Assessment of Nutritive Value of Goa Cowpea 3 (*Vigna unguiculata*) Cultivar and its Synbiotic Application

A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

IN THE SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY

GOA UNIVERSITY



BY

MS. VARSHA NARAYAN SUNIL NAIK

SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY

OCTOBER 2025

DECLARATION

I, Ms. Varsha N S Naik, hereby declare that this represents work which has been carried out by me and that it has not been submitted, either in part or in full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau.

Date:

Varsha N S Naik

School of Biological Sciences
and Biotechnology
Goa University

CERTIFICATE

I hereby certify that the above declaration of the candidate, Ms. Varsha N S Naik, is true and that the work was carried out under my supervision.

Dr. Savita Kerkar (Retd.Senior Professor)

Guide
Discipline of Biotechnology,
School of Biological Sciences and Biotechnology
Goa University, Taleigao plateau, 403206

Dr. Meghanath Prabhu

Assistant Professor, Co-Guide
Discipline of Biotechnology,
School of Biological Sciences and Biotechnology
Goa University, Taleigao plateau, 403206

ACKNOWLEDGEMENT

Dear almighty God, your grace and presence are constant reminders of hope and perseverance, illuminating my path and making this achievement possible. I surrender this success to you, trusting that your divine wisdom will continue to guide me.

Pursuing a Ph.D. on an interdisciplinary topic has been both a challenging and enriching experience, simultaneously perplexing, rewarding, and an incredible learning opportunity.

Achieving my goal would not have been possible without the unwavering support of my incredibly patient, deeply caring, and accomplished advisor and Guide, **Dr. Savita Kerkar,** former Senior Professor. I am profoundly grateful to Madam for accepting me as her student, and she is certainly a constant inspiration. Some bonds are truly meant to last forever!

My heartfelt gratitude to **Dr. Varun Sahni**, former Vice-Chancellor of Goa University, for granting permission to undertake interdisciplinary research, and to **Dr. Harilal Menon**, Vice-Chancellor, Goa University, for providing the necessary resources to support my Ph.D. I am immensely grateful to my Vice Chancellor's nominees, **Prof. Sanjeev Ghadi**, discipline of Biotechnology, School of Biological Sciences and Biotechnology (SBSB), and **Dr. M.K. Janarthanam**, former Senior Professor at SBSB, for their invaluable expertise and constructive feedback in enabling the conduct of research work. My sincere gratitude goes to **Dr. Meghanath Prabhu**, Assistant Professor and Co-guide, for his wholehearted cooperation and ever-helpful nature.

Special thanks to **Mr. Prasad Lolayekar**, IAS, Secretary (Education), Government of Goa; **Mr. Bhushan Sawaikar**, Director, Directorate of Higher Education (DHE), and **Mr. Siddesh Kesarkar**, Assistant Director, DHE, for granting the timely official permissions and desired cooperation to pursue my Ph.D. **Dr. Mahesh Pai**, Officiating Principal, Goa College of Home Science, deserves a special mention for being extremely supportive during this journey.

I wholeheartedly thank **Dr. Rajendran Ananthan**, Scientist E, Food Chemistry Division, ICMR-NIN, Hyderabad, and **Dr. Rakhee Khandeparker**, Principal Scientist, BOD, CSIR-NIO, Goa, for sharing their invaluable and unparalleled expertise, their cheerfulness, and warmth made me feel at ease while conducting experiments. I am also grateful to their

dedicated research staff—**Ms. Pranjali Bansod, Dr. Ujwala Amberkar, Dr. Harshada Kankonkar, Ms. Adhira Mahale,** and **Ms. Kirtani Palekar**, for their committed assistance. My sincere thanks to **Dr. Gopakumar V.**, former Chief Librarian of Goa University, for his steadfast support right from the submission of the application till the culmination of this work. Many thanks to the Assistant Professors of Goa University, **Dr. Shanti Desai**, **Dr. Milind Naik**, **Dr. Vinod Mandrekar**, and **Dr. Nitin Sawant.** Dr. Shanti has been more than a wonderful friend, offering constant encouragement and understanding

I am at a loss for words to fully express my deepest gratitude to the scientific community of ICAR, Old Goa, **Dr. K.K. Manohar**, **Dr. Rajkumar Solomon**, **Dr. Mathla Gupta**, **Dr. Subhradeep Bhattacharjee,** and **Ms. Yogini Shanbaug** for their continuous, invaluable support. Gratitude to **Dr. Flory Pereira**, PES College, Farmagudi, for her support and for extending valuable resources that contributed to this research. Many people deserve extreme appreciation for their immense backing in providing timely facilities wholeheartedly, *viz.* Officials of the Directorate of Food and Drug Administration, Govt. of Goa— **Ms. Jyoti Sardesai**, former Director; **Ms. Shweta Desai**, Director; **Ms. Nilima Mishal**, Retd. Sr. Scientific Officer and **Ms. Sushmita. D'Souza**, Jr., Scientific Officer; my uncle, **Dr. Rajeshwar V. Naik, Cardiologist,** and **Ms. Shraddha Gaonkar, Medical Microbiologist,** at Royal Hospital, Margao**; Dr. Lydia Dias** from GMC, Bambolim**; Mr. Sunil Vishwakarma**, Dr. Willmar Schwabe India, Pvt. Limited: **Mr. Manish Parab**, Syngenta, Goa, **Dr. Ratna Raje Thar** and **Dr. Suneeta Chandorkar**.

I consider myself fortunate to have had **Mr. Anchit Parker** accompany me on this exhilarating journey at every moment of less familiar experimental analysis. No words to express my gratitude to him. I am truly grateful to my research colleagues **Deepti, Delicia, Dviti, Dr. Priti, Dr. Samantha, Dr. Preethi, Dr. Manasi, Dr. Ruchira, Peranto, Dr. Pingal, Dr. Manisha, Nuha Ameen, Fazila, Diksha, Kunal, Linus, Komal, Elaine, Veda, Devika, Snesha, Rakshita, Hetal, Paresh,** and **Trusha** for their timely help at every moment, on numerous occasions.

My sincere appreciation goes to all my colleagues at Goa College of Home Science, particularly **Pratiksha, Darshana**, **Dilip, Shweta** and **Eknath.** I am truly indebted to **Mr. Redualdo Serrao** and **Ms. Nilima Madkaikar**, for their ready and valuable assistance. Many thanks to **Dr. Sandhya, Ms. Jaya, Mr. Sameer,** and **Mr. Ashish** for their continuous support. Appreciation goes to many MSc (Biotechnology) students and to the staff of the

post-graduation section at Goa University for their tremendous support, especially Ms. **Varda, Ms. Aditi,** and **Mr. Kalpesh**. Millions of thanks to **Mr. Sachin and Dr. Shikha Morje** for the immense patience during the most crucial step of formatting the thesis. Life would be incomplete without friends and well-wishers, and my deepest appreciation to **Shilpa and Yadavendra Naik, Rishikesh and Sneha Kulkarni, Lina and Prashant Naik, Ms. Aditi Phadte, Arina Frank, Nita and Late. Guruprasad Rane, Sejal and Yetindra Maralkar, Yogesh Kapadi, Krishnaprasad Rane, Dr. Nilesh Malpure, Samrat Sardesai, Smisha Parrikar, Medha Bhate, Mitzie Fernandes,** and **Mahesh Biradar** for offering tremendous personal support whenever needed and for being wonderful listeners to my emotional moments.

I am deeply grateful to my in-laws**, Mrs. Sharmila** and **Mr. Mahesh M. Naik**, for their constant presence and care toward my children; to my **Maa** and **Pappa,** for empowering me to make my own decisions, and for sincere blessings. My appreciation to my siblings, **Dr. Durgesh**, **Er. Pratiksha, Adv. Harsha** and **Dr.Vijaya,** for their relentless support, both physically and emotionally, in times of stress. Many thanks to **Dinesh Pankar**, my brother-in-law, for constant reminders and for taking pride in my achievements. I am profoundly grateful to all my **Teachers** at Dayanand Bandodkar Memorial High School, at Dhempe Higher Secondary School of Arts and Science, at Goa College of Home Science, and at Nirmala Niketan College of Polytechnic and Home Science, Mumbai for their faith in my abilities and to **Dr. Gauri Pandit** and **Dr. Suman Sharma, former Scientists** at BARC, Deonar, Mumbai for inspiring my interest in environmental research.

Last but certainly not least, a heartfelt salute to the three most important pillars of my life — **Saiccha** and **my techno wizard, Darsh**, to both I apologise for moments of harshness and for not being fully committed during their precious childhood years, and to **Swapnil** for being a soul companion and support in pursuing my dreams. I could not have accomplished this without their understanding and cooperation.

While some say the longest line is of critics, my life has been blessed with an overwhelming number of people who have helped me. If I have inadvertently missed anyone's name, please know it is unintentional.

"सगळ्यांक सुखाचो वांटो आंवडेतां"

Varsha

Dedicated

With love and gratitude

to

My most beloved children

Darsh and Saiccha,

My Guide

and

"**God**", who exists

Contents

ABBREVIATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1: INTRODUCTION		**1-8**
	Objectives and significance	7
CHAPTER 2: REVIEW OF LITERATURE		**9-55**
2.1	Significance of cowpea: Nutrition, health, and food technology	13
2.2	Gut microbiota and diet	22
2.3	Prebiotics: An evolution that cannot be stopped	24
2.4	Probiotics: Beneficial group of microbes	26
2.5	Synbiotics and their modes of action	38
2.6	Legumes as matrices for probiotic delivery	42
2.7	Processing of legumes: A boon to human nutrition	44
2.8	Nutritional and food technological benefits of microbially fermented synbiotic foods	48
2.9	Health benefits of fermented foods	53
CHAPTER 3: MATERIALS AND METHODS		**56-104**
3.1	Screening of saltpan isolates	58-60
3.1.1	Growth and maintenance of the saltpan isolates	58
3.1.2	Characterisation of the halotolerant bacteria	58
3.1.3	Fermentation assay: Carbohydrate utilisation of the isolates	59
3.1.4	Qualitative screening for extracellular hydrolytic enzymes	59
3.2	*In- vitro* studies on safety, desired probiotic attributes, and shelf life of the *Bacillus* isolates	60
3.2.1	Safety studies of *Bacillus* isolates	61-64
3.2.1.1	Study of haemolytic activity	61
3.2.1.2	Detection of enterotoxin genes in the potential probiotic isolates using PCR	61

3.2.1.3 Detection of endotoxin in the prospective probiotic isolates 62
3.2.1.4 Antimicrobial activity of the *Bacillus* isolates 63
3.2.1.5 Determination of antibiotic disc sensitivity of the *Bacillus* isolates 63
3.2.2 Studies on desired probiotic attributes of *Bacillus* isolates 64-69
3.2.2.1 Tolerance to gastric acid and intestinal bile salts in simulated conditions 64
3.2.2.2 Evaluation of bile salt hydrolase activity 66
3.2.2.3 Assessment of adherence capacity 67
3.2.2.4 Assessment of antioxidant activity 69
3.3 Identification of potential probiotic isolates 70-74
3.3.1 Morphological and biochemical characteristics 70
3.3.2 Physiological characteristics (Effect of salinity, pH, and temperature) 73
3.3.3 Molecular identification by 16S r-RNA gene sequencing 73
3.4 Study of shelf life upon storage of *Bacillus* culture at low and room temperature 74
3.5 Analysis of proximate /nutritional composition of Goa cowpea 3 (*Vigna unguiculata*) cultivar seeds 75-79
3.5.3 Estimation of fatty acids 79
3.5.4 Estimation of total amino acids (TAAs) 80
3.5.5 Quantification of water-soluble vitamins 80
3.5.6 Quantification of fat-soluble vitamins 82
3.5.6.1 Quantification of total and individual carotenoid content 82
3.5.7 Estimation of minerals 84
3.5.8 Analysis of oligosaccharides (stachyose, verbascose, raffinose) 84
3.6 Study of physical and cooking properties of GC3 seeds 85-90
3.7 Optimisation of fermentation and evaluation of the efficacy of GC3 as a probiotic carrier 90
3.8 Analysis of the physicochemical properties of GC3 slurry during fermentation 91-94
3.8.1 Measurement of pH 91
3.8.2 Estimation of total titratable acidity (TTA) 92

3.8.3	Estimation of organic acids and short chain fatty acids (SCFAs)	92
3.8.4	Measurement of viscosity during fermentation	92
3.8.5	Determination of protein content	93
3.8.6	Analysis of free amino nitrogen (FAN)	93
3.8.7	Estimation of total carbohydrates and total reducing sugars	94
3.9	Development of a synbiotic powder	95
3.9.1	Study of probiotic cell viability before and after lyophilization of the bacillus cell suspension and GC3 synbiotic formulation	95
3.9.2	Study of probiotic cell viability of the GC3 synbiotic formulation upon storage at low and room temperature	95
3.10	Effect of fermentation by *Bacillus licheniformis PQ098138* on the proximate analysis, peptide formation, nutritional composition, phytate, and raffinose family oligosaccharides content of GC3 formulation	96-100
3.10.1	Proximate analysis (moisture, ash, fat, dietary fiber: TDF, IDF and SDF, crude protein, carbohydrate)	96
3.10.2	SDS-PAGE (Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis)	96
3.10.3	Comparative assessment of the composition of free amino acids	98
3.10.4	Estimation of water-soluble and fat-soluble vitamins and carotenoids	98
3.10.5	Estimation of minerals	98
3.10.6	Analysis of phytic acid	99
3.10.7	Estimation of oligosaccharides	100
3.11	*Bacillus* viability in the synbiotic food formulation in comparison to cell suspension during exposure to simulated gastrointestinal conditions	100
3.12	Assessment of the antibiotic susceptibility, antioxidant activity, and antimicrobial activity of the synbiotic food formulation	101
3.13	Assessing the functional properties of the synbiotic formulation for suitability in food product	102-103
3.13.1	Emulsification capacity	102

3.13.2 Emulsification stability 102
3.13.3 Foam capacity and stability 103
3.13.4 Heat tolerance assay 103
3.14 Statistical analysis of data 104
CHAPTER 4: RESULTS AND DISCUSSION **105-213**
4.1 Screening of saltpan isolates 107
4.1.2 Characterisation and identification of halotolerant bacteria 107
4.1.3 Fermentation ability and carbohydrate utilisation of the isolates 109
4.1.4 Qualitative screening for extracellular hydrolytic enzymes 112
4.2 *In-vitro* studies on safety , desired probiotic attributes, and identification of the isolates 115-143
4.2.1.1 Haemolytic activity 116
4.2.1.2 Enterotoxin genes in the prospective probiotic isolates 117
4.2.1.3 Endotoxin in the prospective probiotic isolates 119
4.2.1.4 Antimicrobial activity of the *Bacillus* isolates 120
4.2.1.5 Antibiotic disc sensitivity of the *Bacillus* isolates 122
4.2.2.1 Gastric acid tolerance (*in- vitro*) 124
4.2.2.2 Tolerance to intestinal bile salts (*in- vitro*) 127
4.2.2.3 Bile salt hydrolase (BSH) activity 130
4.2.2.4 Adherence capacity 134
4.2.2.5 Antioxidant activity 137
4.2.3 Identification of potential probiotic isolates 139
4.3 Shelf life upon storage at low and room temperature of the *Bacillus* isolate 143
4.4 Proximate analysis and nutritional composition of Goa cowpea cultivar 3 (*Vigna unguiculata*) seeds 144-149
4.4.2 Fatty acid profile of GC3 149
4.4.3 Profile of total amino acids 151
4.4.4 Content of water-soluble and fat-soluble vitamins 153
4.4.5 Mineral content 155
4.4.6 Oligosaccharides (stachyose, verbascose, raffinose) in GC3 157
4.5.1 Physical properties of GC3 seeds 158

4.5.2 Cooking properties of GC3 seeds 162
4.6 Fermentation of GC3 and its efficacy as a probiotic carrier 167-182
4.6.1 Influence of *B. licheniformis* on the physicochemical properties of GC3 slurry during fermentation 169
4.6.1.1 pH 169
4.6.1.2 Total titratable acidity (TTA) 171
4.6.1.3 Organic acids and short chain fatty acids (SCFAs) 174
4.6.1.4 Viscosity 176
4.6.1.5 Protein content 177
4.6.1.6 Free Amino Nitrogen (FAN) 178
4.6.1.7 Total carbohydrates (TC) and Total reducing sugars (TRS) 180
4.6.2 Probiotic cell viability before and after lyophilization of *Bacillus* cell suspension and GC3 synbiotic formulation 182
183
4.6.3 Probiotic cell viability of the synbiotic formulation upon storage at low and room temperature 184
4.7 Effect of fermentation on proximate analysis, peptide formation, nutritional composition, phytate, and oligosaccharide content of the synbiotic formulation 186-206
4.7.1 Proximate analysis 186
4.7.2 Protein hydrolysis and peptide formation by SDS – PAGE 189
4.7.3 Comparative assessment of composition of free amino acids (FAAs) 190
4.7.4 Water- and fat-soluble vitamins and carotenoids 192
4.7.5 Mineral content upon fermentation 194
4.7.6 Phytic acid content 196
4.7.7 Oligosaccharide content after fermentation 196
4.8 *Bacillus* viability in the synbiotic food formulation in comparison to its cell suspension during exposure to simulated gastrointestinal conditions 197
4.9.1 Antibiotic susceptibility of the synbiotic formulation 201
4.9.2 Antioxidant activity of the synbiotic formulation 202
4.9.3 Antimicrobial activity of the synbiotic formulation 204

4.10 Functional properties of the synbiotic formulation for suitability in food products 206
4.10.1 Emulsification capacity and stability 207
4.10.2 Foam capacity and stability 208
4.10.3 Heat tolerance 210
SUMMARY **214-223**
CONCLUSION **224**
BIBLIOGRAPHY **225-253**
APPENDICES **254-275**
PUBLICATIONS **276**

ABBREVIATIONS

~	Approximately
α	Alpha
A	Absorbance
AAD	Antibiotic Associated Diarrhoea
AAE	Ascorbic Acid Equivalent
ACE	Angiotensin Converting Enzyme
ACN	Acetonitrile
ACS	Acetyl-coenzyme A Synthetase
ADP	Adenosine-5'-Diphosphate
ALP	Alkaline Phosphatase
AMP	Adenosine-5'-Monophosphate
ANF	Antinutritional Factor
ANOVA	Analysis of Variance
ANSA	3-amino-5-nitrosalicylic acid
AOAC	Association of Official Analytical Collaboration
ATCC	American Type Culture Collection
AU	Anson Unit
ATP	Adenosine Triphosphate
β	Beta
BATH	Bacterial Adhesion to Hydrocarbons
BANA	Benzoyl-DL Arginine-Naphthylamide
BBB	Blood Brain Barrier
BCFAs	Branched Chain Fatty Acids
Beads/mL	Beads per Millilitre
BET	Bacterial Endotoxin Test
BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
bp	Base Pairs
BCAA	Branched Chain Amino Acids

BSA	**Bovine Serum Albumin**
BSH	**Bile Salt Hydrolase**
C	**Concentration**
°C	**Degree Celsius**
Cells/mL	**Cells per Millilitre**
CFU/mL	**Colony Forming Units per Millilitre**
cm	**Centimetre**
CNS	**Central Nervous System**
CoA	**Coenzyme A**
CS	**Citrate Synthase**
CVD	**Cardiovascular Diseases**
d	**Days**
DAD	**Diode Array Detector**
DASH	**Dietary Approaches to Stop Hypertension**
DBT	**Department of Biotechnology**
DGGE	**Denaturing Gradient Gel Electrophoresis**
D-GPT	**D-glutamate-pyruvate Transaminase**
D-LDH	**D-Lactate Dehydrogenase**
DMPD	**N, N-dimethyl-p-phenylene-diamine Sulphate**
DNA	**Deoxyribonucleic Acid**
DNSA	**3,5-dinitrosalicylic Acid**
DPPH	**2,2-diphenyl-1-picrylhydrazyl**
DTT	**Dithiothreitol**
D/W	**Distilled Water**
EFSA	**European Food Safety Authority**
EMS	**Ethyl Methane Sulfonate**
EPS	**Exopolysaccharide**
FA	**Formic Acid**
FAA	**Free Amino Acid**
FAMEs	**Fatty Acid Methyl Esters**
FAN	**Free Amino Nitrogen**
FAO	**Food and Agricultural Organization**

FCS	**Free Cell Suspension**
FID	**Flame Ionisation Detector**
FISH	**Fluorescence In Situ Hybridisation**
FMT	**Fecal Microbial Transplantation**
FODMAP	**Fermentable Oligosaccharides, Disaccharides, Monosaccharides and Polyols**
FOS	**Fructoligosaccharides**
FSSAI	**Food Safety and Standards Authority of India**
γ	**Gamma**
G-6-P	**Glucose-6-phosphate**
G6P-DH	**Glucose-6-phosphate dehydrogenase**
GABA	**Gamma Amino Butyric Acid**
GAE	**Gallic Acid Equivalent**
GC	**Gas Chromatography**
GC3	**Goa Cowpea Cultivar 3**
GRAS	**Generally Recognized As Safe**
GIT	**Gastrointestinal tract**
GI	**Glycaemic Index**
GOS	**Galactooligosaccharides**
h	**Hour (s)**
HK	**Hexokinase**
HMOS	**Human Milk Oligosaccharides**
HPLC	**High-Performance Liquid Chromatography**
HLS	**Haemocyte Lysate Supernatant**
HSD	**Honestly Significant Difference**
IAA	**Indole Acetic Acid**
ICAR	**Indian Council of Agricultural Research**
ICMR	**Indian Council of Medical Research**
IBD	**Inflammatory Bowel Disease**
IBS	**Irritable Bowel Syndrome**
IDF	**Insoluble Dietary Fiber**
IFCT	**Indian Food Composition Table**

IP1	**Inositol Monophosphate**
IP2	**Inositol Diphosphate**
IP3	**Inositol Triphosphate**
IP4	**Inositol tetraphosphate**
IP5	**Inositol Pentaphosphate**
IP6	**Inositol Hexaphosphate**
ISAPP	**International Scientific Association for Probiotics and Prebiotics**
IU	**International Units**
Kb	**Kilo base pairs**
LAB	**Lactic Acid Bacteria**
L-LDH	**L-Lactate Dehydrogenase**
L-MDH	**L-Malate Dehydrogenase**
LC-MS	**Liquid Chromatography Mass Spectrometry**
L-DOPA	**L-3, 4-dihydroxy Phenylalanine**
LPS	**Lipopolysaccharide**
µL	**Micro Litre**
µm	**Micro Meter**
µmol	**Micro mole**
µmol/L	**Micro moles per litre**
M	**Molar**
MAC	**Microbiota-Accessible Carbohydrates**
MHA	**Mueller Hilton Agar**
min	**Minute(s)**
MRS	**de Man, Rogosa and Sharpe**
MRSA	**Methicillin Resistant Staphylococcus Aureus**
MR-VP	**Methyl Red- Voges Proskauer**
MS	**Mass Spectrometry**
MTCC	**Microbial Type Culture Collection and Gene Bank**
MUFA	**Monounsaturated Fatty Acids**
MW	**Molecular Weight**
N	**Normality**
NAD+	**Nicotinamide-adenine-dinucleotide**

NADH	**Nicotinamide-Adenine-Dinucleotide (reduced)**
NADP+	**Nicotinamide-Adenine-Dinucleotide-phosphate**
NADPH	**Nicotinamide-adenine-dinucleotide-phosphate (reduced)**
NAFLD	**Non-Alcoholic Fatty Liver Disease**
NaGC	**Sodium Glycocholate**
NaTC	**Sodium Taurocholate**
nmol	**Nanomoles**
NBT	**Nitro- blue Tetrazolium**
NCBI	**National Center for Biotechnology Information**
NCDs	**Non-Communicable Diseases**
NGP	**Next-generation Probiotics**
NHPs	**Natural Health Products**
OD	**Optical Density**
ONPG	**Ortho Nitro Phenyl Thio Galactosides**
PAMPs	**Pathogen-Associated Molecular Patterns**
PCR	**Polymerase Chain Reaction**
PEG	**Polyethylene Glycol**
PBS	**Phosphate Buffered Saline**
PDA	**Photo Diode Array**
PDCAAS	**Protein Digestibility Corrected Amino Acid Score**
pH	**Potential of Hydrogen**
PLAB	**Probiotic Lactic Acid Bacteria**
PMSF	**Phenylmethylsulfonyl fluoride**
pNPG test	**p-nitrophenyl-α-D-galactopyranoside**
PRPs	**Pattern Recognition Proteins**
psi	**Pounds per square inch**
psu	**Percentile Salinity Units**
PUFA	**Polyunsaturated Fatty Acids**
PVC	**Polyvinyl Chloride**
PVDF	**Polyvinylidene Flourid**
r	**Correlation Coefficient**
rDNA	**Ribosomal Deoxyribonucleic Acid**

RFO	**Raffinose Family Oligosaccharide**
RI	**Refractive Index**
RID	**Refractive Index Detector**
RNA	**Ribonucleic Acid**
rRNA	**Ribosomal Ribonucleic Acid**
ROS	**Reactive Oxygen Species**
rpm	**Rotations Per Minute**
SD	**Standard Deviation**
SDS	**Sodium Dodecyl Sulfate**
SDS PAGE	**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis**
SCFAs	**Short Chain Fatty Acids**
SEM	**Scanning Electron Microscopy**
SDF	**Soluble Dietary Fiber**
sp.	**Species**
spp.	**Species (plural)**
subsp.	**Subspecies**
SPSS	**Statistical Package for the Social Sciences**
SWATH	**Sequential window acquisition of all theoretical fragment-ion spectra**
TAA	**Total Amino Acids**
TC	**Total Carbohydrates**
TCA	**Trichloroacetic Acid**
TCB	**Total Culturable Bacteria**
TCBS	**Thiosulphate Citrate Bile Salts Sucrose**
TDF	**Total Dietary Fibre**
TEMED	**Tetra Methyl Ethylene Diamine**
TGGE	**Temperature Gradient Gel Electrophoresis**
TMAO	**Trimethylamine N-oxide**
TPP	**Total Plasma Protein**
Tris	**Tris(hydroxymethyl)aminomethane**
TRS	**Total Reducing Sugars**
TTA	**Total Titratable Acidity**

TUI	**Trypsin Units Inhibited**
U-HPLC	**Ultra High-Performance Liquid Chromatography**
U/mg	**Units per Milligram**
UPF	**Ultra Processed Food**
UV	**Ultra Violet**
Vis	**Visible**
VOCs	**Volatile Organic Compounds**
W	**Weight**
WAC	**Water Absorbsion Capacity**
WHO	**World Health Organization**
WUR	**Water Uptake Ratio**

List of Tables

Table 1: Average proximate, vitamin, and mineral composition of raw/cooked cowpea grains.

Table 2: Evidence-based health benefits of cowpea and underlying mechanisms.

Table 3: Summary of the common probiotic bacteria used in industry.

Table 4: ICMR–DBT Guidelines for evaluation of probiotics in Food (India).

Table 5: Some commercially available probiotic products.

Table 6: Synbiotics commonly used in human nutrition.

Table 7: Synbiotic products marketed in India.

Table 8: Legumes of importance in Indian diets.

Table 9: Advantages of controlled fermentation.

Table 10: Volatile compounds produced during fermentation of legume-based substrates.

Table 11: Nutritional and health benefits of fermented legumes.

Table 12: Primers for enterotoxin genes.

Table 13: Preparation of various bile salt concentrations.

Table 14: Resolving gel and stacking gel for SDS-PAGE.

Table 15: Gram's nature of saltern isolates.

Table 16: Carbohydrate utilization pattern of isolates.

Table 17: Extracellular enzyme synthesis of *Bacillus* isolates.

Table 18: Antagonistic activity of isolates and zone of inhibition (ZOI) against tested pathogenic bacteria.

Table 19: Antibiotic susceptibility of the saltpan bacterial strains.

Table 20: BSH activity (IU/h) at different pH towards taurocholate.

Table 21: BSH activity (IU/h) at different pH levels towards glycocholate.

Table 22 A: Morphological characteristics of selected isolates.

Table 22 B: Biochemical characterization and carbohydrate utilisation of the isolates**.**

Table 23: Molecular identification of the selected bacterial isolates.

Table 24: The proximate composition of GC3 seeds.

Table 25: Fatty acid profile of GC3.

Table 26: Amino acid composition (g/100g protein) of GC3 seed cultivar in comparison with cowpea Species as per IFCT, 2017.

Table 27: Vitamin and carotenoid content of GC3 seeds.
Table 28: Comparison of mineral content between the GC3 seed cultivar and the values reported for cowpea species by IFCT (2017).
Table 29: Oligosaccharide content of GC3 seeds and cowpea species as per IFCT, 2017.
Table 30: Physical properties of the GC3 cultivar.
Table 31: Cooking and textural properties of GC3 seeds.
Table 32: Concentration of acetic acid and propionic acid at different fermentation hours.
Table 33: Proximate analysis of fermented GC3 synbiotic formulation.
Table 34: Free amino acid profile of GC3 before and after fermentation.
Table 35: Vitamin and Carotenoid content of GC3before and after fermentation.
Table 36: Mineral content of unfermented and fermented GC3.
Table 37: Effect of fermentation on raffinose family oligosaccharide content of GC3.content of GC3.
Table 38: Viability of *Bacillus* cell suspension in comparison to GC3 synbiotic formulation during exposure to simulated gastric juice.
Table 39: Antibiotic susceptibility of the GC3 synbiotic formulation.

List of Figures

Figure 1: Impact and types of microbial foods.

Figure 2: Five stages indicating changes during food fermentation

Figure 3: (A, B, and C): Viability of *Bacillus* isolates (FSK 0.30, FSK 2, and FSK 3) under simulated gastric conditions at different pH levels over 3 hours.

Figure 4: Effect of simulated gastric pH on the survival of *Bacillus* isolates (FSK 0.3, FSK 2, FSK 3) after 3 hours of exposure.

Figure 5: Viability of isolates during exposure to bile salt.

Figure 6: Adhesion capacity of halotolerant *Bacillus* isolates to mucus (in %).

Figure 7: Phylogenetic tree illustrating the 16S rRNA–based sequence similarity of FSK 2 and FSK 3 with related bacterial species.

Figure 8: Viability of *Bacillus licheniformis* PQ098138 during storage at 4 ± 2 °C and 28 ± 2 °C.

Figure 9: Viable cell counts in GC3 slurry during fermentation.

Figure 10 (A, B, C): Changes in pH, TTA, and viscosity of GC3 slurries during fermentation with *B. licheniformis*.

Figure 11: Effect of *Bacillus* fermentation on protein content of Goa cowpea 3.

Figure 12: Effect of *Bacillus* fermentation on free amino nitrogen content of GC3 slurry.

Figure 13: Profile of total carbohydrates (A) and TRS (B) in GC3 slurry during fermentation.

Figure 14: Viability of *B. licheniformis* in cell suspension and in GC3 synbiotic formulation before and after lyophilization.

Figure 15: Viability of *B. licheniformis* in lyophilized GC3 formulation upon storage.

Figure 16: Viability of *Bacill*us cell suspension (BCS) in comparison to GC3 synbiotic formulation during exposure to simulated intestinal juice.

Figure 17: Reducing power of unfermented and fermented GC3 formulation.

Figure 18: Functional properties of unfermented and fermented GC3.

Figure 19: Foaming stability of unfermented and fermented GC3.

Figure 20A: Viable cell counts of *B. licheniformis* cell suspension.

Figure 20 B: Viable cell counts of *B. licheniformis* in GC3 formulation.

List of Plates

Plate 1: Gram staining of isolates: (A) ABSK 55, (B) FSK 0.30, (C) FSK 2, (D) FSK 2.2, (E) FSK 3, and (F) FSK 5.34.

Plate 2: Endospores of isolates (G) FSK 3 and (H) FSK 2.

Plate 3: Growth of isolates on Nutrient agar after heat shock treatment.

Plate 4: Acid and gas production with Glucose (G) by FSK 2, FSK 3, SK 27, and Sucrose (S) by FSK 3.

Plate 5: (C) Positive control and isolates (1) FSK 2, (3) FSK 3, (4) FSK 0.30 showing glucose utilization.

Plate 6: Production of extracellular enzymes - (A)amylase, (B) lipase, (C) cellulase, (D)β-Galactosidase, (E) protease, and (F) phytase by isolates.

Plate 7: Safety evaluation of bacterial isolates by blood agar haemolysis.

Plate 8: *hblA* gene-specific PCR; Lane 1: 1 kb DNA ladder; Lanes 2, 4, 6: Positive control (std. *B. cereus* 2106); Lanes 3, 5, and 7: Test isolates FSK 2, FSK 3, and FSK 0.30, respectively.

Plate 9: *entFM* gene-specific PCR; Lane 1: 1 kb DNA ladder; Lanes 2, 4, 6: Positive control (std. *B. cereus* 2106); Lanes 3, 5, and 7: Test isolates FSK 2, FSK 3, and FSK 0.30, respectively.

Plate 10: *nheB* gene-specific PCR; Lane 1: 1 kb DNA ladder; Lane 2, 4, and 6: Positive control (std. *B. cereus* 2106); Lanes 3, 5, and 7: Test isolates FSK 2, FSK 3, and FSK 0.30, respectively.

Plate 11: *nheA* gene-specific PCR; Lane 1: 1 kb DNA ladder; Lane 2, 4, and 5: test isolates FSK 3, FSK 2, and FSK 0.30, respectively; Lane 3: Positive control (std. *B. cereus* 2106).

Plate 12: Test for presence of endotoxin: (A) Positive control, (B)Negative control, (C) isolate FSK 2, and (D) isolate FSK 3 (both negative for endotoxin).

Plate 13A: Antimicrobial activity of the isolates (A) FSK 3 and (B) FSK 2 with ATCC strains (1) *B. cereus* ATCC 14579, (2) *E. coli* ATCC 25922, (3) *S. Paratyphi* ATCC 14028, (4) *S. Enteric* ATCC 35664, (5) *S. Aureus* ATCC 6538, and (6) *S. Boydei* ATCC 8700.

Plate 13B: Antimicrobial activity of the isolates (C) FSK 3 and (D) FSK 2 with clinical laboratory pathogens: (1) *Methicillin resistant Staphylococcus aureus (MRSA)*, (2) *S. typhi,*

(3) *S. aureus*, (4) *E. coli*, (5) *E. cloacae*, (6) *S. sonnei* and (7) *S. boydei* (Representative images).

Plate 14: Antibiotic susceptibility test of isolates FSK 0.30, FSK 2 and FSK 3 by Kirby-Bauer disk diffusion method to Gentamycin (G), Penicillin (P), Tetracycline (Te), Chloramphenicol (C), Ampicillin (A), Cephalothin (Ch), Kanamycin (K), Erythromycin (E), Streptomycin (St), Trimethoprim and Ciprofloxacin (Cf).

Plate 15: BSH activity of *L. plantarum* (positive control) and halotolerant isolates with (A) sodium glycocholate and (B) sodium taurocholate.

Plate 16A: Primary screening for EPS-producing bacterial isolates [(A) FSK 3, (B) FSK 2, (C) FSK 0.30, (D) *L. Plantarum*] on Congo Red agar showing black colonies.

Plate 16B: Ethanol precipitation of EPS from isolate FSK 3.

Plate 17: IMViC Tests; Negative Indole utilisation test for (A) FSK 2 and FSK 3, (B) positive MR test, (C) positive VP test for FSK 3, and (D) negative VP test for FSK 2, (E) positive citrate utilization test for FSK 2 and FSK 3, and (F) positive catalyse test for FSK 3.

Plate 18: Dry grains of the Goa cowpea 3 cultivar milled into powder.

Plate 19: Dry seeds of the Goa cowpea 3 cultivar milled to powder.

Plate 20: Changes in viscosity of GC3 slurry after 8 hours of fermentation.

Plate 21: Lyophilized GC3 synbiotic powder stored under refrigeration.

Plate 22: Scanning electron micrograph of *B. licheniformis* in GC3 matrix.

Plate 23: SDS-PAGE profile of GC3 before and after fermentation. Lane 1: Unfermented GC3 powder, Lanes 2 and 3: Fermented GC3 powder; Lane 4: Prestained ladder marker.

Plate 24: Antibiotic susceptibility of GC3 synbiotic formulation to Chloramphenicol (C), Kanamycin (K), Ciprofloxacin (Cf), Tetracycline (Te), Erythromycin (Ery), Vancomycin (Va), Gentamycin (G), Cephalothin (Ch), Ofloxacin (O), Trimethoprim (Tr), and Ampicillin(A).

Plate 25: Antibacterial activity of GC3 synbiotic formulation against: (A) *S. aureus* ATCC 6538, (B) *S. enterica* ATCC 35664, (C) *S. typhimurium* MTCC 531, and (D) *E. coli* ATCC 700927.

CHAPTER 1:
INTRODUCTION

Chapter 1

Introduction

The present era is the age of functional foods. Health-conscious consumers are opting for plant-based synbiotic functional foods, which are regarded as advantageous to human health. Consequently, the food industry has increasingly prioritized the development of functional dietary supplements to meet consumer health demands. The high-demand functional food popular among consumers is prebiotic, probiotic, and synbiotic formulations. Probiotic products account for approximately 10% of the global functional food market, representing a rapidly expanding sector of considerable economic significance (Ashaolu, 2020). Rising health awareness among consumers aiming for a better quality of life has stimulated the growth of functional foods, a trend reinforced by technological progress and the creation of new products (Singh, 2017; Song et al., 2023). Functional foods include cereals, pulses, beverages, bakery products, dairy foods, spreads, meat, eggs, and other formulations containing functional constituents, including minerals, vitamins, omega-3 fatty acids, bioactives like antioxidants, phytosterols, prebiotics, and probiotics (Ashaolu et al., 2021; Bangar et al., 2022). A shift toward healthy diets by 2050 will demand major changes, including doubling the intake of plant-based foods and cutting red meat and sugar consumption by more than 50% (Valero et al., 2020; Jideani et al., 2021). Healthy diets are characterized by an optimal caloric intake and a high diversity of plant-based foods, limited quantities of animal-source foods, a preference for unsaturated over saturated fats, and restricted consumption of refined grains, highly processed foods, and added sugars (Owusu et al.,2022; Shu et al., 2023).

Grain processing continues to be a critical aspect of food and nutrition security in the modern world, as it enhances consumer acceptability while preserving nutritional value. Various techniques are employed for the processing and preservation of legumes, including fermentation and germination (Tamang et al., 2020; Verni et al., 2022)

Fermentation leads to biochemical alterations of the primary food matrix through the action of microorganisms and their enzymes (Akin & Ozcan, 2017; Ali et al., 2018). Fermentation is an ancient method that has been used as a means of improving the quality of legumes and might differently influence their composition, nutrients from legumes, improving the bioaccessibility and bioavailability of nutrients, and improve organoleptic and functional attributes as well as

extend the shelf life by inhibiting the growth of pathogenic bacteria due to the antimicrobial activity of organic acids formed (Akanni et al., 2018; Adebo et al., 2022; Affrifah et al., 2022). Fermentation has been reported to lead to depolymerisation of macromolecules like starch and proteins to simpler forms, leading to physicochemical changes and improvement in their nutritional quality (Akin et al., 2017; Zheng et al., 2017). Fermentation reduces antinutritional factors and is regarded as an effective strategy to lower the risk of mineral deficiencies in populations that rely heavily on unrefined cereals and pulses (Aduol et al., 2020; Adebo et al., 2022).

Spontaneous or natural fermentation is often accompanied by microbial proliferation, including yeasts and molds, which may pose food safety concerns, highlighting the need for controlled fermentation (Verni et al., 2022; Naik & Kerkar, 2024). Although evidence suggests that fermented foods typically demonstrate improved nutritional and functional properties compared to their unfermented counterparts, through the activation of endogenous enzymes that reduce antinutritional factors, enhance antioxidant activity, and release health-promoting postbiotics, optimal fermentation conditions must be determined for each legume to maximize both nutritional and health benefits. (Diez et al., 2020; Banwo et al., 2021; Bangar et al., 2022).

Probiotics, the functional constituents most widely endorsed for their health benefits, are the beneficial microorganisms that impart health advantages to the host when delivered in appropriate amounts (Elshaghabee et al., 2017), and thus are applied in the food formulations. Numerous reports demonstrate the health effects of probiotics; most of these benefits have been proven in animal models, and some have been validated in controlled human trials. These include prevention and treatment of infections as well as inflammatory bowel disease, antibiotic associated diarrhoea, constipation, *Helicobacter pylori* infection, lactose intolerance, irritable bowel syndrome, along with stimulation of immune system, reduction in serum cholesterol, reduction in blood pressure, anticarcinogenic activity, improvement of urogenital health, etc. (Kavita et al., 2015; Santos - Sanchez et al., 2024).

