive protein in intestine. Pre-feeding of LFFW and LRFW before colitis induction with DSS reduced the levels of these inflammatory

Table 4.2: Impact of probiotic fermented whey consumption during colitis induction on blood hematological parameters

Hematological parameters	DSS	W+DSS	LRFW+DSS	LRWS+DSS	LFFW+DSS	LFWS+DSS
Hb (g/dL)	7.82±1.6^{b}	11.05±1.9^{a}	13.9±0.9^{a}	12.65±1.2^{a}	13.13±2.44^{a}	11.53±1.82^{ab}
WBC ($10^{3/}mm^{3}$)	12.16±4.7^{a}	11.43±4.4^{a}	8.38±1.4^{a}	9.13±2.8^{a}	9.45±1.87^{a}	10.85±4.65^{a}
RBC ($10^{6/}mm^{3}$)	4.51±0.9^{a}	7.71±1.8^{a}	8.61±0.7^{a}	8.12±0.6^{a}	8.46±1.49^{a}	8.26±1.75^{a}
MCV (Fl)	54.95±2.5^{a}	56.52±1.3^{a}	54.95±1.0^{a}	54.7±1.8^{a}	55.08±1.53^{a}	56.05±1.43^{a}
MCHC (g/dL)	26.75±1.1^{b}	28.83±0.4^{ab}	31.58±1.6^{a}	29.43±2.1^{ab}	29.98±1.18^{c}	28.65±1.00^{bc}
Neutrophil ($10^{3}/\mu L$)	4.7±2.1^{b}	2.9±1.4^{ab}	1.5±0.8^{a}	2.1±1.0^{ab}	3.09±1.89^{b}	2.60±1.78^{b}

Values are mean ± SD of five animals. Values with different alphabet are significantly different at $p<0.05$

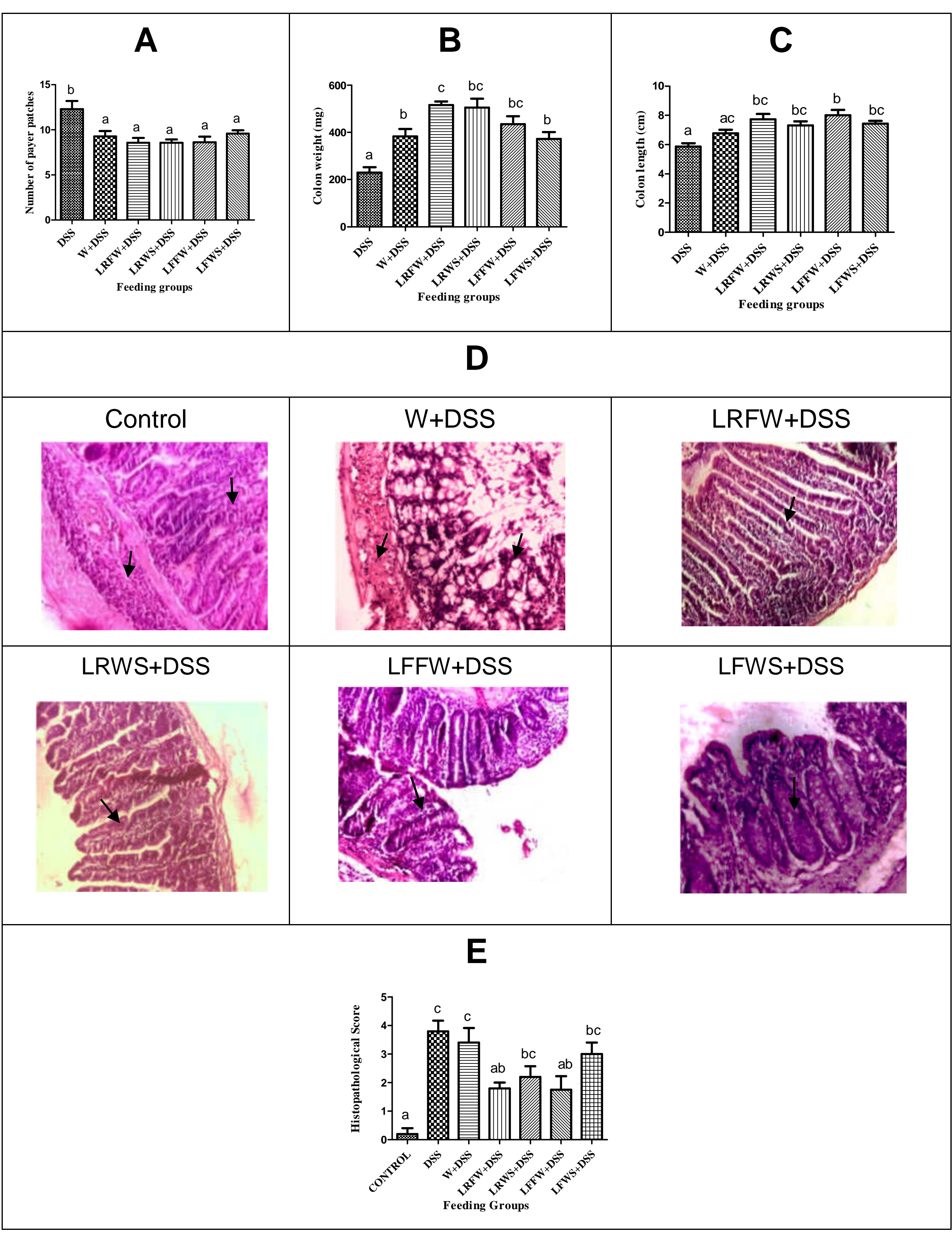

Figure 4.5: Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on intestinal health upon DSS challenge to induce colitis A: Number of payer patches B. Colon weight C. Colon length D. Histological examination of colon after H &E staining (at 400X) E. Histological score

Values are expressed as mean ± SD (n = 5). Superscript letters indicate significant differences at $p<0.05$

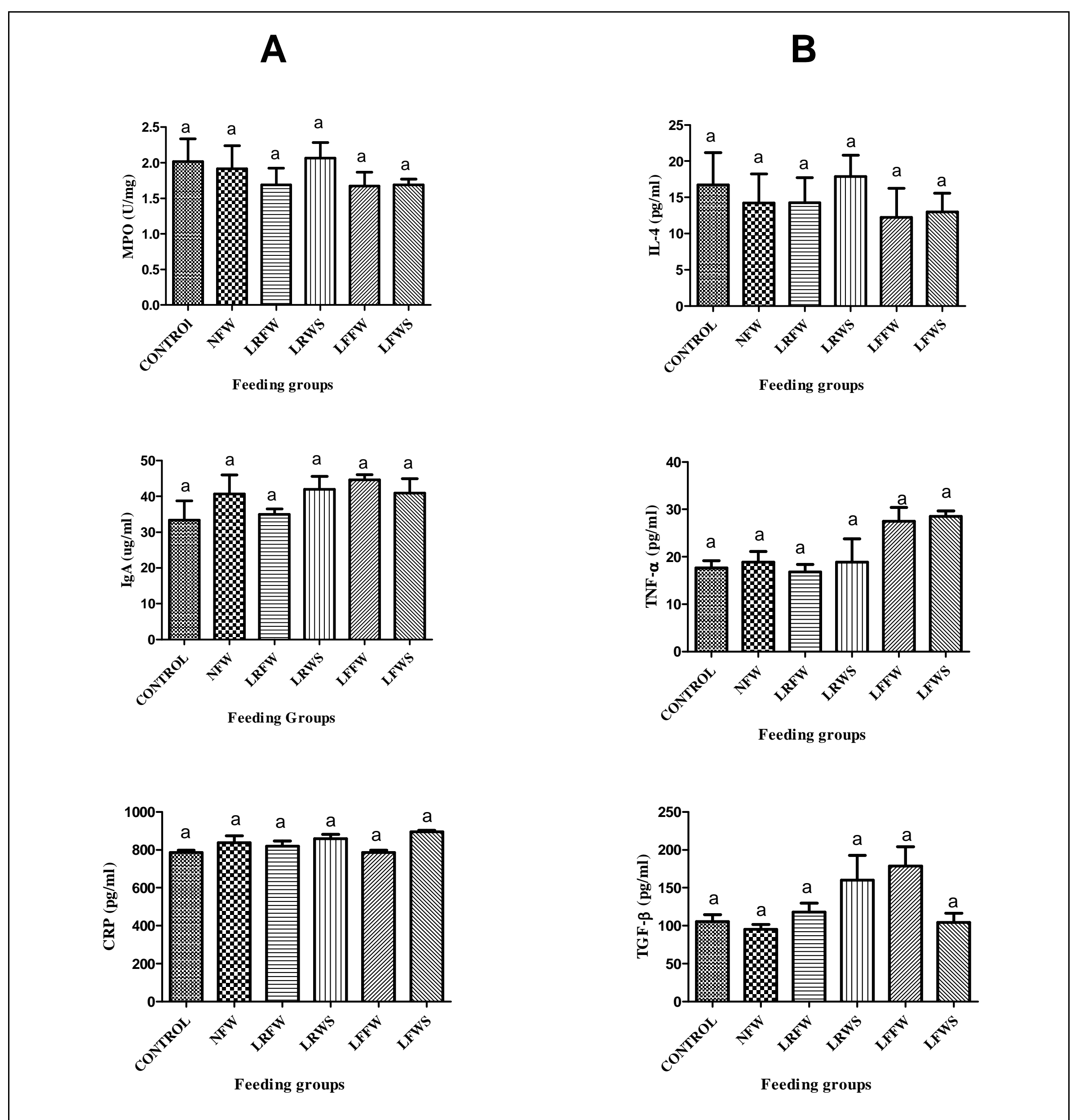

Figure 4.6: Effect of feeding probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey on immune response in the gastrointestinal tract.

A. Inflammatory markers
B**.** Cytokine level
Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at p<0.05

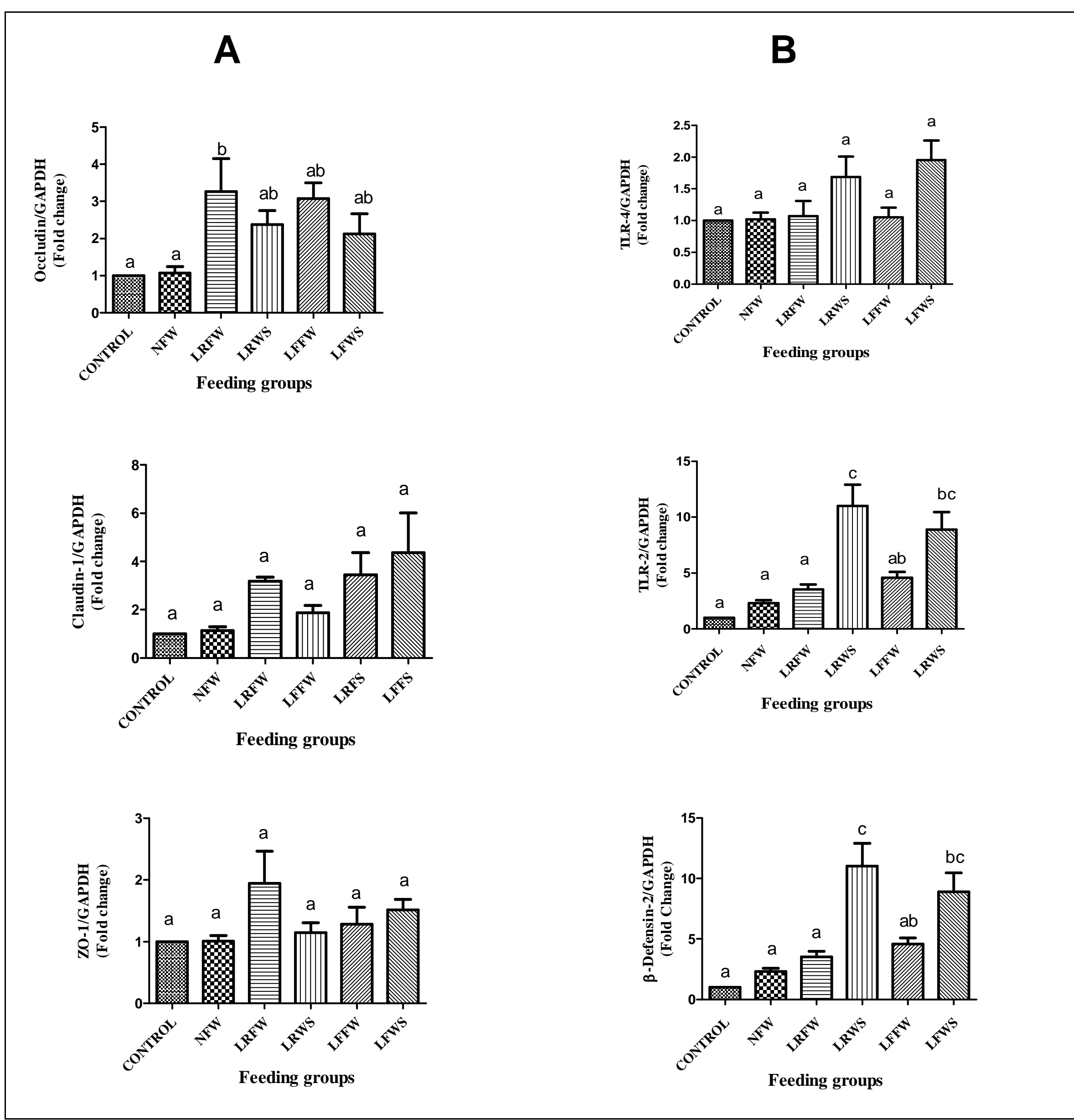

Figure 4.7: Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on restoration of gut barrier functions

A. Tight junctional gene expression

B. Immune gene expression

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

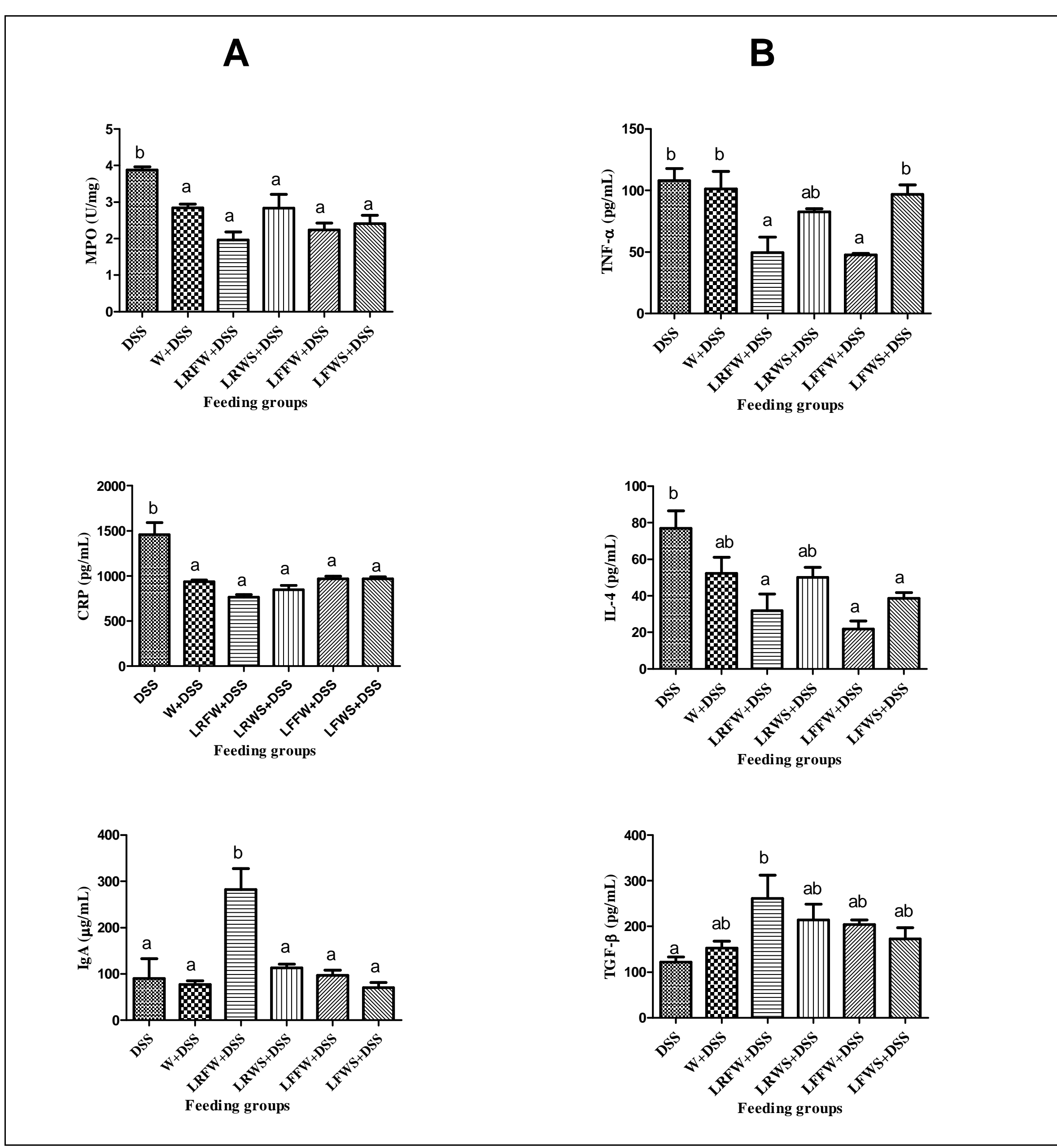

Figure 4.8: Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on immune response in the gastrointestinal tract after DSS challenge to induce colitis

A. Inflammatory markers
B. Cytokine released

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

substances effectively (Fig. 4.8B). Contrarily, the levels of regulatory cytokine TGF-β in intestine increased significantly ($p<0.05$) in LRFW and LFFW fed group than other groups of test animals (Fig. 4.8B). The sIgA levels in intestinal fluid increased tremendously ($p<0.01$) on feeding probiotic *L. rhamnosus* fermented whey as compared to control groups (Fig. 4.8A). Whereas in case of PFWS where live probiotic bacteria were absent, insignificant changes in intestinal sIgA were observed with respect to control animals.

Estimation of gene expression of immune related genes

In case of hBD-2, the RNA expression was reduced significantly ($p<0.05$) upon PFW consumption than DSS control groups which again indicated prevention of inflammation induced by DSS (Fig. 4.9B). Transcriptional expression of pathogen recognition receptor TLR-4 in colon of DSS group increased significantly ($p<0.05$) whereas mRNA expression of TLR-2 was found more in *Lactobacillus* probiotic fermented whey fed animals than control (Fig. 4.9B).

4.8 Restoration of gut barrier functions

Non colitis

Estimation of tight junction gene expression

The expression level of tight junctional genes was estimated by quantitative PCR at different combination (NFW, LRFW, LFFW, LRFS, LFFS). The results showed that LRFW and LFWS increased significantly while LRWS and LFFW not exhibited any significant change in the transcript level of occludin as compared to control and NFW. Claudin-1 and ZO-1 gene expression did not exhibited any significant change among all the experimental groups than control and NFW (Fig. 4.8A).

Visualization of structural changes in tight junctional and cytoskeletal proteins

The immuno-staining of junctional protein actin (Plate 4.3), claudin-1 (Plate 4.4), ZO-1 (Plate 4.5) and occludin (Plate 4.6) in intestinal sections showing their preserved and localized distribution and also established intact

epithelial barriers on consumption of probiotic fermented whey and its supernatant by appearance of regular and intense green fluorescence.

Colitis

Estimation of tight junction gene expression

Transcriptional expression of genes associated with intestinal barrier integrity *occludin, claudin*-1 and *zonula-occluden*-1 were effectively ($p<0.01$) enhanced on PFW consumption having live *L. rhamnosus* than control and PFWS fed animals which was devoid of probiotic microbes consumption respectively (Fig. 4.9A).

Visualization of structural changes in tight junctional and cytoskeletal proteins

The immuno-staining of junctional protein actin (Plate 4.7), claudin-1 (Plate 4.8), ZO-1 (Plate 4.9) and occludin (Plate 4.10) in intestinal sections also established intact epithelial barriers on consumption of probiotic fermented whey and its supernatant by appearance of regular and intense green fluorescence showing their preserved and localized distribution than DSS induced colitis.

Gut permeability assessment

Direct evidence of gut integrity was also confirmed by measuring the leakage of para-cellular tracer FITC-dextran in serum which assessed the stage of severity of infection in mice. The mice treated with DSS showed higher leakage of tracer (548 ± 45.1 ng/mL) in serum and confirmed greater barrier permeability. The oral administration of whey, LRFW, LFFW and LRWS recorded significant lower ($p<0.01$) presence of FITC-dextran ($p<0.05$) in serum (Fig. 4.9C) though LFWS feeding did not appear much effective statistically.

