

**ISOLATION AND CHARACTERIZATION OF
CIRCULAR BACTERIOCIN PRODUCED BY
*LACTOBACILLUS GASSERI***



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

**DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY
BY**

**NEHA PANDEY
M.Sc. (Microbiology)**

**DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA**

2013

Regn. No. 1010801

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Dedicated to my Parents

*For all the love and care they have bestowed upon
me.....*

Isolation and Characterization of Circular Bacteriocin
Produced by *Lactobacillus gasseri*
BY

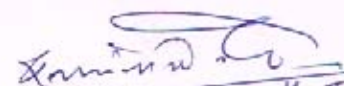
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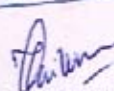
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IN
DAIRY MICROBIOLOGY

Approved by


EXTERNAL EXAMINER


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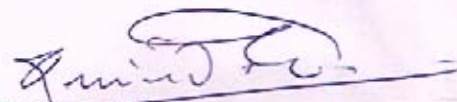
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This is to certify that the thesis entitled, "Isolation and Characterization of Circular Bacteriocin Produced by *Lactobacillus gasseri*" submitted by Ms. Neha Pandey towards the partial fulfilment of the award of the degree of Doctor of Philosophy (Dairy Microbiology) of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: January 23, 2013


(R.K. Malik)
Major Advisor

Acknowledgement.....

“Gratitude makes a sense of our past, brings peace for today and creates a vision for tomorrow”

-Melody Beattie

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(Neha Pandey)

CONTENTS

Chapter	Title		Page No.
1.0.	INTRODUCTION		... 1-3
2.0.	REVIEW OF LITERATURE		... 4-33
2.1.	PROLOGUE		... 4
2.2.	BACTERIOCINS		... 6
	2.2.1.	Bacteriocins of Gram-negative Bacteria	... 7
	2.2.2.	Bacteriocins of Gram-positive Bacteria	... 8
2.3.	BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA		... 8
2.4.	CLASSIFICATION OF BACTERIOCINS		... 10
2.5.	CLASS V (CIRCULAR) BACTERIOCINS		... 12
2.6.	BACTERIOCIN SECRETION		... 14
2.7.	MODE OF ACTION		... 15
2.8.	BACTERIOCIN IMMUNITY AND RESISTANCE		... 16
2.9.	BACTERIOCIN PRODUCED BY THE GENUS <i>LACTOBACILLUS</i>		... 17
	2.9.1.	The Genus <i>Lactobacillus</i>	... 17
	2.9.2.	Ecology	... 18
2.10.	PRODUCTION OF BACTERIOCIN BY <i>LACTOBACILLUS</i> SPS.		... 19
2.11.	ANTIMICROBIAL SPECTRUM OF ACTIVITY		... 20
2.12.	DETECTION OF BACTERIOCIN ACTIVITY		... 22
	2.12.1.	Agar Overlay Method	... 22
	2.12.2.	Flow Cell Detection	... 22
	2.12.3.	PCR Method	... 22
2.13.	DETERMINATION OF SECONDARY STRUCTURE OF BACTERIOCIN- CIRCULAR DICHROISM (CD) SPECTROSCOPY		... 23
2.14.	MICROCIN J25: CIRCULAR BACTERIOCIN FROM GRAM NEGATIVE BACTERIA		... 24
2.15.	GASSERICIN A: FIRST EXAMPLE OF CIRCULAR BACTERIOCIN FROM LAB		... 25
	2.15.1.	Production and Purification of Gassericin A	... 25
	2.15.2.	Properties of Purified Bacteriocin	... 26
	2.15.3.	Determination of Amino Acid Sequence of Gassericin A	... 26
	2.15.4.	Limited Proteolysis of Gassericin A	... 27
	2.15.5.	Structural Gene of Gassericin A	... 28
	2.15.6.	Gassericin A and Reuterin 6	... 28
	2.15.7.	Difference between Gassericin A and Reuterin 6	... 29
2.16.	GASSERICIN T: A LINEAR VARIANT OF GASSERICIN A		... 31
2.17.	APPLICATION OF GASSERICIN A IN FOOD SYSTEM AND HEALTH		... 31
	2.17.1.	Inhibition of Food-borne Pathogenic Bacteria	... 31
	2.17.2.	Antibacterial Activities of Gasserins (A and T) in Combination with Glycine against Spoilage Bacteria (Hurdle Technology)	... 32

	2.17.3.	Gassericin A in Treatment of Mastitis	...	32
	2.18.	Epilogue	...	32
3.0.	MATERIALS AND METHODS			34-63
3.1.	STANDARD CULTURES			34
3.2.	ISOLATION OF <i>LACTOBACILLUS</i> CULTURES			34
	3.2.1.	Collection of Samples	...	34
	3.2.2.	Culture Medium	...	35
	3.2.3.	Procedure for Isolation of <i>Lactobacillus</i> from Fecal Samples	...	35
	3.2.4.	Maintenance of Cultures	...	35
3.3.	MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION			37
	3.3.1.	Morphology	...	37
	3.3.2.	Catalase Test	...	37
	3.3.3.	Growth at 15°C and 45°C	...	37
	3.3.4.	Arginine Hydrolysis	...	38
	3.3.5.	Bile Aesculin Hydrolysis	...	38
	3.3.6.	Carbohydrate Fermentation	...	38
3.4.	PHYLOGENETIC AND GENOTYPIC CHARACTERIZATION			39
	3.4.1.	Isolation of Genomic DNA	...	39
	3.4.2.	Agarose Gel Electrophoresis	...	40
	3.4.3.	Determination of DNA Concentration by Spectrophotometric Analysis	...	40
	3.4.4.	Identification of Isolates by Polymerase Chain Reaction (PCR)	...	40
		3.4.4.1.	Primers	40
		3.4.4.2.	PCR with selected primers	41
		3.4.4.3.	PCR cycling steps	42
			3.4.4.3.1. <i>For genus specific primers</i>	42
			3.4.4.3.2. <i>For species specific primers</i>	42
		3.4.4.4.	Analysis of PCR products by gel electrophoresis	42
3.5.	SCREENING OF <i>LACTOBACILLUS GASSERI</i> CULTURES FOR ANTIMICROBIAL ACTIVITY			43
	3.5.1.	Spot on Lawn Assay	...	43
		3.5.1.1.	Cell free culture supernatant (CFCS)	43
		3.5.1.2.	Assay	43
	3.5.2.	Agar Spot Assay	...	43
	3.5.3.	Agar Well Assay	...	44
3.6.	MEASUREMENT OF ACTIVITY UNIT (AU)			44
3.7.	IDENTIFICATION OF BACTERIOCIN GENE (<i>gaaA</i>) BY PCR AMPLIFICATION			44
	3.7.1.	PCR Cycling Steps	...	45
		3.7.1.1.	Using primer pair	45

			gaA515/gaA61				
		3.7.1.2.	Using primer pair gaA2F/gaA2R		...	45	
		3.7.1.3.	Analysis of PCR product by agarose gel electrophoresis		...	45	
	3.7.2.	PCR Product Purification			...	46	
3.8.	SEQUENCING OF AMPLIFIED PCR GENE PRODUCT					...	46
	3.8.1.	DNA Sequence Similarity Analysis			...	46	
3.9.	ANTIBACTERIAL SPECTRUM OF ACTIVITY					...	46
3.10.	OPTIMIZATION OF THE CULTIVATION CONDITIONS FOR MAXIMAL BACTERIOCIN PRODUCTION BY <i>LACTOBACILLUS GASSERI</i> NBL 18 USING RESPONSE SURFACE METHODOLOGY					...	47
	3.10.1.	Experimental Design			...	47	
	3.10.2.	Optimization of Bacteriocin Production			...	49	
3.11.	PROTEIN ESTIMATION					...	51
	3.11.1.	Determination of Specific Activity			...	51	
3.12.	PURIFICATION OF BACTERIOCIN					...	51
	3.12.1.	Precipitation with Ammonium Sulfate			...	51	
	3.12.2.	Ion-exchange Chromatography			...	52	
		3.12.2.1.	Selection of ion-exchanger and starting pH of the buffer		...	52	
			3.12.2.1.1.	<i>Ion- exchanger</i>	...	52	
			3.12.2.1.2.	<i>Buffers</i>	...	52	
			3.12.2.1.3.	<i>Procedure</i>	...	52	
		3.12.2.2.	Anion-exchange chromatography		...	53	
	3.12.3.	RP-HPLC Profile of Bacteriocin			...	53	
3.13.	DETERMINATION OF MOLECULAR WEIGHT BY TRICINE SDS-PAGE					...	54
	3.13.1.	Sample Preparation			...	54	
	3.13.2.	Preparation of Molecular Weight Markers Solution			...	54	
	3.13.3.	Treatment of Samples Prior to Electrophoresis			...	54	
	3.13.4.	Preparation of Electrophoresis Gels			...	54	
		3.13.4.1.	Gel casting		...	55	
	3.13.5.	Electrophoresis			...	56	
	3.13.6.	Fixing, Staining and Destaining of the Gel			...	56	
	3.13.7.	Detection of Bacteriocin Activity			...	56	
	3.13.8.	Estimation of Molecular Weight			...	57	
3.14.	CIRCULAR DICHROISM SPECTRA OF BACTERIOCIN					...	57
3.15.	CHARACTERIZATION OF BACTERIOCIN PRODUCED BY <i>LACTOBACILLUS GASSERI</i> NBL 18					...	57
	3.15.1.	Crude Bacteriocin			...	57	
	3.15.2.	Partially Purified Bacteriocin			...	57	
	3.15.3.	Inhibitory Spectrum of Activity			...	57	
3.16.	SENSITIVITY OF BACTERIOCIN TO ENZYMES.					...	58

	DETERGENTS, TEMPERATURE AND pH			
	3.16.1.	Enzymes	...	58
		3.16.1.2. Procedure	...	58
	3.16.2.	Detergents	...	58
	3.16.3.	Heat Stability	...	58
	3.16.4.	pH Stability	...	59
3.17.	APPLICATION OF BACTERIOICIN TO INHIBIT FOOD-BORNE PATHOGENS IN WHIPPED CREAM		...	59
	3.17.1.	Determination of Minimum Concentration of Bacteriocin to Inhibit Food-borne Pathogens	...	59
	3.17.2.	Inhibitory Effect of Bacteriocin Against <i>L. monocytogenes</i> ATCC 15303, <i>B. cereus</i> NCDC66, <i>E.coli</i> NCDC 134, <i>P.aeruginosa</i> NCDC 104 and <i>E. faecalis</i> NCDC 114 in Phosphate Buffer Medium	...	59
	3.17.3.	The Inhibitory Effect of Bacteriocin Alone and in Combination with Glycine Against Gram-positive and Gram-negative Organism in Microwell Plate Assay	...	60
	3.17.4.	The Inhibitory Effect of Bacteriocin Alone and in Combination with Glycine against <i>B. cereus</i> , <i>L.monocytogenes</i> , <i>E.coli</i> and <i>P. aeruginosa</i> in Whipped Cream	...	61
3.18.	ANTIBACTERIAL ACTIVITY OF NBL 18: COCULTURE TESTS IN SKIMMED MILK		...	61
3.19.	SAFETY ASSESSMENT OF NBL 18		...	61
	3.19.1.	Liquification of Gelatin	...	61
	3.19.2.	Hemolysis	...	62
	3.19.3.	Antibiotic Susceptibility Test	...	62
	3.19.4.	DNase Test	...	62
3.20.	STATISTICAL ANALYSIS		...	63
	3.20.1.	t-test	...	63
	3.20.2.	Determination of Mean and Standard Error of the Mean (SEM)	...	63
	3.20.3.	Graphical Presentation	...	63
4.0.	RESULT AND DISCUSSION		...	64-118
4.1.	PROLOGUE		...	64
4.2.	ISOLATION OF <i>LACTOBACILLUS</i>		...	65
4.3.	BIOCHEMICAL CHARACTERIZATION OF <i>LACTOBACILLUS</i> ISOLATES		...	69
4.4.	MOLECULAR CHARACTERIZATION OF ISOLATES		...	70
	4.4.1.	PCR with Genus Specific Primer Pair LbLMA1/R161	...	70
	4.4.2.	Identification of the Isolates at Species Level	...	71
		4.4.2.1. Identification of <i>Lactobacillus</i> isolates at species level with carbohydrate fermentation test	...	71

		4.4.2.2.	Identification of <i>Lactobacillus</i> isolates at species level with primer Lgas2/Lgas3	...	71
4.5.	SCREENING OF THE <i>LACTOBACILLUS GASSERI</i> ISOLATES FOR THEIR ANTIMICROBIAL ACTIVITY			...	72
4.6.	SCREENING OF BACTERIOCINOGENIC STRAINS OF <i>LACTOBACILLUS GASSERI</i> FOR <i>gaaA</i> GENE THROUGH PCR			...	75
4.7.	ANTIBACTERIAL SPECTRA OF BACTERIOCINOGENIC (<i>gaaA</i> +) <i>L. GASSERI</i> ISOLATES			...	77
4.8.	SEQUENCING OF PCR PRODUCT			...	81
4.9.	RESPONSE SURFACE OPTIMIZATION OF THE CULTIVATION CONDITIONS FOR MAXIMAL BACTERIOCIN PRODUCTION BY <i>LACTOBACILLUS GASSERI</i> NBL 18			...	85
4.10.	EFFECT OF DIFFERENT LEVELS OF PH, TEMPERATURE, INOCULUM LEVEL AND INCUBATION TIME ON OPTIMAL BACTERIOCIN PRODUCTION			...	88
	4.10.1.	Diagnostic Check of the Quadratic Model		...	88
	4.10.2.	Effect of pH, Incubation Temperature, Inoculum Level and Incubation Time on pH		...	88
	4.10.3.	Effect of pH, Incubation Time, Inoculum Level and Incubation Temperature on OD		...	89
	4.10.4.	Effect of pH, Incubation Time, Inoculum Level and Incubation Temperature on Bacteriocin Activity		...	90
4.11.	OPTIMIZATION OF pH, INCUBATION TEMPERATURE, INOCULATION LEVEL AND INCUBATION TIME ON BACTERIOCIN PRODUCTION			...	91
4.12.	PURIFICATION OF BACTERIOCIN PRODUCED BY <i>LACTOBACILLUS GASSERI</i> NBL 18			...	92
	4.12.1.	Ammonium Sulphate Precipitation		...	92
	4.12.2.	Ion-Exchange Chromatography		...	93
		4.12.2.1.	Selection of pH of the buffer	...	93
		4.12.2.2.	Selection of type of ion-exchanger	...	94
	4.12.3.	RP-HPLC Profile of bacteriocin		...	96
4.13.	CHARACTERIZATION OF BACTERIOCIN			...	97
	4.13.1.	Inhibitory Spectrum		...	98
	4.13.2.	Molecular Weight		...	100
	4.13.3.	Enzyme Sensitivity		...	101
	4.13.4.	Heat Stability of the Bacteriocin		...	102
	4.13.5.	Effect of Detergents on Bacteriocin Activity		...	103
	4.13.6.	pH Stability		...	104
	4.13.7.	Circular Dichroism Spectroscopy of Bacteriocin		...	106
4.14.	DETERMINATION OF BACTERIOCIN (GASSERICIN) CONCENTRATION TO INHIBIT <i>L. MONOCYTOGENES</i> , <i>E. COLI</i> , <i>P. AERUGINOSA</i> AND <i>B. CEREUS</i>			...	109

	4.14.1.	The Inhibitory Effect of Gassericin against <i>Listeria monocytogenes</i> , <i>Bacillus cereus</i> , <i>Enterococcus faecalis</i> , <i>E.coli</i> and <i>P. aeruginosa</i> in Phosphate Buffer Medium		110
	4.14.2.	Microwell Plate Assay	...	110
	4.14.3.	The Inhibitory Effect of Gassericin against <i>B. cereus</i> , <i>E.coli</i> , <i>P. aeruginosa</i> and <i>L. monocytogenes</i> in Whipped Cream Frosting	...	112
4.15.	ANTIBACTERIAL ACTIVITY OF NBL 18: COCULTURE TESTS IN SKIMMED MILK		...	114
4.16.	SAFETY EVALUATION OF NBL 18		...	115
4.17.	EPILOGUE		...	118
5.0.	SUMMARY AND CONCLUSION		...	119-124
	BIBLIOGRAPHY		...	i-xxxi
	ANNEXURE I		...	i-xi
	ANNEXURE II		...	i-viii
	APPENDICES		...	A-J

LIST OF TABLES

Table No.	Title	Page No.
2.1.	Properties of some well characterized bacteriocins from LAB	10
2.2.	Examples of Circular Bacteriocin from Gram positive bacteria	13
2.3.	<i>Lactobacillus</i> species distribution in different parts of the gastrointestinal tract	18
2.4.	Example of some bacteriocins isolated from <i>Lactobacillus</i> species	19
3.1.	Bacterial species and strains used in the study	35
3.2.	Primer pairs used for the identification of genus <i>Lactobacillus</i> and species <i>Lactobacillus gasseri</i>	41
3.3.	Details of the PCR mix used in the study	41
3.4.	Primer pairs used for <i>gaaA</i> gene	45
3.5.	Coded and actual values of variables in RSM experiment	48
3.6.	Full experimental design for optimization experiments for maximum bacteriocin production using response surface methodology	49
3.7.	Composition of gels for Tricine-SDS-PAGE	55
3.8.	Food spoilage and pathogenic bacteria assayed by microwell plate assay method	60
4.1.	Isolation of lactobacilli from infant feces, human milk and saliva as studied on MRS agar	66
4.2.	Antibacterial activity of <i>Lactobacillus gasseri</i> isolates by agar-spot and spot-on-lawn Assay against <i>E.faecalis</i> NCDC 114	72
4.3.	Frequency of bacteriocinogenic <i>Lactobacillus</i> isolates in different samples (Infant faeces human milk and saliva)	74
4.4.	Antibacterial spectrum of activity of <i>Lactobacillus gasseri</i> isolates positive for <i>gaaA</i> gene by spot on lawn method	79
4.5.	Bacteriocin activity of the culture NBL 18 cultivated with different levels of pH, Temperature, Inoculum level and Incubation time	86
4.6.	Regression coefficients and ANOVA of fitted quadratic model for maximum bacteriocin production	87
4.7.	Conditions during optimization of bacteriocin production in CCRD	91
4.8.	Optimized values as compared to predicted values	92
4.9.	Ammonium sulphate precipitation of bacteriocin	93
4.10.	Selection of matrix for ion-exchange chromatography	94
4.11.	Purification of bacteriocin from culture supernatant of NBL 18	96
4.12.	Inhibitory spectrum of bacteriocin from NBL 18 by spot on lawn assay	98
4.13.	Temperature sensitivity of the crude and partially purified bacteriocin against <i>E. faecalis</i> NCDC 114	101
4.14.	Enzymatic sensitivity of the crude and partially purified bacteriocin against <i>E. faecalis</i> NCDC 114	102
4.15.	Effect of detergents on bacteriocin activity	103
4.16.	pH stability of bacteriocin after 120 h of storage at 4°C	105
4.17.	Molar ellipticity of the peptide calculated at different wavelengths	106

4.18.	Minimum Inhibitory Concentration of gassericin against different pathogens	110
4.19.A.	Inhibitory effect of bacteriocin against <i>E.coli</i> NCDC 134 in phosphate buffer medium	after 110
4.19.B.	Inhibitory effect of bacteriocin against <i>P.aeruginosa</i> NCDC 104 in phosphate buffer medium	after 110
4.19.C.	Inhibitory effect of bacteriocin against <i>L.monocytogenes</i> ATCC 15303 in phosphate buffer medium	after 110
4.19.D.	Inhibitory effect of bacteriocin against <i>E.faecalis</i> NCDC 114 in phosphate buffer medium	after 110
4.19.E.	Inhibitory effect of bacteriocin against <i>B.cereus</i> NCDC 66 in phosphate buffer medium	after 110
4.20.	Antimicrobial activity of glycine and culture supernatants of NBL 18 against Gram positive and Gram negative bacteria, as assayed by the microwell plate method	111
4.21.	Changes in cell viability of <i>B. cereus</i> NCDC 66 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasseri</i> NBL 18(Gassericin), or both (GG)	after 112
4.22.	Changes in cell viability of <i>L.monocytogenes</i> ATCC 15303 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasseri</i> NBL 18 (Gassericin), or both (GG)	after 112
4.23.	Changes in cell viability of <i>E.coli</i> NCDC 134 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasseri</i> NBL 18(Gassericin), or both (GG)	after 113
4.24.	Changes in cell viability of <i>P. aeruginosa</i> NCDC 104 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasseri</i> NBL 18 (Gassericin), or both (GG)	after 113
4.25.	Growth of <i>Listeria monocytogenes</i> ATCC 15303 in co-culture with bacteriocin producing <i>Lactobacillus gasseri</i> NBL 18 and non-bacteriocinogenic NBL5	after 114
4.26.	Growth of <i>Staphylococcus aureus</i> NCDC 273 in co-culture with bacteriocin producing <i>Lactobacillus gasseri</i> NBL 18 and non-bacteriocinogenic NBL5	after 114
4.27.	Growth of <i>Enterococcus faecalis</i> NCDC 114 in co-culture with bacteriocin producing <i>Lactobacillus gasseri</i> NBL 18 and non-bacteriocinogenic NBL5	after 114
4.28.	Antibiotic susceptibility of NBL 18	117

LIST OF FIGURES

Figure No.	Title	After page No.
2.1.	Examples of circular proteins isolated from bacteria, mammals, and plants	at 6
2.2.	Comparison of the leader peptide sequences of gassericin A and circularin A with those of nisin, enterocin AS-48 and butyrivibriocin AR10 (putative)	at 14
3.1.	Standard curve for protein estimation by Bradford method	at 51
4.1.	Frequency of bacteriocinogenic <i>L. gasseri</i> isolates in infant feces, human saliva and human milk	74
4.2.	Pie chart showing the frequency occurrence of circular bacteriocin producing isolates from the total isolates	at 76
4.3.	Multiple nucleotide sequence alignment of 16srRNA gene partial of <i>Lactobacillus gasseri</i> NBL 18 with reported sequences of <i>Lactobacillus</i> spp.	at 84
4.4.	UPGMA based tree showing relationship among nucleotide sequence of isolates NBL 18 with reported sequences of <i>Lactobacillus gasseri</i> and other <i>Lactobacillus</i> spp.	at 84
4.5.	Multiple nucleotide sequence alignment of <i>gaaA</i> gene of <i>L. gasseri</i> NBL 18 with reported sequences of gassericin A and acidocinB	at 85
4.6.	UPGMA method based tree showing relationship among nucleotide sequence of <i>gaaA</i> gene of <i>L. gasseri</i> NBL 18 with reported sequences	at 85
4.7.	Response surface curves for pH as influenced by the level of pH, incubation temperature, inoculums level and incubation time	88
4.8.	Response surface curves for optical density as influenced by the level of pH, incubation temperature, inoculums level and incubation time	89
4.9.	Response surface curves for bacteriocin activity as influenced by the level of pH, incubation temperature, inoculums level and incubation time	90
4.10.	Purification of bacteriocin by ion-exchange chromatography	at 95
4.11.	Purification of bacteriocin by RP-HPLC	at 96
4.12.	Effect of temperature on residual activity of crude and partially purified bacteriocin	at 103
4.13.	Effect of detergents on residual activity of crude and partially purified bacteriocin	at 104
4.14.	CD spectra of purified bacteriocin	at 109
4.15.A.	Inhibitory effect of bacteriocin against <i>E. coli</i> NCDC 134 (A) in phosphate buffer medium	110
4.15.B.	Inhibitory effect of bacteriocin against <i>P. aeruginosa</i> NCDC 104 (B) in phosphate buffer medium	110
4.15.C.	Inhibitory effect of bacteriocin against <i>L. monocytogenes</i> ATCC 15303 (C) in phosphate buffer medium	110
4.15.D.	Inhibitory effect of bacteriocin against <i>E. faecalis</i> NCDC 114 (D) in phosphate buffer medium	110
4.15.E.	Inhibitory effect of bacteriocin against <i>B. cereus</i> NCDC 66 (E) in phosphate buffer	110
4.16.	Changes in cell viability of <i>B. cereus</i> NCDC 66 in whipped	112

	cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasser</i> NBL 18(Gassericin), or both (GG)	
4.17.	Changes in cell viability of <i>L.monocytogenes</i> ATCC 15303 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasser</i> NBL 18 (Gassericin), or both (GG)	112
4.18.	Changes in cell viability of <i>E.coli</i> NCDC 134 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasser</i> NBL 18(Gassericin), or both (GG)	113
4.19.	Changes in cell viability of <i>P. aeruginosa</i> NCDC 104 in whipped cream frosting containing glycine, the culture supernatant of <i>Lactobacillus gasser</i> NBL 18 (Gassericin), or both (GG)	113
4.20.	Growth of <i>Listeria monocytogenes</i> ATCC 15303 in co-culture with bacteriocin producing <i>Lactobacillus gasser</i> NBL 18 and non-bacteriocinogenic NBL5	114
4.21.	Growth of <i>Staphylococcus aureus</i> NCDC 273 in co-culture with bacteriocin producing <i>Lactobacillus gasser</i> NBL 18 and non-bacteriocinogenic NBL5	114
4.22.	Growth of <i>Enterococcus faecalis</i> NCDC 114 in co-culture with bacteriocin producing <i>Lactobacillus gasser</i> NBL 18 and non-bacteriocinogenic NBL5	114

LIST OF PLATES

Plate No.	Title	After page no.
4.1.A.	Colonies of lactobacilli showing yellow zone on MRS agar supplemented with 0.004% BCP	69
4.1.B.	Colonies of lactobacilli streaked on MRS agar	69
4.2.	Microscopic observation of lactobacilli isolates under oil immersion (100X)	69
4.3.	Catalase test of lactobacilli isolates	69
4.4.	Aesculin hydrolysis by lactobacilli isolates	69
4.5.	Arginine hydrolysis by lactobacilli isolates	69
4.6.	Growth at 15 and 45 °C by lactobacilli isolates	69
4.7.	Carbohydrate fermentation by lactobacilli isolates	69
4.8.	Genus specific PCR of lactobacilli using primer LpLMA1/161R1	70
4.9.	Species specific PCR of <i>Lactobacillus gasseri</i> using primer Lgas2/Lgas3	70
4.10.	Screening of bacteriocinogenic <i>Lactobacillus gasseri</i> isolates using <i>E.faecalis</i> NCDC 114 as indicator by agar spot assay	73
4.11.	Screening of bacteriocinogenic <i>Lactobacillus gasseri</i> isolates using <i>E.faecalis</i> NCDC 114 as indicator by spot on lawn assay	73
4.12.	Screening of bacteriocinogenic <i>Lactobacillus gasseri</i> isolates using <i>E.faecalis</i> NCDC 114 as indicator by agar well assay	73
4.13.	PCR for <i>gaaA</i> gene using primer gaA515/gaA616	75
4.14.	PCR for <i>gaaA</i> gene using primer gaA2F/gaA2R	75
4.15.	Bacteriocin activity of crude bacteriocin by spot on lawn assay	92
4.16.	Antibacterial activity of partially purified bacteriocin by agar well assay as compared to control containing 60% saturated solution of ammonium sulfate	92
4.17.	Bacteriocin activity of partially purified bacteriocin by spot on lawn assay	at 93
4.18.	Activity of bacteriocin in the HPLC fractions. Activity was observed in fractions collected after 40 ml of buffer B (80 min)	96
4.19.	Molecular weight of bacteriocin as detected by Tricine-SDS-PAGE	100
4.20.	<i>In-situ</i> bacteriocin activity of purified bacteriocin	100
4.21.	Plates showing negative result for Gelatin production (A) and DNase activity (B) by NBL18	116
4.22.	Plate showing vancomycin resistance and sensitivity to other antibiotics by NBL 18	116

ABBREVIATIONS

ATCC	American Type Culture Collection
AU ml ⁻¹	Activity Units per ml
Bac ⁻	Non-bacteriocinogenic variant
Bac ⁺	Bacteriocinogenic variant
BCP	Bromo Cresol Purple
cfu	Colony forming units
d	Days
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
g	Gram
h	Hour
JCM	Japan Collection of Microorganisms
kbp	Kilo base pair
KDa	Kilo Dalton
LAB	Lactic Acid Bacteria
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
N	Normal
NCDC	National Collection of Dairy Culture
ng	Nanogram
OD	Optical Density
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
rpm	Rotations per minute
rRNA	Ribosomal RNA
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
sec	Seconds
SET	Sucrose-EDTA-Tris buffer
spp.	Species
ssp.	Subspecies
TAE	Tris-Acetic acid-EDTA buffer

TE	Tris-EDTA buffer
UV	Ultra violet
V	Volts
v/v	Volume/Volume
vol	Volume
w/v	Weight/Volume
w/w	Weight/Weight
α	Alpha
β	Beta
γ	Gamma
±	Positive and negative to particular value
μl	Microlitre
°C	Degree Centigrade

लैक्टोबैसिलस गैसेराई द्वारा उत्पादित सरकुलर बैक्टिरियोसिन का पृथक्करण एवं निरूपण

सारांश

सरकुलर बैक्टिरियोसिन एन-एवं सी- सिरि से जुड़े हुए ताप एवं पीएच स्थिर ऐसे पैप्टाईड हैं, जो कि लिनियर बैक्टिरियोसिन की तुलना में अधिक सक्रिय पाए गए हैं। इन आशाजनक विशेषताओं को ध्यान में रखते हुए, इस अध्ययन द्वारा लैक्टोबैसिलस गैसेराई से सरकुलर बैक्टिरियोसिन (गैसेरिसिन-ए) को पृथक् एवं चिन्हित करने का प्रयास किया गया है। मानव शिशु अपशिष्ट (50), मानव लार (10), एवं मानव दुग्ध (10) समेत 70 विभिन्न नमूनों में से 523 आइसोलेट चुने गए, जिसमें से 146 आइसोलेट को प्रजाति विशिष्ट पी. सी. आर. द्वारा लैक्टोबैसिलस गैसेराई के रूप में पहचाना गया। इनमें से 42 आइसोलेट ने सूचक जीव एन्टिरोकोकस फिकेलिस एन.सी.डी.सी.114 के विरुद्ध उचित रोगाणुरोधक क्षमता का प्रदर्शन किया। बैक्टिरियोसिन जीन की पहचान पी. सी. आर. द्वारा गैसेरिसिन-ए विशिष्ट प्राइमर इस्तेमाल करके किया गया, जिसमें सिर्फ 6 आइसोलेट ने सकारात्मक परिणाम दिखलाए। इन आइसोलेट में एन. बी. एल. 18 को उसकी अग्रिम जीवाणुरोधि गतिविधि की वजह से आगे के अध्ययन के लिए चयनित किया गया। एन. बी. एल. 18 के 16S-23S rRNA जीन के विरुद्ध पी. सी. आर. प्रोडक्ट को अनुक्रम कराया गया तथा जीनबैंक में अवाप्ति क्रमांक JQ809334.1 से प्रस्तुत किया गया। एन.बी.एल. 18 से बैक्टिरियोसिन के उत्पादन को रिस्पोस सरफेस मैथडोलोजी द्वारा पीएच, तापमान, इनोकुलम स्तर एवं ऊष्मायन समय को स्वतन्त्र चर लेते हुए अनुकूलित किया गया। बैक्टिरियोसिन का अधिकतम उत्पादन 8.0 की पीएच, 37°C के तापमान, 3% की इनोकुलम स्तर और 24 घंटे के उष्मायन समय में प्राप्त किया गया। बैक्टिरियोसिन ताप प्रतिरोधि (121°C/15 मिनट) तथा पीएच की विस्तृत श्रृंखला (2-11) पर सक्रिय पाया गया। इसके अलावा बैक्टिरियोसिन का अमोनियम सल्फेट, एनआयन विनिमय क्रोमैटोग्राफी एवं RP-HPLC द्वारा शुद्धिकरण किया गया। इनका आण्विक वजन 3.5 kDa के रूप में ट्राईसिन एस.डी.एस- पेज द्वारा निर्धारित किया गया। बैक्टिरियोसिन की संरचना में मुख्यतः α - हैलिक्स (78%) शामिल थे, जिसे सी. डी.-स्पैक्ट्रोस्कोपी द्वारा अनुमानित किया गया। खाद्य संरक्षक के रूप में इस्तेमाल करने पर अकेला बैक्टिरियोसिन ग्राम नेगेटिव जीवों को बाधित करने में असमर्थ पाया गया, लेकिन ग्लाइसिन के साथ संयोजन में इस्तेमाल करने पर ग्राम नेगेटिव जीवों को भी सफलतापूर्वक बाधित कर लिया गया। तीस दिनों के अध्ययन में जहाँ 10^3 सी.एफ.यू. प्रति ग्राम रोगजनकों को क्रीम के साथ इनोकुलेट किया गया, वहाँ 1.28×10^4 ए.यू./ग्राम बैक्टिरियोसिन तथा 1% (wt/wt) ग्लाइसीन, दोनों ग्राम पोसिटिव तथा ग्राम नेगेटिव वर्गों के जीवों की गिनती को नियन्त्रित करने में सफल पाए गए। 48 घंटों के को-कलचर अध्ययन में 10^6 सी.एफ.यू. प्रति मिली. एन.बी.एल. 18 कंट्रोल की तुलना में रोकजनक जीवों की गिनती नियन्त्रित करने ($P < 0.05$) में अधिक सफल पाया गया। डाह वर्ण की जाँच करने पर एन.बी.एल. 18 बीटा-हीमोलिसिस, डीएनएस तथा जिलेटिनेस जैसी गतिविधियों से मुक्त पाया गया।

ABSTRACT

Circular bacteriocins are heat and pH stable peptides with linked N and C termini, showing enhanced activity as compared to the linear bacteriocins. Keeping in view these promising attributes, this study was designed to isolate and characterize circular bacteriocin (gassericin A) from *L. gasseri* isolates. From a total number of 523 randomly selected colonies from 70 different samples comprising of infant feces (50), human saliva (10) and human milk (10), 146 isolates were identified as *L. gasseri* by species specific PCR. Among them 42 isolates exhibited antimicrobial activity against the indicator organism *E. faecalis* NCDC 114. Bacteriocin gene was identified by PCR using primers specific for gassericin A and six isolates were tested positive. Among these, NBL 18 was selected for further studies on the basis of its antimicrobial spectrum of activity. The purified PCR product of 16S-23S rRNA gene of NBL 18 was sequenced and submitted to Genbank with Accession. No. JQ809334.1. The production of bacteriocin from NBL 18 was optimized using Response Surface Methodology taking pH, temperature, inoculum level and incubation time as independent variables. Maximum production was attained at a pH of 8.00, temperature of 37°C, inoculum level of 3% and incubation time of 24h. The bacteriocin of NBL 18 was heat resistant (121°C/15 min) and showed activity over a wide range of pH (2-11). Further the bacteriocin was purified by ammonium sulfate precipitation, anion exchange chromatography and RP-HPLC. Molecular weight of the bacteriocin was 3.5 KDa as determined by Tricine-SDS-PAGE. The secondary structure of the bacteriocin consisted mainly of α - helices (78%) which was estimated through Circular Dichroism spectroscopy. When used as preservative, bacteriocin alone was unable to inhibit gram negative organisms but inhibited them when used in combination with glycine. Application of 1.28×10^4 AU/gm of bacteriocin and 1% wt/wt of glycine in whipped cream inoculated with 10^3 cfu/gm of pathogens, significantly decreased ($p < 0.05$) the counts of both Gram positive and Gram negative organisms after 30 days of incubation as compared to control. In co-culture study, performed in skim milk, 10^6 cfu /ml of NBL 18 significantly reduced ($p < 0.05$) the count of pathogens as compared to control after 48h of incubation. Physiological investigation of the isolate NBL 18 for the presence of virulence characters revealed that the isolate does not possess β -hemolytic, DNase and/or gelatinase activity.

Chapter 1

Introduction

1. INTRODUCTION

The phenomenon of microbial antagonism mediated by antimicrobial peptides has gained considerable attention in recent years because of their rapid bactericidal activity over a broad spectrum and the low propensity of bacteria to develop resistance against them. The production of antimicrobial peptides is widespread among both lower and higher organisms. They function as a defense mechanism against infection or, as in the case of bacteria, competition for nutrients in an ecological niche. Bacteriocins are ribosomally synthesized antimicrobial peptides, which are produced by a wide variety of bacteria (De Vugst and Vandamme, 1994). Most bacteriocins from Gram-positive bacteria are grouped into four classes: Class I, lantibiotics (containing posttranslationally modified amino acids such as lanthionine and β -methyllanthionine) (Ryan *et al.*, 1999); Class II – small unmodified thermo stables usually positively charged at a neutral pH peptides (Nes *et al.*, 1996); Class III – large unmodified thermo labile peptides (Atanassova *et al.*, 1999), and Class IV – complex bacteriocins which possess a carbohydrate or lipid component (Miteva *et al.*, 1998). In recent years, a new group of bacteriocins has emerged, the **circular bacteriocins**, which contain an amide bond between the N- and C-termini (Maqueda *et al.*, 2008). These bacteriocins are part of a growing number of ribosomally synthesized peptides, characterized by the head-to-tail cyclization of their backbone. The circular nature of these peptides renders them resistant to many proteases and provides great stability across a wide range of pH and temperatures as compared to their linear counterparts. Such circular peptides have been identified in mammals, plants, fungi and bacteria, and often play a role in host defense (Eisenbrandt *et al.*, 1999; Tang *et al.*, 1999; Jennings *et al.*, 2001; Schmidt *et al.*, 2005; Sivonen *et al.*, 2010; Cascales and Craik, 2010).

In contrast to many cyclic peptides in Eukaryotes, circular bacteriocins do not contain disulfide cross-links. Furthermore, although most other eukaryotic circular peptides are produced as precursors with both N- and C-terminal extensions, the circular bacteriocins are produced with only an N-terminal extension. The classification of circular bacteriocins, however, has been the subject of debate in recent years (Klaenhammer, 1993; Nes *et al.*, 1996; Cotter *et al.*, 2005). Some have classified these peptides as class II bacteriocins (Cotter *et al.*, 2005; Nissen-Meyer *et al.*, 2009), whereas others have proposed that they belong to a separate class- Class V (Kemperman *et al.*, 2003; Maqueda *et al.*, 2004; Heng and Tagg, 2006; Franz *et al.*, 2007; Nes *et al.*, 2007;

van- Belkum *et al.*, 2011). Circular bacteriocins distinguish themselves from class II bacteriocins in several important ways: they are post-translationally modified, are circular in nature, and their biosynthetic machinery is more complex and very different from that of the class II bacteriocins (van-Belkum and Stiles, 2000; Nes *et al.*, 2007). Therefore, it seems appropriate to place circular bacteriocins in a separate class. The stability to high temperatures, pH and degradation by proteases makes circular proteins ideal candidates as biological delivery agents. In recent years, a few circular proteins have been studied in their use as scaffolds in drug design by replacing native residues for biological active peptide sequences (Jagadish *et al.*, 2010). However, to accomplish bioengineering of circular proteins *in vivo* requires a thorough understanding of the biosynthetic mechanism and structure of these circular peptides. The structures of several circular bacteriocins have been elucidated and the genetic machinery is being uncovered.

Circular bacteriocins are able to inhibit the growth of a wide variety of Gram-positive bacteria, including many food spoilage bacteria and pathogens. Some of the circular bacteriocins that have been discovered are produced by lactic acid bacteria, which are generally recognized as safe (GRAS) in food products. Bacteriocin producing lactic acid bacteria have great potential in food preservation (Cotter *et al.*, 2005). *Carnobacterium maltaromaticum* UAL307 that produces a circular bacteriocin carnocyclin A has been approved for use in food products by the US FDA. One of the strain of *Lactobacillus gasserii* (LA 39), the Gram positive bacteria, which fit under general definition of lactic acid bacteria, often isolated from fecal samples as desirable gastrointestinal microbiota is reported to produce the circular bacteriocin gassericin A.

Gassericin A, the circular bacteriocin produced by *L. gasserii* LA 39 is small, hydrophobic, and thermostable with activity over wide range of pH. It is active against many Gram-positive bacteria encompassing undesirable and pathogenic microbes, such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Kawai *et al.*, 2001). This fact stimulates the special interest in gassericin A as a potential food preservative. Due to its highly hydrophobic nature, it deserves to be an excellent option in the biopreservation of high fat dairy products. Although gassericin A has yet not been allowed to be used as such in food products, nevertheless gassericin A producing *L.gasserii* can still be used as starter and/ or adjunct culture for the *in situ* production of

bacteriocin with the additional benefit of increasing the physical, chemical as well as sensory qualities of such products. To study the biochemical and antibacterial properties and to determine its effectiveness in food systems, it is necessary to obtain relatively large quantity of this peptide in pure and concentrated form.

Furthermore, owing to the intestinal origin, *L. gasseri* has been associated with a variety of probiotic activities and roles including reduction of fecal mutagenic enzymes (Pedrosa *et al.*, 1995), adherence to intestinal tissues (Conway *et al.*, 1987; Greene and Klaenhammer, 1994), stimulation of macrophages (Kirjavainen *et al.*, 1999; Kitazawa *et al.*, 1994; Tejada-Simon and Pestka, 1999), and production of bacteriocins (Itoh *et al.*, 1995). Survival in the GI tract, as well as bacteriocin production, are considered as important selection criteria for probiotic organisms and most of the *L.gasseri* strains very well fulfill these requirements. The *in-situ* production of circular bacteriocin by *L.gasseri* strain (LA 39) in reconstituted skim milk has also been studied and it has proved to be an excellent option for combating Gram positive pathogens in the milk (Arakawa *et al.*, 2009). Circular bacteriocin producing strains may have enhanced advantage over linear bacteriocin producers in food system due to their strong stability and broad antibacterial spectrum of activity. These characteristics are encouraging in the search for strains of *L.gasseri* with circular bacteriocin production and further application of these strains in food system as an effective biopreservative. Moreover, to our knowledge, there is hardly any report regarding the circular bacteriocin producing indigenous isolate of *L.gasseri*. Therefore, this area is relatively virgin and needs to be explored.

Keeping in view, the potential of some of the indigenous strains of *L. gasseri* to produce circular bacteriocin(s) and the application of these bacteriocin(s) for the inhibition of food spoilage and food pathogenic organisms, the study had been proposed to be undertaken with the following objectives:-

- i. Isolation of circular bacteriocin producing strains of *Lactobacillus gasseri* and assessment of their antimicrobial activity.
- ii. Optimization of conditions for bacteriocin production through Response Surface Methodology.
- iii. Purification and characterization of bacteriocin produced by the selected strain of *L. gasseri*.
- iv. Application of bacteriocin in the food system to inhibit food-borne pathogens.

Chapter 2

Review of Literature

2. REVIEW OF LITERATURE

2.1. PROLOGUE

A wide variety of protein molecules are produced by micro-organisms for defense, mediation of microbial competitions and signaling purposes. These protein molecules include both small peptides that are assembled non-ribosomally by the enzyme peptide synthetases; and large folded polypeptides that are translated conventionally from genes (ribosomally synthesized) (Kohli *et al.*, 2003). Under physiological conditions proteins have a definite three-dimensional structure which also determines their biological role. The termini of polypeptide chains, however, represent target points for the attack of proteolytic enzymes affecting the stability to the molecule thereby affecting functionality. The non ribosomally synthesized proteins such as toxins, antibiotics, pigments, siderophores etc. that are synthesized by condensation of monomer building blocks by an enzyme- driven process to produce a linear acyl chain; avoid this problem with some post-translational modifications, such as acetylations, hydroxylations and/or glycosylations. Such modifications may give the producing organism an advantage, as the modified molecules are less susceptible to the normal proteolytic cleavage reactions of proteins.

Cyclization is another strategy that has been seen in the non ribosomal peptides to avoid their proteolytic cleavage. It is exemplified by well-known natural products such as antibiotics like cyclosporine A and Gramicidin S. The advantages of such a strategy for stabilizing peptides may be seen by the fact that a significant number of macrocyclic natural products have found pharmaceutical applications, including, for example, the widespread use of cyclosporin A as an immunosuppressive agent. Synthetic cyclic peptides are also widely used as lead molecules in the pharmaceutical industry.

During recent years, several naturally occurring circular proteins fundamentally different from the nonribosomal cyclic peptides have been discovered (Trabi *et al.*, 2002). These molecules are true proteins in that they have a well-folded three dimensional structure and are produced via translation of genes. The structural data available for circular proteins have almost exclusively been derived from NMR analysis and X-Ray diffraction. These proteins contain well-defined and conservative secondary structures. The only difference from conventional proteins is that the gene-encoded precursor proteins must be post-translationally modified to join the N- and C- ends, producing a

seamless circle of peptide bonds. Clearly, cross-linking the termini of a chain decreases the conformational entropy of the highly flexible and disordered linear polypeptides, stabilizing their bioactive conformation and increasing proteolysis-resistance due to the lack of exopeptidase cleavage sites. Another common characteristic of most circular proteins is their strong biological activity (antibacterial, antiviral or pharmacological action). Thus, their biotechnological interest has increased, becoming a prime line of research (Trabi *et al.*, 2002; Craik *et al.*, 2003; Rivas *et al.*, 2003).

Circular proteins were unknown until 1990, but now have been seen to occur in a diverse range of organisms, from bacteria to plants and animals. The discovery of these peptides started in the mid 1990s when circular proteins from plants, now known as the cyclotides, were discovered. Cyclotides are a group of ribosomally synthesized circular proteins in plants belonging to *Rubiaceae*, *Violaceae* and *Cucurbitaceae* families (Craik *et al.*, 2006; Goransson *et al.*, 2004). Their importance is revealed by the fact that they are anti- HIV (Gustafson *et al.*, 1994) insecticidal, anti-tumour, antifouling, anti-microbial, and also acts as neurotensin antagonist (Witherup *et al.*, 1994). Their real discovery was actually in the early 1970s when a tea extract from the plant *Oldenlandia affinis* was reported to give uteronic activities, but the actual structure was not determined until almost thirty years later. Since then several other circular peptides have been isolated.

The circular protein found in mammal is Rhesus theta defensins (RDT-1) (Tang *et al.*, 1999; Selsted *et al.*, 2004). RTD-1 is part of the innate immune system of the macaque monkey, and has microbiocide action against Gram-positive and Gram-negative bacteria, fungi, and also has anti-retroviral activity. Sunflower trypsin inhibitor (SFTI-I) is yet another group of circular protein found in sunflower seeds (Luckett *et al.*, 1999). SFTI-1 and kalata B1 (cyclotide) are examples of peptides with a high content of well conserved cysteines that form cross linking disulfide bonds. These multiple disulfide bonds facilitate the formation of a well-defined and conformational stable three dimensional structure by the peptides. A cyclized backbone together with multiple disulfide bonds gives the peptide an exceptional stability against protease digestion and thermal denaturation. Many cysteine rich peptides display specific biological activities and have been of interest in the field of pharmacology and drug design, but have been commercially limited due to disadvantages with proteolytic degradation *in vivo*. The discovery of increasing

numbers of naturally cyclic peptides has given ideas to develop synthetic techniques to make naturally linear peptides cyclic. This approach has the potential to improve their bioavailability and thus their potential as drug leads. Peptides whose termini are in reasonable close proximity to each other have the potential to be cyclized and still maintain their structure and hence bioactivity. Examples of ribosomally synthesized circular proteins in bacteria include pilins present in some Gram-negative bacteria implicated in the contact between cells during the conjugation process; and **bacteriocins** produced by both Gram positive and Gram-negative bacteria which are involved in microbial defence of the producer organisms.

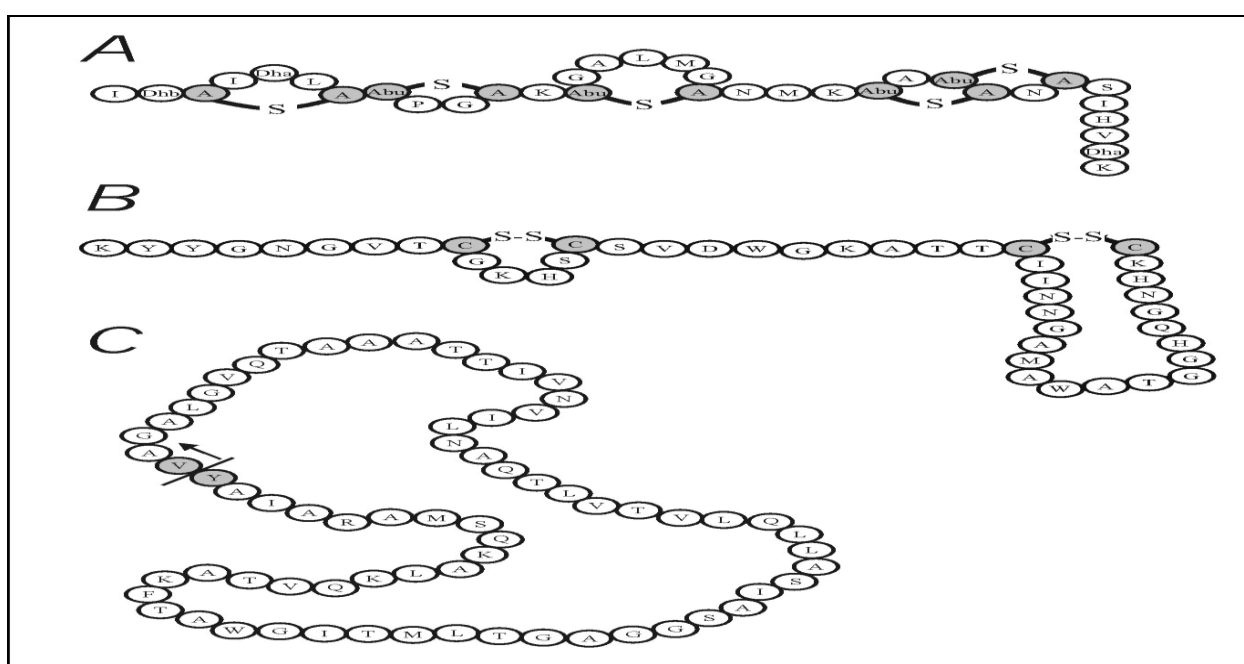


Fig. 2.1: Examples of circular proteins isolated from bacteria, mammals, and plants. A: bacteriocin AS-48 from *E. faecalis*. B: RTD-1 from the leukocytes of macaques. C: SFTI-1 from sunflower seeds

2.2. BACTERIOCINS

Bacteriocins stand out among the wide variety of antimicrobial ribosomal peptides synthesized by bacteria. They have been found in all major lineages of bacteria (Riley *et al.*, 2002). Also it has been observed that some members of the Archaea also produce antimicrobial proteins resembling bacteriocins (O'Connor *et al.*, 2002). Bacteriocins were first discovered by Andre Gratia and his workgroup in 1925 as 'principle V' (produced by a strain of *E. coli* against another culture), however, the term "bacteriocin" was coined in 1953 (Jacob *et al.*, 1953). These molecules are

defined as ribosomally synthesized anti-microbial peptides, proteins or proteinaceous complexes produced by bacteria that act mainly against closely related species. They are characterized by lethal biosynthesis, predominantly intra-species killing activity and adsorption to specific receptors on the surface of bacteriocins sensitive cells.

2.2.1. Bacteriocins Produced by Gram-negative Bacteria

Bacteriocins produced by Gram-negative bacteria, are large proteins (>20 KDa). They can be divided into two groups, namely the colicins and the microcins. Colicins are large (25- to 80-KDa) bactericidal proteins produced by and toxic to some strains of *Escherichia coli*. Their production is induced by conditions triggering the SOS system (Pons *et al.*, 2002). They are found in 30-50% of the strains isolated from human host and are often referred to as virulence factors (Riley and Gordon 1992). Since their discovery the colicins have been the most extensively studied Gram negative bacteriocins, and they now serve as a model system for investigating the mechanisms of bacteriocin structure/function, genetic organization, ecology and evolution (Cascales *et al.*, 2007). Colicins are shown to kill their targets by either membrane permeabilization or nucleic acid degradation (Braun *et al.*, 1994; Riley and Wertz 2002; Smarda and Smajs 1998). Microcins are antimicrobial peptides also produced by members of the family *Enterobacteriaceae* (*E.coli* strains) under conditions of nutrient depletion that target microbes phylogenetically related to the producer strain. These are smaller (<10 KDa) and their synthesis is not SOS-inducible (Kolter *et al.*, 1992). Microcins share more properties with the bacteriocins produced by Gram positive bacteria, including thermostability, resistance to some proteases, relative hydrophobicity and resistance to extreme pH. Except for one *Klebsiella pneumoniae* strain (Pons *et al.*, 2002), until now, all bacteria producing microcins have been identified as natural *E. coli* isolates. Microcins are divided into the following two groups: Class I microcins: are post-translationally modified peptides with a mass of less than 5 kDa with activity against intracellular targets. Members of this group are microcins B17, C7 and J25 (Gaillard-Gendron *et al.*, 2000). Microcin J25 (MccJ25), isolated from *Escherichia coli* AY25 was the first smallest circular protein of bacterial origin (Salomón *et al.*, 1992).