Current research is focused on developing new techniques, processing methods, carrier matrices, and bacterial strains to improve the survival of probiotic strains under heat, bile, acidic conditions, and pressure in the digestive tract. Selecting a probiotic strain with a suitable substrate that ensures high probiotic yield is a key criterion for human application. The most commonly used probiotic strains are: *Bifidobacterium, Lactobacilli, S. boulardii, and B.*

coagulans (Kavita et al., 2015). A key advantage of using *Bacillus* spp. is their ability to form spores, which can survive passage through the digestive tract, resist gastric juices, produce antimicrobial substances, and thrive in a variety of carrier matrices, and maintain viability after storage at room temperature (Feng et al., 2018; Funck et al., 2019). Food fermentation using probiotics is gaining a great deal of attention for its several potential nutritional benefits, such as improved digestibility of starch and protein and reduction in antinutritional factors, including tannins, trypsin inhibitors, and phytic acid, resulting in improved bioavailability of minerals and improved organoleptic properties (Georgieva et al., 2015; Homsuwan et al., 2023). Elevation of vitamin content, formation of postbiotics like organic acids (lactic acid, acetic acid), and volatile compounds like ethanol, acetone, acetaldehyde, and diacetyl is also reported to be a major benefit of probiotic fermentation (Elshghabee et al., 2017; Barat & Ozcan, 2018; Gobetti et al., 2019). Besides, the accumulation of organic acids is also important for food safety and the enhancement of shelf life (Vinderola et al., 2022).

Besides selecting preferred probiotic strains, the choice of food materials as carrier matrices is crucial for the effective production of probiotics. According to ISAAP, probiotics can provide health benefits to the human host when administered in sufficient quantities (Marco et al., 2017). Food is usually the preferred mode for the ingestion of probiotics. Numerous studies have found that these beneficial organisms survive gastrointestinal transit and colonize the gut effectively when administered either through foods or given as supplements in an encapsulated matrix rather than free cell preparations (Funck et al, 2019; Gharibzahedi and Smith, 2021). Synbiotic formulations, a mixture of probiotics and prebiotics, are widely preferred as compared to either of them alone, as they manifest a higher potential for a synergistic health benefit (Kavita, 2015; Keulers et al., 2022).

Originally, dairy foods were the most commonly chosen matrix for probiotic delivery; currently, there is an increasing trend towards incorporating probiotics in different food systems (Min et al., 2017; Merenkova & Zinina et al., 2021). Several foods, like fruits, vegetables, cereals, and legumes, contain prebiotic constituents and have been found to promote the growth of probiotics (Niro et al., 2019; Narli & Ozcan, 2022). Cereals and legumes are key dietary staples worldwide, supplying carbohydrates, proteins, vitamins, minerals, and fiber. A wide array of prebiotic constituents including non-digestible carbohydrates (raffinose family oligosaccharides, galacto oligosaccharides, fructo oligosaccharides, β glucan and resistant starch) and other bioactive compounds (phenolic

compounds, antioxidants,, enzyme inhibitors, phytoestrogens, etc.) present in legumes have been identified to offer protection against numerous chronic diseases such as coronary heart diseases, diabetes, hypertension, cancer, obesity and inflammation (Tomasik & Tomasik, 2020; Xiong et al., 2023). As a result, these foods act as fermentable substrates for probiotic microorganisms in the development of synbiotic functional foods.

The ability of prebiotic substrates to promote the growth of probiotic organisms to above 10^7 cfu/mL in minimum time is an important criterion for their inclusion in synbiotic food product development (Ziar et al., 2014; Bilal et al., 2022; Yoha et al., 2022). A great deal of carriers will help maintain the viability of probiotic strains during storage and administration to the digestive tract. Maintaining probiotic viability in non-dairy products is more demanding compared to dairy matrices, as these products require highly favourable conditions (Distefano et al., 2017; Kaur et al., 2020; Huang et al., 2022). Besides improving probiotic survival, factors such as nutritional value, cost, and sensory acceptability should also be taken into account. Though cereals and common legumes like soybeans and green grams have been successfully used by many scientists as prebiotics and probiotic carriers, many other important legumes have not been widely exploited for their prebiotic potential.

Cowpea is a highly nutritious food, providing carbohydrates, essential nutrients, fiber, and bioactive compounds (Goncalves et al., 2016). Cowpea (*Vigna unguiculata*) is a well-established staple in Goa, and its cultivation occurs in many regions of India as well. Studies have reported different nutritional characteristics and properties in cowpea cultivars and regional diversity (Bai et al., 2020). Exploitation of cowpea for its nutrients, particularly proteins, and as a substrate for probiotics remains to be investigated. The nutritional profiling of the indigenous Goa cowpea 3 cultivar is unknown and was thus studied for its incorporation as a healthy food.

New probiotic genera and strains continue to emerge as research becomes more advanced and targeted. Identifying the unexplored probiotic potential of hypersaline bacterial culture/s would further ascertain their health benefits. The Goan salt pans are highly hypersaline environments, consisting of interconnected multi-pond systems with a continuous influx of seawater, which is evaporated to produce natural salt. As coastal ecosystems, they host microorganisms capable of withstanding a broad range of salinities, classified as halophilic (salt-loving) and halotolerant

(able to tolerate salt but not requiring it for growth) (Ballav et al., 2015; Fernandes et al., 2019).

Salt pans are rich reservoirs of unique microbes that produce novel bioactive compounds with biotechnological potential, including compatible solutes, metal bioremediation agents, biosurfactants, and pharmaceutically important products (Sikkandar et al.,2013; Somplang et al., 2016; Pawaskar et al., 2022). Such microorganisms produce stable compounds capable of functioning under varying salinity, temperature, pH, and other extreme environmental conditions (Gawas & Kerkar, 2023). The application of native hypersaline bacteria as probiotics in humans remains largely unexplored, as does the potential of cowpea as a substrate for probiotic growth and delivery. For effective synbiotic development, the ability of probiotics to utilize prebiotics should be considered a prerequisite, ensuring synergistic interactions that maximize health benefits and enable the design of enhanced functional foods to improve host health. Thus, this research work aimed to assess the nutritive value of the Goa cowpea 3 (*Vigna unguiculata*) cultivar (GC3) and study its synbiotic biotechnological application.

The present study was conducted with the following objectives:

Objectives:

1. Assessing the probiotic potential of selected strains of hypersaline bacteria.
2. Analysing the proximate and selected nutritional composition of the GC3 seed cultivar.
3. Optimizing the method of fermentation and evaluating the effect of selected strain in the fermentation of GC3 seed flour in developing an effective synbiotic food formulation.
4. Evaluating the effect of fermentation by hypersaline bacteria on the physicochemical properties, proximate/nutritional composition, and antinutritional factors of GC3.
5. Determining the suitability of the synbiotic food formulation as a food product

Significance of the thesis

Specific technologies represent the cutting edge of fermentation science in the production of probiotics and postbiotics, offering promising avenues for enhancing gut health. Recent advancements in probiotic fermentation include enhanced strain selection, which identifies high-potency strains with specific health benefits, optimizing strain performance. Precision fermentation approaches allow fine control of pH, temperature, and nutrient levels to maximize probiotic yield and viability, while automation and real-time monitoring ensure consistency and reproducibility.

Advances also involve the use of novel or improved substrates to support probiotic growth and activity, including plant-based, particularly legume-derived prebiotics, or alternative carbon sources, thereby creating balanced and functional products. Synbiotics, or "optibiotics," combine probiotics and prebiotics designed to enhance the survival and colonization of beneficial microbes in the gut. Such mixtures selectively promote the growth or metabolic activity of specific health-promoting bacteria, ultimately enhancing host health (Bilal et al., 2022; Amrutha et al., 2023).

Saltpans serve as a reservoir of diverse microbes with potential applications in food biotechnology. This study highlights the controlled fermentation of the Goa cowpea 3 (GC3) cultivar using saltpan bacteria as a potential probiotic starter culture. Fermentation improved the nutritional and physicochemical properties of GC3, while supporting probiotic viability and metabolite synthesis that could confer health benefits. GC3, rich in proteins, fiber, and carbohydrates, can contribute to cryoprotection by binding water, reducing ice formation, and creating a viscous environment that protects cells from mechanical damage during freezing. Pulse-based matrices like GC3 can thus be used to freeze-dry or cryopreserve probiotics, maintaining their viability and functionality.

The findings provide a basis for scaling up indigenous cowpea production, reducing cost, and developing fermented functional products enriched with probiotics. Furthermore, the study demonstrates the potential of halotolerant probiotics to improve fermentation of plant-based matrices, supporting the development of next-generation plant-based functional foods, considering the challenges of climate change and the goals of sustainable agriculture

CHAPTER 2:

REVIEW OF LITERATURE

Chapter 2

Review of Literature

Plant foods of the family Leguminosae/Fabaceae are amongst the key dietary contributors important for humans (Da Silva et al., 2018). Legumes are generally good sources of slow-release carbohydrates, are rich in proteins, have low levels of lipids, and are an important source of vitamins like B-complex and minerals such as iron, zinc, magnesium, and calcium (Fabbri & Crosby, 2016; Clemente & Olias, 2017). The presence of various phytochemicals and prebiotics makes legumes effective candidates for the development of functional food (Behera et al., 2018). Further, the legume crops are essential for sustainable agriculture, and they can adapt to climate change, being cultivated in arid climates with limited or erratic rainfall.

The desire for enhanced health has led to an interest in formulations to facilitate the proliferation of gut microflora (Dahiya & Nigam, 2022). Plant-origin fermented foods are popular substrates for administering probiotic microorganisms, showing synergistic impact on human health (Rul et al., 2022). With the increase in lactose intolerance, milk allergies, and gluten sensitivity, there is renewed interest in non-dairy and gluten-free sources among health-conscious people (Rasika et al., 2021).

Cowpea (*Vigna unguiculata*) is a centuries-old leguminous crop, having originated in Africa and spread throughout Latin America and Southeast Asia (Oghbaei & Prakash, 2016; Merenkova & Zinina, 2020). Cowpea is primarily important for its unique nutrient composition in addition to its exclusive taste. Studies have reported different nutritional characteristics and properties in cowpea cultivars and regional diversity (Gonçalves et al., 2016; Bai et al., 2020; Affrifah et al., 2022). Indigenous cowpea, grown mainly by subsistent farmers, is preferred owing to its color, fleshy pods, and bold seeds, yielding high biomass, and can serve as a major contributor to food security, nutrition, and health (Manohara et al., 2021; Naik & Kerkar, 2024). Cowpea is also a gluten-free viable option for patients with celiac disease.

Controlled fermentation enables the formulation of legume-based functional foods with enhanced nutritional and health benefits (Verni et al., 2022). It not only reduces or eliminates anti-nutritional compounds but also minimizes the risk of contamination and growth of

pathogenic bacteria through the formation of antimicrobial metabolites and a concomitant decrease in product pH (Di Stefano et al., 2017; Forsido et al., 2020). The accumulation of organic acids and the resulting pH reduction further contribute to food safety (Verni et al., 2022).

Although soybeans and green gram have been extensively studied, data on the use of cowpea as a substrate for probiotic delivery remain scarce. The potential of cowpea and other legumes in functional food production is attributed to their fermentable prebiotic constituents such as oligosaccharides, amylose, glucose, maltose, and other non-digestible components (Dahiya & Nigam, 2022). These compounds not only support the growth of probiotic microorganisms but also enhance their stability, which is an imperative criterion for probiotic food development, as the strains must achieve high viable cell counts (>10^7 cfu/mL) within a short fermentation time (Palanivelu et al., 2022).

Among various etiological factors of diseases, the crucial role of gut microbiota has been recently realised. Since then, several attempts have been made towards modulating the gut microbiota through the use of probiotics. Greater health benefits are obtained when these live cultures of microorganisms (probiotics) are used in combination with prebiotics that are selectively fermented by probiotics. Hence, currently, several attempts have been made to identify probiotic strains and prebiotic ingredients suitable for the development of effective synbiotic food products. Most commonly used probiotic strains, *viz. Bifidobacterium, Lactobacilli, S. boulardii, and B. coagulans* are reported to improve protein and starch digestibility of the product, decrease their viscosity (Kavita et al., 2015; Behera et al., 2018; Forsido et al., 2020), and enhance organoleptic properties due to the formation of postbiotics (Tomasik & Tomasik, 2020).

Salt production is an age-old traditional practice developed by local salt farmers known as "mittkars", with a history spanning over 150 years (Ballav et al*.,* 2016). Salt pans are unique ecosystems that support diverse communities of extremophiles, microorganisms that thrive under high salinity, extreme temperatures, varied pH levels, and limited nutrient availability (Mani et al*.,* 2012). Halophilic bacteria in these environments have evolved specialized strategies to manage salt stress. The rich microbial diversity found in these habitats has drawn significant interest from researchers in studying various biotechnological applications of the saltpan microflora (Fernandes et al*.,* 2019; Gawas & Kerkar, 2024). New genera and strains

of probiotics are continuously emerging with more advanced and focused research efforts. Identifying unexplored probiotic potential of hypersaline bacterial cultures and suitable cost-effective processing techniques to improve the biotic potential of foods would further ascertain their health benefits.

With this in view, saltpan isolates are ascertained for their probiotic attributes, while the Goa cowpea cultivar 3 (GC3) was evaluated for its physical properties and selected nutritional components. Furthermore, the ability of GC3 to support the growth of potential probiotic isolates from the saltpan through fermentation was assessed for the development of a synbiotic food formulation.

2.1 Significance of cowpea: Nutrition, health, and food technology

Cowpea thrives best in lowland areas with temperatures ranging from 25 °C to 35 °C and annual rainfall between 750 and 1100 mm (Verma et al*.,* 2019; Bai et al*.,* 2020; Manohara et al*.,* 2021). It shows greater tolerance to sandy soils and drought conditions compared to soybeans and can grow in various soil types, provided they are well-drained (Carvalho et al*.,* 2012; Verma et al*.,* 2019). As one of the most important legume crops, cowpea plays a major role in the agriculture of the West Coast region. It performs well even in poor soils, largely due to its ability to provide ground cover, fix atmospheric nitrogen up to 80 % and suppress weeds (Bhardwaj et al*.,* 2014; Manohara et al*.,* 2021). These characteristics help reduce the need for nitrogen fertilizers, lower production costs, and enhance soil fertility, making cowpea a key contributor to the sustainability of agricultural systems, particularly in marginal lands (Da Silva et al*.,* 2018).

In recent years, the growing popularity of plant-based diets has increased the demand for legumes and cereals. As accessible sources of vegetable protein, they are gaining renewed attention, offering a practical alternative to animal-based proteins (Akin & Ozcan, 2017; Akweya et al., 2020). Cowpea is primarily cultivated for its seeds, both dry and fresh, as well as its leaves, and, in some less developed regions, for its fresh pods. All parts of the plant, including mature grains, immature green pods, and fresh leaves, are harvested for consumption (Hamid et al., 2016). Owing to its rich nutritional profile and functional properties, cowpea has attracted significant industrial interest as a potential ingredient in various food formulations. The nutritional composition of cowpea is influenced by genetic factors, environmental

conditions, biotic stressors, and post-harvest handling. With its high protein and fiber content and low glycemic index, cowpea stands out as a nutritious food with potential health benefits.

2.1.1 Carbohydrates

As presented in Table 1, cowpea (*Vigna unguiculata*) grains, both in their mature raw and as cooked form, are notably high in carbohydrates, accounting for approximately 50–60 % of the whole grain's dry weight. The predominant form of carbohydrate is starch, which includes a considerable proportion of resistant starch and a higher ratio of slowly digestible to rapidly digestible starch compared to other complex carbohydrate sources. This composition makes cowpea particularly suitable for individuals with insulin resistance or diabetes, as resistant starch induces a lower glycemic response (Appiah et al., 2011; Affrifah et al., 2022). In addition to starch, the presence of simple sugars in relatively small amounts in cowpea significantly contributes to its sensory properties. The sucrose content, in particular, plays a key role in determining the flavor profile of cowpea (Bai et al*.,* 2020). In non-fermented cowpea flour, the major soluble sugars identified include stachyose (3.43 %), sucrose (2.97 %), and raffinose (1.24 %) (Jayathilake et al., 2018; Bai et al., 2020).

Another valuable component of cowpea carbohydrates is prebiotic galactosides, which promote gut health by serving as substrates for beneficial intestinal microbiota (Ziar et al., 2014; Clemente & Olias, 2017; Da Silva et al., 2018). Given its low predicted glycemic index, estimated between 33 and 50, cowpea can be categorized as a low-calorie, functional food. Its consumption is beneficial for maintaining glycemic control in diabetic individuals and may support weight management in obese populations (Tresina & Mohan, 2012; Verma et al., 2019; Yao et al., 2020). Additionally, its carbohydrate composition, particularly its starch content, makes it valuable in food applications where texture and structure are important. Cowpea starch can resist enzymatic and acid hydrolysis and shows a high rate of retrogradation upon modification, making it useful as a natural texture modifier in the food industry (Gondwe et al., 2019; Oyeyinka et al., 2021).

Table 1: Average proximate, vitamin, and mineral composition of cowpea seeds.

Composition	**Mature seeds**		**Immature seeds**
	Raw	**Cooked/Boiled**	**Frozen/Cooked**
Proximate (g/100 g)			
Moisture	11.95	70.00	66.10
Energy (Kcal)	336.00	116.00	132.00
Carbohydrates	60.03	20.80	23.80
Fat	1.26	0.53	0.66
Protein	23.52	7.73	8.49
Total dietary fiber	10.60	6.50	6.40
Total sugar	6.90	3.30	4.46
Ash	3.24	0.94	0.99
Vitamins (per 100 g)			
Vitamin A (µg)	3.00	1.00	4.00
β- Carotene(µg)	30.00	9.00	45.00
Vitamin D_2 (µg)	0.93	ND	ND
Vitamin E (mg α- TE)	0.70	0.48	ND
Vitamin K(µg)	5.00	1.70	36.80
Thiamine (B1) (mg)	0.33	0.28	0.20
Riboflavin(B2) (mg)	0.09	0.06	-
Niacin (B3) (mg)	2.06	0.50	0.73
Pantothenic acid (B5) (mg)	1.50	0.41	0.21
Pyridoxine (B6) (mg)	0.30	0.18	0.16
Biotin (B7) (µg)	3.97	negligible	negligible
Total folate (B9) (µg)	231.00	208.00	141.00
Vitamin C (mg)	1.50	0.40	2.60
Minerals (mg/100 g)			
Calcium	110.00	24.00	23.00
Iron	8.27	2.51	2.12
Magnesium	184.00	53.00	50.00
Phosphorous	424.00	156.00	122.00

Potassium	1112.0	278.00	375.00
Sodium	16.00	4.00	5.00
Selenium	9.00	2.50	3.40
Zinc	3.37	1.29	1.42

TE: Tocopherol equivalent. (Longvah et al., 2017; Verma et al.,2018; USDA 2021; Abebe & Alemayehu 2022; Affrifah et al., 2022).

2.1.2 Proteins

The protein content of cowpea is notably high, contributing significantly to the dry weight of the whole grain, raw mature seeds, and decorticated forms (Table 1). Although protein levels can vary depending on cultivar and growing conditions, whole cowpea seeds generally contain between 16 % and 31% protein (Carvalho et al., 2012; Longvah et al., 2017; Abebe & Alemayehu, 2022; Affrifah et al., 2022). Similarly, studies conducted in South Africa reported that cowpea grain harvested from intercropped plots contained protein levels between 23.7 % and 26.4 %, which were comparable to those obtained from monocropped plots (23.7 %–25.7 %), making it a highly promising food for combating malnutrition (Jayathilake et al., 2018). Studies have reported average protein content at about 25.47 %, comparable to other protein-rich legumes such as soybeans, winged beans, and grams (Anyango et al., 2011; Affrifah et al., 2022).

The majority of cowpea proteins are composed of globulins, albumins, and glutelins, with prolamins present in relatively smaller quantities (Khalid & Elharadallou, 2013; Matemu et al., 2021). The amino acid composition of cowpea is particularly rich in lysine, leucine, tryptophan, and arginine, making it a valuable complement to cereal-based diets that are typically deficient in these essential amino acids (Carvalho et al., 2012; Da Silva et al., 2018; Narli & Ozcan, 2022). However, like many legumes, cowpea is deficient in sulfur-containing amino acids such as methionine and cysteine when compared to animal-derived proteins (Jayathilake et al., 2018; Garrido - Galand et al., 2021).

2.1.3 Lipid content and fatty acid composition

Cowpea whole grain contains lipid content between 0.5 and 3.9 %, with triglycerides (41.2 %), phospholipids (25.1 %), monoglycerides (10.6 %), free fatty acids (7.9 %), diglycerides (7.8 %), sterols (5.5 %), hydrocarbons and sterol esters (2.6 %), and sterol (5.5 %) accounting for the majority of its lipid profile (Hossain et al., 2016). Cowpea whole grain and seeds (raw and dried) have recently been emphasized in nutritional standards for their low-fat content in comparison to other legume grains (soybeans and Bengal gram) (Affrifah et al., 2022). As with other nutrients, the lipid content and fatty acid profile of cowpea seeds are genotype-dependent and can be affected by growth conditions. According to a study (Carvalho, 2012), the lipid content of 30 Brazilian cowpea genotypes ranged from 1.0 to 1.6 %. Polyunsaturated fatty acids (PUFA) make up the majority of total fatty acids and palmitic acid, with percentages ranging from 40.1 to 78.3 % and 20.5 to 67.1 %, respectively. Omega-3 (α-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid) and omega-6 fatty acids (linoleic acid, γ-linolenic acid, arachidonic acids) are two forms of fatty acids that are required for the body's processes (Antova et al., 2014). The level of linoleic acid is between 20.8 and 40.3 %, whereas linolenic acid levels can be as low as 9.6 % and as high as 30.9 % in cowpea. Stearic acid contributes to the fatty acid composition of cowpea seeds, accounting for a relatively minor proportion of 2.9 –14.0 % of the total (Carvalho et al., 2012; Abebe & Alemayehu, 2022). Regular consumption of cowpea may contribute to reducing the risk of non-communicable diseases (NCDs). Given its multiple nutritional and functional benefits, cowpea can rightly be considered a "superfood" and represents an affordable and accessible tool in the fight against malnutrition.

2.1.4 Minerals

Cowpea seeds, whether as whole, decorticated grain, or in their immature (green) or mature (raw or cooked) forms, are rich sources of essential minerals including calcium, magnesium, potassium, iron, zinc, and phosphorus. The concentration of certain minerals, particularly phosphorus, potassium, and manganese, varies significantly based on environmental conditions and cultivation practices (Antova et al*.,* 2014; Aduol et al., 2020).

When compared to common cereals, cowpea leaves, whole grains, and raw seeds exhibit notably higher levels of potassium, calcium, magnesium, and phosphorus (Bhardwaj et al.,

2014; Abebe & Alemayehu, 2022). Trace amounts of iron, sodium, zinc, and selenium are also present, contributing to the overall micronutrient profile of the crop (Da Silva et al., 2018). These characteristics position cowpea as an important food source for improving mineral intake, particularly in regions facing micronutrient deficiencies. However, the mineral bioavailability in cowpea can be limited by anti-nutritional factors (Abbas & Ahmad, 2018; Adebo et al., 2022).

2.1.5 Vitamins

Cowpea whole grains are a valuable source of several essential vitamins, notably vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin A, and vitamin C, with smaller amounts of vitamin B9 (Goncalves et al., 2016; Abebe & Alemayehu, 2022). These vitamins play critical physiological roles in both humans and animals, including muscle contraction, neural function, blood coagulation, gastrointestinal activity, and maintaining acid–base homeostasis (Jayathilake et al., 2018). Notably, the vegetative parts of the cowpea plant, particularly the leaves, contain higher concentrations of vitamins compared to the seeds or germinated grains. Cowpea grains typically contain less than 10 mg/100 g of vitamin C in their dry state, but this value increases substantially after sprouting by approximately 4 to 38-fold, suggesting that processing methods can enhance the content and bioavailability of certain vitamins.

2.1.6 Dietary fiber

Cowpea is a valuable source of both soluble and insoluble dietary fiber, supporting the development and maintenance of a healthy gut microbiota (Davani-Davari et al., 2019; Tomasik & Tomasik, 2020; Ashaolu et al., 2021). While cowpea naturally contains fermentable components, their functionality can be enhanced through extraction and incorporation into food products. Cowpea leaves (aerial parts), immature pods, whole grains, and decorticated grains are all notable sources of total dietary fiber (TDF). The fiber content in whole cowpea grain ranges widely, from approximately 2.5 % to 32 % on a dry matter basis (Abebe & Alemayehu, 2022). Dietary fiber is well recognized for its critical role in human health, particularly in reducing the risk of NCDs such as CVD, diabetes, and cancer (Da Silva et al., 2018).

Genetic diversity among cowpea cultivars also influences fiber content. For instance, a study on 30 Brazilian cowpea genotypes reported dietary fiber levels ranging from 19.5 to 35.6 g per 100 g, reflecting considerable variability (Carvalho et al., 2012). The TDF content is comprised of roughly 6 % cellulose, 3.9 % hemicellulose, 2% lignin, and 1.8 % pectin (Abebe & Alemayehu, 2022). The dry seeds of cowpea plants generally contain TDF in the range of 12.0 to 14.8 g per 100 g (Abebe &Alemayehu, 2022). Another study reported total dietary fiber content of cowpea seeds to be between 13.6 and 15.99 g per 100 g, attributing the high crude fiber primarily to the seed coats and cell walls of the legumes (Affrifah et al., 2022). These findings underscore cowpea's potential as a valuable dietary fiber source, contributing to digestive health and the prevention of chronic diseases.

2.1.7 Anti-nutritional factors in legumes

The nutritional quality of legumes, including cowpea, is often hindered by the presence of various antinutritional factors (ANFs) such as lectins, phytic acid, tannins, trypsin and chymotrypsin inhibitors, amylase inhibitors, proanthocyanidins, haemagglutinins, cyanogenic glucosides, oxalic acid, dihydroxyphenylalanine, saponins and flatulence causing oligosaccharides found in grains and leaves (Tresina & Mohan, 2012; Abebe & Alemayehu, 2022). Regular intake of these compounds can lead to toxin accumulation, which may adversely affect health when consumed in high amounts. Moreover, they interfere with normal metabolism and impair the ability of digestive system to absorb essential nutrients, due to binding various nutrients and reducing protein digestibility (Abbas & Ahmad, 2018; Arbab et al., 2023).

While some cowpea cultivars contain considerable amounts of condensed tannins (e.g., 0.11 % on a dry matter basis), these levels do not negatively impact intake or digestion. Proanthocyanidin content in cowpea seeds typically ranges between 2.2 and 6.3 mg/g, comparable to other legumes (Abebe & Alemayehu, 2022). Phytates present in cowpea grains can reduce the bioavailability of important minerals and proteins by forming insoluble phytate-protein and phytate-mineral complexes, usually divalent and trivalent ions of minerals such as Zn, Mg, Ca, Cu, and Mn (Carvalho et al., 2012). Unprocessed cowpea has been reported to contain phytic acid at concentrations around 836 mg per 100 g (Abdel-Aal, 2016).

Additionally, phytocystatins, protease inhibitors involved in seed development and germination, bind tightly to cysteine proteases, inactivating these enzymes (Abebe &Alemayehu, 2022). Understanding these interactions is crucial for designing nutritional strategies that promote legume consumption while minimizing adverse effects. Oxalic acid forms stable complexes with minerals such as K, Ca, and Mg. While some oxalate salts are water-soluble, others are insoluble; the latter can precipitate and accumulate in the urinary tract, leading to the formation of kidney stones (Adebo et al., 2022). During digestion in the GI tract, oxalic acid interacts with various nutrients, binding essential minerals and thereby reducing their bioavailability (Bora, 2014; Abbas & Ahmad, 2018). Saponins and lectins can reduce protein digestibility and nutrient absorption. Saponins may impart bitterness and cause cell membrane disruption, while lectins can interfere with intestinal function by binding to gut epithelial cells (Kaur et al., 2014; Li & Wang, 2021; Huang et al., 2022).

2.1.8 Bioactive compounds in legumes and their health benefits

Beyond its nutritional value, cowpea is rich in bioactive compounds that provide diverse health benefits. Among these, polyphenols stand out with unique profiles not commonly found in other legumes (Awika & Duodu, 2017). These compounds are mainly concentrated in the seed coat (Abdel Aal, 2016) and influence not only health-promoting properties but also seed coat colour, which affects cowpea selection and cultural utilization (Chua et al., 2025; Banwo et al., 2021).

Major phenolic compounds in cowpea include phenolic acids and flavonoids. Cowpea exhibits distinctive polyphenol profiles, especially in flavanols and flavan-3-ols, suggesting unique bioactive properties complementing other foods (Da Silva et al., 2018; Kumar et al., 2019). Phenolic acid derivatives range from 148 to 1176 µg/g, while flavanol glycosides range from 27 to 1060 µg/g across varieties (Carvalho et al., 2012). Some cultivars contain anthocyanins (875–3860 µg/g) and flavan-3-ols (2155–6297 µg/g). The flavan-3-ols (tannins) are primarily monomeric catechin-7-O-glucoside, which likely enhances bioavailability and reduces antinutritional effects (Owusu et al., 2022). Phenolic acids are important secondary metabolites and serve as structural cell wall components, biosynthetic intermediates, signalling molecules, and defense agents. These compounds largely determine the diverse

coloration in cowpea seeds, ranging from white, red, cream, bronze, purple, to black (Dueñas et al., 2005; Cuvas et al., 2021).

Table 2: Evidence-based health benefits of cowpea and underlying mechanisms.

Health Effect	**Mechanisms / Supporting Evidence**
Glycemic control	High fibre + slowly digestible/resistant starch reduces postprandial blood sugar. Some studies show improved insulin sensitivity.
Cardiovascular health/Anti-hypertensive effects	Fibre helps reduce LDL; phytochemicals may reduce oxidative stress; low-fat content favours lipid profile. Probably via mineral content (potassium, magnesium), fibre, and possibly bioactive compounds.
Antioxidant/Anti-inflammatory	Phenolics, flavonoids, and tannins in cowpea have shown effects.
Anti-cancer / Cell protection	*In vitro* and model organism results show that extracts/constituents in cowpea can protect against oxidative DNA damage, reduce aggregate formation.
Anti-aging and Neuroprotection	Studies (in model organisms and cell lines) show cowpea extracts may upregulate pathways like AMPK, SIRT1, and FOXO, which are implicated in cellular aging and neuroprotection. Also, protective against α-synuclein toxicity in models of Parkinson's disease.
Nutrition security / alleviating micronutrient deficiencies	As a source of iron, folate, etc., contributing to micronutrient intake. A good source of protein, thus can alleviate malnutrition

AMPK – **AMP-**activated protein kinase**; SIRT1 –** Sirtuin 1**; FOXO –** Forkhead box O

(Antova et al., 2014; Aduol et al., 2020; Rengadu et al., 2020; Abebe & Alemayehu, 2022; Affrifah et al., 2022).

In addition to unique polyphenols, cowpea contains bioactive peptides that have been studied for their health benefits (Niro et al., 2019). These peptides are encrypted within major storage proteins—vicilin (7S globulin), legumin (11S globulin), and β-vignin—and are released

through enzymatic processes such as digestion, fermentation, germination, or hydrolysis (Abebe & Alemayehu, 2022). Though most bioactive peptides are liberated during processing, small amounts of bioactive peptide fragments may exist naturally in raw seeds due to mild endogenous proteolytic activity during seed maturation and storage. Peptides derived from β-vignin exhibit antimicrobial and antioxidant activities, and cowpea seeds contain peptides with angiotensin-converting enzyme (ACE) inhibitory and antioxidant properties (Awika & Duodu, 2017; Banwo et al., 2021).

2.2 Gut microbiota and diet

The human body harbors trillions of microorganisms, particularly within the GIT, which play a pivotal role in regulating metabolism, immunity, and overall physiological function. Although earlier estimates suggested that microbial cells vastly outnumber human cells, current research indicates that microbial and human cells exist in roughly equal numbers. Regardless, maintaining a balanced gut microbiota is essential for sustaining health and preventing disease. Diet is the main contributing element that can influence shape and alter gut composition and function across the lifespan, which in turn impacts our nutritional status and overall health (Shanahan et al., 2021). Gut microbiota plays an important role in the gastrointestinal (GI) system as it supplies essential nutrients, aids in digestion, and promotes a healthy digestive system. Gut microbes play a central role in numerous aspects of human health, including immune regulation, metabolism, and neurobehavioral functions (Bagga et al., 2018). Incorporating plant-based fermented microbial foods into our diets has an enormous potential to radically reduce the environmental footprint (Dimidi et al., 2019).

The concentration of gut microbiota varies significantly along different sections of the GIT due to varying physiological conditions. In the stomach and duodenum, microbial populations are relatively low, with fewer than 10^3 bacteria per gram of content (Shanahan et al., 2021). The predominant microorganisms in this region are *Lactobacilli* and *Streptococci* (Yang et al., 2020*).* The harsh acidic environment, along with bile and pancreatic secretions, suppresses the survival of most ingested microbes. Additionally, phasic propulsive motor activity prevents stable colonization of the lumen in these upper sections (Valero et al., 2020). Moving further along the digestive tract, the jejunum and ileum exhibit a gradual increase in microbial density. The bacterial count rises from approximately 10^4 cells per gram of content in the jejunum to

about 10^7 cells per gram in the distal ileum. This increase corresponds with a reduction in digestive secretions and a slower transit time, allowing for more favourable conditions for microbial growth (Yang et al*.,* 2020; Shanahan et al., 2021). The large intestine, in contrast, is the most densely colonized region, harbouring approximately 10^{12} bacterial cells per gram of luminal contents. This region is predominantly populated by anaerobic microorganisms, which thrive in the low-oxygen environment and play a critical role in fermenting undigested dietary components and maintaining gut health (Ashaolu et al., 2021).

The gut microbiota is also part of the intestinal barrier and contributes to mucosal immunity (Yang et al., 2020). The microbiota and its metabolites largely impact the intestinal barrier integrity, host metabolism, and pathogen colonization (Shanahan et al., 2021). In an adult GIT, the microbiota consists of more than 1000 different bacterial species, some of the most abundant are: *Bifidobacterium, Eubacterium, Clostridium, Peptococcus, Firmicutes, Peptostreptococcus, Ruminococcus, Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus, Proteus, proteobacteria, Verrucomicrobia, fusobacteria, cyanobacteria and actinobacteria* (Fan et al., 2015).

Our diet provides not only the nutrients we need but also the material and medium for the sustenance and growth of gut bacteria. The composition of the diet will inevitably affect the selective growth of types of bacteria in the gut (Dahiya & Nigam, 2022). Understanding the specific nutritional mechanisms through which legumes exert their health benefits is key to advancing dietary nutrition. Growing evidence indicates that gut microbiota are central to these effects through their intricate interactions with legumes. Firstly, legumes influence gut microbial composition and activity; secondly, the microbiota transform legume components into bioactive compounds with distinct physiological roles; and thirdly, they mediate interactions among various legume constituents. Hence, exploring the gut microbiota–legume relationship is essential for elucidating the nutritional mechanisms of legumes in future research. Bioactive ingredients, such as polyphenols present in legumes, can modulate microbiota patterns by promoting the growth of beneficial bacteria and inhibiting the reproduction of pathogenic bacteria, exerting anti-inflammatory activity and neuroprotective effects (Clemente & Olias et al., 2017; Leeming et al., 2019). Any disruption of the gut microbial ecosystem triggered by various factors, including poor dietary patterns, antibiotic use, chronic stress, infection, and environmental pollutants, can result in dysbiosis (Leeming et al., 2019; Song et al., 2023).

2.2.1 Manipulating the gut microbiota through diet

Gut microbiota can shift within days of dietary change; notable differences were seen after African Americans and rural Africans swapped diets for two weeks (Keulers et al., 2022). In African Americans, the rural diet increased butyrate-producing bacteria, boosting butyrate levels 2.5-fold and lowering secondary bile acid synthesis (Kumar et al., 2022). Another study examining extreme dietary shifts between plant and animal-based proteins reported significant changes in the gut microbiota after only five days (Leeming et al., 2019). But healthy microbiota is resilient to temporal changes by dietary interventions, meaning that homeostatic reactions restore the original community composition, as recently shown in the case of bread (Dimid et al., 2019). A balanced plant-based diet rich in prebiotics and polyphenols fosters a healthy microbiome, suppressing pathogens, improving intestinal barrier function, and enhancing immune tolerance (Elango et al., 2022).

2.3 Prebiotics: An evolution that cannot be stopped

Food today serves not only to meet basic nutritional needs but also to promote health, leading to the emergence of the concept of functional foods. In South Africa, the prevalence of immunocompromised individuals correlates with high rates of gastrointestinal infections (Dimidi et al., 2019), which has driven increasing consumer interest in functional foods. These foods provide health benefits beyond basic nutrition and may help reduce the risk of chronic conditions such as inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease (Mysonhimer et al., 2022). Among functional food components, prebiotics play a key role in modulating host health.

The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines prebiotics as "selectively fermented ingredients that induce specific changes in the composition and/or activity of the gastrointestinal microbiota, thereby conferring health benefits to the host" (Gibson et al., 2017). While all prebiotics are fermentable dietary fibers or microbiota-accessible carbohydrates (MACs), not all such carbohydrates qualify as prebiotics. Prebiotics are non-digestible food components that selectively stimulate the growth of beneficial gut bacteria while inhibiting harmful microbial activities (Naik & Kerkar, 2024). They resist

enzymatic digestion and acidic gastric conditions, reach the colon intact, and are fermented by gut microbes to generate beneficial metabolites (Dahiya & Nigam, 2022).

The impact of MACs on gut microbiome composition can be profound, enriching specific species that may constitute significant portions of the fecal microbiota. To be classified as prebiotics, substances must meet certain criteria validated by *in vitro* and *in vivo* studies, namely, resistance to digestion and absorption, fermentation by intestinal microbiota, and selective stimulation of beneficial bacteria without degradation during food processing (Peng et al., 2020).

The first recognized prebiotic source was human breast milk, particularly human milk oligosaccharides (HMOs), which selectively nourish Bifidobacterium infantis despite being indigestible by infants (Gibson et al., 2017). Prebiotics have been shown to enhance the absorption of minerals such as calcium, magnesium, zinc, and iron. Additionally, prebiotic substrates synergize with probiotic cultures to improve the efficacy of probiotic formulations (Davani-Davari et al., 2019).

Diets rich in prebiotics increase gut microbial diversity and influence microbial metabolism, promoting the production of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate as well as branched-chain fatty acids (BCFAs), peptides, organic acids, ammonia, phenolic compounds, amines, and gases. Moreover, prebiotics serve as protective agents in probiotic encapsulation, enhancing bacterial viability (Naik & Kerkar, 2024). Observational and intervention studies consistently demonstrate the health benefits of prebiotics, including reduced insulin resistance in T2D, correlated with favorable microbiota shifts and increased beneficial metabolites (Slavin et al., 2013).

Legume starches, characterized by high amylose content, are notable sources of resistant starch, a recognized prebiotic carbohydrate. Resistant starch escapes digestion by human enzymes and acts as a substrate for beneficial colonic bacteria, promoting SCFA production and gas release (hydrogen and carbon dioxide) through fermentation (Kavita et al., 2015; Keulers et al., 2022). Rengadu et al. (2020) reported resistant starch levels ranging from 9.3 % to 12.1 % in cowpea varieties, supporting their prebiotic potential by fostering the growth of beneficial bacteria.

Conventional fibers like pectin and cellulose lack selective fermentability, whereas certain oligosaccharides such as fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides

(GOS), lactitol, lactulose, lactosucrose, isomalto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides (XOS), gentio-oligosaccharides, transgalactooligosaccharides, and polydextrose found in legumes, are effective prebiotics due to their selective metabolism by gut bacteria (Elango et al., 2022).

In a study examining gut microbiota modulation by short-chain fructo-oligosaccharides in human microbiota-associated gnotobiotic mice, FOS consumption increased the relative abundance of beneficial bacteria while reducing taxa from the family Sutterella (Bell et al., 2018). The positive impact of FOS on butyrate-producing bacteria and its ability to increase microbial diversity further reinforces the role of prebiotics in conferring beneficial functions to the host (Leeming et al., 2019).

Low fiber intake diminishes SCFA production and shifts microbial metabolism toward less favorable substrates, resulting in potentially harmful metabolites. This depletion is linked to the degradation of the colonic mucus barrier, increased pathogen susceptibility, and inflammatory mechanisms associated with Western diets and chronic diseases (Song et al., 2023). Supplementation with inulin and other prebiotics mitigates these effects, preserving mucus barrier integrity and improving metabolic functions (Slavin et al., 2013).

2.4 Probiotics: Beneficial group of microbes

Man and Microbes have an evolutionary relationship, and microbes are our living partners since the beginning of life on this earth. Science is making us realise the boundless potential of microbes. Probiotics are a beneficial group of microbes that modulate our gut population on the positive side and keep us healthy. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Gibson et al*.,* 2017). Probiotics (mostly *Bifidobacterium* and *Lactobacillus* spp.) can be included in a variety of produ cts, including foods, dietary supplements, or drugs.

Though the health benefits of probiotics are traditionally associated with the ingestion of live microbial cultures, several studies have demonstrated that non-viable or inactivated cells can also exert beneficial physiological effects (Ashaolu et al., 2020; Bagga et al., 2018; Bilal et al., 2022). Emerging areas in probiotic research include the use of novel microbial strains and combinations, the development of synbiotic formulations that integrate probiotics with complementary prebiotics, and personalized probiotic strategies tailored to individual microbial

and metabolic profiles associated with inflammation, cancer, lipid metabolism, and obesity (Dahiya & Nigam, 2022).

Stable engraftment of a probiotic *Bifidobacterium longum*, for example, has been shown to depe nd on individualised features of the gut microbiota, providing a rationale for the personalisation of probiotic applications (Kavita et al., 2015).

