CONTENTS

		Page No
List of Tables		viii - ix
List of Figur	res	x - xiii
List of Abbr	eviations	xiv - xvi
Chapter 1:	Introduction and Review of Literature	1 - 35
1.1.	Introduction	1
1.2.	Objectives of the Study	1
1.3.	Review of Literature	3 - 35
1.3.1.	Folate Discovery	3
1.3.2.	Folate Structure	4
1.3.3.	Folate Absorption	5
1.3.4.	Bioavailability: Folate Vs Folic acid	5
1.3.5.	Folate Functionality	6
1.3.6.	Health Implications of Folate Deficiency	7
1.3.7.	Folate: Dietary Sources,	9
	Requirements and Fortification Programmes	
1.3.8.	Folate Production by Food Grade	12
	Microorganisms	
1.3.9.	Folate Biosynthesis	17
1.3.10.	Lactic Acid Bacteria	19
1.3.11.	LAB in Food Fermentation	20
1.3.12.	LAB as Starter Cultures	20
1.3.13.	LAB as Probiotics	22
1.3.14.	Development of Probiotic Foods	24
1.3.15.	Probiotic Industry	26

1.3.16.	Nutraceuticals from LAB	30
1.3.17.	Metabolic Engineering of LAB	31
	for Nutraceutical Production	
1.4.	Conclusion	35
Chapter 2:	Materials and General Methods	36 - 53
2.1.	Materials	36
2.1.1.	Microorganisms	36
2.1.2.	Plasmid DNA and Primers	36
2.1.3.	Culture media, Reagent kits and Chemicals	36
2.1.4.	Cell Lines	37
2.2.	General Methodology	37
2.2.1.	Microbiological Methods	37
2.2.2.	Analytical Methods	40
2.2.3.	Molecular Biology Methods	45
2.3.	Major Instruments	53
2.4.	Conclusion	53
Chapter 3:	Isolation, Identification and Probiotic	54 - 83
	of Folate Producing Lactic Acid Bacteria	
3.1.	Introduction	54
3.2.	Materials and Methods	55
3.2.1.	Microorganisms and Culture Conditions	55
3.2.2.	Isolation and Identification of LAB	55
3.2.3.	Screening for Folate Producers	55
3.2.4.	Biochemical and Molecular Identification	56
	of the Selected Folate Producers	
3.2.5.	Probiotic Characterization of the	56

Selected Isolates

3.2.5.1.	Tolerance to Inhibitory Conditions	56
3.2.5.2.	Antibiotic Susceptibility	57
3.2.5.3.	Antimicrobial Activity	57
3.2.5.4.	Antioxidant Activity	57
3.2.5.5.	Surface Binding Properties	58
3.3.	Results and Discussion	60
3.3.1.	Isolation of LAB	60
3.3.2.	Primary Screening for Folate Production and Folate Quantification	64
3.3.3.	Identification of the Selected Isolates	65
3.3.4.	Probiotic Characterization of the Selected Isolates	71
3.3.4.1.	Tolerance to Inhibitory Conditions	71
3.3.4.2.	Antibiotic Susceptibility	73
3.3.4.3.	Antimicrobial activity	75
3.3.4.4.	Antioxidant Activity of the Isolates	77
3.3.4.5.	Surface Binding Properties	78
3.4.	Conclusion	83
Chapter 4:	Fermentative Production of Folate in	84 - 97
	Skim Milk by Lactococcus lactis CM28	
4.1.	Introduction	84
4.2.	Materials and Methods	85
4.2.1.	Bacterial Strains and Growth Conditions	85
4.2.2.	Inoculum for Folate Fortification of Skim Milk	85
4.2.3.	Effect of Culture Conditions and Additives on Folate Production	85

4.2.4.	Batch Fermentation in Bioreactors	85
4.2.5.	Folate Analysis	86
4.2.6.	Storage Stability of the Fermented Milk	86
4.3.	Results and Discussion	87
4.3.1.	Effect of Culture Conditions and Additives on Folate Production	87
4.3.2.	Batch Fermentations in Bioreactors	93
4.3.3.	Storage Stability Studies of the Fermented Milk	96
4.4.	Conclusion	97
Chapter 5:	Folate Production in Skim Milk by	98 - 107
	Co-culturing of <i>Lactococcus lactis</i> CM22 and CM28	
5.1.	Introduction	98
5.2.	Materials and Methods	99
5.2.1.	Microorganisms and Culture Conditions	99
5.2.2.	Compatibility Test	99
5.2.3.	Folate Production Media	99
5.2.4.	Inoculum Preparation and Fermentation	99
5.2.5.	Storage Stability Studies of the Fermented Skim Milk	100
5.3.	Results and Discussion	100
5.3.1.	Compatibility Test	100
5.3.2.	Folate Production by the Co-culture in Fermented Skim Milk	101
5.4.	Conclusion	107
Chapter 6:	Encapsulation of Folate Producing Lactococcus lactis Strains for Enhanced	108 - 118