Class II microcins are unmodified peptides with a molecular mass between 8 and 10 KDa. They exert their function through membrane depolarization. Members of this group of bacteriocins, produced by *Enterobacteriaceae*, are microcins E492, L and colicin V (Gaillard-Gendron *et al.*, 2000).

2.2.2. Bacteriocins Produced by Gram-positive Bacteria

The bacteriocins produced by Gram positive bacteria are abundant and more diverse than those found in Gram negative bacteria. They are generally cationic, amphipathic small molecules that range in size from 2-6 kDa (> 8 k Da). These are often membrane permeabilizing peptides resembling the antimicrobial peptides produced by eukaryotes. They vary a lot in make-up and general structure. They have features in common such as heat stability, protease resistance and the fact that most run faster through an SDS-PAGE gel than predicted on the basis of their sizes. Available 3D-structures of some bacteriocins show that some of them are tightly folded peptides. Internal cross-linking supports the stability of many of these peptides. They differ from bacteriocins of Gram negative bacteria in two fundamental ways. First the bacteriocins produced by Gram positive bacteria are not necessarily lethal to the producing cell. This critical difference is due to the dedicated transport mechanisms Gram positive bacteria encode to release the bacteriocin toxin. Typically their biosynthesis is self regulated with specifically dedicated transport mechanisms facilitating release, although some employ the Sec-dependent export pathway. Second, the Gram positive bacteria have evolved bacteriocin specific regulation, whereas bacteriocins of Gram negative bacteria rely solely on host regulatory networks (Braun *et al.*, 1994; Smarda and Smajs 1998).

2.3. BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) constitute a diverse group of Gram-positive bacteria, characterized by some common morphological, metabolic and physiological traits. They are anaerobic bacteria, non-sporulating, acid tolerant and produce mainly lactic acid as an end product of carbohydrate fermentation. Lactic acid bacteria consist of a number of diverse genera which include both homofermenters and heterofermenters based on the end product of their fermentation. The homofermenters produce lactic acid as the major product of fermentation of glucose and include the genera *Lactococcus*, *Streptococcus* and *Pediococcus*. In contrast, the heterofermenters produce a number of products besides lactic acid, such as carbon dioxide, acetic acid, and ethanol from the fermentation of glucose and includes the genus *Leuconostoc* and a subgroup of the genus *Lactobacillus*, the Betabacteria (Jay 1986; Kandler et al. 1986; Schillinger and Lücke 1987).

Lactic acid bacteria consist of a number of bacterial genera within the phylum Firmicutes. Recent taxonomic studies suggested that the LAB group includes the

following genera; *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Ercolini et al. 2001; Carr et al. 2002). Species of these genera can be found in the GIT of man and animal as well as in fermented food. LAB strains used as probiotics usually belong to species of the genera *Lactobacillus*, *Enterococcus* or *Bifidobacterium*.

The core LAB genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* share a long history of safe usage in the processing of fermented foods. The antimicrobial effects and safety of LAB in food preservation is widely accepted (EFSA, 2005; de Vuyst and Leroy 2007; Sit and Vederas 2008). Their preservative effect is mainly due to the production of lactic acid and other organic acids which results in lowering of pH (Daeschel 1989). Preservation is enhanced by the production of other antimicrobial compounds, including hydrogen peroxide, CO₂, diacetyl, acetaldehyde, and bacteriocins (Stiles and Hastings 1991; Klaenhammer 1988; 1993).

In addition to the antimicrobial effects specific LAB also possess health promoting properties. Evidence from *in vitro* systems, animal models, and human clinical studies suggest that LAB function as immunomodulators and can enhance both specific and nonspecific immune responses (Ouweland et al. 1998; 2002; O'Flaherty and Klaenhammer 2009) justifying their use as health promoting supplements or probiotics both for humans and animals.

Lactic acid bacteria are capable of producing a wide range of ribosomally synthesized proteins and peptides which have antimicrobial activity to compete with other bacteria of the same species (narrow spectrum) or to counteract bacteria of other genera (broad spectrum) (Cotter et al. 2005). Since the initial discovery of bacteriocins in 1925, numerous bacteriocins have been isolated from LAB. A large number of bacteriocins have been isolated and characterized from lactic acid bacteria and some have acquired a status as potential antimicrobial agents because of their potential as food preservatives and antagonistic affect against important pathogens. The important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, plantaricins and gassericsins (Nettles and Barefoot, 1993). Continuous

discovery of new bacteriocins with novel features requires an updating of the classification of bacteriocins.

Table 2.1. Properties of some well characterized bacteriocins from LAB (Soomro *et al.*, 2002)

Bacteriocin	Producer organism	Properties
Nisin	<i>Lactococcus lactis subsp.lactis</i> ATCC 11454	Lantibiotic, broad spectrum, chromosome / plasmid mediated, bactericidal, produced late in the growth cycle
Pediocin A	<i>Pediococcus Pentosaceus</i> FBB61 and L-7230	Broad spectrum, plasmid mediated
Pediocin AcH	<i>Pediococcus acidilactici</i> H	Broad spectrum, plasmid mediated
Leucocin	<i>Leuconostoc gelidum</i> UAL 187	Broad spectrum, plasmid Mediated, bacteriostatic, produced early in the growth cycle
Helveticin J	<i>Lactobacillus helveticus</i> 481	Narrow spectrum, chromosomally mediated, bactericidal
Carnobacteriocin	<i>Carnobacterium piscicola</i> LV17	Narrow spectrum, plasmid mediated, produced early in the growth cycle.
Gassericin A	<i>Lactobacillus gasseri</i> LA 39	Broad spectrum, plasmid mediated, bactericidal

2.4. CLASSIFICATION OF BACTERIOCINS

Bacteriocins produced by lactic acid bacteria, which have a long history of use in fermentation and meat and milk products preservation are the best characterized bacteriocins. The classification of bacteriocins, however, remains controversial. Klaenhammer classified bacteriocins into four classes (Klaenhammer 1993)- the lantibiotics, the unmodified peptides, the large heat labile bacteriolysins and the

complex bacteriocins. By contrast, Cotter proposed two major divisions for bacteriocins (Cotter *et al.*, 2005): Class I -the lantibiotics and Class II- the non-lantibiotic bacteriocins. Klaenhammer's classes IIc and IV were withdrawn in Cotter's scheme since they were unproven entities. The novel post-translationally modified cyclic peptides were relegated to Class IIc. The class III heat labile bacteriolysins proposed by Klaenhammer exhibited all the defining characteristics of bacteriocins (bacteriolytic proteins such as lysostaphin and zoocin A), including genes encoding specific immunity factors. However, it was observed that some class III bacteriocins such as helveticin J do not kill by lytic mechanism and hence cannot be classified as bacteriolysins. Therefore the classification of such enzymes as being bacteriocins has been thrown into doubt (Cotter *et al.*, 2005). To redress this apparently unjustifiable classification directed against class III bacteriocins, a new classification scheme was devised by Heng and Tagg (Heng and Tagg, 2006) that incorporated the aspects of both Klaenhammer and Cotter proposals. It made provisions for all currently known antimicrobials that conform to the original 'Jacobian' definition of a bacteriocin. According to the new classification scheme of Heng and Tagg, Klaenhammer classes IIc and IV were eliminated. However, in contrast to Cotter, Class III (large bacteriocins) was retained and divided into Types IIIa (bacteriolytic) and IIIb (non-lytic). The cyclic post-translationally modified bacteriocins (Class IIc) were upgraded to Class IV. The lantibiotics are divided into class Ia which are elongated molecules with a flexible structure in solution, class Ib, which tends to have a more rigid, globular structure (although there are some exceptions) and class Ic which are multicomponent. Lack of consensus also exists in the distinction between subgroups of the nonlantibiotics belonging to class II, which include a large number of small peptides that only have in common their marked thermostability and a lack of modified residues (Cleveland *et al.*, 2001; Eijsink *et al.*, 2002; Ross *et al.*, 2002; Cotter *et al.*, 2005; Drider *et al.*, 2006). Ness *et al.*, (2007), grouped bacteriocins into two major classes, class I: lantibiotics; and class II: the heat-stable nonlantibiotics. Further in his classification scheme, class II were subdivide into four subclasses: IIa (antilisteria pediocin-like bacteriocins), IIb (two-peptide bacteriocins), IIc (leaderless peptide bacteriocins) and class IId (circular bacteriocins).

Circular bacteriocins have been grouped into different classes by various workers. It has been recommended by Kempermen *et al.*, (2003), to classify circular bacteriocins into a separate class *i.e.* 'Klaenhammer Class V' bacteriocins. This proposal to classify circular bacteriocin into a new class (Class-V) has also been approved by several other authors

(Maqueda *et al.*, 2004; Heng and Tagg, 2006; Franz *et al.*, 2007; Nes *et al.*, 2007; van-Belkum *et al.*, 2011).

2.5. CLASS V (CIRCULAR) BACTERIOCINS

Amongst all the classes of bacteriocins, the class V (Kemperman *et al.*, 2003), comprising circular bacteriocins have attracted increasing attention, since they are the simplest prokaryotic representatives of the ubiquitous circular peptides with various physiological activities (Craik *et al.*, 2006). These bacteriocins range in size from 3400 to 7200 Da and contains 58 to 78 amino acids and are very hydrophobic. These are produced by Gram positive bacteria via gene translation. Their N and C termini are joined by peptide bonds to produce a circular molecule. They render the cell membrane permeable to small molecules and thereby disrupt the PMF, which eventually leads to cell death. Only a few circular bacteriocins have been described to date (Table 2.2) which include the peptide enterocin AS-48 produced by *Enterococcus faecalis* S-48 (Maqueda *et al.*, 2004); circularin A, produced by *Clostridium beijerinckii* ATCC 25752 (Kempermen *et al.*, 2003, Kawai *et al.*, 2004a); gassericin A and reuterin 6, two hydrophobic bacteriocins produced by species of *Lactobacillus* namely *Lactobacillus gasseri* LA39 and *Lactobacillus reuteri* LA6, respectively (Kawai *et al.*, 2004b); acidocin B, produced by *Lactobacillus acidophilus* (Leer *et al.*, 1995); butyrivibriocin AR10, produced by the anaerobic ruminal bacterium *Butyrivibrium fibrisolvens* AR10 (Kalmokoff *et al.*, 2003); uberolysin produced by *Streptococcus uberis* (Wirawan *et al.*, 2007); and subtilisin A produced by *Bacillus subtilis* (Kawulka *et al.*, 2004). Subtilisin A is the only circular lantibiotic described to date. Uberolysin was the first circular bacteriocin to be characterized biochemically and genetically. Enterocin AS-48 is the first and most vigorously characterized member of the class V bacteriocins (Maqueda *et al.*, 2004). It is also active against Gram negative bacteria such as *E. coli*, *Myxococcus xanthus*, *Salmonella choleraesuis* and *Escherichia coli* O157:H7.

The circular bacteriocins are subdivided into two groups based on sequence similarity and biochemical characteristics (Cotter *et al.*, 2005; Martin-Visscher *et al.*, 2009): group I consists of enterocin AS-48, circularin A, uberolysin, lactocyclicin Q, carnocyclin A and garvicin ML, whereas group II consists of gassericin A and butyrivibriocin AR10. Bacteriocins in group I are cationic and have a high isoelectric point (pI-10). Peptides in group II share significant sequence homology, have more

acidic residues compared to group I peptides and their isoelectric point is much lower (pI ~5).

Circular bacteriocins have a high degree of pH and thermal stability, along with increased resistance to denaturation by chaotropes. These all factors improve their biological activity in vivo. In fact, due to their wide spectrum of action and stability across considerable pH and temperature ranges, they constitute one of the most attractive groups of inhibitors described to date. In terms of their three-dimensional structures, circular proteins behave very much like conventional proteins, *i.e.*, their sequence contains all of the information required for folding into a defined three-dimensional shape.

Other bacteriocins, not identified as such, might be Class V circular bacteriocins as well. The amino acid sequence of acidocin B, as derived from the structural gene, is very homologous to that of gassericin A and, like gassericin A, acidocin B has been shown to be blocked at the N-terminus, precluding N-terminal amino acid sequencing. Another bacteriocin, boticin B produced by *C. botulinum*, displays features of a circular bacteriocin: it is resistant to protease digestion and has a blocked N-terminus. Alternatively, putative Class V bacteriocin loci can be identified in the growing number of microbial genomes that have been sequenced to completion.

Table 2.2. Examples of Circular Bacteriocin from Gram positive bacteria

Circular bacteriocin	Producer organism	Leader peptide (amino acid)	Size (amino acid)	Mass (Da)	Citations
Gassericin A	<i>Lactobacillus gasseri</i> LA39	33	58	5652	Toba <i>et al.</i> , 1991
Reuterin 6	<i>Lactobacillus reuteri</i> LA6	33	58	5652	Kawai <i>et al.</i> , 1994
Acidocin B	<i>Lactobacillus acidophilus</i> M46	33	58	5620	Leer <i>et al.</i> , 1995
Butyrovibriocin	<i>Butyrovibrium fibrisolvens</i> AR10	22	58	5981.5	Kalmokoff <i>et al.</i> , 2003
Uberolysin	<i>Streptococcus uberis</i>	3	70	7048	Wirawan <i>et al.</i> ,

					2007
Subtilisin A	<i>Bacillus subtilis</i>	8	35	3399.7	Zheng <i>et al.</i> , 1999
Enterocin AS-48	<i>Enterococcus faecalis</i> S-48	35	70	7150	Galvez <i>et al.</i> , 1986, Maqueda <i>et al.</i> , 2004
Circularin A	<i>Clostridium beijerinckii</i> ATCC 25752	6	69	ND	Kemperman <i>et al.</i> , 2003
Carnocyclin A	<i>Carnobacterium maltaromaticum</i> UAL307	4	60	5862	Martin-Visscher <i>et al.</i> , 2008

2.6. BACTERIOCIN SECRETION

Secretion of LAB bacteriocins has been shown to proceed in either of three ways: some bacteriocins are secreted via the general *sec*-pathway of protein secretion, the LsbA and LsbB bacteriocins of *L. lactis* are externalized via multidrug resistance (MDR) proteins (Gajic *et al.*, 2003), while still other bacteriocins are secreted via dedicated ABC-type transporters, for which the genetic information is located in the direct vicinity of the bacteriocin structural genes. Many class II bacteriocins are released after being cleaved at a conserved Gly-Gly motif at positions -2 and -1 in the processing site by a proteolytically active domain in the C terminus of the dedicated secretion protein (Franke *et al.*, 1999; Venema *et al.*, 1995). Although their leader peptide sequences show similarities, the sequences of the mature bacteriocins differ widely.

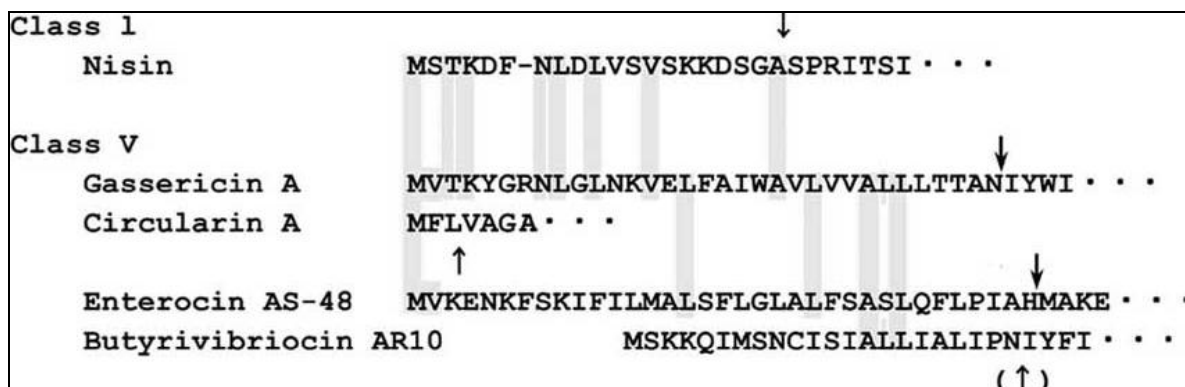


Fig.2.2. Comparison of the leader peptide sequences of gassericin A and circularin A with those of nisin, enterocin AS-48 and butyrivibriocin AR10 (putative). The arrows show processing sites in mature bacteriocins (putative site in parenthesis)

ABC type transporter genes are also found in the direct vicinity of the structural genes of all known and putative circular bacteriocins, suggesting that dedicated transporters are involved in the secretion of these proteins, although none of these transporters have been proven to be involved in bacteriocin secretion as yet. The putative leader peptide sequence of gassericin A contains 33 amino acid residues and does not contain the Gly-Gly motif. It is more similar to the leader sequence of nisin. A gene encoding a protein with a putative ATP binding domain is located downstream of *gaaA*, suggesting that it may be involved in the secretion of gassericin A (Kawai *et al.*, 2004a).

2.7. MODE OF ACTION

Most of the bacteriocins of Gram-positive bacteria studied so far exert their bactericidal effect by piercing the cytoplasmic membrane of sensitive cells. However a few type-B lantibiotics act by enzyme inhibition. Mersacidin e.g. binds to the cell wall precursor lipid II, thereby inhibiting cell wall biosynthesis by the transglycosylase, which subsequently leads to cell lysis (McAuliffe *et al.*, 2001). Membrane-active piercing bacteriocins on the other hand create pores in the membrane, resulting in dissipation of the proton motive force, loss of cellular metabolites and ultimately cell death as a result of energy depletion (Montville *et al.*, 1998). At least in some cases a receptor molecule appears to target the bacteriocins to the membrane, although some bacteriocins are active in experimental conditions where no receptor is available. Nisin, for instance, can interact with the membrane in a voltage-dependent manner (Garcera *et al.*, 1993; McAuliffe *et al.*, 1998; Sahl *et al.*, 1998) but lipid II, as a receptor molecule, enhances nisin activity 1000-fold (Breukink *et al.*, 1999; Hechard *et al.*, 2002). Class II bacteriocins work in a voltage-independent fashion, requiring a (protein) receptor to target them to the cytoplasmic membrane (Chikindas *et al.*, 1993).

However, the class V bacteriocins, gassericin A and reuterin 6 did not cause any cell lysis neither do both the bacteriocins caused ATP efflux from the indicator cells (Kawai *et al.*, 2004b). The effects of gassericin A and reuterin 6 on the viability and lysis of indicator cells of *L. delbrueckii* subsp. *bulgaricus* JCM 1002T and the amounts of the bacteriocins that attached to the cells were examined and found that the level of cell viability was decreased by the actions of both bacteriocins but optical density at 650 nm was maintained after bacteriocin treatment, indicating that no cell lysis was caused by either of the bacteriocins. The level of activity of each bacteriocin detected in the cell

suspension decreased with the advance of time, indicating that the levels of attachment of both bacteriocins increased. Both bacteriocins had caused potassium ion efflux from the indicator strain, and the amount of efflux had decreased with long time of incubation.

2.8. BACTERIOCIN IMMUNITY AND RESISTANCE

Immunity is the ability of a bacteriocin-producing bacterium to protect itself against the produced bacteriocin by way of a dedicated protein. In all other cases protection against bacteriocins is referred as bacteriocin resistance. Resistance therefore is the ability of bacteria to survive in the presence of bacteriocins without the need to express a dedicated immunity protein(s). Immunity proteins differ in size and general make up. The amino acid sequences of immunity proteins do not give a clear indication as to their mode of action. Immunity proteins are generally believed to block formation of a functional pore either by direct binding of the bacteriocin (Quadri *et al.*, 1995) or by binding to the bacteriocin receptors (Quadri *et al.*, 1995; Nissen-Meyer *et al.*, 1993; Venemema *et al.*, 1994).

ABC transporters, often with so-called accessory proteins, transport a cognate bacteriocin(s) and provide resistance by pumping out (active export) the bacteriocin molecules that have invaded from outside of the producing cells (Cheigh *et al.*, 2005; Diaz *et al.*, 2003). Many bacteriocin producers also make specific immunity proteins (peptides) to counteract the activity of the bacteriocin they produce. Generally, immunity proteins (peptides) are diverse in their primary structures (Fimland *et al.*, 2002; Martin-Visscher *et al.*, 2008). Several small peptides have been implicated in providing immunity to circular bacteriocins. The peptides, GaaI (for gassericin A), BviD (for butyrivibriocin), As-48D1 (for enterocin AS-48), CirE (for circularin A), and AblB (for subtilosin A), ranging in size from 49 to 56 amino acid residues, have one or two transmembrane segments (TMSs), except for UblE (three TMSs), the putative immunity peptide for uberolysin, which consists of 89 amino acid residues and is slightly longer than the immunity peptides of other class V bacteriocins (Maqueda *et al.*, 2008). These TMSs may expose positively charged amino acid residues to the outer surface of the cytoplasmic membrane. These predictions suggest that the immunity peptides for class V bacteriocins may be located in the cell membranes of the producing strains. These peptides contain several lysines and arginines outside of the putative TMSs. This suggests that these positively charged residues on the peptides located on the outer and/or inner surface of the cell membrane may electrostatically prevent the attachment of cognate bacteriocins that are also

positively charged. Conversely, many immunity proteins of the class IIa bacteriocins, ranging in size from 81 to 115 amino acid residues, which have been classified into three groups according to sequence homology (leucocin A and enterocin A in group A, piscicolin 126 and sakacin P in group B, and carnobacteriocin B2 and enterocin P in group C) (Fimland *et al.*, 2002), have no deduced TMSs and may be located at the inner surface of the cell membrane and act intracellularly (Delcher *et al.*, 1999; Fimland *et al.*, 2002; Martin-Visscher *et al.*, 2008). The mechanism of immunity peptides working against class V bacteriocins could be different from that of immunity peptides of class IIa bacteriocins (Diep *et al.*, 2007; Martin-Visscher *et al.*, 2008; Soliman *et al.*, 2007), as the modes of action of the two classes of bacteriocins may be different.

2.9. BACTERIOCINS PRODUCED BY THE GENUS *LACTOBACILLUS*

2.9.1. The Genus *Lactobacillus*

Lactobacillus, also called Doderlein's bacillus, is a genus of Gram-positive, non-sporulating, non-motile, facultative anaerobic or microaerophilic and chemo-organotrophic rod-shaped bacteria (Makarova *et al.*, 2006). They are usually straight, although they can form spiral or coccobacillary forms under certain conditions. They are often found in pairs or chains of varying length. Lactobacilli are classified as lactic acid bacteria, and derive almost all of their energy from the conversion of glucose to lactate during homolactic fermentation (Satokari *et al.*, 2003). In this process 85-90% of the sugar utilized is converted to lactic acid. They generate ATP by nonoxidative substrate-level phosphorylation. They are acid-tolerant and may grow at pH as low as 3.5 (Chou *et al.*, 1999). They are catalase negative even if pseudocatalase activity can sometimes be present in some strains. They have a generation time ranging from 25 minutes to several hundred minutes, and grow optimally between the temperatures of 30 and 40°C, although thermophilic strains can be comfortable at temperatures as high as 60°C. Their optimum growth pH ranges between 5.5-6.2. Considering DNA base composition of the genome, they usually show a GC content of lower than 54 mol%. More than 150 species have been identified within the *Lactobacillus* genus (<http://www.bacterio.cict.fr//Lactobacillus.html>), with substantial genetic and phenotypical differences between different groups (Claesson *et al.*, 2007). Like the other members of the *Lactobacillus* genus, *Lactobacillus gasseri* is a gram-positive bacterium that falls into the category of lactic acid bacteria. It is also a rod shaped and of the non-spore-forming type.

2.9.2. Ecology

Lactobacilli are almost ubiquitous and are found in environments where carbohydrates are available, such as food (dairy products, fermented meat, sour doughs, vegetables, fruits, beverages), respiratory, GI tracts of humans and animals, and in sewage and plant material. In human gastrointestinal tract they are symbiotic and make up a small portion of the gut flora. Lactobacilli are found in the gastro-intestinal microbiota of almost all adult individuals (Matsuda *et al.*, 2009), but, with the possible exception of the small intestine, they are not a dominating bacterial group in these habitats. An overview of lactobacilli commonly found in the alimentary microbiota is shown in Table 2.3.

Table 2.3. *Lactobacillus* species distribution in different parts of the gastrointestinal tract

Oral cavity	Stomach	Small intestine	Faeces	Colon epithelial
<i>L. paracasei</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. plantarum</i>
<i>L. rhamnosus</i>	<i>L. reuteri</i>	<i>L. reuteri</i>	<i>L. paracasei</i>	<i>L. rhamnosus</i>
<i>L. fermentum</i>	<i>L. ruminis</i>	<i>L. rhamnosus</i>	<i>L. ruminis</i>	<i>L. paracasei</i>
<i>L. plantarum</i>	<i>L. reuteri</i>			
<i>L. gasseri</i>	<i>L. plantarum</i>			
<i>L. salivarius</i>				

Lactobacilli commonly found in the oral cavity (Ahrne *et al.*, 1998; Colloca *et al.*, 2000; Munson *et al.*, 2004; Dal Bello *et al.*, 2006; Strahinic *et al.*, 2007; Koll *et al.*, 2008), stomach (Reuter *et al.*, 2001; Ryan *et al.*, 2008), small intestine (Reuter *et al.*, 2001) and large intestine (Matsuda *et al.*, 2009; Ahrne *et al.*, 1998; Dal Bello *et al.*, 2006; Reuter *et al.*, 2001; Vaughan *et al.*, 2005).

As natural GI microflora they are believed to perform several beneficial roles including immunomodulation, interference with enteric pathogens, and maintenance of healthy intestinal microflora. Historically, probiotic roles have been ascribed primarily to *Lactobacillus acidophilus*. Recent developments in taxonomy have revealed six different *Lactobacillus* species (on the basis of DNA sequence similarity and cell wall composition) that comprise the acidophilus group. These species include *L. acidophilus* (A1), *L. crispatus* (A2), *L. amylovorus* (A3), *L. gallinarum* (A4), *L. gasseri* (B1) and *L. johnsonii* (B2). Among these, *Lactobacillus gasseri* appears to represent the major

homofermentative *Lactobacillus* species that occupies the human GI tract (Mitsuoka, 1992; Kullen *et al.*, 2000). This *L. gasseri* demonstrates good survival in the GI tract (Conway *et al.*, 1987; Pedrosa *et al.*, 1995) and has been associated with a variety of probiotic activities and roles including reduction of fecal mutagenic enzymes (Pedrosa *et al.*, 1995), adherence to intestinal tissues (Conway *et al.*, 1987; Greene and Klaenhammer, 1994), stimulation of macrophages (Kirjavainen *et al.*, 1999; Kitazawa *et al.*, 1994; Tejada-Simon and Pestka, 1999), and production of bacteriocins (Itoh *et al.*, 1995). There are a diverse number of naturally-occurring *Lactobacillus* species found in the human GI tract and many are considered to exert beneficial roles there. Establishing these roles, individually and in combination, remains a significant challenge. Relative to the other members of the acidophilus complex, there exists a significant platform of work on the *L. gasseri* species related to its transformability by electroporation (Luchansky *et al.*, 1988), expression of heterologous genes (Cho *et al.*, 2000), and the available genome sequence information for one temperate bacteriophage (Altermann *et al.*, 1999; Raya *et al.*, 1992). *L. gasseri* is substantially more amenable to DNA introduction and manipulation which has lead to the development of more genetic tools that will be useful in the functional generation.

Lactobacillus gasseri are not only found in the alimentary tract canal, but also in the vagina of fertile women where they dominate the microbiota. The other most commonly found vaginal lactobacilli in women of reproductive ages are *L. crispatus*, *L. iners* and *L. jensenii* (Vasquez *et al.*, 2002; Burton *et al.*, 2003; Martin *et al.*, 2007). *Lactobacillus gasseri* is also isolated from breast milk (Martin *et al.*, 2007; Heikkila *et al.*, 2003), but the origin of the bacterium is not clear.

2.10. PRODUCTION OF BACTERIOCINS BY *LACTOBACILLUS* SPS.

The inhibitory activity of lactobacilli towards putrefactive organisms is thought to be partially due to the production of bacteriocins. Some of the bacteriocins isolated from lactobacilli are listed in Table 2.4:

Table 2.4. Example of some bacteriocins isolated from *Lactobacillus* species

Bacteriocins	Producing species	References
GassericinA	<i>L. gasseri</i> LA39	Kawai <i>et al.</i> , 1994
Gassericin T/ Gassericin K7 B	<i>Lactobacillus gasseri</i> LA158	Kawai <i>et al.</i> , 2000

Lactacin B	<i>L. acidophilus</i> NCFM	Barefoot and Klaenhammer 1983
Lactacin F	<i>L. acidophilus</i> 11088	Muriana and Klaenhammer, 1991
Acidocin B	<i>L. acidophilus</i> M46	Leer <i>et al.</i> , 1995
Reuterin	<i>L. reuteri</i> LA6	Toba <i>et al.</i> , 1991
Plantaricin A	<i>L. plantarum</i> C11	Daeschel <i>et al.</i> , 1990; Nissen-Meyer <i>et al.</i> , 1993; Diep <i>et al.</i> , 1994
Plantaricin F	<i>L. plantarum</i> BF001	Fricourt <i>et al.</i> , 1994
Helveticin J	<i>L. helveticus</i> 481	Joerger and Klaenhammer, 1990
Lactocin S	<i>Lactobacillus sake</i> L45	Mortvedt <i>et al.</i> , 1991

The bacteriocin from *L. gasseri* which is commonly found in human GI tract is expected to have a beneficial effect on human health. Gassericin A and reuterin 6 are circular peptides produced by *Lactobacillus gasseri* LA39 (Toba *et al.*, 1991) and *Lactobacillus reuteri* LA6 (Kawai *et al.*, 1994) respectively, which possess identical primary amino acid sequences deduced from their structural genes (Kawai *et al.*, 2004b). However, they differ in their circular dichroism (CD), inhibitory spectra as well as killing kinetics against selected indicator bacteria (Kawai *et al.*, 2004b). Gassericin A (5.6 kDa) has a wide antibacterial spectrum only against Gram positive bacteria, including *Lactobacillus* strains and some food pathogen or spoilage bacteria such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* (Kawai *et al.*, 1998b; Kawai *et al.*, 2001). Acidocin B (from *Lactobacillus acidophilus* M46), displays significant amino acid similarity to gassericin A (Kawai *et al.*, 1998a), although the physical properties of acidocin B remain to be confirmed.

Lactobacillus gasseri LA158 isolated from human-infant feces produce bacteriocin named gassericins T. Gassericin T has high heat stability (121°C, 10 min), good pH tolerance (pH 2–11), and strong bactericidal activity against many Gram-positive bacteria, especially lactic acid bacteria, and thus is expected to be effective food preservatives.

2.11. ANTIMICROBIAL SPECTRUM OF ACTIVITY

Based on the antimicrobial spectrum, molecular weight, stability, physical-chemical properties and mode of action of bacteriocins, the producing species form a heterogeneous group. There is the classical type, which has a spectrum of activity only against homologous species, and a second type, though less common, which shows action

against a wide range of Gram-positive microorganisms. One example of the first group is lactacin B, which is produced by some strains of *Lactobacillus acidophilus* (Barefoot and Klaenhammer, 1983). Other group comprises Acidocin CH5, produced by *L. acidophilus* CH5, which exerts wide inhibitory spectrum of activity against Gram positive bacteria and food spoilage bacteria, *Arthrobacter* sp., *Bacillus* sp. and *Corynebacterium* sp. (Chumchalova *et al.*, 1995). In case of circular bacteriocins, gassericin A produced by *L. gasseri* LA 39 shows a broad spectrum antibacterial activity against Gram-positive food-borne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*, but has no inhibitory activity against Gram-negative microorganisms such as *Salmonella typhimurium*, *S. enteritidis*, and *Escherichia coli* (Kawai *et al.*, 1998b). Circularin A, produced by *C. beijerinckii* ATCC 25752, has a wide spectrum of antimicrobial activity, inhibiting all *C. tyrobutyricum* strains and also lactococci, enterococci, and some *Lactobacillus* strains (Kemperman *et al.*, 2003). Acidocin B, a putative circular bacteriocin produced by *L. acidophilus* M46, combines the inhibition of *Clostridium sporogenes* with a very narrow activity spectrum within the genus *Lactobacillus* (Leer *et al.*, 1995). Reuterin 6, a narrow-spectrum bacteriocin produced by *L. reuteri* LA6 has 100% sequence similarity to gassericin A but differs in inhibitory spectra (Kabuki *et al.*, 1997). It shows narrow spectrum of activity against some Gram-positive organisms such as *B. cereus*.

Bacteriocins are not frequently active against Gram-negative bacteria. The outer membrane of this class of bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents and dyes from reaching the cytoplasmic membrane (Stevens *et al.*, 1991). However, some studies have reported bacteriocin activity against this group of bacteria. Examples are plantaricin 35d, produced by *Lactobacillus plantarum* and active against *Aeromonas hydrophila* (Messi *et al.*, 2001); bacteriocin ST151BR, produced by *Lactobacillus pentosus* ST151BR (Torodov and Dicks, 2004) and a bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* active against *Escherichia coli* (Caridi, 2002); Bacteriocins ST28MS and ST26MS, produced by *Lactobacillus plantarum* isolated from molasses (Torodov and Dicks, 2005) inhibited the growth of *Escherichia coli* and *Acinetobacter baumannii* along with some Grampositive bacteria. Lade *et al.*, (2006) have isolated two *Lactobacillus* species (*L. plantarum* and *L. lactis*) from vegetable waste that produced a bacteriocin which inhibited the growth of *E. coli*. Among the circular bacteriocins, only the enterococcal bacteriocin enterocin AS-48 stands

for its peculiar characteristics and broad-spectrum antimicrobial activity including Gram negative bacteria such as *Salmonella* and *E. coli* (Martinez-Bueno *et al.*, 1994; Mendoza *et al.*, 1999).

2.12. DETECTION OF BACTERIOCIN ACTIVITY

2.12.1. Agar Overlay Method

A common mean for screening bacteriocin activity is the use of agar media contained in petri plates. Mayr-Harting *et al.*, (1972), Piddock (1990) and Parente and Riccardi (1999) have reviewed a number of these methods for detection and measurement of bacteriocin activity. There are many variations, most of them derivatives of the “spot-on-lawn” approach (Uhlman *et al.*, 1992), which involve an agar overlay. Kekessy and Piguet (1970) described a procedure in which the producing and indicator strains were grown on different optimal media. The producing culture was spot-inoculated onto a spread plate, and after growth, the agar mass was aseptically dislodged with a spatula from the petri dish bottom and transferred to the lid of the dish. A soft agar overlay seeded with the indicator was then poured over the inverted agar. Following re-incubation, bacteriocin-positive cultures displaying a halo of clearing in the lawn around the original button of growth were considered to be positive for the production of bacteriocin. This assay minimizes the effects of acids and bacteriophage, since the producing and indicator strains are physically separated by an agar layer.

2.12.2. Flow Cell Detection

In a flow cell, Mugochi *et al.* (2001) developed a rapid and sensitive method for detection of bacteriocins in fermentation broth. Low concentrations of potassium ions were measured, so that released potassium ions from a bacteriocin-sensitive indicator strain directly correlated to concentrations of crude bacteriocin present in fermentation broth injected into the cell. This method compared very well to a conventional agar well diffusion assay.

2.12.3. PCR Methods

PCR methods have been used to detect genes responsible for bacteriocin production and regulation in bacterial cultures. The most common approach to determine the identity of new found bacteriocins has been random testing by PCR with primers targeting one or more suspect bacteriocins (Akcelik *et al.*, 2006; Reminger *et al.*, 1996; Rodriguez *et al.*, 1995). Knoll *et al.*, (2008) and Omar *et al.*, (2006) both employed the use of multiple bacteriocin-

related gene targets in characterizing lactic acid bacteria isolated from the malolactic fermentation of wines or from fermented maize, respectively. Both groups used PCR primers targeting many of the 20 or more bacteriocin related genes clustered in 5 different operons in *Lactobacillus plantarum*, a common isolate in the two types of fermentations they examined.

The use of PCR techniques can readily identify bacteriocin genes in bacteriocinogenic LAB. Yi et al. (2010) used a variation of 'colony PCR' to facilitate the screening of colonies for class IIa bacteriocin producing LAB. They used degenerate primers based on the conserved N-terminal regions found in many class IIa bacteriocins and specific downstream primers to design PCR reactions resulting in large amplicons specific for pediocin, enterocin, and plantaricin (Yi *et al.*, 2010). Wieckowicz et al. (2011) used a PCR assay with a custom panel of bacteriocin-related primers to screen metagenomic DNA preparations obtained from the microflora of Polish artisanal cheeses and found they were able to identify class IIa bacteriocin sequences in LAB as well as from non-LAB sources.

Macwana and Muriana (2012) subjected the DNA from Bac⁺ strains to a bacteriocin-specific PCR array in individual reactions with primers representing forty-two known structural genes of bacteriocins from LAB. Sequencing of the amplicons followed by sequence analysis helped to determine if the sequences had identity with others currently in GenBank or were unique sequences.

2.13. DETERMINATION OF SECONDARY STRUCTURE OF BACTERIOCIN: CIRCULAR DICHROISM (CD) SPECTROSCOPY.

Circular Dichroism (CD) spectroscopy is used to gain information about secondary structure of proteins and polypeptides in solution. The far-UV (ultraviolet) CD spectrum of proteins can reveal important characteristics of their secondary structure. CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha-helix conformation, the beta-sheet conformation, the beta-turn conformation, or some other (e.g. random coil) conformation (Whitmore *et al.*, 2008; Greenfield, 2006). These fractional assignments place important constraints on the possible secondary conformations that the protein can be in. CD cannot, in general, say where the alpha helices that are detected are located within the molecule or even completely predict their number. The near-UV CD spectrum

(>250 nm) of proteins provides information on the tertiary structure. The signals obtained in the 250–300 nm region are due to the absorption, dipole orientation and the nature of the surrounding environment of the phenylalanine, tyrosine, cysteine (or S-S disulfide bridges) and tryptophan amino acids. Unlike in far-UV CD, the near-UV CD spectrum cannot be assigned to any particular 3D structure. Rather, near-UV CD spectra provide structural information on the nature of the prosthetic groups in proteins, e.g., the heme groups in hemoglobin and cytochrome c.

Visible CD spectroscopy is a very powerful technique to study metal–protein interactions and can resolve individual d–d electronic transitions as separate bands. CD spectra in the visible light region are only produced when a metal ion is in a chiral environment, thus, free metal ions in solution are not detected. This has the advantage of only observing the protein-bound metal, so pH dependence and stoichiometries are readily obtained. Optical activity in transition metal ion complexes have been attributed to configurational, conformational and the vicinal effects.

Kawai *et al.*, 2003 recorded circular dichroism (CD) spectra for the purified gassericin A between 190 and 300 nm at room temperature by using a 1-mm-pathlength quartz cell on a JMS-700 spectropolarimeter. A positive maximum near 190, negative maxima at 208 and 222 nm, and Θ values indicated that the bacteriocin is peptide consisting mainly of α -helix structure.

2.14. MICROCIN J25: CIRCULAR BACTERIOCIN FROM GRAM NEGATIVE BACTERIA

Microcin J25 (MccJ25), isolated from *Escherichia coli* AY25 was the first smallest circular protein of bacterial origin (Salomó'n *et al.*, 1992). It was originally classified as a Class V bacteriocin (Pillardy *et al.*, 2001) based on a predicted circular nature (Blond *et al.*, 2001). It has been reported to contain a head-to-tail cyclized backbone based on enzyme cleavage data, sequencing, mass spectrometry, and NMR studies (Blond *et al.*, 1999). It is inhibitory against *Escherichia coli* and *Salmonella enterica* serovar Newport. Interestingly, the bioactivity of a thermolysin-linearized form of MccJ25 against *E. coli* strains is significantly reduced compared to the native form (Blond *et al.*, 1999). These findings suggest that the circular structure is probably more crucial for a specific protein-protein interaction than a nonspecific interaction with the bacterial membrane.

It was seen that a synthetic linear analogue of MccJ25 did not fold correctly and did not have antibacterial activity even though a thermolysin-linearized derivative of the native peptide retained some structure and activity.

2.15. GASSERICIN A: FIRST EXAMPLE OF CIRCULAR PROTEIN FROM LAB

Gassericin A was first described in 1991, under the name of reuterin 6 (Toba *et al.*, 1991). It is produced by lactic acid bacteria *Lactobacillus gasseri* LA 39, also known as *L. gasseri* JCM 11657. It carries a head-to-tail cyclization, comprises 58 amino acids with Isoluecine at the N - terminal and Alanine at the C- terminal, and has a mass of 5,652 Da (Kawai *et al.*, 1998b; Kawai *et al.*, 2001). It does not contain any modified residues. In addition to its antimicrobial activity against several *Lactobacillus* species, Gassericin A is also active against several foodborne pathogenic bacteria, including *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus* (Kawai *et al.*, 2001). It has a molecular size of 3.8 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which suggests it has a very compact and stable structure since heating for 60 min at 100°C did not cause any loss in its inhibitory activity (Toba *et al.*, 1991). More than 74% of the residues of Gassericin A are hydrophobic and most probably exposed on the surface of the peptide. It is slightly soluble in water; but solubility increases on adding acetonitrile or alcohol.

2.15.1. Production and Purification of Gassericin A

Gassericin A was produced from *Lactobacillus gasseri* LA39 by propagating it in MRS broth or DO-MRS broth (*i.e.* modified MRS broth in which Tween 80 and beef extract are replaced by oleic acid) (Kawai *et al.*, 1994). The bacteriocin activity of the cell supernatant was confirmed by agar well assay by taking *Lactobacillus delbrueckii subsp bulgaricus* JCM 1002 as the indicator strain. Gassericin A produced in MRS broth associated strongly with Tween 80 and several trials of dissociation were unsuccessful. It has been suggested that gassericin A gets absorbed with Tween 80 and becomes a large molecule like a complex polymer, although direct proof of complex formation is still not apparent. The production of gassericin A from DO-MRS broth resulted in 4500 fold increase in its specific activity. The DO-MRS broth was found to be superior to the MRS broth for producing purified gassericin A. Purified gassericin A was obtained by repetition of RP- chromatography with an octyl column by stepwise elution with water; acetonitrile and 90% 2- propanol.

2.15.2. Properties of the Purified Bacteriocin

The molecular weight of purified bacteriocin was determined by SDS-PAGE by the method of Laemmli *et al.*, by taking 4.5% spacer gel and 20% separating gel (Kawai *et al.*, 2003). After electrophoresis of 20mV for 2 hr, gassericin A was identified as a single band of 3800 Da holding bacteriocin activity. The bacteriocin was further tested for its pH stability (at 4 °C for 48 hr) and it was found that there was no decline in the activity level of gassericin A at pH values in the range from 2-12. The CD spectra of the bacteriocin recorded between 190 and 300 nm at room temperature on a spectropolarimeter revealed that the peptide is mainly composed of α -helices and is nearer the α/β type than $\alpha+\beta$ type. The N-terminal sequencing of HPLC-purified gassericin A did not advance, showing that the molecule has a circular structure linked at N-and C- terminal (Kawai *et al.*, 1998b).

For determination of the mode of action of the bacteriocin on indicator cell, the cell viability and lysis was tested on adding the crude bacteriocin to the indicator cell. The cell population was measured by plating anaerobically on MRS agar and the cell lysis was determined by measuring optical density at 650nm using spectrophotometer. The amount of ATP efflux from indicator cells was determined using ATP assay reagent kit based on the firefly luciferin-luciferase reaction. It was confirmed that no ATP efflux from indicator cells was caused by gassericin A; neither the bacteriocin caused lysis of the cell (Kawai *et al.*, 2004b). To quantify the potassium ion efflux from liposome, lipid vesicles composed of L- α - phosphatidylcholine were prepared and mixed with the purified bacteriocin taking 60% 2- propanol as control. The concentration of potassium ion in external solution was measured by using 692 pH/ion meter. The results indicated that the bacteriocin acts in the cytoplasmic membrane of the target cell and causes death of cell by efflux of potassium ions. The molecular mass of gassericinA analysed using FAB-MS gave the m/z value of $[M+H]^+ = 5651$, which corresponded to the theoretical value calculated from the amino acid sequence of mature circular gassericin A (Arakawa *et al.*, 2010).

2.15.3. Determination of Amino Acid Sequence of Gassericin A

Purified gassericin A migrated as a sharp band in SDS-PAGE with a molecular weight of 3,800 Da. Purified gassericin A was digested by lysylendopeptidase and 3-bromo-3-methyl-2-(2-nitrophenyl-mercapto)-3H-indole, which cleaves the tryptophanyl bonds, and the liberated peptides were sequenced by N-terminal protein sequencing (Kawai *et al.*, 1998b). Digestion of gassericin A with lysylendopeptidase made it possible to

sequence first 27 amino acids (LLDAMASGASLGTAFAAILGVTLPAWA) that contained a tryptophan residue that was located at the C terminal side of gassericin A. The peptide after digestion with lysylendopeptidase migrated as a large molecular component in a band of 5600 Da on SDS-PAGE. This difference in the movement was expected from a change in the cyclic structure to a rod form through enzymatic cleavage at the sole lysyl residue in its structure.

Digestion of gassericin A with MMNM-indole, cleaved the peptide into three bands. These bands were recovered from gel and their amino acid sequenced. Peptide A was composed of 15 amino acid (ALAAAGALGATAAIY) including C and N terminal amino acid moiety in structure which was observed through cloning of structural gene of gassericin A. Presence of bond between Isoleucine and alanine showed that gassericin A is a circular bacteriocin linked at N and C terminal. Band B had its first 25 amino acid sequenced (IADQFGIHLATGTARKLLDAMASGA) including cleavage site via lysylendopeptidase. Band C was considered to be a mixture of two rod form fragments from disruption at each tryptophan residue by MMNM-indole. All amino acid residues of gassericin A were identified in this way and the derived amino acid sequence was the same as that deduced from cloning of structural gene *gaaA*. The molecular weight, 5,652 by mass spectrometry, of gassericin A conformed to the weight expected after the elimination of H₂O from the deduced amino acid sequence. The bacteriocin did not contained any modified amino acid as lanthionine (Kawai *et al.*, 1998b).

2.15.4.Limited Proteolysis of Gassericin A

Three protease (V8 protease, trypsin and chymotrypsin) and two peptidase (arginolendopeptidase and lysylendopeptidase) in their optimal buffers were added to crude bacteriocin to carry out limited proteolysis at 37°C for 4-8 hrs (Kawai *et al.*, 2003). These digests were then subjected to SDS-PAGE and an *in situ* activity assay to detect changes in molecular mass and active intensity. Treatment with chymotrypsin which has five cleavage sites in the molecule resulted in complete loss of bacteriocin activity in agar well diffusion assay. Except for chymotrypsin, the protease tested did not reduce the bacteriocin activity, but trypsin and lysylendopeptidase changed the migration of active bacteriocin (20Leu-18Argor 19Lys) on SDS-PAGE from 3800 to 5600. Arginylendopeptidase did not hydrolyse the molecule. V8 protease cleaved the peptide at only one of the two predicted site (7Glu and 8Gln). These data suggest that the region of

residue 8-19 (theoretical cleavage site of trypsin and lysylendopeptidase) is important in the compact folding of the molecule as predicted in native gassericin A.

2.15.5. Structural Gene of Gassericin A

It was earlier reported that the structural gene of gassericin A (*gaaA*) (DDBJ, EMBL, and GenBank databases, accession number AB007043), were located on the chromosomal DNA of *Lactobacillus gasseri* LA39 (Kawai *et al.*, 1998a). Recently a 33,333-bp plasmid, designated as pLgLA39 has been reported in *L. gasseri* LA 39 that contained 44 open reading frames, including seven genes related to gassericin A production/immunity, as well as genes for replication, plasmid maintenance, and conjugative transfer (Ito *et al.*, 2009). This is opposite to the earlier report of localization of *gaaA* on the chromosome as an ORF encoding a protein of 91 amino acids together with two other ORFs, one of whose function was supposed to be encoding immunity protein (Leer *et al.*, 1995) and the other encoding a protein with putative ATP binding domain involved in the secretion of gassericin A. The plasmid pLgLA39 carries *tra* genes and was shown to conjugally transfer to *L. gasseri* JCM 1131 by filter mating. The conjugant revealed productivity of gassericin A as well as resistance to gassericin A (>30-fold-higher). *Lactobacillus reuteri* LA6, the producer of reuterin 6, was proved to harbor a plasmid indistinguishable from pLgLA39 and carrying seven genes 100% identical to *gaa*. This suggests that pLgLA39 might have been transferred naturally between *L. gasseri* LA39 and *L. reuteri* LA6. The seven *gaa* genes of pLgLA39 were cloned into a plasmid vector pSYE2 to construct pGAA. JCM 1131T transformed with pGAA expressed antibacterial activity and resistance to gassericin A. pGAA was segregationally more stable than a pGAA derivative plasmid from which *gaaA* was deleted and even was more stable than the vector. This suggests the occurrence of postsegregational host killing by the *gaa* genes (Kawai *et al.*, 2009).

2.15.6. Gassericin A and Reuterin 6

The primary amino acid sequence of gassericin A is 100% similar to Reuterin 6, another circular bacteriocin produced by *Lactobacillus reuteri* LA6 (Kawai *et al.*, 2001). Initially both bacteriocins were thought to be the same substance because they have the same structural genes and cannot be distinguished by their molecular weights (MWs) or primary amino-acid sequences (Toba *et al.*, 1991). The MWs of both reuterin 6 and gassericin A, determined by time-of-flight mass spectroscopy, are 5652. PCR amplification of the chromosomal DNA of *L. reuteri* LA6 by using primers that had been

designed on the basis of *gaaA* gene and sequencing revealed that *L. reuteri* LA6 has sequence *gaaA* with no variation between those primers (Kawai *et al.*, 2001). Both encode a 91 amino acid pro-prepeptide consisting of a 33 residue leader peptide that possibly mediates their targeting to and translocation across the bacterial cytoplasmic membrane, and a 58 amino acid mature peptide. They present more than 74% hydrophobic residues on their surfaces and are resistant to several peptidases and proteases, as are the other circular bacteriocins (Toba *et al.*, 1991). Their secondary structures are organized mainly in α -helices and both bacteriocins have been described as acting in the cytoplasmic membrane of target cells and causing the death of the cell via an efflux of potassium ions.

Gassericin A has a wide antibacterial spectrum only against Gram positive bacteria, including *Lactobacillus* strains and some food pathogen or spoilage bacteria (Kawai *et al.*, 1998b; Kawai *et al.*, 2001), whereas reutericin 6 has a spectrum of activity against Gram-positive, Gram-negative bacteria, Protozoa, Virus and Fungi. Interestingly, gassericin A inhibits the growth of *Lactobacillus reuteri* LA6, but reutericin 6, which has a narrower spectrum, does not inhibit the growth of *Lactobacillus gasseri* LA39, and in addition loses 50% of its activity at low pH values. The two bacteriocins also differ in their circular dichroism (*i.e* their spectra did not coincide), inhibitory spectra as well as killing kinetics against selected indicator bacteria (Kawai *et al.*, 2004b).

2.15.7. Differences between Gassericin A and Reutericin 6

To identify the basis for these phenotypic differences, partial composition analysis of the D- and L-amino acids of both peptides was performed (Kawai *et al.*, 2004b). It was reported that Gassericin A and reutericin 6 both contained D- and L-amino acids, a novel finding in itself, but in different D-Ala: L-Ala ratio. In the study, the only difference found between these bacteriocins was the presence of two (gassericin A) or one (reutericin 6) D-Ala residues in the molecule, which was expected to be the cause of the differences in their modes of action and secondary structures (Kawai *et al.*, 2004b). However, it was suggested, that the apparent difference in activity and CD spectra may be due to other structural differences. The two peptides could for instance differ with respect to the configuration (*trans* or *cis*) of the peptide bond on the N-terminal side of a proline residue present in both bacteriocins, as the configuration of such a peptide bond influences the structure and activity of antimicrobial peptides (Leuders *et al.*, 2005). Bacteriocins with the same primary structures produced by different bacterial species

have been reported for other lactic-acid bacteria (LAB) species such as *Pediococcus acidilactici* and *Lactobacillus plantarum* (pediocin AcH) (Motlagh *et al.*, 1992; Ennahar *et al.*, 1996) and *Lactobacillus curvatus* and *Lactobacillus sake* (curvacin A-sakacinA) (Axelsson and Holck, 1995), but the study by Kawai *et al.*, (2004b) was the first to report the different characteristics of identical native primary bacteriocins such as gassericin A and reuterin 6, conferred by a difference in D-Ala contents in the molecules, apparently arising from an L-Ala to D-Ala conversion. Also, it was observed that in case of heterologous expression of gassericin A and reuterin 6 in *Escherichia coli*, the specific activity of the non cyclic bacteriocin expressed with free N and C termini was weaker than those of native bacteriocins and no D-Ala residues were contained in the expressed molecule (Kawai *et al.*, 2003). This finding emphasized the need to analyse the D and L amino acid from microorganisms having same genes which may give interesting results about the difference in structure and function of the peptides.