Probiotics as a concept acquired scientific credibility when Russian scientist Elie Metchnikoff proposed the theory of longevity and attributed the long life of Bulgarians to the consumption of fermented products (Kumar et al., 2022). The discovery of *Lactobacillus casei shirota* by a Japanese scientist, M. Shirota, led to the development of a very popular Japanese dairy-based drink, 'Yakult', for better digestibility and effective containment of gastric disorders, for which the probiotic concept gained further momentum (Keulers et al., 2022). Since then, new research initiatives on establishing the functional and therapeutic efficacy of these bacteria have steadily increased.

2.4.1 Health benefits of probiotics

The scope of probiotic research has expanded beyond traditional microbiology to include disciplines such as food technology, nutrition, and therapeutic sciences. Health benefits from probiotics are achieved through the consumption of foods and supplements formulated with beneficial microorganisms. When ingested in sufficient quantities, probiotics confer a wide range of health advantages to the host. However, these effects are strain-specific and cannot be universally applied to all species or groups (Kumar et al., 2012; Gopikrishna et al., 2021). The human gastrointestinal tract (GIT) harbors nearly 100 trillion microorganisms that are essential for maintaining gut health. This complex microbiota contributes to nutrient absorption, immune defense, infection resistance, and metabolic regulation (Marco et al., 2017). Probiotics complement these functions by reinforcing mucosal barrier integrity, preventing colonization by pathogens, and modulating metabolic and immune activities (Shokryazdan et al., 2014; Diez et al., 2020). They stimulate intestinal epithelial cells to release protective mucins and influence immune signalling pathways through regulatory molecules, microRNAs, and immunomodulatory compounds (Yoha et al., 2022).

Additionally, probiotics improve lactose metabolism, exhibit anti-mutagenic and anti-carcinogenic properties, and reduce the duration and severity of infections. Their ability to

compete with pathogens for nutrients and adhesion sites, enhance mucus secretion, and reinforce epithelial barrier function collectively support intestinal and systemic immune health (Marco et al., 2017).

Therapeutic doses of probiotics are typically established at 10^6–10^7 CFU/g in the final food product, with daily intakes of 10^8–10^9 CFU via 100 g or 100 mL of probiotic-rich foods considered beneficial for human health (Feng et al., 2018). Probiotics have proven effective in managing antibiotic-associated diarrhea (AAD) through microbiota restoration. They have also shown efficacy in suppressing Helicobacter pylori colonization, managing relapses of inflammatory bowel disease (IBD), and reducing certain cancer risks via secretion of antioxidant and anticarcinogenic metabolites. Furthermore, probiotics contribute to oral health by aiding in the management of periodontitis and candidiasis (Naik & Kerkar, 2024).

Through enzymatic activities such as bile salt hydrolase, probiotics detoxify harmful substances and enhance nutrient bioavailability (Shehata et al., 2016; Berebon et al., 2019). Their metabolic activities are associated with improved mineral absorption (iron, calcium, magnesium) and enhanced vitamin D synthesis and uptake (Berebon et al., 2019; Homsuwan, 2023). Beyond gastrointestinal benefits, probiotics influence various physiological systems. They participate in gut–brain axis signalling, contributing to the management of neuropsychiatric and stress-related disorders (Amritha et al., 2018; Yoha et al., 2022). Probiotic interventions have also been linked to reduced serum LDL levels, improved vaginal and urinary tract health, prevention of pancreatitis, enhanced respiratory function, and inhibition of tumorigenesis. Moreover, probiotics help maintain metabolic homeostasis and support the management of diabetes, cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD) (Berebon et al., 2019; Khan et al., 2023).

In addition to their direct physiological effects, probiotics produce postbiotics, soluble bioactive compounds generated during microbial metabolism. These include short-chain fatty acids (SCFAs), vitamins (B_2, K_2), enzymes, lactic acid, bacteriocins, ketones, methyl acetates, carbon disulfides, and antimicrobial peptides that inhibit pathogenic bacteria through non-specific mechanisms (Clement et al., 2018; Tomasik & Tomasik, 2020).

2.4.2 Strains, sources, and reservoirs of probiotic microorganisms

2.4.2.1 Strains and sources

Lactobacillus, Lactococcus, Bacillus, Streptococcus, Bifidobacterium, Pediococcus, and Propionibacterium are familiar probiotic species, with *Lactobacillus and Bifidobacterium* being the most common and known to be inhabitants of the human GIT and are generally regarded as safe (GRAS) (Table 3). Most commonly used probiotic strains are *Bifidobacterium, Lactobacilli, S. boulardii, and B. coagulans* (Kavita et al, 2015; Gopikrishna et al., 2021). Yeast probiotics include *Saccharomyces carisbergensis, S. cerevisiae,* and *S. boulardii,* and *Aspergillus niger* and *A. oryzae* are regarded as fungal probiotics (Elshaghabee et al.,2017; Naik & Kerkar, 2024). Beneficial microbes produce a variety of exoenzymes such as amylase, protease, and lipase, which help to degrade the undigested food material in the GIT.

Table 3: Summary of the common probiotic bacteria used in industry**.**

Lactobacillus **species**	***Bifidobacterium*** **species**	**Other Lactic acid bacteria**	***Bacillus*** **species**	**Other bacteria**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*B. cereus*	*Clostridium butyricum*
L. casei	*B. animalis*	*E. faecium*	*B. clausii*	*E. coli*
L. 'caucasicus' = L. kefiri	*B. animalis subsp. lactis*	*Lactococcus lactis*	*B. coagulans*	*Propionibacterium*
L. crispatus	*B. bifidum*	*Leuconostoc*	*B. licheniformis*	*freudenreichii*
L. delbrueckii subsp. delbrueckii	*B. breve*	*mesenteroides*	*B. mesentericus*	*Saccharomyces cerevisiae subsp. cerevisiae*
L. delbrueckii subsp. bulgaricus	*B. longum subsp. infantis*	*Pediococcus*	*B. subtillis*	*Saccharomyces cerevisiae subsp. boulardii*

L. delbrueckii subsp. lactis	*B. longum subsp. longum*	*acidilactici*		
L. helveticus	*B. coagulans*	*P. pentosaceus*		
L. fermentum, *L. gallinarum* *L. gasseri* *L. johnsonii* *L. leichmanii = L. delbrueckii subsp. lactis* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus* *L. sakei* *L. salivarius*		*Streptococcus salivarius* *S. macedonicus* *S. mitis* *S. sanguis* *S. thermophilus*		

(Marco et al*.,* 2017; Tomasik & Tomasik, 2020; Malmo et al*.,* 2021)

2.4.2.2 Salt pan-derived microbes as potential probiotics

Probiotics, the beneficial microbes, are widely distributed across various natural and industrial sources, each contributing distinct strains with unique probiotic properties. The probiotics most commonly used for human applications originate from the human digestive tract (e.g., *Lactobacillus gasseri and L. reuteri*) (Fontana et al., 2013) or from fermented dairy products. The adult human gut harbors more than 500 different bacterial species. *L. fermentum*, isolated from human colonic mucosal biopsy samples, has demonstrated antimicrobial activity against several food-borne pathogens (Fontana et al., 2013; Ashaolu, 2020).

Probiotics are also isolated from a wide range of other sources, including the feces of breast-fed infants, human breast milk, non-dairy fermented plant-based foods and beverages, non-dairy and non-fermented foods, products of animal origin, and environmental niches such as

soil, water, and salt pans (Table 1.7). Probiotic strains are delivered not only through foods but also via dietary supplements in the form of capsules, tablets, powders, or functional beverages (Gobetti et al., 2019). With ongoing advances in microbial ecology and biotechnology, new genera and strains of probiotics continue to be identified, broadening their potential applications in human and animal health. For richer or poorer, in sickness and in health, our relationship with the microbiota that inhabit the human body lasts a lifetime. Microbial communities in salt pans are dominated by members of the domains Bacteria and Archaea, with halophilic bacteria being particularly abundant. Among these, genera such as *Halomonas* and *Salinivibrio* are commonly found, while lactic acid bacteria (LAB), *Bacillus* spp., and Actinobacteria are frequently isolated and studied for their functional and biotechnological properties (Kamat & Kerkar, 2011; Das et al., 2019).

Salt pans are rich reservoirs of halophilic and halotolerant microorganisms with significant probiotic potential. Their ability to withstand extreme environmental conditions translates into advantages for both industrial applications and gut health management (Pawaskar et al., 2022; Gawas & Kerkar, 2024). Continued exploration of salt-pan microbiota can contribute to the development of next-generation probiotics (NGPs) and support the conservation of these ecologically and economically important habitats (Selvarajan et al., 2017; Das et al., 2019).

Goan salt pans in India have been the focus of several studies, revealing the presence of *B. coagulans*, *B. subtilis*, and *B. licheniformis*, which are known for their spore-forming ability and probiotic potential. LAB such as *L. plantarum* and *Enterococcus faecium* have also been isolated. These strains often exhibit acid and bile salt tolerance, antimicrobial activity, and enzymatic capabilities—key functional criteria for probiotics (Kamat & Kerkar, 2011; Fernandes et al., 2019). The advantages of salt-pan microbes include (Das et al., 2019; Fernandes et al., 2019; Gawas & Kerkar, 2023):

• Environmental stress resistance: These microbes are naturally adapted to harsh conditions, enhancing their resilience during processing, storage, and gastrointestinal transit.
• Spore formation (in *Bacillus* species): Ensures longer shelf life and viability in diverse delivery systems (capsules, powders, food matrices).

• Untapped biodiversity: Salt pans represent relatively unexplored ecosystems with potential for discovering novel strains possessing unique metabolic and health-promoting traits.

2.4.3 Efficacy and safety evaluation of probiotics

Probiotics are experiencing renewed interest and demand, supported by a long history of safe use in fermented dairy foods and growing scientific evidence of their health benefits, particularly for the human gastrointestinal system. For a microorganism to be considered an effective probiotic, it must exhibit a defined set of functional characteristics. Key selection criteria include the ability to withstand harsh gastrointestinal conditions, such as exposure to gastric acid and bile salts, adherence to the intestinal mucosal surfaces, and the capacity to competitively exclude pathogenic microorganisms (Homsuwan et al., 2023). The ICMR-DBT (Indian Council of Medical Research - Department of Biotechnology, 2011) has proposed that a viable probiotic candidate should meet the following essential criteria (Ganguly et al., 2011; Somplang & Piyadeatsoontorn, 2016) as described in Table 4:

Table 4: ICMR–DBT guidelines for evaluation of probiotics in food (India).

S. No.	**Evaluation Criteria**	**Description**	**Mandatory for initial screening**
1	Strain identification	Identify the probiotic at the genus, species, and strain level using validated molecular and phenotypic methods	✓
2	Strain deposition	Strains must be deposited in a recognized culture collection with a valid accession number.	-
3	*In vitro* characterization	Assess for: Resistance to gastric acid and bile Antimicrobial activity Adhesion to intestinal mucosa Bile salt hydrolase activity	✓
4	*In vivo* safety (animal studies)	Conduct toxicity studies (acute, sub-chronic, chronic); monitor for pathogenicity and side effects in animal models.	-
5	Antibiotic resistance profile	Must not contain transferable antibiotic resistance genes.	✓

6	Toxin and Haemolysis testing	Screen for absence of toxin production and hemolytic activity, especially for species with known pathogenic strains.	✓
7	*In vivo* efficacy (animal studies)	Demonstrate functional benefit in appropriate animal models before human trials.	-
8	Human trials	Must show clinically and statistically significant benefits in controlled human studies; required for health claims.	-
9	Effective dose	Clearly define minimum effective dose (CFU/day) to confer health benefits; must be present at end of shelf life.	-
10	Labelling requirements	Label must include: Genus, species, strain – CFU at end of shelf life, recommended dose, storage conditions, health claims	-
11	Manufacturing standards	Must comply with good manufacturing practices (GMP) and Codex General Principles of Food Hygiene.	-
12	Quality control	Implement Hazard Analysis and Critical Control Point (HACCP) protocols for probiotic product safety and consistency.	-

2.4.4 Administration of probiotics

Effective intentional probiotic administration relies on delivering the right strain, dosage in sufficient numbers, and delivery via a stable and targeted method, at the appropriate time. Continued research is helping to refine strategies for strain selection, delivery systems, and personalization of probiotic therapies (Mukherjee et al., 2022). The therapeutic dose of probiotics is generally considered to be 10^8–10^9 CFU per day. To be effective, the probiotic organisms must remain viable and active until they reach the target site, usually the intestine (Gibson et al., 2017; Mathews, 2017). Stability during processing, storage, and passage

through the acidic gastric environment is essential for viability (Pang et al., 2020). Probiotics are often more effective when taken with or just before meals, which helps buffer stomach acid and improves survival (Yoha et al.,2022). There are numerous delivery modes through which probiotics are administered and they include (Golnari et al., 2024):

a. **Food-based delivery systems**

- Fermented Foods: Yogurt, kefir, buttermilk, cheese, sauerkraut, kimchi, miso, kombucha, idli, dosa batter.
- Non-Fermented Functional Foods/ Fortified foods containing added probiotic cultures: Fruit juices, cereal bars, chocolate, ice cream, infant formulas, smoothies.
- Dairy-Based Products: Milk, yogurt, cheese, curd.
- Plant-Based Matrices: Soy milk, oat milk, coconut milk, cereal-based beverages, and legume-based products.

b. **Pharmaceutical / Supplement forms**

- Capsules and Tablets, Powders and Sachets, Lozenges and Chewable Tablets

c. **Using encapsulation and advanced delivery systems**

- Microencapsulation, Nanoencapsulation, Liposome or Emulsion-Based Systems

d**. Specialized medical and clinical forms**

- Enteric-Coated Capsules
- Probiotic-Enriched Infant Formula

e. Fecal microbial transplantation (FMT)

FMT is the term used to describe the delivery of stool or fecal microbiota from a healthy donor into a patient, either by enema, colonoscopy, or via the upper gastrointestinal tract (oral capsules, nasogastric, nasoduodenal or nasoenteric tube, or endoscopy) (Bezkorovainy, 2001). Transmission of fecal microflora from healthy donors to patients to restore the healthy microbial composition of the gut is done. They can be co-administered with antibiotics a few hours apart from antibiotics to avoid microbial destruction or with prebiotics (as synbiotics), which can enhance their efficacy by promoting their growth and activity (Duche et al.,2023). Some commercially available probiotic products are presented in Table 5.

Table 5: Some commercially available probiotic products.

Brand	Food type and probiotic	Manufacturer
Actimel	Yogurt drink with *L. casei Imunitass* as probiotic	Danone, France
Activia	Creamy yogurt having *Bifidus actiregularis*	Danone, France
Aciforce	Freeze-dried items with *Lactococcus lactis, L. acidophilus, E. faecium, Bifidobacterium bifidum*	Bioherma, Netherlands
Bacilac	Freeze-dried product with *L. acidophilus, L. rhamnosus*	THT, Belgium
Bactisubtil	Freeze-dried product with Bacillus sp. strain IP5832	Synthelabo, Belgium
Bififlor	Freeze-dried items with *L. acidophilus, L. rhamnosus, Bifidobacterium bifidum*	Eko-Bio, The Netherlands
Gefilus	An extensive range of LGG products	Valio, Finland
Helus	Dairy products containing *L. fermentum* ME-3	Tallinna Piimatoööstuse AS, Estonia
Jovita Probiotisch	Cereals, fruit and probiotic yogurt blend	HandJ Bruggen, Germany
Pohadka	Pohadka Yogurt milk with probiotic cultures	Valašské Mezirˇícˇí Dairy, CzechRepublic
Proflora	Freeze-dried product with *L. acidophilus, L. delbrueckii subsp. bulgaricus, Streptococcus thermophilus, Bifidobacterium*	Che faro, Belgium
Provie	Fruit drink with *L. plantarum*	Skånemejerier, Sweden
ProViva	Refreshing natural fruit drink and yogurt in many different flavors containing *L. plantarum*	Skånemejerier, Sweden

Rela	Yogurts, cultured milks and juices with *L. reuteri*	Ingman Foods, Finland
Revital Active	Yogurt and drink yogurt with probiotics	Olma, Czech Republic
Snack Fibra	Snacks and bars having natural fibers, extra vitamins, and minerals	Celigüeta, Spain
SOYosa	Soy and oat-based variety products like a refreshing drink and a probiotic soy oat yogurt	Bioferme, Finland
Soytreat	Kefir-like product with six probiotics	Lifeway, USA
Yakult	Milk drink containing	Yakult, Japan
Yosa	Yogurt-like oat product flavored with natural fruits and berries containing probiotic bacteria (*L. acidophilus, B. lactis*)	Bioferme, Finland
Vifit	Yogurt drink with LGG, vitamins, and minerals	Campina, Netherlands
Vitamel	Dairy products (*L. casei GG, L. acidophilus, B. bifidum*)	Campina, Netherlands
Vitality	Yogurt with pre- and probiotics and omega-3	Müller, Germany

Source: Siro et al. (2008); Kolacek et al. (2017)

2.4.5 Regulation of probiotics: need for global harmonization

The regulatory criteria for probiotics vary considerably between countries. In the absence of harmonized international guidelines for probiotic foods, there is significant scope for the marketing of products with unsubstantiated or misleading claims. It is therefore imperative that probiotic products fulfil essential prerequisite conditions before being labelled as "probiotic". Currently, probiotics are regulated under different categories across the globe, such as foods, dietary supplements, drugs, or medical foods, depending on national frameworks. These categorizations are country-specific, resulting in regulatory ambiguity and posing challenges for the probiotic industry in navigating a heterogeneous landscape.

While the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) jointly define probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host," the regulatory implementation of this definition differs. The European Food Safety Authority (EFSA) follows a more restrictive approach and does not currently permit the use of the term "probiotic" in product labelling (Kumar et al., 2019). According to EFSA, the term implies a health claim, and since no probiotic health claims have been approved to date, the use of such terminology is prohibited (Mejía-Caballero et al., 2021).

In contrast, some national authorities, such as those in Italy and Switzerland, have adopted more flexible regulatory frameworks that allow the use of "probiotic" under defined conditions, such as demonstrated viability and strain-level identification (Mukherjee et al., 2022). These discrepancies underscore the pressing need for global harmonization of probiotic regulations to ensure product authenticity, consumer safety, and scientific credibility in health claims.

2.4.5.1 Regulations and regulatory trends on probiotics

In India, the Food Safety and Standards Authority of India (FSSAI) regulates probiotics under the category of "Foods with added probiotic ingredients." Regulations specify that the strains used must be capable of surviving passage through the digestive tract, adhering to and proliferating in the gut, and conferring physiological benefits.

The global regulatory landscape for probiotics remains heterogeneous, highlighting the need for regulatory harmonization. Researchers aim to generate high-quality, evidence-based data, while industry seeks to develop safe, effective, and commercially viable products with substantiated, consumer-relevant claims (Mukherjee et al., 2022). Regulators safeguard public health by ensuring the accuracy of product information and preventing misleading claims, and consumers require reliable information to make informed decisions (Arora and Baldi, 2017). The rapid pace of probiotic research has outstripped regulatory adaptation, making collaboration among scientists, industry, and regulators essential to develop best practices and science-driven, harmonized regulations that ensure compliance and clear communication of complex scientific information to consumers (Garg et al., 2024; Prajapati, 2015).

2.5 Synbiotics and their modes of action

A synbiotic or optibiotics is a combination of probiotics and prebiotics that improves the survival and implantation of live helpful microbes in the gut, hence benefiting the host. Synbiotics are food ingredients or dietary supplements that synergistically combine probiotics and prebiotics, thus the name (Bilal et al.,2022; Dahiya & Nigam et al., 2022). Synbiotics can be prepared by the use of several raw materials like milk, dairy products, cereals, legumes, fruits, vegetables, meat, fish, or a blend of several items. Additionally, several commercially available prebiotic ingredients are also incorporated in the development of synbiotic foods. The individual effect of the probiotic microorganisms and prebiotics can be increased by using them together and synbiotic effects occur (Gibson et al., 2017; Brown et al., 2018; Kumar et al., 2022).

In addition, the use of encapsulation is an effective approach to improve the stability of probiotics through harsh conditions (Malmo et al., 2021). Foods are carriers for the delivery of probiotics to the human body, and the appropriate combination of prebiotics and probiotics manifests a higher potential for a synergistic effect (Gharibzahadi & Smith, 2021). Complementary synbiotics are those in which each component is chosen independently for its potential effect on host health, or synergistic synbiotics, in which the prebiotic component is chosen to assist the function of the probiotic component (Fang et al., 2018).

The first aspect to be taken into account when composing a synbiotic formula should be the selection of an appropriate probiotic and prebiotic, exerting a positive effect on the host's health when used separately (Yang et al., 2022). The determination of specific properties to be possessed by a prebiotic to have a favorable effect on the probiotic seems to be the most appropriate approach (Gibson, 2017; Kumar et al., 2021). A combination of *Bifidobacterium* or *Lactobacillus* genus bacteria with FOS seems to be the most popular synbiotic product (Langa et al., 2019). Table 6 indicates some examples of synbiotics used in human nutrition.

Two modes of synbiotic action are action through the improved viability of probiotic microorganisms and action through the provision of specific health effects (Kumar et al., 2022). Synbiotic consumption has been associated with a reduction in harmful metabolites, as well as the detoxification of nitrosamines and carcinogenic compounds (Kavita et al., 2015).

Additionally, synbiotics contribute to elevated levels of SCFAs, ketones, carbon disulfides, and methyl acetates, all of which are believed to exert positive health effects on the host (Kumar et al., 2022). Table 7 indicates synbiotic products marketed in India**.**

Table 6: Synbiotics commonly used in human nutrition.

Sr. No	**Synbiotics**
1	*Lactobacillus* genus bacteria + inulin
2	*Lactobacillus, Streptococcus* and *Bifidobacterium* genus bacteria + FOS
3	*Lactobacillus, Bifidobacterium, Enterococcus* genus bacteria + FOS
4	*Lactobacillus* and *Bifidobacterium* genus bacteria + oligofructose
5	*Lactobacillus* and *Bifidobacterium* genus bacteria + inulin
6	*Lactobacillus* genus bacteria + inulin
7	*Lactobacillus, Streptococcus* and *Bifidobacterium* genus bacteria + FOS
8	*Lactobacillus, Bifidobacterium, Enterococcus* genus bacteria + FOS
9	*Lactobacillus* and *Bifidobacterium* genus bacteria + oligofructose
10	*Lactobacillus* and *Bifidobacterium* genus bacteria + inulin
11	Lactobacillus genus bacteria + inulin

(Markowiak and liżewska, 2017)

From a therapeutic perspective, synbiotics have demonstrated antibacterial, anticarcinogenic, and anti-allergic properties. They may also mitigate intestinal putrefaction, help prevent constipation and diarrhea, and support gut barrier function. Emerging evidence suggests their potential efficacy in the prevention of osteoporosis, reduction of blood lipid and glucose levels, modulation of immune responses, and even the management of neurological disorders linked to hepatic dysfunction (Bilal et al., 2022; Keulers et al., 2022).

Table 7: Synbiotic products marketed in India**.**

Manufacturers	**Strain**	**Brand**
Food		
Nestle	*L. acidiphilus* NCFM	Nesvita/ Actiplus probiotic curd
Mother dairy	*Bifidobacterium. lactis* BB12 and LA5	Nutrifit probiotic drink
Mother dairy	*Bifidobacterium lactis* BB12	b-activ probiotic curd
YaKULT Danone India	*L. casei* Shirota	Probiotic drink
Amul	*Bifidobacterium lactis* BB12	Prolife ice cream, curd and lassi
	Bifidobacterium lactis BB12	Flaavyo-flavoured probiotic yogurt
Heritage foods	*L.rhamnosus, L. acidophilus* and *Bifidobacterium* + milk.	Probiotic curd
Supplement		
Seven Turns Private Limited	*L.crispatus, L. gasseri, L. jensenii, L. rhamnosus GG* plus prebiotic (Inulin), D-mannose, Cranberry extract, Vitamin C, Zinc gluconate	The Good Bug – Good Down There Synbiotic Supergut Powder
ZeroHarm Sciences Pvt. Ltd.	11 strains, including *L. plantarum, Bifidobacterium lactis, L. acidophilus, Bifidobacterium longum, Streptococcus thermophilus, L. gasseri, Bacillus coagulans, Saccharomyces boulardii, Bifidobacterium breve, L. rhamnosus, Bacillus clausii + prebiotic components (Psyllium husk, peppermint extract etc.)*	Gut Army Prebiotic + Probiotic Capsule (Synbiotic, 25 strains)
RychBiome	Blend of 7 probiotic species + 1	RychBiome Indus

(India)	prebiotic	
Natural Factors via Maple Herbs (USA brand available in India)	10 strains: *L. acidophilus, Lacticaseibacillus casei, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum, Bifidobacterium lactis, L. salivarius, Streptococcus thermophilus, Bifidobacterium longum,* Bifidobacterium bifidum, L. bulgaricus + fermented whole-food prebiotic matrix (apple, blueberry, etc.) including inulin	Natural Factors – Whole Earth and Sea Synbiotic Pre+Probiotic
Pharmaceuticals		
Tablet India Ltd.	*L. rhamnosus* Gr-1 and *L. reuteri* RC-14	Eco flora
Tablet India Ltd.	*Streptococcus faecalis* T-110, *Clostridium butyricu*m TO-A, *Bacillus mesentericus* TO-A, *Lactobacillus sporogens*	Bifilac Bifilac-hp Bio 4
Glenmark	*L. rhamnous* Gr-1 and *L. reuteri* RC-14	Econova
Dr. Reddy's Laboratories	*Streptococcus faecalis* *Clostridium butyricu*m *Bacillus mesentericus* JPC *Lactobacillus*	Becelac PB
Unichem	*L. sporogens*	Vizyl
J B Chem	*Streptococcus faecalis*, *Clostridium butyricu*m, *Bacillus mesentericus, L. sporogens*	Gutpro
Glaxo Smithline	*Saccharomyces boulardii*	Stibs

(Kumar et al., 2022).

2.6 Legumes as matrices for probiotic delivery

The development of functional foods incorporating probiotics has traditionally centered around dairy-based matrices such as yogurt, kefir, cheese, and fermented milk. However, growing awareness of lactose intolerance, milk allergies, veganism, and the demand for plant-based alternatives has led to a substantial shift toward non-dairy food matrices for probiotic delivery. This transition aligns with broader public health recommendations encouraging plant-based diets, particularly in response to the global rise in NCDs, especially in low- and middle-income countries (Owusu et al*.,* 2022). Among non-dairy carriers, legumes, including chickpea, lentil, mung bean, and soybean, have gained attention as promising substrates for probiotic incorporation (Clement & Olias, 2017). Table 8 indicates the legumes of importance in Indian diets. Their appeal lies in their high content of fermentable carbohydrates, plant proteins, resistant starches, GOS, RFOs, various phytochemicals, and dietary fiber, which exhibit prebiotic properties and are ideal carriers for probiotics (Oghbaei et al*.,* 2016; Verni et al*.,* 2022).

In addition to nutritional benefits, legumes possess structural features such as complex plant cell matrices and surface ridges that can protect probiotics from harsh GIT conditions, including stomach acidity and bile salts (Omodara et al*.,* 2018). These properties make legumes dual-function matrices, serving both as nutrient-rich growth media and as protective delivery vehicles for probiotics (Onyenekwe et al*.,* 2000; Santivarangkna, 2016). Recent research has highlighted legume sprouts as particularly effective probiotic carriers. Germination enhances the bioavailability of nutrients and reduces ANFs, thereby improving the overall functional potential of the legume matrix (Panghal et al*.,* 2018). For instance, a probiotic drink developed from sprouted green gram supported *L. acidophilus* viability up to 10^{10}–10^{11} CFU/mL after only 8 hours of fermentation. Similarly, lentil and adzuki bean sprouts have been shown to support *Saccharomyces cerevisiae* var. *boulardii* growth above 10^{7} CFU/g, while also providing protective effects during *in vitro* gastrointestinal digestion (Nkhata et al*.,* 2018). In another study, chickpea sprouts fermented with *L. casei* inhibited the growth of pathogenic microorganisms, enhancing product safety (Li & Wang, 2021). Additionally, *Lactobacillus plantarum* improved starch digestibility in lentil and mung bean sprouts. These findings suggest that legume-based matrices can simultaneously deliver live probiotics and contribute to improved nutritional and functional characteristics (Kaur et al*.,* 2014).

Table 8: Legumes of importance in Indian diets.

English name	**Latin name**	**Local name**
Black gram	*Vigna mungo*	Urid
Chickpea, Bengal gram	*Cicer arietinum*	Chana
Cowpea	*Vigna unguiculata*	Alsande
Grasspea	*Lathyrus sativus*	Khesari
Green gram	*Vigna radiata*	Mung
Horsegram	*Dolichos uniflorus*	Kulith
Hyacinth bean	*Lablab purpureus*	Vaal
Lentil	*Lens culinaris*	Masur
Moth bean	*Vigna aconitifolia*	Matki
Pea	*Pisum sativum*	Matar
Pigeon pea, red gram	*Cajanus cajan*	Tur
Soyabean	*Glycine max*	Soyabean

(Indian Food Composition Tables (IFCT), 2017).

To confer health benefits, probiotic foods should contain a minimum of 10^6–10^7 CFU/g or mL of viable probiotic cells at the point of consumption, though effective daily doses may vary depending on the strain and the health outcome targeted (Elshaghabee et al., 2017). Achieving this target in legume-based systems poses several challenges, including variations in pH, oxygen availability, and nutrient profiles (Mathew, 2017). To address these issues, researchers have employed a variety of technological strategies such as microencapsulation using biopolymers (e.g., alginate, pectin); immobilization techniques; use of protective additives (osmo-, thermo, or cryo-protectants) during drying and storage, and strain selection to ensure that probiotics remain viable through processing, storage, and digestion (Mathews 2017; Behera et al*.,* 2018; Malmo et al*.,* 2021).

Legume-based probiotic products such as fermented pulse beverages, plant-based yogurts, and legume snacks are increasingly being commercialized (Adebo et al*.,* 2022), and the use of legumes as matrices for probiotic delivery represents a promising avenue for both nutrition-sensitive innovation and sustainable food product development. Their integration into probiotic

food systems supports the creation of accessible, functional, and culturally adaptable health-promoting products.

2.7 Processing of legumes: A boon to human nutrition

Food legumes are an important part of diets worldwide due to their high nutritional value, particularly their rich protein, fiber, and micronutrient content, and also for their potential therapeutic roles in managing lifestyle-related conditions such as diabetes, CVD, and certain cancers (Abbas & Ahmad, 2018). However, legumes in their raw form also contain various ANFs, as discussed earlier, that can interfere with nutrient absorption and digestion. To maximize their health benefits, these compounds must be minimized or eliminated through appropriate processing techniques such as de-hulling, milling, soaking, sprouting, boiling, microwave cooking, extrusion, autoclaving, irradiation, fermentation, supplementing with various chemicals and enzymes, etc. (Verni et al*.,* 2022). Notably, some ANFs, such as protease inhibitors and lectins, are heat-labile, whereas others, including phytic acid, raffinose, tannins, and saponins, are more heat-stable (Oghbaei & Prakash, 2016).

In addition to reducing ANFs, there is a growing need to explore synbiotic formulations integrated into legume-based products. These combinations can enhance digestive health**,** modulate gut microbiota**,** and improve the bioavailability of nutrients**.** Microbially produced foods, defined here as ‘‘microbial foods,’’ are created through science-driven microbial fermentations, microbial biomass, or food ingredients derived from microbial cell factories (Sharma et al*.,* 2020) (Figure 1). Fermentation appears most promising in significantly reducing ANFs (Coda et al*.,* 2017).

The fermentative action of selected probiotic strains can help degrade complex carbohydrates and antinutrients, while prebiotics derived from legumes serve as substrates to promote beneficial microbial activity (Shiferaw & Augustin et al., 2020). Therefore, a dual strategy that addresses both the reduction of antinutritional compounds and the incorporation of synbiotic components holds significant promise for developing next-generation legume-based functional foods that offer both enhanced nutritional value and targeted health benefits (Sharma et al., 2020; Verni et al.,2022).

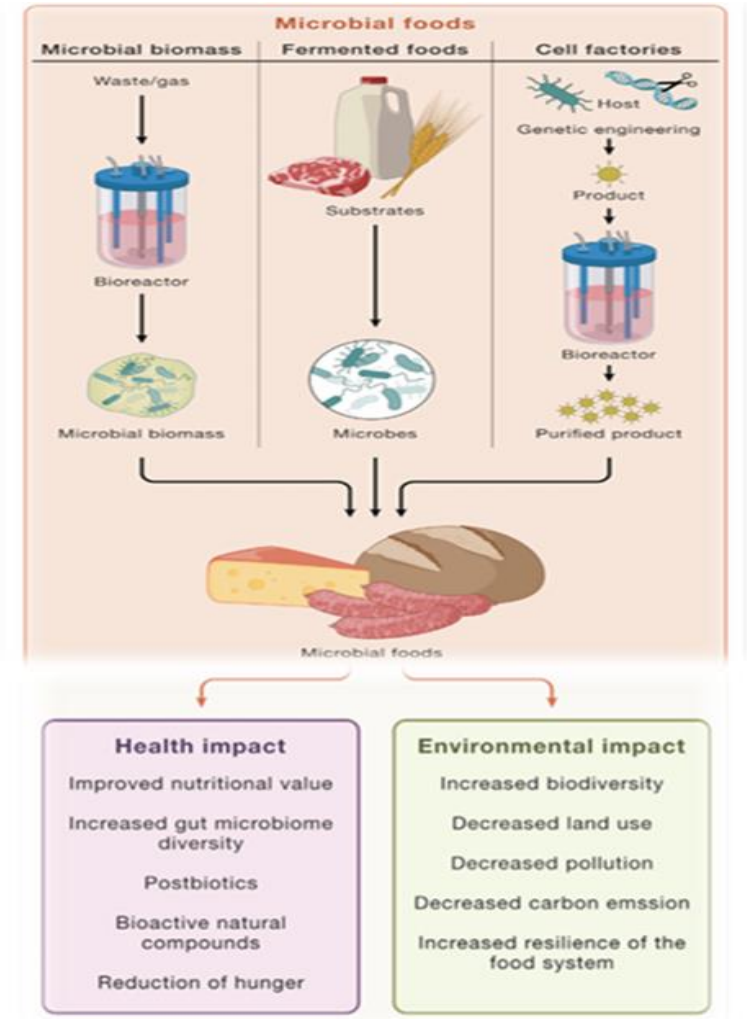


Figure 1: Impact and types of microbial foods

Overall, processing improves nutritional quality by breaking down complex nutrients, synthesizing new bioactive compounds, destroying ANFs, enhancing mineral bioavailability, and improving functional properties (Shiferaw & Augustin et al., 2020; Sharma & Sharma 2021).

2.7.1 Milling

Milling is the mechanical process of grinding grains into flour or meal, primarily to remove the husk and, in some cases, bran layers, producing an edible powder with variable particle sizes (Abebe et al., 2022). It is a key post-harvest step, and the extent of milling directly influences

nutrient content: increased milling reduces essential nutrients while minimally affecting energy density (Affrifah et al., 2022).

Grains consist of the endosperm, germ, and bran, with the endosperm comprising roughly 80% of grain weight; germ and bran proportions vary by grain type (Budhwar et al., 2020). Milling can be whole-grain, retaining all components, or differential, separating the grain into its constituent parts. For example, legumes may be milled into whole flour or refined through roller milling. As nutrients and phytonutrients are concentrated in outer layers, refining or differential milling typically decreases nutrient content, particularly non-starch components. Consequently, finer, refined flours often have lower nutrient density compared to whole-grain products (Abbas & Ahmad, 2018; Ali et al., 2018).

2.7.2 Controlled fermentation

Microorganisms play a pivotal yet often underappreciated role in the food system, spanning production, processing, preservation, and digestion (Dai et al., 2017; Ritter et al., 2018). Fermentation, one of the oldest and most widely practiced food processing techniques, originates from the Latin term *fermentum*. While biochemically defined as "an ATP-generating process with organic compounds serving as both electron donors and acceptors," in the context of food, fermentation refers to microbial-driven biochemical transformations that enhance safety, shelf life, flavour, and nutritional value (Akanni et al., 2018; Adebo et al., 2022).

The microorganisms involved in food fermentations include Bacteria such *as Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Bifidobacterium*, Yeasts (e.g., *Saccharomyces cerevisiae*, *Candida krusei*, *Candida humilis),* and Fungi/Molds (e.g., *Aspergillus oryzae*, *Aspergillus sojae*, *Penicillium roqueforti*, *Penicillium chrysogenum* (Arbab et al., 2023; Amritha & Venkateswaran, 2018; Buta & Emere, 2015). Food fermentations can be broadly classified into spontaneous fermentation, where naturally occurring microbes initiate the process, and controlled fermentation, which involves the deliberate use of selected microbial starter cultures under regulated conditions (e.g., temperature, pH, duration) (Naik & Kerkar, 2024).

Controlled fermentation uses defined starter cultures, which may consist of traditional probiotics or novel strains (Dai et al.,2022). Traditional probiotics, such as *Lacticaseibacillus*

rhamnosus GG and *L. acidophilus*, are widely employed in dairy fermentations like yogurt and kefir due to their acid tolerance and well-documented health benefits (Marco et al*.,* 2017). Emerging probiotics include:

- Next-generation probiotics (NGPs) such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Christensenella minuta*, known for their anti-inflammatory and metabolic functions (Danilova & Sharipova, 2020).
- Non-traditional LAB and spore-formers like *Bacillus coagulans*, *Weissella cibaria*, and *Pediococcus acidilactici*, isolated from fermented plant-based foods (Budhwar et al.,2020).
- Probiotic yeasts, especially *Saccharomyces cerevisiae var. boulardii*, which support gut barrier function and pathogen defense (Dahiya & Nigam 2022).

Table 9: Advantages of controlled fermentation.

Feature	Benefits
Microbial consistency	Ensures standardized product quality and probiotic viability
Targeted functionality	Enables production of desired bioactives (e.g., GABA, peptides)
Safety and traceability	Reduces contamination risks using documented strains
Product optimization	Allows adjustment of pH, oxygen, and fermentation conditions
Compatibility with novel carriers	Supports dairy-free, allergen-free substrates (e.g., legumes, cereals)

(Rodrigues et al*.,* 2014; Saraniya & Jeevaratnam, 2015; Sharma et al., 2020)

Controlled fermentation enables the safe, scalable, and targeted application of probiotic microbes, enhancing metabolite production (e.g., bioactive peptides, vitamins, SCFAs), improving organoleptic properties, and ensuring high probiotic viability in the final product (Naik & Kerkar, 2024). Table 9 indicates the advantages of controlled fermentation. Studies show that this approach can reliably achieve probiotic counts of 10^6–10^9 CFU/g or mL, in line with international guidelines (Rezac et al., 2018). For example, Lacticaseibacillus acidophilus

and Bifidobacterium animalis in fermented plant-based beverages maintain counts above 10^8 CFU/mL for several weeks (Forsido et al., 2020).

Fermented foods such as sauerkraut, tempeh, and soy-based beverages consistently reach probiotic levels sufficient to confer health benefits (Rezac et al., 2018; Marco et al., 2021). This is achieved through careful starter strain selection, optimized fermentation parameters, and proper storage (Ritter et al., 2018). Controlled fermentation has expanded beyond dairy to plant-based substrates like grains, legumes, fruits, and vegetables (Valero et al., 2020), and also supports industrial biotechnology for enzyme and metabolite production, as well as climate-resilient food systems using microbial foods in bioreactors (Shiferaw & Augustin, 2020). By leveraging microbial biodiversity, combining traditional and novel strains, and optimizing fermentation conditions, this approach enables the reliable delivery of health-promoting microbes and bioactives through diverse food systems supporting both human and planetary health.

2.8 Nutritional and food technological benefits of microbially fermented synbiotic foods

Fermented foods are widespread around the world and make up about one-third of foods consumed globally (Akin & Ozcan, 2017). It is increasingly being used for the production of probiotics and synbiotics with high viability and functionality. The probiotic fermented foods have demonstrated positive roles in relief of lactose intolerance, shortening of rotavirus diarrhea, immune modulation, regulation of serum cholesterol levels, treatment of irritable bowel syndrome (IBS), urinary tract infections, bladder cancer, and allergies associated with skin, gut and respiratory tract (Ali et al., 2018; Bagga et al., 2018; Barat & Ozcan, 2018; Akweya et al., 2020). Fermented legume products offer improved digestibility and reduce beany flavor, enhancing their sensory appeal and overall consumer acceptance. Changes happening during the fermentation that include chemical transformation of primary compounds to new secondary metabolites can be categorized into 5 stages (Figure 2).

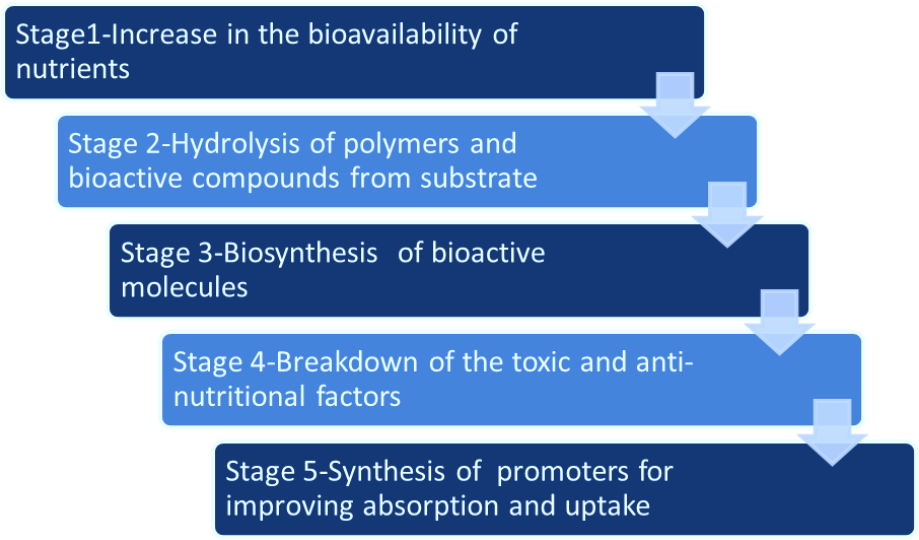


Figure 2: Five stages indicating changes during food fermentation (Naik & Kerkar, 2024).