4.9 Bacterial load in feces

The results of feeding probiotic fermented whey and its supernatant on fecal bacterial count are shown in figure 6A &B. Intestinal gut microbial ecology changed with the state of disease after consumption of probiotic fermented whey. After 28 days of experimental feeding, fecal lactobacilli colonies on MRS agar were significantly higher ($p<0.05$) after LRFW, LFFW, LRWS and LFWS consumption as compared to DSS control group. In contrast, *E. coli* colonies on

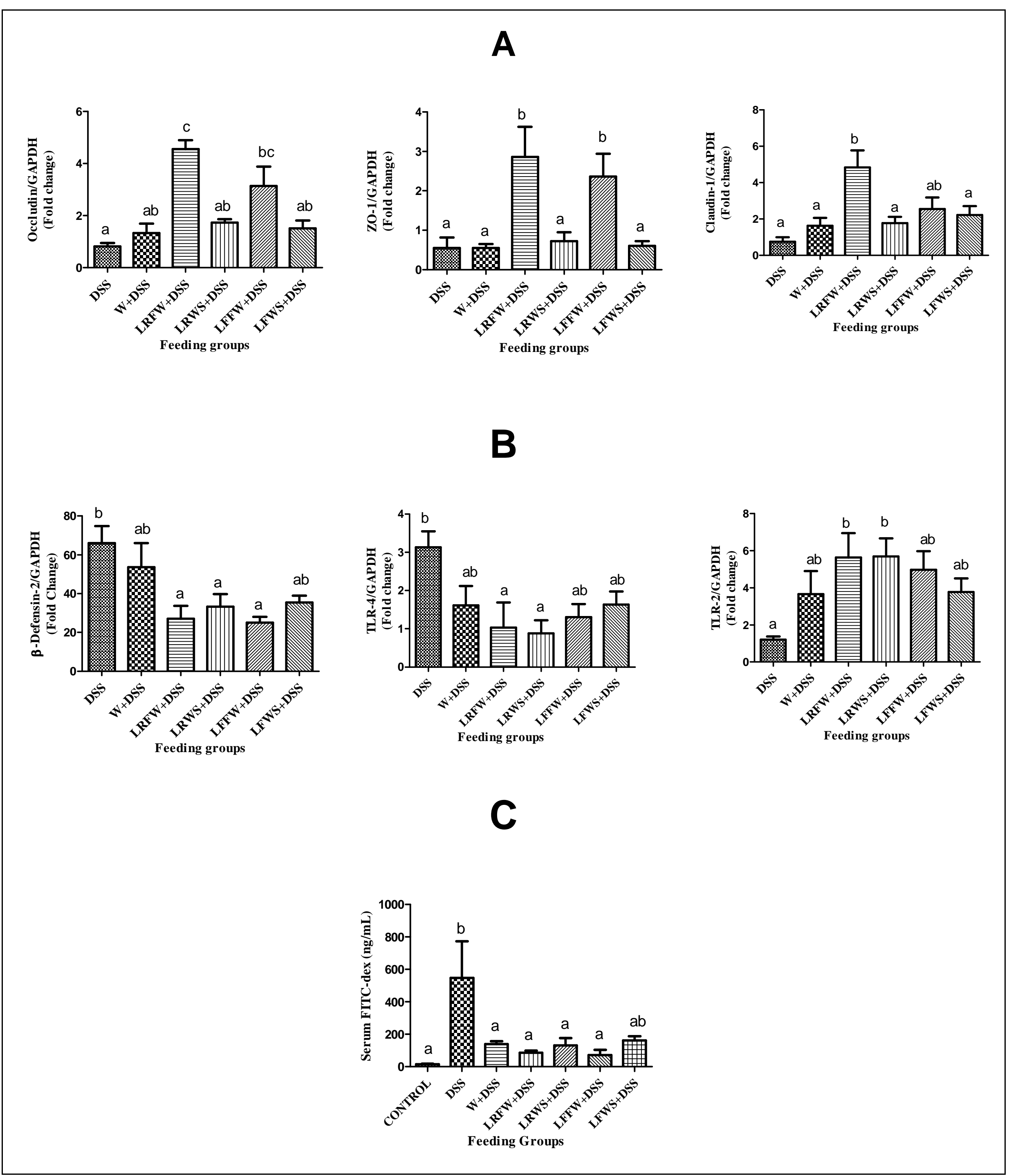

Figure 4.9: Effect of feeding *Lactobacillus* probiotic (*L. fermentum* and *L. rhamnosus*) fermented whey on restoration of gut barrier functions upon DSS challenge to induce colitis.

A. Tight junctional gene expression
B. Immune gene expression
C. FITC-dextran level in serum

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

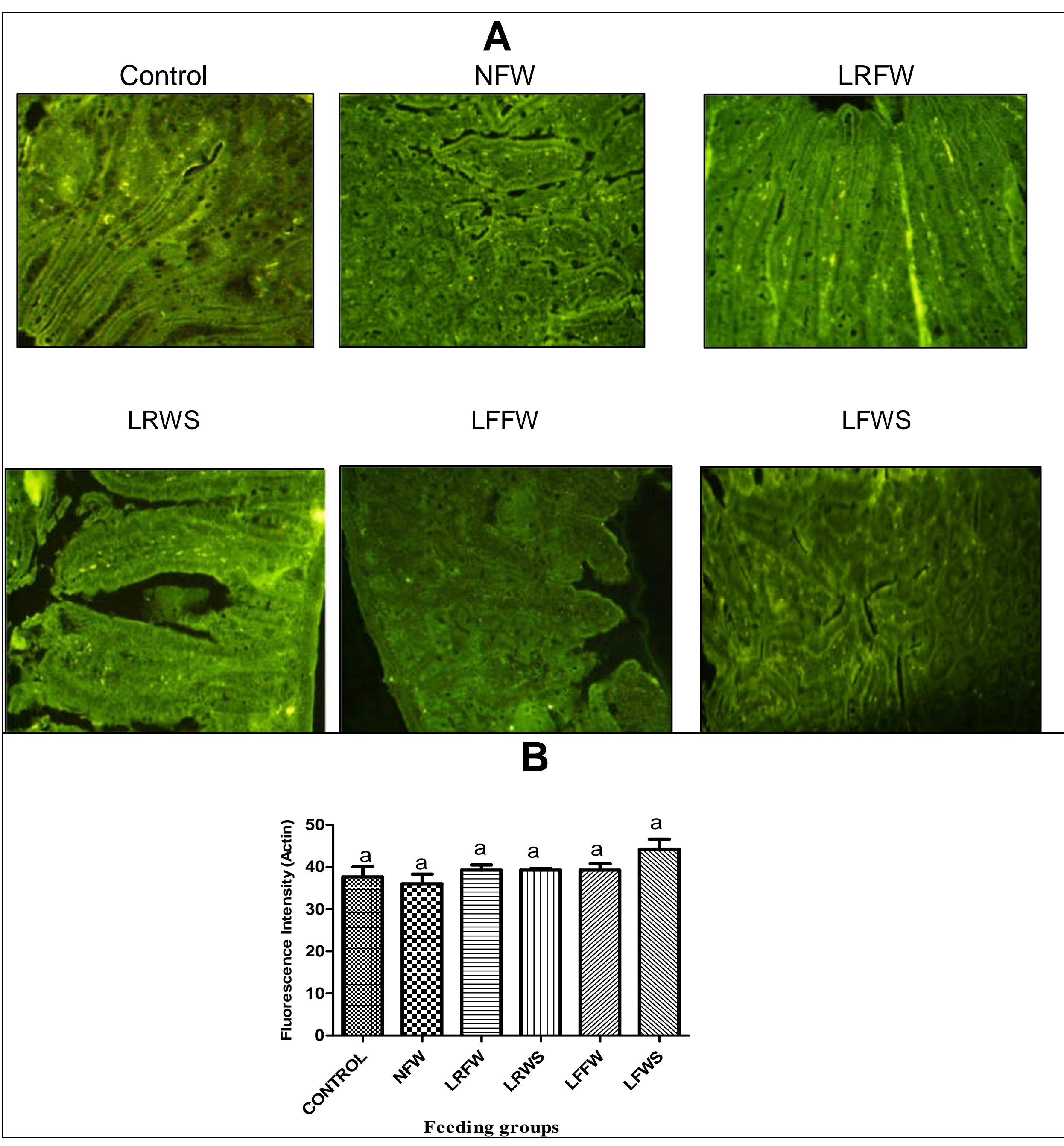

Plate 4.3 Representative images displaying cytoskeleton protein (Actin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey

A. Immunofluorecent Images
B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 2). Superscript letters indicate significant differences at $p<0.05$

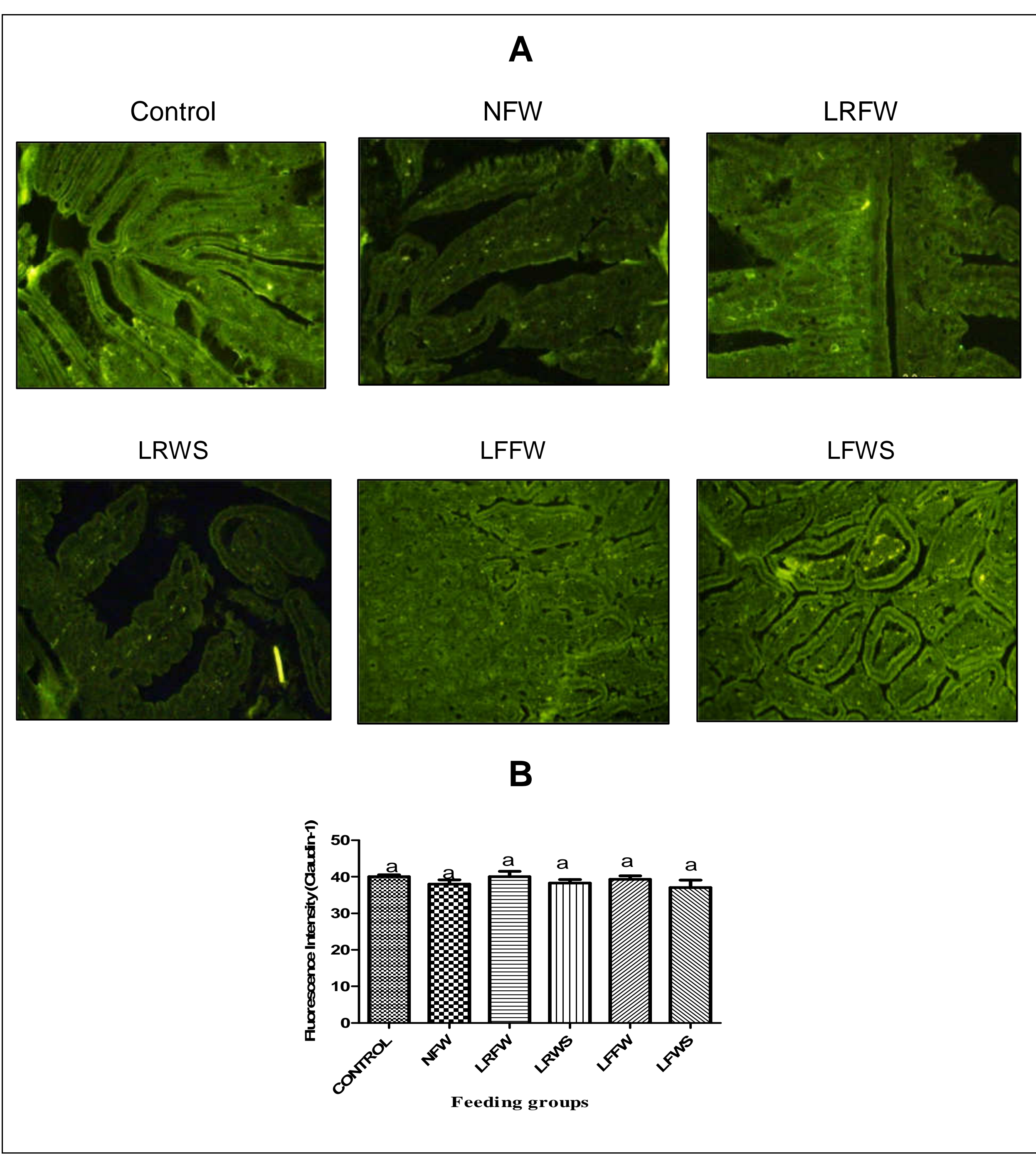

Plate 4.4 Representative images displaying cytoskeleton protein (Claudin-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 2). Superscript letters indicate significant differences at $p<0.05$

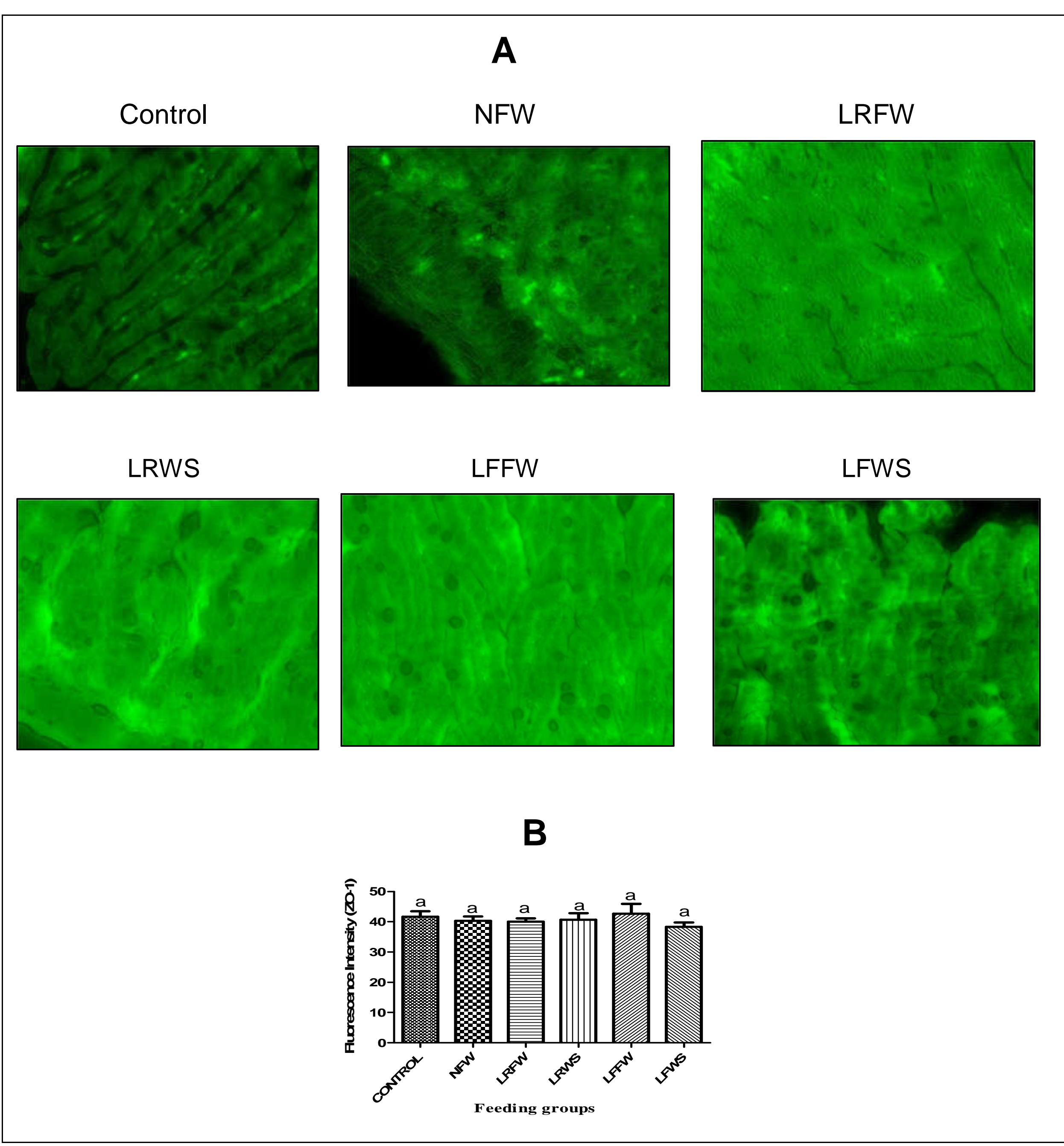

Plate 4.5 Representative images displaying cytoskeleton protein (ZO-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 2). Superscript letters indicate significant differences at $p<0.05$

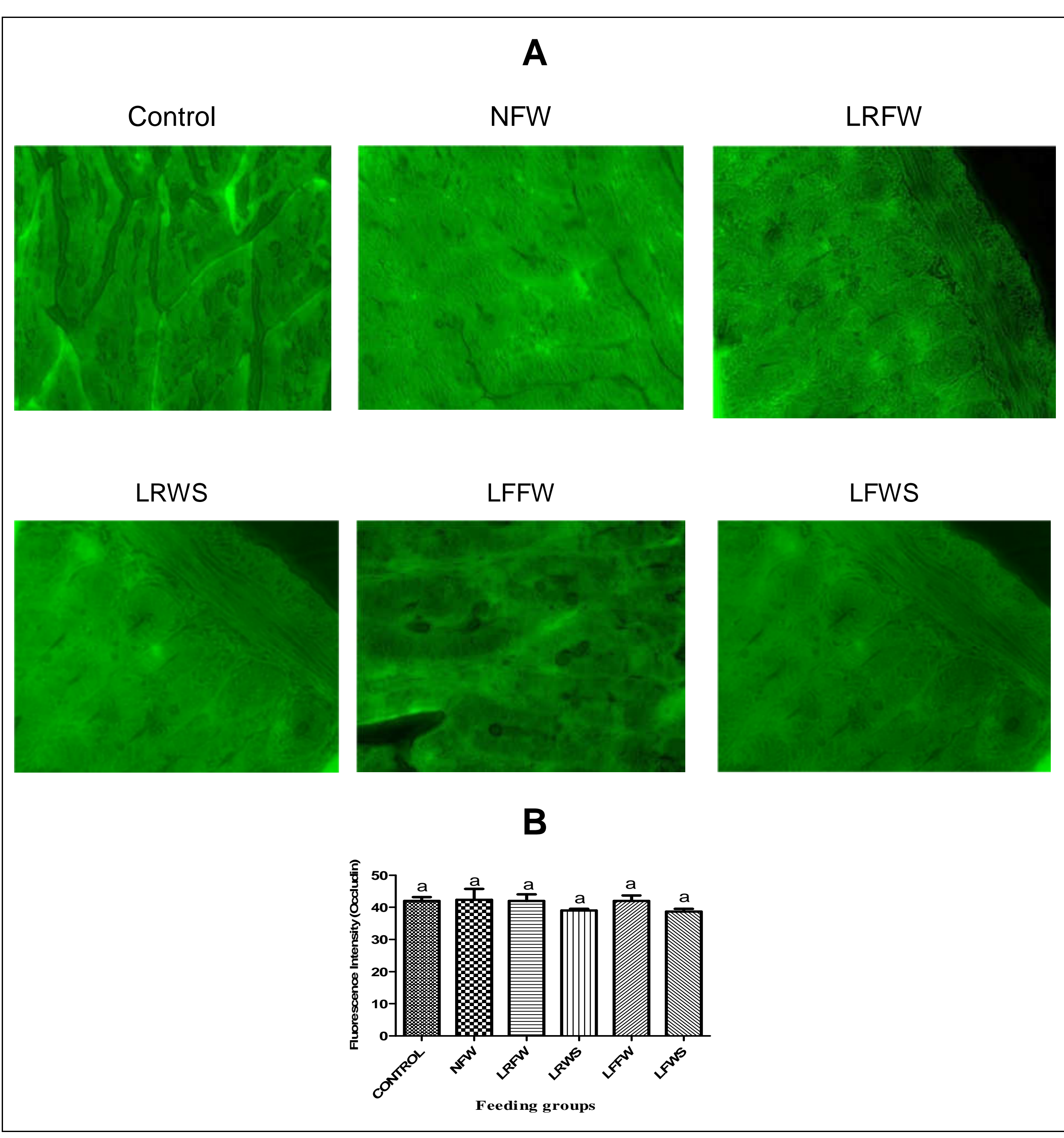

Plate 4.6 Representative images displaying cytoskeleton protein (Occludin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

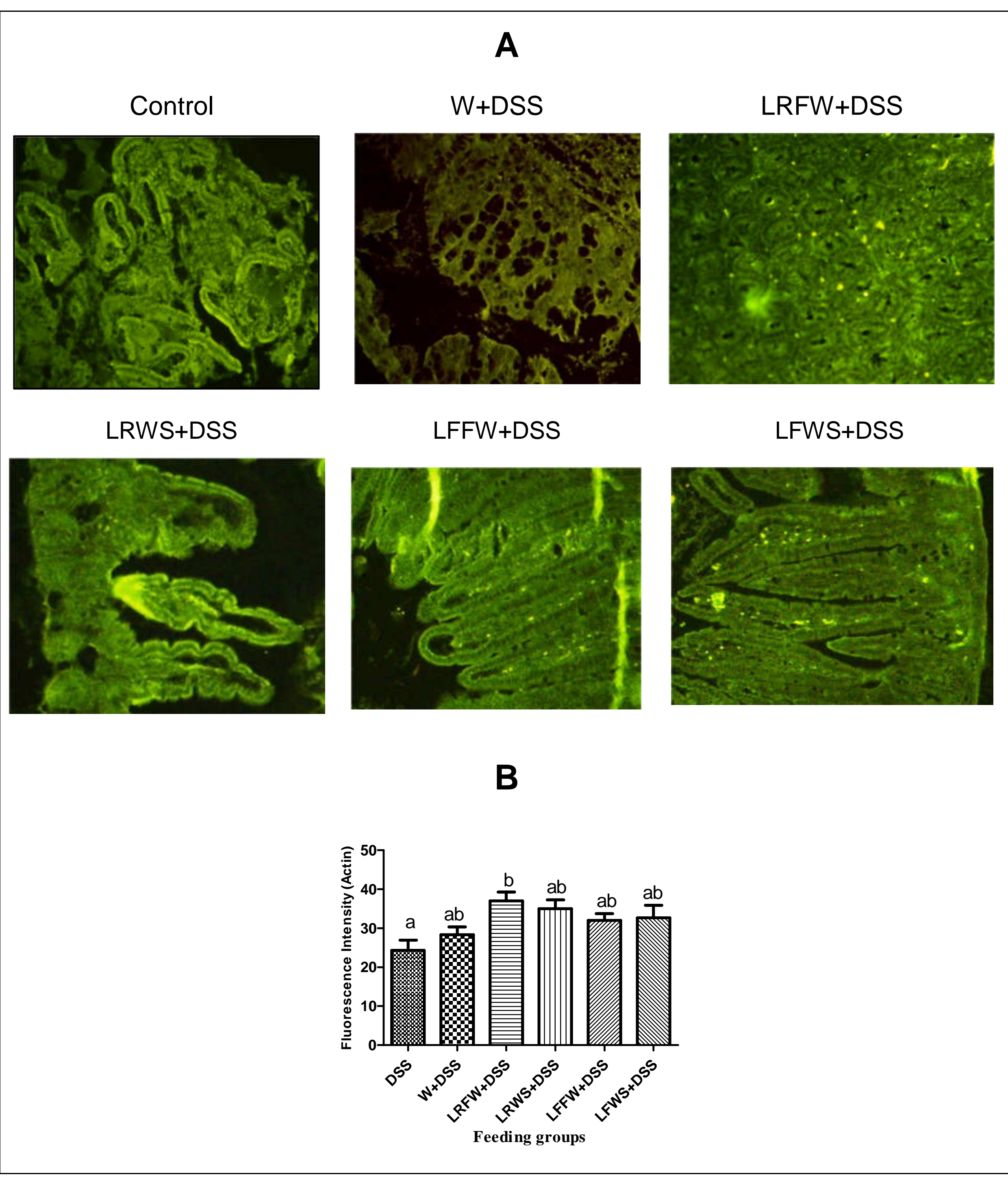

Plate 4.7 Representative images displaying cytoskeleton protein (Actin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis

A. Immunofluorecent Images
B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 2). Superscript letters indicate significant differences at $p<0.05$

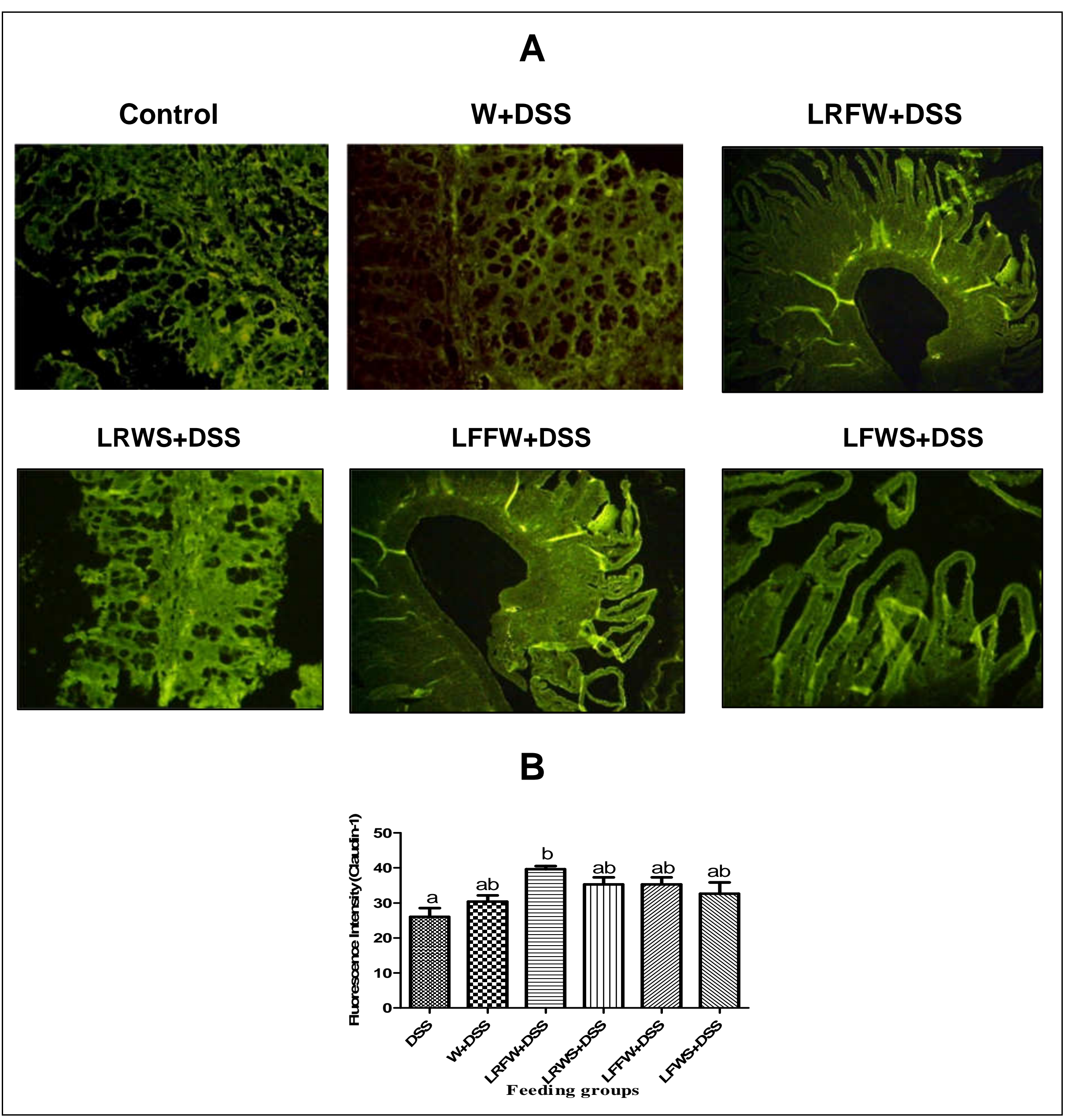

Plate 4.8 Representative images displaying cytoskeleton protein (Claudin-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 2). Superscript letters indicate significant differences at $p<0.05$

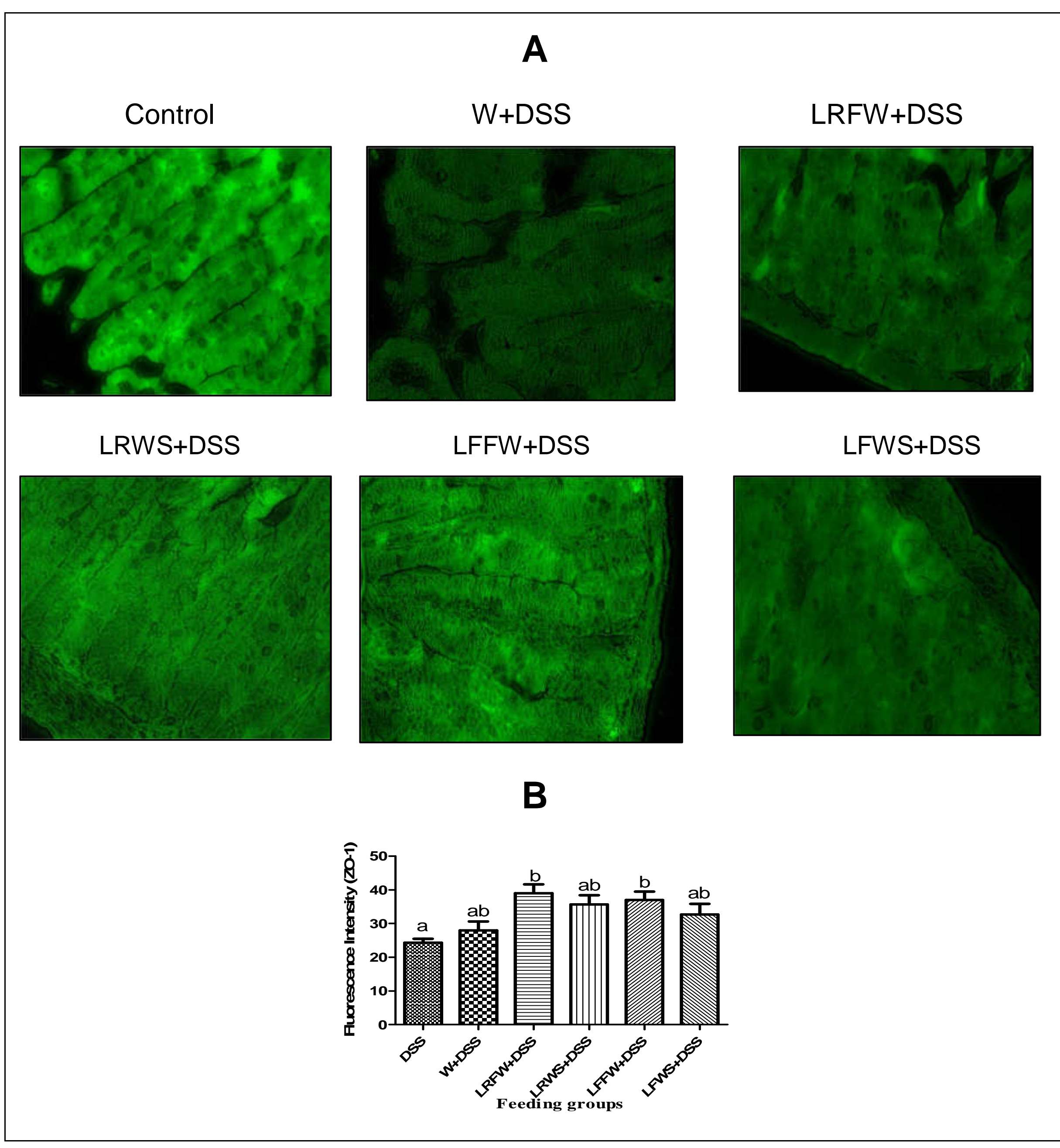

Plate 4.9 Representative images displaying cytoskeleton protein (ZO-1) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

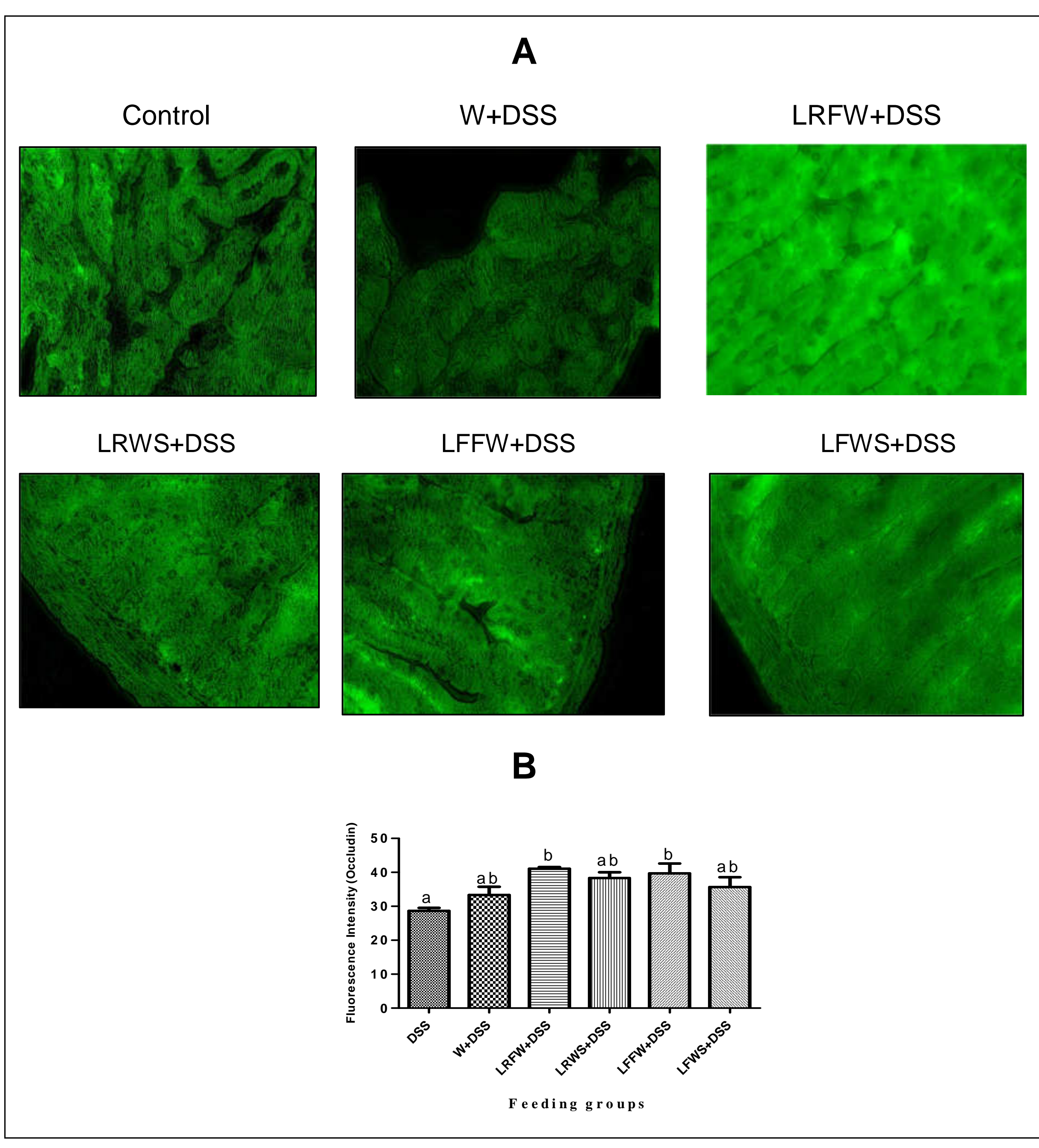

Plate 4.10 Representative images displaying cytoskeleton protein (Occludin) by immunofluorescence after intervention of probiotic *Lactobacillus* (*L. fermentum* and *L. rhamnosus*) fermented whey upon DSS challenge to induce colitis

A. Immunofluorecent Images

B. Fluorescent intensity

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

EMB agar dropped significantly ($p<0.05$) after LRFW and LRWS consumption than DSS induced colitis animals. However, no change was observed in LFFW and LFWS groups.

4.10 Morphological features of intestinal epithelial cells (HT-29 cells)

The human colorectal adenocarcinoma cell line (HT-29) used under present investigation were cultured using RPMI containing antibiotics (penicillin G, streptomycin) and 10% fetal bovine serum (FBS) under humified conditions at 37℃ in an incubator using 5% CO_2 level. Cells adhered to the surface of flask and obtained confluency in a span of 3 days their phenotype morphologically and functionally resemble the enterocytes lining of small intestine**.** Plate 4.11 represent the images of HT-29 cells on different days of culture.

4.11 MTT assay

The MTT assay was based on the reduction of the tetrazolium salt to blue formazan product by mitochondrial dehydrogenase actively of growing cells which revealed no significant changes when confluent HT-29 were treated with *L. fermentum* (1ng-100 µg) and *L. rhamnosus* (1ng-100 µg) simulated digested fermented whey for 4 hr respectively. These results clearly indicated that incubation of both probiotic fermented whey had no major effect on the proliferation and number of HT-29 is maximum at 50 µg during 4 hr incubation when compared to control (Fig. 4.10).

4.12 Impact of simulated digested probiotic fermented whey on expression of genes associated with epithelial barrier function during challenge with inflammatory agent (*E. coli*)

Occludin

Exposure of intestinal cells to lactobacilli (LF and LR) fermented whey enhanced the mRNA expression of occludin significantly ($p<0.05$) than control as well as cells exposed to either of the inflammatory agents. Likewise, both probiotic lactobacilli fermented whey resulted into significantly higher ($p<0.05$) occludin mRNA expression as compared to control. On the other hand, mRNA expression of occludin in intestinal cells were 2.84 ± 0.52 and 2.42 ± 0.59 folds during exclusion of *E. coli* by SDFW and SDRW respectively (Fig. 4.11A).

Claudin-1

The mRNA expression of claudin-1 in HT-29 cells also decreased significantly ($p<0.05$) after *E. coli* incubation by reduction of 0.1833±0.1612 fold respectively (Figure C&D). On the other hand, exposure of intestinal cells to lactobacilli (SDFW and SDRW) fermented whey enhanced the mRNA expression of claudin-1 significantly ($p<0.05$). Similarly, exclusion (pre-treatment) of inflammatory agents by both probiotic lactobacilli fermented whey resulted into significantly higher ($p<0.05$) claudin-1 mRNA expression as compared to control. These results are shown in figure 4.11B.

Zonula occluden-1 (ZO-1)

Time dependent exposure of inflammatory agents (live *E. coli*) to HT-29 cells suppressed the expression of ZO-1 (Fig. 4.11C). However, the suppression was statistically non-significant after treatment of SDRW and SDRW respectively followed by *E. coli* treatment.

Human Beta defensin-2 (hBD-2)

Figure 4.12A represent the results of hBD-2 expression. In this case hBD-2 mRNA showed most dramatic increased expression by 5.436 ± 1.007 fold when HT-29 cells were exposed to live *E. coli* (1×10^8 cfu/ml). Corresponding SDFW showed remarkably lower hBD-2 expression (2.37 ± 0.32 fold) and SDRW (1.85 ± 3.43) during exclusion with *E. coli*. The gene expression data of hBD-2 clearly indicated the presence of healthy environment which lead to its reduced level of expression during presence of probiotic lactobacilli fermented whey than inflammatory alone which may be responsible for better junctional integrity.

4.13 Nuclear translocation of NF-κβ in intestinal cells

NF-κβ is a global transcription factor which involves in expressions of inflammatory response genes. Initially, *in vitro* effect of probiotic *Lactobacillus fermentum* (10 µg) or *Lactobacillus rhamnosus* (10 µg) simulated digested fermented whey and inflammatory agents live *E. coli* ($1\text{x}10^8$ cfu/ml) on nuclear translocation of p-65 subunit of NF-κβ was studied by western blotting. Later impact of probiotic strains on translocation of nuclear factor in the presence of inflammatory agents was studied.

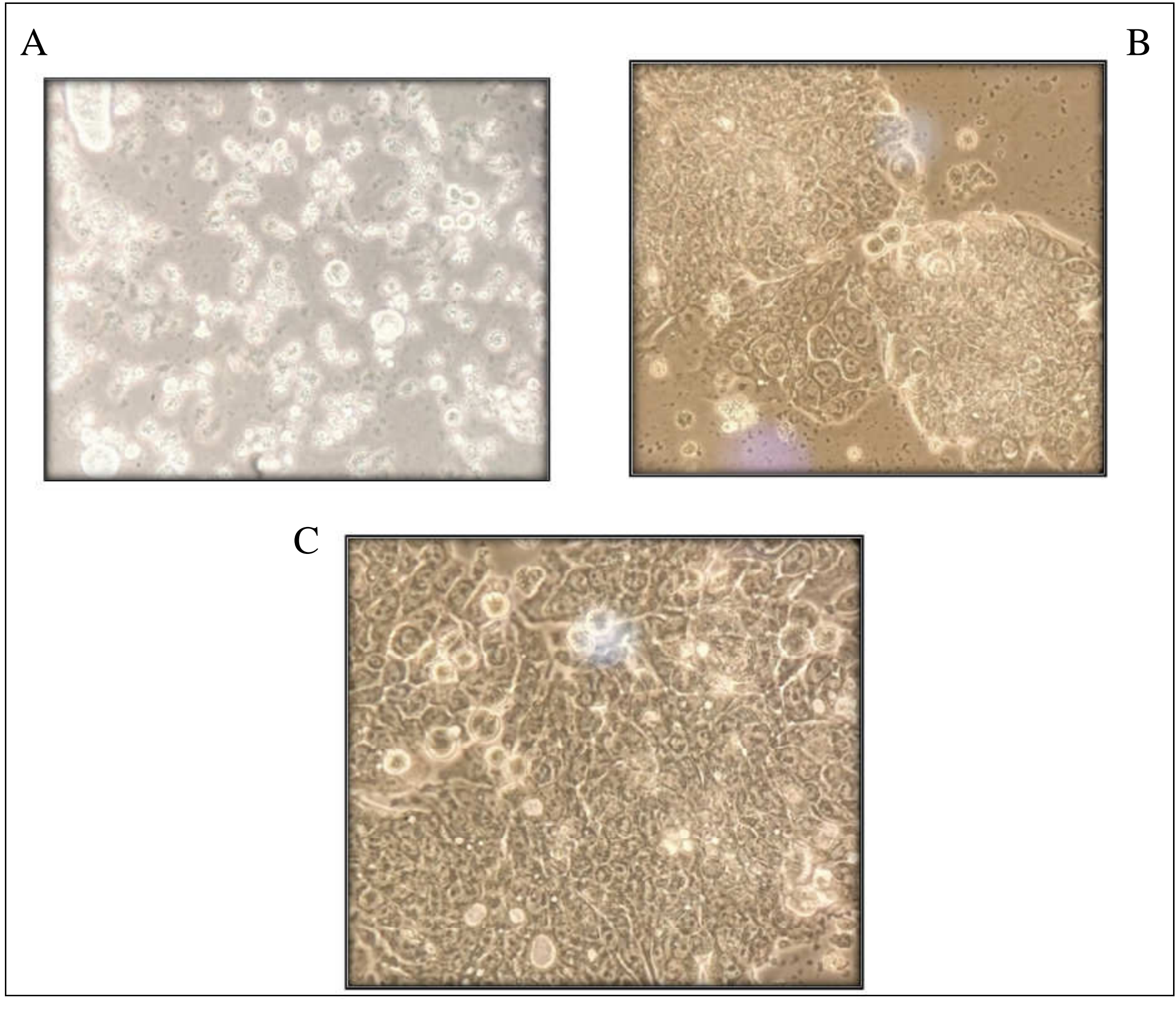

Plate 4.11: Representative images of HT-29 cells under phase contrast inverted microscope (40 X) on different days of culture A. 1st day B. 2nd day C. Confluent cells