Recently, it has been reported that purified gassericin A and reuterin 6 are chemically identical circular molecules containing no D-Ala residue (Arakawa *et al.*, 2010). On analysing the D/L-amino acid composition of the two bacteriocins using two distinct methods with chiral fluorescent derivatization reagents (+)-1-(9-fluorenyl) ethyl chloroformate and o- phthalaldehyde/N-acetyl-L-cystein it was revealed that neither Gassericin A nor Reuterin 6 contained D-Ala residues contrary to their previous findings. They showed that the production of and immunity to the bacteriocins (GassericinA and Reuterin 6) are encoded by seven genes (*gaa* and *reu* genes) identical to each other on two plasmids (LA39/LA6) (Ito *et al.*, 2009). Viewing the identical genes on the plasmid, the authors suggested that the difference in the D-Ala content of both bacteriocins may be dependent on the unidentified host factor(s); otherwise, it may be due to contamination because of incomplete purification (Arakawa *et al.*, 2010). The contaminating peaks, for example, may be small peptides containing D-Ala from peptidoglycan of the bacteriocin producing cells. The newly published data is contrary to their previous reports that GA and R6 had different characteristics inspite of the same primary structure (Kawai *et al.*, 2001) and the two bacteriocins had a structural difference because of the presence of D-Ala residue (Kawai *et al.*, 2004b). Mass analysis, N-terminal sequencing and bacteriocin assay of HPLC purified bacteriocins showed that the two bacteriocins had identical seamless circular structures with the same m/z value (5651) of [M+H]⁺ and both had the same specific activity. The reason for the difference in the production rate of the two bacteriocins reported by Kawai *et al.* (2001), is expected

to be caused by the differences of the growth of plasmid LA39 and LA6 and the copy number of pLgLA39 and pLrLA6. Otherwise, another possibility expected is the difference in transcription and/or translation efficiency of the seven genes in LA39 and LA6. Further analysis and characterization of the bacteriocin production from LA39 and LA6 are in progress (Arakawa *et al.*, 2010).

2.16. GASSERICIN T: A LINEAR VARIANT OF GASSERICIN A

Gassericin T is a broad spectral bacteriocin produced by *L. gasseri* SBT2055 isolated from human feces. It has a molecular weight of 5400 Da and composed of 57 amino acids. On cloning the chromosome DNA, six ORFs including structural gene for gassericin T were deduced through computer analysis and data bases (Kawai *et al.*, 2000). The structural gene of gassericin T (*gatA*) encodes a protein composed of 75 amino acid that included the GG motif of the cleavage site. Chemical sequence analysis of complete amino acid sequence shows that gassericin T has a disulfide bond in the molecule and no modified amino acid residues making it a class II hydrophobic bacteriocin. It has 60% sequence similarity to mature Laf A (57 amino acid, lactacin F, bacteriocin produced by *L. johnsonii* VPI11088) and the sequences around processing site and C terminal area are well conserved. Similar to gassericins A, gassericin T also has high heat stability (121°C, 10 min), good pH tolerance (pH 2–11), and strong bactericidal activity against many Gram-positive bacteria, especially lactic acid bacteria, and thus is expected to be an effective food preservative.

2.17. APPLICATION OF GASSERICIN A IN FOOD SYSTEM AND HEALTH

2.17.1. Inhibition of Food-Borne Pathogenic Bacteria

Several strains of *L. gasseri* shows a wide inhibitory spectrum against food-borne pathogens such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. The action, however, is limited only to Gram-positive bacteria. Bacteriocins produced by many species of *Lactococcus* and *Pediococcus* inhibit the growth of food-borne enteric pathogens (Davidson and Hoover 1993); however, only a few strains of *Lactobacillus* produce such bacteriocins (Schillinger *et al.*, 1991). Gassericin A from *L. gasseri* LA 39 is the first bacteriocin of *L. acidophilus* group which exhibits bactericidal activity against enteric pathogens (Itoh *et al.*, 1995).

2.17.2. Antibacterial Activities of Gasserins (A and T) in Combination with Glycine against Spoilage Bacteria (Hurdle Technology)

Generally, LAB bacteriocins have strong activity against closely related LAB strains, but no activity against Gram-negative bacteria except for some such as enterocin AS-48 (Abriouel *et al.*, 2003), whereas glycine is generally effective against not only Gram-positive species but also Gram-negative species at neutral pH, but has a very weak activity against LAB that inhabit an acidic condition (Tsutsumi and Ohotaka, 1985). The mixed solutions of gasserins and glycine inhibit the growth of all the tested bacterial strains which indicates their effective combination by compensating for each other's lack of active range. The potential for food preservation of Gassericin A and Gassericin T, produced by probiotic *L. gasseri* LA39 and LA158 was examined using the microwell plate method against the 12 bacterial strains isolated from putrid custard cream. In the microwell plate method, Gassericin A and Gassericin T solutions had broader antibacterial spectra against the tested Gram-positive isolates than nisin A solutions with equivalent activities against *Lb. delbrueckii* ssp. *bulgaricus* JCM1002. However, gasserins, were ineffective for the inhibition of the growth of the Gram-negative bacteria. Glycine had a durable antibacterial activity against *Bacillus* spp., *Staphylococcus* spp., and the Gram-negative strains, but had a comparatively weak activity against LAB strains, In the case where the gasserins and 1.0% (wt/vol) glycine were used in combination, the growth of all the spoilage bacterial isolates was inhibited. This combination can be used as hurdle technology to control the overall growth of many food spoilage bacteria (Arakawa *et al.*, 2009).

2.17.3. Gassericin A in Treatment of Mastitis

Gassericin A prevented the growth of wild type of toxic *Staphylococcus aureus* isolated from mastitis milk and breast organs and also is quite useful for medical treatment of mastitis without any inflammation on cow (Kawai *et al.*, 2004a; Arakawa *et al.*, 2008).

2.18. EPILOGUE

Although intensive studies over the last decade have greatly advanced our knowledge base about circular bacteriocins, further work is needed before we are able to fully understand the molecular mechanisms, structure-function relationships, and mechanisms of action of these bacteriocins. Despite present progress in defining the biology of circular bacteriocins, the understanding of the enzymatic mechanisms involved in the circularization processes is still incomplete and lacking experimental

evidence. Nonetheless, considerable attention is being directed towards identifying the posttranscriptional machinery and processing steps involved in the reaction(s). The function of the leader peptides, the method of leader cleavage and the steps involved in the circularization of the peptide remain unknown. Research in these areas is critical for the effective applications of circular bacteriocins and would help develop methods to genetically engineer circular bacteriocins with better activity, solubility, and stability.

Although many circular bacteriocins have been isolated and characterized, only carnocyclin A has been approved for use in food products by the US FDA. Since circular bacteriocins that have been used as food preservatives have relatively narrow activity spectra and are generally not active against Gram-negative bacteria (except AS-48), it can be expected that other antimicrobial agents will continue to be incorporated and developed into hurdle concept technologies for food preservation. The simultaneous application of gasserins and glycine to improve shelf life of foods is attractive since foods usually have better sensory and nutritional qualities compared with products produced using conventional thermal processing methods. As *L. gasseri* is present as a natural inhabitant of the human intestine, the bacteriocin and the producer strain, as a probiotic, may be used safely and to the benefit of human health.

Chapter 3

Materials and Methods

3. MATERIALS AND METHODS

The composition of all the media, buffers and reagents used in this study are given in Appendix I, II and III. The media and AR or reagent grade chemicals and reagents, used for whole of the study (except molecular biology work), were procured from different manufacturers like HiMedia (Mumbai), SRL (Mumbai) and Qualigens (Mumbai), while Molecular Biology Grade chemicals were purchased from Bangalore Genei (Bangalore) and Sigma-Aldrich (CA, USA).

3.1. STANDARD CULTURES

The standard bacterial cultures used in this study are listed in Table 3.1. The standard cultures of *Lactobacillus gasseri* ATCC 19992 and *Lactobacillus gasseri* JCM 11657 were procured from American Type Culture Collection (ATCC), and Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Japan respectively. *Enterococcus faecalis* NCDC 114 used as an indicator culture was obtained from National Collection of Dairy Cultures, Karnal, Haryana. The standard cultures of lactic as well as other non-lactic acid bacteria used in this study were obtained from National Collection of Dairy Cultures (NCDC), NDRI, Karnal.

All the bacterial cultures were propagated twice prior to use by sub culturing in their respective broths (Table 3.1.) with the composition as given in Appendix I. All the cultures used for study were maintained in their respective broths at 4°C and sub-cultured after every 7-15 d interval.

3.2. ISOLATION OF *LACTOBACILLUS* CULTURES

3.2.1. Collection of Samples

A total number 70 samples comprising of 50 infant feces, 10 human milk and 10 adult human saliva samples were collected from Government Civil Hospital, Karnal; rural and urban areas in Karnal and also from other places (Delhi, Panipat and Rewari). Samples were collected in sterile sample bottles.

3.2.2. Culture Medium

The deMann Rogosa Sharpe Medium (MRS) agar (de Mann *et al.*, 1960) supplemented with bromo cresol purple dye @ 0.004% as an indicator was used for the isolation of *Lactobacillus* from different samples. Besides this TGE medium (agar/soft agar) (Biswas *et al.*, 1991) was used for the detection of bacteriocin production from isolates.

3.2.3. Procedure for Isolation of *Lactobacillus* from Fecal Samples

Serial dilutions of the samples (1 gm/ml) were prepared in sterile normal saline. Appropriate dilutions of the samples were plated out on the MRS medium. Plates were incubated at 37°C for 24-48 h. Typical cream coloured well isolated colonies from MRS agar were picked up randomly and transferred to MRS broth (de Mann *et al.*, 1960). On subsequent streaking on MRS supplemented with 0.004% BCP, colonies with yellow zone were selected and transferred again to MRS broth. After growth at 37°C for 24-36 h, the cultures were tested for purity and morphology by microscopic examination.

3.2.4. Maintenance of Cultures

All the preliminarily screened isolates were maintained in MRS at 4°C and sub-cultured once in a week. Also a stock culture of all the isolates was preserved in 15% glycerol stock medium (Appendix I) at -20°C. The cultures were activated prior to use by subculturing twice in MRS broth.

Table 3.1. Bacterial species and strains used in the study

Bacterial Species	Strains and Source	Culture conditions
GRAM POSITIVE		
<i>Lactobacillus gasseri</i>	ATCC 19992, USA	37°C, MRS
<i>Lactobacillus gasseri</i>	JCM 11657, Japan	37°C, MRS
<i>Lactobacillus delbrueckii subsp. Bulgaricus</i>	NCDC 4, Karnal	37°C, MRS
<i>Lactococcus lactis subsp. cremoris</i>	NCDC 86, Karnal	30°C, M17
<i>Lactococcus lactis subsp. lactis</i>	NCDC 278, Karnal	30°C, M17
<i>Lactobacillus casei subsp. casei</i>	NCDC 017, Karnal	37°C, MRS

<i>Lactobacillus brevis</i>	NCDC 001, Karnal	37°C, MRS
<i>Lactobacillus acidophilus</i>	NCDC 291, Karnal	37°C, MRS
<i>Lactococcus cremoris</i>	NCDC 060, Karnal	37°C, MRS
<i>Lactobacillus helveticus</i>	NCDC 005, Karnal	37°C, MRS
<i>Lactobacillus fermentum</i>	NCDC 156, Karnal	37°C, MRS
<i>Lactobacillus rhamnosus</i>	NCDC 018, Karnal	37°C, MRS
<i>Lactobacillus plantarum</i>	NCDC 020, Karnal	37°C, MRS
<i>Lactobacillus paracasei</i>	NCDC 063, Karnal	37°C, MRS
<i>Streptococcus thermophilus</i>	NCDC 074, Karnal	37°C, M17
<i>Streptococcus thermophilus</i>	NCDC 117, Karnal	37°C, M17
<i>Streptococcus thermophilus</i>	NCDC 80, Karnal	37°C, M17
<i>Pediococcus acidilactici</i>	LB-42, USA	37°C, MRS
<i>Enterococcus faecalis</i>	NCDC 114, Karnal	37°C, MRS
<i>Enterococcus faecalis</i>	NCDC 115, Karnal	37°C, MRS
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	37°C, BHI
<i>Listeria monocytogenes</i>	ATCC 15303, USA	37°C, BHI
<i>Micrococcus luteus</i>	NCDC 131, Karnal	37°C, M17
NON-LACTIC BACTERIA		
<i>Escherichia coli</i>	ATCC 25922, USA	37°C, BHI
<i>Staphylococcus aureus</i>	NCDC 273, Karnal	37°C, BHI
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	37°C, BHI
<i>Klebsiella pneumonia</i>	NCDC 138, Karnal	37°C, BHI
<i>Bacillus cereus</i>	NCDC 66, Karnal	37°C, NA
<i>Bacillus subtilis</i>	NCDC 70, Karnal	37°C, NA
<i>Salmonella typhi</i>	NCDC 113, Karnal	37°C, BHI
<i>Shigella dysenteriae</i>	NCDC 107, Karnal	37°C, BHI
<i>Escherichia coli</i>	NCDC 134, Karnal	37°C, BHI
<i>Pseudomonas aeruginosa</i>	NCDC 104, Karnal	37°C, BHI

YEAST		
<i>Rhodotorula glutinis</i>	NCDC 51, Karnal	25°C YPD
<i>Saccharomyces cerevisiae</i>	NCDC 47, Karnal	25°C YPD
<i>Torulopsis candida</i>	NCDC 43, Karnal	25°C YPD
<i>Candidia guilliermondii</i>	NCDC 44, Karnal	25°C YPD
<i>Saccharomyces cerevisiae</i>	NCDC 50, Karnal	25°C YPD

3.3. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

An array of physiological and biochemical tests was performed as per standard methods (Ahmed *et al.*, 2004) to identify the *Lactobacillus* isolates. The composition of media and reagents used are given in Appendix I. Pure cultures were grown in MRS broth at 37°C for 24 h. The activated culture was used as an inoculum for the various physiological and biochemical tests employed to identify the isolated cultures. An inoculum rate of 1.0% was used unless otherwise stated.

3.3.1. Morphology

The morphological characteristics *i.e.* shape, and arrangement of individual organisms were determined by standard Gram staining procedure of heat fixed smears of activated presumptively screened cultures. Positive and negative staining was also performed to confirm the shape of the isolates.

3.3.2. Catalase Test

The catalase test was performed by the slide method. Using an inoculating needle, culture from a well-isolated colony was placed on a clean glass slide. A drop of 3% H₂O₂ solution was added on to this culture and closely observed for the effervescence, indicating positive test.

3.3.3. Growth at 15°C and 45°C

MRS broth tubes were inoculated with the active cultures and incubated at 15°C and 45°C for 3 d. Growth was estimated from the turbidity in the culture broths.

3.3.4. Arginine Hydrolysis

An inoculum from a pure culture is transferred aseptically to a sterile tube of arginine dihydrolase broth (HiMedia). The inoculated tube is incubated at 35-37 °C for 24 hours and the preliminary results are determined. The microbe must first use the glucose present to cause the *pH* to drop. This is indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme arginine dihydrolase is activated. The culture is incubated an additional 24 hours at 35-37 °C to allow the microbe to now use the arginine. The final results are then obtained by observing the tube at 48 hours. Change back to purple from yellow indicates a positive test for arginine dihydrolase. Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicates a negative result.

3.3.5. Bile-aesculin Hydrolysis Test

Bile-aesculin agar (Composition in Appendix I) was prepared by the method of Facklam and Moody (1970). Five microlitres of each of the test cultures were spotted on overnight dried bile-aesculin agar plates and plates were incubated at 37°C for 3 d. Appearance of black zone of hydrolysis around the spots was considered positive.

3.3.6. Carbohydrate Fermentation

Tentative identification of *Lactobacillus* isolates was done on the basis of sugar fermentation pattern. The ability of the cultures to ferment and produce acid from various sugars was tested in a CHL fermentation broth lacking sugar (Composition in Appendix I) containing 0.004% BCP as pH indicator using different carbohydrate (sugar) discs (HiMedia Laboratories Ltd., Mumbai). Carbohydrates tested were: Arabinose, cellobiose, mannose, ribose, salicin, rhamnose, sorbitol, trehalose mannitol and melibiose. The CHL broth tubes with different carbohydrate discs (Hi Media Laboratories Ltd., Mumbai) were inoculated with 30 µl of active cultures and incubated at 37°C and examined up to 3 d. The change of colour from purple to yellow indicated the fermentation of the carbohydrate with acid production.

3.4. PHYLOGENETIC AND GENOTYPIC CHARACTERIZATION

All the biochemically identified *Lactobacillus* isolates were subjected to molecular characterization for definite confirmation of genus and species. The composition of all the reagents used in this study is given in Appendix II.

3.4.1. Isolation of Genomic DNA

All the cultures used in this study along with standard *L. gasseri* culture ATCC 19992 and JCM 11657 were grown for 18 to 20 h in MRS broth at 37°C and the extraction of their genomic DNA was done as described Pospiech and Neumann (1995). The procedure in brief is as follows:

- The cells were harvested from 1.5 ml of overnight grown culture in a microcentrifuge tube by centrifugation in a refrigerated centrifuge (Sigma-Aldrich, St. Louis, Missouri, USA) at 3000 rpm for 10 min.
- The supernatant was discarded carefully without disturbing the pellet and the pellet was resuspended in 0.5 ml of SET buffer (pH 7.5).
- Lysozyme was added to the above cell suspension at a concentration of 1 mg ml⁻¹ and incubated at 37°C /1 h.
- This was followed by the addition of 1/10th (one- tenth) volume of 10% SDS and further incubation at 37°C with occasional inversion for 30 min.
- After this one third volume of 5 M NaCl and one volume of chloroform: iso- amyl alcohol (24:1) were then added to the above mixture and incubated at room temperature for 30 min with frequent inversions for 30 min.
- The samples were centrifuged at 4,000 rpm for 15 min and the aqueous phase was transferred to a new tube.
- The DNA was precipitated by adding one and half volume of isopropanol and the tubes were inverted gently to allow proper mixing and kept for 30 min at -20°C or kept at -20°C for overnight.
- The DNA pellet thus obtained was recovered after centrifugation at 12,000 rpm for 10 min at 4°C in a refrigerated centrifuge.
- The pellet was washed with 70% ethanol and dried for 10 min and finally dissolved in 50 µl of TE buffer (pH 8.0).
- The extracted genomic DNA along with tracking dye was run on 1% agarose in gel electrophoresis unit (Mini submarine, Hoeffer, USA).

3.4.2. Agarose Gel Electrophoresis

Agarose gels (1% concentration) were prepared by dissolving the appropriate quantity of agarose in 1 X TAE buffer (pH 8.0) (Appendix II) in a microwave oven followed by addition of ethidium bromide stock solution (Appendix II) at the rate of $0.5 \mu\text{g ml}^{-1}$ before casting the gel.

Molten agarose was cooled to 50°C and poured into respective mould of minigel using appropriate comb. The surface was leveled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel plate was mounted on respective horizontal electrophoresis tank (Mini submarine, Hoeffer, USA). The electrophoresis tank was filled with 1 X TAE electrophoresis buffer to cover the gel to a depth of about 1 mm. The DNA samples were mixed with $5 \mu\text{l}$ of tracking dye and were loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 100 V (60 mA current) for one hour in mini gel electrophoresis apparatus. After completion of electrophoresis, the gels were taken out of the chamber and examined using UV transilluminator (Foto/UV-21, Fotodyne Inc., USA) and the gels were subsequently photographed by Bio RadGel Doc system (Bio-Rad, Hercules, CA, USA).

3.4.3. Determination of DNA Concentration by Spectrophotometric Analysis

The concentration of DNA samples was determined by the measurement of optical density (OD) in a UV spectrophotometer (DU 640, Beckman, USA) at 260 and 280 nm. The purity of DNA was ascertained by measuring as ratios at 260 to 280 nm. The concentration and quality of DNA was calculated by the following equivalents as suggested by Sambrook *et al.*, (1989).

$$\begin{aligned} \text{DNA mg ml}^{-1} &= A_{260} \times \text{dilution} \times 50.0 \\ A_{260} &= \text{Absorbance as OD at 260 nm.} \\ \text{OD (=1)} &= 50 \mu\text{g ml}^{-1} \text{ (double stranded DNA) or } 30 \mu\text{g ml}^{-1} \\ &\quad \text{(Single stranded DNA)} \end{aligned}$$

3.4.4. Identification of Isolates by Polymerase Chain Reaction (PCR)

3.4.4.1. Primers

The identity of genus of the isolates as *Lactobacillus* was confirmed by PCR using genus specific primers (Dubernet *et al.*, 2002). Also, oligonucleotide primers targeted against strongly conserved sequence of *L. gasseri* were selected from the published literature (Song *et al.*, 2000). All the primers were

custom synthesized from Bangalore Genei Pvt. Ltd., Bangalore. The sequences of the primer pairs along with the target genes used in the present investigation and the size of the PCR amplified products are given in Table 3.2.

Table 3.2. Primer pairs used for the identification of genus *Lactobacillus* and species *Lactobacillus gasseri*

Genus	Target gene	Primer	Sequence	Expected Size
<i>Lactobacillus</i>	16S	(F) (Lb1)	5'-AGA GTT TGA TCA TGG CTC AG -3'	250bp
	rRNA	(R) (Lb2)	5'-CGG TAT TAG CAT CTG TTT CC-3	
Species	Target	Primer	Sequence	Expected Size
<i>L. gasseri</i>	16S-	F(Lgas 2)	5'-TGC TAT CGC TTC AAG TGC TT-3'	360bp
	23S rRNA	R(Lgas 3)	5'-AGC GAC CGA GAA GAG AGA GA-3'	

3.4.4.2. PCR with selected primers

Before setting up of PCR, all the reagents were thawed (except Taq DNA polymerase), mixed and spinned. Reagents were always kept on ice bath during the period of setting up of the reaction. The reaction mixture comprising 10 X PCR buffer (containing MgCl₂), dNTPs and primers was prepared and distributed to reaction tubes according to the requirements shown below (Table 3.3). The final volume of PCR mix was adjusted to 25µl. All the reagents except template DNA were added to the master mix, and then it was given a short spin, and distributed equally into the PCR tubes. The template DNA was added just prior to transferring the tubes into the thermal cycler. PCR tubes were given a final spin again and at once transferred to the heating block of the thermo cycler (Eppendorf Master Cycler, Germany).

Table 3.3. Details of the PCR mix used in the study

Reagents	Concentration	Volume (µl)
Sterile nuclease free deionized	--	Variable

water		
10X Reaction buffer with MgCl ₂ (15 mM)	1X	10
dNTP mix (2.5 mM)	0.2 mM	0.8
Primer forward	0.1-0.5 µM	1.0
Primer reverse	0.1-0.5 µM	1.0
Taq DNA polymerase	0.03 – 0.05 U µl ⁻¹	1.2
Template DNA	200 pg µl ⁻¹	2

3.4.4.3. PCR cycling steps

3.4.4.3.1. *For Genus specific primers*

The PCR programme was the same as given by Dubernet *et al.*, (2002). It comprised one cycle of initial denaturation (5 min at 95°C), followed by 30 cycles each of denaturation (30 s at 95°C), primer annealing (30 s at 55°C) and extension (30 s at 72°C) followed by one cycle of final extension (7 min at 72°C). After the run was over, the amplified PCR products were held at 4°C until further use.

3.4.4.3.2. *For Species specific Primer*

The PCR programme was the same as given by Song *et al.*, (2000). It comprised one cycle of initial denaturation (1 min at 95°C), followed by 35 cycles each of denaturation (20 s at 95°C), primer annealing (2 min at 65°C) and extension (2 min at 55°C) followed by one cycle of final extension (5 min at 74°C). After the run was over, the amplified PCR products were held at 4°C until further use.

3.4.4.4. Analysis of PCR products by agarose gel electrophoresis

PCR amplified products obtained with different templates were electrophoresed on the agarose gels (1.0-2.0%) by following the standard procedure as given by Sambrook *et al.*, (1989). Agarose gel (1 - 2 % concentration) was prepared by dissolving the appropriate quantity of agarose in 1 X TAE buffer (pH 8.0) as described under section 3.4.2. The DNA size markers used were produced by Bangalore Genei (Banglore). Electrophoresis was carried out at 100 V (60 mA current) for one hour in mini gel electrophoresis apparatus. After completion of electrophoresis, the gels were taken out of the chamber and examined under UV transilluminator ((Foto/UV-21, Fotodyne Inc., USA) and photographed using BioRad Gel Doc system (Bio-rad, Hercules, CA, USA).

3.5. SCREENING OF *LACTOBACILLUS GASSERI* CULTURES FOR ANTIBACTERIAL ACTIVITY

One hundred and forty six *Lactobacillus gasseri* cultures isolated in this study were screened for their antibacterial activity against indicator strains- *Enterococcus faecalis* NCDC 114, *Pediococcus acidilactici* LB42, *Lactobacillus delbrueckii subsp .bulgaricus* NCDC 4 and *Escherichia coli* NCDC 134 by agar-spot assay (Fleming *et al.*, 1975) and were further confirmed by spot-on-lawn assay (Uhlman *et al.*, 1992).

3.5.1. Spot-On-Lawn Assay (Uhlman *et al.*, 1992)

3.5.1.1. Cell free culture supernatant (CFCS)

Active *L. gasseri* cultures were grown in MRS broth for 16-18 h at 37°C. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4°C). The culture supernatants thus obtained were heat treated (90°C, 5-7 min) to kill any live cell. The cell-free supernatant was adjusted to pH 6.5 using 1N NaOH and was used as CFCS.

3.5.1.2. Assay

A fresh culture of indicator bacteria grown for 16-18 h was further inoculated for its active growth at optimum temperature for 4 h. Thirty microlitres of this culture was mixed with 7 ml of melted and cooled (45°C) TGE soft agar (Composition given in Appendix I) and poured onto the solidified and dried (one day at 37°C) TGE agar plates. The soft agar was allowed to solidify and 5 µl of CFCS was directly placed on the lawns of indicator organism. A clear zone of 1 mm or more was considered positive inhibition.

3.5.2. Agar-Spot Assay (Fleming *et al.*, 1975)

L. gasseri cultures for spot-inoculation of agar surfaces were activated in MRS broth at 37°C by three consecutive transfers at 24 h interval. Indicator strains for seeding the soft agar for overlay were also grown in appropriate broths by three successive transfers at 24 h intervals at their optimum growth temperatures. All the cultures were used undiluted in the assay.

The surface of the solidified and dried (one day at 37°C) TGE agar (1.5% agar) plates was spot inoculated with 5 µl of *L. gasseri* cultures. The inoculated agar

dishes were incubated at 37°C for 18-24 h and then overlaid with 5 ml of TGE soft agar (0.8% agar) seeded with 30 µl of indicator culture. A clear zone of 2 mm or greater extending laterally from the border of the *Lactobacillus* colonies after incubation for one day at optimum temperature of indicator strains was recorded as positive inhibition.

3.5.3. Agar Well Assay (Barefoot & Klaenhammer, 1983)

Overnight grown culture of the target organism was inoculated into the TGE broth. After incubation at 37°C for 4 h, thirty microlitres of this culture was mixed with 7 ml of melted and cooled (45°C) soft agar and poured into a Petri dish containing 20-25 ml of agar medium. The soft agar was allowed to solidify and the plates were kept at 5°C for one hour before wells of 6.0 mm diameter were punched out of the agar with the help of glass borer. The agar buttons were removed and the base of the well was sealed using melted TGE agar. Wells were filled with 100 µl of CFCS (3.5.1.1.). The plates were once again kept in a refrigerator (5°C) for 3-4 h to facilitate the diffusion of antibacterial compounds and were then incubated at appropriate temperature for 18 h. The diameter of zone of inhibition extending laterally from the edge of wells was recorded.

3.6. MEASUREMENT OF ACTIVITY UNITS (AU)

Five microlitres of serial dilutions (2, 4, 8, 16...) of the CFCS (3.5.1.1.) were used to determine the highest dilution that produced a 1 mm zone of growth inhibition using the spot-on-lawn assay (3.5.1.). The activity units of the culture broth were calculated using the following formula and expressed as activity units per ml:

$$\text{Activity Units per ml (AU/ml)} = 200 \times \text{Reciprocal of highest dilution that gave a clear zone}$$

3.7. IDENTIFICATION OF THE BACTERIOCIN GENE (*gaaA*) BY PCR AMPLIFICATION

Lactobacillus gasseri isolates tested positive for antibacterial activity were screened for circular bacteriocin (gassericin A) producing gene, *gaaA* through PCR with two primers. One of the primer was selected from the published literature (Majhenic *et al.*, 2003), while the other was designed in our

laboratory by using the submitted sequence of *gaaA* gene of *L. gasseri* LA39 from EMBL (European Molecular Biology Laboratory, Nucleotide Sequence Database, European Bioinformatics Institute). The sequence of the primer pair used in the present investigation and the size of PCR amplified products is given in Table.3.4.

Table 3.4. Primer pairs used for *gaaA* gene

S.No.	Target gene	Primer	Sequence	Expected Size
1.	<i>gaaA</i>	gaA515/ gaA61	F- 5'-GAC CAC AGC GAA CAT T-3' R- 5'-AAT GAG GCA CCA GAA G-3'	102 bp
2.	<i>gaaA</i>	gaA2F/ gaA2R	F 5'-GGGCGGTTTTAGTACTTGCT-3' R: 5'AAGCCCATGCAGGTAATGTC-	180bp

3.7.1. PCR Cycling Steps

3.7.1.1. Using primer pair gaA515/gaA61

The PCR programme was the same as given by Majhenic *et al.*, 2003. It comprised initial denaturation at 95°C for 3 min and 35 cycles each of denaturation at 94°C for 1 min followed by annealing at 45°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. After the run was over, the amplified PCR products were held at 4°C until further use.

3. 7.1.2. Using primer pair gaA2F/gaA2R

The PCR cycling steps comprised initial denaturation at 95°C for 3 min and 35 cycles each of denaturation at 94°C for 1 min followed by annealing at 61°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. After the run was over, the amplified PCR products were held at 4°C until further use.

3.7.1.3. Analysis of PCR products by agarose gel electrophoresis

The PCR products of *gaaA* gene were analysed by agarose gel electrophoresis as mentioned in section 3.4.4.4.

3.7.2. PCR Product Purification

The purification of PCR products was carried out by adding 440 µl of 100% alcohol in 180 µl of pooled PCR product. The mixture was kept in ice for half an hour and then was spun at 12,000 rpm for 15 min under refrigerated conditions (4°C). Alcohol was drained carefully and pellet was washed with 70% ethanol (300 µl) and spun at 12,000 rpm for 5 min under refrigerated conditions (4°C). Alcohol was drained and pellet was dried at 37°C for 10 min and stored at -20°C

3.8. SEQUENCING OF AMPLIFIED PCR GENE PRODUCT

The PCR amplification products of 16S-23S rRNA gene and *gaaA* gene of *L. gasseri* NBL18 were got sequenced by Automated DNA Sequencing Services provided by GCC Biotech (India) Pvt. Ltd., New Delhi following the Sanger's Dideoxy chain termination methodology in ABI Prism (Model 3100, Version 3.7).

3.8.1. DNA Sequence Similarity Analysis

Sequences were submitted to the National Centre for Biotechnology Information (Bethesda, Md. USA) for similarity searches in GenBank. The computer program CLUSTAL W (1.82) multiple sequence alignment editor (<http://www.ebi.ac.uk/clustalw/index.html/>) was used for sequence alignment and Nucleotide sequence similarity searches were performed using BLAST tools available through the National Institutes of Health Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) to determine the gene from which each amplicon was most likely derived.

3.9. ANTIBACTERIAL SPECTRUM OF ACTIVITY

Inhibitory spectra of cell free culture supernatants, neutralized to pH 6.5, were determined by using the spot-on-lawn assay against a broad range of Gram-positive and Gram-negative strains (Table 3.1.). Seven millilitres aliquots of TGE soft agar were inoculated with 30 µl of 3-4 h old cultures of the indicator strains (absorbance at 660 nm = 0.01) and poured into a Petridish. Five microlitres of the cell free supernatants were spotted on the cooled TGE agar plates. The plates were kept undisturbed for 2 h and subsequently incubated at 37°C. After 24 h, the diameters (mm) of the growth inhibition zones were measured.

3.10. OPTIMIZATION OF THE CULTIVATION CONDITIONS FOR MAXIMAL BACTERIOICIN PRODUCTION BY *LACTOBACILLUS GASSERI* NBL 18 USING RESPONSE SURFACE METHODOLOGY

3.10.1. Experimental Design

Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 1997). The central composite rotatable design (CCRD), one of the most important experimental designs used in process optimization studies was applied in this study with the objective to develop an empirical model of the process and to obtain a more precise estimate of the optimum operating conditions for the factors involved. To describe the nature of the response surface in the optimum region, a four factor (five levels at each factor) second order central composite rotatable design (CCRD) was adopted. The independent factors viz: pH (A), Incubation temperature (B), Inoculation level (C) and Incubation Time (D) were considered for optimization of processing variables for pH, OD and bacteriocin production. The selected range for the variables was 4-8 for pH, 37-42°C for incubation temperature, 1-3% inoculum level and 6-24 h of incubation period. For the four factors, the CCRD design constituted of 30 experiments as shown in the table.3.5. This design was made up of 2^4 factorial design, six replications of the center points and the eight axial design. The axial distance α was chosen to be 1.68 to make this design rotatable. A center point is a point in which all variables are set at their mid value. Six center experiments were included in factorial designs as repetition so as to minimize the risk of missing non-linear relationships in the middle of the intervals, and also for the determination of confidence intervals. The response function (Y) were pH, OD and bacteriocin produced (AU/ml). These responses were related with the coded factors by a second –degree polynomial equation Eq. (1) using the least square method.

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD + \varepsilon \quad (1)$$

The coefficient of the polynomials were represented by b_0 (constant terms), b_1, b_2, b_3, b_4 (linear terms), $b_{11}, b_{22}, b_{33}, b_{44}$ (quadratic terms), $b_{12}, b_{23}, b_{33}, b_{14}, b_{24}$

b_{34} (interactive terms) and ε (random error). Thus the optimization of bacteriocin production was achieved using a central composite design and surface modelling method.

Adequacy of the model was evaluated using F ratio, model was considered adequate when F-calculated was more than table-F. The analysis of variance (ANOVA) tables were generated and the effect of variables at linear, quadratic and interactive level on individual response was described using significance at 1 and 5% levels of confidence. The magnitude and sign of coefficients in the model indicated the effects of variables on response. The magnitude of coefficient described the extent of dependency of variables on increasing or decreasing the response depending on positive or negative sign of coefficient terms. In the case of negative interaction, the level of one factor could be increased while decreasing the level of other variable. All negative coefficients of quadratic terms indicate maximum response at stationary point, all positive coefficients of quadratic terms indicate minimum response at origin of stationary point, whereas mixed sign of quadratic terms indicate mini-max response (middle point) at origin of stationary point.

Table. 3.5. Coded and actual values of variables in RSM experiment

Level	Coded				Actual			
	Factor 1	Factor 2	Factor 3	Factor 4	pH	Incubation temperature	Inoculum level	Incubation time
-2.0	-2.0	-2.0	-2.0	-2.0	2	34.5	0	3
-1.0	-1.0	-1.0	-1.0	-1.0	4	37	1	6
0	0	0	0	0	6	39.5	2	15
+1.0	+1.0	+1.0	+1.0	+1.0	8	42	3	24
+2.0	+2.0	+2.0	+2.0	+2.0	10	44.5	4	33

3.10.2. Optimization of Bacteriocin Production

Responses were optimized using Design Expert Version 8.1.2 software, Stat-Ease, Inc., USA. The bacteriocin was produced using different levels of pH, incubation temperature, inoculum level and incubation time as depicted in design matrix of CCRD, as shown in table 3.6. Thirty different combinations were evaluated for maximum bacteriocin production. For optimization, both numerical and graphical optimization was done using pH, OD and bacteriocin production (AU/ml) as responses.

Table 3.6. Full experimental design for optimization experiments for maximum bacteriocin production using response surface methodology

S.No.	Factors			
	pH (A)	Temp (B)	Inoculum level (C)	Incubation time (D)
1	4	37	3	24
2	8	42	1	24
3	4	42	3	6
4	6	39.5	2	15
5	8	42	3	24
6	4	37	3	6
7	2	39.5	2	15
8	4	42	3	24
9	8	37	3	6

10	6	39.5	4	15
11	4	42	1	6
12	6	39.5	2	15
13	6	34.5	2	15
14	6	39.5	2	15
15	4	42	1	24
16	4	37	1	24
17	4	37	1	6
18	8	42	1	6
19	6	39.5	2	-3
20	8	37	1	24
21	8	37	3	24
22	8	42	3	6
23	6	44.5	2	15
24	6	39.5	2	33
25	6	39.5	2	15
26	6	39.5	2	15

27	6	39.5	2	15
28	6	39.5	0	15
29	8	37	1	6
30	10	39.5	2	15

3.11. PROTEIN ESTIMATION

Protein content of the bacteriocin samples was estimated by the method of Bradford *et al.*, (1976) using the bovine serum albumin (BSA) as the standard (Fig. 3.1) as well as by absorbance at 280 nm in spectrophotometer (Jenway, Genova, UK).

3.11.1. Determination of Specific Activity

Specific activity of the bacteriocin preparation was defined as activity units of the bacteriocin per milligram of protein.

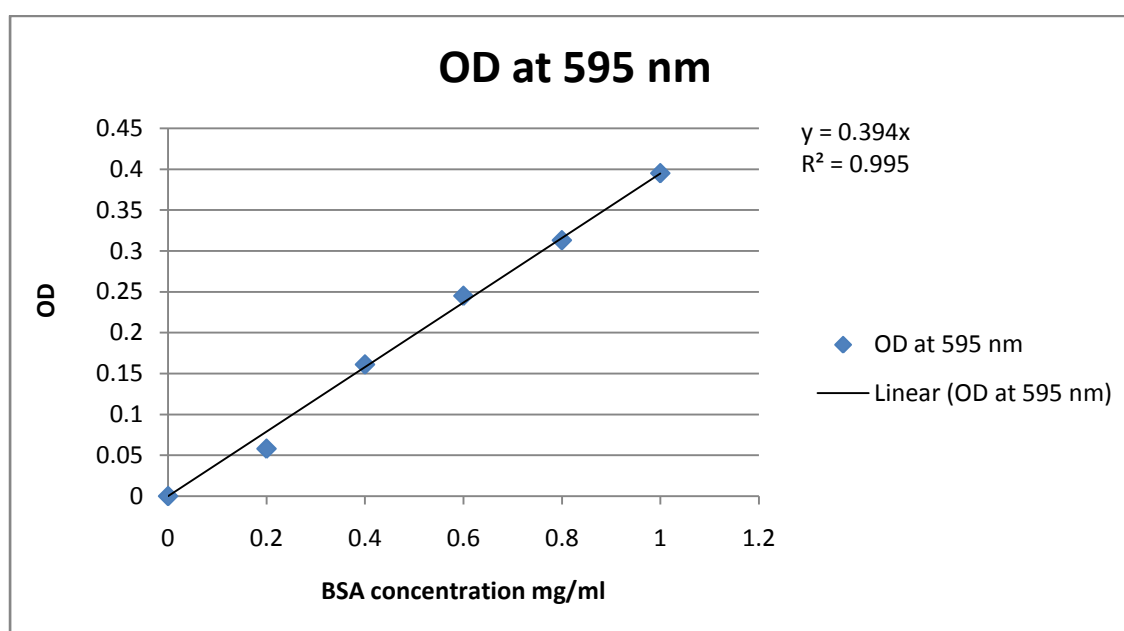


Fig. 3.1. Standard curve for protein estimation by Bradford method

3.12. PURIFICATION OF THE BACTERIOCIN

3.12.1. Precipitation with Ammonium Sulfate

MRS broth having an initial pH 8.0 was inoculated at 3% level with the producer strain *L. gasseri* NBL 18 and incubated at 37°C for 24 h. The cells were then removed by

centrifugation at 10,000 rpm for 20 min. The pH of supernatant was adjusted to 6.5 and it was filter sterilized by passing through a 0.2 μ , 45 mm diameter membrane filter. This supernatant was used for purification studies. The bacteriocin activity (3.6.) and protein content (3.11.) of the supernatant were determined to calculate the specific activity (3.11.1.).

To 1000 ml of supernatant, ammonium sulfate was added slowly with constant stirring to achieve 60% saturation and stirring was continued for another one hour in a cold room at 5-7°C. The mixture was then kept overnight in the cold room. It was then centrifuged at 10,000 rpm for 20 min and the precipitates were recovered. The supernatant was subsequently adjusted to 80% saturation levels by further addition of solid ammonium sulfate. The pellet in each case was dissolved in MilliQ water.

3.12.2. Ion-Exchange Chromatography

Further purification of the ammonium sulfate precipitated (60% saturation) bacteriocin was attempted by ion-exchange chromatography.

3.12.2.1. Selection of ion-exchanger and starting pH of the buffer

3.12.2.1.1. *Ion-Exchanger*: CM-Sephadex C-25 and DEAE-Sephadex A-25 (Pharmacia GE Health Sciences, Uppsala, Sweden) were used as cation and anion exchangers, respectively.

3.12.2.1.2. *Buffers*: Acetate buffers (pH 4.5, 4.9 and 5.5), phosphate buffers (pH 6.5 and 7.0) and Tris-HCl buffers (pH 8.0 and 8.5) were used for the anion-exchanger.

3.12.2.1.3. *Procedure*: Slurry of ion-exchanger matrix CM-Sephadex and DEAE-Sephadex was prepared in excess of water and boiled for 3-4 min. After swelling of the matrix excess of the water was removed and 0.5 ml of the slurry was transferred to eppendorffs. The slurry of ion-exchanger was equilibrated in each eppendorff to a different pH (4.5, 4.9, 5.5, 6.5, 7.0, 8.0 and 8.5) value by washing 10 times with 1 ml of 0.05 M buffer, until the pH of the slurry attained the pH of the buffer used. Fifty microlitres of the bacteriocin solution was added to the eppendorff tubes containing slurry of ion-exchanger at different pH values. The gel slurry was vortexed for 5-10 min and allowed to settle. The supernatant was removed after spinning at 1,000 rpm for a few seconds and assayed for the bacteriocin activity (3.6.) to determine the amount of bacteriocin bound to the ion-exchanger. Gel slurry was washed 2-3 times with respective buffers to

remove out any unbound bacteriocin. Then bound protein was eluted with 1M NaCl in respective buffers of different pH values after mixing well and spinning at 1,000 rpm for few sec. This elution buffer was checked for the activity of bound bacteriocin as described in section 3.6.

Further confirmation of the column was done by calculating the Isoelectric point (pI) of gassericin A through ExPasy (**Expert Protein Analysis System**, Swiss Institute of Bioinformatics (SIB), Bioinformatics Resource Portal). Since the calculated pI was 6.75, and maximum bacteriocin (about 80%) was found to get attached to the anionic exchanger through previous experiment, the purification of bacteriocin was carried out through anion exchange chromatography.

3.12.2.2. Anion-exchange chromatography

Anion-exchange chromatography for the bacteriocin was carried out on Multidimensional Chromatographic system (Akta Prime, GE Health Sciences, Zurich, Switzerland) using QXL Hiload 16/10 Q Sepharose column (GE Health Sciences). Solvent A used was 50 mM Tris-HCl buffer (pH 8.5), and solvent B was Tris-HCl buffer with 1M NaCl (pH 8.5), both filtered through 0.45 µm membrane filter and degassed before use. Column before use was thoroughly washed and equilibrated with 50mM Tris-HCl (pH 8.5). Elution was done by the gradient of 0- 100% 1 M NaCl in 50 mM Tris-HCl buffer (pH, 8.5) at the flow rate of 1.2ml/min. The active fractions were pooled and subjected to RP-HPLC.

3.12.3. RP-HPLC Profile of Bacteriocin

The reverse-phase HPLC analysis of bacteriocin was done on Multidimensional Chromatographic system (Akta Explorer, GE Health Sciences, Zurich, Switzerland) using Resource RPC column (1 ml, GE Health Sciences). Solvent A used was 0.1% trifluoroacetic acid (TFA; Sisco Research Laboratories, Pvt. Ltd., Mumbai) in 2% acetonitrile. Solvent B was 0.05 % TFA in 70% acetonitrile. Both the solvents were filtered through 0.45 µm membrane filter and degassed before use. After centrifugation at 16,000 rpm, for 10 min, the sample was applied to the column. The bacteriocin was eluted

from the column with 85.2% of solvent B [70% acetonitrile + 0.05% (v/v) Tri Fluoro Acetic acid (TFA)] at a flow rate of 0.5 ml/min. Fractions were checked for bacteriocin activity.

3.13. DETERMINATION OF MOLECULAR WEIGHT BY TRICINE SDS-PAGE

The Tris- Tricine SDS-PAGE is the most widely used method for qualitatively analyzing low molecular weight peptides. The molecular weight of the bacteriocin of NBL 18 (gassericin) was determined as per the procedure of Schagger and Jagow (1987). The bacteriocin produced under optimized conditions and purified by RP-HPLC was used for the determination of molecular weight of the bacteriocin.

3.13.1. Sample Preparation

The bacteriocin purified through RP-HPLC was mixed with sample buffer (Appendix III) in the ratio 4:1.

3.13.2. Preparation of Molecular Weight Markers Solution

Dissolved the Molecular weight marker solutions in the sample buffer in the 4:1 ratio.

3.13.3. Treatment of Samples Prior to Electrophoresis

Heated the samples and molecular weight markers at 95°C for 4 min and then allowed to cool to room temperature.

3.13.4. Preparation of Electrophoresis Gels

Composition and preparation of all the components is given in Appendix III. All preparations were made in highest quality of water available.

Table 3.7. Composition of gels for Tricine-SDS-PAGE

S.No.	Gel Solutions	Stalking Gels (4%, 6.25 ml)	Spacer Gel (10%, 7.5 ml)	Separating Gel (16.5%, 15 ml)
1	49.5% Acrylamide	0.5 ml	1.525 ml	5 ml

2	Gel Buffer	1.5 ml	2.5 ml	5 ml
3	Glycerol	-	-	1.6 ml
4	Water	4.2ml	3.47 ml	3.4 ml
5	10% APS	50µl	25µl	50µl
6	TEMED	15µl	10µl	15µl

3.13.4.1. Gel casting

- All the glass plates of the electrophoresis apparatus were cleaned by dipping the glass plates in chromic acid, previous night before experiment.
- The spacers of appropriate thickness were selected and the gel apparatus was assembled.
- A desired 10 well comb was placed completely into the gel cassette. The glass plates was marked 1 cm below the comb teeth, level to which the separating gel poured.
- The resolving gel monomer was prepared in a 50 ml glass beaker as per the recipe given by adding APS and TEMED just before polymerization. The solution was mixed well by shaking the mixture. The solution was poured smoothly by the side of cassette from top taking care of not to allow any air bubble in the gel.
- Immediately the gel was overlaid with 100µl of the water (or water saturated isobutanol). The gel was allowed to polymerize without any disturbance.
- The distilled water over the gel was discarded completely. The top of the separating gel was blotted dry with Whatman No. 1 filter paper strips without disturbing the gel surface.
- The resolving gel monomer solution was prepared as per the recipe and poured between the glass plates until the top of the glass plate was reached.
- The comb was inserted and gel was allowed to polymerize without any disturbance.
- The positions of the wells were marked on the glass plates with a marker. The comb was removed without disturbing the wells and the wells were rinsed thoroughly with running buffer.

3.13.5. Electrophoresis

- The gel cassette was removed from the assembly and placed suitably in the electrophoresis tank.
- The running buffer was poured into electrophoresis tank upto the appropriate level. The buffer of the cathode or upper chamber was in contact with the gel.
- The samples prepared as well as protein molecular weight markers were loaded into the wells as planned for a particular experiment with a micropipette.
- The lid of the electrophoresis tank was put properly over it. The necessary power cord of the power supply was connected to power supply. For SDS-PAGE, the plug of the upper chamber was connected to the cathode (black) and that of the lower chamber was connected to the anode (red).
- The electrophoresis was run with a constant voltage of 40V till the tracking dye (bromophenol blue) reaches near the bottom of the gel.

3.13.6. Fixing, Staining and Destaining of the Gel

- The first half of the gel containing protein markers was transferred to staining solution (Appendix III) for at least 4 h with agitation.
- The gel was then transferred to destaining solution (Appendix III) in the glass tray. After every 30 min, the destaining solution in the tray was replaced with the fresh one until a transparent gel background was seen.
- The destained gel was observed for the protein bands and photographed using Kodak Easy Share Digital Camera (Eastman Kodak Company, New York).

3.13.7. Detection of the Bacteriocin Activity

The second half of the gel containing only bacteriocin samples in the two lanes was washed with deionised water for 4 h. The wash water was replaced every 30 min with the fresh one for effective cleaning of the gel. The washed gel was transferred to a dried TGE agar plate. The gel was overlayed with 10 ml of TGE soft agar (0.8% agar) containing 50 µl of freshly grown indicator strain *E. faecalis* NCDC 114. The plate was incubated overnight at 37°C and examined for the zone of inhibition after 24 h.

3.13.8. Estimation of Molecular Weight

The stained gel was superimposed on the gel used to detect the bacteriocin activity. Approximate molecular weight of the activity band was estimated by comparing the centre of the zone of the inhibition with the molecular weight markers.

3.14. CIRCULAR DICHROISM SPECTRA OF BACTERIOCIN

The Circular dichroism (CD) spectra for the RP-HPLC purified bacteriocin was recorded between 200 and 260 nm at room temperature by using a 1-mm-pathlength quartz cell on a Chirascan spectropolarimeter (Photophysics), (AIRF, JNU, New Delhi). Samples were scanned ten times, and the data were averaged. A peptide-free control spectrum with 70% (vol/vol) acetonitrile + 0.05% TFA was subtracted, providing the mean residue ellipticity of the peptide.

3.15. CHARACTERIZATION OF THE BACTERIOCIN PRODUCED BY *L. GASSERI* NBL 18

The crude as well as partially purified bacteriocin preparations of bacteriocin (gassericin) were used in the characterization studies.

3.15.1. Crude Bacteriocin

L. gasseri NBL 18 was grown in MRS broth under optimized conditions for 24 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4°C). The culture broths were boiled at 90-95°C for 3-5 min in a boiling water bath to kill the living cells. The cell-free supernatant was adjusted to pH 6.5 using 1N NaOH and was used as crude bacteriocin.

3.15.2. Partially Purified Bacteriocin

The bacteriocin precipitated with ammonium sulfate (60% saturation) was dissolved in deionised water and filter sterilized by passing through 0.22 µm membrane filter.

3.15.3. Inhibitory Spectrum of Activity

The antibacterial activity of partially purified bacteriocin preparations of NBL 18 against various lactic and non-lactic cultures as well as against some of the yeasts and molds was determined by spot on lawn assay (3.5.1).

3.16. SENSITIVITY OF BACTERIOCIN TO ENZYMES, DETERGENTS, TEMPERATURE AND pH

3.16.1. Enzymes

Proteolytic enzymes used were: Chymotrypsin, Pepsin, Papain, Proteinase-K and Protease. The non-proteolytic enzymes included: α -amylase, Lipase and Lysozyme. All these enzymes were obtained from Sigma-Aldrich Corporation, St. Louis, Missouri, USA.

All the enzymes, except pepsin, were dissolved in sterile 50 mM phosphate buffer (pH 7.0) at a concentration of 10 mg/ml, whereas pepsin was dissolved in 0.02 N HCl.

3.16.1.2. Procedure

To 100 μ l of crude bacteriocin(3.15.1) containing 2.56×10^4 AU ml⁻¹ and 100 μ l of partially purified bacteriocin (3.15.2) containing 3.2768×10^6 AU ml⁻¹ in a microcentrifuge tube, 100 μ l of enzyme preparation (3.16.1) was added and mixed immediately. The microcentrifuge tubes were allowed to float in a water bath maintained at 37°C for 2-3 h. The microcentrifuge tubes were then transferred to a boiling water bath for 5 min. Before transferring to the boiling water bath, the pH of the pepsin treated samples was brought to 7 with 0.1 N NaOH.