Gastrointestinal Survival

6.1.	Introduction	108
6.2.	Materials and Methods	109
6.2.1.	Bacterial Strains and Culture Conditions	109
6.2.2.	Encapsulation of the LAB strains	109
6.2.3.	Survival of Encapsulated LAB in Simulated Gastric Juice (SGJ)	110
6.2.4.	Survival of Encapsulated LAB in Simulated Intestinal Juice (SIJ)	110
6.3.	Results and Discussion	110
6.3.1	Encapsulation of Lactococcus strains	110
6.3.2.	Survival of Free and Encapsulated LAB in Simulated Gastric Conditions	111
6.3.3.	Survival of Free and Encapsulated LAB in Simulated Intestinal Conditions	115
6.4.	Conclusion	118
6.4. Chapter 7:	Conclusion Folate Fortification with Indigenous	118 119 - 132
	Folate Fortification with Indigenous	
Chapter 7:	Folate Fortification with Indigenous Lactococcus lactis Isolates	119 - 132
Chapter 7: 7.1.	Folate Fortification with Indigenous <i>Lactococcus lactis</i> Isolates Introduction	119 - 132 119
Chapter 7: 7.1. 7.2.	Folate Fortification with Indigenous <i>Lactococcus lactis</i> Isolates Introduction Materials and Methods	119 - 132 119 120
Chapter 7: 7.1. 7.2. 7.2.1.	Folate Fortification with Indigenous Lactococcus lactis Isolates Introduction Materials and Methods Bacterial Strains and Culture Conditions	119 - 132 119 120 120
Chapter 7: 7.1. 7.2. 7.2.1. 7.2.2.	Folate Fortification with IndigenousLactococcus lactis IsolatesIntroductionMaterials and MethodsBacterial Strains and Culture ConditionsFolate fortification of Apple JuiceFolate Fortification of Skim Milk and Ice Cream	119 - 132 119 120 120 120
Chapter 7: 7.1. 7.2. 7.2.1. 7.2.2. 7.2.3.	Folate Fortification with Indigenous Lactococcus lactis Isolates Introduction Materials and Methods Bacterial Strains and Culture Conditions Folate fortification of Apple Juice Folate Fortification of Skim Milk and Ice Cream using Encapsulated Bacteria	119 - 132 119 120 120 120 121

by Free and Encapsulated Bacteria

7.4.	Conclusion	132
Chapter 8:	Isolation, Identification and Cloning of Folate Biosynthetic Genes of <i>Lactococcus lactis</i> CM28	133 - 149
8.1.	Introduction	133
8.2.	Materials and Methods	134
8.2.1.	Microorganisms, Plasmids and Culture Conditions	134
8.2.2.	Isolation of Genomic DNA of L. lactis CM28	134
8.2.3.	PCR Amplification of Folate Biosynthetic Genes	134
8.2.4.	Cloning of Folate Genes into pTZ57R/T Vector	135
8.2.5.	Isolation of Recombinant Plasmids from <i>E. coli</i> DH5α Cells	136
8.2.6.	Cloning of Folate Genes into the Expression Vector pNZ8148	136
8.3.	Results and Discussion	137
8.3.1.	PCR Amplification of Folate Biosynthetic Genes from <i>L. lactis</i> CM28	137
8.3.2.	Cloning of Folate genes into T vector and Sequencing	139
8.3.3.	Cloning of Folate Genes into the Expression Vector pNZ8148	147
8.4.	Conclusion	149
Chapter 9:	Summary and Conclusion	150 - 153
Bibliography		154 - 185
Annexure I		186 - 193
Media Composition		186
Annexure II		194

List of Major Instruments	194
Annexure III	195 - 197
List of Publications	195

List of Tables

No.	Legend	Page No
1.1.	Folate content in selected foods	10
1.2.	Daily folate requirements	11
1.3.	Folate produced by microorganisms in chemically defined folate free medium	15
1.4.	LAB in common fermented foods	21
1.5.	Probiotics in market	28
1.6.	Nutraceuticals from lactic acid bacteria	31
1.7.	Successful metabolic engineering strategies employed in LAB	34
2.1.	Reaction mixture for folate detection by microbiological assay	42
3.1.	LAB isolates and their source of isolation	61
3.2.	Morphological and biochemical characteristics of the isolates	66
3.3.	16S rRNA gene sequence of Lactococcus lactis CM28	69
3.4.	16S rRNA gene sequence of Lactococcus lactis CM22	69
3.5.	16S rRNA gene sequence of Weissella cibaria G4	70
3.6.	16S rRNA gene sequence of Enterococcus sp. P8	70
3.7.	Tolerance of isolates to low pH	71
3.8.	Bile, NaCl and phenol tolerance of the isolates	72
3.9.	Antibiotic susceptibility of the isolates	74
3.10.	Antimicrobial activity of LAB isolates	76
3.11.	Inhibition of ascorbate autoxidation by intracellular cell free extract of the LAB isolates	77
3.12.	Hydrophobicity of the isolates	78