Increasing the intake of fermented foods in the human diet can offer multiple benefits for both human health and environmental sustainability, as outlined below.

2.8.1 Fermentation: food safety and preservation

Fermentation reduces the population of pathogenic microorganisms by lowering pH, by microbial competition for nutrients, or by production of preservatives with antimicrobial and antifungal compounds like bacteriocins and promoting the degradation of toxins (e.g., mycotoxins), promoting mucosal cell differentiation and immune barrier function of the epithelium (e.g., metabolic detoxification of mycotoxins and other endotoxins by *Rhizopus oryzae*) (Pang et al., 2020; Bhangar et al., 2022). As a result, fermented foods are generally safer than their raw counterparts.

Additionally, fermentation extends shelf life, which helps in reducing food loss throughout the supply chain from agricultural overproduction to consumer-level waste, thereby improving resource efficiency and promoting sustainability in food systems (Da Silva et al., 2018; Jahn et al., 2023). The extended shelf life of fermented foods contributes to reducing food loss throughout the entire food production value chain, from overproduction at the farm level to post-harvest waste in markets, retail stores, and households. This enhancement in shelf life leads to more efficient resource utilization.

2.8.2 Fermentation and *de novo* creation of delicious flavors and textures

Microbial fermentation plays a central role in shaping the sensory characteristics of food. The textures of cheese and the distinct flavors of products like chocolate, soy sauce, wine, and many fermented plant-based foods are all a result of complex microbial activity (Akanni et al., 2018; Alli et al., 2018; Jahn et al., 2023). During fermentation, microorganisms generate a variety of volatile and non-volatile compounds, including alcohols, aldehydes, esters, organic acids, ketones, fatty acids, sulfur-containing compounds, and nitrogenous metabolites, which contribute to taste profiles such as bitter, umami, sweet, sour, and salty (Naik & Kerkar, 2024). Through enzymatic hydrolysis and biotransformation reactions, including the degradation of proteins and cell wall polysaccharides, microbial metabolism significantly modifies the structural and chemical properties of raw food materials (Watson et al., 2013; Delgado et al., 2014; Devi et al., 2015). These modifications result in improved texture, digestibility, and nutrient retention, especially when fermentation is carefully controlled.

The choice of microbial strains is critical in defining the flavor and texture of fermented products. Lactic acid bacteria (LAB), for example, produce organic acids mainly lactic acid that impart sourness, lower pH, and influence perceived moisture and mouthfeel. Specific fermentation pathways can enhance or suppress particular flavor notes, depending on the strain and the substrate (Verni et al., 2022; Vinderola et al., 2023). In traditional biotechnologies like sourdough fermentation, microbial activity also impacts rheology, sensory properties, and functional attributes of baked goods (Gobbetti et al., 2018). These examples highlight how fermentation can be strategically applied to produce foods that are both health-promoting and sensorially appealing.

The acceptance of novel synbiotic foods, particularly legume-based products, depends not only on their probiotic and prebiotic benefits but also on taste, aroma, and texture (Aduol et al., 2020). Characterization of fermented legumes has revealed a rich profile of volatile organic compounds (VOCs), including esters, aldehydes, and sulfur compounds, which contribute to flavor development (Verni et al., 2022) as seen in Table 10. Harnessing microbial fermentation for sensory enhancement thus supports the creation of sustainable, functional foods that align with consumer expectations for both health and palatability.

Table 10: Volatile compounds produced during fermentation of legume-based substrates.

Compounds		
(E)-2-Hexen-1-ol	2-Pentanone	Hexanoic acid
(E)-2-Octenal	2-Pentylfuran	Isomyl alcohol
(E)-2-Penten-1-ol	2-Phenyl ethanol	Isoamyl salicylate
(E)-2-Pentenal	2-Undecenal	Isobutanol
(E, E)-2,4-Decadienal	3-Butanediol	Isobutyric acid
(E, E)-2,4-Heptadienal	3-Hydroxy-2-butanone	Linoleic acid
1-(2-Furyl) ethenone	3-Octanol	Linolenic acid
1,2-Heptanediol	5-Hydroxymethylfurfural	Maltol
1,2-Xylene	Acetic acid	Methyl laureate
1,4-Dimethylpiperazine	Acetoin	Methyl linoleate
1-Butanol	Amyl salicylate	Methyl palmitate
1-Hexanol	Benzaldehyde	Myristic acid
1-Nonanol	Benzoic acid	Nonanoic acid
1-Octadecene	Benzyl alcohol	n-Tetradecane
1-Octen-3-ol	Butylated hydroxyl toluene	Octadecenoic acid
1-Pentanol	Butyric acid	Octanoic acid
1-Penten-3-ol	Caproic acid	Oleic acid
2(5H)-Furanone	Ethanol	Palmitic acid
2,2-Dichloroethanol	Ethyl palmitate	Palmitoleic acid
2-Butanol	Ethyl benzene	Propionic acid
2-Cyanoethyl hexyl ether	Furfural	Pyranone
2-Dodecano	Heptanal	Tetrahydropyran
2-Furanmethanol	Heptanoic acid	Valeric acid
2-Methyl-1-butanol	Hexanal	Vanillin

(Xiang et al., 2019; Liu et al., 2021)

2.8.3 Fermentation and production of the "Biotics"

Probiotics, synbiotics, postbiotics, oncobiotics, paraprobiotics, pharmabiotics, and psychobiotics are the emerging 'biotics' as bioactives (e.g., enzyme cofactors, GABA, ACE

peptide inhibitor, antihypertensive peptides, PUFA, isoflavone aglycones, and vitamins) (Vinderola et al., 2023). Fermentation can make foods with specific health benefits by releasing target bioactives or transforming parent substances into these bioactives (Tomasik & Tomasik, 2020). Fermentation has been proven feasible for releasing phenolic compounds from a wide range of natural food resources; as a result, fermented foods and beverages contain different concentrations of simple phenols (e.g., phenolic acids), more complex phenolics (e.g., flavonoids, including monomeric (catechins) and oligomeric flavanols (proanthocyanidins), chalcones (e.g., xanthohumol), lignans, and ellagitannins (Awika & Duodu, 2017; Vinderola et al., 2022). The nutritional and health benefits of probiotic fermented foods can also be enhanced because of the generated beneficial enzymes (e.g., galactosidase, which facilitates lactose hydrolysis and compensates for the reduction of human lactase activity over time), and nutrients or bioactives (Awika & Duodu, 2017; Danilova & Sharapova, 2020). Fermentation of cowpea flour and its incorporation into legume-enriched wheat bread using *L. plantarum* ATCC 14917 has been shown to alter phenolic profiles and enhance antioxidant activity (Dueñas et al., 2005).

2.8.4 Fermentation and modified nutritional composition of food

Fermentation can increase digestibility and accessibility, allowing the nutrients, including initially nondigestible carbohydrates, to be digested and absorbed more easily whilst reducing cooking times (Tamang et al., 2020). Ingredients containing ANFs, which chelate essential metal ions and thereby reduce nutrient bioavailability as well as those with high fiber content or even naturally occurring toxins, can be converted into more palatable and nutritionally enhanced products via microbial fermentation (Amritha & Venkateswaran, 2018). A well-documented example is the fermentation of the starchy tuber cassava (*Manihot esculenta*), a dietary staple in many regions of the Global South, which reduces its cyanogenic glycoside content to safe, consumable levels (Ali et al., 2018).

Fermentation can improve human health outcomes by enhancing nutrient bioavailability, decreasing protein allergenicity, and enriching foods with additional nutrients, including macro and micro-nutrients, vitamins and minerals, essential amino acids, fatty acids (Gobbetti et al; 2019; Arbab et al., 2023). Strategies have been set to transform natural microbial fermentation towards metabolic engineering to achieve more precise and efficient production of amino acids

(Zheng et al., 2017; Gauthankar et al., 2021). The branched-chain amino acids can be manufactured via fermentation with *C. glutamicum, E. coli* and *S. thermophilus*, with those produced using *S. thermophilus* exhibiting positive functions in protein synthesis, muscle performance, and maintenance of lean body mass (Hossain et al., 2016; Matemu et al*.,* 2021; Homsuwan et al., 2023). The organic acids generated during fermentation also exhibit health benefits. Lactic acid, generated, helps regulate the glycemic response through lowering the rate of starch digestion, whilst acetic acid and propionic acid can slow gastric emptying (Shiferaw & Augustin, 2020).

Fermentation can significantly increase the vitamin content of foods and enhance their bioavailability compared to their raw counterparts, primarily due to microbial biosynthesis (Nami et al., 2018; Adebo et al*.,* 2022). For instance, kefir is rich in vitamin B12, while yogurt contains elevated levels of vitamins A, B, and D (Tamang et al., 2020; Aziz et al., 2023). Tempeh, a traditional fermented soybean product, has higher concentrations of B-group vitamins than unfermented soybeans, and natto, fermented with *B. subtilis*, is a notable source of vitamin K2 (Zheng et al., 2017). These vitamin differences are influenced by several factors, including the raw material, the microbial strains used, and fermentation conditions (Marco et al., 2017). However, not all effects are positive. While yeast fermentation may increase vitamin B1 content, *Lactobacillus* fermentation can lead to a reduction (Merenkov & Zinina, 2021). For example, significant losses of vitamin B1 were observed during the fermentation of chickpeas and cowpeas with strains such as *L. casei*, *L. leichmannii*, *L. plantarum*, *Pediococcus acidilactici*, and *P. pentosaceus* (N'Zi et al., 2021). Finally, the microorganisms used in fermentation, their residual EPS, microbial building blocks, proteins, or metabolites, are likely to directly or indirectly interact with human physiology as well as the human gut microbiome, thereby influencing human health. (Korcz & Varga, 2021; Lu et al., 2022).

2.9 Health benefits of fermented foods

Human studies on dietary interventions are still limited; however, available evidence points to the health-promoting effects of fermented foods, notably in metabolism, weight regulation, enhancing mood and cognitive well-being and overall mortality reduction (Funck et al., 2019).

Table 11: Nutritional and health benefits of fermented legumes.

Legume	**Strain**	**Effect**	**Reference**
Lentils	*L.plantarum*	Increase in GABA content, antioxidant, and ACE inhibitory activity	Fan et al., 2015
Mungbean	*L.reuteri, L. casei, L. heleviticus*	Increase in protein content, nitrogen solubility, *in vitro* digestibility, reducing the phytate content and trypsin inhibitor activity	Wu et al., 2015
Navy beans	*L.bulgaricus and L. plantarum*	ACE inhibitory activity	Aziz et al., 2023
Soybean	*L.brevis OPY-1 B. subtilis CS 90*	Yogurt with high levels of GABA, free amino acids, and isoflavones. Biotransformation of phytochemicals. isoflavones, flavonols, and phenolic acids	Dai et al., 2017; Ali et al., 2018
Soymilk	*B. longum BB536 and B. pseudolongum*	A-galactosidase activity leading to the hydrolysis of oligosaccharides (Raffinose and stachyose)	Ali et al., 2018
Soymilk supplemented with FOS, mannitol, inulin, pectin and, maltodextrin	*L.acidophillus, L. casei, B. longum*	ACE inhibitory activity and bioconversion of isoflavones	Buta et al., 2015

A recent study in Western adults demonstrated that a fermented food-enriched diet decreased inflammatory signals and increased gut microbiome diversity (Garrido et al., 2021). These effects may result from microbial transfer from food to the gut, as some microbes can survive gastrointestinal transit, or from interactions between microbial metabolites, the gut microbiota,

and the host (Gopikrishna et al., 2021). Advances in metagenomic sequencing and clinical trials are expected to further clarify these microbial and molecular interactions (Fan et al., 2015).

Beyond health benefits, fermentation promotes the valorization of underutilized plant species and agricultural by-products, reducing food waste and diversifying food sources. It also contributes to climate resilience by facilitating the use of novel or climate-adapted crops (Shiferaw et al., 2020). Table 11 summarizes the nutritional and health benefits of fermented legumes. Some benefits, such as lowering glycaemic index, increasing mineral bioavailability, and reducing gluten content, are well-established, while other potentials are emerging. Evidence also highlights sourdough fermentation for salt reduction, management of IBS, synthesis of bioactive compounds (e.g., phenolic metabolites), and utilization of non-conventional flours and milling by-products (Bascuñán et al., 2020).

Health outcomes can now be influenced and monitored through dietary modulation via the gut microbiome and host metabolites. Legume-based probiotic foods have the potential to contribute significantly to personalized nutrition strategies. Given the inter-individual variability in gut microbiota composition, optimizing dietary interventions may require tailoring probiotic/prebiotic intake to individual microbiome profiles. However, further clinical research, particularly randomized controlled trials using standardized legume-based matrices, is essential to validate health claims and translate findings into clinical and dietary practice.

CHAPTER 3:

MATERIALS AND METHODS

Chapter 3

Materials and Methods

This study was carried out in three phases. Phase I focused on screening saltpan isolates and evaluating their probiotic potential. Phase II dealt with the proximate analysis. nutritional composition and physical and cooking properties of the cowpea cultivar. In phase III, the efficacy of GC3 as a probiotic carrier was evaluated and established after fermentation.

Phase I

3.1 Screening of saltpan isolates

3.1.1 Growth and maintenance of the saltpan isolates

Halotolerant bacteria from the hypersaline environment were selected for the present study. Halotolerant bacterial isolates (46) from biofilms, water and sediments, collected from Batim, Agarwado and Ribandar saltpans in Goa, as part of Dr Savita Kerkar's culture collection, were used. They were maintained on 25 % Nutrient broth and Nutrient agar supplemented with 2 % salt.

3.1.2 Characterisation of the halotolerant bacteria

Individual halotolerant bacterial strains were cultured in 50 mL of 25 % Nutrient broth of Hi-Media (Appendix I) supplemented with 1 % sea salt and grown overnight, and incubated at 28 ± 2 °C with shaking at 120 rpm on an orbital shaker. The purity of selected cultures was checked using the streak plate technique. Pure cultures were maintained on Nutrient agar plates, and their Gram character was assessed by Hans Christian Gram staining (Coico, 2006; Appendix II). The isolates which retained crystal violet and appeared blue were Gram-positive.

A sterile suspension of Gram-positive isolates was subjected to heat shock by incubating it in a water bath maintained at 80 °C for 10-15 min to screen for spore formers. On cooling, a loopful of suspension was isolated on a sterile Nutrient agar plate and incubated at room temperature (RT) for 24 h. The presence of spores was ascertained with Schaeffer and Fulton's method (1933)

(Appendix II). The bacterial isolates were further characterized morphologically as either cocci or rod-shaped.

3.1.3 Fermentation assay: Carbohydrate utilisation of the isolates (Gosh et al., 2017; Ritter et al., 2018)

Each of glucose, fructose, sucrose, lactose, xylose, maltose, mannitol, arabinose, and raffinose (10 g/L) was added to the carbohydrate test broth (10 mL) separately (Appendix I). A Durham's tube was inserted into each tube, ensuring there is no air bubble. The test tubes were autoclaved for 15 min at 121 °C and 15 psi. Aseptically, each test tube was inoculated with the individual test microorganism (25 isolates) and swirled gently to mix the contents. *Lactobacillus plantarum* MTCC 2621 (Microbial Type Culture Collection, Haryana, India) served as a positive control. All the tubes were incubated for 1-7 days at 37 °C. The colour change from reddish orange to yellow was considered to be a positive result for acid production, and a bubble in the Durham's tube indicated fermentation of the respective carbohydrates.

3.1.4 Qualitative screening for extracellular hydrolytic enzymes

The ability of sixteen short-listed halo-tolerant *Bacillus* isolates to produce the following extracellular enzymes was studied. Qualitative assessment of enzyme production was performed using plate assays on minimal media supplemented with specific substrates.

Amylase: Amylolytic activity was assessed by spot inoculating the isolates onto 1% starch agar plates (Appendix I), followed by incubation at 28 ± 2 °C for 24–48 h. Plates were subsequently flooded with 1% Lugol's iodine solution (Sigma L6146) and examined for clear zones surrounding the colonies. A colourless zone around the isolate against a blue background of intact starch indicated a positive test (Banerjee et al., 2017).

Cellulase: Cellulase production was evaluated by spot inoculating bacterial strains onto 1% carboxymethyl cellulose (CMC) agar (Appendix I), followed by incubation at 28 ± 2 °C for 24–48 h. Gram's iodine stain was then used to flood the plates and examined for clear zones around the colonies (Jeon et al., 2018).

Protease: The isolates were inoculated on agar supplemented with 1 % skim milk powder (Appendix I) at 28 ± 2 °C for 24 – 48 h to study protease activity. A zone of clearance around the colony indicated a positive test when visualised with 1% TCA (Das et al., 2019).

β-galactosidase: Ortho-nitrophenyl-β-galactosides (ONPG) were the substrate used to study this activity. To the sterile tubes containing sterile saline (1 mL), ready-to-use discs impregnated with ONPG were transferred, followed by inoculation of the *Bacillus* isolate suspension into these tubes. A change in colour of the disc and saline suspension to yellow after 36 h incubation at 28 ± 2 °C indicated a positive test (http//catalog hardy diagnostic.com, Gottschalk, 1986).

Phytase: A plate assay was performed using phytase-specific medium by following the protocol of Dev et al. (2016) with slight modifications (Appendix I), supplemented with 2 % agar and streaking the pure test culture on it. The media served as a negative control. The incubation of plates was done at 28 ± 2 °C for 72 h, and observed for clear zones around the streaked culture.

Lipase: Tributyrin agar (1%) was used to study lipase activity (Appendix I). A zone of clearance around the colonies after 48 h at 28 ± 2 °C indicated lipase activity (Banerjee et al., 2017).

3.2 *In vitro* studies on safety, desired probiotic attributes, and shelf life of the *Bacillus* isolates

With the advancement in the metagenomics approach and bioinformatics analysis, an opportunity is provided to study complex microbial communities and help ascertain patterns that regulate human microbial ecosystems (Dahiya & Nigam, 2022). This enabled the discovery of new-generation probiotics, which will continue to evolve in modulating human intestinal health.

Seven bacterial isolates were studied *in vitro* for haemolytic activity, and three non-haemolytic isolates were examined for the detection of endotoxin production, enterotoxin genes, antimicrobial activity against pathogens, and antibiotic susceptibility. Their probiotic attributes, *viz.,* acid and bile tolerance, adherence capacity, exopolysaccharide (EPS) production, bile salt hydrolase (BSH), and antioxidant activity, were determined. The shelf life and storage studies of potential probiotic *Bacillus* isolates (02) to be used for cowpea cultivar fermentation were carried out. The isolates were subsequently characterized and identified through biochemical tests, as well as morphological and molecular analyses, following the guidelines of Bergey's Manual of Systematic Bacteriology (Claus, 1986).

3.2.1 Safety studies of *Bacillus* isolates

3.2.1.1 Study of haemolytic activity

The seven isolates shortlisted based on extracellular enzyme production were evaluated for their haemolytic activity. These isolates previously grown in Nutrient broth (25 %) and 2 % salt were spot inoculated on 6 % sterile human blood agar plates (Appendix I). Incubation of the plates was carried out at 37°C for 48h. Results were observed in terms of the colour of the halo around the growth of the culture in the spotted area. α-Haemolysis was indicated by partial lysis of erythrocytes, resulting in a greenish zone around the bacterial colony. β-Haemolysis was characterised by a clear zone of complete haemolysis, whereas γ-haemolysis was recorded when no change in the agar was observed around the colony (Banerjee et al., 2017).

3.2.1.2 Detection of enterotoxin genes in the potential probiotic isolates using PCR

(Das et al., 2009; Ali et al., 2015)

Enterotoxins are bacterial toxins that specifically affect the intestines, causing gastrointestinal symptoms such as diarrhoea, vomiting, and abdominal cramping and are often associated with food-borne illnesses. Thus, the safety of potential probiotics needs to be established when used in food fermentation or as a functional supplement.

Bacillus cereus NCIM 2106, a strain known to produce enterotoxins, was procured from the National Collection of Industrial Microorganisms (NCL), now NCMR (National Centre for Microbial Resource), Pune. *B. cereus* NCIM 2106 was inoculated in Luria Bertani (LB) broth and incubated at 37 °C (120 rpm) for 24 h. The cell mass was harvested after centrifugation (8,000 rpm) for 8 min at 8 °C, and the pelleted cells obtained were washed with phosphate buffer saline (PBS). The three *Bacillus* isolates, showing negative haemolysis (γ-haemolysis), were inoculated individually in Nutrient broth (25 %) and supplemented with 1% salt, incubated at 28 ± 2 °C at 120 rpm for 24 h. Their cell mass was harvested as that of *B. cereus*. The genomic DNA from the three *Bacillus* isolates and the standard culture was purified using the DNA-Sure tissue mini kit from Sigma-Aldrich. The pelleted cultures were suspended in 20 mM Tris /HCL, 1 % Triton X-100, 2 mM EDTA at pH 8, adding 20 mg/mL lysozyme, followed by 1 h incubation at 28 ± 2 °C with a further treatment with proteinase K and RNAase A and incubation at 55 °C for 3 h. The kit protocol was followed for extraction. The DNA from each isolate was subjected

to electrophoresis using agarose gel (1%) to visualise the DNA. The primers for target enterotoxin genes *nheA, nheB, hb1A*, and *entFM* are shown in Table 12. DNA was also quantified by UV spectrophotometry. 5 µl of the extract was mixed with 95 µl of TE buffer. (1:20 dilution). Absorbance was measured at 260nm. This gives the DNA concentration when multiplied by 1000. (1-unit OD_{200} corresponds to 50 µg/ml DNA and 20 is the dilution factor).

PCR Master Mix (2X) from Promega was used to check for the presence of four enterotoxin genes, if present in the 3 *Bacillus* species as well as in the standard *B. cereus* NCIM 2106. Amplification of the test isolates and standard culture was carried out using primers for target genes in separate reaction mixtures. 50 µL PCR reaction mixture, 25 µL of PCR Master Mix, 0.5 µM each of forward and reverse primers, 0.1 µg template DNA and nuclease-free water were mixed to make the volume to 50 µL. The samples were gently vortexed, and PCR amplification was carried out using a Palm Cycler. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 45 s, and extension at 73 °C for 1 min; followed by a final extension at 72 °C for 5 min. The PCR products were then analysed by loading 10 µL of the samples mixed with gel loading dye, on a 2 % agarose gel with Ethidium bromide and visualised using UV light. A 10,000 bp DNA ladder was also loaded on each gel.

Table 12: Primers for enterotoxin genes.

Target gene	Primer	Primer sequence	Reference
nheA	NH1F	GCTCTATGAACTAGCAGGAAAC	Phelps & McKillip, 2002
	NH1R	GCTACTTACTTGATCTTCAACG	
nheB	NH2F	CGGTTCATCTGTTGCGACACG	
	NH2R	GATCCCATTGTGTACCATTGG	
hblA	HblA1	GCTAATGTAGTTTCACCTGTAGCAAC	Das et al., 2009
	HblA2	AATCATGCCACTGCGTGGACATATAA	
entFM	ENT A	ATGAAAAAAGTAATTTGCAGG	
	ENT B	TTAGTATGCTTTTGTGTAACC	

3.2.1.3 Detection of endotoxin in the prospective probiotic isolates

Bacterial endotoxin test (BET), commonly referred to as the Limulus amoebocyte lysate (LAL) test, was used to detect the presence or absence of endotoxin in the potential probiotics. The test is based on the reaction between bacterial endotoxins and a lysate derived from horseshoe crab

blood cells. The test cultures FSK 0.30, FSK 2 and FSK 3 were grown overnight in Nutrient broth, centrifuged at 8,000 rpm at 5 °C for 5 min, and the cells obtained were sonicated in an ice water bath at 4 °C for 10 min and used to carry out the test. The test was performed using kit instructions (Pyrostar-ESF, FUJIFILM Wako Chemicals U.S.A. Corp). Endotoxin from *E. Coli* UKT-B in a lyophilized form, provided in the kit, was used as a control.

3.2.1.4 Antimicrobial activity of the *Bacillus* isolates

The isolates were tested for antibacterial activity against various gastrointestinal tract (GIT) pathogens as well as food pathogens following the protocols of Shokryazdan et al. (2014) and Georgieva et al. (2015). The antibiosis of the isolates was studied on Mueller-Hinton Agar (MHA) plates against ATCC strains of pure culture suspensions of GIT pathogens of *B. cereus* ATCC 14579, *Salmonella enterica* ATCC 35664, *E.coli* ATCC 25922, *Salmonella paratyphi* ATCC 14028, *S. aureus* ATCC 6538, *Shigella boydei* ATCC 8700, and laboratory clinical pathogens, *Salmonella typhi*, *E. coli, Methicillin-resistant Staphylococcus aureus*(MRSA)*, Enterobacter cloacae, S. aureus, Shigella sonnei,* and *Shigella boydei* by the cross-streak method. The antimicrobial potential of the isolates was evaluated by streaking a pure culture as a single line in the centre of the assay plate. Plates were incubated at 28 ± 2 °C for 24 h, after which pathogenic strains were streaked at a 90° angle to the producer strain. The plates were then incubated for an additional 24 h at 37 °C. Antimicrobial activity was assessed by measuring the zone of inhibition in millimetres.

3.2.1.5 Determination of antibiotic disc sensitivity of the *Bacillus* isolates (Somplang, 2016)

Disc diffusion assay was used to study the antibiotic susceptibility of the selected cultures using antibiotic discs obtained from Hi-media at their indicated concentration. The antibiotics selected were of three categories, *viz.,* a) inhibitors of cell wall synthesis, b) inhibitors of protein synthesis, and c) inhibitors of nucleic acid synthesis (Appendix III). A sterile cotton bud was dipped into the suspension of three overnight-grown test isolates and swabbed on the entire surface of MHA (Appendix I) plates. Subsequently, after 5 min, Antibiotic discs were applied to the surface of the plates, which were then incubated (28 ± 2 °C) for 24 h. The diameters of the zone of inhibition of the cultures were measured in mm. Strains were classified as R (resistant),

MS (moderately susceptible), or S (susceptible) according to interpretative standards described by Charteris et al., 1998.

3.2.2 Studies on desired probiotic attributes of *Bacillus* isolates

3.2.2.1 Tolerance to gastric acid and intestinal bile salts in simulated conditions

Probiotics, if taken in appropriate amounts, are beneficial to the host. Probiotic organisms must overcome harsh biological barriers like stomach acid and intestinal bile salts and colonise themselves in the intestine, resulting in health-promoting effects (Kaur et al., 2020).

3.2.2.1.1 Preparation of FSK 0.30, FSK 2, and FSK 3 cell suspensions

Selected isolates (03) were inoculated in Nutrient Broth (25 %) followed by incubation at 28 ± 2 °C for 24 h, and centrifuged at 8,000 rpm for 5 min at 10 °C. The cells were washed with 0.85 % saline and resuspended to obtain an OD of 0.25 corresponding to ~9 log CFU/ mL of each isolate.

3.2.2.1.2 Acid tolerance test (*in vitro)*

Artificial gastric fluid was prepared using 1.56 g NaHCO3, 4.8 g NaCl, 2.2 g KCl, and 0.22 g CaCl2 in one litre of distilled water (D/W) and was divided into 5 portions (Clavel et al., 2004; Ziarno, 2008; Khan et al., 2023). Their pH was adjusted to 1.2, 2.0, 3.0, 4.0, and 7.0, respectively, and autoclaved at 15 psi for 15 min. Pepsin solution (5 %) in D/W was filter sterilised through a 0.45 µ membrane filter. Each of 4.5 mL of sterile artificial gastric juice of the respective pH was mixed using 0.5 mL of 5 % pepsin solution to simulate conditions in the stomach and was incubated at 28 ± 2 °C for 3 h with gentle shaking to mimic peristalsis. Tubes were inoculated with individual test culture suspension (0.5 mL). 0.5 mL of the vortexed mixture was soon transferred to a sterile 4.5 mL saline tube labelled as 0 h and incubated at 28 ± 2 °C for 3 h. Aliquots (0.1 mL) of the samples were drawn at hourly intervals of 1 h, 2 h, and 3 h and 0.1 mL spread plated on agar plates in triplicate after serial dilutions up to 10^{-8} and incubated at 37 °C for 24 h. The average number of organisms surviving at the end of 1 h, 2 h, and 3 h was measured and recorded as log CFU/ mL. The lowest pH that the isolates could tolerate was determined

from the viable count of bacteria. The percentage survival was determined after 3 h exposure to gastric fluid as follows (Ritter et al., 2018):

% Survival = (log CFU of viable cells survived/log CFU of initial viable cells inoculated) × 100

3.2.2.1.3 Bile tolerance test (*in vitro)*

Stock solutions of bile salts (5 % Sodium deoxycholate and 5 % Sodium taurocholate) were prepared, adjusting the pH to 8.0 and appropriately diluted as indicated in Table 13 as per Stasiak-Różańska et al. (2021) with modifications. The stock solutions were filter sterilized using a 0.45 µ membrane filter. Pancreatin (Sigma Chemical Company, USA) was suspended in sterile saline at 1 g/L to prepare simulated small intestinal juices, either in the presence or absence of bile salts (Fluka Analytical), and the pH was adjusted to 8.0. The tubes were inoculated with 0.5 mL of suspension of each of the selected isolates; aliquot of the suspension was immediately drawn, serially diluted up to 10^{-9} and labelled as 0 h and plated on Nutrient agar, incubated at 37 °C for 4 h. Samples were drawn at 1 h, 2 h,3 h and 4 h interval, subjected to serial dilutions, plated and incubated at 37 °C for 24 h and the average viable colony count was determined. If no significant reduction in bacterial survival was observed in the bile salt solution after 4 h (which is the typical transit time for food through the intestine), the isolates were deemed as tolerant to bile. Bacterial survival was expressed as a percentage at a 0.5 % bile concentration after 4 h, considering that 0.3 % represents the average human physiological bile concentration and 4 h simulates the maximum intestinal transit time.

Table 13: Preparation of various bile salt concentrations.

Bile salt concentration in %	**Volume of 5 % Sodium deoxycholate (mL)**	**Volume of 5 % Sodium taurocholate (mL)**	**Volume of diluent 0.2% NaCl (mL)**	**Total Volume (mL)**
0	0	0	5.0	5.0
0.5	0.25	0.25	4.5	
1.0	0.50	0.50	4.0	
1.5	0.75	0.75	3.5	
2.0	1.00	1.00	3.0	

3.2.2.2 Evaluation of bile salt hydrolase activity

Bile salt hydrolysis, a reaction of biological significance, helps to alter bile acids under the influence of bile salt hydrolases (BSHs). They bring about bile acid deconjugation. BSH enzymatically breaks down glycine- and taurine-conjugated bile salts, releasing amino acids and free bile acids (Ritter et al., 2018). Microorganisms exhibiting BSH activity may be beneficial due to their potential to lower serum cholesterol levels (Kaur et al., 2020). Two methods were used:

3.2.2.2.1 Plate method (Sedlackova et al., 2015)

MRS agar plates supplemented with 0.3 % filter-sterilized sodium taurocholate (NaTC) and sodium glycocholate (NaGC) and 0.035 % $CaCl_2$ were used. Wells (10 mm) were bored in the agar plates. Suspension (100 µL) of each of the three previously grown bacterial isolates, FSK 0.30, FSK 2, and FSK 3, and a standard culture, *Lactobacillus plantarum* MTCC 2621, were inoculated individually in the bore wells. The plates were incubated for 72 h at 37 °C. The presence of an opaque zone around wells was considered a positive reaction.

3.2.2.2.2 Modified ninhydrin method (Sridevi & Prabhune, 2009)

All the isolates FSK 0.30, FSK 2, and FSK 3, and the standard culture (positive control) were grown in sodium glutamate broth at 50 °C for 24 h at 120 rpm, followed by centrifugation at 8,000 rpm at 5 °C for 5 minutes. Cell pellets were resuspended in 1.5 mL reaction mixture containing 15 mM PBS (pH 6.5), 15 mM DTT, and 15 mM sodium glycocholate or sodium taurocholate. Incubation of the mixture was done at 37 °C for 1h. The reaction was terminated the reaction by adding 300 µL of TCA (l5 % w/v). The mixture was centrifuged at 8,000 rpm for 5 min; 1 mL of the supernatant was mixed with 1 mL ninhydrin reagent solution (2 %) and, boiled for 15 min after thorough mixing. The absorbance of the sample was recorded at 570 nm after cooling. The BSH activity of whole cells (per gram) was determined using a taurine-based standard curve. One unit of BSH activity corresponds to the enzyme amount required to release 1 µL of amino acid from the substrate per minute. A standard graph was prepared using 2000 µg/mL of taurine. A concentration range of 200-2000 µg/mL was prepared. Ninhydrin (2 %) was added to different concentrations of taurine in the proportion of 1:1.

3.2.2.3 Assessment of adherence capacity

Probiotics are generally expected to colonize the host gut, and a key characteristic is their ability to adhere to mucosal cells (Lu et al., 2022). The efficacy of a probiotic strain is believed to be linked to its ability to adhere to intestinal surfaces, and this hydrophobicity is considered as an *in vitro* biomarker to assess the colonization of potential probionts (Krausova et al., 2019). The adherence capacity was assessed using the bacterial adhesion to hydrocarbons **(**BATH) assay, by evaluating the exopolysaccharides (EPS) production and the mucus adhesion test.

3.2.2.3.1 Bacterial adhesion to hydrocarbons (BATH) test

BATH test of the three isolates was performed using xylene as a hydrocarbon as per Ritter et al., 2018). The isolates were cultured in nutrient broth and incubated at 28 ± 2 °C for 24 h. Following incubation, the cultures were centrifuged at 8,000 rpm for 6 min at 5 °C. The resulting cell pellets were washed with 0.85% saline and resuspended to achieve an optical density of 0.5 at 600 nm. For the assay, 1.5 mL of this cell suspension was mixed with an equal volume of xylene in a test tube. The mixture was vigorously vortexed for 2 min to form a two-phase system. After incubating at room temperature for 45 min, the aqueous phase was carefully collected, and its absorbance was measured at 600 nm. The percentage of bacterial adhesion was then calculated using the following formula:

$$\text{Adherence capacity (\%)} = [(A_0\text{-}A)/A_0] \times 100$$

Where: A_0 = initial absorbance and A = final absorbance

3.2.2.3.2 Exopolysaccharide (EPS) production by hypersaline Bacteria

Bacterial exopolysaccharides comprise a diverse group of carbohydrate polymers that may be covalently linked to the cell surface as a capsule, loosely attached, or entirely secreted into the surrounding environment (Taj et al., 2022). EPS production by probiotic bacteria can enhance organoleptic, technological, nutritional, and health-related properties. Additionally, EPS synthesis may support the survival of potential probiotic strains under the harsh conditions of the gastrointestinal tract (Fang et al., 2004). The two isolates were screened for the production of EPS. Primary screening was done by morphological identification of the mucoidal bacterial colonies by touching a sterile nichrome loop to individual colonies (Ali et al., 2019). The

formation of a string upon lifting of the loop was considered a positive result. EPS production was examined by streaking overnight-grown two test isolates on 25 % Nutrient agar plates with 2 % NaCl containing 0.08 % Congo red dye. After incubation at 28 ± 2 °C for 24 h, the plates were observed for black coloration of colonies, which indicated the production of EPS by the potential probiotics (Fang et al., 2004).

Quantification was carried out by Yasmin et al. (2020) with slight modifications. A loopful of each bacterial isolate [1% (v/v), 10^9 CFU/mL] was inoculated into 100 mL of Media X (1 % Sucrose, 2 % NaCl, 1g Yeast Extract, and 0.5 g Na2HPO4 in 1000 mL D/W) and incubated on a shaker overnight at 140 rpm at 28 ± 2 °C. The overnight-grown culture broth was adjusted to an optical density (OD) of 0.8 at 600 nm to promote EPS production. The culture suspension was centrifuged (10,000 rpm at 4 °C for 15 min). Supernatant was decanted into a separate tube. EPS extraction was carried out by the precipitation method by adding 3 times the volume of cold ethanol (95–100 %) to the supernatant while stirring and incubating at 4 °C overnight. The culture was centrifuged at 10,000 rpm for 20 min at 4 °C to collect EPS pellets, which were subsequently washed with cold ethanol and resuspended in distilled water. For quantification, 2 mL of the supernatant was mixed with 1 mL of 5 % phenol solution, followed by the addition of 5 mL concentrated H_2SO_4. The resulting yellow-orange colour was measured spectrophotometrically at 490 nm. EPS concentration was then determined using a standard curve prepared with glucose, following the phenol–sulfuric acid method (Dubois et al., 1956).

3.2.2.3.3 Mucus adhesion assay

The adherence ability of 2 bacterial isolates to the mucin layer *in vitro* was assessed by crystal violet assay according to Krausova et al. (2019) with slight alterations. A 10 mg/mL solution of Type III porcine gastric mucin (Sigma-Aldrich) in sterile PBS was used to precoat sterile 96-well microtitre plates by adding 200 µL per well and incubating overnight at 4 °C. After removing the excess mucin solution, the plates were gently rinsed with sterile PBS. A 150 µL suspension of potential probiotic cells, grown overnight and adjusted to an OD_{600} of 0.5, was added to the mucin-coated wells. Adhesion was allowed to occur by incubating the plates at 30 °C under mild agitation (100 rpm) for 2 h. Non- adherent cells were removed by washing the wells three times with PBS. The adherent bacteria were then fixed at 60 °C for 15 min and stained with 150 µL of 0.1 % crystal violet for 30 min, followed by three PBS washes to remove excess

dye. The bound stain was solubilized by adding 150 µL of citrate buffer (pH 4.3) and incubating at room temperature for 45 min. Wells coated with mucin but without bacterial cells served as negative controls. Absorbance was measured at 650 nm using a microtitre plate reader (Bio-Rad, iMark™), and adhesion was quantified by subtracting the absorbance of the negative control from that of the test samples.

3.2.2.4 Assessment of antioxidant activity

3.2.2.4.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay (Athavale & Jirankalgikar, 2023)

There is a growing interest in antioxidants, especially to avert the harmful effects of free radicals in human health. Thus, the DPPH Radical-scavenging assay was performed to determine the antioxidant capacity of bacterial isolates. The overnight grown isolates were centrifuged at 8000 rpm for 8 min at 4 °C to obtain cell-free supernatant. Homogenized solution (3 mL) of freshly prepared 0.3 mM DPPH solution in methanol was added to 1 mL of the CFS and incubated for 40 min by keeping in the dark at RT. Distilled water (1 mL) and DPPH radical (3 mL) served as a control, and methanol was used as a blank. The absorbance was recorded using a UV mini 1240 UV-Vis spectrophotometer at 517 nm and compared with the standard (ascorbic acid as stock,0.1 mg/mL stored in an amber bottle and refrigerated). The experiment was carried out in triplicate, and the average absorbance value was calculated. Radical scavenging activity in percentage was calculated as:

$$\text{Scavenging activity } (\%) = (\text{AC} - \text{AS} \times 100)/\text{AC}$$

Where: A_C= Absorbance of the control; A_S=Absorbance of the Sample

3.2.2.4.2 Reducing power assay

The reducing power of the cell suspension was assessed in triplicate following the method of Lin et al. (2006), which measures the capacity of a compound to convert the Fe^{3+}/ferricyanide complex to its ferrous form (Fe^{2+}). An increase in the absorbance at 700nm of the reaction mixtures indicates an increase in antioxidant activity. The antioxidant compound reacts with potassium ferricyanide, TCA, and ferric chloride to form a coloured complex. The inoculum with

24 h individually grown *Bacillus* sp was centrifuged at 8,000 rpm for 6 min at 5 °C. An aliquot (0.25 mL) containing different volumes of the supernatant (20-100 µL) was mixed with 0.25 mL of 50 mM phosphate buffer (pH 7.0) and 0.25 mL of 1 % (w/v) potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. The reaction was stopped by adding 1 mL of 10 % TCA. After centrifugation at 3,000 rpm for 10 min, 0.5 mL of the resultant supernatant was collected and mixed with 0.5 mL of D/W and 0.1 mL of 0.1 % (w/v) ferric chloride. After allowing the mixture to stand at room temperature for 10 min, the absorbance was measured at 700 nm. An equivalent volume of distilled water instead of the sample was used as the blank. A higher absorbance of the reaction mixture signified a greater reducing power. Ascorbic acid was used as the standard, and results were expressed as ascorbic acid equivalent reducing power (µg AAE/mL) based on a standard curve prepared under identical conditions.

3.3 Identification of potential probiotic isolates

The two *Bacillus* isolates to be used for controlled GC3 fermentation were characterized and identified based on morphological, biochemical, physiological, and molecular characteristics according to Bergey ‘s Manual of Systematic Bacteriology (Claus, 1986).