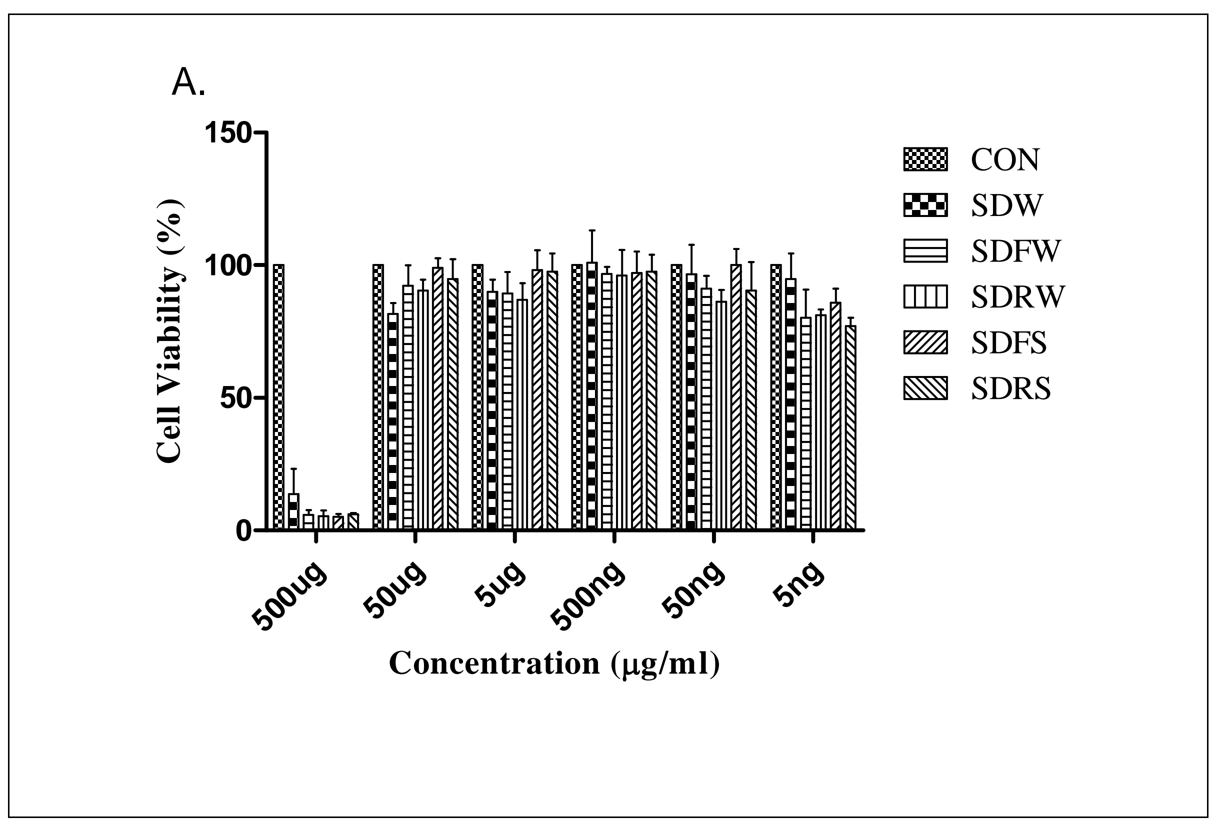

Figure 4.10: HT-29 cell viability upon exposure to variable concentration of simulated digested probiotic fermented whey (*L. fermentum* and *L. rhamnous*) and their supernatant

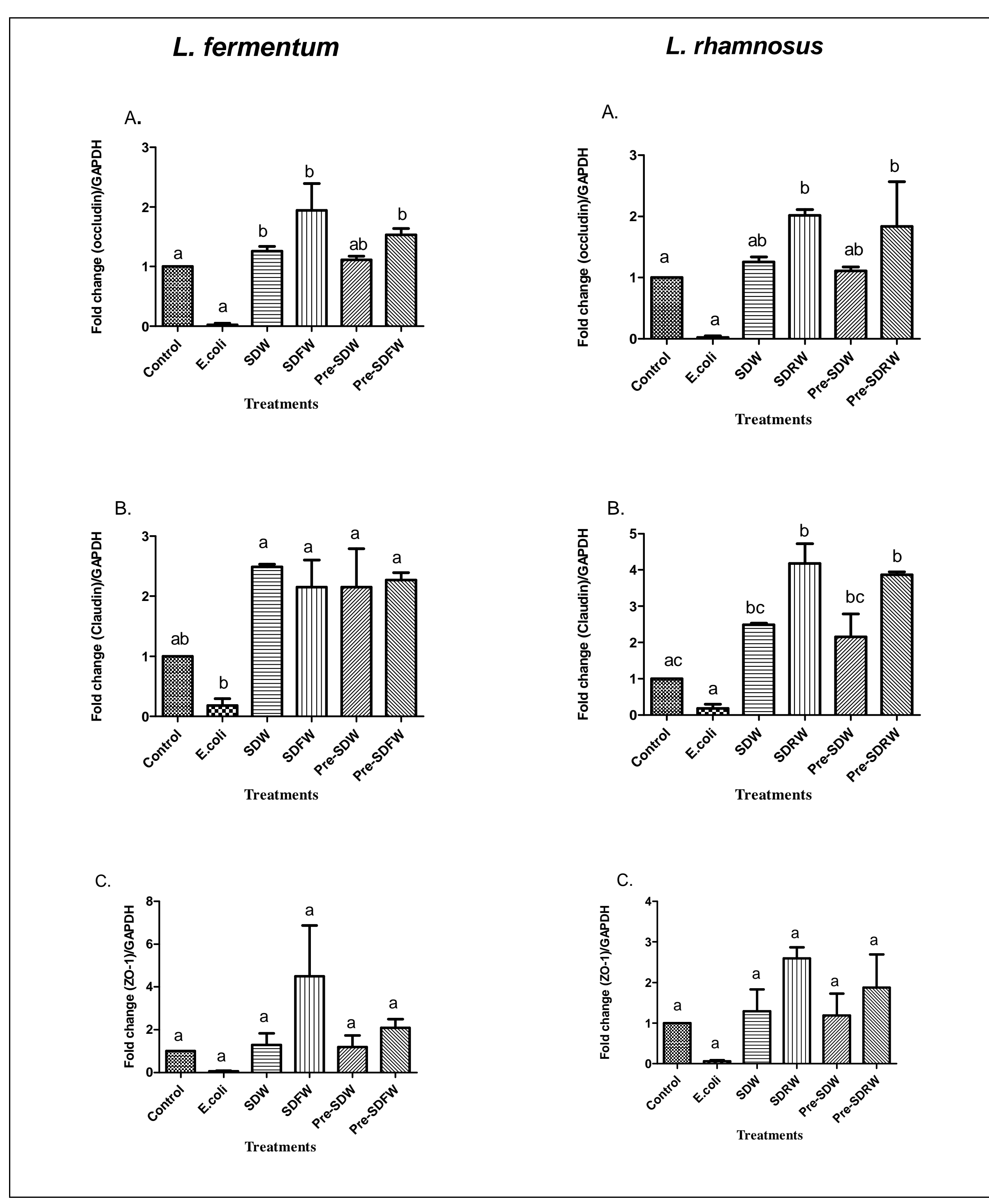

Figure 4.11 Transcriptional modulations in key genes associated with epithelial barrier function by simulated digested probiotic fermented whey (*L. rhamnosus and L. fermentum)*

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

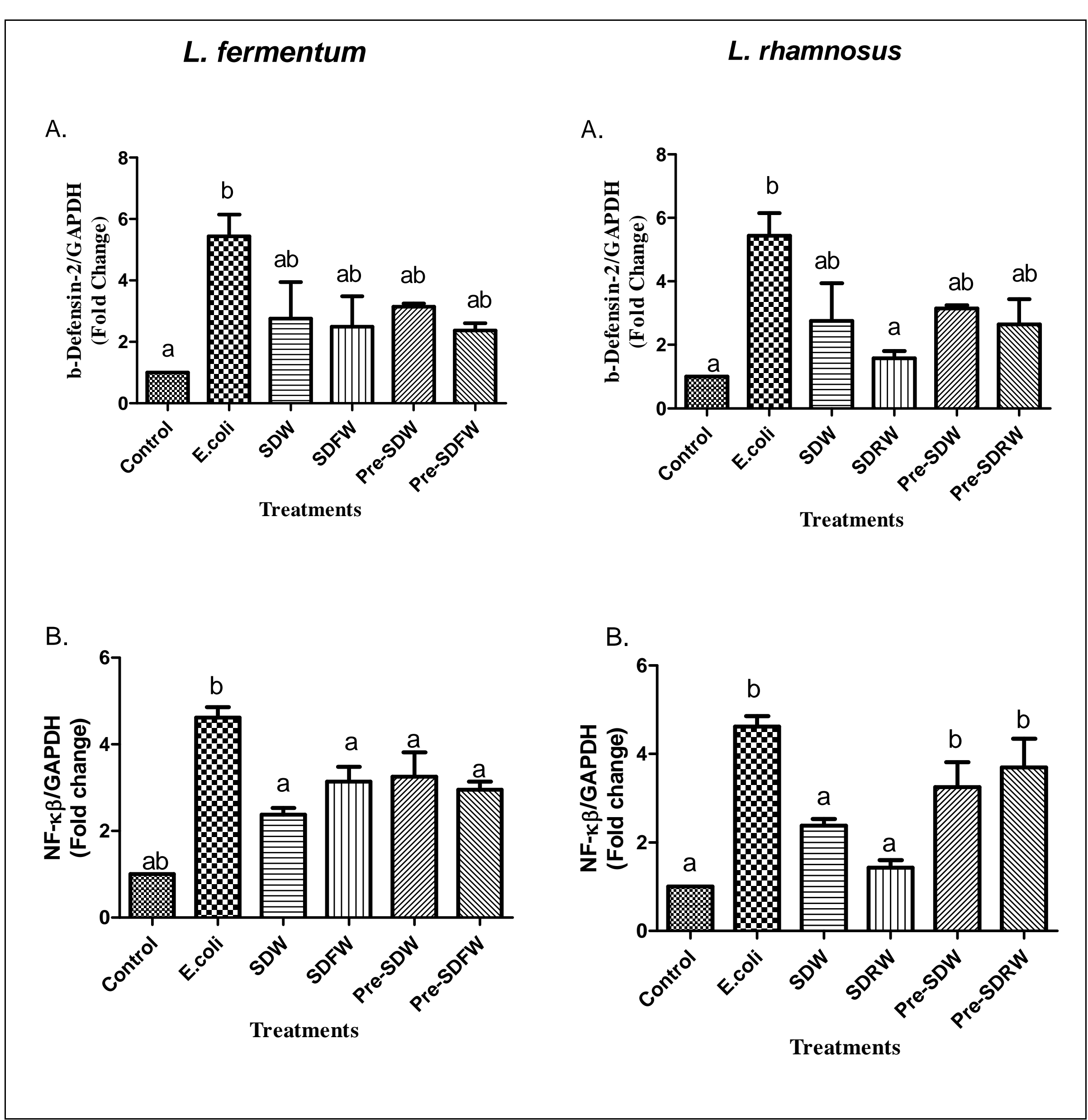

Figure 4.12: Transcriptional modulations in key genes associated with epithelial barrier function by simulated digested probiotic fermented whey (*L. rhamnosus and L. fermentum*) on HT-29 cells during exclusion of *E. coli*

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at p<0.05

4.14 Nuclear translocation of NF-κβ by western blotting NF-κβ (p-65) nuclear translocation in intestinal cells on challenge with inflammatory agents during the presence of simulated digested *L. fermentum* fermented whey

NF-κβ is a downstream transcription factor which regulate secretion of various cytokines. Therefore, to evaluate inhibitory effect of probiotic *L. fermentum* fermented whey NF-κβ (p-65 subunit) translocation from cytosol to nucleus was also determined in intestinal cells by western blotting. The results of NF-κβ (p-65 subunit) translocation during challenge of HT-29 cells with inflammatory agent *E. coli* in the presence of *L. fermentum* fermented whey are depicted in figure 4.13. During treatment of HT-29 cells with inflammatory agent (*E. coli*) translocation of p-65 subunit into nucleus increased significantly as compared to cells treated only with media.

In contrast, intestinal cells treated with probiotic *L. fermentum* fermented whey showed translocation of p-65 subunit similar to unstimulated cells. Likewise, pre-exposure of intestinal cells with *L. fermentum* for 4 hr followed by challenge with *E. coli* (exclusion) resisted the translocation of NF-κβ (p-65 subunit) into nucleus significantly as compared to cells treated with *E. coli* inflamed cells.

4.15 Nuclear translocation of NF-κβ (p-65) in intestinal cells on challenge with inflammatory agents in the presence of probiotic simulated digested *L. rhamnosus* fermented whey

The results of p-65 translocation during various challenge with inflammatory agents in the presence of probiotic *L. rhamnosus* in HT-29 cells are shown in figure 4.14. Different stimulation trigger translocation and activation of NF-κβ which regulate transcription of various inflammatory genes. Consistence with this, we found that exposure of HT-29 cells with inflammatory agents (*E. coli*) for 4 hr increased nuclear translocation of p-65 subunit significantly than control. However, stimulation of intestinal cells with probiotic *L. rhamnosus* alone diminished translocation of this factor to nucleus significantly than inflamed cells. Incubation of intestinal cells with *L. rhamnosus* followed by *E. coli* challenge (exclusion) suppressed nuclear translocation of p-65 subunit significantly in comparison to *E. coli* inflamed cells.

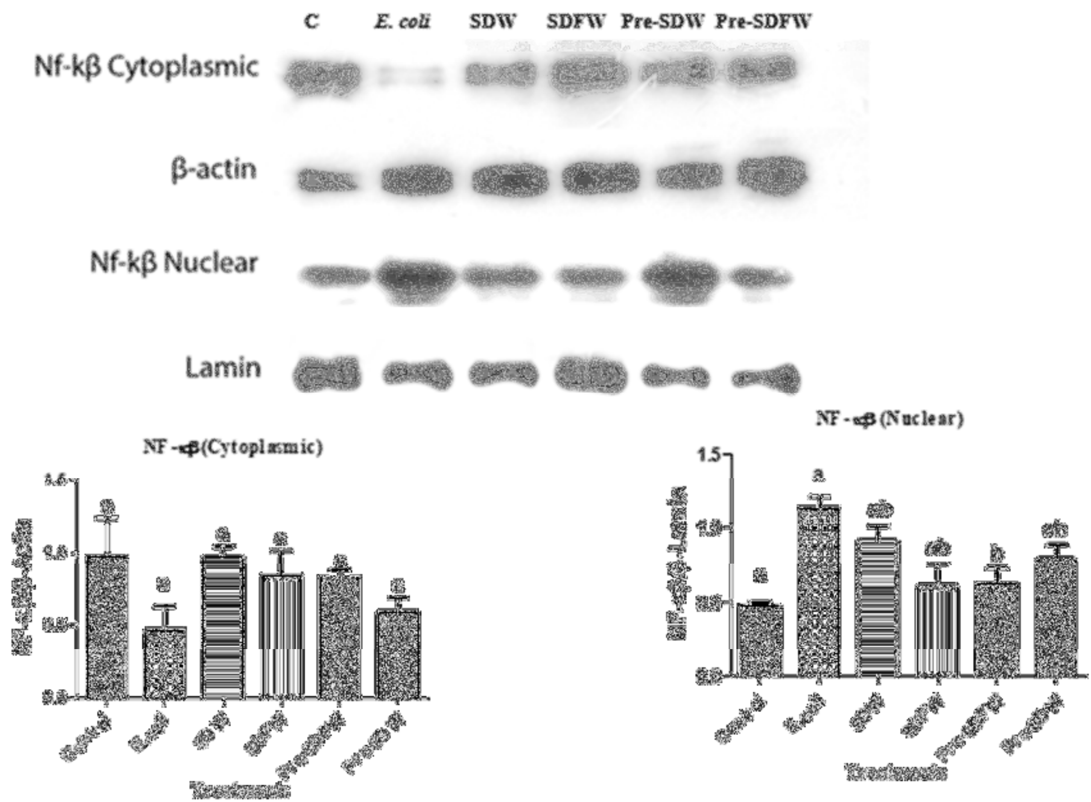

Figure 4.13: Influence of simulated digested fermented whey prepared from *L. fermentum* (MTCC: 5898) on nuclear translocation of NF-κβ p-65 subunit in intestinal epithelial cells (HT-29) during exclusion of *E. coli*

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

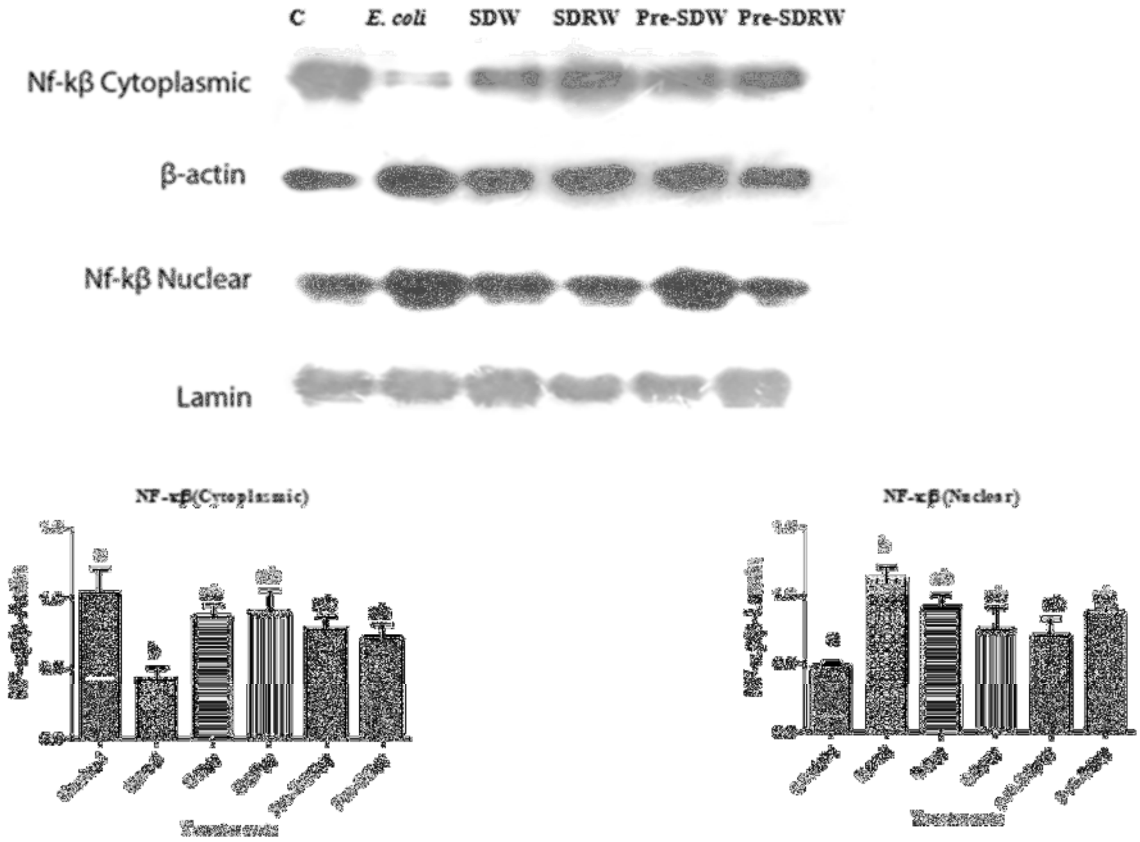

Figure 4.14 Influence of simulated digested fermented whey prepared from *L. rhamnosus* (MTCC:5897) on nuclear translocation of NF-κβ p-65 subunit in intestinal epithelial cells (HT-29) during exclusion of *E. coli*

Values are expressed as mean ± SEM (n = 5). Superscript letters indicate significant differences at $p<0.05$

CHAPTER –5

Discussion

DISCUSSION

Unhealthy lifestyle and unbalanced diet causes dysbiosis of gut microbiota and leads to dysfunction of the gut epithelial barrier. Interaction of microbes with pathogen recognition receptors (PRR's) present on membrane surface initiates immune response. Dysregulation of immune response in intestinal cells underlie disorders like inflammatory bowel diseases. Probiotic bacteria which form indistinguishable part of natural microbiota, retain their activity while passing through the gastrointestinal tract and carrying out their valuable effect on intestinal health. These effect includes inhibition of the pathogenic binding, promote microbial balance, improve barrier integrity and maintain immune homeostasis in intestinal epithelial cells. Deregulation of this mucosal immune system contributes towards pro-inflammatory milieu, causing tissue destruction associated with disease progression (Shi *et al.* 2017). In this regard, the components present in the diet plays a crucial role in pathophysiological outcomes. A recent report identified the presence of 6210 proteins in the milk whey describing the potential implication in the host defence system (Chopra *et al.* 2020). Microbial genomics and proteomics-based studies reveals the presence of hundreds of proteins in lactobacilli for tolerating the harsh condition of the gut (Ali *et al.* 2020). The capability to harbour in gut microenvironment makes them the suitable candidate for microbial fermented food. Therefore components derived from probiotic fermented whey modulate the gut barrier function (De Santis *et al.* 2015 and Nataraj *et al.* 2020).