4.1.	Folate levels in fermented skim milk under	93
	optimized conditions (8 h)	
4.2.	Storage stability studies of the fermented skim milk	96
7.1.	Viable count of bacteria in skim milk and ice cream	130
	after fermentation by free and encapsulated bacteria	
8.1.	Oligonucleotides used for amplifying folate biosynthetic	134
	genes	
8.2.	Optimum PCR conditions for the selected folate genes	135
	of L. lactis CM28	
8.3.	Nucleotide and amino acid sequence of	141
	folKE gene of L. lactis CM28	
8.4.	Nucleotide and amino acid sequence of <i>folC</i> gene	144
	of L. lactis CM28	
8.5.	Nucleotide and the deduced amino acid sequence of <i>folA</i>	146
	gene of L. lactis CM28	

List of Figures

No	Legend	Page No
1.1.	Structure of folic acid and folate derivatives	4
1.2.	Folate functionality	6
1.3 (A) Folate biosynthetic pathway in L. Lactis	18
1.3 (B) Schematic representation of folate gene cluster in <i>L. Lactis</i>	18
1.4.	Health benefits of probiotics	24
1.5.	Schematic representation of the NICE system: components and function	32
2.1.	Preparation of inoculum for microbiological assay	41
2.2.	Standard graph for folate detection by microbiological assay	43
2.3.	Standard graph for lactic acid standard detection	44
3.1.	Isolates from curd in MRS – CaCO ₃ plate	62
3.2.	Lactic acid production by the isolates	63
3.3.	Folate production by the isolates in FAA medium	64
3.4	Extracellular folate production by the selected isolates in skim milk	65
3.5.	Gram staining images of the isolates showing the cell morphology	68
3.6.	Antibiotic susceptibility of G4	74
3.7.	Antagonistic activities of G4 and P8 against S. Aureus	76
3.8.	Autoaggregation of the isolates	80
3.9.	Coaggregation of the isolates with pathogens (5 h)	80
3.10	Mucin adhesion of the isolates	81
3.11.	Adhesion of the LAB isolates on HT-29 cells by SEM analysis	82

3.12.	Adhesion of isolates to Caco-2 cells	83
4.1.	Effect of incubation time on folate production by <i>L. lactis</i> CM28	87
4.2.	Effect of incubation temperature on folate production by <i>L. lactis</i> CM28	88
4.3.	Effect of prebiotics on folate production by L. lactis CM28	89
4.4.	Effect of PABA on folate production by <i>L. lactis</i> CM28 in skim milk	90
4.5.	Effect of glutamate on folate production by L. lactis CM28	91
4.6.	Effect of reducing agents on folate production by L. lactis CM28	92
4.7.	Batch fermentation for folate production in parallel fermenter	93
4.8.	Folate production in parallel fermenter	94
4.9.	Skim milk fermentation for folate production in 5 L fermenter	95
4.10.	Folate production in 5 L fermenter	95
5.1.	Co-existence test (A) cross streaking (B) Co-streaking	101
5.2.	Folate production in skim milk by the co-culture (CM22:CM28) at various inoculum ratios	102
5.3.	Storage stability of folate in fermented skim milk medium	104
5.4	Changes in viable count of bacteria during refrigerated storage	105
5.5	Changes in pH of fermented milk during refrigerated storage	106
5.6	Changes in titratable acidity of fermented milk during refrigerated storage	106
6.1.	Encapsulation of L. lactis CM22	111
6.2.	Survival of free and encapsulated L. lactis CM22 in SGJ	112
6.3.	Survival of free and encapsulated L. lactis CM28 in SGJ	113
6.4.	Percentage survival of the free and encapsulated <i>L. lactis</i> strains in simulated gastric conditions after incubation period (2 h)	114
6.5.	Survival of free and encapsulated L. lactis strains in SIJ	116