3.3.1 Morphological and biochemical characteristics

Gram characteristic

Gram staining of the selected cultures was performed using the procedure described by Hans Christian Gram, as per section 3.1.2.

Spore staining

The Schaeffer–Fulton method (1933) was used to stain the Gram-positive cultures (Appendix II) and were microscopically checked for the presence of spores.

Motility test

The hanging drop method by Collins and Lyne (1984) was used to determine the motility of the potential probiotics.

Catalase test

Bacillus cultures were incubated on nutrient agar plates for 24 h. A loopful of the culture was then transferred onto a clean glass slide, and two drops of 3 % hydrogen peroxide were added. The cultures were observed for the production of effervescence, which denoted a positive result (Das et al., 2019).

Oxidase test

The oxidase test was performed using oxidase discs (Hi-Media, DD018). A 10 µL aliquot of the bacterial suspension was applied onto the disc, and the color change was observed. Development of a purple color within 5–10 seconds indicated a positive oxidase reaction, while no color change or a delayed reaction (beyond 10 seconds) was interpreted as oxidase negative.

Methyl Red- Voges Proskauer (MR-VP) test

A 50 µL aliquot of the bacterial suspension was inoculated into 5 mL of MR-VP (Glucose Phosphate Broth; Appendix I) and incubated in a shaker incubator at 120 rpm for 24 h at 28 ± 2 °C. After incubation, the broth culture was divided equally into two sterile vials. To one vial, a few drops of Methyl Red indicator (Appendix II) were added, and the resulting color change from colorless to red or yellow was recorded for the Methyl Red (MR) test. In the second vial, Barritt's Reagent A and Reagent B (Appendix II) were added, and the appearance of a stable pink coloration indicated a positive Voges–Proskauer (VP) reaction.

Citrate utilisation test

The two potential probiotic isolates were streaked on Simmons Citrate Agar (Appendix I) slants and incubated at 28 ± 2 °C for 60 h. Colour change, to blue from green of the medium, was observed.

ONPG Test (β-galactosidase)

Refer to Section 2.1.4

pNPG Test (α-galactosidase)

Extracellular α-Galactosidase assay was carried out qualitatively by determining the enzymatic hydrolysis of the synthetic chromogenic substrate, p-nitrophenyl-α-D-galactopyranoside (pNPG). pNPG on cleavage by α-galactosidase releases p-nitrophenol, which produces a yellow colour under alkaline conditions.

The substrate solution, 5 mM pNPG was prepared by adding 0.150 g of pNPG in 100 mL of 50 mM Sodium phosphate buffer (Appendix II) and stored in the dark at 4 °C. Stop Solution, 0.5 M Sodium Carbonate (Na2CO3) was prepared by adding 53 g of Na2CO3 in 800 mL of distilled water and volume was made up to 1 L with distilled water. The 24 h-grown bacterial cultures were centrifuged at 8,000 rpm for 15 min at 4 °C twice to separate bacterial cells from the culture supernatant. The supernatant was carefully collected and used as the source of extracellular α-galactosidase enzyme. 800 µL of assay buffer was added to each tube, followed by the addition of 100 µL of 5 mM pNPG substrate solution. The supernatant (100 µL) was added to the tubes, mixed thoroughly, and incubated at 28 ± 2 °C for 60 min. The reaction was terminated after incubation by adding 500 µL of stop solution to each tube.

100 µL of heat-inactivated (after boiling for 10 min) supernatant was used as a negative control. 100 µL of α-galactosidase solution was used as a positive control. The lyophilized form of commercial, purified α-galactosidase was reconstituted as per the manufacturer's instructions in DW and appropriately diluted. The presence of a visible yellow color after adding the stop solution, compared to the negative controls, indicated positive α-galactosidase activity.

Carbohydrate utilization test

Studies on carbohydrate utilization of the bacteria were carried out using KB009 HiCarboTM kits (HiMedia KB009A/ KB009B1 / KB009C), which included a total of 35 carbohydrates. The utilization profile was determined as per the manufacturer‘s instructions (Hi-Media Laboratories Pvt. Ltd, India). A pure colony of the bacteria under study was inoculated in 50 mL of Nutrient Broth and incubated on an orbital shaker at room temperature for 16 h. After incubation, 50 µL of the resulting bacterial culture was carefully added to each well of the test strip under aseptic conditions and incubated for 24–36 h at room temperature. The wells were then examined for

any colour change, and interpretations were made following the guidelines provided by the manufacturer.

3.3.2 Physiological characteristics (Effect of salinity, pH, and temperature)

3.3.2.1 Salinity tolerance test

The growth of FSK 2 and FSK 3 at varying NaCl concentrations (0–20 % w/v) in Nutrient Broth was evaluated. Each isolate was inoculated separately and incubated at 28 ± 2 °C for 48 h. Salt tolerance was assessed by measuring the optical density at 600 nm at intervals up to 24 h.

3.3.2.2 pH tolerance

The tolerance of FSK 2 and FSK 3 towards pH was studied by varying the pH of the Nutrient broth from 5 to 8. FSK 2 and FSK 3 were inoculated individually and incubated at 28 ± 2 °C for 48 h. The tolerance of the isolates to varied pH was assessed by recording the absorbance at 600nm up to 24 h.

3.3.2.3 Effect of temperature

The tolerance of FSK 2 and FSK 3 towards various temperatures was studied by varying the incubation temperature after inoculation of FSK 2 and FSK 3 in the Nutrient broth. The temperatures tested were from 5 °C to 65 °C. The temperature tolerance of the isolates was evaluated by measuring optical density at 600 nm over a 24 h period.

3.3.3 Molecular identification by 16S r-RNA gene sequencing

The genomic DNA extraction of the two cultures was performed according to the manufacturer‘s instructions using a Kit (Himedia, MB505) of Hi-Pur A Bacterial Genomic DNA Purification. The isolated genomic DNA was used as a template to amplify the 16S rRNA gene by Polymerase chain reaction using a Biometra thermal cycler (T-Personal 48). The universal bacterial primers for the 16S rRNA gene utilized for the PCR reaction were: forward primer 27F: AGAGTTTGATCCTGGCTCCAG and reverse primer 1492R: TACGGTTACCTTGTTACGACTT. The PCR reaction mixture contained 20 µL MilliQ water,

3 µL template, 1 µL of each primer (forward and reverse), and 25 µL 2X Taq polymerase mix. PCR was performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and additionally, 1 cycle of 7 min at 72 °C. The PCR amplified products were run on a 0.8 % low-melting agarose gel along with StepUp™ 500 bp DNA ladder (Genei, India). The expected 1.5 kb length band was excised using a sterile scalpel and eluted by AxyPrep DNA Gel Extraction Kit following the manufacturer's instructions (Axygen Biosciences, USA). The DNA sequence of the amplified, excised gene segment was obtained from Agri Genome Labs, Pvt. Ltd, India. The obtained raw sequences were used to assemble the contigs using Bio Edit software, version 7.2.5, and aligned. The contigs (approximately 1.5 kb) were matched with the GenBank database using the nucleotide Basic Local Alignment Search Tool facility of the National Center for Biotechnology Information (NCBI-BLAST). Based on the maximum identity score, the first ten sequences were selected and aligned using the multiple alignment software program Clustal W. A distance matrix was generated, and the phylogenetic tree was constructed using MEGA 11 software (Tamura et al., 2021) using the Neighbour-joining method. The evolutionary distances were computed by the Tamura-Nei method with 1000 bootstrap replicates. The analysis included 21 nucleotide sequences. The positions consisting of less than 95 % site coverage were eliminated. Therefore, fewer than 5 % alignment gaps, ambiguous bases, and missing data were allowed at any position. The sequences of the two isolates have been deposited in GenBank.

3.4 Study of shelf life upon storage of *Bacillus* culture at low and room temperature

Based on the probiotic attributes studied, FSK 3 was shortlisted as a potential probiotic to be used in controlled fermentation of GC3; thus, its shelf life was studied. FSK 3 was inoculated in 50 mL of sterile Nutrient broth (25 %), incubated at 28 ± 2 °C overnight on a shaker at 120 rpm. The broth was centrifuged at 8,000 rpm for 6 min, the pellet obtained was washed twice with sterile PBS, and subjected to lyophilization (Scan Vac Coolsafe Lyophilizer, Labogene, Denmark) to obtain the powdered form. The powder was transferred to tubes to be stored at two different temperatures (4 °C and 28 ± 2 °C) for a shelf-life study. Its viable count (CFU/mL) was determined after resuspending in sterile saline on 0-day, and thereafter at monthly intervals for

twelve months by the spread plating method on Nutrient agar (Davis, 2014). The % survival at the end of 12 months of storage was determined as follows.

Survival % = Log CFU of cells after storage / Log CFU of cells before storage X 100

Phase II

3.5 Analysis of proximate /nutritional composition of Goa cowpea 3 (*Vigna unguiculata*) cultivar seeds

3.5.1 Procurement and milling

Clean and dry seeds of the Goa cowpea Cultivar 3 (GC3) were procured from the Indian Council of Agricultural Research (ICAR), Ela, Old Goa. They were milled into a fine powder using a commercial cyclone mill (UDY Corporation, USA) and stored under refrigeration at 5 °C in airtight containers until further analysis.

3.5.2 Proximate composition

3.5.2.1 Moisture (AOAC 950.46)

In a hot air oven, the water content of the sample gets reduced by heating, resulting in the loss of weight that can be determined by percentage loss. Weighed sample (10 g) was taken in a clean, dry, and pre-weighed petri dish, which was then kept in an air oven at a temperature of 110 ± 5 °C and cooled in a desiccator. The process of heating and cooling was repeated to achieve a constant weight. Moisture content was calculated as follows:

$$\% \text{ Moisture } = (\text{Loss in weight of the sample})/(\text{Weight of sample before drying}) \text{ X } 100$$

3.5.2.2 Ash (dry ashing procedure)

The ash content is determined based on the loss of weight occurring during complete oxidation of the sample at a high temperature by volatilization of organic materials. Pre-weighed (5 g) moisture-free sample was placed in a dry, clean crucible and incinerated at 550 ± 50 °C in a

muffle furnace for 4 h. The crucible containing ash was then cooled in a desiccator and weighed. The ash content was calculated as follows:

$$\% \text{ Ash} = (\text{Weight of ash})/(\text{Weight of sample before ashing}) \text{ X } 100$$

3.5.2.3 Crude protein (Kjeldahl Method) (AOAC 954.01, 2010; Goyal et al., 2022)

The method measures total organic nitrogen in the sample and involves 3 steps of decomposition, distillation, and titration. Protein was estimated using an automated FOSS Kjeltec TM 8400 analyser. A moisture free sample (500 mg) was digested by transferring to a digestion tube with an acidic medium of 5 mL of concentrated sulphuric acid and a pinch of digestion catalyst mixture [Copper sulphate (1 g), Potassium sulphate (5 g), sodium selenite (0.1 g)] to convert nitrogen in the food into ammonia (as ammonium sulphate) and other organic matter to carbon dioxide and water. The contents were then digested at 350 ℃ until the solution became very clear. The digested sample was made alkaline by adding NaOH, which converts ammonium sulphate into ammonia. The solution was then distilled in a Kjeldahl plus distillation unit for 7 min. Ammonia liberated during distillation was trapped in a flask containing 20 mL of 4 % boric acid solution and Kjeldahl indicator [Methyl red (30 mg), Bromocresol green (20 mg) mixed in 40 mL 90 % ethanol]. This boric acid was then back-titrated against a standard sulphuric acid solution (0.06 N) to achieve the end point of light pink. Ammonium sulphate was used as a standard. The crude protein content was then determined by using a conversion factor of 6.25 (equivalent to 0.16 g Nitrogen/ g of protein).

% Nitrogen and % Protein were calculated as follows:

$$\% \text{ Nitrogen} = (14 \times \text{Normality of acid} \times \text{Titre value}) / (\text{Weight of sample (mg)}) \times 100$$

$$\% \text{ Protein} = \% \text{ Nitrogen} \times 6.25$$

3.5.2.4 Fat- Soxhlet's method (AOAC, 2000)

Fat extraction of the sample was carried out in petroleum ether solvent in the Soxhlet's extraction apparatus. A moisture-free sample of 10 g powder was taken in a cellulose extraction thimble (25 mm × 80 mm, Whatman®) that was placed in the Soxhlet's apparatus, and the sample was extracted under reflux for 15 h. Ether was used as a solvent for fat extraction. After the extraction,

the petroleum ether in the flask was evaporated using a rotary vacuum solvent evaporator system. The flask with the residue was then flushed with nitrogen, evaporated till dry, and weighed. Percent fat was calculated as follows:

$$\% \text{ Fat} = (W2 - W1)/W \text{ X } 100$$

Where: W1 = weight of empty flask

W2 = weight of flask after evaporation of petroleum ether

W = weight of sample

3.5.2.5 Dietary fibre

The content of total dietary fibre (TDF), insoluble (IDF), and soluble dietary fibre (SDF) of the dried and defatted GC3 powder using the solvent ether extraction method was determined using an enzymatic kit method of Sigma Aldrich in triplicate. The food sample was gelatinised at ~ 95 °C and then subjected to heat-stable amylase, followed by protease, and finally with amyloglucosidase incubation to hydrolyze carbohydrate, protein, and starch, and treated with ethanol to precipitate soluble fiber and remove depolymerised protein and glucose (from starch). Samples (1 g) in triplicate were accurately weighed into 400 mL beakers, and 50 mL of 0.08 M phosphate buffer (pH 6.2) was added to each beaker. The samples were stirred on a magnetic stirrer until completely dissolved. 50 µL heat-stable α-amylase solution was then added, while stirring at low speed. The samples were covered with aluminum foil and placed in a shaking water bath at 95-100 °C and incubated for 20 min with continuous gentle agitation. After incubation, the beakers were cooled to 60 °C. Protease solution (100 µL) was added to each sample, and the beakers were incubated in a shaking water bath at 60 ± 1 °C, with continuous agitation for 30 min. 5 mL of 0.56 1 N HCl solution was then dispensed into the sample while stirring. This was followed by the addition of 300 µL amyloglucosidase solution while stirring on a magnetic stirrer, and the samples were incubated again in a shaking water bath at 60 °C for 30 min with constant agitation.

IDF: After enzymatic treatment, the filtered residue from the sample was analyzed for protein, and the residue from the triplicate sample for ash. IDF value is obtained after correcting for the protein and ash content of the residue. Crucibles containing residue were dried overnight in a hot air oven at 100 °C, cooled in a desiccator, and weighed to determine the weight of IDF residue.

A duplicate residue was analyzed for protein (N x 6.25) by the Kjeldahl method, and another residue was incinerated at 525 °C for 4 h for ash.

SDF: SDF is precipitated from the leftover filtrate obtained from the above step with the addition of four volumes of 95 % ethanol. Clean crucibles containing 0.5 g Celite were tared to the nearest 0.1 g after cooling in a desiccator. The weight of the celite and the crucible together was recorded. The solution obtained was filtered through sintered glass crucibles containing celite as an even mat. Precipitate was transferred quantitatively from the enzyme digest to the crucible and washed with 20 mL of 78 % ethanol and 10 mL of 95 % ethanol, and finally with 10 mL of acetone. Filtered residue from one sample is analyzed for protein, and the other is incinerated at 525 °C to determine ash. SDF value of the sample is the weight of the residue corrected for the weight of protein and ash.

TDF: The Sum of IDF and SDF gives the TDF content of the sample after subtracting the reagent blank value. A reagent blank was run along with the samples. The contribution of any of the reagents to the residue was determined in the blank without a sample, and the measured blank value was deducted from the sample.

Calculations:

$$\text{Blank (\%)} = \text{Wt. of the blank residue} - [\text{protein* in blank residue} + \text{ash* in blank residue}] \times 100$$

$$\text{IDF (\%)} = ([\text{Wt. of the residue} - [(\text{protein in IDF residue} + \text{ash in IDF residue}) - \text{Blank}])/(\text{Weight of the sample taken (g)}) \times 100$$

$$\text{SDF (\%)} = \frac{[\text{Wt. of the SDF residue-(protein in SDF residue + ash in SDF residue) - Blank}]}{\text{Weight of the sample taken (g)}} \times 100$$

$$\text{TDF (\%)} = \text{IDF} + \text{SDF}$$

* Protein is due to enzymes, and ash is due to the buffer

3.5.2.6 Carbohydrate

The differential method was used for indirect calculation of the total carbohydrate content of the GC3 sample as:

$$\text{Total carbohydrate} = 100 - (\text{moisture} + \text{ash} + \text{fat} + \text{total protein} + \text{total dietary fiber})$$

3.5.3 Estimation of fatty acids

Fatty acid methyl esters (FAMEs) (Sigma Aldrich) were prepared according to the standard of International Union of Pure and Applied Chemistry (IUPAC) method 2.301 (Oluwatosin,1998) with slight modifications. Fat was extracted by using the protocol in section 3.4.2.4. Methanolic HCl (1 mL) was added to the dried lipid extract, followed by the addition of internal standards, and incubated in a water bath at 60 – 80 ℃ for 1 - 2 h in sealed vials. After cooling, 1 mL of hexane and 1 mL of water were added to the reaction mixture. The mixture was vortexed to allow phase separation. The upper hexane phase containing FAMEs was collected and dried over anhydrous sodium sulfate, followed by its concentration under nitrogen to ~100 µL for GC analysis. The fatty acid composition of the sample in triplicate was determined by the AOAC (2000) method using Gas Chromatography with flame ionisation detector (FID) with separation carried out in a fused silica column (HP 88 100 m x 0.250 mm x 0.25 µm) of Agilent, J & W. The temperature of the injection and detection ports was maintained at 260 ℃. A sample volume of 1.0 µL was injected with the split ratio of 1:75. The initial temperature of 100 ℃ was increased at the rate of 4 ℃ per min and maintained at 226 ℃ for 20 min. Individual fatty acids were identified and quantified by comparing the retention time and peak area with authentic FAMEs. The fatty acid composition was reported as a relative percentage of the total peak area, and the results were calculated as mg/100 g of dry matter.

3.5.4 Estimation of total amino acids (TAAs)

Briefly, the estimation of amino acid content in GC3 powder involved the sample preparation followed by the analysis of chromatographic data and was carried out by the method described by Gauthankar et al. (2021) with slight modifications using HPLC Agilent 1260 Infinity System (Agilent Technologies Inc., USA). The HPLC system is equipped with a quaternary pump, an autosampler, a column compartment, and a diode array detector (DAD). The analysis was performed using an Agilent ZORBAX Eclipse plus C18 Column (4.6 × 250 mm with 5 µm packing particle, Agilent Technologies Inc., USA). For the derivatization of the primary amino acids, O-phthalaldehyde was used. The mobile phase A, comprised 10 mM Na_2HPO_4: 10 mM $Na_2B_4O_7$: 5 mM NaN_3 (pH 8.2), whereas the mobile phase B, comprised of the mixture containing acetonitrile: methanol: water in the ratio of 45:45:10 (v: v: v).

During the separation process, a flow rate of 1.5 mL/min was maintained. The primary amino acids in the samples were quantified by using alpha-aminobutyric acid as an internal standard. For the quantification of TAA content, 1g of finely ground seed powder was hydrolysed with 2 mL of 6 N HCl (EMPARTA ~ 36 %, Merck Life Science Pvt. Ltd., India) in an amber-coloured vial. The sample vials were spiked with 200 µL of internal standard (10 nm/µL ABA, Sigma-Aldrich, USA), purged with N_2 gas (2 m), screw-capped, and then kept at 110 °C for 24 h. Post-hydrolysis, the supernatants of GC3 were neutralised by the addition of 2 mL of 6 N NaOH and dried in a rotary vacuum evaporator. Dried samples were reconstituted in 2 mL of deionised water and injected into an HPLC system (Agilent 1260 Infinity, USA for understanding the composition of TAA. Each quantified amino acid was expressed as g/100 g of protein estimated in GC3 powder.

3.5.5 Quantification of water-soluble vitamins

Water-soluble Vitamins (B2, B3, B5, B6, B9) were analysed in the GC3 seed flour sample using Ultra-HPLC (U-HPLC; Dionex Ultimate 3000 RSLC, USA, with Chromeleon software). The standards were of Merck KGaA, Darmstadt, Germany.

3.5.5.1 Vitamin B2 and B3 estimation (Sebastian et al., 2023)

Vitamins B2 and B3 were analysed by extracting 1 g of sample with 0.1 M HCl and centrifuging the samples at 4000 rpm at 10 °C for 8 min, followed by filtration. Supernatant was collected into amber-coloured HPLC auto-sampler vials using a 0.45 μm polyvinylidene fluoride (PVDF) syringe filter. The vitamin was separated on a reverse-phase chromatographic column (Thermo Scientific BDS Hypersil C18 column 250 × 4.6 mm; particle size, 5 μm) by maintaining 40 °C. Phosphate buffer (0.05 M, pH 3.2) and acetonitrile at a ratio of 70:30 (v/v) were used as the mobile phase for the quantification of vitamin B2 with a flow rate of 1 mL/min. A fluorescence detector (FLD) was used with an excitation and emission wavelength of 445 and 522 nm, respectively. Vitamin B3 was quantified using a mobile phase of phosphate buffer (25 mM, pH 3.02) and acetonitrile at the ratio of 95:5 (v/v) with a flow rate of 1 ml/min and detected using a photodiode array (PDA) detector at 260 nm.

3.5.5.2 Vitamin B5 estimation

Vitamin B5 was determined using the method reported by Sebastian et al. (2023), where the sample (1 g) was extracted with 3 % acetic acid. Samples were centrifuged at 4000 rpm for 10 min (10 °C), and the supernatant was collected into HPLC auto-sampler vials after filtration through a 0.45 μm PVDF syringe filter. Phosphate buffer (0.1 M, pH 2.25) and acetonitrile (95:5) were used as mobile phase at a flow rate of 1 mL/min. The sample was passed through a Thermo Scientific BDS Hypersil C18 column (250 × 4.6 mm, 5 μm), maintaining a temperature of 40 °C, and the vitamin was detected using a PDA detector at 205 nm.

3.5.5.3 Vitamin B6 estimation

Vitamin B6 was quantified as per the method reported by Valls et al. (2001) and Chyne et al. (2019). The sample (1 g) was extracted using 5 % metaphosphoric acid, followed by centrifugation at 4000 rpm (~1500 g) for 10 min at 10 °C. The supernatant was collected and injected into the U-HPLC after filtration using a 0.45 μm PVDF syringe filter. Thermo Scientific BDS Hypersil C18 column (250 × 4.6 mm; 5 μm) was used for the separation, while the mobile phase consisted of 25 mM phosphate buffer (pH 3.2) and acetonitrile (97:3 v/v) with a flow rate of 0.8 mL/min. The B6 vitamers, such as pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM), were detected using a fluorescence detector with the excitation and emission wavelengths of 290 and 395 nm, respectively. The total Vitamin B6 was calculated using the formula:

$$\text{T}otal\ B6\ (mg/100\ g) = PM + (1.01 \times PL) + (0.79 \times PN)$$

3.5.5.4 Vitamin B9 estimation

Vitamin B9 was analysed by the method of De Brouwer et al. (2008) with slight modifications. B9 was extracted using a buffer containing dipotassium hydrogen phosphate, ascorbic acid, sodium azide, and 2-mercaptoethanol at pH 7.2. The extract was treated with tri-enzyme (α-amylase, protease & deconjugase) to convert folates into their different forms. Extract was purified through a strong anion exchange cartridge (SEP-PAK cartridge) and analysed by U-HPLC (Dionex Ultimate 3000 RSLC).

3.5.6 Quantification of fat-soluble vitamins

All the chemicals used in the study were of analytical grade, and the solvents such as acetonitrile and methanol were HPLC grade. The chemicals and solvents were procured from Merck (Merck, India). Standards used in the study, namely vitamin E**,** vitamin K1 (viscous liquid) were procured from Sigma Aldrich (Missouri, United States). All-trans β-carotene, lutein, zeaxanthin, ammonium acetate (analytical grade), and HPLC mobile phase Methyl-Tert-Butyl Ether (MTBE) were obtained from Sigma Company (St Louis, MO, USA). Milli Q water was from Water Purification Systems, while Vitamin E standards (α-tocopherol and β-tocopherol) were obtained from Chroma Dex (California, United States). Methanol and Tetrahydrofuran (THF) were purchased from JT Baker Chemical (Philipsburg, NJ), USA.

3.5.6.1 Quantification of total and individual carotenoid content

All the standards were procured from DHI, Denmark. The method described by Rodriguez-Amaya & Kimura (2004) was used to quantify carotenoids with slight modifications. 15 mL of methanolic KOH was used for saponification and extraction of carotenoids from 3 g of the sample. Petroleum ether (15 mL) was added to separate the extracted layer four times. The combined extract was then concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R100) and redissolved in 2 mL of petroleum ether. The absorbance of the redissolved solution was measured at 450nm for the estimation of total carotenoids using a spectrophotometer (Analytikjena U2800 SPECORD S.600, Germany).

The individual carotenoids, lutein, zeaxanthin, and β-carotene, however, were measured using HPLC (Dionex Ultimate 3000 RSLC, USA) with a C18 Thermo BDS column (150 4.6 mm, 5 μm) and a DAD at 450 nm. For the quantification of individual carotenoids, the extracted solution was reconstituted with chloroform (3 mL), filtered using a 0.2 μm PVDF syringe filter, and injected into the U-HPLC for separation. The total carotenoid content was calculated using the equation:

$$\text{Total carotenoid content} = A \times volume \times 10^{4} AE \times s\ ample\ weight \times 100$$

Where: A = absorbance of the sample, volume = total volume of extract,

AE = absorption coefficient of β-carotene in petroleum ether

The outcomes were given in µg/100 g of sample. The quantification of carotenoids was performed by calculating the peak areas in the high-performance liquid chromatograms, calibrated against known concentrations of standards. Each carotenoid peak was identified and confirmed based on the retention time and characteristic absorption spectra of the respective standards.

3.5.6.2 Vitamin E estimation

Methods by McMurray et al. (1980) and Bonvehi et al. (2000), with slight modifications, were followed to quantify the amount of Vitamin E (tocopherols and trienols) in the sample. The methods used were initial saponification of the sample, followed by extraction of the tocopherol into hexane. Milled sample (3 g) was weighed into 125 mL amber colour reagent bottles and heated for 30 min with 20 mL 5 % pyrogallol in ethanol and 5 mL 60 % KOH at 70 °C in a water bath. After cooling, 5 mL of 20 % NaCl and 40 mL hexane were added, and the bottles were kept on a magnetic stirrer for 30 min. The hexane layers were transferred to 150 mL round-bottom flasks, and the samples were re-extracted with an additional 20 mL of hexane by keeping for magnetic stirring for 15 min, and the hexane layers were combined with those from the first extraction. Hexane was then evaporated under a rotary evaporator and reconstituted with 2 mL of hexane. These concentrated extracts were used for HPLC (U-HPLC; Dionex Ultimate 3000 RSLC, USA, with Chromeleon software). The standards were obtained from Chroma Dex (California, United States).

3.5.6.3 Vitamin K estimation (Jakob & Elmadfa, 1996)

Vitamin K_1 (Phylloquinone) was quantified using HPLC 2 g of a wet fresh sample was placed into 250 mL amber-coloured bottles, to which 100 mL of dichloromethane: methanol mixture (2:1) was added and kept for stirring (30 min). After 5 min. of centrifuging the samples at 3,000 rpm, the supernatant was collected and dried using a flash evaporator. The dried sample was redissolved in 20 mL of 100 % n-hexane to which an equal volume of a water: methanol mixture in the ratio of 1:9 was added, and vortexed for 2 min. From this, the upper hexane layer was collected and evaporated till dry using a flash evaporator. The extract was reconstituted by adding 2 mL of 25 % 2-propanol in methanol (mobile phase) and filtered through a 0.2-micron syringe filter and injected into HPLC.

3.5.7 Estimation of minerals

The mineral content was analysed by the AOAC (968.08) method. Milled samples were digested in a microwave (MARS 6, CEM Corporation, USA) using Suprapure nitric acid (65 %) and hydrogen peroxide (30 %) at a ratio of 3:1 (v/v). The contents were made up to 25 mL using Milli-Q water in volumetric flasks. Elements including zinc (Zn), iron (Fe), copper (Cu), calcium (Ca), magnesium (Mg), manganese (Mn), and potassium (K) were quantified using a flame-atomic absorption spectrophotometer (Analytikjena ContrAA 700, Germany) operated with Aspect CS 2.2.1.0 tech software with absorbance taken at wavelengths 248.32 nm (Fe), 213.86 nm (Zn), 324.75 nm (Cu), 279.48 nm (Mn), 766.49 nm (K), 285.21 nm (Mg) and 422.67 nm (Ca). Linearity range and limits of detection were ascertained using a multi-element standard diluted up to 5 µg/mL. Phosphorus was analysed by the modified Fiske and Subbarow method (AOAC 931.01, 2010) using a spectrophotometer (Analytikjena–U-2800 SPECORD S.600, Germany) at 660 nm.

3.5.8 Analysis of oligosaccharides (stachyose, verbascose, raffinose)

The method of Sanchez–Meta et al. (1998), with some modifications, was used. Separation of oligosaccharides was achieved on an HPLC system (Waters 2695 module, Milford, Massachusetts, United States) equipped with a refractive index detector (RID), controlled by Empower version 3.0 software fitted with a Supelco NH2 polymer column (5µm, 25cm x 46mm) and maintained at 40 °C. GC3 powder (0.5 g) was mixed with 10 mL, 70 % ethanol, vortexed, and centrifuged (4,000 rpm, 10 min). The supernatant was collected, and again 70 % ethanol was added, and the same procedure was repeated thrice. The pooled supernatant was mixed with 10 % lead acetate to precipitate protein. The supernatant was again centrifuged (4000 rpm, 10 min at 10 °C), and 0.5 mL of 10 % oxalic acid was added. The solution was evaporated using a rotary vacuum evaporator (BUCHI, R-215, Switzerland). Milli Q water (5 mL) was added to the residue and filtered with PVDF (0.2 micron) filters into HPLC vials. The samples were run in HPLC with ACN: Water (60:40) as mobile phase with a flow rate of 1 mL/min.

3.6 Study of physical and cooking properties of GC3 seeds

3.6.1 Physical Properties

A month-old harvested, undamaged, clean, and dry GC3 seeds were studied for physical properties.

3.6.1.2 Seed dimensions

The main seed dimensions, viz. length (L), width (W), and thickness (T) of dry seeds,3 h soaked seeds, and pressure-cooked seeds (10 min), were studied using a SSEA stainless iron nickel vernier calliper to an accuracy of 0.05 mm. Measurements were recorded as the average of 20 determinations of dry seeds before and after soaking and cooking.

3.6.1.3 Geometric mean diameter

The Geometric mean diameter of the dry, soaked, and cooked seeds as above was determined based on the average of 20 seed measurements using the three principal axial dimensions: length (L), width (W), and thickness (T). The following formula was used:

$$Dg = (LWT)^{(1/3)}$$

Where: Dg = Geometric mean diameter (mm)

L = Length (mm)

W = Width (mm)

T = Thickness (mm)

3.6.1.4 Sphericity

The sphericity of the seed indicates the ratio of the surface area of the sphere with a volume similar to the seed, to the surface area of the seed. Sphericity (ϕ) of raw seeds was calculated as a function of the three principal dimensions using the following formula, following Mohsenin (1986), and reported as a percentage.

$$\phi = \frac{Dg}{L} x\ 100$$

Where: Dg = geometric mean diameter

L = length

ϕ = a dimensionless number between 0 and 1.

3.6.1.5 Length: Breadth ratio

Length: breadth ratio of 10 randomly selected seeds (raw and soaked seeds) was ascertained by dividing the cumulative length by breadth (AACC, 2000) as measured using callipers. The average of ten determinations is reported.

3.6.1.6 Thousand seed weight

Thousand seed weight was estimated by manually counting and weighing 1000 seeds using an electronic balance, as reported by Sebastian et al. (2023). Knowing the 1000 seed weight is important in agriculture and seed science, which helps to determine the seed processing and handling; seeding rate calculation, seed quality, as heavier seeds often indicate better filling and higher vigor, suggesting better germination potential and seedling strength. It also helps estimate potential grain yield, breeding, and selection.

3.6.1.7 Seed bulk density

Loose seed bulk density (Pb) in g/mL was measured using the mass-to-volume ratio, following the procedure of Hamid et al. (2016). A known volume of seeds (100 mL) was poured gently into a graduated cylinder without tapping. The mass of the seeds was recorded using a digital balance, and bulk density was calculated as:

Pb = Mass of seeds (g) / Volume (mL)

3.6.1.8 Colour

The surface colour of seeds was analysed by CIE color scales in the values of Hunter *L* (lightness), *a* (redness to greenness), and *b* (yellowness to blueness) using a portable Hunter Lab Spectro colorimeter (Miniscan XETM, Hunter Associates Laboratory Inc., Reston, Virginia, USA) according to AACC, 2000. The degree of lightness or darkness was indicated by L ∗ with values varying from 0 (black) to 100 (white). The degree of redness to greenness was indicated by a ∗ from (+a) to (-a) respectively, while the degree of yellowness to blueness was indicated by b ∗ from (+b) to (-b) respectively. Auto measurement mode was followed at 30 s, 60 s, and 120 s intervals, and the average value was given.

3.6.2 Cooking properties

Cooking and textural properties of the GC3 seeds were evaluated to assess their culinary quality and consumer acceptability.

3.6.2.1 Hydration capacity and Hydration index

Cooking properties depend on hydration. The hydration capacity and index were determined by soaking 100 seeds in 500 mL of distilled water in a measuring beaker at room temperature and covered with an aluminium foil for 12 h. The increase in weight was calculated after every 3 h after draining, and excess water was removed using tissue paper. Hydration capacity and hydration index were calculated using the following formulas (Hamid et al., 2016).

$$\text{Hydration Capacity (g/seed)} = (W1 - W0)/N$$

Where: W_0 = Initial weight of dry seeds (g) , W_1 = Weight of seeds after soaking (g) and N = Number of seeds

The hydration index shows the relative increase in weight due to water absorption.

$$\text{Hydration Index} = (W1 - W0)/W0$$

Where: W_0 = Initial weight of dry seeds (g) and W_1 = Weight after soaking (g)

3.6.2.2 Swelling capacity and Swelling index

Swelling capacity and swelling index of GC3 seeds were determined using a volumetric method based on a count of 100 seeds by modifying the protocol of Hamid et al (2016). Dry seeds were placed in a 500 mL graduated cylinder, and the initial volume (V_0) occupied by the seeds was recorded without tapping. Then, 100 mL of distilled water was added to the cylinder, ensuring the seeds were completely submerged. The seeds were soaked at room temperature (25 ± 2 °C) for 24 h. After soaking, the excess water was carefully decanted, and the final volume (V_1) of the swollen seeds was recorded. The experiment was conducted in triplicate to ensure accuracy and reproducibility.

The swelling capacity (mL/seed) and swelling index were calculated using the formula:

$$\text{Swelling capacity} = \frac{V1-V0}{100}$$

$$\text{Swelling index} = \frac{V1}{V0}$$

Where V_0 is the initial volume of the 100 dry seeds (in mL) and V_1 is the volume after soaking.

3.6.2.3 Cooking time

Unsoaked and soaked GC3 seeds were cooked, and the cooking time was determined as suggested by Hamid et al. (2016) with slight modifications using the standard boiling method and recording the time required for the seeds to become soft under consistent heating conditions. GC3 seeds (1000 seeds) were taken in a 500 mL flask with 400 mL of distilled water and pressure-cooked. Time taken for pressure cooking for one whistle and three whistles was also noted. Each treatment was conducted in triplicate to ensure accuracy.

3.6.2.4 Cooked length–breadth ratio

The cumulative length and breadth of 10 GC3 seeds were measured after cooking the soaked seeds to the minimum cooking time as per section 3.5.2.3.

3.6.2.5 Water uptake ratio (WUR)

The water uptake ratio of GC3 seeds was determined as per Sebastian et al. (2023) with slight modification before and after cooking both unsoaked and soaked seeds. Unsoaked seeds were cooked for 25 min, while soaked seeds were cooked for 10 min under standardized conditions. After cooking, excess water was drained, and the surface moisture was gently removed using filter paper. The seeds were then immediately weighed. The water uptake ratio was calculated as the ratio of the weight gained after cooking to the initial weight before cooking, using the following formula:

$$\mathrm{WUR} = \frac{\mathrm{Wc} - \mathrm{Wi}}{\mathrm{Wi}}$$

Where: Wi = Initial weight of seeds before cooking, and Wc = Weight of seeds after cooking.

3.6.2.6 Gruel solid loss

Gruel solid loss (%) was estimated by pressure-cooking 100 seeds (both soaked and unsoaked) in 100 mL of double-distilled water for 10 min by modifying the method of Hamid et al. (2016). After cooking, the resulting gruel (cooking water) was carefully transferred into pre-weighed beakers and evaporated in a hot air oven at 90 °C until the solids were completely dried. The dried residue was then weighed, and the gruel solid loss was calculated as a percentage of the initial seed weight using the following formula.

$$\text{Gruel Solid Loss (\%)} = \frac{(\text{Initial weight of seeds} - \text{Weight of dried solids})}{100}$$

3.6.2.7 Water absorption capacity (WAC)

The WAC of GC3 whole seed powder was determined using a standard centrifugation method. GC3 powder (1.0 g) was weighed into a clean, dry centrifuge tube, and 10 mL of distilled water was added. The mixture was stirred thoroughly to ensure uniform wetting and allowed to stand at room temperature for 30 min, with gentle mixing every 10 min. After the standing period, the mixture was centrifuged at 3000 rpm for 15 min with careful decanting of the supernatant. The tube containing the hydrated sample was weighed. All measurements were performed in triplicate, and the average value was recorded. WAC was calculated as the amount of water retained per gram of dry powder using the formula:

$$\text{WAC (g /g)} = \frac{W2-W1}{W0}$$

Where: W0 = Weight of dry GC3 powder (g)

W1 = Weight of empty tube (g)

W2 = Weight of tube + hydrated sample after decanting (g)

3.6.2.8 Texture analysis

Textural properties such as hardness, chewiness, cohesiveness, and adhesiveness of 3 h soaked and 10-min pressure-cooked GC3 seeds were evaluated following the method of Hamid et al. (2016) using a Texture Analyser (IMADA, Model FRTS-5N) equipped with a cylindrical probe (P/36R) and a 5 kg load cell. Seed samples were cooled to room temperature (25 ± 2 °C) before testing. Intact seeds of uniform size were individually placed on the analyser platform. The test was performed in compression mode with a two-cycle sequence, using pre-test, test, and post-test speeds of 1.0 mm/s. Each seed was compressed to 50 % of its original height with a 5 g trigger force, and a 5-second interval was maintained between the two compressions. Force–time curves generated were analyzed using Texture Exponent software to determine hardness, cohesiveness, chewiness, adhesiveness, and springiness. Each treatment was replicated ten times, and results were expressed as mean ± standard deviation.

Phase III

3.7 Optimisation of fermentation and evaluation of the efficacy of GC3 as a probiotic carrier

3.7.1 Slurry preparation and inoculation of the starter culture

A slurry was prepared using GC3 powder with different concentrations for fermentation. The final quantity of powder was set to 10 % level (w/v) by mixing with distilled water and supplemented with 0.5 % natural salt (pH 7). The slurry was then autoclaved at 15 psi for 15 min at 121 °C and cooled before inoculation**.**

3.7.2 Potential probiotic microorganism, inoculation, and fermentation

The potential probiotic *Bacillus licheniformis* PQ098138 (test culture) isolated from the Goan saltpan was used to ferment the GC3 slurry. The standard probiotic microorganism *Lactobacillus plantarum* MTCC 2621 (Microbial Type Culture Collection, Chandigarh, Haryana, India) was used as a positive control based on its efficacy reported in literature to determine viable count and pH only. The standard organism was inoculated in MRS broth, whereas the test culture was inoculated in Nutrient broth (25 %), incubated at 28 ± 2 °C for 24 h in an orbital shaker incubator at 140 rpm, and centrifuged at 8000 rpm at 10 °C for 8 min. The cells were washed using saline (0.85 %) and then re-suspended in the same. The individual cultures were appropriately diluted and inoculated in slurries to obtain an initial cell count of approximately 4 log CFU/mL slurry (4.28 log CFU/mL). The slurries were incubated at 28 ± 2 °C for 24 h in an orbital shaker incubator at 160 rpm to facilitate fermentation.

3.7.2.1 Enumeration of viable count

The enumeration of potential probiotic and standard probiotic microorganisms in the fermented slurries was carried out by the spread plating technique at 4 h intervals up to 24 h on Nutrient agar (HiMedia Laboratories, India). Fermented slurry (1 mL) was transferred aseptically into a test tube containing 9 mL saline (0.85 %). Decimal dilutions were prepared according to the microbial load expected. Diluted inocula (0.1 mL) were used to spread plate in triplicate. The plates were then incubated at 28 ± 2 °C for 24 h, and the colonies obtained on each plate were counted and recorded as log CFU/ mL slurry.

3.8 Analysis of the physicochemical properties of GC3 slurry during fermentation

3.8.1 Measurement of pH

The pH of the slurry was measured during fermentation using a digital pH meter (EUTECH, Singapore) at 4 h intervals up to 24 h and thereafter at 24 h intervals up to 72 h.