3.16.2. Detergents

The surfactants tested were Sodium Dodecyl Sulphate (SDS), Tween 80, Tritone X-100 and Tween 20 at final concentration of 1% (all from Hi Media Laboratory Pvt Ltd. India). Two controls were used: one consisted of phosphate buffer and detergent solution and the other consisted of crude or partially purified bacteriocin and phosphate buffer without detergent, treated in the similar manner. All samples and controls were incubated at 30°C for 5 h and tested for activity. The residual activity of the detergent treated sample was estimated as given in 3.6.

3.16.3. Heat Stability

One ml crude bacteriocin (3.15.1) and partially purified bacteriocin (3.15.2) was subjected to different heat treatments *viz.* 63°C/30 min, 85°C/10 and 15 min, 90°C/10 and 15 min, 100°C (boiling)/5, 10, 30 and 60 min and autoclaving at

121°C/15 min, cooled on ice immediately after heat treatment and assayed for the residual activities (3.6.).

3.16.4. pH Stability

The 100 µl aliquots of crude bacteriocin (3.15.1) and partially purified bacteriocin (3.15.2) were mixed with 100 µl of 50 mM buffers of different pH values 1-10. The bacteriocin activity was assayed after 2, 8, 24, 48, 72 and 120 h of storage at 4°C.

The buffers used were: HCl-KCl buffer for pH 1 and 2, glycine HCl buffer for pH 3, acetate buffer for pH 4 and 5, phosphate buffer for pH 6 and 7, Tris-HCl buffer for pH 8 and 9 and glycine-NaOH buffer for pH 10.

3.17. APPLICATION OF BACTERIOCIN (GASSERICIN) TO INHIBIT FOOD-BORNE PATHOGENS IN WHIPPED CREAM FROSTING

3.17.1. Determination of Minimum Concentration of Bacteriocin to Inhibit Food Borne Pathogens

The pathogenic strains (*E. coli* NCDC 134, *P. aeruginosa* NCDC 104, *L. monocytogenes* ATCC 15303 and *B. cereus* NCDC 66) were grown in nutrient broth for 24 h, harvested, washed twice with phosphate buffer (pH 7.2, 50 mM), suspended in the same buffer, and diluted to an A₄₂₀ of 0.2 measured with a spectrophotometer (Jenway, Genova, UK). This suspension was diluted 1/10, which corresponds to about 1X10⁶ CFU/ml. This inocula of the cultures was used (0.1 ml) to inoculate aliquots of the diluted bacteriocin (0.2 ml) in 96 wells microplates. Bacteriocin was two-fold serially diluted in Muller-Hinton medium. The microplate was incubated at 37°C and observed for growth if any after 24 h.

3.17. 2. Inhibitory Effect of Bacteriocin Against *L. monocytogenes* ATCC 15303, *B. cereus* NCDC66, *E.coli* NCDC 134, *P.aeruginosa* NCDC 104 and *E. faecalis* NCDC 114 in Phosphate Buffer Medium

Inocula of the cultures were prepared (≈10⁶ CFU/ml) as mentioned above and used to inoculate MRS broth containing crude bacteriocin (1.28 X 10⁴ AU/ml). The exposure times were 1, 2, 3, 4 and 5 hours. Viable cells were determined by the pour plate method in Nutrient agar.

3.17. 3. The Inhibitory Effect of Bacteriocin Alone and in Combination with Glycine Against Gram positive and Gram negative bacteria in Microwell Plate Assay

The antibacterial activities of the bacteriocin alone and in combination with glycine against the 12 strains of food spoilage and pathogenic bacteria was assayed using the microwell plate method described by Toba *et al.*, (1991) and Kawai *et al.*, (1997) (Table 3.8). An appropriate culture broth (140 µl) and an antibacterial solution (50 µl) were added to a well of a 96-well plate, which was then inoculated with 10 µl of a target strain suspension (10^3 CFU/mL). The overnight culture supernatants of *L. gasseri* NBL 18 and 1.0% (wt/vol) glycine solution were neutralized, filter sterilized, and individually or jointly used as antibacterial substances. Antibacterial activity was evaluated as 4 levels (A, B, C, and no effect) by visual comparison to preservative-free wells after 6, 12, 18, 24, and 48 h of incubation at the strain's optimum temperature (26, 30, or 37°C). Level A (strong and durable inhibition) means complete inhibition of the indicator's growth, which is judged from clarity of the solution in the well for 48 h. The levels B (durable inhibition) and C (weak inhibition) mean delay of the indicator's growth after the long (24 h) and short (6–18 h) incubation, respectively, compared with the preservative-free control.

Table 3.8. Food spoilage and pathogenic bacteria assayed by microwell plate assay method

Bacterial species	Strains and Source
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NCDC 86, Karnal
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NCDC 278, Karnal
<i>Lactobacillus casei</i> subsp. <i>casei</i>	NCDC 017, Karnal
<i>Lactobacillus brevis</i>	NCDC 001, Karnal
<i>Enterococcus faecalis</i>	NCDC 114, Karnal
<i>Enterococcus faecalis</i>	NCDC 115, Karnal
<i>Staphylococcus aureus</i>	NCDC 110, Karnal
<i>Staphylococcus aureus</i>	NCDC 273, Karnal
<i>Listeria monocytogenes</i>	ATCC15303, USA
<i>Pseudomonas aeruginosa</i>	NCDC104, Karnal
<i>Escherichia coli</i>	NCDC134, Karnal
<i>Bacillus cereus</i>	NCDC66, Karnal

3.17. 4. The Inhibitory Effect of Bacteriocin Alone and in Combination with Glycine Against *B. cereus*, *L.monocytogenes*, *E.coli* and *P. aeruginosa* in Whipped Cream Frosting

Ten gram of cream samples were aseptically weighted into sterile bags and pasteurized by heating at 85°C for 30 min. These were then inoculated with bacterial suspensions to give ca 10³ CFU/g. Bacteriocin stock solution (1.28 X 10⁴ AU/gm) and 1% glycine (wt/wt) was added alone as well as in combination in contaminated cream samples. Untreated cream sample was used as control. The samples were placed in 30°C and survival of organisms was monitored on 0, 2nd, 5th, 15th and 30th days.

3.18. ANTIBACTERIAL ACTIVITY OF NBL 18: COCULTURE TESTS IN SKIMMED MILK

L. gasseri NBL 18 and NBL 5 (a non bacteriocinogenic isolate of *L.gasseri*) were separately cocultured with *L. monocytogenes* ATCC 15303, *S.aureus* NCDC 273 and *E. faecalis* NCDC 114 in 100mL of 12.5% reconstituted skimmed milk (RSM) at 37°C for 48 h. NBL 18 and NBL 5 was inoculated at about 10⁶ CFU/ml, and pathogens had a final count of 10³ CFU/ml. In each experiment, control inoculated with pathogen alone, and control inoculated with NBL 18 and NBL 5 alone were included. After 0, 3, 6, 12, 24 and 48 h, samples were taken, serially diluted in sterile saline solution, and plated onto MRS agar plates for the enumeration of *L. gasseri* NBL 18 and NBL 5; and on PALCAM agar, Baird Parker agar and Citrate azide agar for the enumeration of *L.monocytogenes*, *S.aureus* and *E.faecalis* respectively. Values of pH and bacteriocin activity (AU/ml) were also monitored.

3.19. SAFETY ASSESSMENT OF NBL 18

3.19.1. Liquefaction of Gelatin

Gelatinase production by *L. gasseri* NBL 18 isolate was studied by using TND agar (Appendix D) containing 0.4% gelatin. Petriplates containing the medium were spot inoculated with the test culture and incubated at 37°C for 3 d. The petriplates were then flooded with saturated ammonium sulphate solution. Development of clear zones around the spots against opaque background indicated a positive reaction.

3.19.2. Hemolysis

L. gasseri NBL 18 strain was grown overnight in MRS medium at 37°C, and then streaked onto Blood Agar Base (HiMedia Laboratories Ltd., Mumbai) plates containing 5% of fresh calf blood obtained from Animal Health Complex of the Institute. The plates were incubated for 3 d at 37°C. The hemolytic reaction was recorded by observation of a clear zone of hydrolysis around the colonies (β -hemolysis), a partial hydrolysis and greening zone (α -hemolysis) or no reaction (γ -hemolysis).

3.19.3. Antibiotic Susceptibility Test

Antibiotic susceptibility testing was carried out by disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, 2010, however, Mueller- Hinton agar was replaced by TGE agar. The following antibiotics (Himedia) were tested: vancomycin (VA; 30 μ g) (inhibitors of cell wall synthesis), streptomycin (S; 10 μ g), tetracycline (T; 10 μ g), gentamicin (G; 10 μ g), kanamycin (K; 30 μ g), erythromycin (E; 10 μ g), ciprofloxacin (CF; 5 μ g), Amoxycillin (AM; 30 μ g), Bacitracin (B; 10 μ g), Penicillin-G (P; 10 μ g), Polymyxin-B (Pb; 300 μ g) Chloramphenicol (C; 30 μ g), Ampicillin (A; 10 μ g), Cefazolin (Cz; 30 μ g), Cefuroxime (Cu; 30 μ g), Amikacin (Ak; 30 μ g), Co-Trimoxazole (Co; 25 μ g) and Rifampicin (R; 30 μ g).

A suspension from fresh overnight cultures with a density of McFarland 0.5 in buffered saline was plated on MRS agar plates; then, antibiotic discs were dispensed onto the plates. After incubation at 37°C for 24 h in anaerobiosis, the diameters of the bacterial-free zone were measured, and results were expressed in terms of resistance according to the interpretative criteria issued by the CLSI, 2010.

3.19.4. DNase Test

L. gasseri NBL 18 was streaked on DNase agar medium (HiMedia Laboratories Ltd., Mumbai) to check production of DNase enzyme. Plates were incubated at 37°C for 48 h to observe any appearance of halos surrounding colonies.

3.20. STATISTICAL ANALYSIS

The data were subjected to various statistical analysis as and when needed, using SYSTAT 6.0.1. (Statistical Software Package, 1996, SPSS, Inc., USA), Microsoft R excel 2000 Software Package, Microsoft Corporation, USA and SAS (Statistical Analysis System, version 9.2, 2009), SAS Institute Inc., USA. The statistical model used was one way ANOVA. Differences among means were compared by Turkey multiple range test.

3.20.1. t-test

t-test was employed to test the significance of differences between the predicted and observed value of different responses in optimization study using Microsoft Excel 2000 Software Package, Microsoft Corporation, USA. A p value < 0.05 was considered to be statistically significant.

3.20.2. Determination of Mean and Standard Error of the Mean (SEM)

The experimental data, as and when necessary, is presented as the mean and standard deviation of different parameters studied in the present investigation. The mean and standard deviation were determined running Microsoft Excel 2000 Software Package, Microsoft Corporation, USA.

3.20.3. Graphical Presentation

The mean $\pm$ standard deviations of different parameters studied were graphically presented using Microsoft Excel 2000 Software Package, Microsoft Corporation, USA.

Chapter 4

Results and Discussions

4. RESULT AND DISCUSSION

4.1. PROLOGUE

Circular bacteriocins are special molecules, whose precursors must be post-translationally modified to join the N and C termini with a head-to-tail peptide bond. This feature appears to have a range of potential advantages over their linear counterparts, since they are less susceptible to proteolytic cleavage and show enhanced pH and thermal stability. The advantages of circularization are reflected by the fact that a significant number of macrocyclic natural products have found pharmaceutical applications. Thus, their biotechnological interest has increased during the last few years, becoming a prime line of research. Circular bacteriocins are different from other nonribosomal cyclic peptides produced by bacteria and fungi, such as cyclosporin A, which are synthesized in a peptide synthetase-driven process. Circular bacteriocins were unknown about two decades ago, and the field is still in its infancy. There are now a few examples of bacterially produced circular proteins. The circular bacteriocins produced by Gram-positive bacteria described to date, include enterocin AS-48 from *Enterococcus faecalis* S-48, gassericin A from *Lactobacillus gasseri* LA39, and a similar one, reutericin 6 from *Lactobacillus reuteri* LA6, butyrivibriocin AR10 from the ruminal anaerobe *Butyrivibrio fibrisolvens* AR10, uberolysin from *Streptococcus uberis*, circularin A from *Clostridium beijerinckii* ATCC 25752, and subtilosin A from *Bacillus subtilis*. Circular bacteriocins from lactic acid bacteria have been examined extensively in the recent years. Much of the interest involves the lethal activity of these peptides against important food pathogens such as *Listeria* and *Clostridium* spp.

LAB are indigenous flora of mammals, including humans. They are widely used in food and feed production. They have "Food grade quality" and are industrially important. Strains of the *Lactobacillus acidophilus* group of homofermentative LAB (Johnson *et al.*, 1980; Fujisawa *et al.*, 1992) are found in human and animal intestines and are added as dietary adjuncts to commercial fermented milk products, such as acidophilus yogurt, because of their physiological functions as "probiotics": they can survive and grow in the gastrointestinal tract (Sanders *et al.*, 2001). The intake of these bacteria may have beneficial effects on human and animal health (Sanders *et al.*, 2001). There are a number of reports on the production of bacteriocins by strains of the six subgroups of *L. acidophilus*. *L. johnsonii* VPI11088 (lactacin F) (Muriana *et al.*, 1991; Fremaux *et al.*, 1993) *L. amylovorus* LMG P-13139 (lactobin A) (Contreras *et al.*, 1997) *L. amylovorus*

DCE471 (amylovorin L471) (Callewaert *et al.*, 1999) and *L. gasseri* SBT2055 (gassericin T) (Kawai *et al.*, 2000) are some of the potent bacteriocin producers. Among these subgroups of *L. acidophilus*, *L. gasseri* LA39 is a known producer of circular bacteriocin Gassericin A. Gassericin A, a circular bacteriocin produced by *Lactobacillus gasseri* LA 39 shows antibacterial activity against a number of Gram positive food borne pathogenic bacteria including *L.monocytogenes*, *S.aureus* and *B. cereus*.

The increased emergence of antibiotic resistant pathogens due to inadequate use of antibiotics may in the near future leave us insufficient to cope with at least certain bacterial infections. Therefore it has been hypothesized that non pathogenic bacteriocin producing strain of apparent low virulence (LAB) can be entrenched into the indigenous microflora in order to interfere with colonization and infection of pathogenic species. Also, applications of bacteriocins as food preservatives have been so far limited because of their low antimicrobial activity in foods. Nisin is the only bacteriocin of significant use, but applications are restricted principally because of its very low activity at neutral or alkaline pH. Thus, the isolation of new bacteriocins stable in different food system is desired. In view of this, the present investigation is taken to isolate, optimize and characterize the heat and pH stable circular bacteriocin (gassericin A) from lactic acid bacteria *Lactobacillus gasseri* and to study its effect in the preservation of high fat food (cream).

The results obtained in the present investigation broadly include isolation of *Lactobacillus gasseri* from infant faeces, saliva and human milk; screening of *L. gasseri* isolates for gassericin A (circular bacteriocin) production, optimization of cultural conditions for bacteriocin production, purification and characterization of the bacteriocin and application of bacteriocin in the preservation of high fat food.

4.2. ISOLATION OF *LACTOBACILLUS*

In an attempt to obtain some promising circular bacteriocin producing strains of *Lactobacillus* with broad spectrum of antagonistic activity, a total number of 70 samples comprising infant faeces (50), human milk (10), and human saliva (10) were obtained from rural and urban areas near Karnal, Rewari and Panipat. Samples of infant faeces (50) were collected from healthy infants of 0-6 months, human milk samples (10) were collected from healthy lactating mothers and saliva samples (10) were collected from

healthy individuals. Decimal dilution of these samples were plated on MRS agar (de Mann *et al.*, 1960) and incubated at 37°C for 24h. Typical cream colored oval shaped colonies (Plate 4.1 a&b) after 24-48 h of incubation at 37°C were counted. The *Lactobacillus* counts ranged from 10^3 - 10^4 cfu ml⁻¹ in human saliva and 10^1 - 10^2 cfu ml⁻¹ in human milk and 10^9 - 10^{10} cfu ml⁻¹ in infant fecal samples (Table 4.1).

Table 4.1. Isolation of lactobacilli from infant feces, human milk and saliva as studied on MRS agar

Source	No. of samples	Sources	CFU ml ⁻¹ /g ⁻¹	No. of isolates picked randomly
Infant feces	50	Government hospital, Maternity homes and residential area of Karnal, Panipat and Rewari	10^9 - 10^{10}	423
Human milk	10	Healthy lactating mothers	10^1 - 10^2	50
Human saliva	10	Healthy individuals	10^3 - 10^4	50
Total	70			523

Lactobacilli are normal inhabitant of human GI tract. In a study conducted by Kimura *et al.*, (2009), the strain composition of *Lactobacillus* of fecal samples collected from 10 human subjects was examined. The results showed that each subject harbored a unique collection of *Lactobacillus* strains. Among all the lactobacilli, *L. gasseri* and *L. fermentum* were most commonly detected species in the fecal samples. Song *et al.*,

(1999), also reported that *L. gasseri* and *L. fermentum* were the dominant *Lactobacillus* species in the feces of Japanese women. According to their study, the predominant species in stools were *L. gasseri*, *L. fermentum*, and *L. paracasei* subsp. *paracasei* for women and *L. gasseri*, *L. crispatus*, and *L. salivarius* subsp. *salivarius* for infants. Benno *et al.*, 1989 reported that *L. gasseri* was the dominant species among lactobacilli isolated from the intestinal tracts of elderly Japanese people. Mitsuoka, (1992), reported that *L. crispatus*, *L. gasseri*, *L. salivarius* and *L. reuteri* were the major *Lactobacillus* species. Reuter, (2001), found *L. gasseri* and *L. reuteri* to be predominant autochthonous *Lactobacillus* species in infants as well as in adult feces. Meanwhile, in the studies in Western countries, the *L. acidophilus* group, *L. salivarius*, and *L. fermentum* were usually recovered from stools of adults and infants (Cooperstock *et al.*, 1983) and the largest taxa in the rectal mucosa of healthy adults were *L. plantarum*, *L. rhamnosus*, and *L. paracasei* subsp. *paracasei* (Ahrne *et al.*, 1998). These results indicate that the intestinal lactobacilli may be different between Asian and Western people. Although the reasons for this dissimilarity are unclear, it may be speculated that the inhabiting *Lactobacillus* species in stools and in the intestinal mucosa are different and that differences between Asian and Western diets or race may influence the *Lactobacillus* species resident in the intestine.

It has been reported that isolated bacteria from breast milk commonly included staphylococci, streptococci, micrococci, enterococci and lactobacilli such as *Lactobacillus gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum* and *L. fermentum* (Abrahamsson *et al.*, 2009; Heikkila and Saris, 2003; Martin, 2007; West *et al.*, 1979). The presence of lactobacilli in breast milk has been attributed to the existence of prebiotic oligosaccharides in this fluid (Kralj *et al.*, 2002; Pridmore *et al.*, 2004; Tieking *et al.*, 2003; van Hijum *et al.*, 2002).

Lactobacilli are absent from the oral cavity of newborns, but appear during the first year of the life. McCarthy *et al.*, 1965 observed the presence of lactobacilli in 50% of newborns during their first year with a rate from 2.0×10^2 to 3.0×10^4 cfu ml⁻¹ of saliva. In children without caries, the rate of salivary lactobacilli varied among the different studies. Carlsson *et al.*, 1975 considered that lactobacilli became regularly present in 50% of children since the age of two. Later, Köhler *et al.*, 1984, indicated that 40% of a population of 3-year old children harboured lactobacilli in rates varying from 2.10^3 to

4.10^4 cfu/ml of saliva. For older children (from 6 to 16 years old), this rate is slightly higher (54.6%) (Kailis *et al.*, 1965). On the other hand, other authors reported the presence of lactobacilli in 100% of sampled children (Munoz-Jeldrez *et al.*, 1980). One factor that could influence the rate of salivary lactobacilli during childhood is the carbohydrate intake (Beighton *et al.*, 1996). In young adults, their presence is not unconditional and it depends on appropriate ecological niches. Pits and fissures or partially erupted third molars provide a retentive environment favourable to the growth of these microorganisms (Tanzer *et al.*, 2001). Other studies have shown a significant correlation between smoking habits and salivary rates of lactobacilli (Heintze *et al.*, 1984; Sakki *et al.*, 1996). In old subjects, the lactobacilli rate tends to increase again (Fure *et al.*, 2003). Higher counts have been found in subjects harbouring removable prostheses (Narhi *et al.*, 1999) and in subjects consuming lot of medicines (Beighton *et al.*, 1991; Guivante-Nabet *et al.*, 1999).

A total number of five hundred and twenty three typical colonies (Table 4.1) with yellow zone on MRS plate supplemented with 0.004% BCP were randomly selected and transferred to MRS broth, incubated at 37°C for 24 h. The broth cultures thus obtained were purified by streaking twice on the MRS Agar. Well separated colonies thus obtained were again inoculated into MRS broth and incubated at 37°C for 24 h and were examined microscopically for shape and arrangement of cells after Gram's staining to ascertain the purity of cultures. The Gram-positive rods or coccobacilli in chains or pairs and the absence of any cocci confirmed purity of *Lactobacillus* cultures (Plate 4.2 a&b).

Several media have been reviewed for the isolation, enumeration and identification of lactobacilli from dairy and fecal samples. deMan, Rogosa and Sharpe (1960), developed MRS for isolation of lactobacilli to replace the tomato juice medium and the meat extract tomato juice medium used initially to isolate lactobacilli. Since then, it has been used enormously in the enumeration and isolation of lactobacilli. The MRS culture media contain polysorbate (Tween 80), acetate, magnesium and manganese which are known to act as special growth factors as well as a rich nutrient base for lactobacilli. As these media show a very low degree of selectivity *Pediococcus* and *Leuconostoc* species as well as other secondary bacteria may also grow on them.

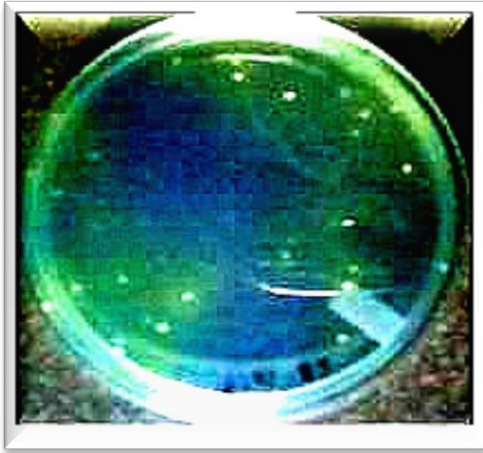
Other possible media that can be used instead of MRS include LAMVAB, Rogosa agar, and LBS. The "*Lactobacillus* Anaerobic MRS with Vancomycin and Bromocresol green"

(LAMVAB) agar contains vancomycin and features a low pH as selective agents, and is now widely used in isolating *Lactobacillus* species particularly from human faeces, as well as other species that inhabit dairy products. Due to the presence of bromocresol in LAMVAB, *Lactobacillus* colonies are differentiated by their blue or green colour. Other bacterial genera may be observed as white or yellow colonies (Jackson *et al.*, 2002). Furthermore, cystein-HCl plays a role as a booster for lactobacilli to sustain their growth under anaerobic conditions (Hartemink *et al.*, 1997). Rogosa agar, developed by Rogosa, Mitchell and Wiesman (1951), contains high acetate and is of a low pH, which characteristically selects lactobacilli and suppresses the growth of other lactic acid bacteria. However, Rogosa agar is not very selective since it can still support other bacterial species (Jackson *et al.*, 2002), moreover, it is not satisfactory for dairy isolates. Kurtzman *et al.*, (1971) found that LBS (*Lactobacillus* selective) agar was the most suitable media for maximizing growth and plate counts in isolating *L. fructivorans* and *L. plantarum* from mayonnaise and salad dressing. Atlas (1993) described the composition of LBS agar, and the procedure required to produce this agar medium. LBS media is widely used to support the growth of *Lactobacillus* species from tooth surfaces and internal flora (Wiseman *et al.*, 1956; Ellis & Sarles, 1958). However, MacFaddin (1985) suggested that this medium should not be used to isolate *L. lactis* and *L. bulgaris* from milk since it contains high levels of salt.

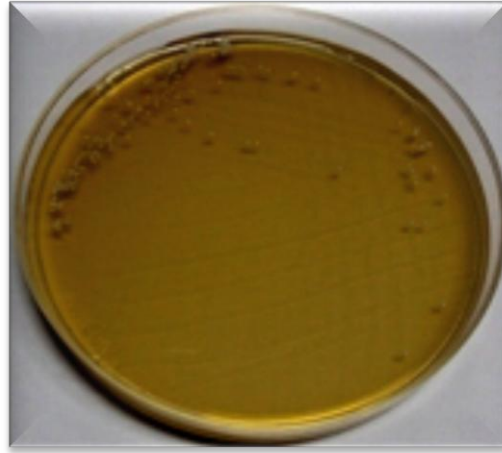
4.3. BIOCHEMICAL CHARACTERIZATION OF *LACTOBACILLUS* ISOLATES

The detailed taxonomic studies involving various physiological and biochemical characteristics to identify the five hundred twenty three isolates to genus (*Lactobacillus*) level are documented in Annexure I.

It may be observed from the Annexure I that out of five hundred twenty three isolates, two hundred eighty four isolates were selected for molecular studies based on the following results. All exhibited growth at 45°C but not at 15°C. They consisted of Gram-positive, catalase-negative rods. They hydrolysed aesculin but were unable to hydrolyse arginine, also did not produce gas from glucose. These characteristics suggested that the isolates belonged to the *Lactobacillus* and are homofermentative.



(A)



(B)

Plate 4.1. (A). Colonies of lactobacilli showing yellow zone on MRS agar supplemented with 0.004% BCP

(B). Colonies of lactobacilli streaked on MRS agar



(A)



(B)

Plate 4.2. Microscopic observation of lactobacilli isolates under oil immersion (100X)

(A) Positive staining

(B) Negative staining

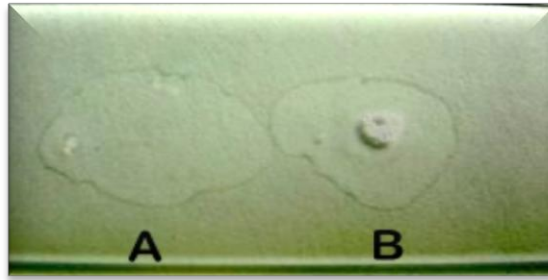


Plate 4.3. Catalase test of lactobacilli isolates
A=Negative; B=Positive

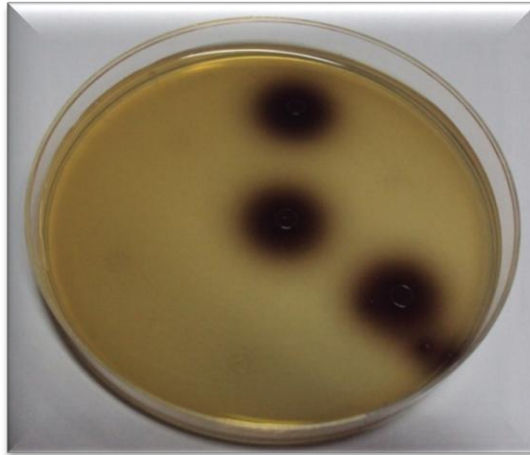


Plate 4.4. Aesculin hydrolysis by lactobacilli isolates

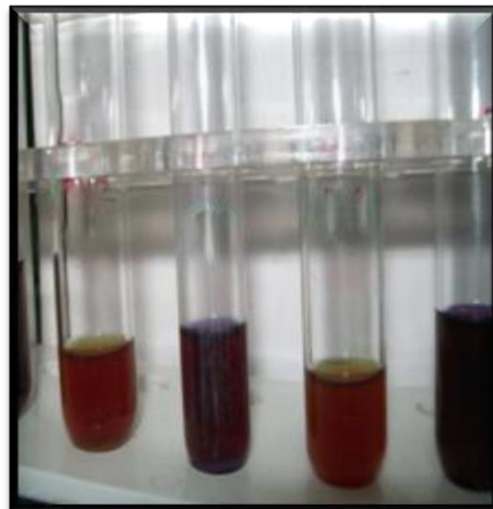


Plate 4.5. Arginine hydrolysis by lactobacilli isolates
Violet Colour:-Positive
Yellow Colour: - Control

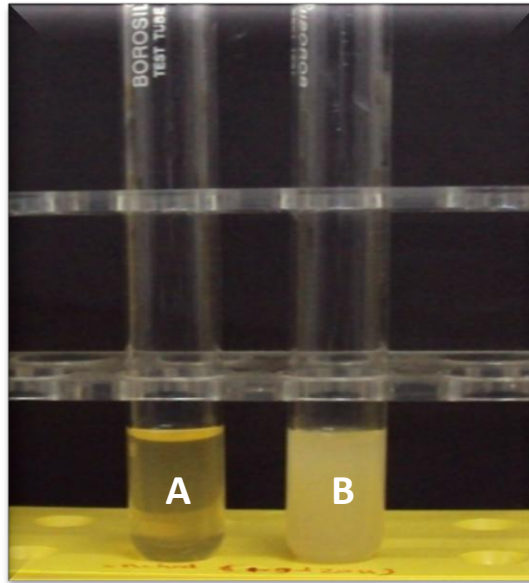


Plate 4.6. Growth at 15 and 45°C by lactobacilli isolates

A=15°C B=45°C



Plate 4.7. Carbohydrate fermentation by lactobacilli isolates

Traditionally, *Lactobacillus* species have been identified on the basis of cell morphology, analysis of fermentation products and associated enzyme activities, and the ability to utilise various carbohydrate substrates. The application of these approaches in the classification and identification of Lactic acid bacteria has been the subject of several reviews (Hammes *et al.*, 1995; Pot *et al.*, 1994; Sgorbati *et al.*, 1995). In general, phenotypic methods, however, suffer from a lack of reproducibility generated by conditions of culture related to different laboratories, and to the diversity of strains (biotypes) that comprise the recognised species (Ballongue *et al.*, 1993; Hammes *et al.*, 1995; Sgorbati *et al.*, 1995).

4.4. MOLECULAR CHARACTERIZATION OF ISOLATES

Nucleic acids are universal in cellular biology, and the nucleotide base sequence of these molecules is not influenced by culture conditions. Analysis of nucleic acids thus provides a basis for identification methods that are reproducible from one laboratory to another. Use of molecular techniques may, therefore, be helpful in removing the confusion regarding their precise identification. Genotypic approaches hold the most promise for the rapid and accurate identification of lactobacilli. In the present study, total DNA was used as template for molecular identification of *Lactobacillus* isolates.

4.4.1. PCR with Genus Specific Primer Pair LbLMA1/R161

Amplification of 16S-rRNA gene of *Lactobacillus* was carried out using primer pairs LbLMA1/R161 devised by Dubernet *et al.*, 2002. Reaction mixture 20, 50 or 100 µl consisted of 1X PCR buffer (Banglore Genei, Banglore), 200-µM concentration of deoxynucleoside triphosphate mix (Banglore Genei, Banglore), a 0.25 µM concentration of each primer (Banglore Genei, Banglore), 50 ng bacterial DNA, and 0.04 U µl⁻¹ Taq DNA polymerase (Banglore Genei, Banglore). The PCR cycling conditions used in this study for *Lactobacillus* included initial denaturation at 95°C for 5 min and 35 cycles each of denaturation at 94°C for 30 sec followed by annealing at 55°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 7 min. The results obtained showed a specific amplification product of 250 bp for the isolates of *Lactobacillus* along with the positive control (Plate 4.8).

On the basis of this technique, 198 (out of 284) biochemically identified isolates were confirmed as *Lactobacillus*. Our results in this regard are in complete agreement with those of Dubernet *et al.*, (2002), who also demonstrated amplification of 250 bp product

in PCR assay with all the standard cultures as well as wild isolates of lactobacilli used in their study.

4.4.2. Identification of the Isolates at Species Level

4.4.2.1. Identification of *Lactobacillus* isolates at species level with carbohydrate fermentation test

The *Lactobacillus* isolates were further identified on the basis of their capability to ferment different sugars (Annexure II). While all the isolates were able to ferment cellobiose, mannose and salicin, only a few were able to ferment ribose, mannitol and melebiose (Annexure II). Majority of the isolates were identified as *L.gasseri* (77.77%). Seven strains (3.53%) viz., NBL 55, NBL 59, NBL 61, NBI 498, NBL502, NBL 503 and NBL 504 that fermented ribose, mannitol and melebiose but not sorbitol and trehalose; were tentatively identified as *L. plantarum*. However, nine (4.54%) isolates viz. NBL 70, NBL 71, NBL 72, NBL 73, NBL 74, NBL 83, NBL 84, NBL85 and NBL 365 were identified as *L. rhamnosus* as they were rhamnose and mannitol positive. Rest of the twenty eight isolates (14.14%), based on their abilities to ferment both arabinose and mannitol, were tentatively identified as *L. acidophilus*. None of the isolates from the samples under study could be identified as *L. paracasei* subsp. *paracasei*, *L. crispatus* or *L. salivarius* subsp. *salivarius*.

4.4.2.2. Identification of *Lactobacillus* isolates at species level with primer Lgas2/Lgas3

After ascertaining the identity of 198 isolates as *Lactobacillus*, on the basis of genus specific PCR assays, and 154 isolates as *L. gasseri* on the basis of carbohydrate fermentation tests, further efforts were made to confirm the identity of these isolates as *L. gasseri* by subjecting them to species specific PCR assay using primer pair Lgas2/Lgas3 as reported by Song *et al.*, (2000). Reaction mixture 20, 50 or 100 µl consisted of 1X PCR buffer (Banglore Genei, Bangalore), 200-µM concentration of deoxynucleoside triphosphate mix (Banglore Genei, Bangalore), a 0.25 µM concentration of each primer (Banglore Genei, Bangalore), 50 ng bacterial DNA, and 0.04 U µl⁻¹ Taq DNA polymerase (Banglore Genei, Bangalore). The PCR cycling conditions used in this study included initial denaturation at 95°C for 1 min and 35 cycles each of denaturation at 94°C for 20 sec followed by annealing at 65°C for 2 min and extension at 55°C for 2 min followed by final extension at 74°C for 5 min. The results obtained showed a specific amplification product of 360 bp for the isolates of *Lactobacillus gasseri* along with

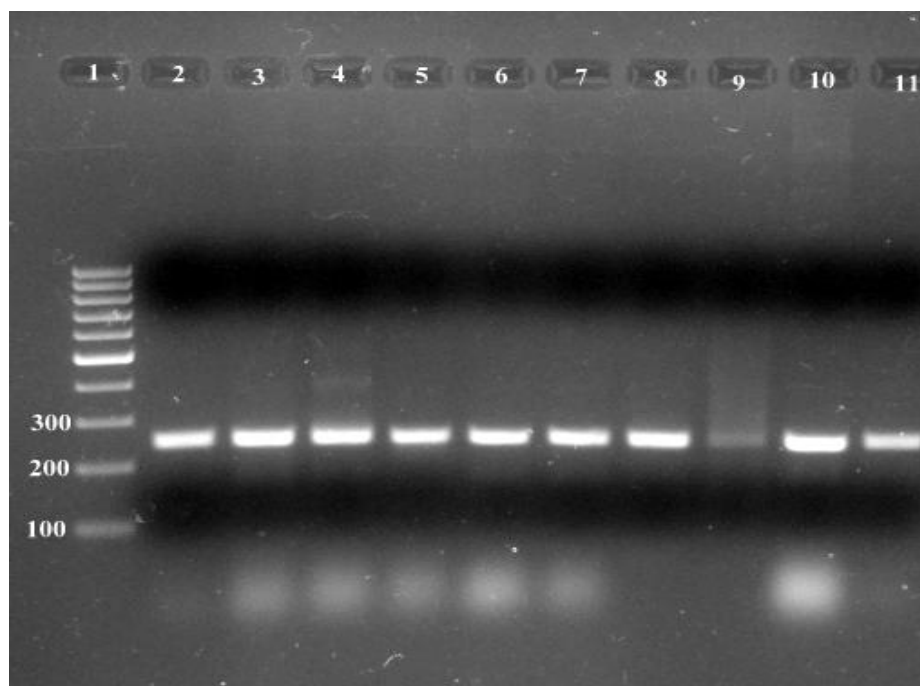


Plate 4.8. Genus specific PCR of lactobacilli using primer LpLMA1/161R1.

Lane 1:100bp ladder

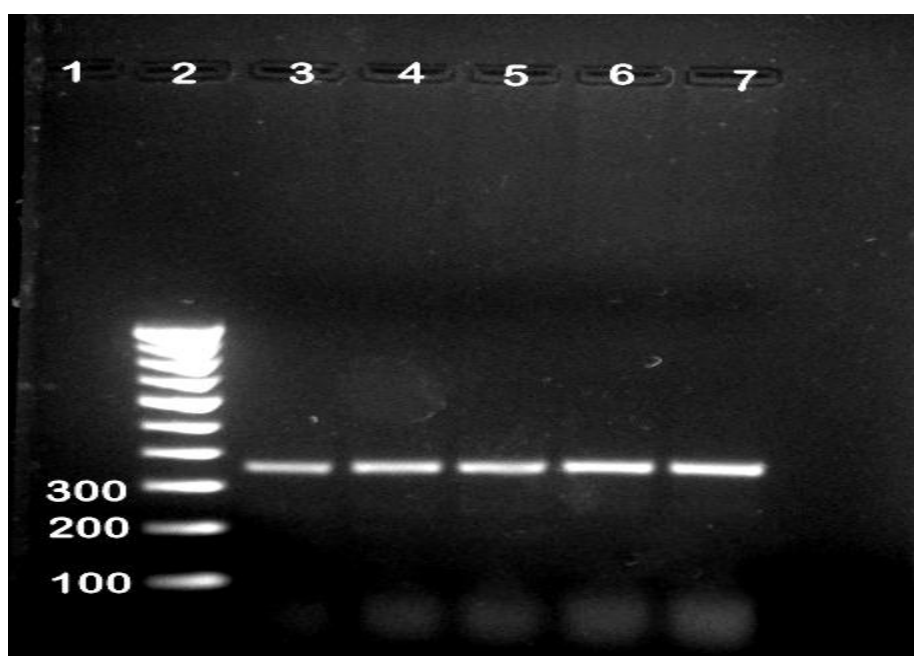


Plate 4.9. Species specific PCR of *Lactobacillus gasseri* using primer Lgas2/Lgas3.

Lane 2:100bp ladder

positive control (Plate 4.9). Our results in this regard are in complete agreement with those of Song *et al.*, (2000), who also demonstrated amplification of 360 bp product in PCR assay with all the standard cultures as well as wild isolates of *Lactobacillus gasseri* used in their study. Out of 198 isolates, 146 isolates were identified as *L. gasseri* using species specific PCR.

4.5. SCREENING OF THE *LACTOBACILLUS GASSERI* ISOLATES FOR THEIR ANTIMICROBIAL ACTIVITY

All the 146 isolates were screened for the antibacterial activity by agar spot assay and spot-on-lawn assay (Plate 4.10; Plate 4.11) using four different indicator strains *viz.* *Pediococcus acidilactici* LB42, *Enterococcus faecalis* NCDC 114, *Lactobacillus delbrueckii subsp.bulgaricus* NCDC 4 and *E. coli* NCDC 134. Out of total number of 146 *L.gasseri* isolates, only seventy eight isolates (53.42%) were found to inhibit at least one test organism by agar spot assay. Ability of these 78 isolates to inhibit the indicator strain was also examined by spot on lawn assay. Out of 78 isolates, only 42 isolates (28.76%) were found to inhibit at least one of the four test cultures by this assay too. The difference in the results obtained by these two methods may be due to the production of acidity by the isolates during agar spot assay in which 5 µl of culture is spotted, whereas in case of spot on lawn assay, culture supernatant neutralized to pH 6.5 is tested as antimicrobial agent. Among the 42 bacteriocinogenic isolates, all the 42 isolates (100%) showed antagonistic activity against *Enterococcus faecalis* NCDC 114 by spot-on-lawn assay, whereas, 35.7% of the isolates were effective against *Pediococcus acidilactici* LB42, and 52.3% of the isolates were effective against *Lactobacillus delbrueckii subsp.bulgaricus* NCDC 4. None of the 42 isolates tested were able to inhibit *E. coli* NCDC 134 by spot-on-lawn as well as by agar spot assays. Therefore, on the basis of number of isolates inhibiting the indicator organism and the diameter of the zone of inhibition, *E.faecalis* NCDC 114 was selected as the indicator organism for this study. Results of antibacterial activity of these isolates against *E.faecalis* NCDC 114 are shown in Table 4.2.

4.2. Antibacterial activity of *Lactobacillus gasseri* isolates by agar-spot and spot-on-lawn Assay against *E.faecalis* NCDC 114

S.No.	Isolates of <i>Lactobacillus gasseri</i>	Agar spot assay	Spot on lawn assay
1	NBL 1	++++	+++
2	NBL 4	++++	+++
3	NBL 11	++++	+++

4	NBL 18	++++	++++
5	NBL 25	++++	++
6	NBL 52	++	+++
7	NBL 56	+++	++++
8	NBL 60	++	++++
9	NBL 65	++	++
10	NBL 71	++	+
11	NBL 89	+++	+++
12	NBL 106	+++	++
13	NBL 124	+++	+
14	NBL 132	++	+
15	NBL 135	+++	+
16	NBL 154	++++	+++
17	NBL 170	++++	++
18	NBL 177	++++	+++
19	NBL 184	++++	+
20	NBL245	++++	++++
21	NBL247	++++	++++
22	NBL255	++	+
23	NBL260	++	+
24	NBL261	++	++
25	NBL279	++++	+++
26	NBL283	++++	++++
27	NBL285	++	++
28	NBL286	++++	+
29	NBL287	++	+
30	NBL328	++++	++
31	NBL 329	++++	++++
32	NBL 363	++++	++++
33	NBL367	++++	++
34	NBL386	+++	++
35	NBL388	+++	++++
36	NBL389	++	+++
37	NBL477	++++	++++

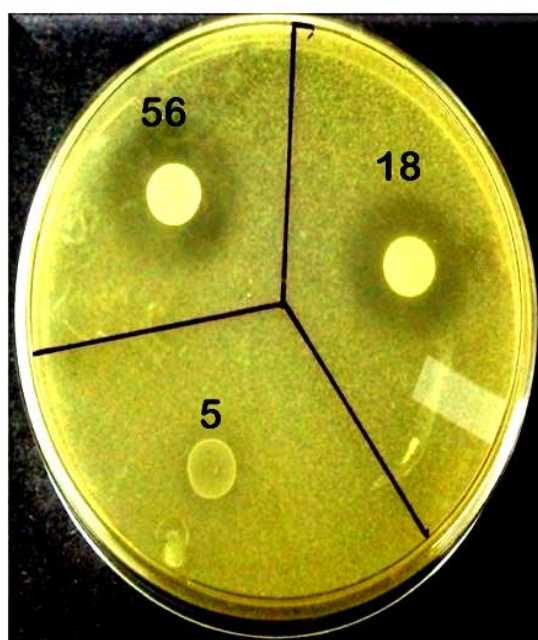


Plate 4.10. Screening of bacteriocinogenic *Lactobacillus gasseri* isolates using *E.faecalis* NCDC 114 as indicator by agar spot assay



Plate 4.11. Screening of bacteriocinogenic *Lactobacillus gasseri* isolates using *E.faecalis* NCDC 114 as indicator by spot on lawn assay

38	NBL483	++++	++++
39	NBL496	++++	++
40	NBL505	++++	++++
41	NBL516	++++	++++
42	NBL518	++	+

Diameter of inhibitory Zone (mm):

Spot-on-Lawn Assay

More than 15 = +++++;

13- 15 = +++;

10-13 = ++;

Less than 10 = +;

No inhibition = -

Agar spot Assay

More than 25 = +++++;

20-25 = +++;

10-20 = ++;

Less than 10 = +;

No inhibition = -

Bacteriocinogenic strains of *Lactobacillus* were encountered in all the samples analyzed but frequency of their incidence varied. Incidence of bacteriocin production trait was found to be highest among the *Lactobacillus* strains isolated from the saliva samples (16%) followed by fecal samples (7.32%) and breast milk (6%) (Fig.4.1.).

Table 4.3. Frequency of bacteriocinogenic *Lactobacillus* isolates in different samples (Infant faeces human milk and saliva)

S.No.	Source	No. of isolates picked randomly	Isolates tested positive as <i>L.gasseri</i>	No. of Bac ⁺ isolates	Frequency
1	Infant feces	423	125	31	7.32%
2	Human milk	50	4	3	6%
3	Saliva	50	17	8	16%
Total		523	146	42	8.03%

There is an increasing interest in the research of antimicrobial peptides (bacteriocins and bacteriocin-like compounds) produced by lactic acid bacteria (LAB) because of their

potential use as antimicrobial agents for improving the safety of food products (Yildirim *et al.*, 1999). Among lactobacilli, strains belonging to species of the *L. acidophilus* complexes are most frequently used as probiotics (Klaenhammer and Kullen, 1999). Bacteriocin production is often proposed as a beneficial characteristic of probiotic bacteria (Klaenhammer and Kullen, 1999; Fooks and Gibson, 2002). It may contribute to the colonisation resistance of the host and its protection against gastrointestinal pathogens (Reid, *et al.*, 2001; Bourlioux, 1997). The first study on the production of bacteriocins by *L. acidophilus* group was reported by Barefoot and Klaenhammer (1983) who mentioned that *L. acidophilus* produces bacteriocin which he named as Lactacin B. *L. gasseri* found in the GI tract has been associated with a variety of probiotic activities and roles including production of bacteriocins (Itoh *et al.*, 1995). *Lactobacillus gasseri* LA158 isolated from human-infant feces produce bacteriocin named gassericins T. It has a high heat stability (121 °C, 10 min), good pH tolerance (pH 2-11), and strong bactericidal activity against many Gram-positive bacteria, especially lactic acid bacteria, and thus is expected to be effective food preservatives. *Lactobacillus gasseri* K7 isolated from healthy infant produces wide-inhibitory spectra bacteriocins: gassericin K7 A and gassericin K7 B. Gassericin A is a circular bacteriocin produced by *Lactobacillus gasseri* LA39 isolated from infant faeces, which shows antibacterial activity against a number of Gram-positive food-borne pathogenic bacteria. Isolation of bacteriocinogenic *Lactobacillus gasseri* from faeces of human has been well reported. Our findings are in agreement with these studies showing that bacteriocin producing *Lactobacillus gasseri* can exist in diverse environments and are also found associated with gastrointestinal tract of human (Itoh *et al.*, 1995).

4.6. SCREENING OF BACTERIOCINOGENIC STRAINS OF *LACTOBACILLUS GASSERI* FOR *gaaA* GENE THROUGH PCR

Specific primers, amplifying segment of bacteriocin encoding gene (*gaaA*) of selected *Lactobacillus gasseri*, were applied in PCR reaction. The bacteriocin producing *L. gasseri* strains were selected, and bacteriocin specific primers were constructed within the structural genes of gassericin A produced by *L. gasseri* LA 39. The usefulness of a PCR based approach as the first step in the characterization of and screening for circular bacteriocin producing *Lactobacillus gasseri*, avoids, to some extent, time consuming and expensive methods, such as protein isolation and amino acid sequencing.



Plate 4.12. Screening of bacteriocinogenic *Lactobacillus gasseri* isolates using *E.faecalis* NCDC 114 as indicator by agar well assay

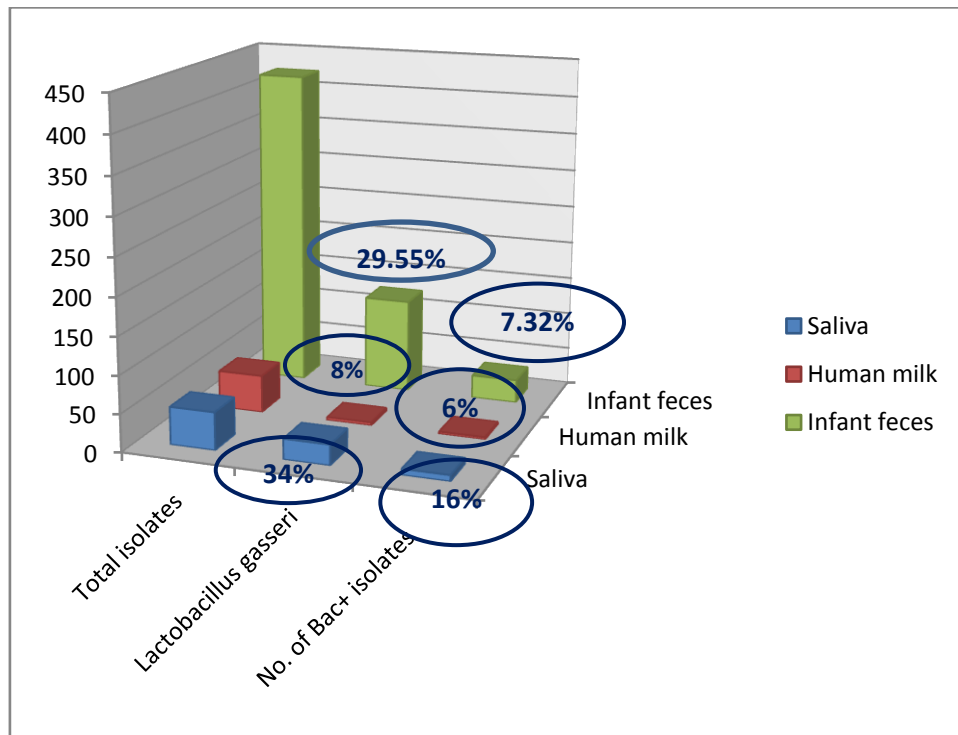


Fig. 4.1. Frequency of bacteriocinogenic *L. gasseri* isolates in infant feces, human saliva and human milk



Plate 4.13. PCR for *gaaA* gene using primer gaA515/gaA616

Lane 1:100bp ladder

Lane 2:JCM11657

Lane3: NBL 18

Lane4: NBL386

Lane5: NBL 25

Lane6: NBL388

Lane7: NBL 154

Lane8: NBL389

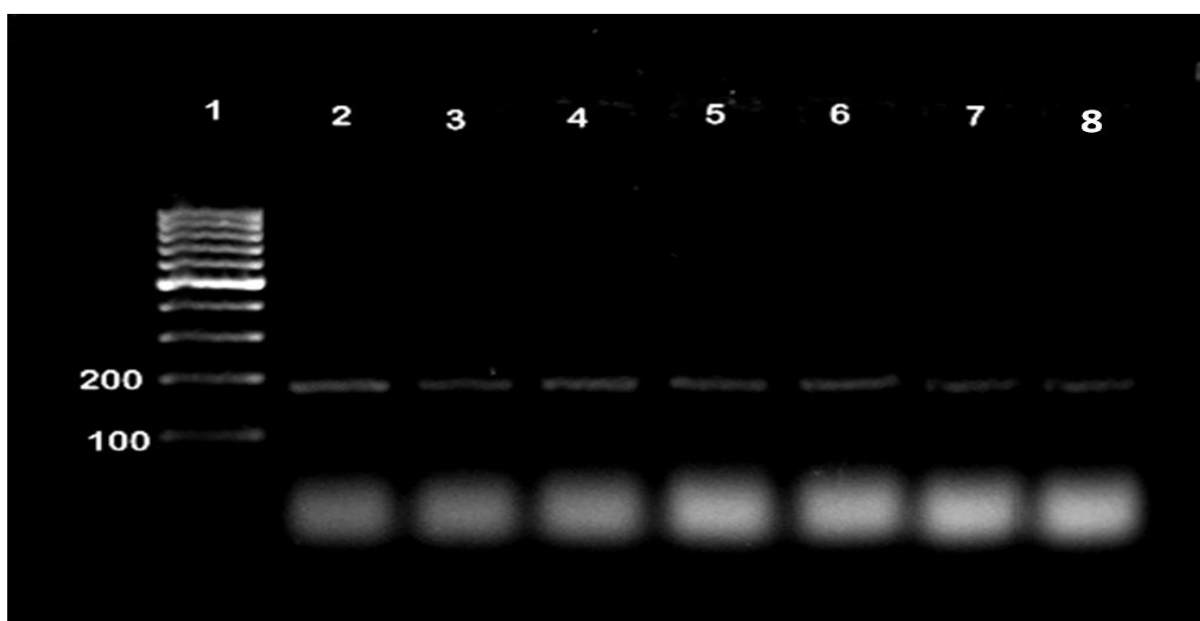


Plate 4.14. PCR for *gaaA* gene using primer gaA2F/gaA2R

Lane 1:100bp ladder

Lane 2:JCM11657

Lane3: NBL 18

Lane4: NBL386

Lane5: NBL 25

Lane6: NBL388

Lane7: NBL 154

Lane8: NBL389

All the 42 bacteriocinogenic isolates of *L. gasseri* were subjected to PCR assay based on gassericin A primers gaA-515/gaA-616 targeted against *gaaA* gene (structural gene for gassericin A) (Majhenic *et al.*, 2003). Reaction mixture 20, 50 or 100 µl consisted of 1X PCR buffer (Banglore Genei, Banglore), 200-µM concentration of deoxynucleoside triphosphate mix (Banglore Genei, Banglore), a 0.25 µM concentration of each primer (Banglore Genei, Banglore), 50 ng bacterial DNA, and 0.04 U µl⁻¹ Taq DNA polymerase (Banglore Genei, Banglore). The PCR cycling conditions used in this study for *Lactobacillus* included initial denaturation at 95°C for 3 min and 35 cycles each of denaturation at 94°C for 1 min followed by annealing at 45°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. The results obtained showed a specific amplification product of 102 bp for the isolates along with positive control (Plate 4.13). However, the bands obtained through primer gaA-515/gaA-616 were faint, so for further confirmation PCR was done with the second set of primer gaA2F/gaA2R. A second set of primer was synthesized gaA2F/gaA2R with the submitted sequence of gassericin A in EMBL through Primer3 Input (version 0.4.0) software. For PCR with primer set gaA2F/gaA2R, reaction mixture 20, 50 or 100 µl consisted of 1X PCR buffer (Banglore Genei, Banglore), 200-µM concentration of deoxynucleoside triphosphate mix (Banglore Genei, Banglore), a 0.25 µM concentration of each primer (Banglore Genei, Banglore), 50 ng bacterial DNA, and 0.04 U µl⁻¹ Taq DNA polymerase (Banglore Genei, Banglore). The PCR cycling conditions used in this study for *Lactobacillus* included initial denaturation at 95°C for 3 min and 35 cycles each of denaturation at 94°C for 1 min followed by annealing at 61°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. The results obtained showed a specific amplification product of 180 bp for the isolates along with positive control (Plate 4.14). Six of the bacteriocinogenic isolates of *L. gasseri* NBL 18, NBL 25, NBL 154, NBL 386, NBL 388, NBL 389 were screened positive for *gaaA* gene through PCR.