5.1 Probiotic *Lactobacillus* fermented whey improves the general health status

The present study intended to make a strategy shift from treatment to prevention of the colitis infection through the use of potential probiotic *Lactobacillus (L. rhamnosus and L. fermentum)* fermented whey in order to enhance the intestinal defense which is a unique preventive measure against colitis injury. Twenty-eight-day repeated dose of probiotic *Lactobacillus* fermented whey provided information on the main health effects. Body weight

and relative organ indices are good indicators of adverse effects of a substrate in animal studies. Our results showed no major change in body weight of mice fed with probiotic *Lactobacillus* fermented whey MTCC-5898 compared to the control group. Besides, liver, kidney, and spleen indices of the mice in the respective treatment groups were not statistically different from the control group. The potentially supportive health-modulating preventive effects of probiotic *Lactobacillus* fermented whey were observed, with no adverse change in liver and kidney indices in all the treatment groups even after colitis induction with DSS. Moreover, probiotic *Lactobacillus* fermented whey feeding during colitis induction significantly normalized the increased spleen weight/index equally to healthy control. Increased spleen weight during colitis induction may be explained by immune activation and infection leading to hyperactivity in the spleen, as this organ is the primary site of immune response and filter for unwanted foreign substances and aging cells (Jadhav *et al.* 2013). Similar, results showing a decrease in spleen weight were observed earlier after administration of *L. paracasei* NTU 101 to C57BL/6 mice and confirmed their recovery even after DSS colitis induction (Chen *et al*. 2019). In the present study, hematological parameters of probiotic *Lactobacillus* fermented whey animals were also found within the standard reference limits. These results were in consonance with prior observations (Park *et al.* 2005) on probiotic *Lactobacillus fermentum* where experimental mice were fed daily for 28 without any major changes on hematological parameters (Hb, WBC count, RBC count, MCHC, MCH). Furthermore, hematological blood analysis also reflected the level of infection after DSS treatment that caused severe inflammatory response with symptoms of anemia, decreased RBC, hemoglobin (Hb) and increased WBC, including neutrophils which were brought to normal range in probiotic *Lactobacillus* fermented whey and its supernatant fed animals. These results are also in consonance with previous findings (Herias *et al*. 2005), where *Lactobacilli casei* strain shirota (LcS) consumption improved the clinical condition of mice by normalization of Hb, RBC, WBC, neutrophil and lymphocyte in DSS induced colitis mice.

5.2 Probiotic *Lactobacillus* fermented whey maintain the immune response in gastrointestinal tract

Inflammatory response provoked by dextran sodium sulfate (DSS) imparted cytotoxicity to intestinal epithelial cells that caused dysbiosis, reproduce many immunological hallmarks in colonic colitis and finally led to impairment in mucosal epithelial barrier integrity (Ohtsuka *et al.* 2003). A remarkable enhanced activity of MPO also conveyed the increased neutrophil infiltration, which has been used as a histopathological marker and directly correlated with intestinal tissue injury (Zhao *et al.* 2013). Thus, it seems plausible that the consumption of probiotic *Lactobacillus* fermented whey decreased the MPO activity that was also evidenced in the histopathological evaluation of colonic segments marked with complete intact epithelium with reduced neutrophil infiltration. Likewise, reduced MPO activity indicating less inflammation was confirmed through histological examination after oral administration of *L. plantarum* HY115 and *L. brevis* HY7401 supernatant in mice with DSS colitis (Lee *et al.* 2008) which also supported present observations. These results are also consistent with a recent study (Yoon *et al.* 2019) showing enhanced anti-inflammatory and antioxidative activity with decreased MPO activity in colon tissue of Balb/c mice after consumption of probiotic fermented yogurt.

Pro-inflammatory mediators and cytokines have been reported to induce pathogenesis of ulcerative colitis. Whey proteins hydrolysates showed immunomodulatory anti-inflammatory effects involving reduced binding of bacterial components to TLR-4 with the down-regulation of inflammatory response (Ma *et al.* 2016 and Iskandar *et al.* 2013). Furthermore, probiotics components influence the cytokine production via TLR-2 signaling axis and promote regulatory T-cell response (Llewellyn *et al.* 2017). Present results after pre-consumption of probiotic *Lactobacillus* fermented whey followed by colitis induction displayed significant reduction in the pro-inflammatory cytokines IL-4, TNF-α and increased regulatory TGF-β secretions and thus established its immune-protective function having major role in attenuation of pathogenesis. However, a similar shift in pro-inflammatory and regulatory cytokines was also observed after probiotic fermented whey supernatant consumption. However, it was statistically insignificant may be due to the absence of live probiotic bacteria

in it. Previous investigations have firmly established that T-regulatory TGF-β annihilates the pro-inflammatory release and established a state of immune tolerance in colitis and other infectious diseases (Himmel *et al.* 2008). Thus earlier findings related to mitigation of *Salmonella typhimurium* induced inflammation by probiotic *L. acidophilus* in the gut via TGF-β signaling (Kanmani and Kim, 2020) supported present observations. Imaoka *et al.* (2008) proved that daily consumption of LAB fermented milk reduced pro-inflammatory cytokine IL-4, TNF-α and increased anti-inflammatory IL-10, which helped in the prevention of IBD and provide intestinal immunity in SAMP1/Yit mice. Instead, feeding of probiotic *Lactobacillus* fermented whey to mice did not bring any major variations in IL-4, TNF-α and TGF-β levels in experimental animals compared to control group of non-colitis.

On the other hand, additional inflammatory marker CRP is an acute-phase protein whose concentration rise remarkably in infection. It has also been implicated that IL-4 and CRP induce the release of TNF-α an essential mediator of colitis and may lead to progression of the disease (Sproston *et al.* 2018 and Van Kampen *et al.* 2005). In the current study, a statistically significant increase in CRP was found in colitis while after probiotic *Lactobacillus* fermented whey and its supernatant administration to animals, a slight, gradual significant decrease was observed. Instead, no change was observed in animals with probiotic *Lactobacillus* fermented whey feeding in non-colitis group animals. Anti-inflammatory cytokine, TGF-β was confirmed as the main driving force in the IgA class switch recombination and plasma cell differentiation to provide defense mechanism at the mucosal surface (Cerutti *et al.* 2008). Under present investigation, administration of probiotic *Lactobacillus* fermented whey significantly induced IgA production in the intestinal fluid, which prevented the invasion of pathogens through intestinal epithelial cells. Thus present results are in accordance with Sakai *et al.* (2014) who reported similar observation where dietary intake of probiotic *L. gasseri* SBT2055 showed TLR-2 and TGF-β signals were critical for enhanced production of IgA and IgA (+) cell population.

5.3 Probiotic *Lactobacillus* fermented whey improves the gut barrier function

Epithelial surface of gastrointestinal tract acts as a barrier between exterior environment and body and offer the first line of defense against infection that allows the absorption of appropriate nutrient and water while limiting the uptake of harmful substances. Selective permeability in intestine depends upon specialized transmembrane proteins such as claudin, ZO-1, occludin that maintains the gut barrier integrity located at the apical end of the lateral membrane (Castro and De Souza, 2015). DSS colitic mice induced the exaggerated pro-inflammatory response, which can be linked with the disruption of epithelial barrier junctions made of occludin, claudin-1 and ZO-1. However, as evident from the present investigation, prior feeding of probiotic *Lactobacillus* fermented whey significantly increased the transcriptional activity of tight junctional genes (*Occludin, Claudin-1* and *ZO-1*) and also restored architecture of intestinal villi epithelium in colitis induced mice. However basal expression was observed in non-colitis animals. It indicates that live bacteria of fermented whey might reinforce the epithelial barrier or immune system against pathogens. Earlier feeding of *L. plantarum* MTCC-5690 fermented formulation in *Salmonella* challenged animals displayed up-regulation in expression of barrier genes (ZO-1, Claudin-1, Occludin) also supported our observations (Rokana *et al.* 2016). Kim, (2014) also reported that pro-inflammatory cytokine (TNF-α) induced the release of hBD-2 antimicrobial peptide which was directly linked with inflammatory ulcerative colitis. The present study also revealed that inflammatory condition caused by DSS remarkably elevated mRNA level of *hBD-2* gene while pre-administration of probiotic *Lactobacillus* fermented whey to animals followed by induction of colitis significantly decreased transcription of *hBD-2* mRNA showing the presence of the healthy gut environment. While no change was observed in the non-colitis animal. Similar to our results, prior studies observed a remarkable increase in mRNA levels of hBD-2 gene in inflammatory bowel diseased condition and in the colon of T84 carcinoma cells infected with enterohemorrhagic *E. coli* (Ho *et al.* 2013 and Lewis *et al.* 2016). Furthermore, prophylactic administration of probiotic *Lactobacillus* fermented whey significantly reduced *TLR-4* and up-regulated the *TLR-2* mRNA expression in

DSS induced colitis mice. It indicates that live bacteria or components released during fermentation of whey with *L. rhamnosus* may act as TLR-2 ligand that plays a crucial role in activating its transcription and suppressing the TLR-4 mediated inflammatory response induced during colitis. Previous studies also noticed that probiotics and their fermented biogenic components enhance the epithelial resistance by activating TLR-2 (Ulluwishewa. *et al.* 2011). Likewise, Rokana *et al*. 2016) revealed that probiotic *L. plantarum* MTCC-5690 fermented milk suppressed the inflammatory process by modulating TLR-4 and TLR-2 expression. The pre-treatment of *L. rhamnosus* also protects intestinal epithelial cells against F4+ETEC infection partly through the anti-inflammatory mediated TLR-2 expression and counteracting TNF-α increase and thus enhanced barrier function (Zhang *et al*. 2015). Moreover, pre-administration of probiotic *Lactobacillus* fermented whey and its supernatant and then induction of colitis under present study was able to induce a fecal bacterial population of lactobacilli and lowers *E. coli* count in feces. These results are consistence with previous findings where oral administration of cheese whey rich in cysteine, threonine in Wistar rats increased the intestinal lactobacilli count under chronic inflammation of DSS induced colitis (Sprong *et al.* 2010). Induction of IgA production by probiotic *Lactobacillus* fermented whey may also play an essential role in the control of intestinal microbiota as it was shown earlier that IgA secretion is critical in the regulation of microbial community in the gut (Kawamoto *et al.* 2012). In this investigation, DSS administration for six days not only led to infection but also increased the gut permeability as indicated by FITC-dextran fluorescent marker which crossed the epithelium due to higher permeability. However, improved intestinal function by reduced leakage of FITC-dextran upon probiotic *Lactobacillus* fermented whey consumption in weanling mice established its protective role against intestinal epithelial damage. While administration of in non-colitis groups did not show any changes of FITC-dextran level in their serum as compared to control animals. However, it is interesting to note that under present investigation pre-treatment of animals with probiotic *Lactobacillus* fermented whey having probiotic lactobacilli appeared much effective in providing the barrier protection in intestinal tissue even after colitis induction over its supenatant when bacteria were removed from the fermented product. It was confirmed by tight junctional transcriptional as well as intestinal barrier leakage

data through FITC-dextran permeability. Transcriptional data showed that in probiotic fermented whey supernatant group restitution of mRNA synthesis after DSS stimulation did not occur like probiotic fermented whey animals, but there is no change in FITC-dextran permeability. It may have happened because the previously synthesized tight junctional proteins may not be dislodged yet in probiotic fermented whey supernatant fed group. These results conform with the protective effects of probiotic *L. plantarum* (MTCC 5690) fermented milk depicting reduced FITC-dextran permeability across the epithelium in *Salmonella* treated animals (Rokana *et al.* 2016).

5.4 Probiotic *Lactobacillus* fermented whey augmented the modulation of genes associated with the epithelial barrier and immune function

The protective effects of fermented foods against pathogen driven epithelial barrier dysfunction or strengthening of these functions may potentially involve the epithelial tight junctions signalling. The data available in the literature have often attributed the barrier enhancement property of probiotics to improve the expression of tight junctional proteins (Walker *et al.* 2008). Interaction of intestinal epithelial cells with pathogenic bacteria caused stimulation of various immune signals such as several cytokines, chemokines or other signalling molecules (Onyiah *et al.* 2016). Under present study augmentation in epithelial barrier function was demonstrated during the treatment of probiotic *Lactobacillus* simulated digested whey in HT-29 cells. During the exclusion study, the treatment of epithelial HT-29 cells with *L. rhamnosus* and *L. fermentum* simulated digested whey for 4 hr upregulated the expression of important barrier genes (occludin, claudin-1, ZO-1 and HBD-2). However, results also showed that the treatment of HT-29 cells with probiotic *L. fermentum* simulated digested whey decreased the expressions of NF-κβ at transcriptional levels. Similarly, Habil *et al.* (2014) reported that treatment of Caco-2 cells with heat killed probiotic lactobacilli and their wall components also stimulated the differential expression of a human beta-defensin-2 by epithelial intestinal cells. Yang *et al.* (2000) observed that oral administration of *L. reuteri* I5007 for 14 days significantly boosted the protein abundance of jejunal epithelial Claudin-1, Occludin and ZO-1 compared to non-fed new born piglets. Similarly, Liu *et al.* (2017a) recently reported that the modulation in expression of variable host

defense peptides of porcine small intestinal epithelial cells and neonatal piglets under *in-vivo* and *in-vitro* trials when exposed to probiotic *L. reuteri* I5007 for different durations.

5.5 NF-κβ signalling in host intestinal epithelial cells by probiotic *Lactobacillus* fermented whey during exclusion of *E. coli*

Regulated NF-κβ dependent signalling is critical for efficient immune response, but prolonged activation contribute to generation of inflammatory diseases (Yan and Polk, 2010). NF-κβ is a transcription factor, present in inactive form in cytosol by binding to inhibitory protein Iκβ-α and control the expression of inflammatory cytokines in non-stimulatory conditions. On stimulation, inhibitor of NF-κβ (Iκβ-α) undergo phosphorylation followed by ubiquitination degradation and unbound NF-κβ undergoes nuclear translocation which induce activation of immune related genes (Oeckinghaus *et al*. 2009). Under present study, nuclear translocation of p-65 subunit during exclusion was analysed by western blotting. It was observed that stimulation of intestinal cells with *E. coli* show enhanced translocation of p-65 subunit into nucleus. These findings are consistent with previous studies showing that inflammatory agents enhanced p-65 translocation to nucleus. Lesueur *et al*. (2012) observed that stimulation of intestinal cells (Caco-2/TC7) with *E. coli* augmented excessive activation of p-65 subunit. Likewise, stimulation of RAW264.7 cells with LPS enhanced nuclear translocation of p-65 subunit (Seo *et al*. 2011). In contrast, incubation of intestinal cells (HT-29) with simulated digested probiotic fermented whey individually or during exclusion with inflammatory agents showed protective effect through inhibiting excessive translocation of p-65 subunit into nucleus in intestinal cells. Likewise, pre-exposure of intestinal cells (Caco-2BBe) with *Lactobacillus acidophilus* before TNF-α diminished NF-κβ activation due to inhibition of nuclear translocation of the p-65 subunit (Borthakur *et al*. 2013). Roselli *et al*. (2016) demonstrated that probiotic *L. amylovorus* cell wall component S-layer proteins displayed anti-inflammatory response to *E. coli* infection by decreasing NF-κβ activation in intestinal cells. Likewise, exposure of *L. sakei* K17 displayed protective effect by potentially preventing LPS stimulated NF-κβ activation in DCs and peritoneal macrophages (Eun *et al*. 2016). Kaci *et al*. (2011) demonstrated that metabolites present in *Streptococcus salivarius*

supernatant also inhibited NF-κβ activation induced by stimulation of intestinal cells HT-29 with pro-inflammatory cytokines IL-1β. Similarly, *L. fermentum* IM12 attenuated inflammation by reducing the activation of NF-κβ in LPS stimulated peritoneal macrophage (Nam *et al.* 2017). Similarly, present investigation showed protective effects of *Lactobacillus* (*L. rhamnosus* and *L. fermentum*) simulated digested whey on inflammation induced by *E. coli* in intestinal epithelial cells. Probiotic strain *L. rhamnosus* fermented whey recovered the inflammatory conditions more effectively than *L. fermentum* during exclusion assays by reducing the NF-κβ signalling through p-65 nuclear translocation.

CHAPTER –6

Summary and Conclusions

SUMMARY AND CONCLUSIONS

The intestinal epithelium cells act as a physical barrier that prevents the interaction of millions of microbes with internal milieu and play a crucial role in the regulation of intestinal immune homeostasis. In the presence of pathogens, unregulated or uncontrolled immune response lead to tissue damage and disruption of tight junction which play important role in the progression of gastrointestinal disorders such as inflammatory bowel diseases, celiac diseases, and allergies. Fermented food contain beneficial bioactive components that act as good alternative to treat these conditions. Therefore, the present study was executed to assess the role of *Lactobacillus* (*Lactobacillus rhamnosus* (LR: MTCC 5897) and *Lactobacillus fermentum* (LF: MTCC 5898) fermented sweet whey on epithelial function. This study was divided into two objectives. Under the first objective, the effect of lactobacilli fermented whey on protection of gut barrier integrity in colitis induced mice was assessed. The second objective focussed to elucidate the mode of action of lactobacilli fermented whey on human intestinal epithelial barrier integrity, where expression of tight junctional genes and immune related genes and nuclear translocation of NF- kβ (p-65) were determined. The results of both the two objectives are summarized below.

6.1 The effect of lactobacilli fermented whey on protection of gut barrier integrity in colitis induced mice

- The *L. rhamnosus* and *L. fermentum* both were able to obtain a population of 9.3 log CFU/mL and 8.7 log CFU/mL respectively with increase in titarable acidity and decrease in pH in 12 h of fermentation.
- The *L. rhamnosus fermented whey* (LRFW) showed the more proteolytic activity as compared to *L. fermentum fermented whey* (LFFW) based on the N-terminals measured by OPA method. While a significant decrease ($p<0.05$) in lactose on fermenting whey with either of *Lactobacillus* strains (LR and LF) during 12 h fermentation.
- Induction of colitis with DSS maintained on basal diet showed significantly ($p<0.01$) higher loss in percent body weight than animal groups fed with whey, probiotic fermented whey and its supernatant respectively.

- Higher spleen index ($p<0.01$) and its larger size in DSS-colitis group indicated systemic inflammatory response than non-colitis control. However, mice pre-fed with probiotic *Lactobacillus* fermented whey have significantly reduced ($p<0.05$) the spleen index even after challenge with DSS.
- Impact of DSS administration on induction of colitis like symptoms by stimulating inflammatory response were also evidenced by significantly higher neutrophils ($p<0.05$) and lymphocyte ($p<0.01$) cell count with reduced ($p<0.01$) hemoglobin content which appeared to reverse upon consumption of either of probiotic *Lactobacillus* fermented whey.
- DSS challenged animals in colitis induced group showed higher ($p<0.01$) number of payer patches in intestine, whereas they remained similar to healthy control animals even when colitis was induced after feeding whey, probiotic fermented whey and its supernatant to respective animal groups.
- The sIgA levels in intestinal fluid increased tremendously ($p<0.01$) on feeding probiotic *L. rhamnosus* fermented whey (LRFW) even after their subsequent challenge with DSS-to induce colitis.
- Animals exposed to DSS also enhanced the inflammatory response by significantly higher release ($p<0.01$) of pro-inflammatory cytokines such as IL-4 and TNF-α along with C reactive protein in intestine. The feeding of either of probiotic *Lactobacillus* fermented whey prepared with *L. rhamnosus* and *L. fermentum* significantly reduced the levels of these pro-inflammatory markers.
- Reduction in inflammatory bio-markers on pre-feeding of whey, probiotic fermented whey and its supernatant respectively prior to colitis induction were also confirmed by significantly reduced MPO activity as a direct measure of neutrophils infiltration in intestinal tissue.
- Transcriptional expression of genes associated with intestinal barrier integrity (occludin, claudin-1 and zonula-occluden-1) were effectively ($p<0.01$) enhanced on consumption of probiotic *Lactobacillus* fermented whey having live *L. rhamnosus* and *L. fermentum* than their supernatant fed animals which was devoid of probiotic microbes respectively.

- In case of hBD-2, the RNA expression was reduced significantly ($p<0.05$) upon live *L. rhamnosus* and *L. fermentum* fermented whey consumption than DSS control groups which again indicated prevention of inflammation induced by DSS.
- Transcriptional expression of pathogen recognition receptor TLR-4 in colon of DSS group increased significantly ($p<0.05$) whereas mRNA expression of TLR-2 was found more in *L. rhamnosus* fermented whey and its supernatant fed animals.
- The oral administration of respective fermented whey, having live *L. rhamnosus* and *L. fermentum*, recorded significant lower release of FITC-dextran ($p<0.05$) in serum as compared to DSS-induced colitis control establishing their protective effect on gut.
- Fecal lactobacilli colonies were significantly higher ($p<0.05$) after consumption of *L. rhamnosus* and *L. fermentum* fermented whey respectively as compared to DSS control group. In contrast, *E. coli* colonies in feces dropped significantly ($p<0.05$) after probiotic fermented whey consumption than DSS induced colitis animals.
- The immuno-staining of junctional protein claudin-1, occludin, ZO-1 and cytoskeleton actin filament in intestinal sections also established intact epithelial barriers on consumption of probiotic *Lactobacillus* fermented whey having live microbes than their respective supernatants by appearance of regular and intense green fluorescence showing their preserved and localized distribution than DSS induced colitis.