3.8.2 Estimation of total titratable acidity (TTA) (AOAC, 2000)

It was measured by reacting the acids present in the food sample with a base (NaOH) to an end point, close to neutrality, as indicated by a pH-sensitive colour indicator at 4 h intervals p to 24 h and thereafter at 24 h intervals up to 72 h. The slurry (1 mL) was diluted with 9 mL of distilled water and titrated against a standard solution of 0.01 N NaOH using phenolphthalein (0.2 % in ethanol) as the indicator. Lactic acid was calculated as follows:

$$\text{Lactic acid } (\text{g } \%) = (\text{A x N x } 0.090 \text{ x } 100)/V$$

Where: A= titre value (mL), N= normality of NaOH used and V = volume of sample (mL)

3.8.3 Estimation of organic acids and short-chain fatty acids (SCFAs)

The metabolic activity of *Bacillus* during fermentation results in the production of organic acids and SCFAs (Elshaghabee et al., 2017). Lactic acid, acetic acid, propionic acid, butyric acid and valeric acid are the major acids produced; hence their content in the fermented slurries was determined at 4 h interval up to 24 h following Zhang et al. (2020) using Gas Chromatography (GC) (Agilent 8890) with DB-FFAP column; a nitro terephthalic acid modified polyethylene glycol (PEG) column of high polarity for the analysis of volatile fatty acids, 30 m long with FID at a flow rate of 1 mL/min. The standard was prepared by weighing 50 mg of the compound and dissolving it in 10 mL of ACN to make a 5000-ppm solution. From a 5000-ppm solution, different concentrations of the solution were prepared by pipetting out 0.2 mL of the 5000-ppm solution into the 10 mL flask and 9.8 mL of ACN to make 100 ppm. Similarly, solutions of 200 ppm, 300 ppm, 400 ppm, and 500 ppm concentrations were prepared. Each sample was weighed (~ 500 mL), vortexed with 1 mol L−1 HCl, and sonicated for 10 min in an ice water bath. Subsequently, the sample was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was pipetted into a tube, extracted with 2 mL ethyl acetate for 3 min, and recentrifuged at 12,000 rpm. Lastly, the supernatant was filtered with a 0.22 μm organic filter membrane and injected into the GC.

3.8.4 Measurement of viscosity during fermentation (Rodrigues et al., 2014)

A Brookfield Viscometer (DV-I + Viscometer, Brookfield Engineering Laboratories, MA, USA) with a 7-number spindle set at 60 RPM at 28 ± 2 °C was used to measure the viscosity of GC3

slurry at different fermentation times. The spindle was introduced into the slurry (approximately 80 mL), placed in a 100 mL capacity beaker, taking care to see that it does not touch the sides and base of the beaker. The viscosity was recorded in millipascal second (mPa.s) at 4 h intervals up to 24 h and thereafter at 24 h intervals up to 72 h.

3.8.5 Determination of protein content (Lowry et al., 1951)

This method is a well-established colorimetric assay that relies upon the reaction of proteins with copper ions and the Folin-Ciocalteu reagent. Lowry Reagents (Reagent A, Reagent B, Reagent C, and Folin-Ciocalteu) and standard protein solution of Bovine Serum Albumin (BSA) at known concentrations were prepared (Appendix II). 2 mL of the homogenized fermented GC3 slurry was added to 20 mL of PBS buffer, and centrifuged at around 10,000 rpm for 10 min at 4 °C. The supernatant containing the soluble protein fractions was collected. 0.1 mL of supernatant and BSA standards was pipetted into separate test tubes. Reagent A (2 mL) was added to the tubes and gently mixed. Unfermented slurry served as a control. The tubes were incubated at room temperature for 10 min to allow the reaction to proceed. Reagent B (0.2 mL) was then added to each tube, followed by gentle mixing and incubation at RT for 30 min until a blue-purple color developed. 0.2 mL of diluted Folin-Ciocalteu reagent was added to each tube and mixed with further final incubation for an additional 30 min at RT in the dark. The absorbance was measured at 750 nm using a spectrophotometer (UV-2450, Shimadzu, Japan), and using the standard curve, the absorbance was plotted against the known concentrations of the BSA standards to determine the protein concentration. The protein concentration of the bacillus was subtracted from that of the fermented slurry at different time intervals (0 - 24 h). All the analyses were carried out in triplicate.

3.8.6 Analysis of free amino nitrogen (FAN) (Hill et al., 2019)

Nitrogen assimilation from the substrate encourages swift multiplication and growth of bacteria, and thus, FAN was measured. The technique is based on the capability of free amino acids to react with ninhydrin to produce a pink color, which is measured spectrophotometrically. Ninhydrin (0.2 g) was placed in an amber coloured bottle and dissolved in 10 mL of distilled ethanol and glacial acetic acid (5 mL). Cadmium chloride (0.3 g) was mixed in 0.5 mL of distilled water and added to the contents in the bottle. The reagent was prepared fresh for each use. GC3

slurry was centrifuged at 10,000 rpm for 8 min at 4 °C. An aliquot of the supernatant (200 µL) was mixed with the ninhydrin reagent (800 µL) in a microfuge tube. The contents in the tube were boiled for 4 min and cooled. The absorbance was measured at 504 nm in a spectrophotometer (UV-1800, Shimadzu, Japan). The concentration was calculated using glycine as the standard.

3.8.7 Estimation of total carbohydrates and total reducing sugars

Sugars present provide easily metabolizable substrates as prebiotics for the rapid growth of starter cultures. Total carbohydrates (TC) were estimated by the phenol sulphuric acid method as described by Dubois et al. (1956) with a slight modification as reported by Ezeonu et al. (2014). The Dubois method quantifies total carbohydrates by colorimetric determination at 490 nm after their dehydration by sulfuric acid and reaction with phenol. The homogenized fermented GC3 sample was centrifuged at 12,000 rpm for 6 min at 4 °C. The supernatant obtained was diluted appropriately. Diluted sample extract (1.0 mL) was reacted with 1.0 mL 5 % phenol and 5.0 mL concentrated H_2SO_4. Absorbance was measured at 490 nm after 30 min. TC content on a dry weight basis was expressed as:

Carbohydrate (mg/g) = (Concentration from curve (mg/L) × Dilution factor) / (Sample weight (g) × (Dry weight % / 100)

As the ability of microbial cultures to metabolize different sugars varies widely, the profile of total reducing sugars (TRS) was studied during fermentation. TRS was estimated by the dinitrosalicylic acid method (DNSA) (Miller, 1959). 3, 5-DNSA reacts with the free carbonyl group to form 3-amino-5-nitro salicylic acid (ANSA), which under alkaline conditions is converted to a reddish-brown coloured complex. DNSA reagent was prepared by mixing DNSA (500 mg), crystalline phenol (100 mg), and sodium sulfite (25 mg) in 1 % NaOH (50 mL). GC3 slurries were centrifuged at 12,000 rpm for 6 min at 4 °C. An aliquot of the supernatant (500 µL) was mixed with DNSA reagent (500 µL) in a microfuge tube, and the tube contents were boiled for 5 min, cooled, and 200 µL of potassium sodium tartrate (40 %) was added to it. The absorbance was measured at 575 nm in a spectrophotometer (UV-1800, Shimadzu, Japan). The concentration was calculated using glucose as the standard.

3.9 Development of a synbiotic powder

3.9.1 Study of probiotic cell viability before and after lyophilization of the *Bacillus* cell suspension and GC3 synbiotic formulation

The 12 h fermented GC3 slurry was subjected to lyophilization for 24 h to get the right consistency of powder, without using any cryoprotectant. Lyophilization may drastically affect the survivability of probiotics due to the damage during the freezing process, owing to the mechanical stresses of the ice crystals formed, thereby causing injury to the cells (Pandey et al., 2017). To determine the influence of the food matrix on cell viability, the viable cell count of the bacillus cell suspension and synbiotic formulation was carried out before and after lyophilization. The percentage survival of cells on lyophilization in the bacillus suspension and in the food matrix was carried out using the formula below.

$$\text{Survival (\%)} = ((\text{Viable count after lyophilization}) / (\text{Initial viable count}))\text{x } 100$$

SEM was used to view *B. licheniformis* PQ098138 in the fermented GC3. The lyophilised fermented GC3 was sputter-coated and then viewed using a scanning electron microscope (JEOL JSM-5800 LV SEM) at x 5000 magnification.

3.9.2 Study of probiotic cell viability of the GC3 synbiotic formulation upon storage at low and room temperature

The survival of the probiotic during storage is important for synbiotic formulations to maintain an adequate number, if the goal is to provide a product with health benefits to the host upon consumption. The lyophilized GC3 synbiotic formulation was stored in sterile 50 mL capacity capped polypropylene tubes (BD Falcon). The tubes were stored at 4 °C and 28 ± 2 °C, and the viability of *B. licheniformis* cells in the lyophilized formulation was assessed after 12 months of storage using the method described previously (Section 3.3). For analysis, 1 g of the freeze-dried synbiotic powder was reconstituted in 1 mL of sterile saline prior to enumeration.

3.10 Effect of fermentation by *Bacillus licheniformis* PQ098138 on the proximate analysis, peptide formation, nutritional composition, phytate, and raffinose family oligosaccharides content of GC3 formulation

3.10.1 Proximate analysis (moisture, ash, fat, dietary fiber: TDF, IDF and SDF, crude protein, carbohydrate)

Analysis was carried out as per section 3.5.2 in Phase II.

3.10.2 SDS-PAGE (Sodium dodecyl sulphate- polyacrylamide gel electrophoresis)

The SDS-PAGE profile of GC3 before and after fermentation was studied in relation to protein hydrolysis and peptide formation using the method of Laemmli (1970), Sell et al. (2005), and Chevallet et al. (2006). SDS-PAGE was carried out in a 2-mm vertical slab gel (10 cm × 8 cm). The resolving gradient gel (10-15 %) and stacking gel (5 %) were prepared as described in Table 14. The resolving gels were placed in a gradient mixer and poured in the gap between the plates, leaving sufficient space for the stacking gel (the length of the teeth of the comb plus 1cm). The gel was allowed to polymerize at room temperature, and the stacking gel was gently poured over the resolving gel. A clean Teflon comb was immediately inserted into the gel, avoiding any air bubbles. The gel was allowed to polymerize at room temperature.

Meanwhile, the sample and protein molecular weight markers (3.5-240 kDa mBLUefl) were prepared for loading. 100 mg of each of sample was mixed with 800 µLof1x gel loading buffer prepared of 4 x using 16 mL distilled water,5 mL 0.5 M Tris-Cl buffer (pH 6.8), 8 mL 50 % Glycerol, 8 mL 10 % SDS, 2 mL 2-β mercaptoethanol (added immediately before use) and 1 mL 0.1 % bromophenol blue and heated at 100 °C for 5 min. The protein molecular weight marker (50 µL) was mixed with an equal volume of gel loading buffer (2x) and heated at 100 °C for 5 min. After complete polymerization of the gel, the comb was removed, and the wells were washed with distilled water. The distilled water was removed, and the electrophoresis unit was filled with the running buffer. 10 µL of the protein molecular weight marker and 15 µL of the samples, boiled in gel loading buffer upon cooling, were loaded in wells immersed in the gel running buffer.

Table 14: Resolving gel and stacking gel for SDS-PAGE.

Resolving gel	**10 % gel (mL)**	**15 % gel (mL)**
Distilled water (DW)	4.0	1.3
Glycerol -	-	1.0
30 % acrylamide (29 g acrylamide + 1g N, N'-Methylene-bis-Acrylamide in 100 mL DW)	3.3	5.0
1.5 M Tris-Cl buffer, pH 8.8	2.5	2.5
10 % SDS	0.1	0.1
10 % Ammonium persulfate	0.1	0.1
TEMED (Tetramethyl ethylenediamine)	0.004	0.004
Stacking gel	**5 % gel (mL)**	
Distilled water	3.4	
30 % acrylamide (29 g acrylamide + 1g N, N'-Methylene-bis-Acrylamide in 100 mL DW)	0.83	
1.0 M Tris-Cl buffer, pH 6.8	0.63	
10 % SDS	0.63	
10 % Ammonium persulfate	0.05	
TEMED	0.005	

Gel running buffer (10 x) was prepared using Tris base (30 g), glycine (190 g), SDS (10 g), and 1 L of distilled water. An equal volume of gel loading buffer (1x) was loaded in unused wells. Electrophoresis was carried out at 15 mA for 4 h. After careful removal of the plates, the gel was immersed in 150 mL of the fixation solution prepared by mixing distilled water, methanol, and acetic acid in a ratio of 4.5: 4.5: 1 for 30 min. The gel was then washed with 50 % methanol (150 mL) for 10 min to remove residual fixatives, followed by a second wash with distilled water (100 mL) for 5 min. The gel was incubated for a minute in 0.02 % sensitizing solution (30 mg of sodium thiosulfate pentahydrate dissolved in 150 mL of water) and washed twice with distilled water for 1 minute each. Silver solution was prepared by dissolving 150 mg of silver nitrate in 150 mL of water and 120 µL of 37 % formaldehyde. The gel was submerged in the silver solution for 25 min, followed by rinsing the gel twice with distilled water for 1 min each. The gel was immersed in the developer solution (6 g of sodium carbonate dissolved in 300 mL of water, adding 120 µL of 37 % formaldehyde just before use) until the desired staining intensity was

achieved. The gel was washed in the stop solution prepared with 7.5 mL of acetic acid and 142.5 mL of water for 10 min, followed by a final wash using distilled water for 5 min. Bands were then visualized.

3.10.3 Comparative assessment of the composition of free amino acids

In brief, free amino acid (FAA) content in both 12 h fermented and unfermented GC3 was determined following sample preparation and chromatographic analysis, based on the method of Gauthankar et al. (2021) with slight modifications, using the Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., USA). The system was equipped with a quaternary pump, autosampler, column compartment, and diode array detector (DAD). Separation was carried out on an Agilent ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 µm particle size). Primary amino acids were derivatized using O-phthalaldehyde. The mobile phases consisted of 10 mM Na_2HPO_4:10 mM $Na_2B_4O_7$:5 mM NaN_3 (pH 8.2) as phase A, and acetonitrile: methanol: water (45:45:10, v/v/v) as phase B, with a flow rate of 1.5 mL/min. Alpha-aminobutyric acid was used as an internal standard for quantification.

For FAA extraction, GC3 samples were treated with 15 mL of 10 % (w/v) ice-cold trichloroacetic acid (≥ 99 % purity, Sigma-Aldrich, Germany) to partially unfold proteins. The mixture was then extracted with an equal volume of diethyl ether (99.9% purity, Hi-Media, India), a process repeated five times to maximize FAA recovery. The collected aqueous fraction was evaporated to dryness at temperatures below 40 °C using a rotary vacuum evaporator (ROTEVA EQUITRON). The dried samples were spiked with 200 µL of internal standard solution (10 nmol/µL ABA, Sigma-Aldrich, USA), purged with nitrogen for 2 min, sealed, and incubated at 110 °C for 24 h. FAA concentrations were expressed as g per 100 g of GC3 powder.

3.10.4 Estimation of water-soluble and fat-soluble vitamins and carotenoids

Analysis carried out as per sections 3.4.5 and 3.4.6

3.10.5 Estimation of minerals

As per section 3.4.7

3.10.6 Analysis of phytic acid (986.11, AOAC, 2010)

Cereals, millets, and legumes contain high amounts of antinutritional factors (ANFs) that impede the availability of nutrients (Abbas et al., 2018). Phytate is an ANF that can bind to essential minerals, thereby reducing their bioavailability. It can form complexes with proteins and digestive enzymes, thereby affecting protein digestion and overall nutrient absorption (Tresina et al., 2012). The effect of fermentation on phytate levels was thus evaluated.

3.10.6.1 Sample extraction

Phytate was extracted from duplicate samples using dilute HCl. A moisture-free sample (1 g) was accurately weighed and placed into a 100 mL conical flask with 20 mL of 0.66 M HCl added to it. The flask, covered with parafilm, was kept on a shaker at 140 rpm for 3 h at RT. The extract was removed and centrifuged at 12,000 rpm for 8 min to obtain a clear supernatant. 1 mL of the extract was centrifuged. 0.5 mL of the resultant supernatant was neutralized by the addition of 0.5 mL of NaOH (0.75 M) in a fresh tube and was then used in the enzymatic dephosphorylation reaction.

3.10.6.2 Elution of phytic acid and colorimetric determination of phosphorus

Aliquots of 1.0, 3.0, and 5.0 mL of the standard phosphorus solution (80 µg P/mL) were transferred into a 50 mL volumetric flask, followed by the addition of 20 mL of distilled water and thorough mixing. Subsequently, 2 mL of molybdate solution and 1 mL of sulfonic acid reagent were added, mixed well, and incubated for 15 min. Absorbance was measured at 650 nm. Phytic acid was eluted using 0.7 M NaCl solution and digested with a mixture of concentrated HNO_3 and H_2SO_4 to release inorganic phosphorus. To this digest, 2.0 mL of molybdate solution and 1.0 mL of sulfonic acid reagent were added, mixed, and incubated at 40 °C for 90 min. The resulting solution was measured colorimetrically at 640 nm using a UV–visible spectrophotometer (Analytikjena U-2800 SPECORD S.600, Germany). Phytic acid content was then calculated using the formula, considering that phytic acid contains 28.2 % phosphorus.

$$\text{Phytic acid (mg/g sample)} = \frac{\text{Weight of sample (mg)}}{\text{Content volume}} \times \text{mean K} \times \text{Absorbance} \times 0.282 \times 1000$$

Where: mean K = concentration of phosphorus standard ÷ absorbance of standard

3.10.7 Estimation of oligosaccharides

GC3 synbiotic powder was dissolved in 10 mL of 70 % ethanol, vortexed, and centrifuged (4,000 rpm) for 10 min. The analysis was performed as per section 3.5.8.

3.11 *Bacillus* viability in the synbiotic food formulation in comparison to cell suspension during exposure to simulated gastrointestinal conditions

Probiotics should colonize the large intestine and provide health benefits to the host. Probiotics should be able to survive the harsh transit through the upper GIT for successful colonization. Hence, the survivability of *B. Licheniformis* PQ098138 in the GC3 synbiotic food formulation upon exposure to simulated gastrointestinal conditions was evaluated and compared to the cell suspension alone performed earlier (3.2.2.1).

3.11.1 Tolerance to simulated gastric juice

Simulated gastric fluid was prepared as per the protocol in section 3.2.2.1.2. Freeze-dried synbiotic powder (1 g) was reconstituted in 1 mL of sterile saline. An aliquot (0.5 mL) of fermented GC3 with an initial cell count of ~9.5 log CFU/ mL of each isolate was mixed with 5 mL of simulated gastric fluid and incubated at 37 °C. Tolerance to the simulated conditions of gastric acid (1.2 - 4 pH) up to 3 h was carried out by determining the viable cell counts as per the method in section 3.2.2.1.2.

3.11.2 Tolerance to simulated small intestinal juice

Artificial small intestinal juice was prepared for the assay as per the protocol in section 3.2.2.1.3. Freeze-dried synbiotic powder (1 g) was reconstituted in 1 mL of sterile saline. An aliquot (0.5 mL) of fermented GC3 with an initial cell count of ~9.5 log CFU/ mL of each isolate was mixed with 5 mL of simulated small intestinal juice and incubated at 37 °C. Tolerance to simulated conditions of small intestinal juice with or without only 0.5 % bile salts at 1 h and 4 h was carried out by determining the viable cell counts as per the method in section 3.2.2.1.3.

3.12 Assessment of the antibiotic susceptibility, antioxidant activity, and antimicrobial activity of the synbiotic food formulation

3.12.1 Antibiotic susceptibility

Studying the antibiotic resistance patterns of probiotics is a mandatory test indicated as per the ICMR-DBT guidelines (Ganguly et al., 2011) to establish that the probiotic strain poses no significant risk of transferable antibiotic resistance. Further, the fermented formulation can display different susceptibility patterns, and performing these tests in the context of fermented food products is essential for ensuring safety. Lyophilised GC3 synbiotic formulation as powder (0.5 g) was reconstituted in 1 mL sterile saline, and the resultant suspension was spread on MHA plates. The antibiotic susceptibility of the formulation was determined as per section 3.2.1.5.

3.12.2 Antioxidant activity

The antioxidant activity was measured by the DPPH and reducing power assay. The radical scavenging action of the stable DPPH free radical was used to evaluate the antioxidant activity of GC3 synbiotic powder and compared to that of unfermented GC3 powder. A moisture-free sample was weighed (1 g) and transferred to a conical flask, and then mixed with 25 mL of 80 % methanol. The flask was stoppered and placed on an orbital shaker set to 160 rpm. The extraction was carried out for 4 h at 30 °C. The different concentrations (10 µL to 100 µL) of the fractions of the methanol extract were taken, and the volume was made up to 1 mL with methanol, with the addition of 3 mL of DPPH. Absorbance was measured as per the protocol discussed in section 3.2.2.4.1. Analysis was done in triplicate.

The reducing power of the methanolic extracts in triplicate was determined by the method of Lin et al. (2006). Unfermented and fermented GC3 formulations were centrifuged individually at 12,000 rpm for 5 min at 4 °C to obtain supernatant. In brief, a 0.75 mL reaction mixture was prepared by combining 0.25 mL of sample solution, containing varying volumes of supernatant (20–100 µL) in 0.25 mL of 50 mM phosphate buffer (pH 7.0), with 0.25 mL of 1% (w/v) potassium ferricyanide. The further analysis was performed as per the protocol discussed in section 3.2.2.4.2.

3.12.3 Antimicrobial activity

Gastrointestinal infections are widely caused by food borne pathogens. Evaluation of the antimicrobial activity of GC3 synbiotic formulation can be of great therapeutic significance. 2 g of lyophilized powder was reconstituted in 10 mL of sterile saline to obtain a 200 mg/mL concentration of the lyophilized synbiotic (100 µL concentration is expected to have 20 mg of powder). The antimicrobial activity of the reconstituted GC3 synbiotic formulation of different concentrations (25 µL,50 µL,75 µL, 100 µL) was tested against *E. coli* (ATCC 700927), *S. Typhimurium* (MTCC 531), *S. aureus* (ATCC 6538), and *S. Enteric* (ATCC 35664) by well diffusion assay following Aziz et al. (2023). These pathogens were individually grown overnight in Luria Bertani broth and incubated at 37 °C for 10 h till moderate turbidity developed, and were then used to spread on MHA plates for further study as per the method discussed in section 3.2.1.4. Sterile saline was used as a negative control.

3.13 Assessing the functional properties of the synbiotic formulation for suitability in food product

3.13.1 Emulsification capacity (Chandra et al., 2015)

The emulsification capacity of the unfermented and fermented GC3 formulation was determined. Individual powders (1g) were diluted with 10 mL of distilled water and mixed with 10 mL vegetable oil in a centrifuge tube (50 mL tube) and homogenized for 5 min at speed 4 (~5000 rpm). The mixture was then centrifuged at 2000 rpm for 4 min at 25 °C. The ratio of the height of the emulsion layer to the total height of the mixture was calculated as the emulsion activity in percentage.

Emulsification activity (%) = (Height of emulsion layer / Height of whole layer) × 100

3.13.2 Emulsification stability (Yasumatsu et al., 1972)

Emulsion stability was assessed both before and after heating. The emulsion, placed in a calibrated centrifuge tube, was heated at 80 °C for 30 min in a water bath, cooled under running tap water for 15 min, and then centrifuged at 2,000 rpm for 15 min.

The emulsion stability was determined by allowing the emulsion to stand, and the volume of oil separated at 30 min was noted. The percentage of emulsion stability was determined as follows:

$$\text{Emulsion stability (\%)} = (\text{Height of emulsified layer})/(\text{ Height of whole layer})\ x100$$

3.13.3 Foam capacity and stability (Narayana & Narasinga Rao, 1982)

Foaming properties of both fermented and unfermented GC3 powder were evaluated. A 1.0 g sample of the powder was added to 50 mL of distilled water at 28 ± 2 °C in a graduated cylinder. The mixture was thoroughly shaken for 5 min to generate foam. Foam volume measured 30 seconds after whipping was used to calculate foam capacity using the following formula:

$$\text{Foam capacity (\%)} = [(\text{Volume of foam AW} - \text{Volume of foam BW}) \,/\, \text{Volume of foam BW}] \times 100$$

Where: AW = after whipping, BW = before whipping

The whipped sample was allowed to stand at 28 ± 2 °C for 30 min before measurement of drainage volume. The foam stability was expressed in terms of percentage of initial foam volume as follows:

$$\text{Foam stability (\%)} = [(\text{Initial volume of foam} - \text{Drainage volume}) \,/\, \text{Initial volume of foam}] \times 100$$

3.13.4 Heat tolerance assay

The heat tolerance assay for probiotics is typically used to assess how well a probiotic strain can survive and remain viable under elevated temperatures, which is important for determining its stability during processing, storage, and transportation. Also, the food matrix can influence the survival of the microbial cells. Hence, the viability of *Bacillus* under heat stress (40 - 70 °C) was evaluated for a synbiotic formulation and compared to that of the cell suspension of a potential probiotic. Cell suspensions were prepared as per 3.2.2.1.1.

Suspension (1 mL) of fermented formulation after appropriate dilution and cell suspension was dispensed into microfuge tubes and placed in a water bath set to the desired temperature from 40 - 70 °C. The viable counts were then enumerated after 10 min and 15 min of heat exposure by spread plating on Nutrient agar plates.

3.14 Statistical analysis of data

Statistical analyses were conducted using IBM SPSS version 25.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 10.5.0 (GraphPad Software, San Diego, CA, USA). Paired t-tests were applied to compare means between two related samples, while independent t-tests and Welch's two-sample t-tests were used for comparisons between two independent groups, as appropriate. Differences among multiple groups were assessed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A significance level of 0.05 was considered for all analyses.

CHAPTER 4:
RESULTS AND DISCUSSION

Chapter 4

Results and Discussion

Phase I

4.1 Screening of saltpan isolates

4.1.1 Growth and maintenance of the saltpan isolates

The previously isolated 46 halotolerant bacterial cultures from water and sediments from Ribandar, Batim, and Agarwado saltpans of Goa and preserved in Dr. Savita Kerkar's laboratory collection were used in the present study. They were maintained in 25 % Nutrient broth and Nutrient agar of analytical grade with 0.5 - 2 % salt.

4.1.2 Characterisation and identification of halotolerant bacteria

Out of 46 isolates, 39 were found to be Gram-positive rods (Table 15, Plate 1). Isolates that were Gram-negative and gave a coccobacillary appearance were not selected for further study. Heat Shock treatment revealed that 25 isolates were endospore-forming bacteria, as seen in Table 15.

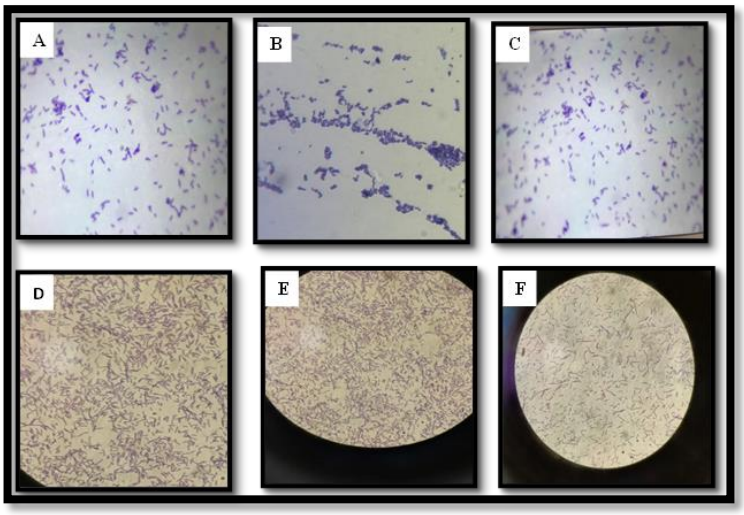


Plate 1: Gram staining of isolates: (A) ABSK 55, (B) FSK 0.30, (C) FSK 2, (D) FSK 2.2, (E) FSK 3 and (F) FSK 5.34.

Table 15: Gram's nature of saltern isolates.

Gram's Nature and Morphology	Isolates	Endospore-forming Isolates
Gram-positive, short, and thick rods.	FSK 0.30, SK 27, ABSK 55, TSK 71, SK 07, SBSK 2, FSK 5.2, FSK 1.12, FSK 1.16, FSK 1.19, FSK 1.35, FSK 1.7, FSK 2.10, FSK 2.30, FSK 2.41, FSK 2.42, FSK 3.19, TSK 32, FSK 3.27, FSK 3.35, FSK 3.56, FSK 3.6, FSK 4.3, FSK 3.43, FSK 2	FSK 0.30, SK 27, ABSK 55, SK 07, SBSK 2, FSK 0.34, FSK 1.12, FSK 1.16, FSK 1.35, FSK 1.7, FSK 2.10, FSK 2.30, FSK 2.41, FSK 2.42, FSK3.27, FSK 3.35, FSK 3.56, FSK 3.6, FSK 2
Gram positive, long and slender rods.	FSK 1.40, FSK 3.43, FSK 2.42, FSK 3.19, FSK 3.27, SK 3.35, SK 3.5, SK 4.11, SK 4.43, SK 5.13, FSK 5.34	TSK 71, FSK 2.27, FSK 3.19, FSK 5.34
Gram positive, short and thin rods.	FSK 2.2, SK 2.21(B), FSK 3	FSK 2.2, FSK 3

Plate 2 indicates the endospores at subterminal ends in isolates FSK 2 and FSK 3. The growth of these isolates was visible on 0.5 % to 2 % supplemented Nutrient agar plates after streaking pure colonies for 24 h of incubation at 28 ± 2 °C (Plate 3).

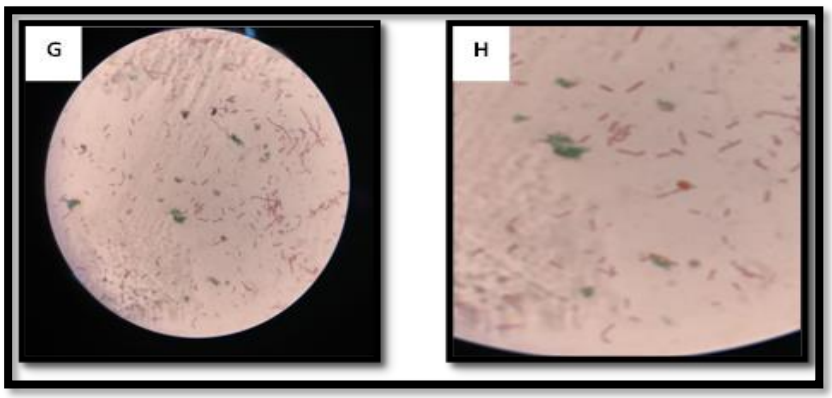


Plate 2: Endospores of isolates (G) FSK 3 and (H) FSK 2.

Studying endospores in *Bacillus* probiotics helps optimize their formulation, enhance safety, and maximize their health benefits (Biermann & Beutel, 2023). The protective shell of endospores ensures that probiotic bacteria remain viable, allowing them to survive the stomach acidity and bile salts, which is crucial for their effectiveness (Pawar et al., 2025). Additionally, endospores enable *Bacillus* probiotics to endure extreme conditions such as high temperatures, desiccation, and UV radiation, making them more resilient than non-spore-forming probiotics like *Lactobacillus* and *Bifidobacterium* (Biermann & Beutel, 2023).

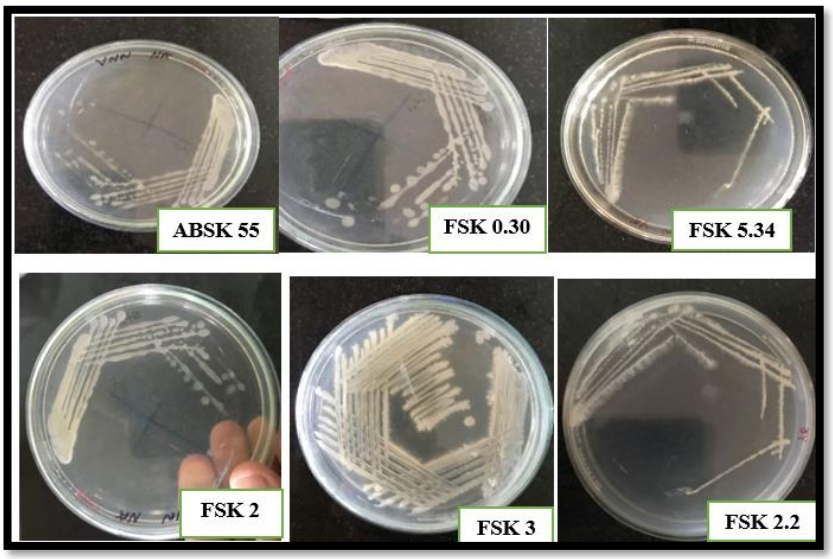


Plate 3. Growth of isolates on Nutrient agar after heat-shock treatment.

4.1.3 Fermentation ability and carbohydrate utilisation of the isolates

Out of 46 salt pan isolates, 25 isolates were checked for their fermentation potential. The ability of isolates to ferment a specific carbohydrate (glucose, fructose, sucrose, lactose, xylose, maltose, mannitol, arabinose, raffinose) was studied. The colour change to reddish orange at neutral (about 7.4 pH), yellow at acidic pH (at and below 6.8 pH), and pink-red at alkaline pH (at or above 8.4 pH), if any was observed. Twelve isolates (SK 07, TSK 71, FSK 0.34, FSK 1.12, FSK 1.16, FSK 2.10, FSK 2.2, FSK 2.30, FSK 2.41, FSK 2.42, FSK 3.27, FSK 5.34) exhibited positive reaction for acid production whereas 4 isolates demonstrated acid and gas production as seen in Plate 4 (FSK 2, FSK 3, SK 27, FSK 0.30).

The incubation period varied for each isolate from 1–5 days. Isolates FSK 2, FSK 3, SK 27, and SK 07 displayed the change in color from reddish orange to yellow after a day of incubation, whereas 7 isolates indicated a color change after 5 days. All the isolates exhibited different patterns of sugar utilization as seen in Table 16. Plate 5 indicates glucose utilization. The isolates exhibited differences in their ability to metabolize dietary sugars into acids. Different probiotic strains produce varying amounts and types of acids. Probiotics ferment carbohydrates to produce short-chain fatty acids (SCFAs) like acetic acid, propionic acid, lactic acid, and butyric acid; their type and amount produced indicate how well the probiotic can utilize specific carbohydrates (Watson et al., 2013). Strains with higher acid production are generally more effective in gut colonization and inhibition of pathogens (Ziar et al., 2014). High acid-producing strains can enhance fermentation efficiency and preservation (Bangar et al., 2022).

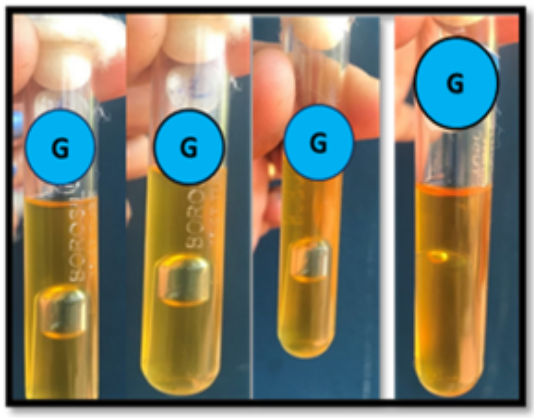


Plate 4: Fermentation assay showing acid and gas production with Glucose (G) by FSK 2, FSK 3, SK 27 and FSK 0.30.

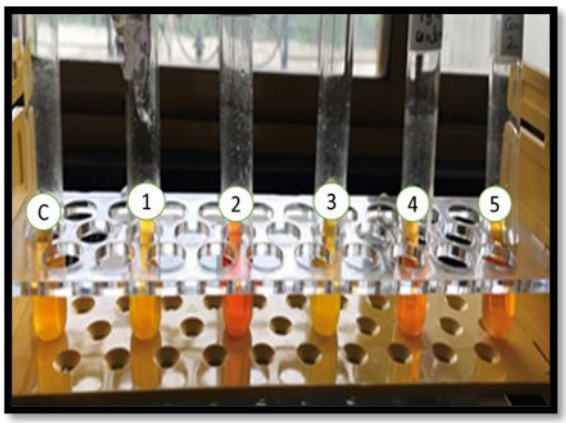


Plate 5: Carbohydrate (Glucose) utilization by (C) Positive control, (1) FSK 2, (3) FSK 3 after 24 h; isolates (2) FSK 0.34, (4) FSK 0.30 and (5) TSK 71 not showing acid production after 24 h.

Table 16: Carbohydrate utilization pattern of isolates.

Isolate code	Test								
	glucose	**fructose**	**sucrose**	**lactose**	**xylose**	**maltose**	**mannitol**	**arabinose**	**raffinose**
FSK 0.30	+	+	+	-	-	-	+	+	+
FSK 0.34	+	+	+	-	-	+	-	-	+
FSK 1.12	+	-	+	-	-	+	-	-	-
FSK 1.16	+	+	+	-	-	+	-	-	+
FSK 2.2	+	+	+	-	+	-	+	+	+
FSK 2.41	+	+	+	-	-	+	+	-	-
FSK 3.27	+	+	-	+	-	+	-	-	-
FSK 3.35	+	+	-	-	-	-	+	+	+
FSK 3.56	+	+	-	+	-	+	-	-	-
FSK 3.6	+	+	+	-	+	-	+	+	+
FSK 2	+	+	+	+	-	+	+	-	+
FSK 3	+	+	+	+	-	+	+	-	+
SK 27	+	+	+	-	-	-	+	-	+
SK 07	+	+	+	-	-	+	+	-	-
TSK 71	+	-	-	-	+	+	+	+	-
FSK 5.34	+	-	-	-	+	+	-	-	+

+ = positive, - = negative

Probiotics must efficiently utilize carbohydrates to thrive in the gastrointestinal tract (GIT). Different strains have varying abilities to ferment dietary fibers, prebiotics, and other carbohydrates (Watson et al., 2013). Understanding carbohydrate utilization helps in designing synbiotic formulations, and knowledge of carbohydrate metabolism guides the formulation of functional foods and supplements enriched with probiotics (Peng et al., 2020).

4.1.4 Qualitative screening for extracellular hydrolytic enzymes

Sixteen shortlisted halotolerant *Bacillus* isolates were further evaluated for qualitative extracellular production of amylase, cellulase, protease, β-Galactosidase, phytase, and lipase by plate assay on minimal media using specific substrates. The production of extracellular enzymes by probiotics is a beneficial trait, as it can facilitate the digestion of complex nutrients and also enhance the production of desirable metabolites (Delgado-García et al., 2014).

The enzyme activity of the short-listed isolates is indicated in Table 17. The results demonstrated that the halotolerant isolates were capable of producing a diverse array of extracellular enzymes. Each isolate exhibited a distinct enzymatic profile, with significant differences ($p < 0.05$) observed in enzyme production among the various halotolerant strains. Extracellular amylase and protease production were observed in 13 isolates. As seen in Plate 6, halotolerant bacteria FSK 3 exhibited the highest amylase production at 24 h (20.0 ± 1.0 mm), with SK 07 (19.13 ± 1.5 mm) and FSK 2 (18.20 ± 1.0 mm) showing slightly lower activity. Amylases are a fascinating group of enzymes with significant biotechnological potential. They are particularly valuable in clinical and analytical chemistry, as well as in various industrial applications, including starch saccharification, textiles, food production, brewing, and distilling (Samanta, 2022). Among them, halophilic amylases stand out for their remarkable stability and versatility (Fernandes et al., 2019; Samanta, 2022). Maximum protease activity was seen in isolate SK27 (20.32 ±1.5 mm) followed by FSK 3 (20.17 ±1.5 mm) and FSK 2 (19.37 ±1.0 mm). Cellulase production was observed in 14 isolates, while 10 isolates produced lipase. Isolate FSK 2 showed maximum cellulase (22.57 ± 1.5 mm) (Plate 6) followed by FSK 3 (21.23 ± 0.5 mm) and FSK 5.34 (20.46 ± 0.5 mm), with SK 27 showing maximum lipase production (20.32 ±1.0 mm), followed by FSK 5.34 with 15.0 ± 1.2 mm clearance zone on tributyrin agar.

Phytase production was shown by seven isolates (Table 17), with FSK 2 & FSK 3 showing maximum clearance. Phytate, commonly found in plant-based foods, is a strong inhibitor of mineral absorption in humans. The use of Bacillus strains that produce phytate-degrading enzymes (phytases) to lower phytate levels represents a promising but relatively underexplored area of research (Saraniya & Jeevaratnam, 2015).

Amritha and Venkateswaran (2018) reported that *Lactobacillus plantarum* MTCC 1325 can serve as a starter culture for cereal–legume fermentations and act as a potential probiotic capable of hydrolysing phytate in both food systems and the GIT. Fermenting cereal and legume flours with this strain for 24 h resulted in a reduction of phytate content, with sorghum showing the greatest decrease (73 %) and horse gram the least (34 %). Beta-galactosidase production was observed in nine isolates, suggesting that if used as probiotics, these cultures could aid in lactose metabolism for individuals with lactose intolerance. Previous research has shown that *Bacillus* species produce a variety of exoenzymes, including proteases, carbohydrases, and lipases, which effectively break down proteins, carbohydrates, and lipids into smaller molecules. Non-pathogenic strains of *Bacillus* are widely applied in food processing and industrial fermentation (Delgado-García et al., 2014) and are also employed in the production of commercially valuable enzymes (Danilova & Sharipova, 2020).