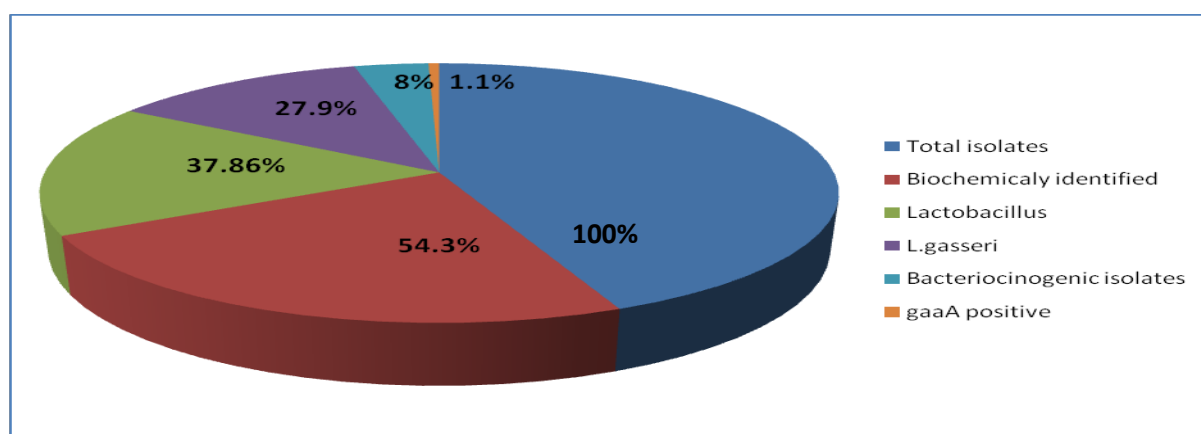


Fig.4.2. Pie chart showing the frequency occurrence of circular bacteriocin producing isolates (6) from the total isolates (523)

4.7. ANTIBACTERIAL SPECTRA OF BACTERIOCINOGENIC (*gaaA*+) *L. GASSERI* ISOLATES

Out of the 42 bacteriocinogenic *L. gasseri* isolates, six isolates, (14.28%) were found to be positive for *gaaA* gene and quite potent and stable producers of bacteriocins. Therefore, for further studies, six potent and stable bacteriocin producers were used and their antibacterial spectra were determined against a large number of lactic acid and non-lactic acid bacteria by spot-on-lawn assay (Table 4.4). Gram-negative pathogenic bacteria viz., *Shigella dysenteriae* NCDC 107, *Salmonella typhi* NCDC 113 and *Pseudomonas aeruginosa* NCDC 104 were also included in the study, but none of these Gram negative organisms were found to be susceptible to the potent bacteriocins of these six *Lactobacillus* isolates.

All the six isolates of *L.gasseri* inhibited *Lactobacillus brevis*, *Lactobacillus acidophilus* and *Enterococcus faecalis* strongly, but showed weak or no inhibition against other LAB tested. Inhibition of pathogens was the most prevalent type of antagonism as all the bacteriocinogenic isolates showed inhibition against *Listeria monocytogenes*, *Micrococcus luteus* and *Staphylococcus aureus*. Amongst all the pathogens tested *L. monocytogenes*, *S. aureus* and *E. faecalis* were found to be most sensitive to the antagonistic activity of the selected bacteriocinogenic strains.

GassericinA has been described as acting in the cytoplasmic membrane of target cells and causing the death of the cells via an efflux of potassium ions (Kawai *et al.*, 2004b). Its inhibition spectra has been reported against a number of Gram-positive, foodborne, pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, but not against Gram-negative organisms (Itoh *et al.*, 1995; Kawai *et al.*, 2004b). Acidocin B (58 amino acids) is another hydrophobic class V bacteriocin produced by *Lactobacillus acidophilus* M46, which is active against *Listeria monocytogenes*, *Clostridium sporogenes*, *Brochothrix thermosphacta*, *Lactobacillus fermentum* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, but inactive against most other *Lactobacillus species* (Leer *et al.*, 1995). According to in silico analysis, although this peptide was not identified as a circular protein (Leer *et al.*, 1995) it might be considered to be a natural variant of gassericin A (Kawai *et al.*, 2004a) because of its high sequence similarity (98%), only differing in one residue (M24 V).

Table 4.4. Antibacterial spectrum of activity of *Lactobacillus gasseri* isolates positive for *gaaA* gene by spot on lawn method

Bacterial species	Strains and Source	NBL18	NBL25	NBL154	NBL386	NBL388	NBL389
Gram-positive							
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NCDC 86, Karnal	+	+	–	+	+	+
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NCDC 278, Karnal	+	+	+	+	+	+
<i>Lactobacillus casei</i> subsp. <i>casei</i>	NCDC 017, Karnal	+	+	+	–	+	+
<i>Lactobacillus brevis</i>	NCDC 001, Karnal	++	++	–	–	++	+
<i>Lactobacillus acidophilus</i>	NCDC 291, Karnal	++	+	–	+	–	–
<i>Lactococcus cremoris</i>	NCDC 060, Karnal	+	+	+	+	+	+
<i>Lactobacillus helveticus</i>	NCDC 005, Karnal	+	+	+	+	+	+
<i>Lactobacillus fermentum</i>	NCDC 156, Karnal	+	+	+	+	+	+
<i>Lactobacillus rhamnosus</i>	NCDC 018, Karnal	+	+	+	+	+	+
<i>Lactobacillus plantarum</i>	NCDC 020, Karnal	+	++	+	+	+	+
<i>Lactobacillus paracasei</i>	NCDC 063, Karnal	+	+	–	+	+	+

<i>Streptococcus thermophilus</i>	NCDC 074, Karnal	+	-	-	-	+	-
<i>Streptococcus thermophilus</i>	NCDC 117, Karnal	+	-	-	-	+	-
<i>Streptococcus thermophilus</i>	NCDC 80, Karnal	+	-	-	-	-	-
<i>Pediococcus acidilactici</i>	LB-42, USA	++	++	-	++	++	+
<i>Enterococcus faecalis</i>	NCDC 114, Karnal	++++	++	+++	+++	++++	++++
<i>Enterococcus faecalis</i>	NCDC 115, Karnal	++++	+++	+++	++++	++++	++++
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	++++	+++	++	++	++	++
<i>Listeria monocytogenes</i>	ATCC 15303	++++	++	++	+	+	++
<i>Micrococcus luteus</i>	NCDC 131, Karnal	+++	+	++++	+	++	++
Non-lactic Bacteria							
<i>E. coli</i>	ATCC 25922	-	-				
<i>Staphylococcus aureus</i>	NCDC 273, karnal	++++	+	++	++	++	++
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	++++	+	++	++	+++	+++
<i>Klebsiella pneumoniae</i>	NCDC 138	-	-	-	-	-	-
<i>B. subtilis</i>	NCDC 70	+++	++	++	++	+	++
<i>Salmonella typhi</i>	NCDC 113, Karnal	-	-	-	-	-	-

<i>Shigella dysenteriae</i>	NCDC 107, Karnal	–	–	–	–	–	–
<i>E. coli</i>	NCDC 134, Karnal	–	–	–	–	–	–
<i>Pseudomonas aeruginosa</i>	NCDC 104, Karnal	–	–	–	–	–	–
Yeast							
<i>Rhodotorula glutinis</i>	NCDC 51, Karnal	–	–	–	–	–	–
<i>Saccharomyces cerevisiae</i>	NCDC 47, Karnal	–	–	–	–	–	–
<i>Torulopsis candida</i>	NCDC 43, Karnal	–	–	–	–	–	–
<i>Candida guilliermondii</i>	NCDC 44, Karnal	–	–	–	–	–	–
<i>Saccharomyces cerevisiae</i>	NCDC 50, Karnal	–	–	–	–	–	–

More than 15mm= +++; 13-15mm= ++; 10-13mm= ++; Less than 10= +; No inhibition= -

Our findings are very much in accordance with the bacteriocins of other lactic acid bacteria (LAB) that are mainly active against Gram-positive organisms but cannot penetrate through the lipopolysaccharide layer of Gram-negative bacteria (Aymerich *et al.*, 1996; Nes *et al.*, 1996; Franz *et al.*, 2003). Toba *et al.*, 1991 examined the antimicrobial activity of gassericin A and found that it inhibited the growth of *L. acidophilus* JCM 2125, *L. delbrueckii* subsp. bulgaricus JCM 1002, *L. delbrueckii* subsp. lactis JCM 1248 and JCM 1148, but was not active against heterofermentative lactobacilli. Neutralized cell-free supernatant fluid of *L. gasseri* did not inhibit the growth of *E. coli* and other Gram negative organisms.

Importantly, LAB showing a strong activity against *Listeria* and *Staphylococcus* spp. can be of practical use in the food industry and strains possessing such properties can be used as starter cultures and/or protective cultures. The bacteriocinogenic isolates antagonistic to *Listeria monocytogenes* but insensitive or less sensitive to other LAB may be successfully exploited as co-cultures or protective cultures.

4.8. SEQUENCING OF PCR PRODUCT

The sequencing was performed for *Lactobacillus gasseri* NBL 18 (16S-rRNA gene), the strain showing the best antimicrobial spectrum of activity and the PCR product of *gaaA* gene of *L. gasseri* NBL 18. The primers used for 16S-rRNA of *L.gasseri* NBL 18 was Lgas2 (forward) and Lgas3 (reverse) and that for *gaaA* gene of *L.gasseri* NBL18 was gaA2F (forward) and gaA2R (reverse). The sequences of both the genes were aligned properly to get a clean readable sequence of 353 and 179 bp respectively. These sequences of *L.gasseri* NBL 18 have been submitted to Genbank with Accession No. JQ809334

On BLAST search of 16srRNA gene of *L.gasseri* NBL 18, a homology of 98% at the DNA level was obtained with the nucleotide sequence of available gene of *L.gasseri* isolate L1, and *L.gasseri* ATCC33323 with Accession No. DQ445775.1 and CP000413.1 respectively. *L.gasseri* strain with accession number AF182721.1 showed 97% sequence homology with the strain NBL 18, whereas 95% sequence homology was observed with *Lactobacillus johnsonii* FI9785, *Lactobacillus delbrueckii* subsp. *lactis* strain:ATCC 4797, *Lactobacillus acidophilus* strain:ATCC 4356, *Lactobacillus johnsonii*

NCC 533, *Lactobacillus kefiranofaciens* ZW3 with accession number FN298497.1, AB092635.1, AB092634.1, AE017198.1 and CP002764.1 respectively. A sequence homology of 89% and 87% was obtained with the sequences of *Lactobacillus jensenii* (AB035486.1) and *Lactobacillus kefiranofaciens* ZW3 (CP002764.1) respectively, whereas 86% sequence homology was obtained with *Lactobacillus amylovorus* (strain GRL1118, GRL 1112) and *Lactobacillus acidophilus* (strain 30SC, isolate A4,GP1B,GP4A) with accession number CP002609.1, CP002338.1, CP002559.1, AB092632.1, AB092631.1 and AB092630.1 respectively.

CLUSTAL 2.1 multiple sequence alignment

```

Lamylovorus_AF182732.1_      -----GCAGTCCTACAAC
Lacidophilus_AB092632.1_    -----GCAGTCCTACAAC
LamylovorusGRL              -----GCAGTCCTACAAC
Lacidophilus30SC            -----GCAGTCCTACAAC
Lacidophilus_AB092631.1_    -----GCAGTCCTACAAC
LamylovorusGRL1118          -----GCAGTCCTACAAC
LkefiranofaciensZW3         -----TGCCTTATTGCAGTCCTACAAC
Ljensenii                   -----
L.gasseri                   -----TGCTATCGCTTCAAGTACTTTATTGCAGTCCTACTAC
LgasseriATCC                -----TGCTATCGCTTCAAGTGCCTTTCCTGCAGTCCTACTAC
Lgasseri16srRNA             -----TGCTATCACTTCAAGTACTATATCGCAGTCCTACAAC
Lacidophilus16srRNA_AB092634.1 -----TGCTATCACTTCAAGTACTATATCGCAGTCCTACAAC
LjohnsoniINCC               -----TGCTATCACTTCAAGTACTATATCGCAGTCCTACAAC
Lactobacillus               GCGACCGAGAAGAGAGAGAACTCAACTTGAAATAGGTCAAGTAGAAAAG

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Lamylovorus_AF182732.1_      CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
Lacidophilus_AB092632.1_    CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
LamylovorusGRL              CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
Lacidophilus30SC            CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
Lacidophilus_AB092631.1_    CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
LamylovorusGRL1118          CCCAAATGATAAATCA-CTTGGT-TTGGGCTCTTTCCTC-T----TCGCT
LkefiranofaciensZW3         CCCAATTGATAAATCA-ACTGGT-TTGGGCTCTTTCCTC-T----TCGCT
Ljensenii                   -----TGGT-TTGGGCTCTTTCCTC-T----TCGCT
L.gasseri                   CCCAACTGGCAAGCCA-GCTGGT-TTGGGCTCTTTCCTG-T----TCGCT
LgasseriATCC                CCCAACTGGCAAGCCA-GCTGGT-TTGGGCTCTTTCCTG-T----TCGCT
Lgasseri16srRNA             CCCAACTGGCAAGCCA-GCTGGT-TTGGGCTCTTTCCTG-T----TCGCT
Lacidophilus16srRNA_AB092634.1 CCCAACTGGCAAGCCA-GCTGGT-TTGGGCTCTTTCCTG-T----TCGCT
LjohnsoniINCC               CCCAACTGGCAAGCCA-GTTGGT-TTGGGCTCTTTCCTG-T----TCGCT
Lactobacillus               GGCGCATGGTGAATGCCTGAGGCACTAGAAGCCGATGAAGTGACGTGACG
                                **  *  *  *  *  *  *

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Lamylovorus_AF182732.1_      CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lacidophilus_AB092632.1_    CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
LamylovorusGRL              CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lacidophilus30SC            CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lacidophilus_AB092631.1_    CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
LamylovorusGRL1118          CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
LkefiranofaciensZW3         CGCCGCTA----CTAAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Ljensenii                   CGCCGCTA----CTTAGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
L.gasseri                   CGCCGCTA----CTTGGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
LgasseriATCC                CGCCGCTA----CTTGGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lgasseri16srRNA             CGCCGCTA----CTTGGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lacidophilus16srRNA_AB092634.1 CGCCGCTA----CTTGGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
LjohnsoniINCC               CGCCGCTA----CTTGGGAAATCGATTTTTCTTCTCTTCTCCTGCAGCTAC
Lactobacillus               AACCACGAAAGGCTTCGGGGAGCAGTAAGTAAGCGATGATCCGGAGATAT
                                * * *  * * *  *  *  *  *  *  *

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Lamylovorus_AF182732.1_      TTAGATGTTTCAGTTCA---CTGCGTATTCCTTTGAAT-GACT-ATCTAT
Lacidophilus_AB092632.1_    TTAGATGTTTCAGTTCA---CTGCGTATTCCTTTGAAT-GACT-ATCTAT
LamylovorusGRL              TTAGATGTTTCAGTTCA---CTGCGTATTCCTTTGAAT-GACT-ATCTAT
Lacidophilus30SC            TTAGATGTTTCAGTTCA---CTGCGTATTCCTTTGAAT-GACT-ATCTAT

```


LjohnsoniiNCC
Lactobacillus

TTTTCTACTTGACCTATTCAAGTTGAGTTTCTCTCTCTCTCGGTGCG
TAAAGCACTTGAAGCATAGCAA-----
* * * *

Fig.4.3. Multiple nucleotide sequence alignment of 16srRNA gene partial of *Lactobacillus gasseri* NBL 18 with reported sequences of *Lactobacillus* sps.

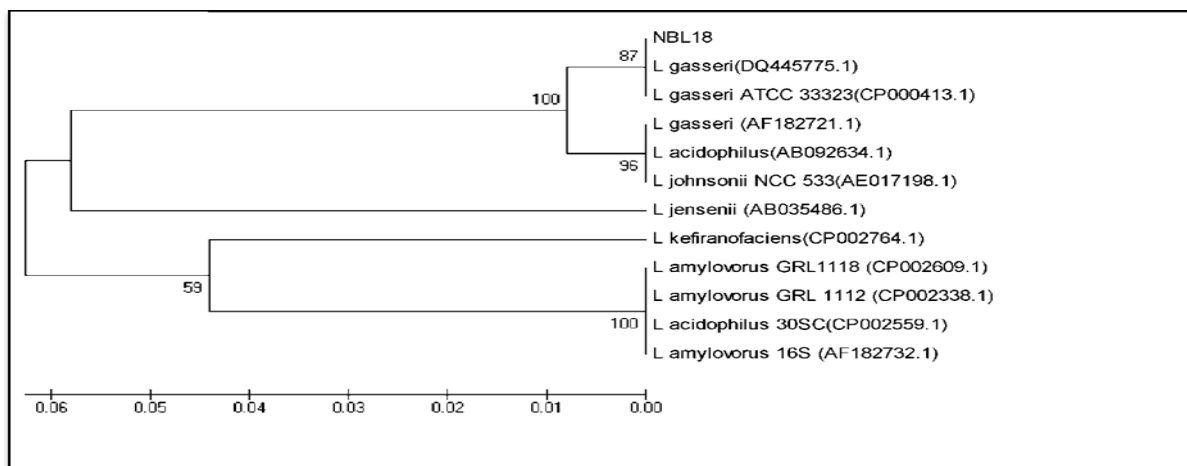


Fig 4.4. UPGMA based tree showing relationship among nucleotide sequence of isolates NBL 18 with reported sequences of *Lactobacillus gasseri* and other *Lactobacillus* sps.

The sequence similarity search (Blast, NCBI), of *gaaA* gene of *L.gasseri* NBL 18 showed 100% homology with the nucleotide sequence *Lactobacillus gasseri* plasmid pLgLA39 DNA and *Lactobacillus gasseri* *gaaB*, *gaaC*, *gaaA*, *gaaD*, *gaaI*, *gaaT*, *gaaE* genes, with accession number AB436615.1 and AB007043.2 respectively. A sequence homology of 99% was obtained with the submitted sequence of *L. acidophilus* *acdB* gene for acidocin B with accession number Z34920.1.

CLUSTAL 2.1 multiple sequence alignment

```
gassericin>      GGGCGGTTTTAGTAGTTGCTCTTTTATTGACCACAGCGAACATTTATTGGATTGCTGATC
pLgLA39>         GGGCGGTTTTAGTAGTTGCTCTTTTATTGACCACAGCGAACATTTATTGGATTGCTGATC
gaaBCADITE>      GGGCGGTTTTAGTAGTTGCTCTTTTATTGACCACAGCGAACATTTATTGGATTGCTGATC
acidocinB>       GGGCGGTTTTAGTAGTTGCTCTTTTATTGGCCACAGCGAACATTTATTGGATTGCTGATC
*****.*****
```

```
gassericin>      AATTCGGGATTCATTTAGCGACTGGAACAGCCCGTAAGTTATTAGATGCAATGGCTTCTG
pLgLA39>         AATTCGGGATTCATTTAGCGACTGGAACAGCCCGTAAGTTATTAGATGCAATGGCTTCTG
gaaBCADITE>      AATTCGGGATTCATTTAGCGACTGGAACAGCCCGTAAGTTATTAGATGCAATGGCTTCTG
acidocinB>       AATTCGGGATTCATTTAGCGACTGGAACAGCCCGTAAGTTATTAGATGCAATGGCTTCTG
*****.*****
```

```
gassericin>      GTGCCTCATTGGGAAGTGCCTTTGCTGCTATTTTGGGCGTGACATTACCTGCATGGGCTT
pLgLA39>         GTGCCTCATTGGGAAGTGCCTTTGCTGCTATTTTGGGCGTGACATTACCTGCATGGGCTT
gaaBCADITE>      GTGCCTCATTGGGAAGTGCCTTTGCTGCTATTTTGGGCGTGACATTACCTGCATGGGCTT
```

```
acidocinB>      GTGCCTCATTGGGAAGTGCCTTTGCTGCTATTTTGGGCGTGACATTACCTGCATGGGCTT
                *****

gassericin>      -----
pLgLA39>         -----
gaaBCADITE>      -----
acidocinB>      TGGCAGCTGCAGGAGCATTGGGAGCGACTG
```

Fig. 4.5. Multiple nucleotide sequence alignment of *gaaA* gene of *L. gasseri* NBL 18 with reported sequences of gassericin A and acidocinB

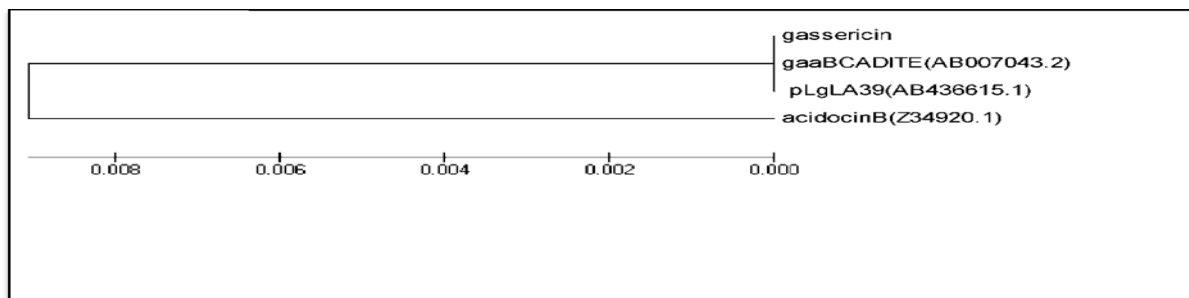


Fig.4.6. UPGMA method based tree showing relationship among nucleotide sequence of *gaaA* gene of *L. gasseri* NBL 18 with reported sequences

4.9. RESPONSE SURFACE OPTIMIZATION OF THE CULTIVATION CONDITIONS FOR MAXIMAL BACTERIOCIN PRODUCTION BY *LACTOBACILLUS GASSERI* NBL 18

The optimization of bacteriocin production was carried out using Central Composite Rotatable Design (CCRD) in realistic vicinity to locate the true optimal value of multiple compositional variables. Besides optimization, combined effect of these variables on bacteriocin production was demonstrated. The central values of four factors/variables i.e. pH (6), incubation temperature (39.5), inoculation level (2), incubation time (15) were coded as A, B, C and D respectively. A total of 30 experiments were carried out in randomized order which include; 16 factorial, 8 star and 6 replicate points. It was assumed that response (y) is a function of experimental factors (A, B, C, D) or $y = f(A, B, C, D)$. Second order polynomial models were developed by multiple regression technique for each of the response using software package, Design Expert (Trial Version 8.0.7.1).

$$y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD + \epsilon$$

where β_0 is the intercept, $\beta_1, \beta_2, \beta_3, \beta_4$ are the first order coefficients, $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$ are cross product coefficients, and $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}$ are the second order coefficients.

The design matrix representing different combinations of the four factors along with responses are delineated in Table. 4.5. Regression coefficient and ANOVA of fitted quadratic model for bacteriocin production are shown in Table. 4.6. The results of the RSM experiments are discussed hereunder.

Table 4.5. Bacteriocin activity of the culture NBL 18 cultivated with different levels of pH, Temperature, Inoculum level and Incubation time

S.N.	Factors				Responses		
	pH	Temperature	Inoculum level	Time	pH	OD	Bacteriocin activity
	(A)	(B)	(C)	(D)			($\times 10^4$ AU/ml)
1	4.00	37.00	3.00	24.00	3.18	0.79	0
2	8.00	42.00	1.00	24.00	5.73	4.17	1.28
3	4.00	42.00	3.00	6.00	3.84	0.21	0
4	6.00	39.50	2.00	15.00	5.23	2.04	0.16
5	8.00	42.00	3.00	24.00	5.48	4.25	1.28
6	4.00	37.00	3.00	6.00	3.95	0.23	0
7	2.00	39.50	2.00	15.00	1.95	0.12	0
8	4.00	42.00	3.00	24.00	3.64	0.93	0
9	8.00	37.00	3.00	6.00	5.54	3.24	0.32
10	6.00	39.50	4.00	15.00	4.89	3.78	0.32
11	4.00	42.00	1.00	6.00	3.93	0.11	0
12	6.00	39.50	2.00	15.00	5.23	2.04	0.16
13	6.00	34.50	2.00	15.00	5.45	1.83	0.08
14	6.00	39.50	2.00	15.00	5.23	2.04	0.16
15	4.00	42.00	1.00	24.00	3.72	0.85	0
16	4.00	37.00	1.00	24.00	3.61	0.98	0
17	4.00	37.00	1.00	6.00	3.97	0.14	0
18	8.00	42.00	1.00	6.00	5.67	3.1	0.32
19	6.00	39.50	2.00	-3.00	6	0.01	0
20	8.00	37.00	1.00	24.00	5.43	5.07	2.56
21	8.00	37.00	3.00	24.00	5.01	5.72	2.56

22	8.00	42.00	3.00	6.00	5.67	3.66	0.32
23	6.00	44.50	2.00	15.00	4.97	2.45	0.32
24	6.00	39.50	2.00	33.00	4.76	3.73	0.64
25	6.00	39.50	2.00	15.00	5.23	2.04	0.16
26	6.00	39.50	2.00	15.00	5.23	2.04	0.16
27	6.00	39.50	2.00	15.00	5.23	2.04	0.16
28	6.00	39.50	0.00	15.00	6	0	0
29	8.00	37.00	1.00	6.00	7.14	2.92	0.32
30	10.00	39.50	2.00	15.00	9.85	0.37	0

Table 4.6. Regression coefficients and ANOVA of fitted quadratic model for maximum bacteriocin production

Partial coefficients	pH	OD	Bacteriocin activity
Intercept	5.23	2.04	1.60
A-pH	1.32**	1.18**	3.73**
B-Incubation temperature	-0.046	-0.024	-0.87
C-Inoculation level	-0.21	0.39	0.27**
D- Incubation time	-0.27	0.69*	3.20
A ²	0.040	-0.33	0.25
B ²	-0.13	0.15	0.75
C ²	-0.074	0.085	0.65
D ²	-0.090	0.080	1.05
AB	-0.062	-0.11	-1.60
AC	-0.10	0.096	0.00
AD	-0.052	0.21	4.00**
BC	0.13	-3.25X10 ⁻³	0.00
BD	0.18	-0.18	-1.60
CD	0.033	-0.028	0.00
Model F value	5.48**	2.50*	3.02*
R ²	0.84	0.70	0.74
APV	9.58	7.4	7.16
Lack of fit	NS	NS	NS

**Significant at 1% level (P<0.01)

*Significant at 5% level (P<0.05)

4.10.EFFECT OF DIFFERENT LEVELS OF PH, TEMPERATURE, INOCULUM LEVEL AND INCUBATION TIME ON OPTIMAL BACTERIOCIN PRODUCTION

4.10.1. Diagnostic Check of the Quadratic Model

The quadratic model for various responses, namely pH, Optical Density and Bacteriocin activity (AU/ml) was obtained through successive regression analysis. The dependence of these responses with respect to levels of four factors (pH, Temperature, Inoculum level and Incubation time) in the form of correlation is presented in Table. 4.6. The model F values for all attributes were more than the Table F values at 5% level of confidence and it indicated the significance of model terms. The lack of fit test, which measure the fitness of the model obtained, did not result in a significant F value, indicating that the model is sufficiently accurate for predicting the bacteriocin production by *L. gasser* NBL 18 from any combination of factor levels within the range evaluated.

4.10.2. Effect of pH, Incubation Temperature, Inoculum Level and Incubation Time on pH

The average pH after growth varied from 1.95 to 9.85 (Table 4.5). The minimum value of pH was found in the bacteriocin produced with combination of Experiment No. 7, whereas, the bacteriocin produced with the combination of Exp. No. 30 had the highest pH. The regression analysis of the data presented in Table 4.6 reveals that the coefficient of determination (R^2) was 0.84. The ANOVA of quadratic model indicated that model F value of 5.48 was more than the tabulated F value. Furthermore the adequate precision value (APV) was found to be 9.58, which was higher than the minimum desirable (4) for high prediction ability. All this showed that the model can be used to describe the effect of variables on pH.

pH after growth of NBL 18 was highly significantly ($p < 0.01$) positively affected by the pH of broth, negatively affected by incubation time, incubation temperature and inoculums level at linear level, but statistically non-significant. At quadratic level, pH was having positive effect and incubation temperature, incubation time and inoculum level had negative effect, but statistically non-significant. Interaction of pH and incubation temperature, pH and inoculum level and pH and incubation time had negative effect, whereas incubation temperature and inoculum level, incubation temperature and time and inoculum level and incubation time had positive effect, but statistically non-significant. Multiple regression equation generated to predict the

bacteriocin production as affected by different factors in terms of actual factors is as follows:

$$\begin{aligned} \text{pH} = & -25.35219 + 1.17381 * \text{pH} + 1.50882 * \text{Temperature} - 1.68854 * \text{Inoculum level} \\ & - 0.29687 * \text{Incubation time} - 0.012375 * \text{pH} * \text{Temperature} - 0.051562 * \text{pH} * \text{Inoculum} \\ & \text{level} \\ & - 2.88194\text{E-}003 * \text{pH} * \text{Incubation time} + 0.051250 * \text{Temperature} * \text{Inoculum level} + \\ & 7.86111\text{E-}003 * \text{Temperature} * \text{Incubation time} + 3.68056\text{E-}003 * \text{Inoculum level} * \\ & \text{Incubation time} + \\ & 0.010026 * \text{pH}^2 - 0.021183 * \text{Temperature}^2 - 0.073646 * \text{Inoculum level}^2 - 1.10983\text{E-} \\ & 003 * \text{Incubation time}^2 \end{aligned}$$

4.10.3. Effect of pH, Incubation Time, Inoculum Level and Incubation Temperature on OD

The average OD after growth varied from 0.00 to 5.72 (Table. 4.5). The minimum value of OD was found in the bacteriocin produced with combination of Experiment No.28, whereas, the bacteriocin produced with the combination of Exp. No. 21 had the highest overall acceptability score.

The regression analysis of the data presented in Table 4.6 reveals that the coefficient of determination (R^2) was 0.70. The ANOVA of quadratic model indicated that

model F value of 2.50 was more than tabulated F value. Furthermore the adequate precision value (APV) was found to be 7.4, which was higher than the minimum desirable (4) for high prediction ability. All this showed that the model can be used to describe the effect of variables on maximum bacteriocin production.

Optical density after growth was highly significantly positively ($p < 0.01$) affected by pH and positively affected by incubation time at linear level. It was negatively affected by temperature and positively affected by inoculum level, but statistically non significant at linear level. At quadratic level, pH has negative effect, whereas, temperature, inoculum level and incubation time had positive effect, but all are non-significant. The interactive effect of all parameters was found to be non-significant. Multiple regression equation generated to predict the bacteriocin production as affected by different factors in terms of actual factors is as follows:

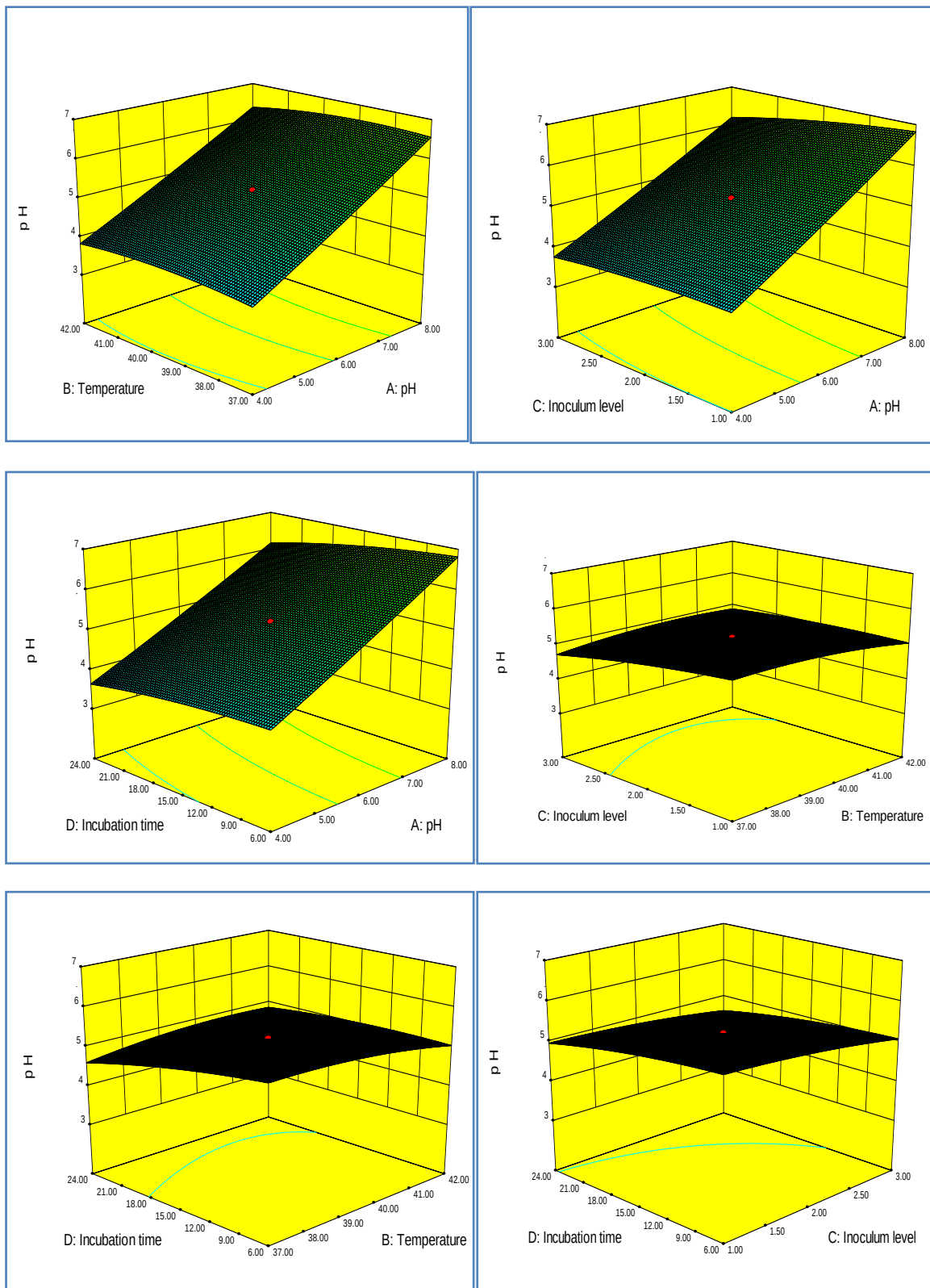


Fig. 4.7. Response surface curves for pH as influenced by the level of pH, incubation temperature, inoculums level and incubation time

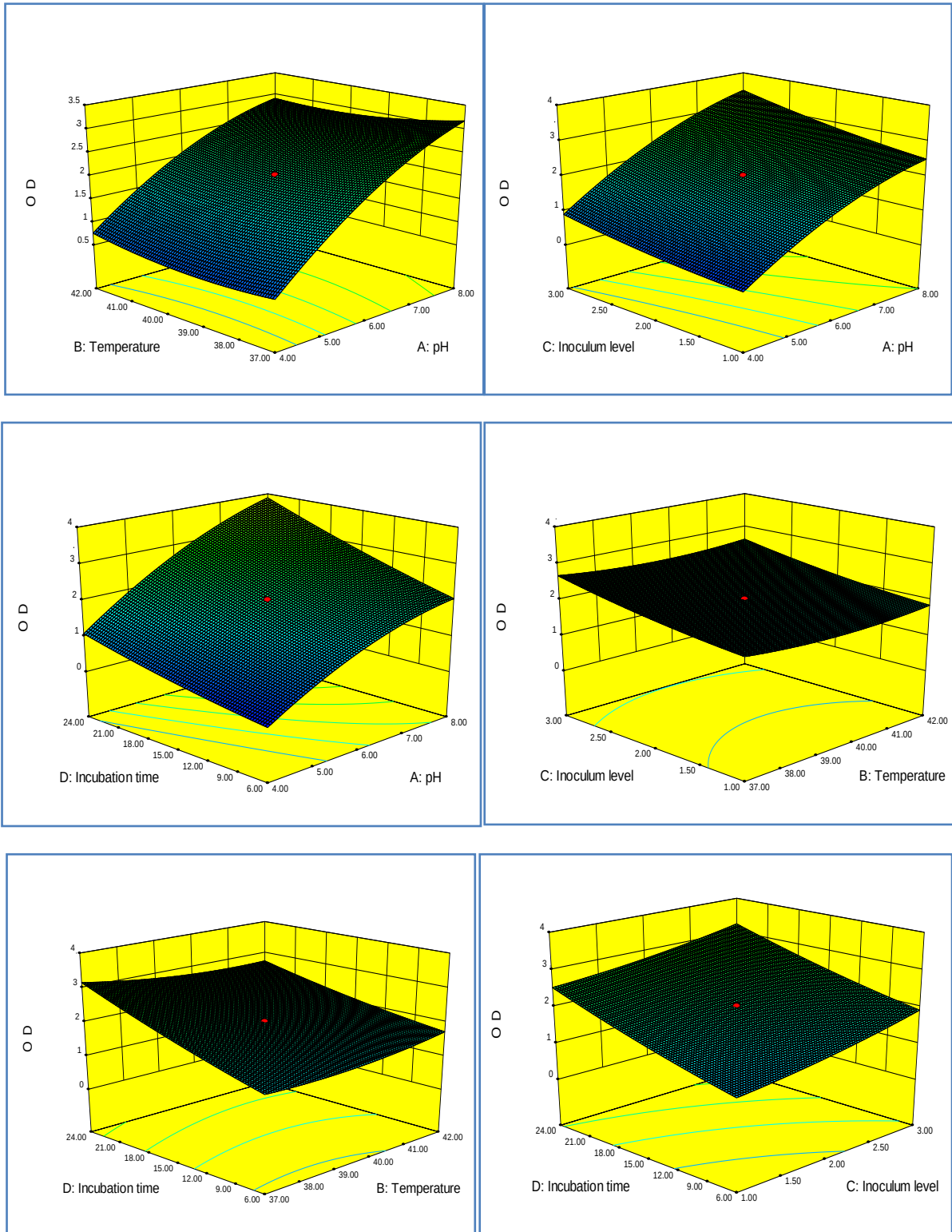


Fig. 4.8. Response surface curves for Optical Density as influenced by the level of pH, incubation temperature, inoculums level and incubation time

$$\begin{aligned} \text{OD} = & + 23.01302 + 2.14919 * \text{pH} - 1.62435 * \text{Temperature} - 0.14646 * \text{Inoculum level} \\ & + 0.30114 * \text{Incubation time} - 0.021625 * \text{pH} * \text{Temperature} + 0.047813 * \text{pH} * \text{Inoculum} \\ & \text{level} + 0.011910 * \text{pH} * \text{Incubation time} - 1.25000\text{E-}003 * \text{Temperature} * \text{Inoculum level} - \\ & 8.08333\text{E-}003 * \text{Temperature} * \text{Incubation time} - 3.12500\text{E-}003 * \text{Inoculum level} * \\ & \text{Incubation time} - 0.081484 * \text{pH}^2 + 0.023650 * \text{Temperature}^2 + 0.085313 * \text{Inoculum} \\ & \text{level}^2 + 9.91512\text{E-}004 * \text{Incubation time}^2 \end{aligned}$$

4.10.4. Effect of pH, Incubation Time, Inoculum Level and Incubation Temperature on Bacteriocin Activity

The average bacteriocin activity varied from 0 to 2.56×10^4 AU/ml (Table. 4.5). The minimum value was found in the bacteriocin produced with combination of Experiment No. 1, 3, 6, 7, 8, 11, 15, 16, 17, 19, 28 and 30, whereas, the bacteriocin produced with the combination of Exp. No. 20 and 21 had the highest overall acceptability score.

The regression analysis of the data presented in Table 4.6 reveals that the coefficient of determination (R^2) was 0.74. The ANOVA of quadratic model indicated that model F value of 3.02 was more than tabulated F value. Furthermore the adequate precision value (APV) was found to be 7.16, which was higher than the minimum desirable (4) for high prediction ability. All these results showed that the model can be used to describe the effect of variables on maximum bacteriocin production.

Bacteriocin activity after growth was highly significantly positively ($p < 0.01$) affected by pH of the broth and inoculum level and negatively affected by incubation temperature and positively affected by incubation time, but statistically non significant at linear level. At quadratic level, all parameter had positive effect, but all are non-significant. The interactive effect of pH* incubation time had highly positive significant effect, pH and incubation temperature, and incubation temperature and incubation time had negative effect, but statistically non-significant. Other parameters were found to have no interactive effect.

Multiple regression equation generated to predict the bacteriocin production as affected by different factors in terms of actual factors is as follows:

$$\begin{aligned} \text{Bacteriocin activity} = & + 95.25000 + 10.42333 * \text{pH} - 6.84000 * \text{Temperature} - 2.33333 * \\ & \text{Inoculum level} + 1.44222 * \text{Incubation time} - 0.32000 * \text{pH} * \text{Temperature} + 3.24740\text{E-} \\ & 015 * \text{pH} * \text{Inoculum level} + 0.22222 * \text{pH} * \text{Incubation time} - 2.66454\text{E-}015 * \text{Temperature} \\ & * \text{Inoculum level} - 0.071111 * \text{Temperature} * \text{Incubation time} - 2.59052\text{E-}016 * \text{Inoculum} \end{aligned}$$

level * Incubation time +0.062500 * pH²+0.12000 * Temperature² +0.65000* Inoculum level²+0.012963* Incubation time²

4.11. OPTIMIZATION OF pH, INCUBATION TEMPERATURE, INOCULATION LEVEL AND INCUBATION TIME ON BACTERIOICIN PRODUCTION

The optimization of levels of pH, incubation temperature, inoculation level and incubation time was attempted using CCRD response surface design and conditions were set as presented in Table 4.7.

Table 4.7. Conditions during optimization of bacteriocin production in CCRD

Name	Goal	Low limit	Upper limit
pH	In range	4	8
Incubation temperature	In range	37	42
Inoculation level	In range	1	3
Incubation time	In range	6	24
pH	In range	1.95	9.85
OD	In range	0.00	5.72
Bacteriocin activity	Maximize	0	25.6

The optimum solution obtained as a result of numerical optimization was verified and the optimum level of pH (8.00), incubation temperature (37°C), inoculation level (3%) and incubation time (24h) were used for maximum bacteriocin production. The actual values of the optimization were compared with the predicted values given by the software using t-test as shown in Table 4.8. The t-test indicated that there were no significant differences between the predicted and the observed values of bacteriocin produced. This indicated that the model was significant and fitted to the data perfectly, so the bacteriocin produced was maximum from possible combinations of variables.

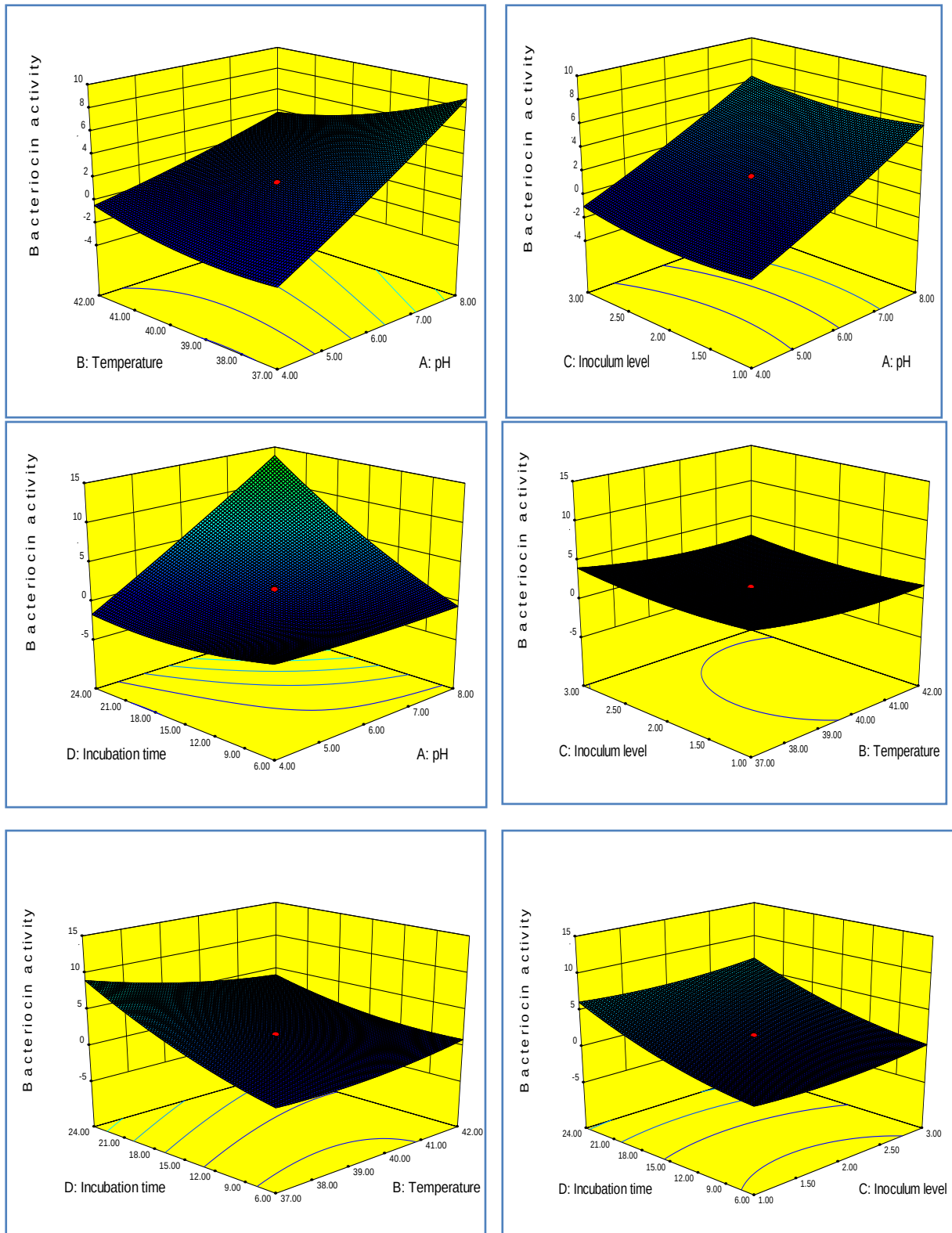


Fig. 4.9. Response surface curves for bacteriocin activity as influenced by the level of pH, incubation temperature, inoculum level and incubation time

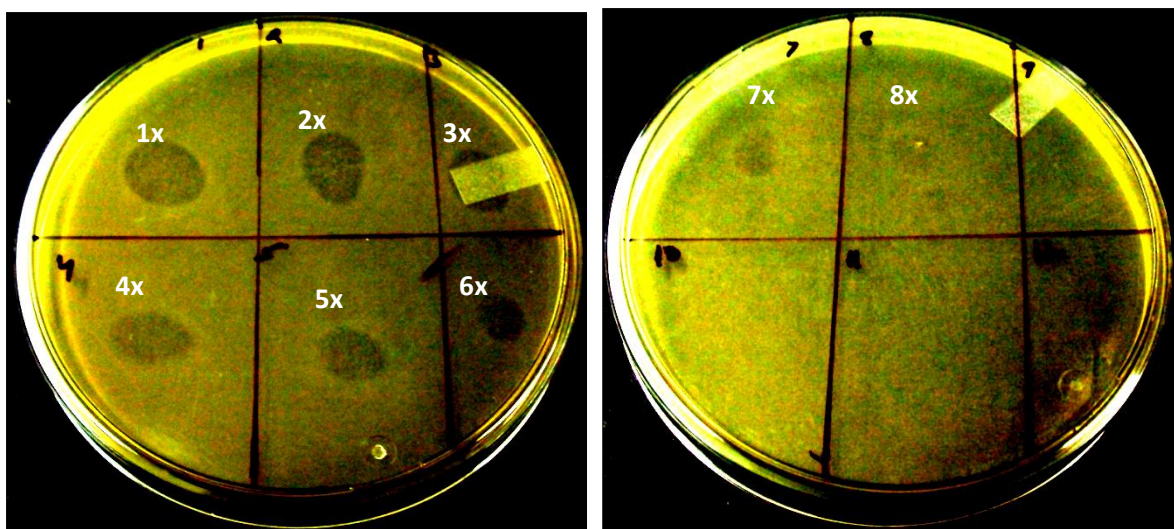


Plate 4.15. Bacteriocin activity of supernatant (CFCS of *L. gasseri* NBL 18 adjusted to pH 6.5) by spot on lawn assay

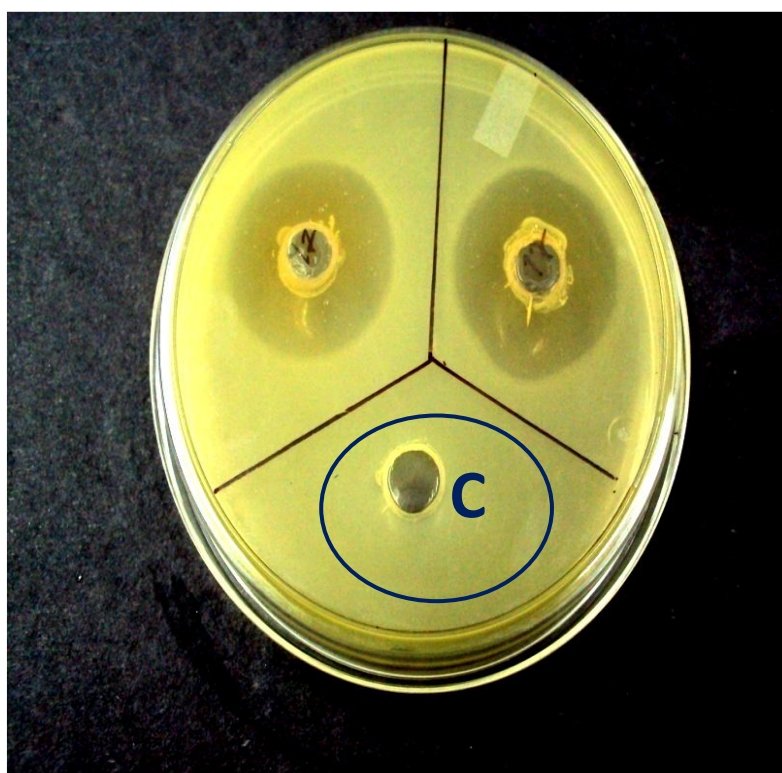


Plate 4.16. Antibacterial activity of partially purified bacteriocin by agar well assay as compared to control (C) containing 60% saturated solution of ammonium sulfate

Table 4.8. Optimized values as compared to predicted values

Responses	Predicted score*	Observed score@	t-value#
pH	5.49	5.53	0.62 ^{NS}
OD	4.88	4.84	1.88 ^{NS}
Bacteriocin production	19.56	21.33	0.41 ^{NS}

*predicted values of Design Expert 8.0.7.1 package

@actual values (mean of three trials)

#p<0.05

^{NS}nonsignificant

4.12. PURIFICATION OF BACTERIOCIN PRODUCED BY *LACTOBACILLUS GASSERI* NBL 18

The purification of bacteriocin from the culture broth was attempted by different methods viz., ammonium sulphate precipitation followed by ion-exchange chromatography and RP-HPLC.