6.6.	Percentage survival of the free and encapsulated LAB strains	117
	in simulated gastrointestinal conditions	
7.1	Folate fortification of apple juice by <i>L. lactis</i> strains	123
7.2.	Storage stability of folate in fermented apple juice	125
7.3.	Folate fortified apple juice	126
7.4.	Changes in pH of fermented apple juice during cold storage	127
7.5.	Variation in titratable acidity of fermented apple juice during cold storage	127
7.6.	Changes in viability of bacteria in fermented apple juice during cold storage	128
7.7.	Fermentative folate fortification skim milk by free and encapsulated <i>L. lactis</i> strains	129
7.8.	Fermentative folate fortification of ice cream by free and encapsulated bacteria	131
8.1.	Vector map of pTZ57R/T	136
8.2.	Vector map of pNZ8148	137
8.3.(A)	Genomic DNA of L. lactis CM28	138
8.3 (B)	PCR amplification of folate biosynthesis genes	138
8.4 (A)	T vector clone of <i>folKE</i>	139
8.4 (B)	Clone confirmation by PCR	139
8.5.	BLAST analysis of <i>folKE</i> gene sequence	140
8.6 (A)	T vector clone of <i>folC</i>	142
8.6 (B)	Clone confirmation by PCR	142
8.7.	BLAST analysis of <i>folC</i> gene sequence	143
8.8(A)	T vector clone of <i>folA</i>	145
8.8(B)	Clone confirmation by PCR	145
8.9.	BLAST analysis of <i>folA</i> gene sequence	146

8.10.	Preparation of insert and vector for cloning of <i>folKE</i> gene	147
	into pNZ8148	
8.11.	Cloning of <i>folKE</i> gene into pNZ8148	148

8.12. Cloning of *folC* gene into pNZ8148 148

List of Abbreviations

%	Percentage
&	And
°C	Degree Celsius
μ	Micro
μg	Microgram
μg/μL	Microgram per microlitre
μL	Microlitre
μΜ	Micro molar
10-formyl-THF	10-formyltetrahydrofolate
5,10-methenyl-THF	5,10-methenyltetrahydrofolate
5,10-methylene-THF	5,10-methylenetetrahydrofolate
5-formimino-THF	5-formiminotetrahydrofolate
5-formyl-THF	5-formyltetrahydrofolate
5-MTHF	5- methyltetrahydrofolate
APP	Amyloid precursor protein
ATCC	American Type Culture Collection
bp	Base pair
CAGR	Compound annual growth rate
CFU	Colony forming unit
CFU/mL	Colony forming units per millilitre
Conc.	Concentrated
CSF	Cerebrospinal fluid
DHF	Dihydrofolate
DNA	Deoxyribo nucleic acid
dNTPs	Deoxynucleotide phosphates
DO	Dissolved oxygen
DRI	Daily recommended intake
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPS	Exopolysaccharides
FAA medium	Folic acid assay medium

FAC medium	Folic acid casei medium
FBP	Folate binding proteins
FDA	U.S. Food and Drug Administration
FOS	Fructooligosaccharides
g	Gram
GM LAB	Genetically modified lactic acid bacteria
GOS	Galactooligosaccharides
GRAS	Generally recognized as safe
GTP	Guanosine triphosphate
h	Hour
HDL	High density lipoprotein
IBDs	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
ICFE	Intracellular cell free extract
IDF	International Dairy Federation
Kb	Kilo base
KDa	Kilo Dalton
L	Litre
LAB	Lactic acid bacteria
LB broth	Luria Bertani broth
LDL	Low density lipoprotein
М	Molar
MBV	Minimum of biovalue
mg	Milligram
mg/mL	Milligram per millilitre
min	Minute(s)
mL	Millilitre
MRS medium	de Man Rogosa Sharpe medium
mMRS medium	Modified MRS medium
MTCC	Microbial Type Culture Collection and Gene Bank
MTHFR	Methylene tetrahydrofolate reductase
NCIM	National Collection of Industrial Microorganisms
ng	Nanogram

ng/mL	Nanogram per millilitre
NICE	Nisin controlled gene expression system
NK cells	Natural killer cells
NTDs	Neural tube defects
PABA	Para aminobenzoic acid
PGA	Pteroyl glutamic acid
pМ	Pico molar
RDA	Recommended dietary allowance
ROS	Reactive oxygen species
rpm	Revolutions per minute
S	Seconds
SAM	S-adenosyl methionine
SCFA	Short chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SGJ	Simulated gastric juice
SIJ	Simulated intestinal juice
ТА	Titratable acidity
TAE buffer	Tris-Acetic acid-EDTA buffer
TE buffer	Tris -EDTA buffer
THF	5,6,7,8- tetrahydrofolate
UHT	Ultra high temperature
USDA	The United States Department of Agriculture
UTI	Urinary tract infection
v/v	Volume / Volume
w/v	Weight / Volume
w/w	Weight / Weight