Ghosh et al. (2017) have reported of *Bacillus* species supporting the digestive function of the gut by producing essential enzymes and could stimulate the normal microflora of the gut. High amylolytic activity is known for bacilli which produce acid-resistant enzymes (Delgado-García et al., 2014). Taj et al. (2022) has reported some *Bacillus* strains to have lipolytic and cellulolytic activity. Several *Bacillus* species produce cellulase and contribute to food fermentation by breaking down cellulose into glucose and other fermentable sugars, making nutrients more bioavailable and improving texture, and enhancing fermentation efficiency (Malik & Javed, 2021).

Proteolytic enzymes from *Bacillus* species are essential in food fermentation, as they break down proteins into peptides and amino acids (Fan et al., 2015). This enzymatic activity impacts the texture, flavour in cheeses (umami flavor), soy sauce, and fish sauce, nutritional profile, and safety of fermented foods (Danilova & Sharipova, 2020). Proteases represent one of the most significant classes of industrial enzymes and currently account for the majority of global enzyme sales. They have long-standing applications across various industries, including detergents, baking, brewing, cheese production, and leather tanning (Behera et al., 2018; Li et al., 2023). *Bacillus* proteolytic enzymes stimulate regeneration processes and enhance the fibrinolytic activity in the plasma even after oral administration (Ghosh et al., 2017).

Table 17: Extracellular enzyme synthesis of *Bacillus* isolates.

Isolate code	Zone of clearance (mm)					
	Amylase	**Lipase**	**Cellulase**	**Protease**	**Phytase**	**Beta galactosidase**
FSK 0.30	11.42 ±0.5	11.0 ± 1.2	14.62 ± 0.5	17.30 ±1.0	+	+
FSK 0.34	10.20 ±0.5	11.0 ± 1.2	12.60 ± 0.5	14.0 ±1.0	ND	ND
FSK 1.12	10.14 ±0.4	ND	10.77 ± 0.5	14.0 ±1.0	ND	ND
FSK 1.16	9.02 ±0.4	ND	10.13 ± 0.5	08.0 ±1.0	ND	+
FSK 2.2	9.00 ±0.5	ND	10.22 ± 0.5	ND	ND	ND
FSK 2.41	ND	10.0 ± 1.2	11.62 ± 0.5	11.0 ±1.0	ND	+
FSK 3.27	9.09 ±0.5	ND	ND	ND	ND	ND
FSK 3.52	ND	ND	12.67 ± 0.5	ND	ND	ND
FSK 3.56	ND	11.2 ± 1.0	13.26 ± 0.5	11.0 ±1.0	ND	ND
FSK 3.6	11.12 ±0.5	ND	ND	13.0 ±1.0	+	ND
FSK 2	18.20 ± .0	12.0 ± 1.4	**22.57 ± 1.5**	19.37 ±1.0	++	+
FSK 3	**20.0 ± 1.0**	14.0 ± 1.0	21.23 ± 0.5	20.17 ±1.5	++	+
SK 27	15.12 ± .5	**20.32 ± 1.0**	12.46 ± 0.5	**20.32 ±1.5**	+	+
SK 07	19.13 ±1.5	14.5 ± 1.0	16.28 ±1.5	17.7±1.0	+	+
TSK 71	15.23 ±1.0	14.0 ± 1.0	18.67 ± 0.5	17.0 ±1.0	+	+
FSK 5.34	14.12 ±0.5	15.0 ± 1.2	20.46 ± 0.5	14.0 ±1.0	+	+

ND=not detected, += positive, ++= maximum clearance

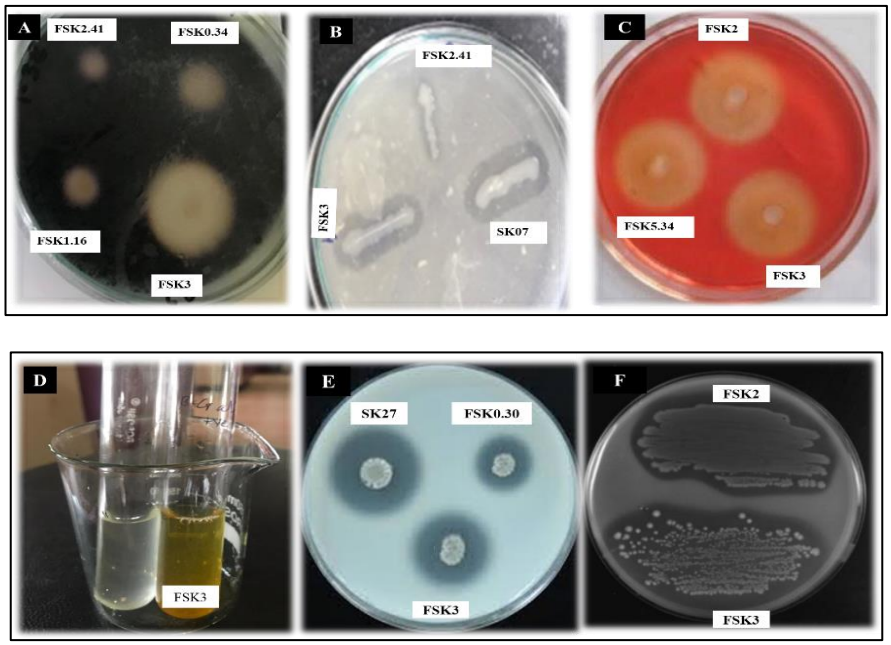


Plate 6: Production of extracellular enzymes - (A)amylase, (B) lipase, (C) cellulase, (D)β-Galactosidase,(E) protease and (F) phytase by isolates.

4.2 *In-vitro* studies on safety and desired probiotic attributes, identification, and shelf life of the isolates

4.2.1 Safety studies

4.2.1.1Haemolytic activity

Haemolysis is not a desirable characteristic in a probiotic. The European Food Safety Authority (EFSA) advises that bacterial isolates intended for food applications should be evaluated for haemolytic activity, even when they are classified as GRAS or hold QPS (Qualified Presumption of Safety) status (FAO/WHO, 2006). Hence, the seven isolates shortlisted based on extracellular enzyme production were evaluated for their haemolytic activity. Strains exhibiting no visible hemolysis (γ-haemolytic or non-haemolytic) were chosen as potential probiotics, whereas those displaying a clear zone of hemolysis (β-haemolytic or fully haemolytic) or a greenish halo (α-haemolytic or partially haemolytic) were excluded from further evaluation.

Two isolates (SK 07 and TSK 71) showed partial haemolysis, another two isolates (FSK 5.34 and SK 27) were completely haemolytic, whereas three of the seven isolates showed no

haemolytic activity (FSK 0.30, FSK 2 and FSK 3) (Plate 7). Hence, they were further screened for the presence of enterotoxin and endotoxin. Our results are in agreement with the findings of Pawaskar et al. (2022) and Fernandes et al. (2019), who reported non-haemolytic activity of some *Bacillus* species isolated from Goan saltpans. The results of the current findings are also consistent with the results of Wang et al. (2018), who evaluated the probiotic potential of *Bacillus spp*. from spontaneously fermented Chinese non-dairy food products and revealed no haemolytic activity of the probiotic. Several studies have also documented the non-haemolytic nature of novel potential probiotics isolated from natural sources (Vijayakumar et al., 2015; Wang et al., 2018; Golnari et al., 2024).

Certain bacterial metabolites can be harmful to host cells, making it essential to evaluate their presence when determining the safety of strains intended for consumption. Notably, haemolysin production is considered a major virulence factor (Banerjee et al., 2017), as it can lyse host cells and limit haemoglobin availability as an iron source (Golnari et al., 2024). Evaluating the haemolytic activity is highly recommended, as haemolysis, the destruction of red blood cells, can signal potential pathogenicity. Ensuring that probiotic strains lack haemolytic activity is a crucial selection criterion, as non-haemolytic strains are generally regarded as non-virulent (FAO/WHO, 2006). Assessing haemolytic activity thus helps ensure consumer safety by identifying risks associated with toxin production or harmful host interactions.

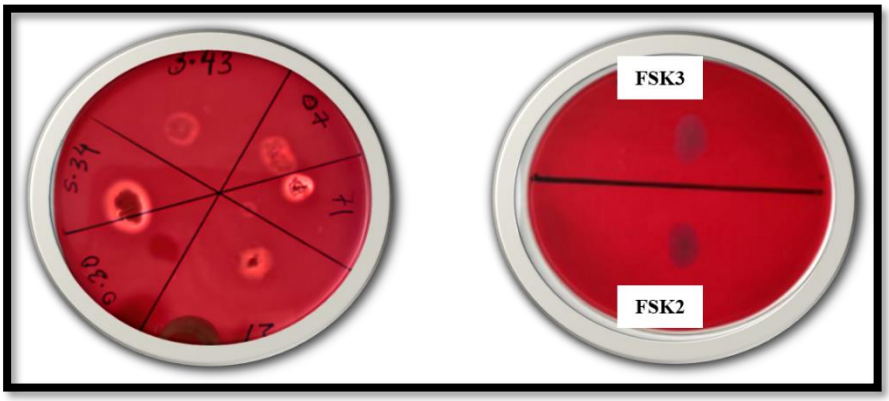


Plate 7: Safety evaluation of bacterial isolates by blood agar haemolysis.

Several bacterial species, such as *Bacillus cereus* and Group A *Streptococcus*, are considered pathogenic because they produce haemolysins, haemolysin BL and streptolysin O,

respectively, which exhibit strong haemolytic activity (Ballav et al., 2015; Aziz et al., 2023). While the precise mechanism of haemolysis in *Bacillus* species remains unclear, research has identified specific genes associated with this activity (Li et al., 2023; Nithya & Halami, 2013).

4.2.1.2 Enterotoxin genes in the prospective probiotic isolates

The three *Bacillus* species (FSK 0.30, FSK 2 and FSK 3) and the standard *B. cereus,* NCIM 2106, exhibited a band when observed in a UV trans-illuminator just a little below the wells, indicating successful extraction of DNA. The concentration of DNA extracted from the three isolates FSK 0.30, FSK 2 and FSK 3 was 25.58 µg/mL, 46.46 µg/mL and 67.14 µg/mL respectively. *B. cereus* NCIM 2106 amplified with primers targeted for *nheA*, *nheB*, *hblA* and *entFM* enterotoxin genes, exhibited bands indicating the presence of the enterotoxin genes.

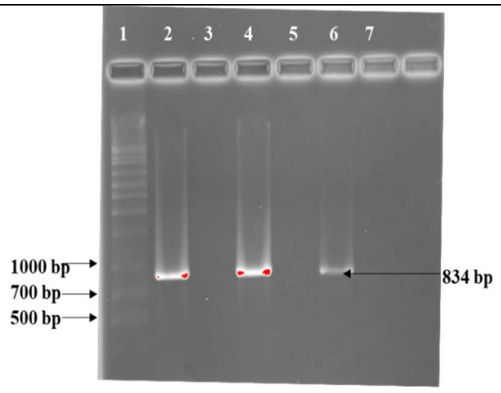


Plate 8: hblA gene-specific PCR;
Lane 1: 1 kb DNA ladder;
Lanes 2, 4, 6: Positive control (std. *B. cereus* 2106) showing amplified gene;
Lanes 3, 5 and 7: Test isolates FSK 2, FSK 3, and FSK 0.30, respectively showing no amplification of target gene.

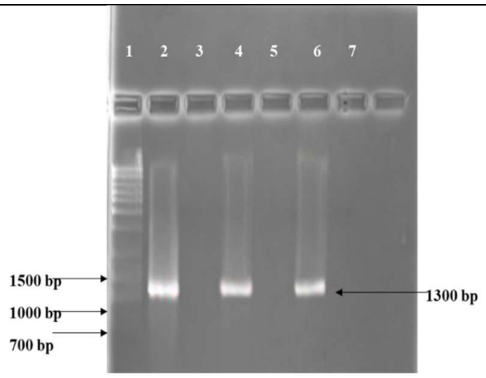


Plate 9: entFM gene-specific PCR;
Lane 1: 1 kb DNA ladder;
Lanes 2,4,6: Positive control (std. *B. cereus* 2106) showing amplified gene; Lanes 3, 5 and 7: Test isolates FSK 2, FSK 3, and FSK 0.30, respectively, showing no amplification of target gene.

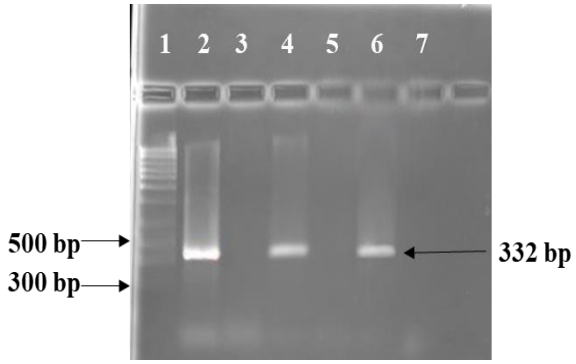


Plate 10: *nheB* gene specific PCR;
Lane 1: 1 kb DNA ladder;
Lanes 2,4,6: Positive control (std. *B. cereus* (2106) showing amplified gene;

Lanes 3, 5 and 7: Test isolates FSK 2, FSK 3 and FSK 0.30, respectively showing no amplification of target gene.

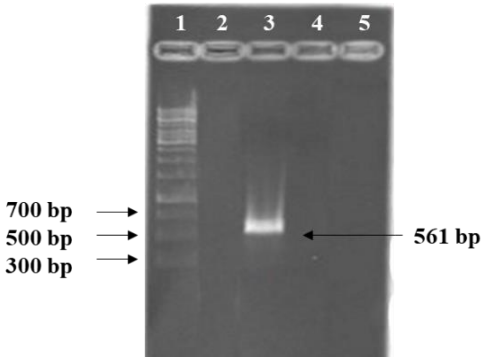


Plate 11: *nheA* gene specific PCR;
Lane 1: 1 kb DNA ladder;
Lane 2, 4 and 5: test isolates FSK 3, FSK 2 and FSK 0.30, respectively showing no amplification of the target gene;
Lane 3: Positive control (std. B. cereus 2106).showing amplified gene.

The three test *Bacillus* species, FSK 0.30, FSK 2, and FSK 3, did not reveal any amplified gene products (Plates 8, 9, 10 and 11), indicating the absence of these specific enterotoxin genes and their safety as potential probiotic strains in food fermentation. Our findings are in agreement with Sorokulova et al. (2008), who reported that *B. licheniformis did* not produce *HblA* or *Nhe* enterotoxins. Enterotoxins are toxins produced by certain bacteria that specifically target the intestinal tract and are commonly linked to foodborne illnesses. Both enterotoxins and emetic toxins are major contributors to food poisoning as well as some non-gastrointestinal infections (Abdulmawjood et al., 2019).

However, pathogenicity among *Bacillus* species is strain-dependent, and not all strains are harmful (Ehling-Schulz et al., 2019). Certain species, including *B. cereus, B. thuringiensis, B. anthracis, B. weihenstephanensis* and *B.pseudomycoides,* are capable of producing toxins (Nithya & Halami, 2012).

Although some *Bacillus* species are currently used as probiotics, the search for new, effective, and safe probiotic strains remains a key focus in both microbiology and the food industry. Thus, the safety of our isolates was established for their use as a potential probiotic and for the fermentation of GC3 seed powder.

4.2.1.3 Endotoxin in the prospective probiotic isolates

The test isolates FSK 0.30, FSK 2 and FSK 3, grown overnight in Nutrient broth, were tested negative for endotoxin (Plate 12), indicating their safety for use in food fermentation as well as a potential probiotic. The Endotoxins, also known as pyrogens, are lipopolysaccharides commonly present in food, the environment, and bacterial-derived clinical products, making them inevitable and widespread microbiological contaminants (Das et al., 2014). Endotoxins are located solely in the outer membrane of Gram-negative bacteria (Dullah & Ongkudon, 2017). Food poisoning caused by endotoxins is usually associated with Gram-negative bacteria. While not all bacteria do not need endotoxin testing, sterility and contamination checks for various microorganisms, including the Gram-positive bacteria, are a must. This is essential for overall safety, and strain-specific safety evaluations are thus recommended.

Robust compliance for probiotics is being carefully examined, and the FAO and WHO have collaboratively developed guidelines for evaluating probiotics in food. This indicates the need for global harmonisation of regulations on probiotics.

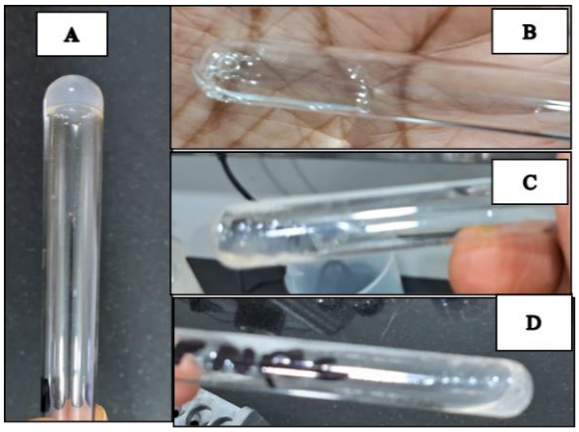


Plate 12. Test for presence of endotoxin: (A) Positive control, (B)Negative control, (C) isolate FSK 2 and (D) isolate FSK 3 (both negative for endotoxin).

Novel probiotics are thus being introduced into the market with their benefits being highlighted; however, safety aspects, including the true identity of the culture, are not revealed to the consumer. Such guidelines would help to create best practices and harmonised regulations, allowing consumers to make informed decisions and obtain the intended health benefits. Thus, the safety and stability of our isolates were established in the present study.

4.2.1.4 Antimicrobial activity of the *Bacillus* isolates

The isolates were tested for antibacterial activity against various gastrointestinal tract as well as common food pathogens. FSK 2 and FSK 3 inhibited all the tested pathogens (ATCC strains). FSK 3 displayed the maximum inhibition to *B. cereus* followed by *E. coli*, *S. aureus* and *S. Enterica* with the least inhibition to *S. boydi* (Plate 13A). Isolate FSK 2 showed maximum inhibition to *E. coli* with a minimum inhibitory effect on *S. paratyphi*. FSK 0.30 did not inhibit ATCC strains of *E. coli* and *S. enterica*. Isolates FSK 2 and FSK 3 also showed inhibition zones against all the tested clinical pathogens; however the inhibition zones were smaller as compared to the inhibitory pattern of ATCC pathogens tested (Plate 13 B). The zones of inhibition are presented in Table 18. Comparable studies by Monteiro et al. (2019) and Ritter et al. (2018) also demonstrated that probiotic strains exhibit antagonistic activity against common pathogens, including *P. aeruginosa, E. coli, S. aureus, Y. enterocolitica,* and *L. monocytogenes*.

Table 18: Antagonistic activity of isolates and zone of inhibition against tested pathogenic bacteria.

Pathogenic ATCC strains (Zone of inhibition in mm)							
Isolate	***1*** ***B. cereus***	***2*** ***E. coli***	***3*** ***S. paratyphi***	***4*** ***S. enterica***	***5*** ***S. aureus***	***6*** ***S. boydei***	
FSK 0.30	2 ± 0.1	NI	2 ± 0.2	NI	2 ± 0.3	2 ± 0.2	
FSK 2	3 ± 0.2	7 ± 0.2	4 ± 0.2	6 ± 0.2	6 ± 0.1	3 ± 0.2	
FSK 3	**12 ± 1.0**	**8 ± 0.5**	**6 ± 0.3**	**7 ± 0.5**	**8 ± 0.5**	3 ± 0.2	
Clinical pathogens							
	1 ***MRSA***	***2*** ***S. typhi***	***3*** ***S. aureus***	***4*** ***E. coli***	***5*** ***E. cloacae***	***6*** ***S. sonnei***	***7*** ***S. boydei***
FSK 0.30	NI	NI	NI	2 ± 0.2	1 ± 0.2	2 ± 0.3	NI
FSK 2	5 ± 0.2	8 ± 0.3	6 ± 0.2	8 ± 0.2	7 ± 0.2	6 ± 0.2	7 ± 0.3
FSK 3	5 ± 0.2	8 ± 1.0	6 ± 0.2	**10 ± 1.0**	**8 ± 0.5**	6 ± 0.2	6 ± 0.5

NI = no inhibition

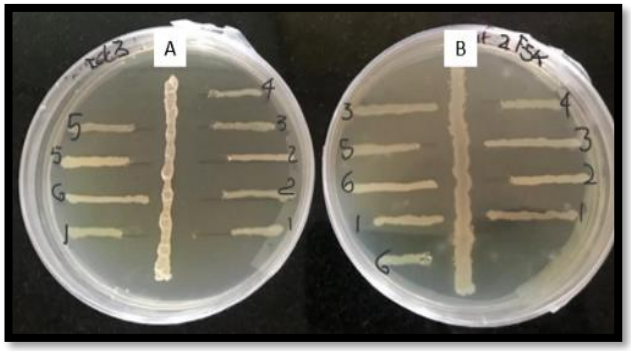


Plate 13 A: Antimicrobial activity of the isolates (A) FSK 3 and (B) FSK 2 with ATCC strains:(1) *B. cereus* ATCC 14579, (2) *E. coli* ATCC 25922, (3) *S. paratyphi* ATCC 14028, (4) *S. enterica* ATCC 35664, (5) *S. aureus* ATCC 6538 and (6) *S. boydei* ATCC 8700.

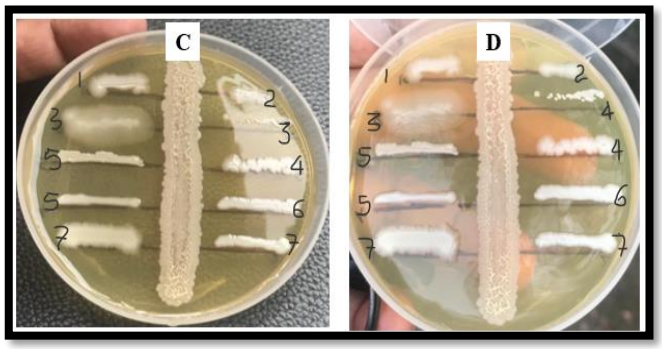


Plate 13 B: Antimicrobial activity of the isolates (C) FSK 3 and (D) FSK 2 with clinical laboratory pathogens: (1) *Methicillin resistant Staphylococcus aureus (MRSA)*, (2) *S. typhi*, (3) *S. aureus*, (4) *E. coli*, (5) *E. cloacae*, (6) *S. sonnei* and (7) *S. boydei* (Representative images).

Antimicrobial activity is a crucial criterion in selecting potential probiotic strains, as it helps prevent the attachment and colonization of putrefactive or pathogenic bacteria in the gut. The antagonistic properties of probiotics play a key role in protecting the host by inhibiting or preventing the invasion of harmful microorganisms. This inhibitory effect may result from the production of various antimicrobial substances, including hydrogen peroxide; organic acids such as lactic, formic, and acetic acids; ethanol; diacetyl; acetaldehyde; fatty acids; acetoin; reutericyclin; reuterin; bacteriocins and bacteriocin-like compounds; peptide and lipopeptide antibiotics; as well as other low–low-molecular-weight compounds with

antimicrobial properties. Bacillus species are widely recognized for producing diverse antimicrobial metabolites (Ritter et al., 2018). Our findings are in accordance with Fernandes et al. (2019) and Pawaskar et al. (2022

The study findings indicate that the isolates possess potential applications in the food industry as starter cultures, co-cultures, or bioprotective cultures to enhance food quality and safety. Furthermore, they may serve as probiotic therapeutics suitable for clinical use.

4.2.1.5 Antibiotic disc sensitivity of the *Bacillus* isolates

The antibiotic susceptibility also influences the probiotic bacteria's survival in the GIT. The increasing use of antibiotics for the treatment of GIT infections could disturb the balance of gut microflora. An essential approach for differentiating intrinsic from acquired antibiotic resistance in probiotic bacteria involves analysing and comparing the antibiotic susceptibility profiles of multiple representative strains from each species (Aziz et al., 2023). The susceptibility of the three salt pan isolates to 13 antibiotics from various classes, *viz*. inhibitors of cell wall synthesis, protein synthesis, and nucleic acid synthesis, is represented in Table 19. The isolate FSK 3 was found to be susceptible to nine antibiotics, FSK 2 to seven antibiotics, and FSK 0.30 to five antibiotics. FSK 3 was moderately susceptible to Penicillin and Trimethoprim, whereas FSK 2 was moderately susceptible to Kanamycin, Penicillin, Streptomycin, and Trimethoprim (Plate 14). The isolate FSK 0.30 was found to be resistant to most of the antibiotics (06).

All isolates exhibited resistance to Ampicillin and Cephalothin, which suggests intrinsic or acquired mechanisms that enable their survival. This resistance allows them to persist in the gut during antibiotic treatment, potentially benefiting gut microbiota restoration (Berebon et al., 2019). However, if resistance is acquired, there is a risk of horizontal gene transfer to pathogens. Further research is needed to assess safety and probiotic efficacy. Additionally, all isolates showed the highest susceptibility to Vancomycin, Erythromycin, Tetracycline, Gentamycin, and Ciprofloxacin, indicating a lack of resistance mechanisms, making them vulnerable during antibiotic treatment but reducing the risk of resistance gene transfer (Georgieva et al., 2015). Tetracycline resistance genes are among the most frequently identified resistance determinants in Lactobacilli (Banerjee et al., 2017; Aziz et al., 2023).

Several species of other probiotic cultures, like *Bifidobacterium* and *Bacillus* species like *B. subtilis* and *B. clausii,* have been reported to harbor antibiotic resistance genes, posing a potential risk of transfer to other bacteria within the intestinal microbiota (Georgieva et al., 2015). These results suggest that antibiotic resistance patterns differ between studies, likely reflecting variations in experimental conditions. The susceptibility of probiotic bacteria to antibiotics can influence their survival in the gastrointestinal tract, and widespread antibiotic use for treating GIT infections may disturb the balance of gut microbiota (Berebon et al., 2019).

Table 19: Antibiotic susceptibility of the saltpan bacterial strains.

Antibiotic Name/ Dosage	**Zone Diameter (mm) and interpretation***			**Interpretative zone diameter (mm)***		
	FSK 0.30	**FSK2**	**FSK 3**	**R**	**MS**	**S**
Inhibitors of cell wall synthesis						
Vancomycin [Va](30µg)	**20^{S}**	**18^{S}**	**18^{S}**	≤ 14	15-16	≥ 17
Ampicillin [A] (10 µg)	0^{R}	2^{R}	05^{R}	≤ 12	13-15	16
Cephalothin [Ch] (30 µg)	14^{R}	4^{R}	14^{R}	≤ 14	15-17	≥ 18
Inhibitors of protein synthesis						
Erythromycin [Ery](15 µg)	**30^{S}**	**25^{S}**	**30^{S}**	≤ 13	14-17	≥ 18
Kanamycin [Ka](30 µg)	15^{MS}	14^{MS}	**25^{S}**	≤13	14 - 17	≥18
Tetracycline [Te] (30 µg)	**30^{S}**	**25^{S}**	**30^{S}**	≤ 14	15-18	≥ 19
Chloramphenicol [C](30 µg)	0^{R}	**35^{S}**	**20^{S}**	≤ 13	14-17	≥ 18
Gentamycin [G] (10 µg)	**22^{S}**	**18^{S}**	**25^{S}**	≤ 12	13-14	≥ 15
Penicillin [P](10 µg)	12^{MS}	13^{MS}	16^{MS}	≤ 11	12-21	≥22
Streptomycin [St] (10 µg)	08^{R}	12^{MS}	**16^{S}**	≤ 11	12-14	≥15
Inhibitors of nucleic acid synthesis						
Ciprofloxacin [Cf] (5µg)	**27^{S}**	**25^{S}**	**29^{S}**	≤ 13	14-18	≥ 19
Ofloxacin [O] (5µg)	12^{R}	**18^{S}**	**16^{S}**	≤ 12	13-14	≥ 15
Trimethoprim [Tr] (5µg)	10^{R}	13^{MS}	13^{MS}	≤ 11	12-14	≥ 15

***R (resistant), MS (moderately susceptible), or S (susceptible) (Charteris et al., 1998)**

When consumed, probiotics can serve as a reservoir for genes that may be transferred to both pathogenic bacteria and normal flora. Therefore, it is essential to assess whether these genetic elements can be transmitted within the food and gut environment (Selvin et al., 2020). Additionally, animal probiotics contain live bacteria, and in the European Union, policies aim to eliminate transmissible resistance in these products. This consideration is crucial before consuming human probiotics.

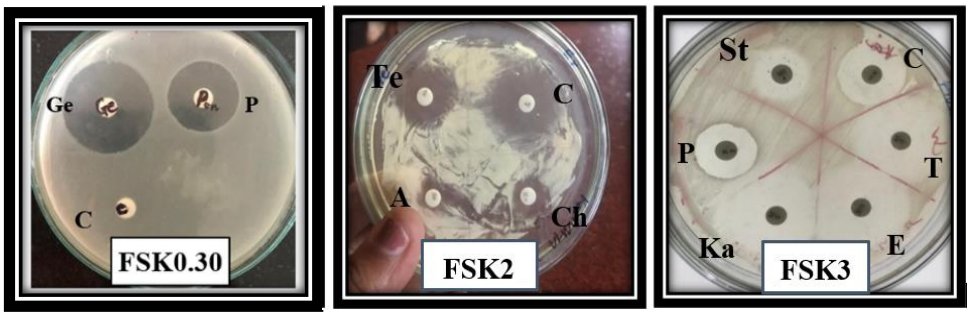


Plate 14. Antibiotic susceptibility test of isolates FSK 0.30, FSK 2, and FSK 3 by the Kirby–Bauer disk diffusion method against Gentamicin (G), Penicillin (P), Tetracycline (Te), Chloramphenicol (C), Ampicillin (A), Cephalothin (Ch), Kanamycin (K), Erythromycin (E), Streptomycin (St), Trimethoprim (Tm), and Ciprofloxacin (Cf).

Since the prospective probiotic cultures in the present study showed sensitivity to most of the tested antibiotics, it can be concluded that they may not harbour genes for antibiotic resistance, thus proving their safety to be administered without the fear of transmitting antibiotic resistance, and can be used in the development of synbiotic foods.

4.2.2 Desirable probiotic attributes of *Bacillus* isolates

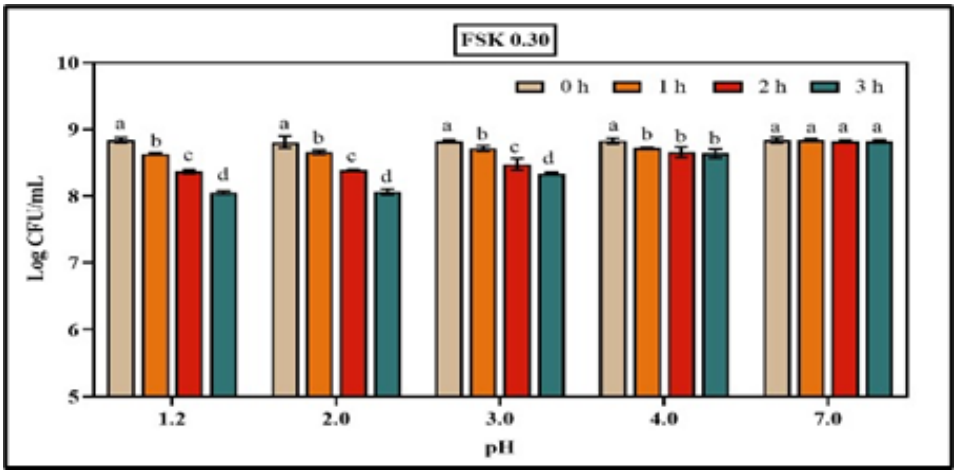
4.2.2.1 Gastric acid tolerance (*in-vitro*)

Probiotic bacteria must withstand stressful environments to remain viable (Keller et al., 2019). Before reaching the intestines, probiotic bacteria must endure the stomach's highly acidic environment, where the pH can drop to 1.5–2.0, and survive for at least 4 hours before progressing to the intestinal tract (Akweya et al., 2020). Gastric acid tolerance is crucial for their passage to the small intestine and colon, aiding in gut microbiota balance (Behera et al., 2018).

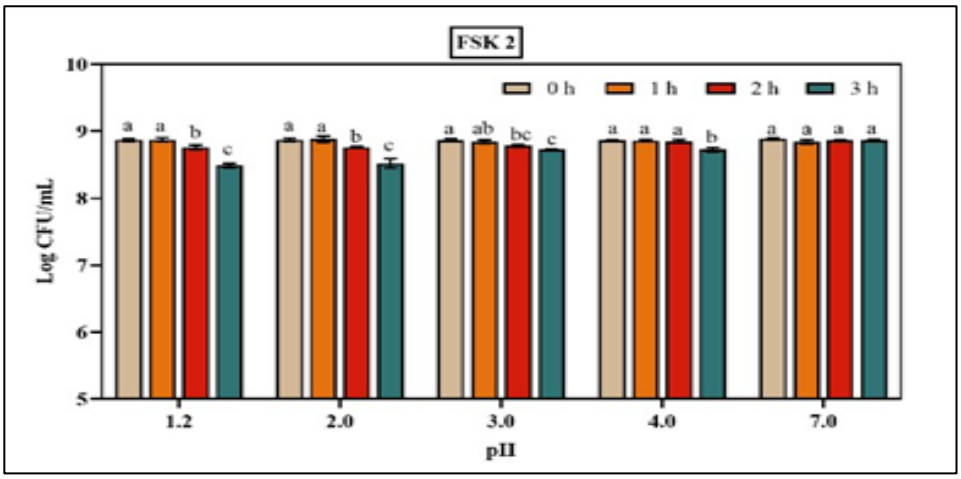
It also ensures survival in high-acid carrier foods and resistance to gastric stresses (Champagne & Gardner et al., 2008). Gastric acid tolerance is an important criterion when evaluating probiotics for use as supplements, as it determines a strain's ability to survive in highly acidic carriers for extended periods without significant loss in cell numbers (Ganguly et al., 2011). The capacity of probiotic bacteria to withstand acidic conditions varies depending on the species and strain (Patra & Duary, 2022). Therefore, assessing acid tolerance is essential to ensure the viability and functionality of potential probiotic isolates. The three investigated isolates could survive at low pH for 3 h (Figure 3 A, B, and C). The viability of FSK 0.30 significantly decreased ($p< 0.05$) with time at pH 1.2, 2.0, and 3.0, with a significant reduction at 3 h. At pH 4.0, a significant reduction ($p < 0.05$) was observed in the viable count after 1 h with no further significant reduction, indicating a tolerance at this pH value. The viable cell counts FSK 0.30 decreased at pH 1.2 and 2.0 ($p < 0.05$) after 1 h of incubation, as compared to FSK 2 and FSK 3, indicating its poor tolerance to acid. FSK 2 showed a slight but statistically significant decline ($p < 0.05$) in viability with time at pH 1.2 to 3.0 after 2 h, whereas at pH 4.0 a significant difference was observed after 3 h, indicating its strong acid tolerance, especially at moderate pH. FSK 2 shows moderate acid tolerance, with better survival than FSK 0.30.

FSK 3 showed a small but significant reduction ($p < 0.05$) in viability at pH 1.2 and 2.0. from 2 h to 3 h. The viability significantly altered ($p < 0.05$) at pH 3.0 and 4.0 after 3 h and 2 h respectively. However, FSK 3 suggests best acid tolerance among the three isolates with an appreciable viable count and more than 90 % survival at acidic pH with a significant higher ($p < 0.05$) percentage survival as compared to FSK 0.30 and FSK 2 (Figure 4). Our findings align with the observations of Ritter et al. (2018), who reported a significant reduction in viability of the *Bacillus* isolates at low pH with appreciable viable count. The viability of all the three bacterial isolates remained unaltered at pH 7. *Bacillus* cells are sensitive to acidic stress, showing significant loss of viability during simulated gastrointestinal conditions, but with appreciable counts. Khan et al. (2023) reported that *B. licheniformis* QAUBL1902 maintained 91 % survival after 4 hours of exposure to acidic conditions. Similarly, Elshaghabee et al. (2017) demonstrated that *L. sporogenes, B. laevolacticus,* and *B. racemilacticus* exhibited significant survival rates at pH 2.5, indicating the presence of *Bacillus* species capable of tolerating highly acidic environments.

A



B



C

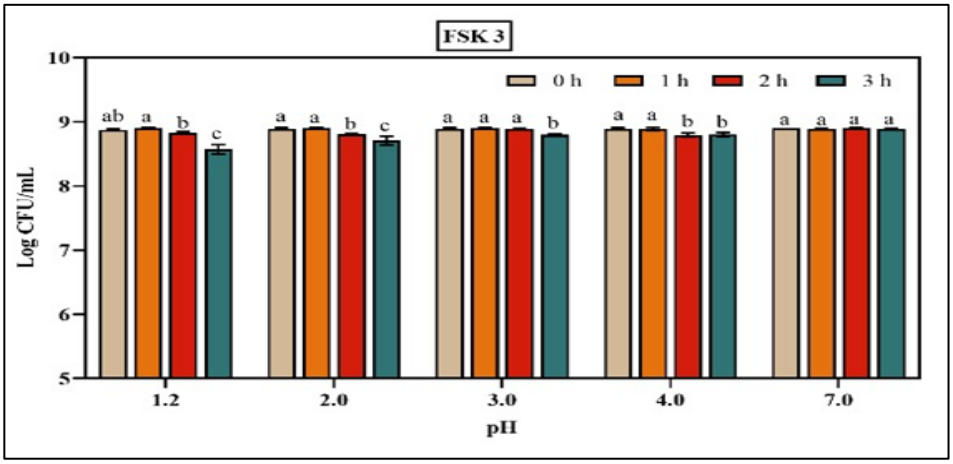


Figure 3 (A, B, and C): Viability of *Bacillus* isolates (FSK 0.30, FSK 2, and FSK 3) under simulated gastric conditions at different pH levels over 3 hours.

Each value is the mean ± SD of three replicates (n = 3). Different lowercase letters above bars indicate statistically significant differences ($p < 0.05$) as determined by ANOVA followed by Tukey's post hoc test for each pH level.

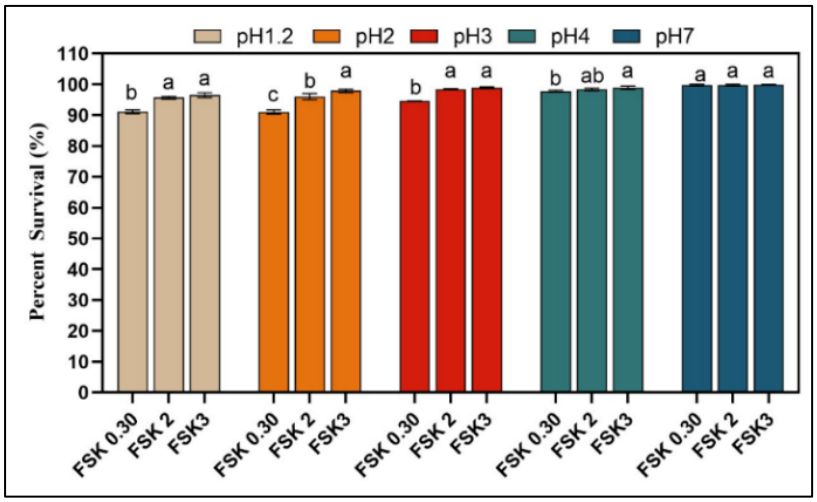


Figure 4: Effect of simulated gastric pH on the survival of *Bacillus* isolates (FSK 0.3, FSK 2, and FSK 3) after 3 hours of exposure.

Values are expressed as the mean ± SD of three replicates. Different letters in lowercase above the bars denote statistically significant differences ($p < 0.05$), determined by ANOVA followed by Tukey's post hoc test for each pH level.

4.2.2.2 Tolerance to intestinal bile salts (*in vitro*)

Resistance to bile is an important characteristic of probiotic bacteria, enabling them to survive and grow in the GIT without being destroyed (Clavel et al., 2004; Akweya et al., 2020). Bile concentrations in the human GIT fluctuate, averaging 0.3 % w/v, with a typical retention time of around 4 h (Ritter et al., 2018). Bile secreted in the duodenum can negatively affect bacterial survival, as the lipid and fatty acid components of the bacterial cell membrane are susceptible to damage by bile salts, which can alter cell permeability and viability (Hernández-Gómez et al., 2021). Thus, tolerance to bile salts is essential for sustaining metabolic activity and facilitating bacterial colonization in the host's small intestine (Akweya et al., 2020).

Figure 5 presents the survivability (log CFU/mL) of cell suspensions of three isolates under simulated small intestinal conditions at pH 8.0 after exposure to various bile salt concentrations (0.5 % - 2 %) and time intervals (0 - 4 h). The abundance of FSK 2 and FSK 3 organism remained unaltered (~9 log CFU/mL) without bile salts with FSK 2 isolate showing a non-significant increase ($p > 0.05$) in viable count without bile salts. This slight increase may be attributed to the isolate's adaptation to non-stressful environment lacking bile salts,

that may have allowed the cells to recover, stabilize, or undergo limited metabolic activity, resulting in improved survival or modest growth. However, FSK 0.30 showed a significant reduction ($p<0.05$) in its viable count (~8.5 log CFU/mL) after 3 h of exposure.