4.12.1. Ammonium Sulphate Precipitation

The results presented in the Table 4.9 show that the protein fraction precipitated between 0-60% ammonium sulphate saturation exhibited a recovery of 64% of the total bacteriocin activity. Ammonium sulphate saturation at higher levels i.e. 60-80% however, resulted in very low bacteriocin recovery (1.56%) besides decrease in specific activity. Almost three fold (2.65) increase in the purity of bacteriocin was observed after 60% saturation of the CFCS.

Ammonium sulphate precipitation is the most common and widely used method to concentrate the antibacterial proteins from the culture supernatants of bacteriocin producing lactic cultures and the procedure results in varying degrees of recovery and purity of different bacteriocins. However, the variations observed in the degree of purity among various bacteriocins of LAB upon concentration with ammonium sulphate is probably due to the differences in the concentration of contaminating proteins which lead to decrease in the specific activity of the bacteriocin preparations.

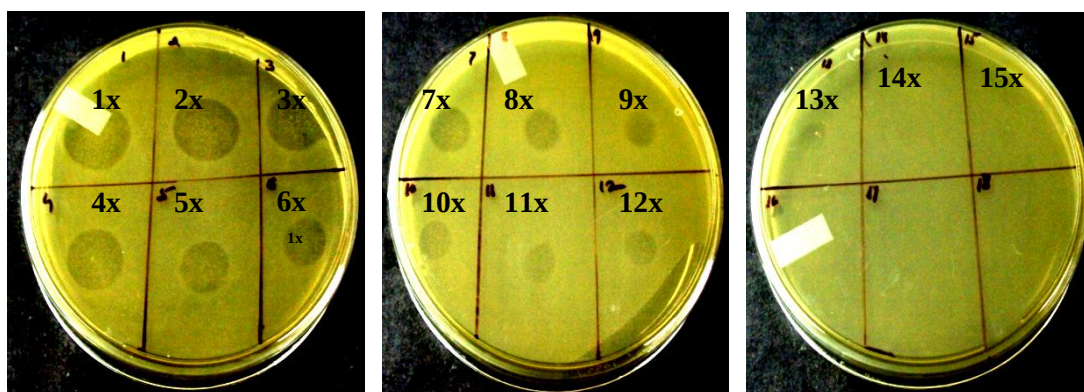


Plate 4.17. Bacteriocin activity of partially purified bacteriocin by spot on lawn assay

Table 4.9. Ammonium sulphate precipitation of bacteriocin

S.No.	Sample	Total activity ($\times 10^3$ AU/ml)	Total protein (mg)	Specific activity ^a (AU/mg)	Purification fold ^b	Yield ^c (%)
1	CFCS	25600	736.04	34780.71	1	100
2	ASP (0-60%)	16384	177.67	92215.90	2.65	64
3	ASP (60-80%)	400	55.30	7272.727	0.20	1.56

^a total activity units divided by total proteins,

^b purification fold relative to specific activity of culture supernatant

^c Activity Units ratios

4.12.2. Ion-Exchange Chromatography

4.12.2.1. Selection of pH of the buffer

The partially purified bacteriocin preparation of NBL 18 obtained after 60% ammonium sulphate precipitation were dissolved in various buffers such as acetate, phosphate, and Tris-HCl having different pH values of 4.4, 4.9 and 5.5; 6.5 and 7.0; and 7.5, 8.0 and 8.5, respectively retained 100% of activity both at refrigeration (5°C) as well as at room temperature (37°C) even after 3 days of storage.

The bacteriocin remained stable both at room as well as at refrigeration temperatures in all the buffers in the pH range 4-8 thus allowing the use of any

such buffer in the further purification studies. Kawai *et al.*, (1994), purified gassericin A with a DEAE-Toyopearl column that has been previously equilibrated with Tris-HCl (pH 8.5).

4.12.2.2. Selection of type of ion-exchanger

The partially purified bacteriocin obtained by ammonium sulphate precipitation was added to both cation and anion exchange resins equilibrated to different pH values in the 1.5 ml capacity Eppendorff tubes. It may be seen from the Table 4.10. that 66% of the total bacteriocin failed to absorb to cation exchanger (CM-Sephadex C-25) at all the pH values in range of 4-8 and remained in the supernatant. However, very less activity (0.04 %) of the initial activity of bacteriocin in supernatant of anion-exchanger slurry showed that most of the bacteriocin was bound by the anion-exchanger (DEAE-Sephadex A-25) equilibrated with Tris buffer (pH 8.5). This was further confirmed by the elution of bound bacteriocin from anion exchange matrix with 1M NaCl in the respective buffers. Further, 83% of bound bacteriocin could be eluted from anion-exchanger matrix and only 0.195% was eluted from the cation-exchange matrix (Table 4.10). Some activity of bacteriocin on elution in cation-exchanger observed may be due to the binding of few peptide molecules with positively charged component of crude media. The bacteriocin showed anionic nature as observed by their interaction with anion-exchange matrix.

Table. 4.10. Selection of matrix for ion-exchange chromatography

Resins used	Residual activity (AU/ml) in unbound fraction	Residual activity (AU/ml) in supernatant after elution with 1M NaCl
DEAE Sephadex A-25	1.6×10^3 ($\approx 0.04\%$)	2.73×10^6 ($\approx 83\%$)
CM Sephadex C-25	2.19×10^6 ($\approx 66\%$)	6.4×10^3 ($\approx 0.195\%$)

* Activity Unit of partially purified bacteriocin = 3.2768×10^6 AU/ml

In another attempt to select the column for ion exchange chromatography, the Isoelectric Point (pI) of gassericin A was calculated From ExPASy (SIB Bioinformatics Resource Portal) by submitting the reported amino acid sequence of Gassericin A and

was found to be 6.75. Since the pI of the peptide was lower than 7, it was considered to be anionic. The purification of bacteriocin by ion-exchange chromatography was, therefore, carried out by using QXL column (Hiload 16/10 Q Sepharose) equilibrated to starting pH 8.5 with Tris-HCl buffer, as per observations of their anionic nature obtained through these two preliminary experiments. The elution profile of bacteriocin with linear gradient of 1M NaCl is presented in Fig.4.10. Bacteriocin was eluted at higher ionic strength of the salt (>60% concentration of eluant). The recovery of the protein in terms of activity was quite less i.e. 0.31%.

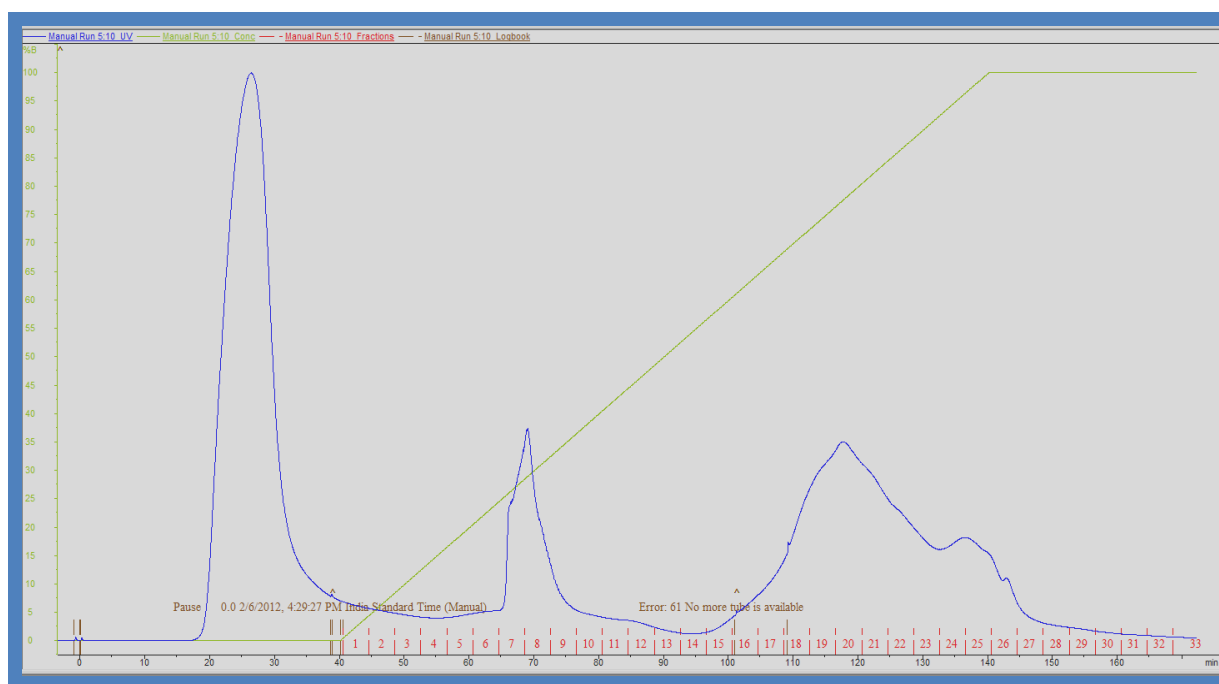


Fig. 4.10. Purification of bacteriocin by ion-exchange chromatography

The failure of the bacteriocins to adsorb onto the cation exchanger (CM-Sephadex C-25) indicated that the bacteriocin carries a net negative charge and such bacteriocin must be rich in acidic amino acid residues like aspartate and glutamate.

The circular bacteriocins are subdivided into two groups based on sequence similarity and biochemical characteristics (Cotter *et al.*, 2005; Martin-Visscher *et al.*, 2008): Group I consists of cationic peptides with high pI *viz.* enterocin AS-48, circularin A, uberolysin, lactocyclin Q, carnocyclin A and garvicin ML, whereas Group II consists of gassericin A and butyrivibriocin AR10. Peptides in group ii have more acidic

residues compared to Group I peptides and their isoelectric point is much lower. These peptides also share a significant sequence homology.

4.12.3. RP-HPLC Profile of bacteriocin

The partially purified bacteriocin obtained by ammonium sulphate precipitation and ion exchange chromatography was further subjected to reverse-phase HPLC. This chromatography revealed one major peak after 80 min but activity was observed in fractions collected after 80 to 110 min (Fig.4.11) (Plate 4.18). The total protein content was estimated to be 19.25 mg and total activity unit was estimated to be 4.4×10^4 . The bacteriocin was eluted with 85.2% of buffer B.

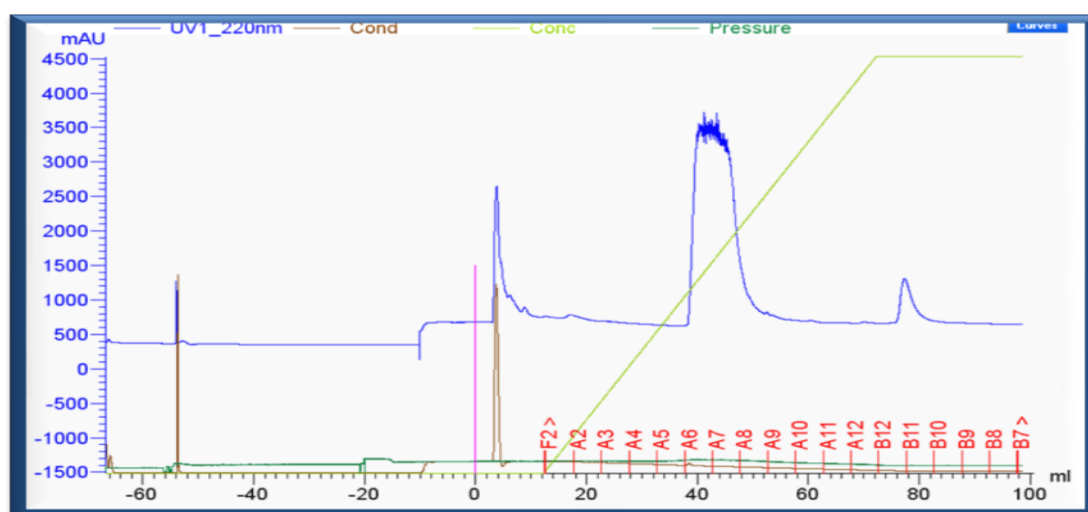


Fig. 4.11. Purification of bacteriocin by RP-HPLC

Table 4.11. Purification of bacteriocin from culture supernatant of NBL 18

S.No.	Sample	Total activity ($\times 10^3$)	Total protein (mg)	Specific activity ^a (AU/mg)	Purification fold ^b	Yield ^c (%)
1	CFCS	25600	736.04	34780.71	1	100
2	ASP (60%)	16384	177.67	92215.90	2.65	64
3	Ion-Exchange chromatography	80	30.00	2666.66	0.076	0.312
4	RP-HPLC	44	19.25	2285.71	0.065	0.171

^a total activity units divided by total proteins

^b purification fold relative to specific activity of culture supernatant

^c Activity Units ratios

In the present study it was observed that the inhibitory activity of the protein on further purification by ion-exchange chromatography and RP-HPLC decreased to a larger extent as shown by their very low total activities and specific activity values. These results are contradictory to the results obtained by various other workers. In a study carried out by Kawai *et al.*, (1994), an 8 fold increase in activity of bacteriocin from *L.gasseri* LA 39 after dialysis and concentration of CFCS was observed and a further 150 and 4500 fold purification was attained after RP-HPLC and re-chromatography respectively. Similarly, Kabuki *et al.*, (1997) reported 1584 fold increase in the purification fold of reuterin 6 after RP- chromatography on butyl Toyopearl and 6870 fold increase in the purification fold after RP-HPLC on phenyl cellulofine. Sawa *et al.*, (2009) purified lactocyclin Q from *Lactococcus* sp. strain QU 12 by ion-exchange chromatography and RP-HPLC and attained 2.84 and 10,100 fold purification with 80 and 25% yield.

Carolissen-Mackay (1997) has reviewed several problems encountered in the purification of bacteriocins of lactic acid bacteria that usually lead to low-yields. Hechard *et al.*, (1999) tried to purify Mesentericin Y105, a 37 amino-acid long peptide produced by *Leuconostoc mesenteroides* Y105 by affinity chromatography, ultrafiltration and reverse-phase high-pressure liquid chromatography but could recover only 0.7% of the antibacterial peptide. Whereas, several other authors have reported >100% intermediate or final yields of recovered bacteriocin activity (Coventry *et al.*, 1996; Kaiser and Montville, 1996; Venema *et al.*, 1997). Ganzle *et al.*, (1999) demonstrated that ecological factors, such as protein content, pH, salinity, and/or salt composition of the medium, modulate the activity of bacteriocins. Moreover, the surfactant Tween 80 may form miscelles with the proteins in the growth medium and is described to interfere with bacteriocins, extending or diminishing their activities according to the peptide tested.

4.13. CHARACTERIZATION OF BACTERIOCIN

The partially purified bacteriocin was characterized with respect to its inhibitory spectrum, enzyme sensitivity, heat and pH stability. The purified bacteriocin obtained through RP-HPLC was characterized for its molecular weight and circular dichroism spectra.

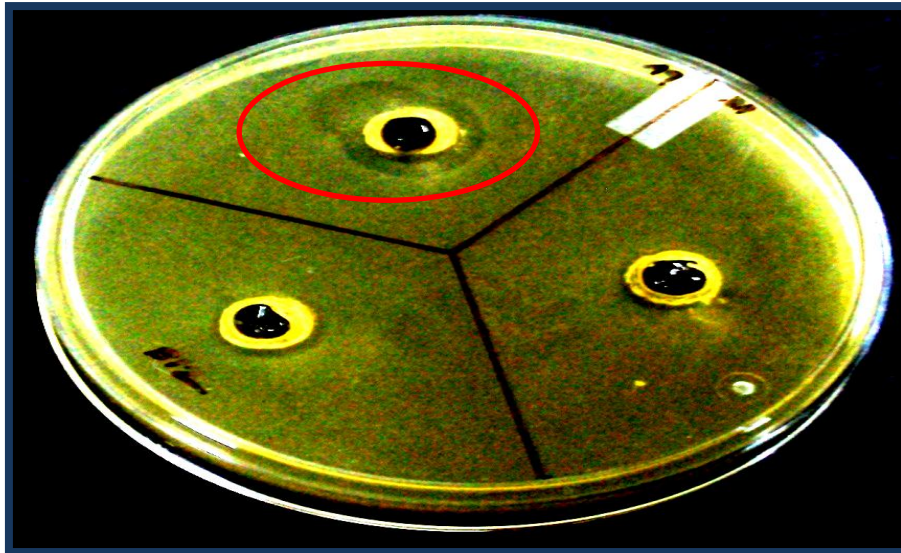


Plate 4.18. Activity of bacteriocin in the HPLC fractions. Activity was observed in fractions collected after 40 ml of buffer B (80 min). Clear zone indicates antibacterial activity of the fractions as tested against *E.faecalis* NCDC 114

4.13.1. Inhibitory Spectrum

The inhibitory spectrum of partially purified bacteriocin was determined by spot on lawn assay (3.5.1) against several bacterial species and a few yeasts. It may be seen from the Table 4.12 that the bacteriocin showed relatively poor inhibitory activity against all the lactic cultures tested as compared to the pathogens. Also, the bacteriocin was not found to be active against any of the yeast cultures tested as well as Gram-negative bacteria.

From application point of view, bacteriocins that antagonize undesirable bacteria with less effects on useful starter and non starter LAB would be an ideal food preservative. From the Table 4.12 it could be observed that relatively weakly inhibited bacteria, such as *Lactocobacillus casei* and *Lactobacillus plantarum* can be considered sufficiently insensitive in bacteriocin containing food systems in which a high concentration of bacteriocin is never reached. As far as gassericin A is concerned, taking into account the high bacteriocin concentration required to inhibit these bacteria, the bacteriocinogenic NBL 18 can be employed with these *Lactobacillus* strains. Moreover, these bacteriocinogenic lactobacilli can be efficiently used with other bacteriocinogenic strains of *Pediococcus* spp. and *Lactobacillus* spp. for flavour development or for enhancing the shelf-life.

Table 4.12. Inhibitory spectrum of bacteriocin from NBL 18 by spot on lawn assay

Bacterial species	Strains and Source	Zone in mm
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	NCDC 86, Karnal	+
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	NCDC 278, Karnal	++
<i>Lactobacillus casei</i> subsp. <i>casei</i>	NCDC 017, Karnal	+
<i>Lactobacillus brevis</i>	NCDC 001, Karnal	+
<i>Lactobacillus acidophilus</i>	NCDC 291, Karnal	+
<i>Lactococcus cremoris</i>	NCDC 060, Karnal	+
<i>Lactobacillus helveticus</i>	NCDC 005, Karnal	+
<i>Lactobacillus fermentum</i>	NCDC 156, Karnal	+
<i>Lactobacillus rhamnosus</i>	NCDC 018, Karnal	++

<i>Lactobacillus plantarum</i>	NCDC 020, Karnal	+
<i>Lactobacillus paracasei</i>	NCDC 063, Karnal	++
<i>Streptococcus thermophilus</i>	NCDC 074, Karnal	++
<i>Streptococcus thermophilus</i>	NCDC 117, Karnal	+
<i>Streptococcus thermophilus</i>	NCDC 80, Karnal	++
<i>Pediococcus acidilactici</i>	LB-42, USA	++
<i>Enterococcus faecalis</i>	NCDC 114, Karnal	+++
<i>Enterococcus faecalis</i>	NCDC 115, Karnal	+++
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	+++
<i>Listeria monocytogenes</i>	ATCC 15303	+++
<i>Micrococcus luteus</i>	NCDC 131, Karnal	+++
<i>E. coli</i>	ATCC 25922	-
<i>Staphylococcus aureus</i>	NCDC 273, karnal	+++
<i>Staphylococcus aureus</i>	NCDC 110, Karnal	+++
<i>Klebsiella pneumonia</i>	NCDC 138	-
<i>B. subtilis</i>	NCDC 70	++
<i>Salmonella typhi</i>	NCDC 113, Karnal	-
<i>Shigella dysenteriae</i>	NCDC 107, Karnal	-
<i>E. coli</i>	NCDC 134, Karnal	-
<i>Pseudomonas aeruginosa</i>	NCDC 104, Karnal	-
<i>Rhodotorula glutinis</i>	NCDC 51, Karnal	-
<i>Saccharomyces cerevisiae</i>	NCDC 47, Karnal	-

<i>Torulopsis candida</i>	NCDC 43, Karnal	-
<i>Candidia guilliermondii</i>	NCDC 44, Karnal	-
<i>Saccharomyces cerevisiae</i>	NCDC 50, Karnal	-

More than 15mm= ++++; 13-15mm= +++; 10-13mm= ++; Less than 10= +;

No inhibition= -

4.13.2. Molecular Weight

The purified bacteriocin obtained through stepwise purification including ammonium sulphate precipitation, ion-exchange chromatography, and RP-HPLC was run on Tricine-SDS-PAGE. One part of the gel with molecular weight markers were stained with Brilliant Blue G and one part of the gel was used to determine the bacteriocin activity.

It may be observed from the Plate 4.19 that the gel stained with the dye had shown one protein band with molecular weight 3.5 KDa for bacteriocin. The unstained other part of the gel overlaid with the indicator strain *E. faecalis* NCDC 114 gave clear zone of inhibition (Plate 4.20). Superimposing of stained gel over the gel used to detect the bacteriocin activity revealed that the 3.5 KDa protein bands detected in the stained gel corresponded to bacteriocin.

The bacteriocin in this study exhibited low molecular weight of approximately 3.5 kDa. The low molecular weight of bacteriocin in this study is in accordance with the molecular weight of gassericin A reported by Kawai *et al.*, (1994, 2004a) who reported a single band of 3.8kDa through Tricine SDS-PAGE showing *in-situ* bacteriocin activity.

All the circular bacteriocin reported till dates are small molecular weight peptides and range in size from 3.4 to 7.8 KDa. However, the molecular weight for gassericin reported in our study are approximations, as size determination by SDS-PAGE is not very accurate.

4.13.3. Enzyme Sensitivity

The residual activities of the bacteriocin after treatment with various proteolytic and non-proteolytic enzymes as determined by critical dilution assay (3.6) against *E. faecalis* NCDC 114 are shown in Table. 4.13. It may be seen from the Table 4.13 that the bacteriocins were sensitive to all the 5 proteolytic enzymes *viz.*, proteinase K, pepsin, papain, protease XIII and chymotrypsin. However, the bacteriocin was resistant to non-proteolytic enzymes such as α -amylase, lysozyme, and lipase, both in crude as well as in partially purified forms.

Sensitivity of the bacteriocin towards a large number of proteolytic enzymes suggested its proteinaceous nature, which is the usual character of bacteriocins. The bacteriocin was, however, not susceptible to the action of lipase thus indicating that no lipid moiety is associated with the antibacterial property and insensitivity to lysozyme reveals that there is no role for peptidoglycan moieties in the inhibitory activity.

The sensitivity of circular bacteriocins to various proteolytic enzymes has been reported widely. Gassericin A has been reported to be sensitive to proteolytic enzymes (actinase E and trypsin) and insensitive to non-proteolytic enzymes such as catalase (Toba *et al.*, 1991). The circular bacteriocin Carnocyclin A lost its activity completely when treated with the proteases trypsin, pepsin, and protease VIII (Martin-Visscher *et al.*, 2008). The inhibitory activity of lactocyclin Q was completely lost by pepsin and proteinase K when the molar ratio of the enzyme to lactocyclin Q was 1:1 (Sawa *et al.*, 2009). Here, our findings are in accordance with the works of other authors as the inhibitory activity of bacteriocin was completely lost when treated with proteolytic enzymes.

Table 4.13. Enzymatic sensitivity of the crude and partially purified bacteriocin against *E. faecalis* NCDC 114

Treatment	Concentration mg/ml	Residual activity of crude bacteriocin (X 10 ⁴ AU/ml)	Residual activity of partially purified bacteriocin (X 10 ⁶ AU/ml)
Control*	-	2.56	3.2768
Proteinase K	10 mg/ml	-	-
α -amylase	10 mg/ml	2.56	3.2768

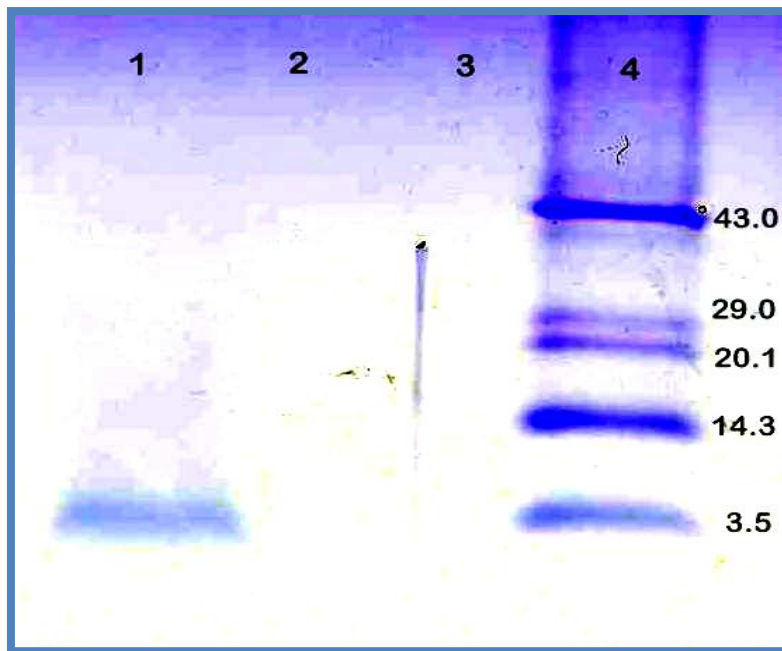


Plate 4.19. Molecular weight of bacteriocin as detected by Tricine-SDS-PAGE



Plate 4.20. *In-situ* bacteriocin activity of purified bacteriocin

Protease	10 mg/ml	-	-
Papain	10 mg/ml	-	-
Chymotrypsin	10 mg/ml	-	-
Pepsin	10 mg/ml	-	-
Lipase	10 mg/ml	2.56	3.2768
Lysozyme	10 mg/ml	2.56	3.2768

***Without treatment**

4.13.4. Heat Stability of the Bacteriocin

The stability of the bacteriocin to different heat treatments is shown in Table 4.15. It may be seen from the Table 4.14 that bacteriocin of the isolate NBL 18 was quite heat stable and retained its full activity even upto 100°C for 60 min, however crude bacteriocin lost almost 60% of its activity after heating at 121°C for 15 min, while almost 50% activity was lost in case of partially purified bacteriocin.

The retention of the bacteriocin activity after different heat treatments shows that the bacteriocin of NBL 18 is extremely heat stable. Stability of bacteriocins of circular bacteriocins over wide temperature range is a general characteristic. Toba *et al.*, (1991) reported that the activity of Gassericin A was not lost by heating at 100°C for 60 min but was completely lost after heat treatment at 120°C for 20 min. The extreme heat stability is believed to be because of their circular structure. Most of the circular bacteriocins are reported to be low molecular weight heat stable peptides.

Table 4.14. Temperature sensitivity of the crude and partially purified bacteriocin against *E. faecalis* NCDC 114

Treatment	Residual activity of crude bacteriocin (X 10 ⁴ AU/ml)	Residual activity of partially purified bacteriocin (X 10 ⁶ AU/ml)
Control*	2.56	3.2768
63°C/30 min	2.56	3.2768
85°C /10 min	2.56	3.2768
85°C /15min	2.56	3.2768
90°C /15min	2.56	3.2768

100°C /5min	2.56	3.2768
100°C /30min	2.56	3.2768
100°C /60min	2.56	3.2768
121°C /15min	1.0 (39.06%)	1.62 (49.43 %)

Values presented in parentheses show percent bacteriocin activity obtained after treatment in comparison to initial activity

*Without treatment

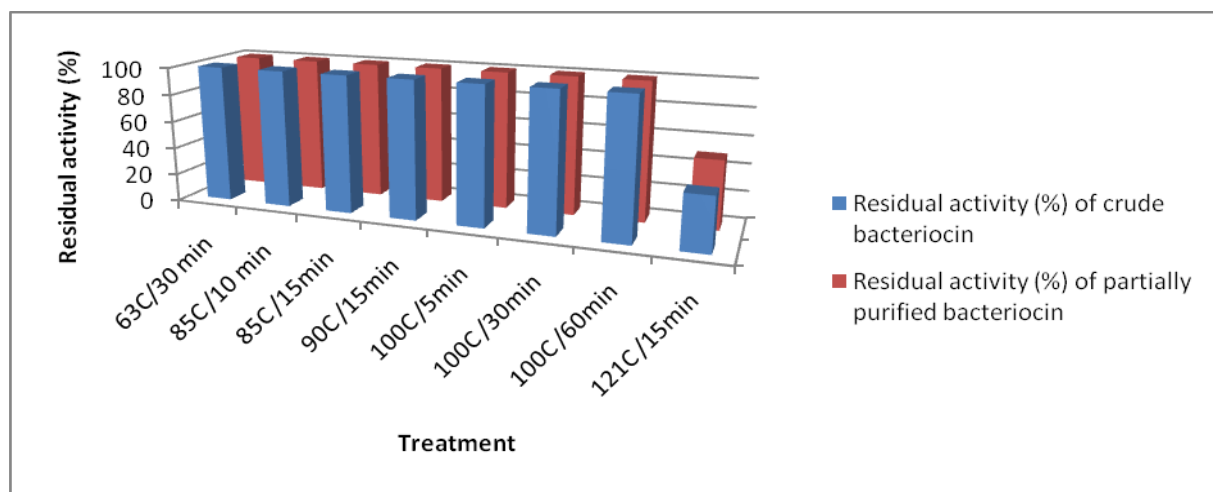


Fig. 4.12. Effect of temperature on residual activity of crude and partially purified bacteriocin

4.13.5. Effect of Detergents on Bacteriocin Activity

To examine the hydrophobic nature of bacteriocin, the neutralized supernatant was treated with surfactants at a final concentration of 1 % (w/v). The sensitivity to the surfactants is given in Table 4.15. Treatment with Tween 80, Tween 20 and Triton X-100 had no effect on the bacteriocin activity. However treatment with anionic detergent like SDS resulted in 37% and 100% increase in residual activity (%) of crude and partially purified bacteriocin respectively.

Table 4.15. Effect of detergents on bacteriocin activity

Treatment	Residual activity of crude bacteriocin (X 10 ⁴ AU/ml)	Residual activity of partially purified bacteriocin (X 10 ⁶ AU/ml)
Control*	2.56	3.2768
Tween 20	2.56	3.2768

Tween 80	2.56	3.2768
Tritone X-100	2.56	3.2768
SDS	3.50 (136.71%)	6.5536 (200%)

***Without treatment**

The effect of detergents on different bacteriocins gives information about the structure of the active molecules. Ivanova *et al.*, (2000) reported that anionic detergent SDS are known to unfold proteins by complexing to the interior hydrophobic core of their native structure thus affecting their three dimensional conformation. In our findings, addition of SDS detergent resulted in an increased bacteriocin effect, which might be due to the solubilization of insoluble aggregates.

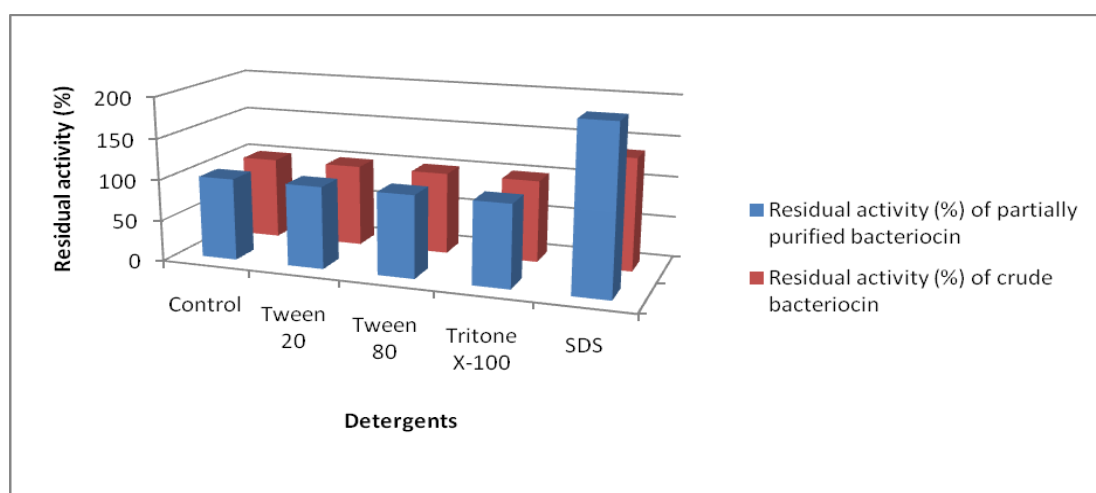


Fig. 4.13. Effect of detergents on residual activity of crude and partially purified bacteriocin

Bacteriocins of LAB have a tendency to form large macro-molecular complexes after aggregating with other bacteriocin molecules or medium components. Non-ionic detergents such as Tween 80, Tween-20 and Tritone X-100 at 1% level did not result in increase in bacteriocin activity indicating that these agents are not capable of dissociating aggregates of *L.gasseri* NBL 18 bacteriocin. The increase in bacteriocin activity by treatment with 1% SDS can be attributed to dispersion of bacteriocin complex thereby releasing more units for activity.

4.13.6. pH Stability

The results pertaining to the pH stability of crude bacteriocin are shown in Table 4.16. The estimation of the residual activities of the pH adjusted culture broths of NBL 18 revealed that the bacteriocin was extremely stable at pH in the range 2-11 retaining

100% of the activity even after 120 h at 4°C of storage. The stability of circular bacteriocin over a wide range of pH values have been well documented in literature (Toba *et al.*, 1991; Martin-Visscher *et al.*, 2008)

Table 4.16. pH stability of bacteriocin after 120 h of storage at 4°C

pH	Residual activity of crude bacteriocin (X 10⁴ AU/ml)	Residual activity of partially purified bacteriocin (X 10⁶ AU/ml)
Control*	2.56	3.2768
pH 2	2.56	3.2768
pH 3	2.56	3.2768
pH 4	2.56	3.2768
pH 5	2.56	3.2768
pH 6	2.56	3.2768
pH 7	2.56	3.2768
pH 8	2.56	3.2768
pH 9	2.56	3.2768
pH 10	2.56	3.2768
pH 11	2.56	3.2768

***Without treatment**

Our observations are similar to those reported by Toba *et al.*, (1991) showing that Gassericin A was a heat stable proteinaceous compound that withstood exposure to pH values between 2-11 for 24 h at 15°C and its antimicrobial activity was not lost by freezing and lyophilization.

The fact that circular bacteriocins maintain their inhibitory activity at different pH values (pH 2-11), shows their potential usefulness in fermented foods which are subjected to significant pH variations during processing (Arakawa *et al.*, 2009). Arakawa *et al.*, (2009) suggested that the activity of gassericin (both A and T) over a wide pH and temperature may be advantageous when produced in gastrointestinal tract, where the pH levels are known to vary from pH 3.0 in the stomach to pH>7.0 in the large intestine.

4.13.7. Circular Dichroism Spectroscopy of Bacteriocin

Circular dichroism (CD) spectra for the purified bacteriocins were recorded between 200 and 260 nm at room temperature by using a 10-mm-pathlength quartz cell on a Chirascan CD spectrometer (Photophysics). Samples were scanned ten times, and the data were averaged. A peptide-free control spectrum with 70% (vol/vol) acetonitrile + 0.05% TFA was subtracted, providing the mean residue ellipticity of the peptide. The contents of α -helix, β -sheet, and unordered structures were estimated according to the software K2D.

Table. 4.17. Molar ellipticity of the peptide calculated at different wavelengths

S.N.	Wavelength	Ellipticity	Molar ellipticity
1	260	0.003795	291.9415
2	259	0.004457	342.8731
3	258	0.005905	454.1923
4	257	0.007115	547.2738
5	256	0.005192	399.3615
6	255	0.006815	524.2146
7	254	0.003901	300.1015
8	253	0.003781	290.8131
9	252	0.003822	294.0224

10	251	0.002885	221.8923
11	250	0.001334	102.6007
12	249	0.001776	136.5923
13	248	0.000132	10.17762
14	247	-0.00594	-456.981
15	246	-0.00535	-411.345
16	245	-0.00594	-456.822
17	244	-0.01679	-1291.24
18	243	-0.02102	-1617.28
19	242	-0.03806	-2927.73
20	241	-0.03722	-2863.27
21	240	-0.03933	-3025
22	239	-0.03815	-2934.23
23	238	-0.0302	-2323.38
24	237	-0.04261	-3277.62
25	236	-0.05217	-4012.77
26	235	-0.06832	-5255.15
27	234	-0.09836	-7565.94
28	233	-0.14698	-11306
29	232	-0.19326	-14866.2
30	231	-0.21503	-16540.7
31	230	-0.239	-18384.8
32	229	-0.26697	-20535.9
33	228	-0.28211	-21701.1
34	227	-0.28315	-21780.7
35	226	-0.32918	-25321.8

36	225	-0.34411	-26470
37	224	-0.36643	-28186.8
38	223	-0.36473	-28056.2
39	222	-0.37897	-29151.8
40	221	-0.37568	-28898.3
41	220	-0.38858	-29891.1
42	219	-0.38807	-29851.5
43	218	-0.38305	-29465
44	217	-0.36854	-28349.2
45	216	-0.36916	-28397.2
46	215	-0.36712	-28240.3
47	214	-0.36762	-28278.7
48	213	-0.34722	-26709.2
49	212	-0.34275	-26365.5
50	211	-0.32595	-25073.1
51	210	-0.30718	-23629.5
52	209	-0.25151	-19346.6
53	208	-0.19344	-14879.8
54	207	-0.08982	-6909.15
55	206	-0.05184	-3987.62
56	205	-0.03032	-2332.54
57	204	0.050859	3912.2
58	203	0.083085	6391.115
59	202	0.093085	7160.346
60	201	0.103085	7929.577
61	200	0.103085	7929.577

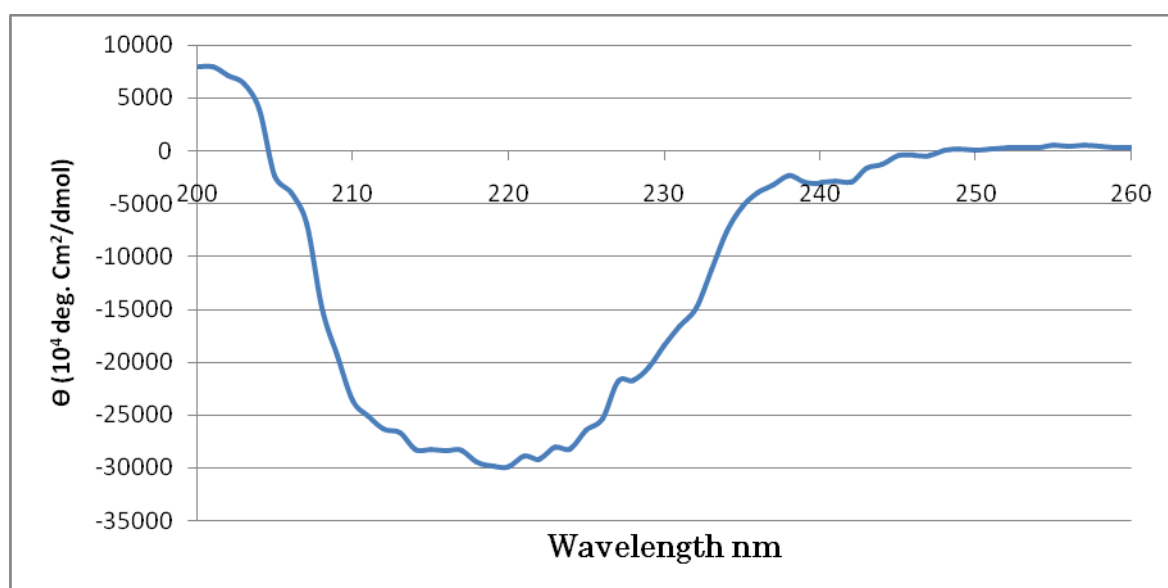


Fig. 4.14. CD Spectra of purified bacteriocin

Fig. 4.14 shows CD spectra of bacteriocin in 70% acetonitrile + 0.05% TFA. A positive maximum near 200, negative maxima at 220 nm, and Θ values indicate that the bacteriocin is a peptide consisting mainly of α -helix structure. On analysing the Θ values through K2D software, the peptide was found to consist 78% of α - helices, 1% of β -sheet and 21% random coil.

CD spectra of the purified gassericin A has been earlier reported by Kawai *et al.*, (2004b). Here our results are in complete agreement with the previous workers who reported that Gassericin A is mainly composed of α - helices (Kawai *et al.*, 2004b). The circular bacteriocins AS-48, gassericin A, reuterin 6 and carnocyclin A are largely composed of α -helices (Martin-Visscher *et al.*, 2008).

4.14. DETERMINATION OF BACTERIOCIN (GASSERICIN) CONCENTRATION TO INHIBIT *L.MONOCYTOGENES*, *E.COLI*, *P.AERUGINOSA* AND *B.CEREUS*

The bacteriocin concentration required to inhibit the growth of *Bacillus cereus* and *Listeria monocytogenes* were 6.4 and 3.2×10^3 AU/ml respectively (Table 4.18). Arakawa *et al.*, (2009) reported 15,750 AU/ ml of gassericin T and 985 AU/ml of gassericin A for the inhibition of these organisms, although durable or weak inhibition was observed in case of 985 AU/ ml of gassericin A. In our case 1.28×10^4 AU/ml of bacteriocin led to strong inhibition of both the Gram positive organism tested, but had no effect on the Gram negative organisms.

Table 4.18. Minimum Inhibitory Concentration of gassericin against different pathogens

Organism	Minimum Inhibitory Concentration (X10 ³ AU/ml)
<i>Pseudomonas aeruginosa</i> NCDC 104	-
<i>Escherichia coli</i> NCDC 134	-
<i>Bacillus cereus</i> NCDC 66	6.4
<i>Listeria monocytogenes</i> ATCC 15303	3.2

4.14.1. The Inhibitory Effect of Gassericin against *Listeria monocytogenes*, *Bacillus cereus*, *Enterococcus faecalis*, *E.coli* and *P. aeruginosa* in Phosphate Buffer Medium

Survival curves of the mentioned strains in phosphate buffer (pH 7.2, 50 mM) with 1.28 X 10⁴ AU/ml bacteriocin added are shown in Fig. 4.15 (A, B, C, D and E). No significant reduction was observed in the counts of Gram negative pathogens and their counts were same as that of the control samples (without bacteriocins), whereas during the same 5h period, bacteriocin resulted in reduced viability of all Gram positive organisms. All Gram positive organisms tested reduced to nondetectable limit within 5h. Statistical analysis showed that the antimicrobial agent had made significant differences on the microbial counts. It is clear from the figure that viability of all Gram positive pathogens decreased with increasing contact time with cell free supernatant. The impact on bacterial cells was almost immediate for all Gram positive pathogens. It is interesting to note that *E. faecalis* was the most susceptible and the number of viable cells steeply declined as the contact with cell free supernatant continued, resulting in a reduction of 6 log cycles (100% inhibition) of the cells after 4 h of treatment followed by *Listeria monocytogenes* with 6 log cycles reduction of the cells after the 5 h of treatment. *Bacillus cereus* also lost its viability during the cell free supernatant treatment. However, it exhibited higher viability with only 5 log cycles reduction of the cells after 5 h of treatment.

4.14.2. Microwell Plate Assay

The antibacterial activity of bacteriocin and glycine against the 12 bacterial strains is shown in Table 4.20. In this method, bacteriocin along with glycine solutions (1.28 X 10⁴ AU/ml) showed broader antibacterial spectra than alone against all the organisms tested. The bacteriocin alone was ineffective for the inhibition of the growth of the Gram-negative bacteria. Glycine had a durable antibacterial activity against all the

Table 4.20. Antimicrobial activity of glycine and culture supernatants of NBL 18 (gassericin) against Gram positive and Gram negative bacteria as assayed by the microwell plate method

Combinations	AU/ml (X10 ⁴)	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>		<i>Enterococcus faecalis</i>		<i>Listeria monocytogenes</i>	Lactic acid bacteria				Gram negative bacteria	
		NCDC 66	NCDC 273	NCDC 110	NCDC114	NCDC115	ATCC 15303	<i>La</i>	<i>Lll</i>	<i>Lb</i>	<i>Lc</i>	<i>P.aeruginosa</i> NCDC 104	<i>E.coli</i> NCDC 134
1% glycine	-	C	C	C	C	C	C	-	-	-	-	C	C
Gassericin	1.28	B	B	B	A	A	A	C	C	C	B	-	-
Gassericin + Glycine	1.28	A	B	B	A	A	A	B	B	C	B	B	B

La = *Lactobacillus acidophilus* NCDC 291; *Lll* = *Lactobacillus lactis* subsp. *lactis* NCDC 278; *Lb* = *Lactobacillus bulgaricus* NCDC 42; *Lc* = *Lactococcus cremoris* NCDC 060

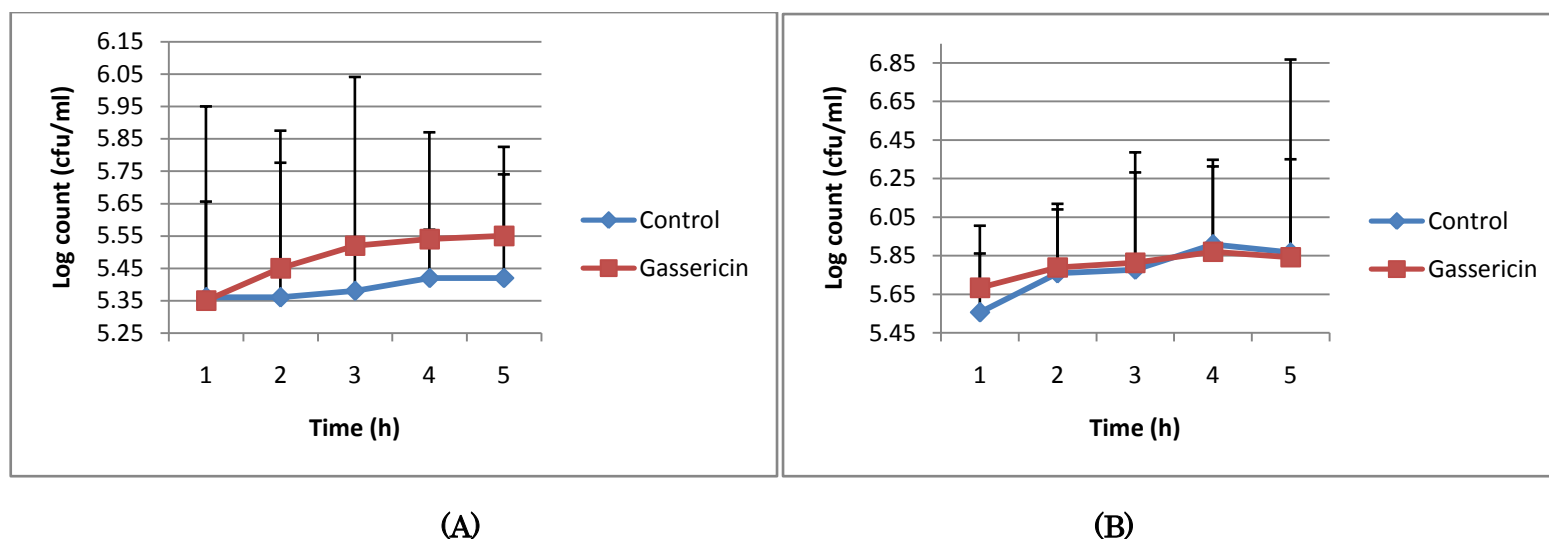


Fig.4.15. Inhibitory effect of bacteriocin against *E.coli* NCDC 134 (A) and *P.aeruginosa* NCDC 104 (B) in phosphate buffer medium

Table.4.19. Inhibitory effect of bacteriocin against *E.coli* NCDC 134 (A) and *P.aeruginosa* NCDC 104 (B) in phosphate buffer medium All values are expressed as mean ± standard deviation with three replicate

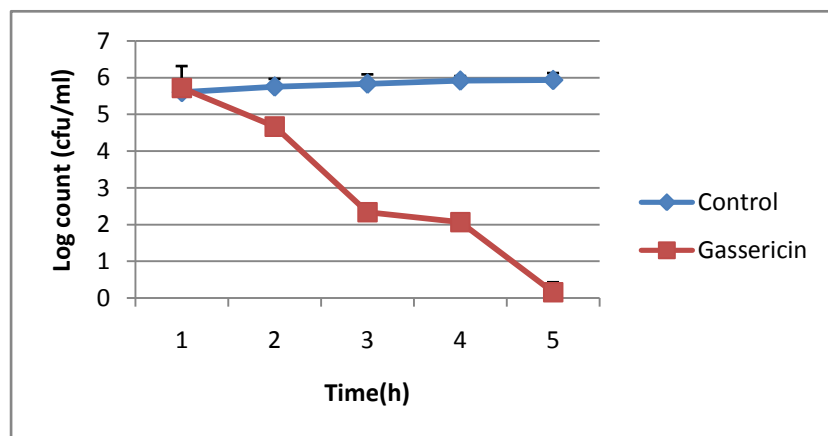
Time (h)	Control	Gassericin
1	5.36±0.590 ^a	5.35±0.306 ^a
2	5.36±0.416 ^a	5.45±0.425 ^a
3	5.38±0.145 ^a	5.52±0.521 ^a
4	5.42±0.149 ^a	5.54±0.330 ^a
5	5.42±0.405 ^a	5.55±0.190 ^a

(A)

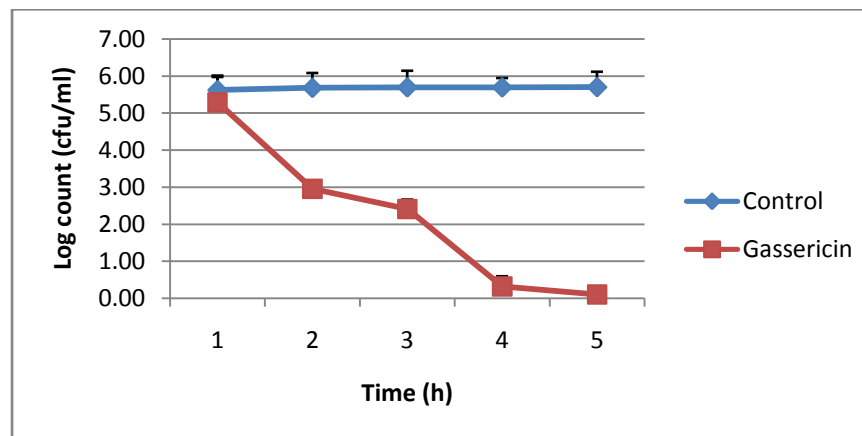
Time (h)	Control	Gassericin
1	5.56±0.306 ^a	5.68±0.322 ^a
2	5.76±0.332 ^a	5.79±0.330 ^a
3	5.78±0.505 ^a	5.81±0.573 ^a
4	5.91±0.440 ^a	5.87±0.443 ^a
5	5.87±1.000 ^a	5.84±0.508 ^a

(B)

^{a,b} values (Mean ± SD) with different superscript in a column are significantly different at the level of p<0.05



(C)



(D)

Fig.4.15. Inhibitory effect of bacteriocin against *L.monocytogenes* ATCC 15303 (C) and *E.faecalis* NCDC 114 (D) in phosphate buffer medium

Table 4.19. Inhibitory effect of bacteriocin against *L.monocytogenes* ATCC 15303 (C) and *E.faecalis* NCDC 114 (D) in phosphate buffer medium. All values are expressed as mean ± standard deviation with three replicates

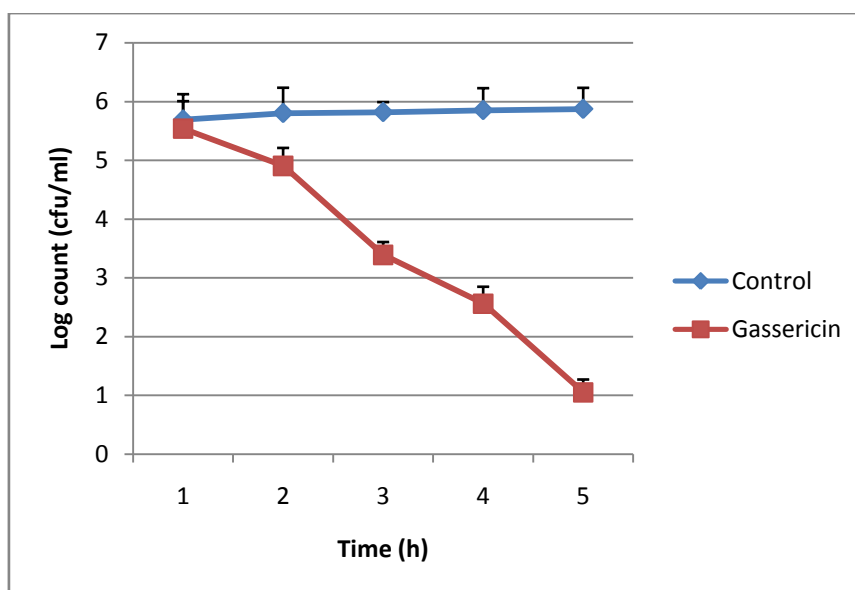
Time (h)	Control	Gassericin
1	5.60±0.228 ^a	5.72±0.600 ^a
2	5.75±0.216 ^a	4.67±0.141 ^b
3	5.83±0.261 ^a	2.33±0.178 ^b
4	5.91±0.130 ^a	2.07±0.100 ^b
5	5.93±0.196 ^a	0.16±0.275 ^b

(C)

Time (h)	Control	Gassericin
1	5.62±0.355 ^a	5.28±0.726 ^a
2	5.68±0.404 ^a	2.95±0.178 ^b
3	5.69±0.450 ^a	2.41±0.252 ^b
4	5.69±0.255 ^a	0.32±0.275 ^b
5	5.70±0.417 ^a	0.10±0.173 ^b

(D)

^{a,b} values (Mean ± SD) with different superscript in a column are significantly different at the level of $p < 0.05$



(E)

Fig.4.15 . Inhibitory effect of bacteriocin against *B.cereus* NCDC 66 (E) in phosphate buffer medium

Table 4.19. Inhibitory effect of bacteriocin against *B.cereus* NCDC 66 (E) in phosphate buffer medium. All values are expressed as mean $\pm$ standard deviation with three replicates

Time (h)	Control	Gassericin
1	5.69 $\pm$ 0.435 ^a	5.54 $\pm$ 0.467 ^a
2	5.80 $\pm$ 0.432 ^a	4.90 $\pm$ 0.305 ^b
3	5.82 $\pm$ 0.172 ^a	3.39 $\pm$ 0.218 ^b
4	5.85 $\pm$ 0.375 ^a	2.56 $\pm$ 0.287 ^b
5	5.87 $\pm$ 0.360 ^a	1.05 $\pm$ 0.216 ^b

(E)

^{a,b} values (Mean $\pm$ SD) with different superscript in a column are significantly different at the level of $p < 0.05$

organisms tested (both Gram positive and Gram negative), but had a comparatively weak activity against LAB strains.