6.2 The mode of action of lactobacilli fermented whey on human intestinal epithelial barrier integrity

- Considerably higher viability of intestinal cells (HT-29) with 50µg/ml of simulated digested whey prepared after fermentation with *L. rhamnosus* and *L. fermentum* respectively was observed at as compared to higher amount based upon the N-terminals measured with OPA method.
- mRNA expression of tight junctional genes *Occludin*, *Claudin-1* in intestinal cells increased significantly ($p<0.05$) in presence of simulated digested fermented whey prepared after fermentation with *L. rhamnosus*

and *L. fermentum* respectively during exclusion assay with inflammatory agent (*E. coli*) than *E. coli* control.

- Incubation of cells with simulated digested fermented whey prepared after fermentation with *L. fermentum* reduced NF-κβ expression significantly ($p<0.05$). Likewise, exclusion of *E. coli* with whey prepared using *L. fermentum* appeared to resist the increased NF-κβ mRNA production caused by inflammatory agent.
- Western blotting showed significant ($p<0.05$) nuclear translocation of p-65 NF-κβ to nucleus from cytoplasm in HT-29 cells on treatment with *E. coli* as compared to control.
- Presence of simulated digested whey prepared after fermentation with probiotic *L. fermentum* significantly resisted NF-κβ translocation of (p-65) from cytoplasm to nucleus during exclusion challenge with *E. coli* as shown by western blotting.
- Similarly, treatment with simulated digested fermented whey prepared after fermentation with probiotic *L. rhamnosus* under exclusion assay with *E. coli* also appeared to resist nuclear translocation of NF-κβ.

Conclusion

Thus the strategy of consuming fermented whey with probiotic *L. rhamnosus* and *L. fermentum* not only added value to the dairy by-product but also appeared to be useful as a suitable delivery vehicle for administration of probiotics. Moreover, presence of live probiotic bacteria in fermented whey appeared more effective in contracting the infection and pathogenesis induced by colitis.

Bibliography

BIBLIOGRAPHY

Acosta, M.P., Ruzal, S.M. and Cordo, S.M., 2016. S-layer proteins from *Lactobacillus* sp. inhibit bacterial infection by blockage of DC-SIGN cell receptor. *International Journal of Biological Macromolecules*, ***92***(12): 998-1005.

Adebolu, T.T. and Olorunfemi, O.B., 2016. Effects of Fermented Cheese Whey on the Cells of the Immune System of Apparently Healthy Albino Rats. *Journal of Immuno Biology*, ***1***(109): 1-2.

Agustina, R., Kok, F.J., Van De Rest, O., Fahmida, U., Firmansyah, A., Lukito, W., Feskens, E.J., Van Den Heuvel, E.G., Albers, R. and Bovee-Oudenhoven, I.M., 2012. Randomized trial of probiotics and calcium on diarrhea and respiratory tract infections in Indonesian children. *Pediatrics*, **129**(5): 1155-1164.

Albert, E.J., Duplisea, J., Dawicki, W., Haidl, I.D. and Marshall, J.S., 2011. Tissue eosinophilia in a mouse model of colitis is highly dependent on TLR2 and independent of mast cells. *The American Journal of Pathology*, ***178***(1): 150-160.

Ali, S.A., Kumar, S., Mohanty, A.K. and Behare, P., 2018. Draft genome sequence of *Lactobacillus fermentum* NCDC 400, isolated from a traditional Indian dairy product. *Genome Announcements*, ***6***(2): 1-2.

Ali, S.A., Singh, P., Tomar, S.K., Mohanty, A.K. and Behare, P., 2020. Proteomics fingerprints of systemic mechanisms of adaptation to bile in *Lactobacillus fermentum*. *Journal of Proteomics*, ***213***: 1-17.

Alm, L., 1982. Effect of fermentation on L (+) and D (−) lactic acid in milk. *Journal of Dairy Science*, ***65***(4): 515-520.

Al-Sadi, R., Boivin, M. and Ma, T., 2009. Mechanism of cytokine modulation of epithelial tight junction barrier. *Frontiers in Bioscience: a Journal and Virtual Library*, ***14***: 27-65.

Amer, M., Nadeem, M., Nazir, R., Ur, S., Fakhar, M., Abid, F., Asif, E., Nazir, S.U.R. and Ain, Q.U., 2018. Probiotics and Their Use in Inflammatory Bowel Disease. *Alternative Therapies in Health and Medicine*, ***24***(3): 6-23.

Aneja, S., Sousa, C, Den Yan, E.P., and Giacca P, I.M., 1990. The technology of traditional milk products in developing countries**,** *Food Research International*, ***31***(9): 1322-1329.

Antoni, L., Nuding, S., Wehkamp, J. and Stange, E.F., 2014. Intestinal barrier in inflammatory bowel disease. *World Journal of Gastroenterology,* ***20***(5): 11-65.

AOAC, 1990. Official Methods of Analysis, 15th ed. *Association of Official Analytical Chemists*, Inc., Arlington.

Araújo, D.F., Guerra, G.C., Pintado, M.M.E., Sousa, Y.R., Algieri, F., Rodriguez-Nogales, A., Araújo Jr, R.F., Gálvez, J., Queiroga, R.D.C.R. and Rodriguez-Cabezas, M.E., 2017. Intestinal anti-inflammatory effects of goat whey on DNBS-induced colitis in mice. *PloS One*, ***12***(9): 1-19.

Bauché, D. and Marie, J.C., 2017. Transforming growth factor β: a master regulator of the gut microbiota and immune cell interactions. *Clinical & Translational Immunology*, ***6***(4): 1-8.

Beaulieu, J., Dupont, C. and Lemieux, P., 2007. Anti-inflammatory potential of a malleable matrix composed of fermented whey proteins and lactic acid bacteria in an atopic dermatitis model. *Journal of Inflammation*, ***4***(1): 1-10.

Begum, P.S., Madhavi, G., Rajagopal, S., Viswanath, B., Razak, M.A. and Venkataratnamma, V., 2017. Probiotics as functional foods: potential effects on human health and its impact on neurological diseases. *International Journal of Nutrition, Pharmacology*, *Neurological Diseases*, **7**(2): 1-11.

Beh, B.K., Mohamad, N.E., Yeap, S.K., Ky, H., Boo, S.Y., Chua, J.Y.H., Tan, S.W., Ho, W.Y., Sharifuddin, S.A., Long, K. and Alitheen, N.B., 2017. Anti-obesity and anti-inflammatory effects of synthetic acetic acid

vinegar and Nipa vinegar on high-fat-diet-induced obese mice. *Scientific Reports*, ***7***(1): 1-9.

Bermudez-Brito, M., Muñoz-Quezada, S., Gomez-Llorente, C., Matencio, E., Bernal, M.J., Romero, F. and Gil, A., 2012, a. Human intestinal dendritic cells decrease cytokine release against *Salmonella* infection in the presence of *Lactobacillus paracasei* upon TLR activation. PLoS One, **7**(8): 1-11

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C. and Gil, A., 2012, b. Probiotic mechanisms of action. *Annals of Nutrition and Metabolism*, ***61***(2): 160-174.

Bhat, M.I., Sowmya, K., Kapila, S. and Kapila, R., 2019. Potential probiotic *Lactobacillus rhamnosus* (MTCC-5897) inhibits *Escherichia coli* impaired intestinal barrier function by modulating the host tight junction gene response. *Probiotics and Antimicrobial Proteins*, ***12***(11): 1-12.

Bhat, M.I., Kapila, S. and Kapila, R., 2020. *Lactobacillus fermentum* (MTCC-5898) supplementation renders prophylactic action against *Escherichia coli* impaired intestinal barrier function through tight junction modulation. *Food Science and Technology*, ***123***(12): 109-118.

Bian, R., Joseph, S., Cui, L., Pan, G., Li, L., Liu, X., Zhang, A., Rutlidge, H., Wong, S., Chia, C. and Marjo, C., 2014. A three-year experiment confirms continuous immobilization of cadmium and lead in contaminated paddy field with biochar amendment. *Journal of Hazardous Materials*, ***272***(10): 121-128.

Bischoff, M., Nowitzki, T., Voß, O., Wilbrandt, S. and Stenzel, O., 2014. Postdeposition treatment of IBS coatings for UV applications with optimized thin-film stress properties. *Applied Optics*, ***53***(4): 212-220.

Bonvicini, F., Pagnotta, E., Punzo, A., Calabria, D., Simoni, P., Mirasoli, M., Passerini, N., Bertoni, S., Ugolini, L., Lazzeri, L. and Gentilomi, G.A., 2020. Effect of *Lactobacillus acidophilus* Fermented Broths Enriched with Eruca sativa Seed Extracts on Intestinal Barrier and Inflammation in a Co-Culture System of an Enterohemorrhagic *Escherichia coli* and Human Intestinal Cells. *Nutrients*, ***12***(10): 30-64.

Borthakur, A., Bhattacharyya, S., Kumar, A., Anbazhagan, A.N., Tobacman, J.K. and Dudeja, P.K., 2013. *Lactobacillus acidophilus* alleviates platelet-activating factor-induced inflammatory responses in human intestinal epithelial cells. *PloS One*, ***8***(10): 56-64.

Boumba, V.A., Voutsinas, L.P. and Philippopoulos, C.D., 2001. Composition and nutritional value of commercial dried whey products from feta cheese manufacture. *International Journal of Dairy Technology*, ***54***(4): 141-145.

Bourlioux, P. and Pochart, P., 1988. Nutritional and health properties of yogurt. *World Review of Nutrition and Dietetics,* ***56***(7)*: 217-258.*

Cario, E., 2010. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflammatory Bowel Diseases*, ***16***(9): 1583-1597.

Carvalho, P.B. and Cotter, J., 2017. Mucosal healing in ulcerative colitis: a comprehensive review. *Drugs*, ***77***(2): 159-173.

Castillo, N.A., Perdigón, G. and de LeBlanc, A.D.M., 2011. Oral administration of a probiotic *Lactobacillus* modulates cytokine production and TLR expression improving the immune response against *Salmonella enterica serovar Typhimurium* infection in mice. *BMC Microbiology*, ***11***(1): 1-12.

Castro, F. and de Souza, H.S., 2019. Dietary composition and effects in inflammatory bowel disease. *Nutrients*, ***11***(6): 13-98.

Cerutti, A., 2008. The regulation of IgA class switching. *Nature Reviews Immunology*, ***8***(6): 421-434.

Chapat, L., Chemin, K., Dubois, B., Bourdet-Sicard, R. and Kaiserlian, D., 2004. Lactobacillus casei reduces CD8+ T cell-mediated skin inflammation. *European Journal of Immunology*, ***34***(9): 2520-2528.

Chassaing, B., Aitken, J.D., Malleshappa, M. and Vijay-Kumar, M., 2014. Dextran sulfate sodium (DSS)-induced colitis in mice. *Current Protocols in Immunology,* ***104***(1)*:* 15-25.

Chen, B., She, S., Li, D., Liu, Z., Yang, X., Zeng, Z. and Liu, F., 2013. Role of miR-19a targeting TNF-α in mediating ulcerative colitis. *Scandinavian Journal of Gastroenterology*, ***48***(7): 815-824.

Chen, C.L., Hsu, P.Y. and Pan, T.M., 2019. Therapeutic effects of *Lactobacillus paracasei* subsp. paracasei NTU 101 powder on dextran sulfate sodium-induced colitis in mice. *Journal of Food and Drug Analysis*, ***27***(1): 83-92.

Chen, J., Luo, X., Jiang, L., Jin, P., Wei, W., Liu, D. and Fang, W., 2009. Molecular characteristics and virulence potential of *Listeria monocytogenes* isolates from Chinese food systems. *Food Microbiology*, ***26***(1): 103-111.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. and Zhao, L., 2018. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, ***9***(6): 1-9.

Chen, S., Einspanier, R. and Schoen, J., 2015. Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports. *Histochemistry and Cell Biology*, ***144***(5): 509-515.

Chen, Y.P., Hsiao, P.J., Hong, W.S., Dai, T.Y. and Chen, M.J., 2012. *Lactobacillus kefiranofaciens* M1 isolated from milk kefir grains ameliorates experimental colitis *in vitro* and *in vivo*. *Journal of Dairy Science*, **95**(1): 63-74.

Chen, Y.P., Hsu, C.A., Hung, W.T. and Chen, M.J., 2016. Effects of *Lactobacillus paracasei* 01 fermented milk beverage on protection of intestinal epithelial cell *in vitro*. *Journal of the Science of Food and Agriculture*, ***96***(6): 2154-2160.

Chiu, Y.H., Lin, S.L., Ou, C.C., Lu, Y.C., Huang, H.Y. and Lin, M.Y., 2013. Anti-inflammatory effect of lactobacilli bacteria on HepG2 cells is through cross-regulation of TLR4 and NOD2 signalling. *Journal of Functional Foods*, ***5***(2): 820-828.

Chopra, A., Ali, S.A., Bathla, S., Rawat, P., Vohra, V., Kumar, S. and Mohanty, A.K., 2020. High-Resolution Mass Spectrometer–Based Ultra-Deep Profile of Milk Whey Proteome in Indian Zebu (Sahiwal) Cattle. *Frontiers in Nutrition*, **7**(9): 322-358.

Chun, S.H., Lee, H.A., Lee, K.B., Kim, S.H., Park, K.Y. and Lee, K.W., 2016. Effects of glycated whey protein concentrate on pro-inflammatory cytokine expression and phagocytic activity in RAW264. 7 macrophages. *Biological and Pharmaceutical Bulletin*, ***39***(2): 199-206.

Clancy, R., 2003. Immunobiotics and the probiotic evolution. *FEMS Immunology and Medical Microbiology*, ***38***(1): 9-12.

Clayburgh, D.R., Shen, L. and Turner, J.R., 2004. A porous defense: the leaky epithelial barrier in intestinal disease. *Laboratory Investigation*, ***84***(3): 282-291.

Cordeiro, M.A., Souza, E.L.S., Arantes, R.M.E., Balthazar, C.F., Guimarães, J.T., Scudino, H., Silva, H.L.A., Rocha, R.S., Freitas, M.Q., Esmerino, E.A. and Silva, M.C., 2019. Fermented whey dairy beverage offers protection against *Salmonella enterica* ssp. enterica serovar Typhimurium infection in mice. *Journal of Dairy Science*, ***102***(8): 6756-6765.

Cross, M.L. and Gill, H.S., 1999. Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology*, ***77***(4): 345-350.

Cruz, A.G., Antunes, A.E., Sousa, A.L.O., Faria, J.A. and Saad, S.M., 2009. Ice-cream as a probiotic food carrier. *Food Research International*, ***42***(9): 1233-1239.

Cunliffe, R.N., Rose, F.R.A.J., Keyte, J., Abberley, L., Chan, W.C. and Mahida, Y.R., 2001. Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut*, ***48***(2): 176-185.

Dahlqvist, A., Asp, N.G., Burvall, A. and Rausing, H., 1977. Hydrolysis of lactose in milk and whey with minute amounts of lactase. *Journal of Dairy Research*, ***44*(**3): 541-548.

Dai, C., Zheng, C.Q., Meng, F.J., Zhou, Z., Sang, L.X. and Jiang, M., 2013. VSL# 3 probiotics exerts the anti-inflammatory activity via PI3k/Akt and NF-κB pathway in rat model of DSS-induced colitis. *Molecular and Cellular Biochemistry*, ***374***(1), pp.1-11.

Das, D. and Goyal, A., 2015. Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim. *LWT-Food Science and Technology*, ***61***(1): 263-268.

Davidson, G.P. and Butler, R.N., 2000. Probiotics in pediatric gastrointestinal disorders. *Current Opinion in Pediatrics*, ***12***(5): 477-481.

Davis, C.E., Rifkind, B.M., Brenner, H. and Gordon, D.J., 1990. A single cholesterol measurement underestimates the risk of coronary heart disease: an empirical example from the Lipid Research Clinics Mortality Follow-up Study. *Jama*, ***264***(23): 3044-3046.

de LeBlanc, A.D.M., Dogi, C.A., Galdeano, C.M., Carmuega, E., Weill, R. and Perdigón, G., 2008. Effect of the administration of a fermented milk containing *Lactobacillus casei* DN-114001 on intestinal microbiota and gut associated immune cells of nursing mice and after weaning until immune maturity. *BMC Immunology*, ***9***(1): 1-12.

de Oliviera Nascimento, L., Massari, P. and Wetzler, L.M., 2012. The role of TLR2 in infection and immunity. *Frontiers in Immunology*, ***3***: 1-17.

De Santis, S., Cavalcanti, E., Mastronardi, M., Jirillo, E. and Chieppa, M., 2015. Nutritional keys for intestinal barrier modulation. *Frontiers in Immunology*, ***6***(10): 1-12.

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y. and Roy, D., 2008. Immunomodulatory effects of probiotics in the intestinal tract. *Current Issues in Molecular Biology*, ***10***(2): 25-37.

Deng, J., Li, Y., Zhang, J. and Yang, Q., 2013. Co-administration of *Bacillus subtilis* RJGP16 and *Lactobacillus salivarius* B1 strongly enhances the intestinal mucosal immunity of piglets. *Research in Veterinary Science*, ***94***(1): 62-68.

Dennis-Wall, J.C., Culpepper, T., Nieves Jr, C., Rowe, C.C., Burns, A.M., Rusch, C.T., Federico, A., Ukhanova, M., Waugh, S., Mai, V. and Christman, M.C., 2017.Probiotics (*Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1, and *Bifidobacterium longum* MM-2) improve rhinoconjunctivitis-specific quality of life in individuals with seasonal allergies: a double-blind, placebo-controlled, randomized trial. *The American Journal of Clinical Nutrition*, ***105***(3): 758-767.

Eun, S.H., Lim, S.M., Jang, S.E., Han, M.J. and Kim, D.H., 2016. *Lactobacillus sakei* K17, an inducer of IL-10 expression in antigen-presenting cells, attenuates TNBS-induced colitis in mice. *Immunopharmacology and Immunotoxicology*, ***38***(6): 447-454.

Ewaschuk, J.B., Diaz, H., Meddings, L., Diederichs, B., Dmytrash, A., Backer, J., Looijer-van Langen, M. and Madsen, K.L., 2008. Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, ***295***(5): 1025-1034.

Forssten, S.D., Sindelar, C.W. and Ouwehand, A.C., 2011. Probiotics from an industrial perspective. *Anaerobe*, ***17***(6): 410-413.

Galdeano, C.M. and Perdigon, G., 2006. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. *Clinical and Vaccine Immunology*, ***13***(2): 219-226.

Galdeano, C.M., Núñez, I.N., de LeBlanc, A.D.M., Carmuega, E., Weill, R. and Perdigón, G., 2011. Impact of a probiotic fermented milk in the gut ecosystem and in the systemic immunity using a non-severe protein-energy-malnutrition model in mice. *BMC Gastroenterology*, ***11***(1): 1-14.

Garza, C., 1979. Appropriateness of milk use in international supplementary feeding programs. *Journal of Dairy Science*, ***62***(10): 1673-1684.

Gogineni, V.K., Morrow, L.E. and Malesker, M.A., 2013. Probiotics: mechanisms of action and clinical applications. *Journal of Probiotic Health*, ***1***(1): 1-11.

Gomi, A., Iino, T., Nonaka, C., Miyazaki, K. and Ishikawa, F., 2015. Health benefits of fermented milk containing *Bifidobacterium bifidum* YIT 10347 on gastric symptoms in adults. *Journal of Dairy Science*, **98**(4): 2277-2283.

Granier, A., Goulet, O. and Hoarau, C., 2013. Fermentation products: immunological effects on human and animal models. *Pediatric Research*, ***74***(2): 238-244.

Gueniche, A., Philippe, D., Bastien, P., Reuteler, G., Blum, S., Castiel-Higounenc, I., and Benyacoub, J., 2014. Randomised double-blind placebo-controlled study of the effect of *Lactobacillus paracasei* NCC 2461 on skin reactivity. *Beneficial Microbes*, ***5***(2), 137-145.

Gupta, R., Mikhaylenko, G., Balasubramaniam, V.M. and Tang, J., 2011. Combined pressure–temperature effects on the chemical marker (4-hydroxy-5-methyl-3 (2H)-furanone) formation in whey protein gels. *LWT-Food Science and Technology*, ***44***(10): 2141-2146.

Gupta, T., Kaur, H**.**, Kapila, S. and Kapila, R., 2021. Potential probiotic *Lactobacillus fermentum* (MTCC-5898) mediated modulation in immune genes and NF-κβ signaling events in epithelial. *Journal of Applied Microbiology*. (*In Press*)

Guyonnet, D., Woodcock, A., Stefani, B., Trevisan, C. and Hall, C., 2009. Fermented milk containing *Bifidobacterium lactis* DN-173 010 improved self-reported digestive comfort amongst a general population of adults. A randomized, open-label, controlled, pilot study. *Journal of Digestive Diseases*, ***10***(1): 61-70.

Guzman, J.R., Conlin, V.S. and Jobin, C., 2013. Diet, microbiome, and the intestinal epithelium: an essential triumvirate?. *BioMed Research*

International, ***87***(6): 1-14.

H. S. Cooper, S. N. Murthy, R. S. Shah and D. J., 1993. Sedergran, Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Laboratory Investing*, ***69***(9): 238-249.

Ha, E. and Zemel, M.B., 2003. Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people. *The Journal of Nutritional Biochemistry*, ***14***(5): 251-258.

Habil, N., Abate, W., Beal, J. and Foey, A.D., 2014. Heat-killed probiotic bacteria differentially regulate colonic epithelial cell production of human β-defensin-2: dependence on inflammatory cytokines. *Beneficial Microbes*, ***5***(4): 483-495.

Harb, H., Van Tol, E.A.F., Heine, H., Braaksma, M., Gross, G., Overkamp, K., Hennen, M., Alrifai, M., Conrad, M.L., Renz, H. and Garn, H., 2013. Neonatal supplementation of processed supernatant from *Lactobacillus rhamnosus* GG improves allergic airway inflammation in mice later in life. *Clinical and Experimental Allergy*, ***43***(3): 353-364.

Hauggaard-Nielsen, H. and Jensen, E.S., 2001. Evaluating pea and barley cultivars for complementarity in intercropping at different levels of soil N availability. *Field Crops Research*, ***72***(3): 185-196.

Heller, K.J., 2001. Probiotic bacteria in fermented foods: product characteristics and starter organisms. *The American Journal of Clinical Nutrition*, **73**(2): 374-379.

Herias, M.V., Koninkx, J.F.J.G., Vos, J.G., In't Veld, J.H. and Van Dijk, J.E., 2005. Probiotic effects of *Lactobacillus casei* on DSS-induced ulcerative colitis in mice. *International Journal of Food Microbiology*, ***103***(2): 143-155.

Hering, N.A., Andres, S., Fromm, A., van Tol, E.A., Amasheh, M., Mankertz, J., Fromm, M. and Schulzke, J.D., 2011. Transforming growth factor-β, a whey protein component, strengthens the intestinal barrier by upregulating claudin-4 in HT-29/B6 cells. *The Journal of Nutrition*, ***141***(5): 783-789.

Himmel, M.E., Hardenberg, G., Piccirillo, C.A., Steiner, T.S. and Levings, M.K., 2008. The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease. *Immunology*, ***125***(2): 145-153.

Ho, S., Pothoulakis, C. and Wai Koon, H., 2013. Antimicrobial peptides and colitis. *Current Pharmaceutical Design*, ***19*(**1): 40-47.

Homayouni, A., Alizadeh, M., Alikhah, H. and Zijah, V., 2012. Functional dairy probiotic food development: trends, concepts, and products. *Immunology and Microbiology:"Probiotics", ed. E. Rigobelo (Rijeka: InTech)* ***17***(2) :197-212.

Huang, I.F., Lin, I.C., Liu, P.F., Cheng, M.F., Liu, Y.C., Hsieh, Y.D., Chen, J.J., Chen, C.L., Chang, H.W. and Shu, C.W., 2015. *Lactobacillus acidophilus* attenuates Salmonella-induced intestinal inflammation via TGF-β signaling. *BMC Microbiology*, ***15***(1): 1-9.

Imaoka, A., Shima, T., Kato, K., Mizuno, S., Uehara, T., Matsumoto, S., Setoyama, H., Hara, T. and Umesaki, Y., 2008. Anti-inflammatory activity of probiotic *Bifidobacterium*: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. *World Journal of Gastroenterology,* ***14***(16): 15-25.

IS: (SP: 18), "ISI Handbook of Food Analysis, Part XI, 1981: Dairy Products. Indian Standards Institution, New Delhi.

Iskandar, M.M., Dauletbaev, N., Kubow, S., Mawji, N. and Lands, L.C., 2013. Whey protein hydrolysates decrease IL-8 secretion in lipopolysaccharide (LPS)-stimulated respiratory epithelial cells by affecting LPS binding to Toll-like receptor 4. *British Journal of Nutrition*, ***110***(1): 58-68.

Jadhav, S.R., Shandilya, U.K. and Kansal, V.K., 2013. Exploring the ameliorative potential of probiotic Dahi containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum* on dextran sodium sulphate induced colitis in mice. *The Journal of Dairy Research*, ***80***(1): 11-21.

Jiang, Y., Lü, X., Man, C., Han, L., Shan, Y., Qu, X., Liu, Y., Yang, S., Xue, Y. and Zhang, Y., 2012. *Lactobacillus acidophilus* induces cytokine and chemokine production via NF-κB and p38 MAPK signaling pathways in intestinal epithelial cells. *Clinical and Vaccine Immunology,* ***20***(4): 10-21.

K. J. Livak and T. D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method, *Methods.* **25**(2): 402–408.

Kaci, G., Lakhdari, O., Doré, J., Ehrlich, S.D., Renault, P., Blottière, H.M. and Delorme, C., 2011. Inhibition of the NF-κB pathway in human intestinal epithelial cells by commensal *Streptococcus salivarius*. *Applied and Environmental Microbiology*, ***77***(13): 4681-4684.

Kaji, R., Kiyoshima-Shibata, J., Nagaoka, M., Nanno, M. and Shida, K., 2010. Bacterial teichoic acids reverse predominant IL-12 production induced by certain lactobacillus strains into predominant IL-10 production via TLR2-dependent ERK activation in macrophages. *The Journal of Immunology*, ***184***(7): 3505-3513.

Kamdar, K., Nguyen, V. and DePaolo, R.W., 2013. Toll-like receptor signaling and regulation of intestinal immunity. *Virulence*, ***4***(3): 207-212.

Kanmani, P. and Kim, H., 2020. Beneficial effect of immunobiotic strains on attenuation of *Salmonella* induced inflammatory response in human intestinal epithelial cells. *Plos One*, ***15***(3): 12-24.

Karimi, R., Mortazavian, A.M. and Da Cruz, A.G., 2011. Viability of probiotic microorganisms in cheese during production and storage: a review. *Dairy Science and Technology*, ***91***(3): .283-308.

Karimi, R., Sohrabvandi, S. and Mortazavian, A.M., 2012. Sensory characteristics of probiotic cheese. *Comprehensive Reviews in Food Science and Food Safety*, ***11***(5): 437-452.

Karlsson, J., Pütsep, K., Chu, H., Kays, R.J., Bevins, C.L. and Andersson, M., 2008. Regional variations in Paneth cell antimicrobial peptide

expression along the mouse intestinal tract. *BMC Immunology*, ***9***(1): 1-11.

Kaur, G., Ali, S.A., Kumar, S., Mohanty, A.K. and Behare, P., 2017. Label-free quantitative proteomic analysis of *Lactobacillus fermentum* NCDC 400 during bile salt exposure. *Journal of Proteomics*, ***167***(3): 36-45.

Kaur, H., Gupta, T., Kapila, S. and Kapila, R., 2020. Role of fermented dairy foods in human health. *Indian Journal of Dairy Science*, ***73***(2): 1-14.

Kaushal, D. and Kansal, V.K., 2011. Age-related decline in macrophage and lymphocyte functions in mice and its alleviation by treatment with probiotic Dahi containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. *The Journal of Dairy Research*, ***78***(4): 390-404.

Kawamoto, S., Tran, T.H., Maruya, M., Suzuki, K., Doi, Y., Tsutsui, Y., Kato, L.M. and Fagarasan, S., 2012. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science*, ***336***(6080): 485-489.

Khmaladze, I., Butler, É., Fabre, S. and Gillbro, J.M., 2019. *Lactobacillus reuteri* DSM 17938—A comparative study on the effect of probiotics and lysates on human skin. *Experimental Dermatology*, ***28***(7): 822-828.

Khor, B., Gardet, A. and Xavier, R.J., 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature*, ***474***(7351): 307-317.

Kiewiet, M.G., Dekkers, R., Gros, M., van Neerven, R.J., Groeneveld, A., de Vos, P. and Faas, M.M., 2017. Toll-like receptor mediated activation is possibly involved in immunoregulating properties of cow's milk hydrolysates. *PLoS One*, ***12***(6): 81-91.

Kim, J.M., 2014. Antimicrobial proteins in intestine and inflammatory bowel diseases. *Intestinal Research*, ***12***(1): 1-20.

Kolacek, S., Hojsak, I., Canani, R.B., Guarino, A., Indrio, F., Pot, B., Shamir, R., Szajewska, H., Vandenplas, Y., Van Goudoever, J. and Weizman, Z., 2017. Commercial probiotic products: a call for improved quality control. A position paper by the ESPGHAN Working Group for

Probiotics and Prebiotics. *Journal of Pediatric Gastroenterology and Nutrition*, ***65***(1): 117-124.

Koller, M., Salerno, A., Muhr, A., Reiterer, A., Chiellini, E., Casella, S., Horvat, P. and Braunegg, G., 2012. Whey lactose as a raw material for microbial production of biodegradable polyesters. *Polyester*, ***347***(2): 51-92.

Kordjazy, N., Haj-Mirzaian, A., Haj-Mirzaian, A., Rohani, M.M., Gelfand, E.W., Rezaei, N. and Abdolghaffari, A.H., 2018. Role of toll-like receptors in inflammatory bowel disease. *Pharmacological Research*, ***129***(3): 204-215.

Krisanaprakornkit, S., Kimball, J.R., Weinberg, A., Darveau, R.P., Bainbridge, B.W. and Dale, B.A., 2000. Inducible expression of human β-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infection and Immunity*, ***68***(5): 2907-2915.

Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T. and Zilmer, M., 2003. Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects. *British Journal of Nutrition*, ***90***(2): 449-456.

Kumar, A., Chatterjee, I., Gujral, T., Alakkam, A., Coffing, H., Anbazhagan, A.N., Borthakur, A., Saksena, S., Gill, R.K., Alrefai, W.A. and Dudeja, P.K., 2017. Activation of Nuclear Factor− κB by Tumor Necrosis Factor in Intestinal Epithelial Cells and Mouse Intestinal Epithelia Reduces Expression of the Chloride Transporter SLC26A3. *Gastroenterology*, ***153***(5): 1338-1350.

Kusumaningsih, T., Subijanto, M.S., Indrawati, R. and Devijanti, R.R., 2016. The level of beta defensin-2 in saliva and its expression in parotid gland epithelial cells after probiotic (*Lactobacillus reuteri*) induction to inhibit Streptococcus mutants in caries. *European Journal of Dentistry*, ***10***(4): 556-571.

L Koltsova, E., 2019. *Prediction of THMs formation potential in disinfection of drinking water* (Master's thesis, Norwegian University of Life Sciences, Ås).

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**(5259): 680-685.

Laffineur, E., Genetet, N. and Leonil, J., 1996. Immunomodulatory activity of β-casein permeate medium fermented by lactic acid bacteria. *Journal of Dairy Science*, ***79***(12): 2112-2120.

Lagouri, V. ed., 2019. Functional Foods. ed. 3. New York: *National Hellenic Research Foundation*, 573-631.

Lee, H.S., Han, S.Y., Bae, E.A., Huh, C.S., Ahn, Y.T., Lee, J.H. and Kim, D.H., 2008. Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice. *International Immunopharmacology*, ***8***(4): 574-580.

Lee, J., Rheem, S., Yun, B., Ahn, Y., Joung, J., Lee, S.J., Oh, S., Chun, T., Rheem, I., Yea, H.S. and Lim, K.S., 2013. Effects of probiotic yoghurt on symptoms and intestinal microbiota in patients with irritable bowel syndrome. *International Journal of Dairy Technology*, ***66***(2): 243-255.

Lee, J.H., Lee, B., Lee, H.S., Bae, E.A., Lee, H., Ahn, Y.T., Lim, K.S., Huh, C.S. and Kim, D.H., 2009. *Lactobacillus suntoryeus* inhibits pro-inflammatory cytokine expression and TLR-4-linked NF-κB activation in experimental colitis. *International Journal of Colorectal Disease*, ***24***(2): 231-237.

Lee, S.H., eun Kwon, J. and Cho, M.L., 2018. Immunological pathogenesis of inflammatory bowel disease. *Intestinal Research*, ***16***(1): 15-26.

Lesueur, C., Bôle-Feysot, C., Bekri, S., Husson, A., Lavoinne, A. and Brasse-Lagnel, C., 2012. Glutamine induces nuclear degradation of the NF-κB p65 subunit in Caco-2/TC7 cells. *Biochimie*, ***94***(3): 806-815.

Lewis, S.B., Prior, A., Ellis, S.J., Cook, V., Chan, S.S., Gelson, W. and Schüller, S., 2016. Flagellin induces β-defensin 2 in human colonic ex vivo infection with enterohemorrhagic *Escherichia coli. Frontiers in Cellular and Infection Microbiology*, ***6***(2): 68-81.

Liao, S.F. and Nyachoti, M., 2017. Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition*, ***3***(4): 331-343.

Liao, Y., Alvarado, R., Phinney, B. and Lönnerdal, B., 2011. Proteomic characterization of human milk fat globule membrane proteins during a 12 month lactation period. *Journal of Proteome Research*, ***10***(8): 3530-3541.

Liu, H., Hou, C., Wang, G., Jia, H., Yu, H., Zeng, X., Thacker, P.A., Zhang, G. and Qiao, S., 2017a. *Lactobacillus reuteri* I5007 modulates intestinal host defense peptide expression in the model of IPEC-J2 cells and neonatal piglets. *Nutrients*, ***9***(6): 500-559.

Liu, M. Y., Yang, Z. Y., Dai, W. K., Huang, J. Q., Li, Y. H., Zhang, J., and Zheng, Y. J. 2017b. Protective effect of *Bifidobacterium infantis* CGMCC313-2 on ovalbumin-induced airway asthma and β-lactoglobulin-induced intestinal food allergy mouse models. *World Journal of Gastroenterology*, **23**(12): 21-49.

Liu, Q., Yu, Z., Tian, F., Zhao, J., Zhang, H., Zhai, Q. and Chen, W., 2020. Surface components and metabolites of probiotics for regulation of intestinal epithelial barrier. *Microbial Cell Factories*, ***19***(1): 1-23.

Liu, Z., Zhang, Z., Qiu, L., Zhang, F., Xu, X., Wei, H. and Tao, X., 2017. Characterization and bioactivities of the exopolysaccharide from a probiotic strain of *Lactobacillus plantarum* WLPL04. *Journal of Dairy Science*, ***100***(9): 6895-6905.

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, ***25***(4): 402-408.

Llewellyn, A. and Foey, A., 2017. Probiotic modulation of innate cell pathogen sensing and signaling events. *Nutrients*, ***9***(10): 11-56.

Lourens-Hattingh, A. and Viljoen, B.C., 2001. Growth and survival of a probiotic yeast in dairy products. *Food Research International*, ***34***(9): 791-796.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, ***193***(11): 265-275.

Pescuma, M., Hébert, E. M., Mozzi, F., and de Valdez, G. F., 2008. Whey fermentation by thermophilic lactic acid bacteria: Evolution of carbohydrates and protein content. *Food Microbiology*, ***25***(3): 442-451.

Prado-Rebolledo, O.F., Delgado-Machuca, J.D.J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X. and Tellez, G., 2017. Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica serovar Enteritidis* colonization and intestinal permeability in broiler chickens. *Avian Pathology*, ***46***(1): 90-94.

Ma, Y., Liu, J., Shi, H. and Yu, L.L., 2016. Isolation and characterization of anti-inflammatory peptides derived from whey protein. *Journal of Dairy Science*, ***99***(9): 6902-6912.

Madureira, A.R., Pereira, C.I., Gomes, A.M., Pintado, M.E. and Malcata, F.X., 2007. Bovine whey proteins–Overview on their main biological properties. *Food Research International*, ***40***(10): 1197-1211.

Magalhães, K.T., Pereira, M.A., Nicolau, A., Dragone, G., Domingues, L., Teixeira, J.A., de Almeida Silva, J.B. and Schwan, R.F., 2010. Production of fermented cheese whey-based beverage using kefir grains as starter culture: Evaluation of morphological and microbial variations. *Bioresource Technology*, ***101***(22): 8843-8850.

Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligné, B., Gänzle, M., Kort, R., Pasin, G., Pihlanto, A. and Smid, E.J., 2017. Health benefits of fermented foods: microbiota and beyond. *Current Opinion in Biotechnology*, ***44***(6): 94-102.

Martinez, R.C.R., Bedani, R. and Saad, S.M.I., 2015. Scientific evidence for health effects attributed to the consumption of probiotics and prebiotics: an update for current perspectives and future challenges. *British Journal of Nutrition*, ***114***(12): 1993-2015.

Matsumoto, S., Watanabe, N., Imaoka, A. and Okabe, Y., 2001. Preventive effects of Bifidobacterium-and *Lactobacillus*-fermented milk on the development of inflammatory bowel disease in senescence-accelerated mouse P1/Yit strain mice. *Digestion*, ***64***(2): 92-99.

McGuckin, M.A., Eri, R., Simms, L.A., Florin, T.H. and Radford-Smith, G., 2009. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflammatory Bowel Diseases*, ***15***(1): 100-113.

Meyer, A.L., Elmadfa, I., Herbacek, I. and Micksche, M., 2007. Probiotic, as well as conventional yogurt, can enhance the stimulated production of proinflammatory cytokines. *Journal of Human Nutrition and Dietetics*, ***20***(6): 590-598.

Miyazawa, K., Harata, G., Yoda, K., Yamazaki, K., He, F. and Hiramatsu, M., 2018. Effects of intake of *Lactobacillus rhamnosus GG* on intestinal environment and skin condition in healthy adults: a randomized, double-blind, placebo-controlled study. *International Journal of Probiotics and Prebiotics*, ***13***(1): 11-18.

Mollea, C., Marmo, L. and Bosco, F., 2013. Valorisation of cheese whey, a by-product from the dairy industry. In *Food Industry*. ***11***(6): 172-185.

Möndel, M., Schroeder, B.O., Zimmermann, K., Huber, H., Nuding, S., Beisner, J., Fellermann, K., Stange, E.F. and Wehkamp, J., 2009. Probiotic *E. coli* treatment mediates antimicrobial human β-defensin synthesis and fecal excretion in humans. *Mucosal Immunology*, ***2***(2): 166-172.

Muniz, L.R., Knosp, C. and Yeretssian, G., 2012. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Frontiers in Immunology*, ***3***(4): 300-310.

Murakami, K., Lagarde, M. and Yuki, Y., 1998. Identification of minor proteins of human colostrum and mature milk by two-dimensional electrophoresis. *Electrophoresis*, ***19***(14): 2521-2527.

Nagao, F., Nakayama, M., Muto, T. and Okumura, K., 2000. Effects of a fermented milk drink containing *Lactobacillus casei* strain *Shirota* on the immune system in healthy human subjects. *Bioscience,*

Biotechnology, and Biochemistry, ***64***(12): 2706-2708.

Nam, T.G., Lim, T.G., Lee, B.H., Lim, S., Kang, H., Eom, S.H., Yoo, M., Jang, H.W. and Kim, D.O., 2017. Comparison of anti-inflammatory effects of flavonoid-rich common and tartary buckwheat sprout extracts in lipopolysaccharide-stimulated RAW 264.7 and peritoneal macrophages. *Oxidative Medicine and Cellular Longevity*, ***17***(5): 1-22

Nataraj, B.H., Ali, S.A., Behare, P.V. and Yadav, H., 2020. Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. *Microbial Cell Factories*, ***19*** (1):1-22.

Neurath, M.F., 2014. Cytokines in inflammatory bowel disease. *Nature Reviews Immunology*, ***14***(5): 329-342.

Nguyen, D.N., Jiang, P., Stensballe, A., Bendixen, E., Sangild, P.T. and Chatterton, D.E., 2016. Bovine lactoferrin regulates cell survival, apoptosis and inflammation in intestinal epithelial cells and preterm pig intestine. *Journal of Proteomics,* ***139***(2): 95-102.

Nicoletti, M., 2012. Nutraceuticals and botanicals: overview and perspectives. *International Journal of Food Sciences and Nutrition*, **63**(1): 2-6.

Nielsen, P.M., Petersen, D. and Dambmann, C., 2001. Improved method for determining food protein degree of hydrolysis. *Journal of Food Science*, ***66***(5): 642-646.

Nyeko, R., Kalyesubula, I., Mworozi, E. and Bachou, H., 2010. Lactose intolerance among severely malnourished children with diarrhoea admitted to the nutrition unit, Mulago hospital, Uganda. *BMC Pediatrics*, ***10***(1): 1-9.

O'Keeffe, M. and St-Onge, M.P., 2013. Saturated fat and cardiovascular disease: A review of current evidence. *Current Cardiovascular Risk Reports*, **7**(2): 154-162.

O'neil, J., Hughes, S., Lourie, A. and Zweifler, J., 2008. Effects of echinacea on the frequency of upper respiratory tract symptoms: a randomized, double-blind, placebo-controlled trial. *Annals of Allergy, Asthma and Immunology*, ***100***(4): 384-388.

Oeckinghaus, A. and Ghosh, S., 2009. The NF-κB family of transcription factors and its regulation. *Cold Spring Harbor Perspectives in Biology*, ***1***(4): 24-34.

Ohtsuka, Y. and Sanderson, I.R., 2003. Dextran sulfate sodium—Induced inflammation is enhanced by intestinal epithelial cell chemokine expression in mice. *Pediatric Research*, ***53***(1): 143-147.

Ong, L., Henriksson, A. and Shah, N.P., 2006. Development of probiotic Cheddar cheese containing *Lactobacillus acidophilus, Lb. casei, Lb. paracasei* and *Bifidobacterium spp.* and the influence of these bacteria on proteolytic patterns and production of organic acid. *International Dairy Journal*, ***16***(5): 446-456.

Onyiah, J.C. and Colgan, S.P., 2016. Cytokine responses and epithelial function in the intestinal mucosa. *Cellular and Molecular Life Sciences*, ***73***(22): 4203-4212.

Ørskov, E.R., 1995. A Traveller's view of Outer Mongolia. *Outlook on Agriculture*, ***24***(2): 127-129.

Ostadrahimi, A., Taghizadeh, A., Mobasseri, M., Farrin, N., Payahoo, L., Gheshlaghi, Z.B. and Vahedjabbari, M., 2015. Effect of probiotic fermented milk (kefir) on glycemic control and lipid profile in type 2 diabetic patients: a randomized double-blind placebo-controlled clinical trial. *Iranian Journal of Public Health*, ***44***(2): 228-237.