4.14.3. The Inhibitory Effect of Gassericin against *B. cereus*, *E.coli*, *P. aeruginosa* and *L. monocytogenes* in Whipped Cream Frosting

Results of the inhibitory activity of bacteriocin (1.28×10^4 AU/ml), glycine alone and their combination against *B. cereus* and *L. monocytogenes* in cream are given in Fig. 4.16 and 4.17. The bactericidal effect of both gassericin and glycine was evident from the 2nd day of incubation as there were 1.07 and 2.15 log cycle reduction in the counts of *Bacillus cereus* treated with glycine and gassericin respectively as compared to control. In case where the combination of both glycine and gassericin was used there was a reduction of 2.39 log cycles as compared to control. Similar trend were observed on 5th, 15th and 30th day where treatment with glycine lead to a reduction of 1.05, 1.98 and 1.77 log cycles respectively of *Bacillus cereus* as compared to control. Treatment with gassericin lead to a reduction of 2.22, 3.22 and 3.97 log cycles on 5th, 15th and 30th day, respectively. Combination of both the compounds, however, resulted in the most significant reduction in the log cycle of organism (a reduction of 6.71 log cycles) on 30th day.

In case of *L. monocytogenes* there was a reduction of 0.98 and 1.06 log cycles when treated with glycine and gassericin, respectively, on the 2nd day of incubation as compared to control. The combination of both, however, resulted in 1.43 log cycle reduction of the organism. The highest log reduction of 7.30 log cycles was observed on 30th day of incubation, in case where a combination of bacteriocin and glycine were used.

The cell populations of both the Gram-negative inoculated organisms, *E. coli* and *P. aeruginosa*, reached upto a level of 10^9 cfu ml⁻¹ in additive free whipped cream frosting after 30 days of incubation (Fig. 4.18 & 4.19). In the whipped cream frosting containing glycine (alone), an inhibitory effect was observed for both the pathogens. A reduction of 5.03 and 4.48 log cycles of *P. aeruginosa* and *E. coli* was observed after 30 days of incubation where the cream was treated with glycine. Gassericin treatment in cream containing both pathogens had no significant effect in the reduction in log counts of organisms. A similar result was obtained in microwell plate assay method where

gassericin had no inhibitory effect on both the organisms. However, in whipped cream frosting containing the combination of gassericin and glycine, a reduction of 6.70 log cycle was observed in case of *P. aeruginosa*, whereas a reduction of 6.95 log cycles was observed in case of *E.coli*.

In our study a synergistic action of the two antibacterial compounds gassericin and glycine (GG) was found to be effective in inhibiting all the pathogens tested. The data for the growth of the strains in the custard creams, correlated well with those found in the microwell plate assay (Table 4.19). This suggests that the growth of all spoilage bacteria tested in whipped cream frosting can be completely inhibited by the mixed supplementation of Gassericin and glycine, which is impossible by either gassericin or glycine alone. Synergistic antimicrobial effects of gassericin in combination with glycine against Gram-positive bacteria and Gram negative bacteria in custard creams has also been reported earlier (Arakawa *et al.*, 2009).

The use of bacteriocins and/or bacteriocin-producing strains of LAB are of great interest as they are generally recognized as safe organisms and their antimicrobial products as biopreservatives. Several authors (Leistner, 1999; Leistner, 2000; Chawla *et al.*, 2004) have recommended the use of bacteriocins combined with other preservation methods to create a series of hurdles during the manufacturing process to reduce food spoilage by microorganisms. In fact, it has been proven that the application of chemical, physical, or mild non-thermal physical methods, which increase the permeability of cell membranes, positively affects the activity of many bacteriocins (Garriga *et al.*, 2002; Ananou *et al.*, 2005; Abriouel *et al.*, 1998; Zhang *et al.*, 1999; Ananou *et al.*, 2004). Notably, combined treatments of bacteriocins with selected hurdles affecting outer-membrane permeability increase the effectiveness of some LAB bacteriocins against Gram-negative cells, which are generally resistant. Glycine is the simplest amino acid, and its structure has no asymmetric carbon atom. As it is a metabolite of serine, it is not an essential amino acid for bacteria (Gillissen *et al.*, 1991). The major mechanism of glycine's antimicrobial activity is its inhibition of cell wall synthesis. High concentrations of glycine induce bacteriolysis or morphological alterations in various bacteria (Gillissen *et al.*, 1991; Schwartz *et al.*, 1979; Stronminger *et al.*, 1965). A possible mechanism of this effect may be inhibition of the reaction in which L-alanine is added to UDP-acetylmuramic acid by UDP-*N*-acetylmuramate-alanine ligase (Ito *et al.*, 1962). This study shows that when bacteriocin from *Lactobacillus gasserii* NBL 18 is used in combination with glycine, it not

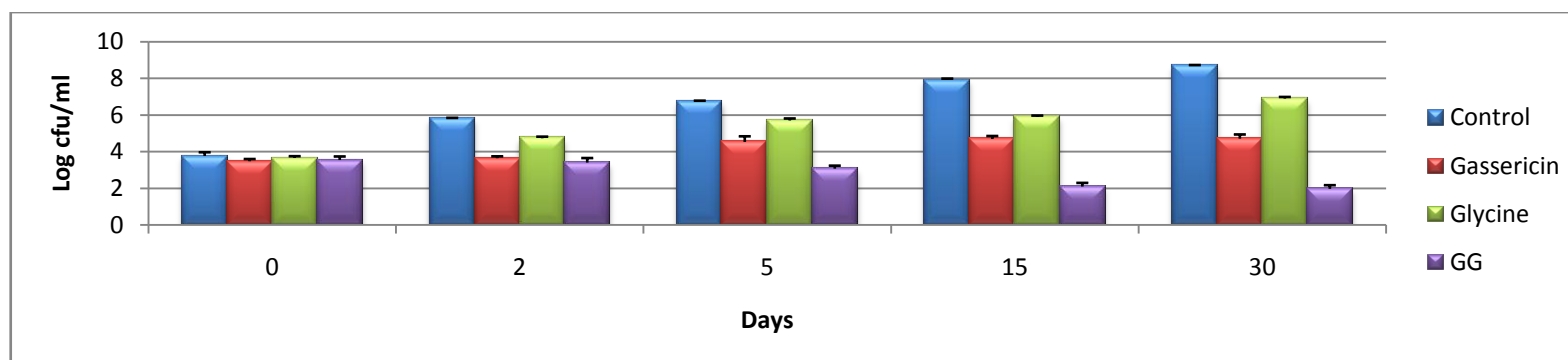


Fig.4.16. Changes in cell viability of *B. cereus* NCDC 66 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasserii* NBL 18(Gassericin), or both (GG). The data show the average values of triplicate determinations and the error bars are standard deviations.

Table 4.21. Changes in cell viability of *B. cereus* NCDC 66 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasserii* NBL 18(Gassericin), or both (GG).

Days		0	2	5	15	30
Combinations	Control	3.76±0.202 ^a	5.80±0.044 ^a	6.74±0.035 ^a	7.91±0.073 ^a	8.68±0.045 ^a
	Gassericin	3.48±0.121 ^a	3.64±0.102 ^{bd}	4.53±0.307 ^{bd}	4.69±0.165 ^b	4.70±0.228 ^b
	Glycine	3.67±0.078 ^a	4.73±0.087 ^c	5.70±0.111 ^{bc}	5.92±0.047 ^c	6.90±0.080 ^c
	GG	3.54±0.194 ^a	3.40±0.248 ^d	3.07±0.163 ^d	2.09±0.199 ^d	1.97±0.199 ^d

^{a,b,c,d} values (Mean ± SD) with different superscript in a row are significantly different at the level of p<0.05

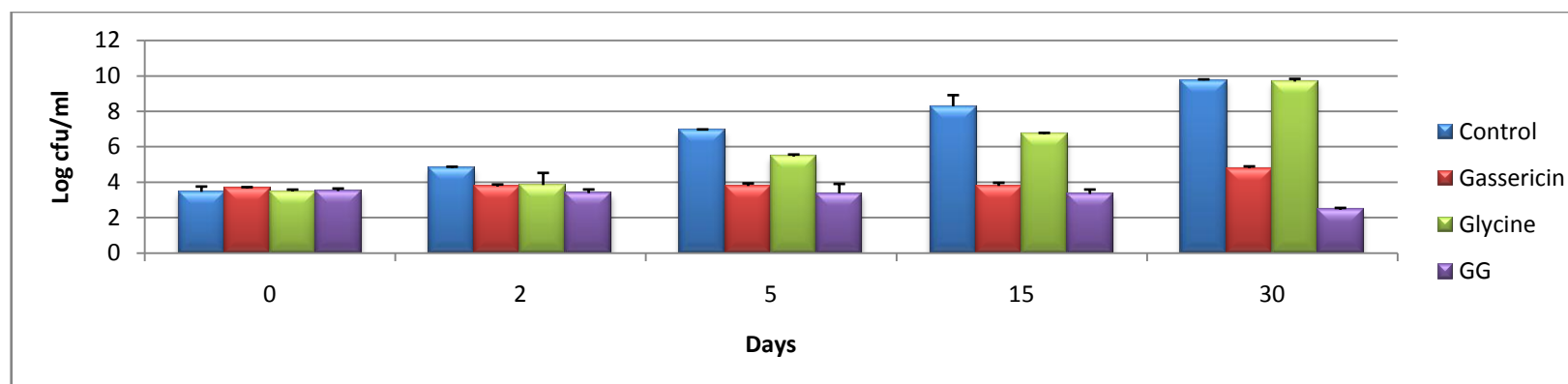


Fig.4.17. Changes in cell viability of *L.monocytogenes* ATCC 15303 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasseri* NBL 18 (Gassericin), or both (GG). The data show the average values of triplicate determinations and the error bars are standard deviations.

Table.4.22. Changes in cell viability of *L.monocytogenes* ATCC 15303 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasseri* NBL 18 (Gassericin), or both (GG).

Days		0	2	5	15	30
Combinations	Control	3.46±0.297 ^a	4.81±0.057 ^a	6.96±0.018 ^a	8.28±0.627 ^{ac}	9.74±0.055 ^{ac}
	Gassericin	3.68±0.045 ^a	3.75±0.116 ^a	3.78±0.148 ^{bd}	3.79±0.177 ^b	4.78±0.111 ^b
	Glycine	3.48±0.103 ^a	3.84±0.684 ^a	5.45±0.111 ^c	6.71±0.069 ^c	9.67±0.151 ^c
	GG	3.50±0.143 ^a	3.38±0.207 ^a	3.37±0.529 ^d	3.34±0.246 ^d	2.44±0.105 ^d

^{a,b,c,d} values (Mean ± SD) with different superscript in a row are significantly different at the level of p<0.05

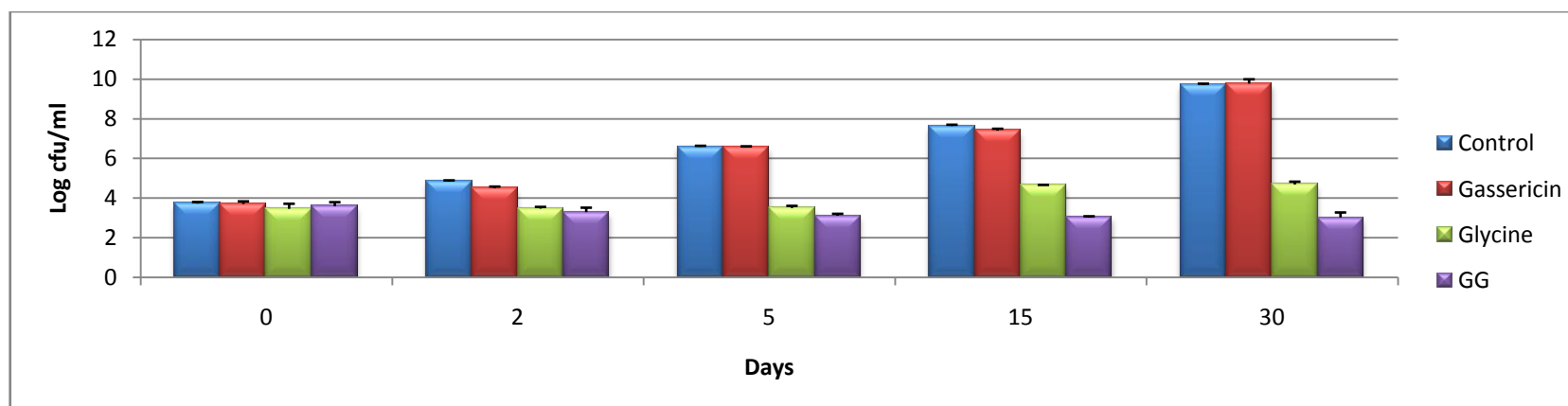


Fig. 4.19. Changes in cell viability of *P. aeruginosa* NCDC 104 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasseri* NBL 18 (Gassericin), or both (GG). The data show the average values of triplicate determinations and the error bars are standard deviations

Table. 4.24. Changes in cell viability of *P. aeruginosa* NCDC 104 in whipped cream frosting containing glycine, the culture supernatant of *Lactobacillus gasseri* NBL 18 (Gassericin), or both (GG).

Days		0	2	5	15	30
Combinations	Control	3.72±0.079 ^a	4.84±0.053 ^a	6.58±0.051 ^a	7.63±0.065 ^a	9.71±0.053 ^a
	Gassericin	3.69±0.136 ^a	4.5±0.075 ^a	6.56±0.043 ^a	7.4±0.096 ^b	9.77±0.219 ^a
	Glycine	3.45±0.256 ^a	3.46±0.088 ^c	3.49±0.108 ^{bc}	4.64±0.016 ^c	4.68±0.141 ^b
	GG	3.59±0.201 ^a	3.29±0.224 ^c	3.11±0.092 ^c	3.05±0.031 ^d	3.02±0.25 ^c

^{a,b,c,d} values (Mean ± SD) with different superscript in a row are significantly different at the level of p<0.05

only controls the growth of Gram positive organisms but the growth of Gram negative pathogens such as *E. coli* and *Pseudomonas aeruginosa* can also be controlled. This combination can be applied in high fat food system to provide a broad spectrum of antimicrobial activity covering both Gram positive and Gram negative organisms and enhance the shelf life of food.

4.15. ANTIBACTERIAL ACTIVITY OF NBL 18: COCULTURE TESTS IN SKIMMED MILK

In reconstituted skim milk, *L. gasseri* NBL 5 reached a count of $8.63 \log \text{ cfu ml}^{-1}$ after 24 h, followed by a drop of $0.67 \log \text{ cfu ml}^{-1}$ after 48 h. During bacterial growth, the pH of the milk decreased from 6.7 to 4.8 after 48 h. *L. gasseri* NBL 18 reached a count of $8.76 \log \text{ cfu ml}^{-1}$ after 24 h in RSM, followed by a drop of $0.63 \log \text{ cfu ml}^{-1}$ after 48 h. During bacterial growth, the pH of the milk decreased from 6.3 to 5.1 after 48 hr. The bacteriocin production by *L. gasseri* NBL 18 was influenced by the culture medium (RSM). Bacteriocin activity was observed only after 6 h (200 AU ml^{-1}), reached a maximum of 1600 AU/ml after 24 hr. However, a decrease in activity was observed after 48 hr and only 400 AU/ml was detected until the end of the incubation period. Arakawa *et al.*, 2009, evaluated *L. gasseri* strain LA 39 and obtained a similar result using reconstituted skim milk supplemented with proteose peptone. The Gassericin A activities in RSM were low—about 20% of that from MRS broth. It was speculated that this low activity was caused not by poor Gassericin A production but by the inactivation of Gassericin A molecules. Because Gassericin A is produced proportionally to the exponential growth of *L. gasseri* LA39 (Kawai *et al.*, 1994), LA39 growth in RSM was not markedly different from that in MRS broth (Arakawa *et al.*, 2009). Furthermore, it was speculated that hydrophobic Gassericin A molecules may be inactivated by reversible hydrophobic aggregation with milk proteins and each other in defatted milk-based media.

The effects of the bacteriocin-producing strain (NBL 18) on the growth of three pathogens *L. monocytogenes*, *S. aureus* and *E. faecalis* in skimmed milk is presented in Fig. 4.20, 4.21 & 4.22. Growth of pathogens increased from 10^6 cfu ml^{-1} to 10^8 cfu ml^{-1} within 24 h in control samples, reaching about 10^9 cfu/ml after 48 h of incubation. When pathogens were co-cultured with the bacteriocin-producing strain (NBL 18), different trends in the growth were obtained. As can be seen, NBL 18 reduced pathogen population within 48 h, although differences in the degree of inhibition were observed among the pathogens. The *L. monocytogenes* counts were reduced by

approximately 6.46 log units compared to the positive control and by 0.96 log unit compared to the initial inoculum. In coculture with NBL 5, the counts of *L. monocytogenes* increased from 3.28 to 8.36 log cfu ml⁻¹ within 48 hr of incubation, which is 0.47 log cfu ml⁻¹ less than the control. Similarly, 6.30 log reduction was observed in case of *S. aureus* (cocultured with NBL18) as compared to the control and 0.81 log reduction as compared to the initial inoculum, whereas, the counts of *S. aureus* increased from 2.75 to 6.95 log cfu ml⁻¹ when it was cocultured with NBL 5. In case of *E. faecalis*, 6.49 log reductions was observed when compared to positive control and 1.20 log reduction with the initial inoculum. The counts of *E. faecalis* increased from 3.46 to 8.56 log cfu ml⁻¹ within 48 h when cocultured with NBL 5. In all the cases, bacteriocin activity against pathogens was observed from 6 h of incubation, peaked at 24h of incubation after which a decline in the activity was observed. The inhibition in all the cases did not seem to be correlated with the reduction in pH, confirming that the antimicrobial activity of the strain is not due to the production of organic acid.

Our results suggest that this bacteriocinogenic strain could be potentially applied in food system to control the growth of *L. monocytogenes* and other pathogens. Bacteriocins of lactobacilli, less examined *in-situ* in food, have usually a very narrow spectrum of activity. *Lactobacillus sakei* and its bacteriocin sakacin K were tested against *Listeria innocua* in sausages (Leroy & De Vuyst, 2005). More studies are infact focused on the application of intestinal isolates of lactobacilli as probiotic cultures for cheese (Bergamini *et al.*, 2005; Collins *et al.*, 1998).

4.16. SAFETY EVALUATION OF NBL 18

In order for a strain to be used as a bioprotective culture, it should be carefully evaluated for the presence of virulence factors, to determine what potential risks might be involved in its use. Therefore, to ensure the safety of NBL 18 for its safe exploitation, it was tested for its virulence potential. Investigation of virulence potential was determined physiologically with the help of various tests *e.g.* Gelatinase production, Haemolysin production, antibiotic susceptibility and DNase production. The isolate was found to be negative for gelatin liquefaction (Plate 4.21a) and DNase production (Plate 4.21b). Haemolytic activity is a trait associated with virulence in some food-associated microorganisms, such as enterococci, but it has not been frequently reported for lactobacilli (Petros *et al.*, 2006). In our study the strain NBL 18

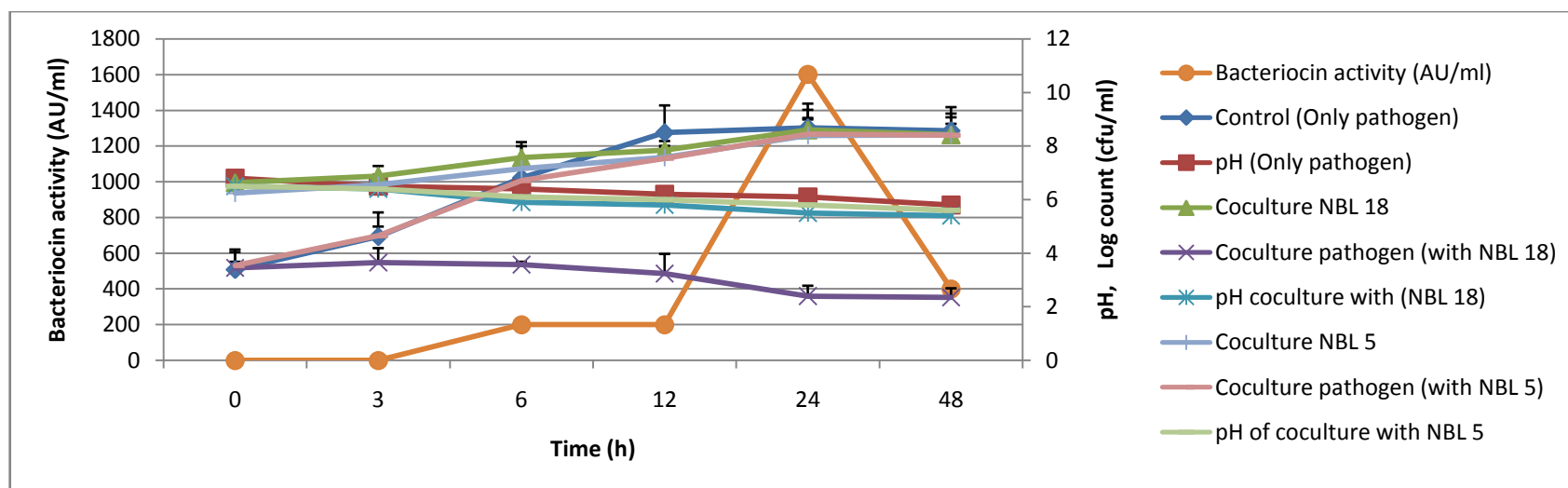


Fig. 4.20. Growth of *Listeria monocytogenes* ATCC 15303 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5

Table 4.25. Growth of *Listeria monocytogenes* ATCC 15303 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5. All values (log counts) are expressed as mean $\pm$ standard deviation with three replicates

Time (h)	Control (Only pathogen)	pH (Only pathogen)	Coculture NBL 18	Coculture pathogen (with NBL 18)	pH coculture with (NBL 18)	Bacteriocin activity (AU/ml)	Coculture NBL 5	Coculture pathogen (with NBL 5)	pH of coculture (with NBL 5)
0	3.39 $\pm$ 0.29 ^{ac}	6.8	6.63 $\pm$ 0.43 ^{bc}	3.45 $\pm$ 0.59 ^c	6.5	0	6.24 $\pm$ 0.22 ^{bd}	3.54 $\pm$ 0.6 ^{acd}	6.5
3	4.62 $\pm$ 0.38 ^{ab}	6.5	6.88 $\pm$ 0.37 ^{bde}	3.65 $\pm$ 0.54 ^{ac}	6.4	0	6.55 $\pm$ 0.38 ^{ad}	4.65 $\pm$ 0.87 ^{ae}	6.4
6	6.81 $\pm$ 0.53 ^a	6.4	7.56 $\pm$ 0.44 ^{bde}	3.57 $\pm$ 0.11 ^{cd}	5.9	200	7.15 $\pm$ 1.0 ^{ad}	6.71 $\pm$ 0.64 ^{ae}	6.1
12	8.50 $\pm$ 1.02 ^{abc}	6.2	7.84 $\pm$ 0.35 ^{be}	3.24 $\pm$ 0.73 ^c	5.8	200	7.57 $\pm$ 0.35 ^{ad}	7.54 $\pm$ 0.46 ^{ace}	6.0
24	8.68 $\pm$ 0.9 ^a	6.1	8.60 $\pm$ 0.75 ^{ab}	2.40 $\pm$ 0.39 ^c	5.5	1600	8.39 $\pm$ 0.6 ^{ad}	8.45 $\pm$ 0.6 ^{ae}	5.8
48	8.57 $\pm$ 0.88 ^a	5.8	8.42 $\pm$ 0.65 ^{ab}	2.35 $\pm$ 0.34 ^c	5.4	400	8.41 $\pm$ 0.42 ^{ad}	8.40 $\pm$ 0.81 ^{ae}	5.6

a,b,c,d,e values (Mean $\pm$ SD) with different superscript in a column are significantly different at the level of $p < 0.05$

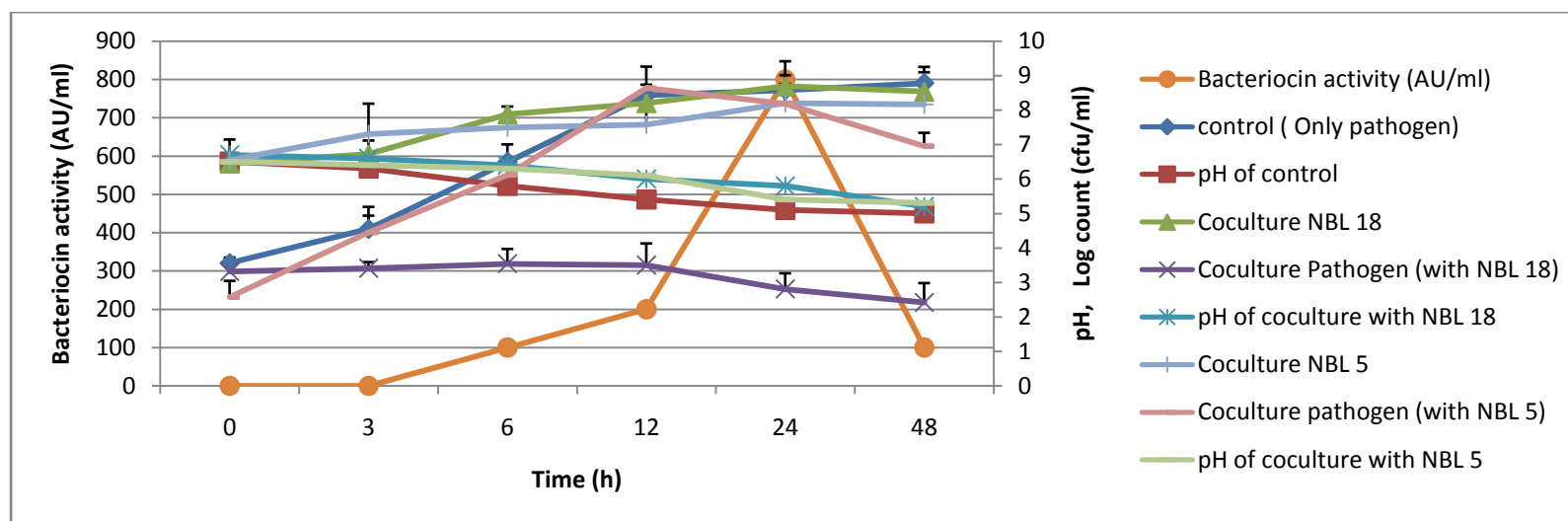


Fig. 4.21. Growth of *Staphylococcus aureus* NCDC 273 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5

Table 4.26. Growth of *Staphylococcus aureus* NCDC 273 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5. All values (log counts) are expressed as mean $\pm$ standard deviation with three replicates

Time (h)	Control (Only pathogen)	pH of control	Coculture NBL 18	Coculture Pathogen (with NBL 18)	pH of coculture with NBL 18	Bacteriocin activity (AU/ml)	Coculture NBL 5	Coculture pathogen (with NBL 5)	pH of coculture with NBL 5
0	3.56 $\pm$ 0.86 ^{ae}	6.5	6.45 $\pm$ 0.242 ^{be}	3.32 $\pm$ 0.401 ^{ac}	6.7	0	6.55 $\pm$ 0.595 ^d	2.57 $\pm$ 0.475 ^{be}	6.5
3	4.55 $\pm$ 0.641 ^{ac}	6.3	6.72 $\pm$ 0.395 ^b	3.41 $\pm$ 0.180 ^c	6.6	0	7.30 $\pm$ 0.886 ^{bd}	4.45 $\pm$ 0.486 ^{ace}	6.4
6	6.50 $\pm$ 0.505 ^a	5.8	7.88 $\pm$ 0.222 ^b	3.54 $\pm$ 0.430 ^c	6.4	100	7.49 $\pm$ 0.375 ^{bd}	6.12 $\pm$ 0.46 ^e	6.3
12	8.43 $\pm$ 0.306 ^{ae}	5.4	8.20 $\pm$ 0.327 ^{ab}	3.50 $\pm$ 0.630 ^c	6.0	200	7.58 $\pm$ 0.755 ^{ad}	8.64 $\pm$ 0.62 ^{be}	6.1
24	8.58 $\pm$ 0.431 ^a	5.1	8.69 $\pm$ 0.725 ^{ab}	2.81 $\pm$ 0.455 ^c	5.8	800	8.20 $\pm$ 0.555 ^{ad}	8.18 $\pm$ 0.292 ^{ae}	5.4
48	8.78 $\pm$ 0.472 ^a	5.0	8.53 $\pm$ 0.274 ^{ab}	2.42 $\pm$ 0.560 ^c	5.2	100	8.16 $\pm$ 0.935 ^{ad}	6.95 $\pm$ 0.39 ^{bde}	5.3

a,b,c,d,e values (Mean $\pm$ SD) with different superscript in a column are significantly different at the level of $p < 0.05$

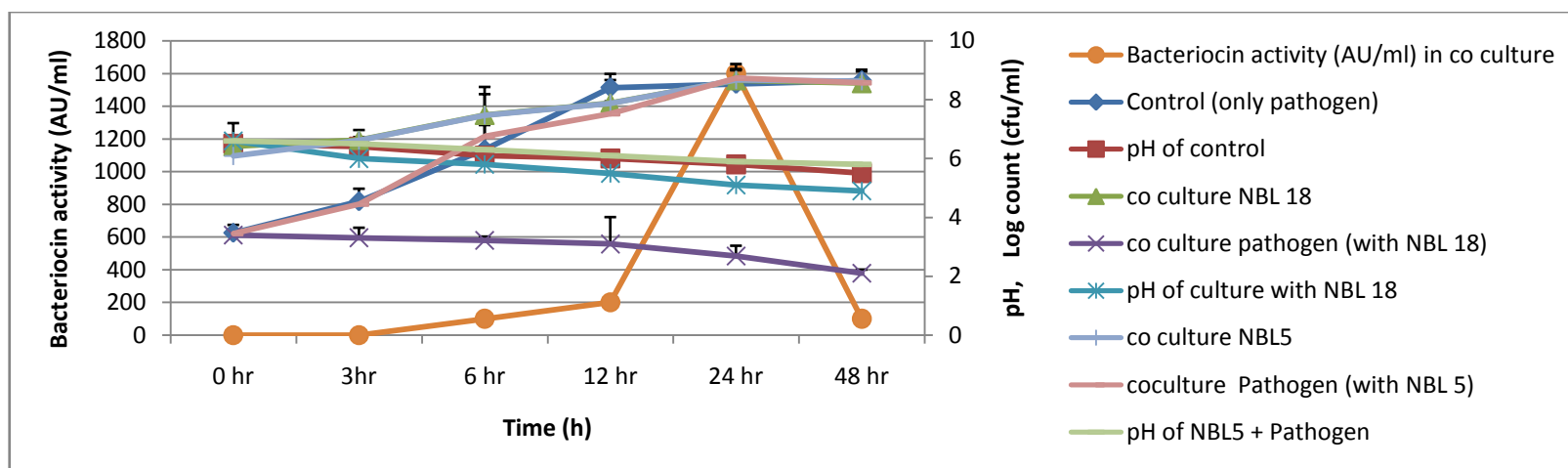


Fig. 4.22. Growth of *Enterococcus faecalis* NCDC 114 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5

Table 4.27. Growth of *Enterococcus faecalis* NCDC 114 in co-culture with bacteriocin producing *Lactobacillus gasseri* NBL 18 and non-bacteriocinogenic NBL5. All values (log counts) are expressed as mean $\pm$ standard deviation with three replicates

Time (h)	Control (Only pathogen)	pH of control	Co culture NBL 18	Co culture pathogen (with NBL 18)	pH of culture with NBL 18	Bacteriocin activity (AU/ml) in co culture	Co culture NBL5	Coculture Pathogen (with NBL 5)	pH of NBL5 + Pathogen
0	3.48 $\pm$ 0.267 ^a	6.5	6.42 $\pm$ 0.782 ^{ab}	3.4 $\pm$ 0.178 ^{ac}	6.6	0	6.09 $\pm$ 0.134 ^b	3.45 $\pm$ 0.121 ^c	6.6
3	4.55 $\pm$ 0.425 ^{ac}	6.4	6.63 $\pm$ 0.100 ^{bd}	3.31 $\pm$ 0.340 ^{ce}	6.0	0	6.62 $\pm$ 0.348 ^d	4.45 $\pm$ 0.098 ^{ae}	6.5
6	6.31 $\pm$ 0.896 ^{ac}	6.1	7.48 $\pm$ 0.950 ^{bc}	3.22 $\pm$ 0.130 ^c	5.8	100	7.47 $\pm$ 0.715 ^{db}	6.75 $\pm$ 0.387 ^{abe}	6.3
12	8.41 $\pm$ 0.462 ^{abd}	6.0	7.89 $\pm$ 0.775 ^{ad}	3.10 $\pm$ 0.911 ^b	5.5	200	7.87 $\pm$ 0.445 ^{ac}	7.52 $\pm$ 0.420 ^d	6.1
24	8.53 $\pm$ 0.465 ^a	5.8	8.66 $\pm$ 0.549 ^a	2.69 $\pm$ 0.349 ^c	5.1	1600	8.67 $\pm$ 0.355 ^{ad}	8.73 $\pm$ 0.301 ^{ae}	5.9
48	8.65 $\pm$ 0.095 ^a	5.5	8.56 $\pm$ 0.120 ^a	2.11 $\pm$ 0.120 ^c	4.9	100	8.63 $\pm$ 0.325 ^{ad}	8.57 $\pm$ 0.445 ^{ae}	5.8

a,b,c,d,e values (Mean $\pm$ SD) with different superscript in a column are significantly different at the level of $p < 0.05$

showed no haemolytic activity on blood agar. Such strain may not, therefore, pose any problem for its use in food industry. Cytolysin/hemolysin activity is seen as a potent virulence factor in several animal model studies (Ike *et al.*, 1984). Fortunately, no β -hemolysis was observed in NBL 18 of faecal origin and thus apparently showing absence of cytolysin gene amongst the strain.

The antimicrobial resistance of NBL 18 is reported in Table 4.20 (Plate 4.22). Within the group of antimicrobial agents that inhibit the cell wall synthesis, it was susceptible to ampicillin but not to vancomycin. Also susceptibility to tetracycline, erythromycin and chloramphenicol was observed. Regarding the aminoglycosides, the strain was sensitive to streptomycin, kanamycin and gentamycin. As for the antibiotics that inhibit the nucleic acids synthesis, the strain was sensitive to ciprofloxacin.

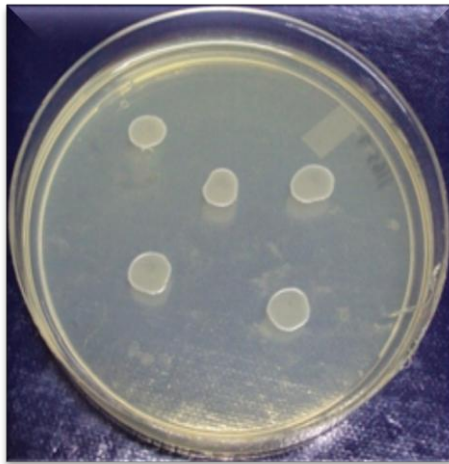
Because of their long-time use in various food and feed products, LAB have been given the GRAS status (Borriello *et al.*, 2003; Salminen *et al.*, 1998) however, several studies have recently documented the presence and expression of antibiotic resistance genes in food associated LAB, including probiotics (Mathur *et al.*, 2005; Perreten *et al.*, 1997), even though this trait is not commonly found in dairy LAB species (Ammor *et al.*, 2008). Recently, the European Food Safety Authority (EFSA) has introduced the Qualified Presumption of Safety (QPS) concept, which is similar to the GRAS system in the United States and would allow microorganisms for which there are no special safety concerns to enter the market without extensive testing requirements (EFSA, 2004). The presence of acquired antibiotic resistance is considered by EFSA an important safety criterion for determining a strain's QPS status (EFSA, 2008). In this study, NBL 18 analysed was resistant only to vancomycin and sensitive to other clinically important antibiotics such as ampicillin, tetracycline, and chloramphenicol, in agreement with other findings (Swenson *et al.*, 1990; Bayer *et al.*, 1978; Holliman *et al.*, 1988), but in contrast with some reports where a high frequency of vancomycin sensitivity was observed in probiotic strains of *L. acidophilus* and *L. delbruekii* (Hamilton –Miller *et al.*, 1998). A variance in our results from some other reports may be explained by the lack of standardization in phenotypic antibiotic resistance testing in LAB food isolates, including differences in methods and media used. Resistance to some antibiotics such as vancomycin has been reported to be intrinsic for lactobacilli (Swenson *et al.*, 1990); but particular attention should be paid to the presence of transferable resistance, since strains of lactobacilli have been reported to harbor different plasmid-encoded resistance

determinants (Chopra & Roberts, 2001; Roberts, 2005; Ammor *et al.*, 2007; Gevers *et al.*, 2003; Klare *et al.*, 2007; van Hoek *et al.*, 2008). According to EFSA (2008), antibiotic resistance in lactobacilli has been heavily debated during the last decade. Lactobacilli intentionally added to the food chain should not carry transferable antibiotic resistance genes. Thus, such traits are currently a ‘no go area’ in the development of new strains. Alternatively, curative strategies could be applied to health promoting or starter lactobacilli strains to remove plasmids carrying unwanted antibiotic resistance genes (Huys *et al.*, 2006; Rosander *et al.*, 2008). One such example is the commercial probiotic *L. reuteri* strain DSM 17938, which was derived from *L. reuteri* ATCC 55730 by the removal of two resistance plasmids without losing any probiotic characteristics (Rosander *et al.*, 2008).

The *in-situ* production of bacteriocins by *Lactobacillus* strains in dairy foods provides a very attractive alternative to the use of purified bacteriocin, since many of them also generate specific aromas and flavors, but several issues including safety and adequate technological properties of the selected strains need to be addressed. As the *L. gasseri* NBL 18 strain studied in the present investigation originated from infant feces and exhibited a strong inhibitory activity against several Gram positive pathogens, they may be useful in controlling the growth of these pathogens in dairy fermentation. The low level of antibiotic resistance observed in our strain could be of interest for a possible technological application since it has been demonstrated that *Lactobacillus* isolates displaying properties of technological interest generally exhibited a low-resistance phenotype (less than two antibiotics). Application of this bacteriocin-producing strain in food system studies is necessary to determine its effectiveness.

Table 4. 28. Antibiotic susceptibility of NBL 18

Antibiotic	Susceptibility
Vancomycin	R
Streptomycin	I
Gentamycin	S
Rifampicin	S
Bacitracin	S
Kanamycin	I
PolymyxinB	S
Tetracycline	S
PenicillinG	S



(A)



(B)

Plate 4.21. Plates showing negative result for Gelatinase production (A) and DNase activity (B) by NBL18

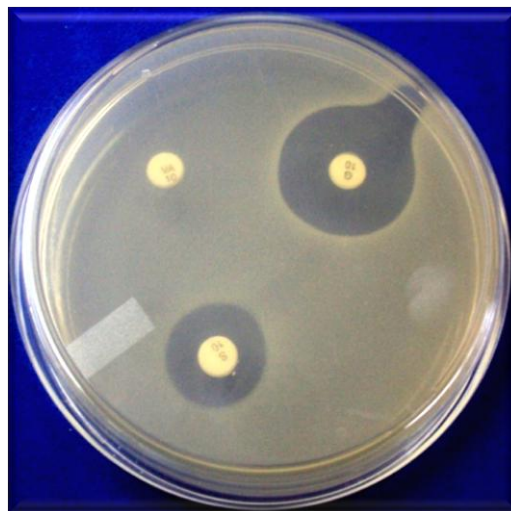


Plate 4.22. Plate showing vancomycin resistance and sensitivity to other antibiotics by NBL 18

Erythromycin	S
Amoxycillin	S
Ciprofloxacin	I
Chloramphenicol	S
Ampicillin	S
Cefazolin	S
Cefuroxime	S
Amikacin	S
Co-Trimoxazole	S

R-Resistant, I-Intermediate sensitive, S-Sensitive

4.17. EPILOGUE

The present investigation was focused on the isolation and identification of circular bacteriocin producing strains of *Lactobacillus gasseri* from infant feces, human saliva and human milk. The bacteriocinogenic isolates were screened for their antimicrobial spectrum of activity against a number of Gram positive and Gram negative bacteria and yeasts. One potent circular bacteriocin producer *L. gasseri* NBL 18 was selected for further studies and its bacteriocin production was optimized using Response Surface Methodology besides the purification and characterization of bacteriocin. Results pertaining to the application of the bacteriocin for preservation of whipped cream frosting and *in-situ* production of bacteriocin in reconstituted skim milk by *L. gasseri* NBL 18 showed that both the isolate (*L. gasseri* NBL 18) and its bacteriocin can be applied in the food system for effective preservation.

Circular bacteriocin production provides an additional advantage to the producing strain for its establishment and competition as compared to linear bacteriocin producing strain. Significant reduction in the count of pathogens by the bacteriocin produced by *L. gasseri* NBL 18, may provide valuable alternatives to the use of chemical preservatives for food preservation. Further study regarding safety aspect of the *L. gasseri* NBL 18 revealed that the isolate may be safe for consumption, however, a thorough investigation of the probiotic attributes and toxicological effects of the isolate on animal model and human volunteers is required.

Chapter 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

5.1. SUMMARY

The salient findings of the present investigation encompassing the isolation of *Lactobacillus gasseri* from infant feces, human saliva and human milk, screening of *L. gasseri* isolates for bacteriocin activity, screening of bacteriocinogenic isolates for *gaaA* gene through PCR, optimization of cultural and environmental parameters for bacteriocin production by the finally selected *L. gasseri* isolate, NBL 18, purification and characterization of the bacteriocin NBL 18, determination of its antimicrobial activity in whipped cream and skimmed milk (*in-situ* production), and safety evaluation of the isolate are summarized in this section.

- i. Lactobacilli are almost ubiquitous as they could be isolated from GI tract and oral cavity of humans and also from human milk. The counts of lactobacilli ranged from 10^9 - 10^{10} CFU/g in infant feces, 10^3 - 10^4 CFU/ml in human saliva and 10^1 - 10^2 CFU/ml in human milk.
- ii. Out of 523 isolates selected randomly, 198 isolates were found to belong to genus *Lactobacillus* through genus specific PCR, which accounts for 37.8% of total isolates.
- iii. 146 isolates were further confirmed as *Lactobacillus gasseri*, by species specific PCR, which accounts for 73.73% of total isolated *Lactobacillus* strains. Among them 29.55% were from infant feces, 8% from human milk and 34% from saliva samples.
- iv. *Enterococcus faecalis* NCDC 114 was found to be the most sensitive amongst the four indicator strains tested for screening bacteriocinogenic *L. gasseri*, whereas *E.coli* NCDC 134 was completely resistant.
- v. The overall frequency of bacteriocin production by *L. gasseri* isolates was found to be 28.76% by spot on lawn assay.
- vi. The incidence of bacteriocinogenic *Lactobacilli* was highest in saliva (16%) followed by infant feces (7.32%) and human milk (6 %).
- vii. Six out of 42 bacteriocinogenic *L. gasseri* were found to be positive for *gaaA* gene through PCR.

- viii. These six potent and stable producers of bacteriocin were found to have antagonistic activity against a wide range of Gram positive organisms, however, none could inhibit any of the Gram negative organism or yeasts tested.
- ix. Isolates showed strong inhibitory activity against all the Gram positive pathogens tested such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Enterococcus faecalis*.
- x. Isolate NBL 18 showing the best antimicrobial spectrum of activity was selected for further studies.
- xi. The PCR product of 16S-23S rRNA of NBL 18 was sequenced and submitted under the accession number JQ809334.1.
- xii. Sequencing of the PCR product of *gaaA* gene of NBL 18 and subsequent analysis through NCBI BLAST search confirmed that the gene sequence of PCR product showed 100% sequence similarity with the submitted nucleotide sequence of gassericin A produced by *L. gasseri* LA 39.
- xiii. A sequence similarity of 99% was obtained with the nucleotide sequence of acidocin B (produced by *L. acidophilus* M 46) which is considered as a putative circular bacteriocin.
- xiv. The optimization of bacteriocin production was performed through response surface methodology using Central Composite Rotatable Design (CCRD). For the diagnostic check of the model, the quadratic model for various responses was obtained through successive regression analysis.
- xv. The model F values for all attributes were more than the table F values at 5% level of confidence and it indicated the significance of model terms.
- xvi. The lack of fit test, which measure the fitness of the model obtained, did not result in a significant F value, indicating that the model is sufficiently accurate for predicting the bacteriocin production by *L. gasseri* NBL 18 from any combination of factors levels within the range evaluated.
- xvii. pH after growth of NBL 18 was highly significantly ($p < 0.01$) positively affected by the pH of broth, negatively affected by incubation time, incubation temperature and inoculums level at linear level, but statistically non-significant.

- xviii. Optical density after growth was highly significantly positively ($p < 0.01$) affected by pH and positively affected by incubation time at linear level. It was negatively affected by temperature and positively affected by inoculum level, but statistically non significant at linear level.
- xix. Bacteriocin activity after growth was highly significantly positively ($p < 0.01$) affected by pH of the broth and inoculum level and negatively affected by incubation temperature and positively affected by incubation time, but statistically non significant at linear level.
- xx. The optimum solution obtained as a result of numerical optimization was verified and the optimum level of pH (8.00), incubation temperature (37°C), inoculation level (3%) and incubation time (24h) were used for maximum bacteriocin production.
- xxi. The actual values of the optimization were compared with the predicted values given by the software using t-test. The t-test indicated that there were no significant differences between the predicted and the observed values of bacteriocin produced.
- xxii. Bacteriocin from NBL 18 was partially purified by 60% ammonium sulphate saturation. This process yielded 64 % recovery of the bacteriocin activity.
- xxiii. Further purification of partially purified bacteriocin with other techniques *viz.*, ion-exchange chromatography and RP-HPLC yielded very poor (<1%) recovery of the bacteriocin activity.
- xxiv. The SDS-PAGE and *in-situ* gel activity revealed that the protein band corresponding to the inhibition zone has a molecular weight of about 3.5kDa.
- xxv. The partially purified bacteriocin showed broad spectrum of activity against all the Gram positive pathogens, but had no activity against Gram negative organisms.
- xxvi. The CD-spectra of the peptide revealed that it is mainly composed of α - helices (78%).
- xxvii. The bacteriocin was sensitive to all the proteolytic enzymes but insensitive to all the non-proteolytic enzymes tested. It was quite heat stable and active over a

wide range of temperature and pH, a characteristic quite common of class V bacteriocins.

- xxviii. The bacteriocin concentration required to inhibit the growth of *Staphylococcus aureus* and *Listeria monocytogenes* was respectively 6.4 and 3.2 X 10³ AU/ml.
- xxix. In the microtitre plate assay, mixed solutions of bacteriocin of NBL 18 and glycine inhibited all the strains tested, which is not possible by either of them alone. This combination was also successful in inhibiting both Gram positive and Gram negative pathogens in whipped cream frosting.
- xxx. The coculture study and the safety evaluation of the isolate suggests that the isolate NBL 18 could be potentially applied in the food system to inhibit the growth of food borne pathogens.

5.2. CONCLUSIONS

Circular bacteriocins are antimicrobial peptides produced by a variety of Gram-positive bacteria. They are part of a growing family of ribosomally synthesized peptides with a head-to-tail cyclization of their backbone that are found in mammals, plants, fungi and bacteria and are exceptionally stable as compared to linear bacteriocins. They are able to inhibit the growth of a wide variety of Gram-positive bacteria, including many food spoilage bacteria and pathogens. Current interest is focused in the isolation of circular bacteriocin producing Lactic acid bacteria as these are generally recognized as safe (GRAS) in food products. Present investigation has afforded a bacteriocinogenic strain of *Lactobacillus gasseri*, NBL 18 that is quite unique for its very high antibacterial activity and inhibition towards *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other spoilage and pathogenic bacteria. Bacteriocin of NBL 18 is quite heat stable and active over a wide range of pH indicating that it can be exploited in the biopreservation of acidic as well as non-acidic foods. A careful evaluation of these strains for their technological performance may help in their successful exploitation as starter/adjunct/protective cultures. The *in-situ* production of bacteriocins by NBL 18 in reconstituted skim milk provides a very attractive alternative to the use of purified bacteriocin, since many strains also generate specific aromas and flavors during their growth, but several issues including safety and adequate technological properties of the selected strains need to be addressed. The low level of antibiotic resistance observed in our strain could be of interest for a possible technological application since it has been demonstrated that isolates displaying properties of technological interest generally exhibit a low-resistance phenotype (less than two antibiotics). This bacteriocinogenic strain may be the valuable alternative to the chemical preservative and antibiotics used for food preservation or treatment of human and animal infections. But, there is still a great deal of knowledge required to learn about the strain and its interaction with its respective hosts.

5.3. FUTURE PROJECTIONS

- Use of *L. gasseri* NBL 18 as a functional starter/ adjunct culture/ protective culture needs to be explored in other food and dairy products for its successful application in the food and dairy industry.
- *L. gasseri* NBL 18 could be used as emerging biotherapeutic agent in human and animal health promotion programs. Therefore, further *in-vitro* and *in-vivo* studies may be conducted for its establishment as a potential probiotic organism.

Chapter 6

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Annexures

ANNEXURE I

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *LACTOBACILLUS* ISOLATES

SNo	Isolate NBL	Gram staining	Growth		Catalase	Arginine hydrolysis	Aesculin hydrolysis	Presumptive identification
			15°C	45°C				
1	NBL 1	+	—	+	-	-	+	<i>Lactobacillus</i>
2	NBL 2	+	—	+	-	-	+	<i>Lactobacillus</i>
3	NBL 3	+	—	+	-	-	+	<i>Lactobacillus</i>
4	NBL 4	+	—	+	-	-	+	<i>Lactobacillus</i>
5	NBL 5	+	—	+	-	-	+	<i>Lactobacillus</i>
6	NBL 6	+	—	+	-	-	+	<i>Lactobacillus</i>
7	NBL 7	+	—	+	-	-	+	<i>Lactobacillus</i>
8	NBL 8	+	—	+	-	-	+	<i>Lactobacillus</i>
9	NBL 9	+	—	+	-	-	+	<i>Lactobacillus</i>
10	NBL 10	+	—	+	-	-	+	<i>Lactobacillus</i>
11	NBL 11	+	—	+	-	-	+	<i>Lactobacillus</i>
12	NBL 12	+	—	+	-	-	+	<i>Lactobacillus</i>
13	NBL 13	+	—	+	-	-	+	<i>Lactobacillus</i>
14	NBL 14	+	—	+	-	-	+	<i>Lactobacillus</i>
15	NBL 15	+	—	+	-	-	+	<i>Lactobacillus</i>
16	NBL 16	+	—	+	-	-	+	<i>Lactobacillus</i>
17	NBL 17	+	—	+	-	-	+	<i>Lactobacillus</i>
18	NBL 18	+	—	+	-	-	+	<i>Lactobacillus</i>
19	NBL 19	+	—	+	-	-	+	<i>Lactobacillus</i>
20	NBL 20	+	—	+	-	-	+	<i>Lactobacillus</i>
21	NBL 21	+	—	+	-	-	+	<i>Lactobacillus</i>
22	NBL 22	+	—	+	-	-	+	<i>Lactobacillus</i>
23	NBL 23	+	—	+	-	-	+	<i>Lactobacillus</i>
24	NBL 24	+	—	+	-	-	+	<i>Lactobacillus</i>
25	NBL 25	+	—	+	-	-	+	<i>Lactobacillus</i>
26	NBL 26	+	—	+	-	-	+	<i>Lactobacillus</i>
27	NBL 27	+	—	+	-	-	+	<i>Lactobacillus</i>
28	NBL 28	+	—	+	-	-	+	<i>Lactobacillus</i>
29	NBL 29	+	—	+	-	-	+	<i>Lactobacillus</i>
30	NBL 30	+	—	+	-	-	+	<i>Lactobacillus</i>
31	NBL 31	+	—	+	-	-	+	<i>Lactobacillus</i>
32	NBL 32	+	—	+	-	-	+	<i>Lactobacillus</i>
33	NBL 33	+	—	+	-	-	+	<i>Lactobacillus</i>
34	NBL 34	+	—	+	-	-	+	<i>Lactobacillus</i>
35	NBL 35	+	—	+	-	-	+	<i>Lactobacillus</i>
36	NBL 36	+	—	+	-	-	+	<i>Lactobacillus</i>
37	NBL 37	+	—	+	-	-	+	<i>Lactobacillus</i>
38	NBL 38	+	—	+	-	-	+	<i>Lactobacillus</i>
39	NBL 39	+	—	+	-	-	+	<i>Lactobacillus</i>
40	NBL 40	+	—	+	-	-	+	<i>Lactobacillus</i>
41	NBL 41	+	—	+	-	-	+	<i>Lactobacillus</i>
42	NBL 42	+	—	+	-	-	+	<i>Lactobacillus</i>
43	NBL 43	+	—	+	-	-	+	<i>Lactobacillus</i>

44	NBL 44	+	—	+	-	-	+	<i>Lactobacillus</i>
45	NBL 45	+	—	+	+	+	-	ND
46	NBL 46	+	—	+	+	+	-	ND
47	NBL 47	+	—	+	+	+	-	ND
48	NBL 48	+	—	+	+	+	-	ND
49	NBL 49	+	—	+	+	+	-	ND
50	NBL 50	+	—	+	+	+	-	ND
51	NBL 51	+	—	+	+	+	-	ND
52	NBL 52	+	—	+	-	-	+	<i>Lactobacillus</i>
53	NBL 53	+	—	+	-	-	+	<i>Lactobacillus</i>
54	NBL 54	+	—	+	-	-	+	<i>Lactobacillus</i>
55	NBL 55	+	—	+	-	-	+	<i>Lactobacillus</i>
56	NBL 56	+	—	+	-	-	+	<i>Lactobacillus</i>
57	NBL 57	+	—	+	-	-	+	<i>Lactobacillus</i>
58	NBL 58	+	—	+	-	-	+	<i>Lactobacillus</i>
59	NBL 59	+	—	+	-	-	+	<i>Lactobacillus</i>
60	NBL 60	+	—	+	-	-	+	<i>Lactobacillus</i>
61	NBL 61	+	—	+	-	-	+	<i>Lactobacillus</i>
62	NBL 62	+	—	+	-	-	+	<i>Lactobacillus</i>
63	NBL 63	+	—	+	-	-	+	<i>Lactobacillus</i>
64	NBL 64	+	—	+	-	-	+	<i>Lactobacillus</i>
65	NBL 65	+	—	+	-	-	+	<i>Lactobacillus</i>
66	NBL 66	+	—	+	-	-	+	<i>Lactobacillus</i>
67	NBL 67	+	—	+	-	-	+	<i>Lactobacillus</i>
68	NBL 68	+	—	+	-	-	+	<i>Lactobacillus</i>
69	NBL 69	+	—	+	-	-	+	<i>Lactobacillus</i>
70	NBL 70	+	—	+	-	-	+	<i>Lactobacillus</i>
71	NBL 71	+	—	+	-	-	+	<i>Lactobacillus</i>
72	NBL 72	+	—	+	-	-	+	<i>Lactobacillus</i>
73	NBL 73	+	—	+	-	-	+	<i>Lactobacillus</i>
74	NBL 74	+	—	+	-	-	+	<i>Lactobacillus</i>
75	NBL 75	+	—	+	-	-	+	<i>Lactobacillus</i>
76	NBL 76	+	—	+	-	-	+	<i>Lactobacillus</i>
77	NBL 77	+	—	+	-	-	+	<i>Lactobacillus</i>
78	NBL 78	+	—	+	-	-	+	<i>Lactobacillus</i>
79	NBL 79	+	—	+	-	-	+	<i>Lactobacillus</i>
80	NBL 80	+	—	+	-	-	+	<i>Lactobacillus</i>
81	NBL 81	+	—	+	-	-	+	<i>Lactobacillus</i>
82	NBL 82	+	—	+	-	-	+	<i>Lactobacillus</i>
83	NBL 83	+	—	+	-	-	+	<i>Lactobacillus</i>
84	NBL 84	+	—	+	-	-	+	<i>Lactobacillus</i>
85	NBL 85	+	—	+	-	-	+	<i>Lactobacillus</i>
86	NBL 86	+	—	+	-	-	+	<i>Lactobacillus</i>
87	NBL 87	+	—	+	-	-	+	<i>Lactobacillus</i>
88	NBL 88	+	—	+	-	-	+	<i>Lactobacillus</i>
89	NBL 89	+	—	+	-	-	+	<i>Lactobacillus</i>
90	NBL 90	+	—	+	-	-	+	<i>Lactobacillus</i>
91	NBL 91	+	—	+	-	-	+	<i>Lactobacillus</i>
92	NBL 92	+	—	+	-	-	+	<i>Lactobacillus</i>
93	NBL 93	+	—	+	-	-	+	<i>Lactobacillus</i>

94	NBL 94	+	—	+	-	-	+	<i>Lactobacillus</i>
95	NBL 95	+	—	+	-	-	+	<i>Lactobacillus</i>
96	NBL 96	+	—	+	-	-	+	<i>Lactobacillus</i>
97	NBL 97	+	—	+	-	-	+	<i>Lactobacillus</i>
98	NBL 98	+	—	+	-	-	+	<i>Lactobacillus</i>
99	NBL 99	+	—	+	-	-	+	<i>Lactobacillus</i>
100	NBL 100	+	—	+	-	-	+	<i>Lactobacillus</i>
101	NBL 101	+	—	+	-	-	+	<i>Lactobacillus</i>
102	NBL 102	+	—	+	-	-	+	<i>Lactobacillus</i>
103	NBL 103	+	—	+	-	-	+	<i>Lactobacillus</i>
104	NBL 104	+	—	+	-	-	+	<i>Lactobacillus</i>
105	NBL 105	+	—	+	-	-	+	<i>Lactobacillus</i>
106	NBL 106	+	—	+	-	-	+	<i>Lactobacillus</i>
107	NBL 107	+	—	+	-	-	+	<i>Lactobacillus</i>
108	NBL 108	+	—	+	-	-	+	<i>Lactobacillus</i>
109	NBL 109	+	—	+	-	-	+	<i>Lactobacillus</i>
110	NBL 110	+	—	+	-	-	+	<i>Lactobacillus</i>
111	NBL 111	+	—	+	-	-	+	<i>Lactobacillus</i>
112	NBL 112	+	—	+	-	-	+	<i>Lactobacillus</i>
113	NBL 113	+	—	+	-	-	+	<i>Lactobacillus</i>
114	NBL 114	+	—	+	-	-	+	<i>Lactobacillus</i>
115	NBL 115	+	—	+	-	-	+	<i>Lactobacillus</i>
116	NBL 116	+	—	+	-	-	+	<i>Lactobacillus</i>
117	NBL 117	+	—	+	-	-	+	<i>Lactobacillus</i>
118	NBL 118	+	—	+	-	-	+	<i>Lactobacillus</i>
119	NBL 119	+	—	+	-	-	+	<i>Lactobacillus</i>
120	NBL 120	+	—	+	-	-	+	<i>Lactobacillus</i>
121	NBL 121	+	—	+	-	-	+	<i>Lactobacillus</i>
122	NBL 122	+	—	+	-	-	+	<i>Lactobacillus</i>
123	NBL 123	+	—	+	-	-	+	<i>Lactobacillus</i>
124	NBL 124	+	—	+	-	-	+	<i>Lactobacillus</i>
125	NBL 125	+	—	+	+	-	+	ND
126	NBL 126	+	—	+	+	-	+	ND
127	NBL 127	+	—	+	+	-	+	ND
128	NBL 128	+	—	+	+	-	+	ND
129	NBL 129	+	—	+	+	-	+	ND
130	NBL 130	+	—	+	+	-	+	ND
131	NBL 131	+	—	+	+	-	+	ND
132	NBL 132	+	—	+	-	-	+	<i>Lactobacillus</i>
133	NBL 133	+	—	+	-	-	+	<i>Lactobacillus</i>
134	NBL 134	+	—	+	-	-	+	<i>Lactobacillus</i>
135	NBL 135	+	—	+	-	-	+	<i>Lactobacillus</i>
136	NBL 136	+	—	+	-	-	-	ND
137	NBL 137	+	—	+	-	-	-	ND
138	NBL 138	+	—	+	-	-	-	ND
139	NBL 139	+	—	+	-	-	-	ND
140	NBL 140	+	—	+	-	-	-	ND
141	NBL 141	+	—	+	-	-	-	ND
142	NBL 142	+	—	+	-	-	-	ND
143	NBL 143	+	—	+	-	-	-	ND

144	NBL 144	+	—	+	-	-	-	ND
145	NBL 145	+	—	+	-	-	-	ND
146	NBL 146	+	—	+	-	-	-	ND
147	NBL 147	+	—	+	-	-	-	ND
148	NBL 148	+	—	+	-	-	-	ND
149	NBL 149	+	—	+	-	-	-	ND
150	NBL 150	+	—	+	-	-	-	ND
151	NBL 151	+	—	+	-	-	-	ND
152	NBL 152	+	—	+	-	-	-	ND
153	NBL 153	+	—	+	-	-	-	ND
154	NBL 154	+	—	+	-	-	+	<i>Lactobacillus</i>
155	NBL 155	+	—	+	-	-	+	<i>Lactobacillus</i>
156	NBL 156	+	—	+	-	-	+	<i>Lactobacillus</i>
157	NBL 157	+	—	+	-	-	+	<i>Lactobacillus</i>
158	NBL 158	+	—	+	-	-	+	<i>Lactobacillus</i>
159	NBL 159	+	—	+	-	-	+	<i>Lactobacillus</i>
160	NBL 160	+	—	+	-	-	+	<i>Lactobacillus</i>
161	NBL 161	+	—	+	-	-	+	<i>Lactobacillus</i>
162	NBL 162	+	—	+	-	-	+	<i>Lactobacillus</i>
163	NBL 163	+	—	+	-	-	+	<i>Lactobacillus</i>
164	NBL 164	+	—	+	-	-	+	<i>Lactobacillus</i>
165	NBL 165	+	—	+	-	-	+	<i>Lactobacillus</i>
166	NBL 166	+	—	+	-	-	+	<i>Lactobacillus</i>
167	NBL 167	+	—	+	-	-	+	<i>Lactobacillus</i>
168	NBL 168	+	—	+	-	-	+	<i>Lactobacillus</i>
169	NBL 169	+	—	+	-	-	+	<i>Lactobacillus</i>
170	NBL 170	+	—	+	-	-	+	<i>Lactobacillus</i>
171	NBL 171	+	—	+	-	-	-	ND
172	NBL 172	+	—	+	-	-	-	ND
173	NBL 173	+	—	+	-	-	-	ND
174	NBL 174	+	—	+	-	-	-	ND
175	NBL 175	+	—	+	-	-	-	ND
176	NBL 176	+	—	+	-	-	-	ND
177	NBL 177	+	—	+	-	-	+	<i>Lactobacillus</i>
178	NBL 178	+	—	+	-	-	+	<i>Lactobacillus</i>
179	NBL 179	+	—	+	-	-	+	<i>Lactobacillus</i>
180	NBL 180	+	—	+	-	-	+	<i>Lactobacillus</i>
181	NBL 181	+	—	+	-	-	+	<i>Lactobacillus</i>
182	NBL 182	+	—	+	-	-	+	<i>Lactobacillus</i>
183	NBL 183	+	—	+	-	-	+	<i>Lactobacillus</i>
184	NBL 184	+	—	+	-	-	+	<i>Lactobacillus</i>
185	NBL 185	+	—	+	-	-	-	ND
186	NBL 186	+	—	+	-	-	-	ND
187	NBL 187	+	—	+	-	-	-	ND
188	NBL 188	+	—	+	-	-	-	ND
189	NBL 189	+	—	+	-	-	-	ND
190	NBL 190	+	—	+	-	-	-	ND
191	NBL 191	+	—	+	-	-	-	ND
192	NBL 192	+	—	+	-	-	-	ND
193	NBL 193	+	—	+	-	-	-	ND

194	NBL 194	+	—	+	-	-	-	ND
195	NBL 195	+	—	+	-	-	-	ND
196	NBL 196	+	—	+	-	-	-	ND
197	NBL 197	+	—	+	-	-	-	ND
198	NBL 198	+	—	+	-	-	-	ND
199	NBL 199	+	—	+	-	-	-	ND
200	NBL 200	+	—	+	-	-	-	ND
201	NBL 201	+	—	+	-	-	-	ND
202	NBL 202	+	—	+	-	-	-	ND
203	NBL 203	+	—	+	-	-	-	ND
204	NBL 204	+	—	+	-	-	-	ND
205	NBL 205	+	—	+	-	-	-	ND
206	NBL 206	+	—	+	-	-	-	ND
207	NBL 207	+	—	+	-	-	-	ND
208	NBL 208	+	—	+	-	-	-	ND
209	NBL 209	+	—	+	-	-	-	ND
210	NBL 210	+	—	+	-	-	-	ND
211	NBL 211	+	—	+	-	-	-	ND
212	NBL 212	+	—	+	-	-	-	ND
213	NBL 213	+	—	+	-	-	-	ND
214	NBL 214	+	—	+	-	-	-	ND
215	NBL 215	+	—	+	-	-	-	ND
216	NBL 216	+	—	+	-	-	-	ND
217	NBL 217	+	—	+	-	-	-	ND
218	NBL 218	+	—	+	-	-	-	ND
219	NBL 219	+	—	+	-	-	-	ND
220	NBL 220	+	—	+	-	-	-	ND
221	NBL 221	+	—	+	-	-	-	ND
222	NBL 222	+	—	+	-	-	-	ND
223	NBL 223	+	—	+	-	-	-	ND
224	NBL 224	+	—	+	-	-	-	ND
225	NBL 225	+	—	+	-	-	-	ND
226	NBL 226	+	—	+	-	-	-	ND
227	NBL 227	+	—	+	-	-	-	ND
228	NBL 228	+	—	+	-	-	-	ND
229	NBL 229	+	—	+	-	-	-	ND
230	NBL 230	+	—	+	-	-	-	ND
231	NBL 231	+	—	+	-	-	-	ND
232	NBL 232	+	—	+	-	-	-	ND
233	NBL 233	+	—	+	-	-	-	ND
234	NBL 234	+	—	+	-	-	-	ND
235	NBL 235	+	—	+	-	-	-	ND
236	NBL 236	+	—	+	-	-	-	ND
237	NBL 237	+	—	+	-	-	-	ND
238	NBL 238	+	—	+	-	-	-	ND
239	NBL 239	+	—	+	-	-	-	ND
240	NBL 240	+	—	+	-	-	-	ND
241	NBL 241	+	—	+	-	-	-	ND
242	NBL 242	+	—	+	-	-	-	ND
243	NBL 243	+	—	+	-	-	-	ND

244	NBL 244	+	—	+	-	-	-	ND
245	NBL 245	+	—	+	-	-	+	<i>Lactobacillus</i>
246	NBL 246	+	—	+	-	-	-	ND
247	NBL 247	+	—	+	-	-	+	<i>Lactobacillus</i>
248	NBL 248	+	—	+	-	-	-	ND
249	NBL 249	+	—	+	-	-	-	ND
250	NBL 250	+	—	+	-	-	-	ND
251	NBL 251	+	—	+	-	-	-	ND
252	NBL 252	+	—	+	-	-	-	ND
253	NBL 253	+	—	+	-	-	-	ND
254	NBL 254	+	—	+	-	-	-	ND
255	NBL 255	+	—	+	-	-	+	<i>Lactobacillus</i>
256	NBL 256	+	—	+	-	-	+	<i>Lactobacillus</i>
257	NBL 257	+	—	+	-	-	+	<i>Lactobacillus</i>
258	NBL 258	+	—	+	-	-	+	<i>Lactobacillus</i>
259	NBL 259	+	—	+	-	-	+	<i>Lactobacillus</i>
260	NBL 260	+	—	+	-	-	+	<i>Lactobacillus</i>
261	NBL 261	+	—	+	-	-	+	<i>Lactobacillus</i>
262	NBL 262	+	—	+	-	-	+	<i>Lactobacillus</i>
263	NBL 263	+	—	+	-	-	+	<i>Lactobacillus</i>
264	NBL 264	+	—	+	-	-	+	<i>Lactobacillus</i>
265	NBL 265	+	—	+	-	-	+	<i>Lactobacillus</i>
266	NBL 266	+	—	+	-	-	+	<i>Lactobacillus</i>
267	NBL 267	+	—	+	-	-	+	<i>Lactobacillus</i>
268	NBL 268	+	—	+	-	-	+	<i>Lactobacillus</i>
269	NBL 269	+	—	+	-	-	+	<i>Lactobacillus</i>
270	NBL 270	+	—	+	-	-	+	<i>Lactobacillus</i>
271	NBL 271	+	—	+	-	-	+	<i>Lactobacillus</i>
272	NBL 272	+	—	+	-	-	+	<i>Lactobacillus</i>
273	NBL 273	+	—	+	-	-	+	<i>Lactobacillus</i>
274	NBL 274	+	—	+	-	-	+	<i>Lactobacillus</i>
275	NBL 275	+	—	+	-	-	+	<i>Lactobacillus</i>
276	NBL 276	+	—	+	-	-	+	<i>Lactobacillus</i>
277	NBL 277	+	—	+	-	-	+	<i>Lactobacillus</i>
278	NBL 278	+	—	+	-	-	+	<i>Lactobacillus</i>
279	NBL 279	+	—	+	-	-	+	<i>Lactobacillus</i>
280	NBL 280	+	—	+	-	-	+	<i>Lactobacillus</i>
281	NBL 281	+	—	+	-	-	+	<i>Lactobacillus</i>
282	NBL 282	+	—	+	-	-	+	<i>Lactobacillus</i>
283	NBL 283	+	—	+	-	-	+	<i>Lactobacillus</i>
284	NBL 284	+	—	+	-	-	+	<i>Lactobacillus</i>
285	NBL 285	+	—	+	-	-	+	<i>Lactobacillus</i>
286	NBL 286	+	—	+	-	-	+	<i>Lactobacillus</i>
287	NBL 287	+	—	+	-	-	+	<i>Lactobacillus</i>
288	NBL 288	+	—	+	-	-	-	ND
289	NBL 289	+	—	+	-	-	-	ND
290	NBL 290	+	—	+	-	-	-	ND
291	NBL 291	+	—	+	-	-	-	ND
292	NBL 292	+	—	+	-	-	-	ND
293	NBL 293	+	—	+	-	-	-	ND

294	NBL 294	+	—	+	-	-	-	ND
295	NBL 295	+	—	+	-	-	-	ND
296	NBL 296	+	—	+	-	-	-	ND
297	NBL 297	+	—	+	-	-	-	ND
298	NBL 298	+	—	+	-	-	-	ND
299	NBL 299	+	—	+	-	-	-	ND
300	NBL 300	+	—	+	-	-	-	ND
301	NBL 301	+	—	+	-	-	-	ND
302	NBL 302	+	—	+	-	-	-	ND
303	NBL 303	+	—	+	-	-	-	ND
304	NBL 304	+	—	+	-	-	-	ND
305	NBL 305	+	—	+	-	-	-	ND
306	NBL 306	+	—	+	-	-	-	ND
307	NBL 307	+	—	+	-	-	-	ND
308	NBL 308	+	—	+	-	-	-	ND
309	NBL 309	+	—	+	-	-	-	ND
310	NBL 310	+	—	+	-	-	-	ND
311	NBL 311	+	—	+	-	-	-	ND
312	NBL 312	+	—	+	-	-	-	ND
313	NBL 313	+	—	+	-	-	-	ND
314	NBL 314	+	—	+	-	-	-	ND
315	NBL 315	+	—	+	-	-	-	ND
316	NBL 316	+	—	+	-	-	-	ND
317	NBL 317	+	—	+	-	-	-	ND
318	NBL 318	+	—	+	-	-	-	ND
319	NBL 319	+	—	+	-	-	-	ND
320	NBL 320	+	—	+	-	-	-	ND
321	NBL 321	+	—	+	-	-	-	ND
322	NBL 322	+	—	+	-	-	-	ND
323	NBL 323	+	—	+	-	-	-	ND
324	NBL 324	+	—	+	-	-	-	ND
325	NBL 325	+	—	+	-	-	-	ND
326	NBL 326	+	—	+	-	-	-	ND
327	NBL 327	+	—	+	-	-	-	ND
328	NBL 328	+	—	+	-	-	+	<i>Lactobacillus</i>
329	NBL 329	+	—	+	-	-	+	<i>Lactobacillus</i>
330	NBL 330	+	—	+	-	-	+	<i>Lactobacillus</i>
331	NBL 331	+	—	+	-	-	+	<i>Lactobacillus</i>
332	NBL 332	+	—	+	-	-	+	<i>Lactobacillus</i>
333	NBL 333	+	—	+	-	-	+	<i>Lactobacillus</i>
334	NBL 334	+	—	+	-	-	+	<i>Lactobacillus</i>
335	NBL 335	+	—	+	-	-	+	<i>Lactobacillus</i>
336	NBL 336	+	—	+	-	-	+	<i>Lactobacillus</i>
337	NBL 337	+	—	+	-	-	+	<i>Lactobacillus</i>
338	NBL 338	+	—	+	-	-	+	<i>Lactobacillus</i>
339	NBL 339	+	—	+	-	-	+	<i>Lactobacillus</i>
340	NBL 340	+	—	+	-	-	+	<i>Lactobacillus</i>
341	NBL 341	+	—	+	-	-	+	<i>Lactobacillus</i>
342	NBL 342	+	—	+	-	-	+	<i>Lactobacillus</i>
343	NBL 343	+	—	+	-	-	+	<i>Lactobacillus</i>

344	NBL 344	+	—	+	-	-	+	<i>Lactobacillus</i>
345	NBL 345	+	—	+	-	-	-	ND
346	NBL 346	+	—	+	-	-	-	ND
347	NBL 347	+	—	+	-	-	+	<i>Lactobacillus</i>
348	NBL 348	+	—	+	-	-	+	<i>Lactobacillus</i>
349	NBL 349	+	—	+	-	-	+	<i>Lactobacillus</i>
350	NBL 350	+	—	+	-	-	+	<i>Lactobacillus</i>
351	NBL 351	+	—	+	-	-	+	<i>Lactobacillus</i>
352	NBL 352	+	—	+	-	-	+	<i>Lactobacillus</i>
353	NBL 353	+	—	+	-	-	+	<i>Lactobacillus</i>
354	NBL 354	+	—	+	-	-	+	<i>Lactobacillus</i>
355	NBL 355	+	—	+	-	-	+	<i>Lactobacillus</i>
356	NBL 356	+	—	+	-	-	+	<i>Lactobacillus</i>
357	NBL 357	+	—	+	-	-	+	<i>Lactobacillus</i>
358	NBL 358	+	—	+	-	-	+	<i>Lactobacillus</i>
359	NBL 359	+	—	+	-	-	+	<i>Lactobacillus</i>
360	NBL 360	+	—	+	-	-	+	<i>Lactobacillus</i>
361	NBL 361	+	—	+	-	-	+	<i>Lactobacillus</i>
362	NBL 362	+	—	+	-	-	+	<i>Lactobacillus</i>
363	NBL 363	+	—	+	-	-	+	<i>Lactobacillus</i>
364	NBL 364	+	—	+	-	-	+	<i>Lactobacillus</i>
365	NBL 365	+	—	+	-	-	+	<i>Lactobacillus</i>
366	NBL 366	+	—	+	-	-	+	<i>Lactobacillus</i>
367	NBL 367	+	—	+	-	-	+	<i>Lactobacillus</i>
368	NBL 368	+	—	+	-	-	+	<i>Lactobacillus</i>
369	NBL 369	+	—	+	-	-	+	<i>Lactobacillus</i>
370	NBL 370	+	—	+	-	-	+	<i>Lactobacillus</i>
371	NBL 371	+	—	+	-	-	+	<i>Lactobacillus</i>
372	NBL 372	+	—	+	-	-	+	<i>Lactobacillus</i>
373	NBL 373	+	—	+	-	-	+	<i>Lactobacillus</i>
374	NBL 374	+	—	+	-	-	+	<i>Lactobacillus</i>
375	NBL 375	+	—	+	-	-	+	<i>Lactobacillus</i>
376	NBL 376	+	—	+	-	-	+	<i>Lactobacillus</i>
377	NBL 377	+	—	+	-	-	+	<i>Lactobacillus</i>
378	NBL 378	+	—	+	-	-	+	<i>Lactobacillus</i>
379	NBL 379	+	—	+	-	-	+	<i>Lactobacillus</i>
380	NBL 380	+	—	+	-	-	+	<i>Lactobacillus</i>
381	NBL 381	+	—	+	-	-	+	<i>Lactobacillus</i>
382	NBL 382	+	—	+	-	-	+	<i>Lactobacillus</i>
383	NBL 383	+	—	+	-	-	+	<i>Lactobacillus</i>
384	NBL 384	+	—	+	-	-	+	<i>Lactobacillus</i>
385	NBL 385	+	—	+	-	-	+	<i>Lactobacillus</i>
386	NBL 386	+	—	+	-	-	+	<i>Lactobacillus</i>
387	NBL 387	+	—	+	-	-	+	<i>Lactobacillus</i>
388	NBL 388	+	—	+	-	-	+	<i>Lactobacillus</i>
389	NBL 389	+	—	+	-	-	+	<i>Lactobacillus</i>
390	NBL 390	+	—	+	-	-	-	ND
391	NBL 391	+	—	+	-	-	-	ND
392	NBL 392	+	—	+	-	-	-	ND
393	NBL 393	+	—	+	-	-	-	ND

394	NBL 394	+	—	+	-	-	-	ND
395	NBL 395	+	—	+	-	-	-	ND
396	NBL 396	+	—	+	-	-	-	ND
397	NBL 397	+	—	+	-	-	-	ND
398	NBL 398	+	—	+	-	-	-	ND
399	NBL 399	+	—	+	-	-	-	ND
400	NBL 400	+	—	+	-	-	-	ND
401	NBL 401	+	—	+	-	-	-	ND
402	NBL 402	+	—	+	-	-	-	ND
403	NBL 403	+	—	+	-	-	-	ND
404	NBL 404	+	—	+	-	-	-	ND
405	NBL 405	+	—	+	-	-	-	ND
406	NBL 406	+	—	+	-	-	-	ND
407	NBL 407	+	—	+	-	-	-	ND
408	NBL 408	+	—	+	-	-	-	ND
409	NBL 409	+	—	+	-	-	-	ND
410	NBL 410	+	—	+	-	-	-	ND
411	NBL 411	+	—	+	-	-	-	ND
412	NBL 412	+	—	+	-	-	-	ND
413	NBL 413	+	—	+	-	-	-	ND
414	NBL 414	+	—	+	-	-	-	ND
415	NBL 415	+	—	+	-	-	-	ND
416	NBL 416	+	—	+	-	-	-	ND
417	NBL 417	+	—	+	-	-	-	ND
418	NBL 418	+	—	+	-	-	-	ND
419	NBL 419	+	—	+	-	-	-	ND
420	NBL 420	+	—	+	-	-	-	ND
421	NBL 421	+	—	+	-	-	-	ND
422	NBL 422	+	—	+	-	-	-	ND
423	NBL 423	+	—	+	-	-	-	ND
424	NBL 424	+	—	+	-	-	-	ND
425	NBL 425	+	—	+	-	-	-	ND
426	NBL 426	+	—	+	-	-	-	ND
427	NBL 427	+	—	+	-	-	-	ND
428	NBL 428	+	—	+	-	-	-	ND
429	NBL 429	+	—	+	-	-	-	ND
430	NBL 430	+	—	+	-	-	-	ND
431	NBL 431	+	—	+	-	-	-	ND
432	NBL 432	+	—	+	-	-	-	ND
433	NBL 433	+	—	+	-	-	-	ND
434	NBL 434	+	—	+	-	-	-	ND
435	NBL 435	+	—	+	-	-	-	ND
436	NBL 436	+	—	+	-	-	-	ND
437	NBL 437	+	—	+	-	-	-	ND
438	NBL 438	+	—	+	-	-	-	ND
439	NBL 439	+	—	+	-	-	-	ND
440	NBL 440	+	—	+	-	-	-	ND
441	NBL 441	+	—	+	-	-	-	ND
442	NBL 442	+	—	+	-	-	-	ND
443	NBL 443	+	—	+	-	-	-	ND

444	NBL 444	+	—	+	-	-	-	ND
445	NBL 445	+	—	+	-	-	-	ND
446	NBL 446	+	—	+	-	-	-	ND
447	NBL 447	+	—	+	-	-	-	ND
448	NBL 448	+	—	+	-	-	-	ND
449	NBL 449	+	—	+	-	-	-	ND
450	NBL 450	+	—	+	-	-	-	ND
451	NBL 451	+	—	+	-	-	-	ND
452	NBL 452	+	—	+	-	-	-	ND
453	NBL 453	+	—	+	-	-	-	ND
454	NBL 454	+	—	+	-	-	-	ND
455	NBL 455	+	—	+	-	-	-	ND
456	NBL 456	+	—	+	-	-	-	ND
457	NBL 457	+	—	+	-	-	-	ND
458	NBL 458	+	—	+	-	-	-	ND
459	NBL 459	+	—	+	-	-	-	ND
460	NBL 460	+	—	+	-	-	-	ND
461	NBL 461	+	—	+	-	-	-	ND
462	NBL 462	+	—	+	-	-	-	ND
463	NBL 463	+	—	+	-	-	-	ND
464	NBL 464	+	—	+	-	-	-	ND
465	NBL 465	+	—	+	-	-	-	ND
466	NBL 466	+	—	+	-	-	-	ND
467	NBL 467	+	—	+	-	-	-	ND
468	NBL 468	+	—	+	-	-	-	ND
469	NBL 469	+	—	+	-	-	-	ND
470	NBL 470	+	—	+	-	-	-	ND
471	NBL 471	+	—	+	-	-	-	ND
472	NBL 472	+	—	+	-	-	-	ND
473	NBL 473	+	—	+	-	-	-	ND
474	NBL 474	+	—	+	-	-	-	ND
475	NBL 475	+	—	+	-	-	-	ND
476	NBL 476	+	—	+	-	-	-	ND
477	NBL 477	+	—	+	-	-	+	<i>Lactobacillus</i>
478	NBL 478	+	—	+	-	-	+	<i>Lactobacillus</i>
479	NBL 479	+	—	+	-	-	+	<i>Lactobacillus</i>
480	NBL 480	+	—	+	-	-	+	<i>Lactobacillus</i>
481	NBL 481	+	—	+	-	-	+	<i>Lactobacillus</i>
482	NBL 482	+	—	+	-	-	+	<i>Lactobacillus</i>
483	NBL 483	+	—	+	-	-	+	<i>Lactobacillus</i>
484	NBL 484	+	—	+	-	-	+	<i>Lactobacillus</i>
485	NBL 485	+	—	+	-	-	+	<i>Lactobacillus</i>
486	NBL 486	+	—	+	-	-	+	<i>Lactobacillus</i>
487	NBL 487	+	—	+	-	-	+	<i>Lactobacillus</i>
488	NBL 488	+	—	+	-	-	+	<i>Lactobacillus</i>
489	NBL 489	+	—	+	-	-	+	<i>Lactobacillus</i>
490	NBL 490	+	—	+	-	-	+	<i>Lactobacillus</i>
491	NBL 491	+	—	+	-	-	+	<i>Lactobacillus</i>
492	NBL 492	+	—	+	-	-	+	<i>Lactobacillus</i>
493	NBL 493	+	—	+	-	-	+	<i>Lactobacillus</i>

494	NBL 494	+	—	+	-	-	+	<i>Lactobacillus</i>
495	NBL 495	+	—	+	-	-	+	<i>Lactobacillus</i>
496	NBL 496	+	—	+	-	-	+	<i>Lactobacillus</i>
497	NBL 497	+	—	+	-	-	+	<i>Lactobacillus</i>
498	NBL 498	+	—	+	-	-	+	<i>Lactobacillus</i>
499	NBL 499	+	—	+	-	-	+	<i>Lactobacillus</i>
500	NBL 500	+	—	+	-	-	+	<i>Lactobacillus</i>
501	NBL 501	+	—	+	-	-	+	<i>Lactobacillus</i>
502	NBL 502	+	—	+	-	-	+	<i>Lactobacillus</i>
503	NBL 503	+	—	+	-	-	+	<i>Lactobacillus</i>
504	NBL 504	+	—	+	-	-	+	<i>Lactobacillus</i>
505	NBL 505	+	—	+	-	-	+	<i>Lactobacillus</i>
506	NBL 506	+	—	+	-	-	+	<i>Lactobacillus</i>
507	NBL 507	+	—	+	-	-	+	<i>Lactobacillus</i>
508	NBL 508	+	—	+	-	-	+	<i>Lactobacillus</i>
509	NBL 509	+	—	+	-	-	+	<i>Lactobacillus</i>
510	NBL 510	+	—	+	-	-	+	<i>Lactobacillus</i>
511	NBL 511	+	—	+	-	-	+	<i>Lactobacillus</i>
512	NBL 512	+	—	+	-	-	+	<i>Lactobacillus</i>
513	NBL 513	+	—	+	-	-	+	<i>Lactobacillus</i>
514	NBL 514	+	—	+	-	-	+	<i>Lactobacillus</i>
515	NBL 515	+	—	+	-	-	+	<i>Lactobacillus</i>
516	NBL 516	+	—	+	-	-	+	<i>Lactobacillus</i>
517	NBL 517	+	—	+	-	-	+	<i>Lactobacillus</i>
518	NBL 518	+	—	+	-	-	+	<i>Lactobacillus</i>
519	NBL 519	+	—	+	-	-	-	ND
520	NBL 520	+	—	+	-	-	-	ND
521	NBL 521	+	—	+	-	-	-	ND
522	NBL 522	+	—	+	-	-	-	ND
523	NBL 523	+	—	+	-	-	-	ND

ANNEXURE II. CARBOHYDRATE FERMENTATION PROFILE OF *LACTOBACILLUS* ISOLATES

S.No.	Isolates	Carbohydrate tested										Presumptive Identification
		Arabinose	Cellobiose	Mannose	Ribose	Salicin	Rhamnose	Sorbitol	Trehalose	Mannitol	Melibiose	
1	NBL 1	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
2	NBL 2	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
3	NBL 4	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
4	NBL 8	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
5	NBL 9	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
6	NBL 10	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
7	NBL 11	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
8	NBL 12	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
9	NBL 16	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
10	NBL 17	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
11	NBL 18	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
12	NBL 19	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
13	NBL 21	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
14	NBL 22	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
15	NBL 24	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
16	NBL 25	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
17	NBL 29	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
18	NBL 30	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
19	NBL 31	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
20	NBL 32	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
21	NBL 37	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
22	NBL 38	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

23	NBL 39	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
24	NBL 40	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
25	NBL 52	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
26	NBL 55	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
27	NBL 56	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
28	NBL 59	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
29	NBL 60	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
30	NBL 61	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
31	NBL 64	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
32	NBL 65	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
33	NBL 66	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
34	NBL 70	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
35	NBL 71	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
36	NBL 72	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
37	NBL 73	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
38	NBL 76	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
39	NBL 77	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
40	NBL 78	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
41	NBL 79	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
42	NBL 80	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
43	NBL 81	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
44	NBL 82	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
45	NBL 83	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
46	NBL 84	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
47	NBL 85	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
48	NBL 86	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
49	NBL 87	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

50	NBL 88	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
51	NBL 89	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
52	NBL 90	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
53	NBL 91	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
54	NBL 92	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
55	NBL 98	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
56	NBL 99	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
57	NBL 100	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
58	NBL 103	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
59	NBL 104	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
60	NBL 105	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
61	NBL 106	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
62	NBL 107	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
63	NBL 111	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
64	NBL 112	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
65	NBL 116	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
66	NBL 117	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
67	NBL 118	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
68	NBL 122	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
69	NBL 123	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
70	NBL 124	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
71	NBL 132	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
72	NBL 133	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
73	NBL 134	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
74	NBL 135	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
75	NBL 154	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

76	NBL 155	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
77	NBL 156	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
78	NBL 162	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
79	NBL 163	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
80	NBL 164	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
81	NBL 167	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
82	NBL 168	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
83	NBL 169	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
84	NBL 170	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
85	NBL 177	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
86	NBL 178	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
87	NBL 179	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
88	NBL 180	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
89	NBL 183	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
90	NBL 184	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
91	NBL 245	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
92	NBL 247	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
93	NBL 255	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
94	NBL 256	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
95	NBL 257	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
96	NBL 258	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
97	NBL 259	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
98	NBL 260	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
99	NBL 261	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
100	NBL 262	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
101	NBL 265	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>

102	NBL 266	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
103	NBL 267	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
104	NBL 269	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
105	NBL 270	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
106	NBL 272	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
107	NBL 274	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
108	NBL 277	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
109	NBL 278	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
110	NBL 279	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
111	NBL 280	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
112	NBL 281	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
113	NBL 282	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
114	NBL 283	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
115	NBL 284	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
116	NBL 285	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
117	NBL 286	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
118	NBL 287	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
119	NBL 328	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
120	NBL 329	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
121	NBL 330	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
122	NBL 331	+	+	+	-	+	-	-	+	+	-	<i>L.acidophilus</i>
123	NBL 332	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
124	NBL 336	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
125	NBL 337	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
126	NBL 338	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
127	NBL 342	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

128	NBL 343	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
129	NBL 344	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
130	NBL 345	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
131	NBL 347	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
132	NBL 348	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
133	NBL 350	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
134	NBL 351	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
135	NBL 353	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
136	NBL 354	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
137	NBL 355	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
138	NBL 356	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
139	NBL 357	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
140	NBL 358	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
141	NBL 359	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
142	NBL 360	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
143	NBL 361	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
144	NBL 362	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
145	NBL 363	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
146	NBL 365	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
147	NBL 366	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
148	NBL 367	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
149	NBL 368	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
150	NBL 369	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
151	NBL 370	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
152	NBL 371	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
153	NBL 372	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
154	NBL 373	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>

155	NBL 374	-	+	+	-	+	+	+	+	+	-	<i>L. rhamnosus</i>
156	NBL 375	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
157	NBL 376	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
158	NBL 377	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
159	NBL 378	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
160	NBL 381	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
161	NBL 382	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
162	NBL 383	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
163	NBL 384	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
164	NBL 385	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
165	NBL 386	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
166	NBL 387	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
167	NBL 388	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
168	NBL 389	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
169	NBL 477	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
170	NBL 478	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
171	NBL 479	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
172	NBL 480	+	+	+	-	+	-	-	+	+	-	<i>L. acidophilus</i>
173	NBL 481	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
174	NBL 482	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
175	NBL 483	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
176	NBL 484	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
177	NBL 485	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
178	NBL 490	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
179	NBL 491	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
180	NBL 492	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
181	NBL 493	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

182	NBL 494	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
183	NBL 495	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
184	NBL 496	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
185	NBL 497	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
186	NBL 498	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
187	NBL 502	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
188	NBL 503	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
189	NBL 504	-	+	+	+	+	-	-	-	+	+	<i>L. plantarum</i>
190	NBL 505	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
191	NBL 506	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
192	NBL 507	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
193	NBL 508	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
194	NBL 509	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
195	NBL 514	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
196	NBL 515	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
197	NBL 516	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>
198	NBL 518	-	+	+	-	+	-	+	+	-	-	<i>L. gasseri</i>

Appendices



APPENDIX I

Composition of different types of Media and Buffers, used in the study, are given below. Sterilization of media and buffers were performed at 121 °C for 20 min under 15 psi pressure.

COMPOSITION OF DIFFERENT CULTURE MEDIA AND BUFFERS

1. DEMANN ROGOSA SHARPE MEDIUM (MRS) (de Mann *et al.*, 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Dipotassium hydrogen phosphate	2.0 g
Sodium acetate	5.0 g
Ammonium citrate	2.0 g
Magnesium sulfate	0.1 g
Manganese sulfate	0.05 g
Tween 80	1.0 g
Distilled water	1000 ml
pH 6.5 ± 0.2	

2. M-17 MEDIUM (Terzaghi and Sandine, 1975)

Tryptone	5.0 g
Peptone	5.0 g
Yeast extract	2.5 g
Beef extract	5.0 g
Lactose	5.0 g
Sodium-β-glycerophosphate	19.0 g
Ascorbic acid	0.5 g
Magnesium sulfate	0.25 g
Distilled water	1000 ml
pH 7.0 ± 0.1	

3. CITRATE AZIDE AGAR MEDIUM (CAA) (Saraswat *et al.*, 1963)

Tryptone	10.0 g
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Yeast Extract	10.0 g
Sodium Citrate	20.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH 7.0 ± 0.2

One ml of 0.1% sterile Triphenyl Tetrazolium Chloride (TTC) and 1 ml of 4% sterile Sodium azide (NaN_3) was added to aliquots (100 ml) of autoclaved medium base tempered to 48-50 °C .

4. NUTRIENT MEDIUM

Peptone	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Sodium chloride	5.0 g
Distilled water	1000 ml

pH 7.4 ± 0.2

5. BILE ESCULINE AGAR

Tryptone	5.0 g
Beef extract	3.0 g
Oxgall	40.0 g
Esculine	1.0 g
Sodium acetate	5.0 g
Ferric citrate	0.5 g
Agar	15.0 g
Distilled water	1000 ml

pH 6.6 ± 0.2

6. POTASSIUM TELLURITE AGAR

Peptone	10.0 g
Lactose	5.0 g
NaCl	5.0 g
K_2HPO_4	2.0 g
Agar	15.0 g
Distilled Water	1000 ml

pH 6.5 ± 0.2

Stock solution of 4% K-tellurite was prepared. One ml of K-tellurite was added to 100 ml aliquots of sterilized medium base tempered to 48-50 °C .

7. ARGININE BROTH

Tryptone	5.0 g
Yeast Extract	5.0 g
K ₂ HPO ₄	2.0 g
L-Arginine monohydrochloride	3.0 g
Dextrose	0.5 g
Distilled Water	1000 ml

pH 7.0 ± 0.1

8. BAIRD PARKER AGAR

Tryptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Distilled water	950 ml

pH 7.0 ± 0.2

50 ml of Egg Yolk Tellurite Emulsion was added to sterilized medium base tempered to 48-50 °C.

9. BRAIN HEART INFUSION MEDIUM (BHI)

Infusion from Beef heart	250.0 g
Infusion from Calf brain	200.0 g
Dextrose	2.0 g
Dipotassium hydrogen phosphate	2.5 g
Sodium chloride	5.0 g
Peptone	10.0 g

Distilled water	1000 ml
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pH 7.4 ± 0.2

10. YEAST EXTRACT LACTATE AGAR (YELA)

Tryptic digest of casein (Trypticase)	10.0 g
Yeast Extract	10.0 g
Sodium Lactate	10.0 g
DiPotassium hydrogen phosphate	0.25 g
Cysteine-HCl	0.5 g
Manganese sulfate. 2H ₂ O	0.005 g
Tween 80	1.0 ml
Resazurin	1.0 mg
Distilled water	1000 ml

pH 7.0 ± 0.2

11. TRYPTOSE GLUCOSE EXTRACT AGAR (TGE) (Biswas *et al.*, 1991)

Tryptone	10.0 g
Glucose	10.0 g
Yeast extract	10.0 g
Magnesium sulfate	0.05 g
Manganese sulfate	0.05 g
Tween 80	2.0 ml
Agar	15.0 g
Distilled water	1000 ml

pH 6.5 ± 0.2

For soft agar 7 g agar was added instead of 15 g per 1000 ml medim.

12. CHL BROTH

Peptone	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Dipotassium phosphate	2.0 g
Sodium acetate	5.0 g
Ammonium citrate	2.0 g
Magnesium sulfate	0.05 g
Manganese sulfate	0.12 g
Bromocresol purple	0.17 g
Tween 80	1.0 g

Distilled water	1000 ml
pH 6.5 ± 0.2	

13. MUELLER HINTON AGAR

Tryptone	17.50 g
Beef heart infusion	2.0 g
Sterch	1.5 g
Agar	17.0 g
Distilled water	1000 ml
pH 7.3 ± 0.2	

14. PHOSPHATE UREA MAGNESIUM SULPHATE (PUM) BUFFER

K ₂ HPO ₄	22.2 g
KH ₂ PO ₄	7.26 g
Urea	1.8 g
MgSO ₄ .7H ₂ O	0.2 g
Distilled water	1000 ml
pH 7.1	

15. PHOSPHATE BUFFERED SALINE (PBS)

NaCl	8.00 g
KCl	0.20 g
Na ₂ HPO ₄	1.15 g
KH ₂ PO ₄	0.20 g
Distilled Water	1000 ml
pH 7.0	

16. TND MEDIUM

Tryptone	17.0 g
Neopeptone	3.0 g
Dextrose	2.5 g
NaCl	5.0 g
K ₂ HPO ₄	2.5 g
Distilled Water	1000 ml

pH 7.2

17. GLYCEROL STOCK

Glycerol	15.0 ml
Distilled water	85.0 ml

Both were mixed and were sterilized by autoclaving, added in equal amount to the culture in each cryovial, at the time of preservation.

18. PEPTONE WATER

Peptone	1g
Distilled Water	1000 ml

pH 7.0

APPENDIX II

1. REAGENTS FOR ISOLATION OF GENOMIC DNA

1.1. SET Buffer

Sucrose (6.7%)	6.70 g
Tris (50 mM)	0.606 g
Di-Sodium EDTA (1mM)	0.0372 g
Deionized Water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6N NaOH and volume was made to 100 ml. Buffer was autoclaved (121 °C /15 min) after preparation.

1.2. SDS Solution (10%)

SDS	10 g
Deionized water	100 ml

Solution was autoclaved before use

1.3. 5M NaCl

NaCl	29.22 g
Deionized water	100 ml

1.4. Chloroform: Isoamyl alcohol (24:1)

Isoamyl Alcohol	4.0 ml
Chloroform	96.0 ml

1.5. TE Buffer

Tris (10mM)	0.1212 g
Di-Sodium EDTA (1mM)	0.0372 g
Deionized water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6N NaOH and volume was made to 100 ml. Buffer was autoclaved (121 °C /15 min) after preparation.

2. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS OF DNA

2.1. TAE Buffer (50 X) (Stock Solution)

Tris	24.20 g
0.5 M Di-Sodium EDTA (pH 8.0)	10.00 ml
Glacial Acetic Acid	5.71 ml
Deionized Water	100 ml

pH of this solution was not adjusted. The solution was autoclaved at 121 °C /15min

2.1.1. Working Solution (1X)

Working solution was prepared by diluting 1 ml of stock solution to 50 ml with distilled water.

2.2. Gel Loading Solution

Bromophenol Blue	0.05 g
Sucrose	40.00 g
Di-Sodium EDTA	3.72 g
SDS	0.50 g
Deionized Water	100 ml

2.3. Staining Solution

2.3.1. Stock Solution (10 mg ml⁻¹):

Ethidium bromide (EtBr) was prepared as a stock solution of 10 mg/ml in water, stored at room temperature in screw cap tubes wrapped in aluminium foil.

2.3.2. Working Solution (0.5 µg ml⁻¹):

The working solution was prepared by adding 50 µl of stock solution to 1l of distilled water.

APPENDIX III

REAGENTS FOR TRICINE-SDS-PAGE

All the solutions were prepared in highest quality of water available.

1. Acrylamide Solution: (49.5% T; 3% C)

Acrylamide	48.0 g
N, N' Methylene-bis-acrylamide	1.5 g

Solution made up to a total volume of 100 ml. Filtered through Whatman # 1 filterpaper and stored in an amber coloured bottle at 4°C for use within one month.

2. Gel Buffer : (pH 8.45)

Tris	36.34 g
SDS	0.3 g

Dissolved in 100 ml with double distilled water. pH was adjusted to 8.45 with conc.

3. SDS: (20%)

SDS	20 g
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Dissolved completely in 90 ml water with gentle stirring and brought the volume to 100 ml with deionized water. Stored at room temperature.

4. Tris-HCl: (1 M, pH6.8)

12.12 g tris was dissolved in 60ml of water. pH was adjusted to 6.8 with conc. HCl, total volume was made to 100ml with water and stored at 4°C.

5. Ammonium Persulphate: (APS) (10% w/v)

The solution was freshly prepared each time by dissolving 10 mg ammonium persulfate in 100 µl of distilled water.

6. Sample Buffer (4X):

20% SDS Solution	4.0 ml
Glycerol	2.4 ml
2-Mercaptoethanol	0.4 ml
Bromophenol Blue	2.0 mg

Tris-HCl, 1.0 M, pH 6.8

1.0 ml

The final volume was made up to 20 ml with water.

7. Anode Buffer: (2 M Tris, pH 8.9)

11.12g of Tris base was dissolved in 300-400 ml of distilled water and pH was adjusted to 8.9 with conc. HCl and total volume made to 500 ml and was stored at 4°C.

8. Cathode Buffer: (1 M Tris, 1 M Tricine, SDS)

Tris base	6.05 g
Tricine	8.96 g
SDS	0.5 g

Dissolved in 300-400 ml of double distilled water and final volume made up to 500 ml. The pH of this solution was around 8.2 ± 0.2 . The buffer was stored at 4 °C.

9. Gel Staining Solution:

50% Methanol, 10% glacial acetic acid, 0.25% Coomassie Brilliant Blue R-250

S.No.	Name of the component	Required final concentration of the component	Amount of component required for 500 ml
1.	Methanol	50%	250 ml
2.	Glacial acetic acid	10%	50 ml
3.	Coomassie Brilliant Blue R-250	0.25%	1.25 g
4.	Distilled water upto	-	500 ml

10. Gel Destaining Solution:

25% Methanol, 10% glacial acetic acid

S.No.	Name of the component	Required final concentration of the component	Amount of component required for 2000 ml
1.	Methanol	25%	500 ml
2.	Glacial acetic acid	10%	200 ml
3.	Distilled water upto	-	1300 ml