Panduru, M., Panduru, N.M., Sălăvăstru, C.M. and Tiplica, G.S., 2015. Probiotics and primary prevention of atopic dermatitis: a meta-analysis of randomized controlled studies. *Journal of the European Academy of Dermatology and Venereology*, ***29***(2): 232-242.

Panicker, A.S., Ali, S.A., Anand, S., Panjagari, N.R., Kumar, S., Mohanty, A.K. and Behare, P.V., 2018. Evaluation of some *in vitro* probiotic properties of *Lactobacillus fermentum* Strains. *Journal of Food Science and Technology*, ***55***(7): 2801-2807.

Park, JH., Lee, YE., Moon, EN., Seok, SH., BaekMW, LeeHY., Kim, DJ., Kim, CH., Park, JH., 2005. Safety assessment of *Lactobacillus fermentum*

PL9005, a potential probiotic lactic acid bacterium, in mice. *Journal of Microbiology Biotechnology, **15***(7): 603–608.

Pérez-Cano, F.J., Marín-Gallén, S., Castell, M., Rodríguez-Palmero, M., Rivero, M., Franch, A. and Castellote, C., 2007. Bovine whey protein concentrate supplementation modulates maturation of immune system in suckling rats. *British Journal of Nutrition*, ***98***(1): 80-84.

Peterson, L.W. and Artis, D., 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology*, ***14***(3): 141-153.

Pfaffl, M.W. and Hageleit, M., 2001. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. *Biotechnology Letters*, ***23***(4): 275-282.

Pinto, M.G., Gomez, M., Seifert, S., Watzl, B. and Holzapfel, W.H., 2009. Lactobacilli stimulate the innate immune response and modulate the TLR expression of HT29 intestinal epithelial cells *in vitro*. International *Journal of Food Microbiology*, ***133***(4): 86–93.

Prado-Rebolledo, O.F., Delgado-Machuca, J.D.J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X. and Tellez, G., 2017. Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica* serovar Enteritidis colonization and intestinal permeability in broiler chickens. *Avian pathology*, ***46***(1): 90-94.

Pragya, P., Kaur, G., Ali, S.A., Bhatla, S., Rawat, P., Lule, V., Kumar, S., Mohanty, A.K. and Behare, P., 2017. High-resolution mass spectrometry-based global proteomic analysis of probiotic strains *Lactobacillus fermentum* NCDC 400 and RS2. *Journal of Proteomics*, ***152***(4): 121-130.

Qin, H., Zhang, Z., Hang, X. and Jiang, Y., 2009. *L. plantarum* prevents enteroinvasive *Escherichia coli*-induced tight junction proteins changes in intestinal epithelial cells. *BMC Microbiology*, ***9***(1): 1-9.

Rakoff-Nahoum, S. and Comstock, L.E., 2014. Starve a fever, feed the microbiota. *Nature*, ***514***(4): 576-577.

Reinhardt, T. A., Lippolis, J. D., Nonnecke, B. J., and Sacco, R. E., 2012. Bovine milk exosome proteome. *Journal of Proteomics*, ***75***(5): 1486-1492.

Rezac, S., Kok, C.R., Heermann, M. and Hutkins, R., 2018. Fermented foods as a dietary source of live organisms. *Frontiers in Microbiology*, ***9***(1): 75-85.

Rodriguez-Palacios, A., Aladyshkina, N. and Cominelli, F., 2015. Stereomicroscopy and 3D-target myeloperoxidase intestinal phenotyping following a fecal flora homogenization protocol. *Protocol Exchange*, ***6***(3): 1-24.

Rokana, N., Singh, R., Mallappa, R.H., Batish, V.K. and Grover, S., 2016. Modulation of intestinal barrier function to ameliorate Salmonella infection in mice by oral administration of fermented milks produced with *Lactobacillus plantarum* MTCC 5690–a probiotic strain of Indian gut origin. *Journal of Medical Microbiology*, ***65***(12): 1482-1493.

Rong, J., Zheng, H., Liu, M., Hu, X., Wang, T., Zhang, X., Jin, F. and Wang, L., 2015. Probiotic and anti-inflammatory attributes of an isolate *Lactobacillus helveticus* NS8 from Mongolian fermented koumiss. *BMC Microbiology*, ***15***(1): 1-11.

Roselli, M., Finamore, A., Hynönen, U., Palva, A. and Mengheri, E., 2016. Differential protection by cell wall components of *Lactobacillus amylovorus* DSM 16698 T against alterations of membrane barrier and NF-kB activation induced by enterotoxigenic F4+ *Escherichia coli* on intestinal cells. *BMC Microbiology*, ***16***(1): 1-10.

Ross, R.P., Fitzgerald, G., Collins, K. and Stanton, C., 2002. Cheese delivering biocultures--probiotic cheese. *Australian Journal of Dairy Technology*, ***57***(2): 61-71.

Round, J.L., Lee, S.M., Li, J., Tran, G., Jabri, B., Chatila, T.A. and Mazmanian, S.K., 2011. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science*, ***332***(2): 974-977.

Rusu, D., Drouin, R., Pouliot, Y., Gauthier, S. and Poubelle, P.E., 2010. A bovine whey protein extract stimulates human neutrophils to generate bioactive IL-1Ra through a NF-κB-and MAPK-dependent mechanism. *The Journal of Nutrition*, ***140***(2): 382-391.

Sakai, F., Hosoya, T., Ono-Ohmachi, A., Ukibe, K., Ogawa, A., Moriya, T., Kadooka, Y., Shiozaki, T., Nakagawa, H., Nakayama, Y. and Miyazaki, T., 2014. *Lactobacillus gasseri* SBT2055 induces TGF-β expression in dendritic cells and activates TLR2 signal to produce IgA in the small intestine. *PloS One*, ***9***(8): 53-70.

Saliganti, V., Kapila, R., Sharma, R. and Kapila, S., 2015. Feeding probiotic *Lactobacillus rhamnosus* (MTCC 5897) fermented milk to suckling mothers alleviates ovalbumin-induced allergic sensitisation in mice offspring. *British Journal of Nutrition*, ***114***(8): 1168-1179.

Saliganti, V., Kapila, R., .and Kapila, S., 2016. Consumption of probiotic *Lactobacillus rhamnosus* (MTCC: 5897) containing fermented milk plays a key role in development of the immune system in newborn mice during the suckling–weaning transition. *Microbiology and Immunology* **60**(4): 261-267.

Santiago-López, L., Aguilar-Toalá, J.E., Hernández-Mendoza, A., Vallejo-Cordoba, B., Liceaga, A.M. and González-Córdova, A.F., 2018. Invited review: Bioactive compounds produced during cheese ripening and health effects associated with aged cheese consumption. *Journal of Dairy Science*, ***101***(5): 3742-3757.

Sarkar, S., 2008. Innovations in Indian fermented milk products—a review. *Food Biotechnology*, ***22***(1): 78-97.

Schlee, M., Harder, J., Köten, B., Stange, E.F., Wehkamp, J. and Fellermann, K., 2008. Probiotic lactobacilli and VSL# 3 induce enterocyte β-defensin 2. *Clinical and Experimental Immunology*, ***151***(3): 528-535.

Sendra, E., Fayos, P., Lario, Y., Fernández-López, J., Sayas-Barberá, E. and Pérez-Alvarez, J.A., 2008. Incorporation of citrus fibers in fermented milk containing probiotic bacteria. *Food Microbiology*, **25**(1): 13-21.

Seo, M.B., Lee, S.K., Jeon, Y.J. and Im, J.S., 2011. Inhibition of p65 nuclear translocation by baicalein. *Toxicological Research*, ***27***(2): 71-76.

Sharma, R., Kapila, R., Dass, G. and Kapila, S., 2014,a. Improvement in Th1/Th2 immune homeostasis, antioxidative status and resistance to pathogenic *E. coli* on consumption of probiotic *Lactobacillus rhamnosus* fermented milk in aging mice. *Age*, ***36***(4): 1-17.

Sharma, R., Kapila, R., Kapasiya, M., Saliganti, V., Dass, G. and Kapila, S., 2014,b. Dietary supplementation of milk fermented with probiotic *Lactobacillus fermentum* enhances systemic immune response and antioxidant capacity in aging mice. *Nutrition Research*, **34**(11): 968-981

Shen, Q., Shang, N., and Li, P. 2011. *In vitro* and *in vivo* antioxidant activity of *Bifidobacterium animalis* 01 isolated from centenarians. *Current Microbiology*, ***62***(4): 1097-1103.

Shi, N., Li, N., Duan, X. and Niu, H., 2017. Interaction between the gut microbiome and mucosal immune system. *Military Medical Research*, ***4***(1): 1-7.

Silviya, R.M., Bhumika, K., Dabhi Parmar, S.C. and Aparnathi, K.D., 2016. Whey and its utilization. *International Journal of Current Microbiology and Applied Sciences,* **5**(8): 134-155.

Sprong, R.C., Schonewille, A.J. and Van der Meer, R., 2010. Dietary cheese whey protein protects rats against mild dextran sulfate sodium–induced colitis: Role of mucin and microbiota. *Journal of Dairy Science*, ***93***(4): 1364-1371.

Sproston, N.R. and Ashworth, J.J., 2018. Role of C-reactive protein at sites of inflammation and infection. *Frontiers in Immunology*, ***9***(6): 44-54.

Stanton, C., Desmond, C., Coakley, M., Collins, J.K., Fitzgerald, G. and Ross, R.P., 2003. Challenges facing development of probiotic-containing functional foods. *Handbook of Fermented Functional Foods*, ***27***(2): 11-22

St-Onge, M.P., Farnworth, E.R. and Jones, P.J., 2000. Consumption of fermented and nonfermented dairy products: effects on cholesterol concentrations and metabolism. *The American Journal of Clinical Nutrition*, ***71***(3): 674-681.

Suwal, S., Wu, Q., Liu, W., Liu, Q., Sun, H., Liang, M., Gao, J., Zhang, B., Kou, Y., Liu, Z. and Wei, Y., 2018. The probiotic effectiveness in preventing experimental colitis is correlated with host gut microbiota. *Frontiers in Microbiology*, ***9***(5): 26-75.

Reinhardt, T. A., Lippolis, J. D., Nonnecke, B. J., & Sacco, R. E. (2012). Bovine milk exosome proteome. *Journal of Proteomics*, ***75***(5): 1486-1492.

Tagliazucchi, D., Helal, A., Verzelloni, E., Bellesia, A. and Conte, A., 2016. Composition and properties of peptides that survive standardised *in vitro* gastro-pancreatic digestion of bovine milk. *International Dairy Journal*, ***61***(2): 196-204.

Takahashi, A., Wada, A., Ogushi, K.I., Maeda, K., Kawahara, T., Mawatari, K., Kurazono, H., Moss, J., Hirayama, T. and Nakaya, Y., 2001. Production of β-defensin-2 by human colonic epithelial cells induced by *Salmonella enteritidis* flagella filament structural protein. *Federation of European Biochemical Societies*, ***508***(3): 484-488.

Tang, Q. and Bluestone, J.A., 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology*, ***9***(3): 239-244.

Tay, E.P. and Gam, L.H., 2011. Proteomics of human and the domestic bovine and caprine milk. *Journal of Molecular Biology Biotechnology*, ***19***(1): 45-53.

Tellez, A., Corredig, M., Brovko, L.Y. and Griffiths, M.W., 2010. Characterization of immune-active peptides obtained from milk fermented by *Lactobacillus helveticus*. *Journal of Dairy Research*, ***77***(2): 121-129.

Thoreux, K. and Schmucker, D.L., 2001. Kefir milk enhances intestinal immunity in young but not old rats. *The Journal of Nutrition*, ***131***(3): 807-812.

Tsutsumi, R. and Tsutsumi, Y.M., 2014. Peptides and proteins in whey and their benefits for human health. *Austin Journal of Nutrition Food Science*,

1(1): 1-20.

Tuohy, K.M., Conterno, L., Gasperotti, M. and Viola, R., 2012. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. *Journal of Agricultural and Food Chemistry*, ***60***(36): 8776-8782.

Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., Wells, J. M., and Roy, N. C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of Nutrition*, ***141***(5): 769-776.

Van Kampen, C., Gauldie, J. and Collins, S.M., 2005. Proinflammatory properties of IL-4 in the intestinal microenvironment. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, ***288***(1): 111-117.

Vinderola, G., Matar, C. and Perdigón, G., 2007. Milk fermentation products of *L. helveticus* R389 activate calcineurin as a signal to promote gut mucosal immunity. *BMC Immunology*, ***8***(1): 1-10.

Vinderola, G., Perdigon, G., Duarte, J., Thangavel, D., Farnworth, E. and Matar, C., 2006,a. Effects of kefir fractions on innate immunity. *Immunobiology*, ***211***(3): 149-156.

Vinderola, G., Perdigón, G., Duarte, J., Farnworth, E. and Matar, C., 2006,b. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. *Cytokine*, ***36***(6): 254-260.

Visser, J., Rozing, J., Sapone, A., Lammers, K. and Fasano, A., 2009. Tight junctions, intestinal permeability, and autoimmunity celiac disease and type 1 diabetes paradigms. *Annals of the New York Academy of Sciences*, ***65***(4): 14-22.

Volynets, V., Reichold, A., Bárdos, G., Rings, A., Bleich, A. and Bischoff, S.C., 2016. Assessment of the intestinal barrier with five different permeability tests in healthy C57BL/6J and BALB/cJ mice. *Digestive Diseases and Sciences*, ***61***(3): 737-746.

Walker, W.A., 2008. Mechanisms of action of probiotics. *Clinical Infectious Diseases*, ***46***(9): 87-91.

Wang, J. and Wang, H., 2017. Oxidative stress in pancreatic beta cell regeneration. *Oxidative Medicine and Cellular Longevity,* ***12***(2): 1-12.

Wang, L., Llorente, C., Hartmann, P., Yang, A.M., Chen, P. and Schnabl, B., 2015,a. Methods to determine intestinal permeability and bacterial translocation during liver disease. *Journal of Immunological Methods*, ***421***(1): 44-53.

Wang, L., Wang, Y.Y., Wang, D.Q., Xu, J., Yang, F., Liu, G., Zhang, D.Y., Feng, Q., Xiao, L., Xue, W.B. and Guo, J., 2015,b. Dynamic changes in the bacterial community in Moutai liquor fermentation process characterized by deep sequencing. *Journal of the Institute of Brewing*, **121**(4): 603-608.

Wehkamp, J., Schwind, B., Herrlinger, K.R., Baxmann, S., Schmidt, K., Duchrow, M., Wohlschläger, C., Feller, A.C., Stange, E.F. and Fellermann, K., 2002. Innate immunity and colonic inflammation: enhanced expression of epithelial α-defensins. *Digestive Diseases and Sciences*, ***47***(6): 1349-1355.

Weill, F.S., Cela, E.M., Paz, M.L., Ferrari, A., Leoni, J. and Maglio, D.H.G., 2013. Lipoteichoic acid from *Lactobacillus rhamnosus* GG as an oral photoprotective agent against UV-induced carcinogenesis. *British Journal of Nutrition*, ***109***(3): 457-466.

Wells, J.M., 2011. Immunomodulatory mechanisms of lactobacilli. In *Microbial Cell Factories*, ***10***(1): 1-15.

Widodo, W., Sakti, A.P., Sukarno, A.S., Wahyuni, E. and Nurliyani, N., 2019. The Effect of Different Starter Cultures of *Lactobacillus paracasei* M104 and *Pediococcus pentosaceus* M103 on the Physicochemical and Microbial Qualities of Fermented Goat Milk. *Jurnal Ilmu dan Teknologi Hasil Ternak*, ***14***(2): 70-77.

Wong, C.W., Seow, H.F., Liu, A.H., Husband, A.J., Smithers, G.W. and Watson, D.L., 1996. Modulation of immune responses by bovine β-

casein. *Immunology and Cell Biology*, ***74***(4): 323-329.

Xavier, R.J. and Podolsky, D.K., 2007. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*, ***448***(2): 427-434.

Xiao, L., Rao, J.N., Cao, S., Liu, L., Chung, H.K., Zhang, Y., Zhang, J., Liu, Y., Gorospe, M. and Wang, J.Y., 2016. Long noncoding RNA SPRY4-IT1 regulates intestinal epithelial barrier function by modulating the expression levels of tight junction proteins. *Molecular Biology of the Cell*, ***27***(4): 617-626.

Xu, C.Q., Liu, B.J., Wu, J.F., Xu, Y.C., Duan, X.H., Cao, Y.X. and Dong, J.C., 2010. Icariin attenuates LPS-induced acute inflammatory responses: involvement of PI3K/Akt and NF-κB signaling pathway. *European Journal of Pharmacology*, ***642***(3): 146-153.

Yadav, J.S.S., Bezawada, J., Ajila, C.M., Yan, S., Tyagi, R.D. and Surampalli, R.Y., 2014. Mixed culture of *Kluyveromyces marxianus* and *Candida krusei* for single-cell protein production and organic load removal from whey. *Bioresource Technology*, ***164***(1): 119-127.

Yadav, R., Dey, D.K., Vij, R., Meena, S., Kapila, R. and Kapila, S., 2018. Evaluation of anti-diabetic attributes of *Lactobacillus rhamnosus* MTCC: 5957, *Lactobacillus rhamnosus* MTCC: 5897 *and Lactobacillus fermentum* MTCC: 5898 in streptozotocin induced diabetic rats. *Microbial pathogenesis*, ***125***(4): 454-462.

Yang, D., Chen, Q., Chertov, O. and Oppenheim, J.J., 2000. Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *Journal of Leukocyte Biology*, ***68***(1): 9-14.

Yang, D., Fu, X., He, S., Ning, X. and Ling, M., 2017. Analysis of differentially expressed proteins in *Mycobacterium avium*-infected macrophages comparing with *Mycobacterium tuberculosis*-infected macrophages. *BioMed Research International, **20***(3): 1-9.

Yang, L.T., Qi, Y.P., Lu, Y.B., Guo, P., Sang, W., Feng, H., Zhang, H.X. and Chen, L.S., 2013. iTRAQ protein profile analysis of Citrus sinensis roots in response to long-term boron-deficiency. *Journal of*

Proteomics, ***93***(7): 179-206.

Yang, X., Fu, Y., Liu, J. and Ren, H.Y., 2013. Impact of probiotics on toll-like receptor 4 expression in an experimental model of ulcerative colitis. *Journal of Huazhong University of Science and Technology*, ***33***(5): 661-665.

Yan, F. and Polk, D.B., 2010. Disruption of NF-κB signalling by ancient microbial molecules: novel therapies of the future?. *Gut*, ***59***(4): 421-426.

Yoda, K., Miyazawa, K., Hosoda, M., Hiramatsu, M., Yan, F. and He, F., 2014. *Lactobacillus* GG-fermented milk prevents DSS-induced colitis and regulates intestinal epithelial homeostasis through activation of epidermal growth factor receptor. *European Journal of Nutrition*, **53**(1): 105-115.

Yoon, J.W., Ahn, S.I., Jhoo, J.W. and Kim, G.Y., 2019. Antioxidant activity of yogurt fermented at low temperature and its anti-inflammatory effect on DSS-induced colitis in mice. *Food Science of Animal Resources*, ***39***(1): 16-28.

Yu, Y.J., Amorim, M., Marques, C., Calhau, C. and Pintado, M., 2016. Effects of whey peptide extract on the growth of probiotics and gut microbiota. *Journal of Functional Foods*, ***21***(2): 507-516.

Zagato, E., Mileti, E., Massimiliano, L., Fasano, F., Budelli, A., Penna, G. and Rescigno, M., 2014. *Lactobacillus paracasei* CBA L74 metabolic products and fermented milk for infant formula have anti-inflammatory activity on dendritic cells in vitro and protective effects against colitis and an enteric pathogen *in vivo*. *PloS One*, ***9*(**2): 1-15.

Zhai, Z., Torres-Fuentes, C., Heeney, D.D. and Marco, M.L., 2019. Synergy between probiotic *Lactobacillus casei* and milk to maintain barrier integrity of intestinal epithelial cells. *Journal of Agricultural and Food Chemistry*, ***67***(7): 1955-1962.

Zhang, W., Zhu, Y.H., Yang, J.C., Yang, G.Y., Zhou, D. and Wang, J.F., 2015. A selected *Lactobacillus rhamnosus* strain promotes EGFR-independent Akt activation in an enterotoxigenic *Escherichia coli* K88-infected

IPEC-J2 cell model. *PloS one*, *10*(4), p.e0125717.and dietary components. *The Journal of Nutrition*, ***141***(5): 769-776.

Zhao, J., Hong, T., Dong, M., Meng, Y. and Mu, J., 2013. Protective effect of myricetin in dextran sulphate sodium-induced murine ulcerative colitis. *Molecular Medicine Reports*, ***7***(2): 565-570.

Zheng, B., van Bergenhenegouwen, J., Overbeek, S., van de Kant, H.J., Garssen, J., Folkerts, G., Vos, P., Morgan, M.E. and Kraneveld, A.D., 2014. *Bifidobacterium breve* attenuates murine dextran sodium sulfate-induced colitis and increases regulatory T cell responses. *Plos One*, ***9***(5): 9-14.

Zhou, Y., Qin, H., Zhang, M., Shen, T., Chen, H., Ma, Y., Chu, Z., Zhang, P. and Liu, Z., 2010. *Lactobacillus plantarum* inhibits intestinal epithelial barrier dysfunction induced by unconjugated bilirubin. *British Journal of Nutrition*, ***104***(3): 390-401.