A slight decline ($p < 0.05$) in the viable count of FSK 0.30 after 4 h was seen at 0.5 % bile salt concentration, however no significant decline was seen at 1 % concentration indicating good tolerance. The isolate showed reduced survival at 1.5 % concentration after 4 h exposure with poor tolerance to the highest 2 % concentration of bile salts with a highly significant reduction in viable count ($p < 0.001$) as seen in Figure 9.

FSK 2 exhibited a moderate tolerance to bile salts, with minor reductions in viable count, but better than isolate FSK 0.30. FSK 2 survived longer but still showed some viability loss ($p < 0.05$) at higher concentrations and time points (4 h) of exposure. Isolate FSK 3 showed consistently high bile salt tolerance even at 2 % over 4 h, with good viability, followed by FSK 2 at 3 h and 4 h. These findings align with observations reported in previous studies (Funck et al., 2019; Fernandes et al., 2019). These results indicate that our isolates are capable of surviving in the gastrointestinal tract and may play a key role in both specific and non-specific host immune defense mechanisms. Nithya & Halami. (2012) reported *Bacillus* sp., namely *B. flexus* Hk1, *B. licheniformis* Me1, and *B. subtilis* Bn1, previously isolated from milk, cheese, and fermented beans, respectively, to possess better acid and bile tolerance.

The percentage survival of the three bacterial isolates was evaluated on exposure to 0.5 % bile salt concentration for 4 h. All the isolates showed > 90 % survival with FSK 3 showing 97.8 % survival, while FSK 2 and FSK 0.30 exhibited 96.37 % and 92.18 % survival, respectively which is required for persistence in the small intestine and is a satisfying key selection criterion for probiotic application. Khan et al. (2023) observed that *B. licheniformis* QAUBL1901 exhibited a 92 % survival rate in 0.45 % bile salts after 4 hours of exposure, while *B. mycoides* BM1901 showed a survival rate of 61 %. FSK 3 was significantly more tolerant ($p < 0.05$) than FSK 2 and FSK 0.30, indicating its superior resistance to bile salt stress.

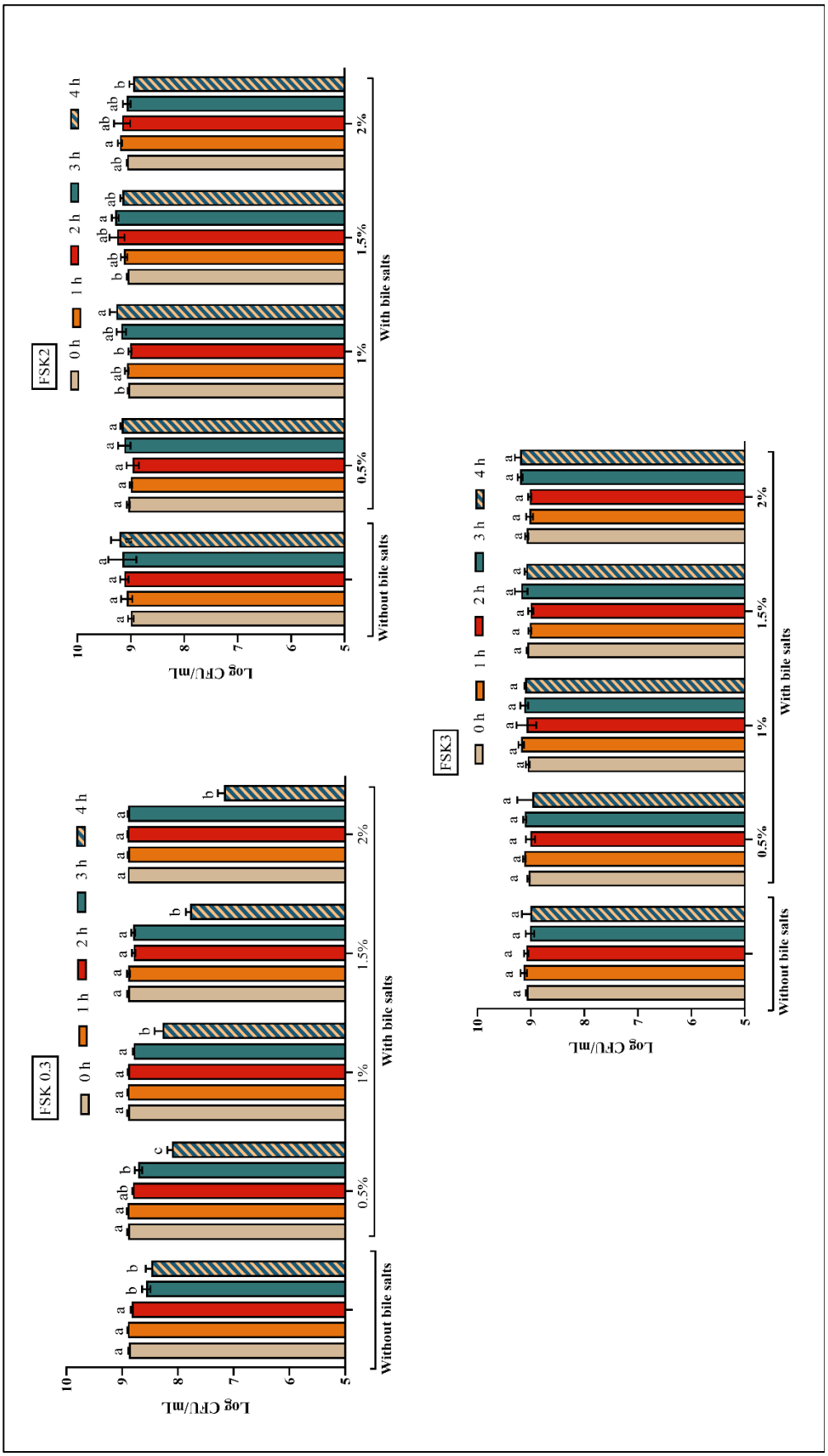


Figure 5: Viability of isolates during exposure to bile salts.

Each value is the mean ± SD of three replicates (n = 3). Different lowercase letters above bars indicate statistically significant differences ($p < 0.05$) as determined by ANOVA followed by Tukey's post hoc test for each pH level.

4.2.2.3 Bile salt hydrolase (BSH) activity

4.2.2.3.1 BSH activity by plate method

Isolates were grown in the presence of Soft Mann Rogosa Sharpe (MRS) agar plates supplemented with 0.3 % sodium taurocholate (NaTC) or glycocholate (NaGC) to assess their ability to hydrolyse bile salts. Taurocholate or glycocholate are the two main bile salts of human body produced in the liver. They should be deconjugated for overall reduction in cholesterol levels (Kumar et al., 2012). FSK 2 and FSK 3 showed precipitation zones towards both the salts whereas FSK 0.30 did not show zone towards NaGC salt. As seen in Plate 15, FSK 3 exhibited a 14 ±1 mm opaque halo zone on glycocholate-supplemented MRS agar, indicating strong BSH activity. Whereas, the zone size of standard probiotic *L. plantarum* MTCC 2621 was bigger (20 ±1 mm). FSK 2 showed a moderate zone of 10 mm on NaGC salt.

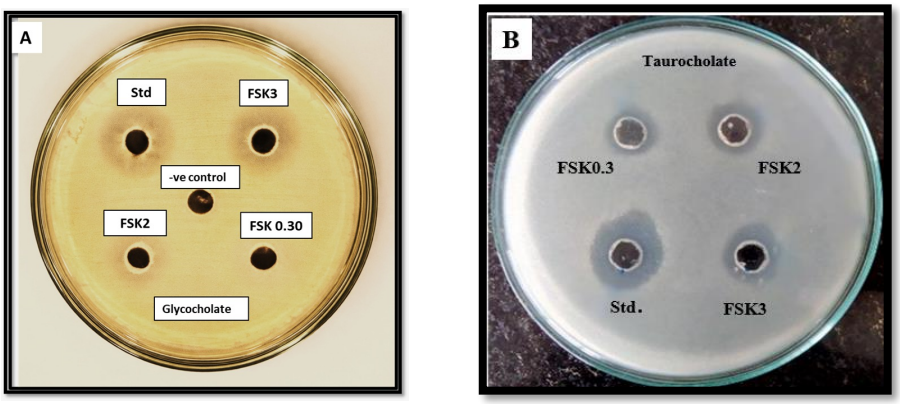


Plate 15: BSH activity of *L. plantarum* (positive control) and halotolerant isolates with (A) sodium glycocholate and (B) sodium taurocholate.

The isolates FSK 0.30, FSK 2, and FSK 3 displayed precipitation zones around the wells, indicating visible BSH activity caused by the precipitation of deconjugated bile acids following taurocholate hydrolysis. As shown in Figure B, all isolates exhibited BSH activity to varying degrees. The standard positive control produced the largest halo (12 ±1 mm), followed by FSK 3 (12 ±1 mm), reflecting strong BSH activity, while FSK 2 and FSK 0.30 showed moderate (10 ±1mm) and smaller (8 ±1 mm) zones, respectively. These results suggest that all strains can deconjugate bile salts, with FSK 3 demonstrating the highest activity, closely approaching that of the reference strain. This enzymatic ability likely contributes to bile tolerance in the GIT and may support cholesterol-lowering effects (Shehata et al., 2016).

The taurocholate hydrolysis pattern reinforces the probiotic potential of FSK 3 in bile salt metabolism. FSK 2 and FSK 3 not only survived the toxicity of these salts but also deconjugated them, potentially facilitating intestinal colonization. Deconjugation of bile salts in the intestine is a known mechanism for probiotic resistance to high bile concentrations (Funck et al., 2019), and our findings align with Khan et al. (2023), who reported BSH activity in *Bacillus* strains isolated from curds. Strain-specific differences in BSH activity could enhance probiotic potential, warranting further investigation.

BSH enzymes have generally been found to be intracellular, oxygen-insensitive, and have slightly acidic pH optima (pH 5–6) (Hernández-Gómez et al., 2021). In *Bifidobacterium* sp., BSH activity is closely linked to bile salt gelation, which, if occurring under physiological conditions in the human gut, may enhance bacterial colonization and survival (Liang et al., 2020).

4.2.2.3.2 BSH activity by modified ninhydrin method

The ability of probiotic strains to produce BSH is one of the *in vitro* tests specified by ICMR-DBT (2011) guidelines for use of probiotics in food. Tables 20 and 21 shows BSH activity of 3 bacterial isolates (FSK 0.30, FSK 2, FSK 3) and *L. plantarum* at different pH levels (4 to 9) when tested against taurocholate and glycocholate salts. Two clear BSH activity trends emerged, with FSK 2 and FSK 3 showing increase in activity as the pH increased, preferring alkaline conditions, whereas the standard *L. plantarum* showed a decrease in activity with increased pH, thus showing its preference to acidic conditions.

The positive standard culture, *L. plantarum,* expressed the maximum BSH activity towards taurocholate at low pH 4 (41.40 ± 0.32 IU/h), but the activity declined steadily as pH increased. The activity dropped to 24.66 IU/h at pH 9, suggesting BSH enzyme of *L. plantarum* as acidophilic, best functioning in the acidic range, and likely adapted to the upper GIT. A similar consistent trend was shown for glycocholate, but with higher BSH activity (pH 4: 51.48 51.48 ± 0.5 IU/h). While activity increased with rising pH for all the test isolates, L. plantarum exhibited maximal activity under acidic conditions (pH 4–5), indicating that its BSH enzymes are more adapted to low pH environments typical of fermented substrates and the upper gastrointestinal tract.

Table 20: BSH activity (IU/h) at different pH levels towards taurocholate.

Isolates	**BSH activity (IU/h) towards taurocholate**					
pH ⟶	**4**	**5**	**6**	**7**	**8**	**9**
FSK 0.30	**05.10 ± 0.07^{e}**	06.06 ± 0.08^{d}	07.26 ± 0.14^{c}	08.09 ± 0.72^{b}	09.36 ± 0.08^{a}	09.48 ± 0.80^{a}
FSK 2	14.60 ± 0.31^{f}	17.86 ± 0.41^{e}	**20.70 ± 0.70^{d}**	**21.97 ± 0.52^{c}**	**25.21 ± 0.60^{b}**	**25.80 ± 0.76^{a}**
FSK 3	13.02 ± 0.71^{f}	14.58 ± 0.62^{e}	**24.52 ± 0.40^{d}**	**25.22 ± 0.54^{c}**	**27.78 ± 0.70^{b}**	**29.19 ± 0.80^{a}**
L.plantarum	**41.40 ± 0.32^{a}**	32.34 ± 0.70^{b}	31.44 ± 0.80^{b}	30.75 ± 0.71^{b}	26.08 ± 0.60^{c}	24.66 ± 0.80^{d}

Values are expressed as the mean ± SD of three replicates (n = 3). Superscript letters within the same row denote statistically significant differences ($p < 0.05$), determined by one-way ANOVA followed by Tukey's post hoc test for each isolate across different pH levels.

Among the test isolates, FSK 3 performed best at alkaline pH, with the highest BSH activity at pH 4 towards taurocholate, possibly suggesting its better suitability for colon or larger intestine. Activity increased sharply from pH 4 (13.02 ± 0.71 IU/h) to pH 9 (29.19 ± 0.80 IU/h) with a steep rise between pH 5 to 6 (from 14.58 ± 0.62 to 24.52 ± 0.40 IU/h), indicating a pH-sensitive enzyme.

At all pH values, BSH activity toward glycocholate was significantly higher ($p < 0.01$) than for taurocholate, with maximum BSH activity towards glycocholate at pH 8 (41.07 IU/h**),** by isolate FSK 3. Since the pH of the small intestine ranges from 6 to 8, the peak BSH activity of FSK 3 in this range indicates its physiological compatibility with the human gut. FSK 2 also showed consistently high BSH activity across all pH values, with a maximum activity at pH 9 (32.78 ± 0.56 IU/h) towards glycocholate, significantly higher ($p < 0.05$) than taurocholate activity at the same pH (25.80 ± 0.76 IU/h).

Table 21: BSH activity (IU/h) at different pH towards glycocholate.

Isolates	**BSH activity (IU/h) at different pH levels towards glycocholate**					
pH ⟶	**4**	**5**	**6**	**7**	**8**	**9**
FSK 0.30	11.13 ± 0.2^{e}	13.45 ± 0.40^{d}	15.36 ± 0.4^{c}	15.58 ± 0.5^{c}	17.48 ± 0.4^{b}	**18.12 ± 0.60^{a}**
FSK 2	25.56 ± 0.3^{f}	27.47 ± 0.10^{e}	29.81 ± 0.4^{d}	30.24 ± 0.6^{c}	31.29 ± 0.4^{b}	**32.78 ± 0.56^{a}**
FSK 3	34.0 6± 0.4^{c}	37.46 ± 0.34^{b}	38.31 ± 0.5^{b}	39.16 ± 0.8^{a}	**41.07 ± 0.5^{a}**	40.01 ± 0.60^{a}
L. plantarum	**51.48 ± 0.5^{a}**	48.29 ± 0.3 ^{b}	40.86 ± 0.7^{c}	40.44 ± 0.8^{c}	35.12 ± 0.5^{d}	31.72 ± 0.70^{e}

Each value is presented as the mean ± SD of three replicates (n = 3). Superscript letters within the same row indicate statistically significant differences ($p < 0.05$), determined using one-way ANOVA followed by Tukey's post hoc test for each isolate across the pH levels.

The least BSH activity was given by FSK 0.30 across all pH levels, overall, making it a weak BSH producer and limiting its capability of deconjugating bile salts, potentially reducing its ability to survive in the bile-rich small intestine functional significance. The trend remained similar to other isolates, showing a gradual increase in BSH activity towards taurocholate and glycocholate, peaking at pH 9 to 9.48 ± 0.80 IU/h and 18.12 ± 0.60 IU/h, respectively. The pH-dependent variation in BSH activity was significant ($p < 0.001$).

The test isolates demonstrated higher substrate specificity for glycoconjugates as compared to tauro-conjugates ($p < 0.05$), indicating that our *Bacillus* isolates are particularly effective at deconjugating this specific bile salt. As glycocholate is the major bile salt in the human intestine, Brashears et al. (1998) proposed that a BSH-active strain preferentially hydrolysing sodium glycocholate may have an enhanced ability to reduce serum cholesterol, assuming that in vivo bile salt hydrolysis contributes to cholesterol lowering.

If *Bacillus* isolates deconjugate taurocholate more efficiently than glycocholate, it suggests a substrate preference for BSH enzymes, influencing bile metabolism. This may impact gut microbiota, fat digestion, and cholesterol reduction by promoting bile salt excretion (Hernández-Gómez et al., 2021).

The ability of these salt pan bacteria to deconjugate bile salts may serve as a detoxification mechanism, with BSH contributing to bile tolerance and supporting bacterial survival in the GIT. Collectively, the data indicate that FSK 3 is the most promising novel strain based on BSH activity, especially for applications targeting the lower GIT or alkaline environments. FSK 2 also shows consistent, high activity and potential utility. Absorbance at 570 nm after ninhydrin reaction of the test isolates and standard culture, along with estimation of taurine liberated, is evident in the graph and indicated in Appendix III.

4.2.2.4 Adherence capacity

The ability of these salt pan bacteria to deconjugate bile salts may serve as a detoxification mechanism, with BSH contributing to bile tolerance and supporting bacterial survival in the GIT with hydrophobicity serving as an *in-vitro* biomarker for evaluating the colonization potential of probionts. This adherence is crucial for probiotic colonization and long-term persistence in the gut, playing a key role in their health benefits (Korcz & Varga, 2021).

4.2.2.4.1 Bacterial adhesion to hydrocarbons (BATH)

BATH test is a widely used *in vitro* method for evaluating the hydrophobicity of bacterial cell surfaces. Cell surface hydrophobicity is closely linked to the ability of bacteria to adhere to host tissues, including epithelial cells and mucosal surfaces of the GIT (Funck et al., 2019). In this study, the percentage adhesion of three halotolerant *Bacillus* isolates (FSK 0.30, FSK 2, and FSK 3) to mucus was assessed to determine significant differences in adhesion capacity. One-way ANOVA revealed a statistically significant difference among the isolates ($p < 0.001$). Subsequent Tukey's HSD post hoc test indicated significant pairwise differences ($p < 0.01$), with isolate FSK 3 exhibiting the highest adhesion (40.75 ± 1.05 %), followed by FSK 2 (35.75 ± 0.96 %), and FSK 0.30 the lowest (26.31 ± 0.27 %).

These findings demonstrate considerable variability in surface hydrophobicity among the tested halotolerant isolates. Similar adhesion capacities have been reported in other *Bacillus* species, isolated from various food sources (Fernandes et al., 2019; Nitya & Halami, 2019). Moreover, extracellular polysaccharides (EPS) influence cell surface hydrophobicity and charge, thereby enhancing the adhesion of probiotic bacteria to host cells and mucosal linings (Korcz & Varga, 2021).

4.2.2.4.2 Exopolysaccharide (EPS) production by hypersaline bacteria

EPS are carbohydrate-rich polymers secreted by bacteria, often containing proteins and other molecules, that play a vital role in bacterial aggregation, adhesion, and colonization (Asgher et al.,2020; Wang et al., 2020). Their production improves probiotic survival, functionality, and technological properties, making them valuable in food and pharmaceutical industries (Xu et al., 2019; Korcz & Varga, 2021). EPS also enhances the texture and stability of fermented foods and may act as prebiotics.

The three bacterial isolates were qualitatively screened for (EPS) production, which plays a key role in enhancing probiotic adhesion to intestinal cells. EPS production was confirmed by the formation of viscous strings and the appearance of black colonies on Congo Red agar (Plate 16 A). Further quantification of EPS among the two halotolerant isolates exhibiting high (BSH) activity (FSK 2 and FSK 3) revealed that FSK 3 produced the highest amount of EPS (1.42 ± 0.05 mg/mL), followed by FSK 2 (1.05 ± 0.04 mg/mL). Plate 16 B presents the precipitation of EPS from isolate FSK 3. Asgher et al. (2020) reported that *B. licheniformis* produced 3.4 g/L of EPS during cost-effective solid-state fermentation. High EPS-producing strains demonstrate better resistance to gastrointestinal stresses such as acid and bile, promoting probiotic persistence in the gut (Xu et al., 2019; Yasmin et al., 2020). Furthermore, EPS protect against phagocytosis and bacteriophage attack and serve as substrates for gut microflora (Patel et al., 2012).

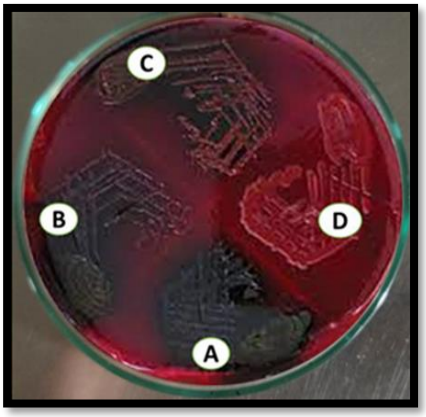


Plate 16 A: Primary screening for EPS-producing bacterial isolates [(A) FSK 3, (B) FSK 2, (C) FSK 0.30, (D) *L. plantarum*] on Congo Red agar showing black colonies.



Plate 16 B: Ethanol precipitation of EPS from isolate FSK 3.

4.2.2.4.3 Mucus adhesion

The halotolerant bacteria (02) were further screened for the mucus adhesion assay. Figure 6 illustrates the two halotolerant isolates showing more than 80 % mucus adhesion. FSK 3 showed the highest mucus adhesion (86.92 ± 3.85 %), followed by FSK 2 (83.08 ± 4.22 %). The mucus layer lining the gastrointestinal tract (GIT) serves as the initial interface between gut microbiota and the host (Collado et al., 2008; Krausova et al., 2019). Probiotic surfaces are composed of diverse biological molecules and structures, including exopolysaccharides (EPS), pili, and lipoteichoic acids, which play a key role in their adhesion to this mucus layer (Lu et al., 2022).

The results of our study demonstrated their capacity to adhere to gut mucus, suggesting their potential to colonize the GIT. Their adherence to probiotic surfaces *in vitro* indicates their potential to survive in the gastrointestinal tract for extended periods and to support pathogen removal, digestion, and the promotion of a healthy gut environment (Lu et al., 2022). This close association with the intestinal lining enables interaction with the host and may allow them to compete with harmful bacteria for binding sites on the mucosal surface (Lu et al., 2022).

This adhesion property is regarded as a critical factor in selecting potential probiotic strains. Another study on food-derived *Bacillus spp*. with probiotic potential reported mucus adhesion values ranging from 30 to 80 % (Ritter et al., 2018). Many EPS-producing probiotics can interact with mucins, the glycoproteins in the gut mucus layer, enhancing their ability to remain attached to the intestinal wall (Ritter et al., 2018). Several studies indicate that bacteria with greater adherence to the mucus layer tend to remain longer in the intestinal tract, suggesting enhanced probiotic potential (Sanchez et al., 2013; Xu et al., 2021)

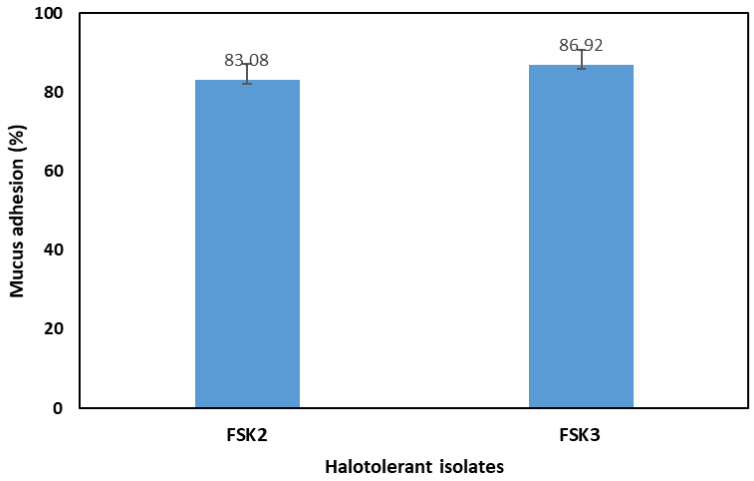


Figure 6: Adhesion capacity of halotolerant *Bacillus* isolates to mucus (in %).

4.2.2.5 Antioxidant activity

The interest in identifying potential probiotics with antioxidant activity stems from their ability to offer a natural, multifunctional, and safe approach to combating oxidative stress, while aligning with the increasing consumer demand for functional and sustainable food products. Oxidative stress is exacerbated by factors such as poor diet, pollution, smoking, and psychological stress, thereby creating a need for natural antioxidant interventions (Jideani et al., 2021). Free radicals are produced in the body as a result of various endogenous processes, environmental exposures, and pathological states. The resulting oxidative stress can damage lipids, proteins, and DNA, contributing to the development of various chronic diseases (Ritter et al., 2018).

Although synthetic antioxidants have been widely used, concerns regarding their potential toxicological effects and health risks when consumed in excess have led to a growing

preference for natural alternatives (Kim et al., 2020). In this context, probiotics with inherent antioxidant capabilities have gained attention.

We assessed the antioxidant activity of two halotolerant bacterial isolates, FSK 2 and FSK 3, in this study using the DPPH radical scavenging assay. The percent scavenging activity was recorded as 60.2 ± 0.6 % for FSK 2 and 66.8 ± 0.72 % for FSK 3. These values were lower than those reported for standard probiotic strains (78 ± 0.7 %) (Ali et al., 2018). The antioxidant potential of probiotic strains varies widely depending on their ability to produce antioxidative enzymes (e.g., superoxide dismutase, catalase), bioactive compounds (e.g., exopolysaccharides, glutathione), and other metabolites (Ritter et al., 2018).

While a DPPH scavenging activity of 70 % or higher is typically considered optimal for probiotic candidates, strains with lower activity may still be valuable due to complementary probiotic properties such as gut adhesion, immune modulation, or pathogen inhibition (Das & Goyal, 2015). Using probiotic bacteria with antioxidant properties can enhance food quality and extend shelf life by reducing oxidative spoilage, preserving sensory attributes, and improving microbial stability (Ritter et al., 2018; Xu et al., 2019).

The reducing power of isolates FSK 2 and FSK 3 was also evaluated, with values recorded at 12.23 µg/mL AAE and 14.78 µg/mL AAE, respectively. However, studies by Kim et al. (2020) & Kim et al. (2021) have reported reducing power of *Bacillus* strains in the range of 30 to 46 µg/mL AAE. Reducing power is a key indicator of antioxidant capacity, reflecting the strain's ability to donate electrons and neutralize free radicals. Probiotics with strong reducing power not only offer health-promoting benefits but also show increased resilience against oxidative stress during food processing and storage (Nithya & Halami, 2013; Ritter et al., 2018). These findings highlight the potential of FSK 2 and FSK 3 as functional probiotics with moderate antioxidant capacity and suggest further exploration of their applications in health-supportive food systems.

4.2.3 Identification of potential probiotic isolates

4.2.3.1 Morphological, biochemical, and physiological characterization of isolates

The two potential *Bacillus* isolates shortlisted for controlled fermentation of GC3 were characterized using morphological, biochemical, and physiological tests. Both isolates were tested negative for indole production, indicating the absence of the enzyme tryptophanase, and therefore the inability to hydrolyse tryptophan. This was confirmed by the lack of red ring formation upon addition of Kovac's reagent. The isolates were positive for citrate utilization, suggesting they can metabolize citrate as a sole carbon source. A positive methyl red (MR) test in both isolates indicated the production of stable acidic end-products during glucose fermentation. However, isolate FSK 2 showed a negative result for the Voges-Proskauer (VP) test, suggesting it does not produce neutral end products such as acetoin. The results of the IMViC tests are presented in Plate 17 and Table 22 B.

Both isolates exhibited catalase activity, suggesting they possess the catalase enzyme, which breaks down hydrogen peroxide into water and oxygen. This trait is significant for potential probiotics, as catalase contributes to oxidative stress resistance, especially in the oxygen-rich regions of the GIT. The ability to neutralize reactive oxygen species (ROS) enhances the survival of probiotic strains under harsh gut conditions. Moreover, catalase-positive strains may offer cross-protection to catalase-deficient beneficial bacteria (e.g., *Bifidobacterium spp*), thereby supporting microbial community stability and promoting host gut health.

Carbohydrate fermentation profiles revealed that both isolates were capable of utilizing glucose, fructose, sucrose, maltose, arabinose, and raffinose (Table 22 B). This broad sugar metabolism supports their adaptability to diverse dietary environments and enhances their probiotic potential by contributing to energy production and colonization efficiency in the host gut. The two isolates demonstrated robust physiological adaptability, growing across a wide pH range of 5 – 8 (Table 22 A), with optimal growth at pH 7, indicating their ability to survive in both food matrices and the fluctuating pH of the gastrointestinal tract. Both the isolates tolerated NaCl concentrations from 0.5 % to 20 %, with optimal growth at 1 %, highlighting their halotolerance and suitability for fermented or high-salt foods.

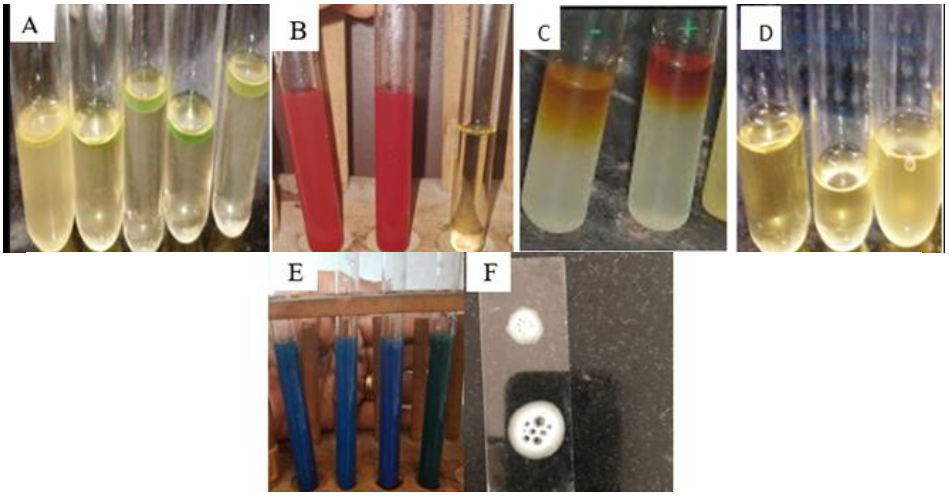


Plate 17: IMViC tests: negative indole utilization test for (A) FSK 2 and FSK 3, (B) positive MR test, (C) positive VP test for FSK 3, and (D) negative VP test for FSK 2. (E) positive citrate utilization test for FSK 2 and FSK 3; and (F) positive catalase test for FSK 3.

Table 22 A: Morphological characteristics of the isolates**.**

Characteristics	**Isolates**	
Morphological	**FSK 2**	**FSK 3**
Gram characteristics	Gram +	Gram +
Morphology	Bacilli	Bacilli
Colour, texture	white, mucoid	white, mucoid
Spores	+	+
Motility	Motile	Motile
% NaCl	0.5-20	0.5-20
Temp °C	15-65	15-65
pH	5-8 (7)	5-8 (7)

‘+’positive, ‘-’negative

Temperature tolerance studies further revealed that the isolates could grow between a broad range of 15 °C and 65 °C, with an optimum at 28 °C, reflecting both psychrotolerant and thermotolerant traits. Collectively, these findings underscore the environmental resilience of the isolates and support their potential as starter cultures or probiotics in functional food systems, capable of withstanding physiological and industrial stresses.

Table 22 B: Biochemical characterization and carbohydrate utilisation of the isolates**.**

Characteristics	**Isolates**	
Biochemical and Carbohydrate Utilization	**FSK 2**	**FSK 3**
Indole	-	-
MR	+	+
VP	-	+
Citrate	+	+
Oxidase	+	-
Catalase	+	+
Urease	-	-
ONPG (β-Galactosidase)	+	+
pNPG (α-Galactosidase)	+	+
Glucose	+	+
Fructose	+	+
Sucrose	+	+
Lactose	-	+
Xylose	-	-
Maltose	+	+
Mannitol	+	-
Arabinose	+	+
Raffinose	+	+

'+'positive, '-'negative

4.2.3.2 Molecular identification

Molecular identification details of the isolates FSK 2 and FSK 3 is presented in Table 23. Salt pan isolate FSK 2 (1484 bp) showed 99.19 % nBLAST similarity with *Bacillus licheniformis* strain ATCC 14580**.** FSK 3 (1487 bp) showed 97.02 % similarity with *Bacillus licheniformis* ATCC 14580 and DSM 13 based on nucleotide homology and phylogenetic

analysis. These sequences have been submitted to the GenBank database under the accession numbers PQ113773 (FSK 2) and PQ098138 (FSK 3).

The phylogenetic placement of the two salt pan bacteria and their closest relatives is depicted in the tree in Figure 7. The figure validates the results of identification, as the bootstrap values were found to be close to 100. The evolutionary relationships of the isolates were determined using the Neighbor-Joining method. The percentage of replicates in which the related taxa clustered together in the bootstrap test (1000 replicates) is depicted next to the nodes. The tree has been drawn to scale, using the same units of branch lengths as those of the evolutionary distances used to deduce the phylogenetic tree.

Table 23: Molecular identification of the selected bacterial isolates.

Sr. No	**Culture code**	**GenBank accession number**	**Length of the sequence**	**Closest match**	**Percent similarity**
1	FSK 2	PQ113773	1484	*Bacillus licheniformis* strain ATCC 14580 and DSM 13	**99.19 %**
2	FSK 3	PQ098138	1487	*Bacillus licheniformis* strain ATCC 14580 and DSM 13	**97.07 %**

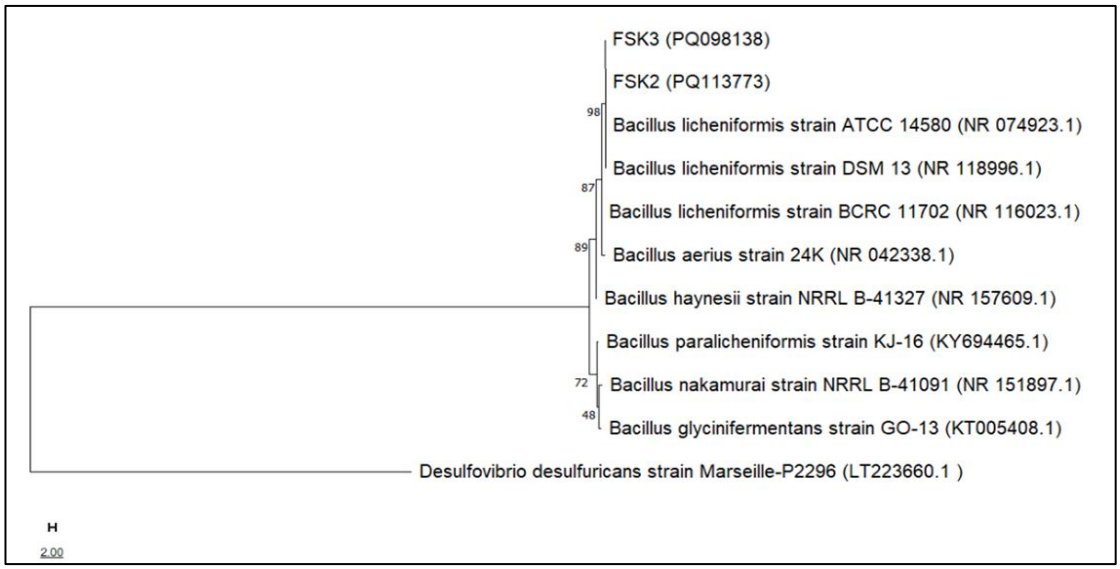


Figure 7. Phylogenetic tree illustrating the 16S rRNA–based sequence similarity of FSK 2 and FSK 3 with related bacterial species.

4.3 Shelf life upon storage at low and room temperature of the *Bacillus* isolate

The shelf life of lyophilized *B. licheniformis* PQ098138, shortlisted as a potential probiotic, was tested at storage temperatures of 4 °C and 28 ± 2 °C (Figure 8). The lyophilized cells of *B. licheniformis* remained viable for 360 d; however, a significant reduction in the viable count was observed ($p < 0.05$). A desirable viable cell count of > 8 log CFU/mL was maintained at 4 °C up to 60 d and at 28 ± 2 °C up to 30 d. A non-significant decrease by 1 log cycle in the viable count of the potential probiotic was seen when stored at RT after 60 d of storage ($p > 0.05$) as compared to the 1 log cycle decrease at 4 °C after 90 d of storage ($p > 0.05$). The percentage survival of *B. Licheniformis* stored at 4 °C and 28 ± 2 °C was 46.74 ± 2 % and 37.34 ± 1.62 % respectively, at the end of 360 d. A storage temperature of 28 ± 2 °C in the present study results in a higher metabolic activity with faster substrate utilization and a lower cell count. Our findings corroborate those of Golnari et al. (2024), who, during the characterization of novel *Bacillus* strains with superior probiotic potential, reported the survivability of cells of some spore-forming species at room temperature, with higher viability at refrigeration temperature. However, the findings imply a need for a suitable cryoprotectant or a food matrix to maintain the viability.

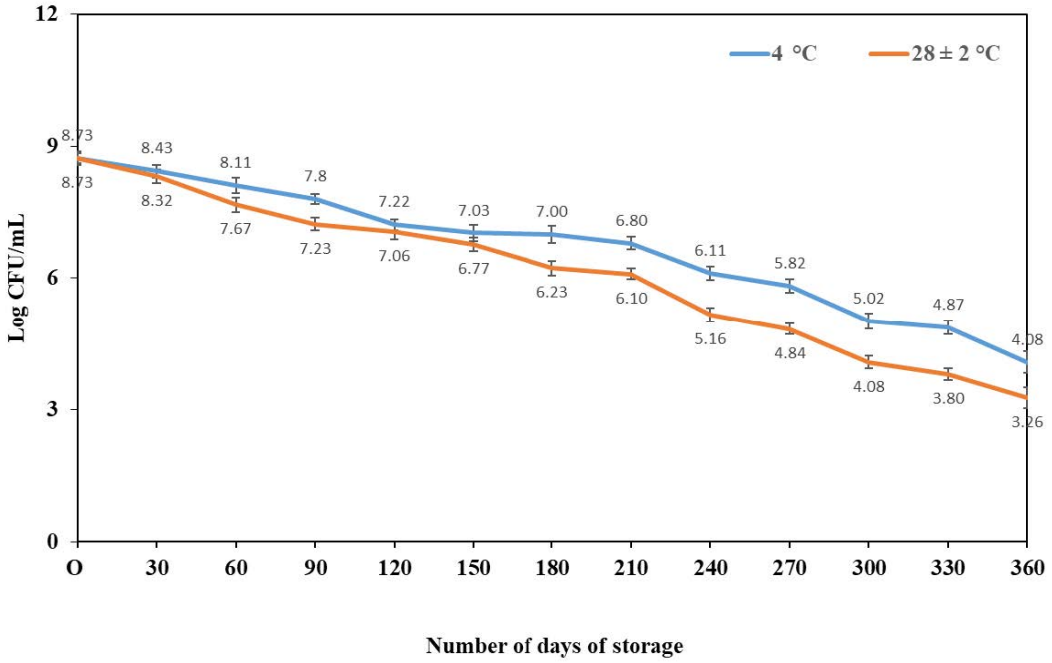


Figure 8: Viability of *Bacillus licheniformis* PQ098138 during storage at 4°C and 28 ± 2 °C

Phase II

4.4 Proximate analysis and nutritional composition of Goa cowpea cultivar 3 (*Vigna unguiculata)* seeds

4.4.1 Proximate composition

Goa Cowpea Cultivar 3 (GC3) seeds, procured from the Indian Council of Agricultural Research (ICAR), Ela Old Goa, Goa (Plate 18), were refrigerated for subsequent use. The seeds were milled into a fine powder and stored under refrigeration at 4 °C in airtight containers for further analysis (Plate 19). Evaluating the nutritional composition of cowpea cultivars is essential, particularly because cowpea serves as a valuable dietary staple in many regions. Understanding the compositional differences among cultivars is critical for developing functional foods, infant formulas, or dietary interventions targeting malnutrition. Legumes such as cowpea contribute significantly to nutritional security because of their rich protein and carbohydrate levels (Eashwarage et al., 2017).

The nutritional quality of cowpea seeds is known to vary by cultivar and is influenced by environmental conditions, soil fertility, season, and agronomic practices (Manohara et al., 2019). In Goa, cowpea is primarily cultivated as a rabi crop in rice-fallow fields under residual moisture conditions, making it a strategically important pulse for the region.



Plate 18: Field view of the Goa cowpea 3 cultivar with an inset showing harvested seeds.



Plate 19: Dry grains of the Goa cowpea 3 cultivar milled into powder.

The moisture content of GC3 seeds was estimated to be 9.97 ± 0.24 g/100 g, indicating low water activity that contributes to extended shelf life, consistent with previous findings by Hossain et al. (2016). This moisture level is slightly lower than the range reported for 24 cowpea cultivars (10.24 – 12.15 g/100 g) by Bai et al. (2020), suggesting better storage stability for GC3. A slightly non-significant difference ($p > 0.05$) was observed compared to the reported values for cowpea species in the Indian Food Composition Table (IFCT), 2017. The ash content, which reflects the total mineral composition, was estimated at

3.36 ± 0.06 g/100 g, suggesting a substantial mineral presence in GC3 seeds, but was significantly higher ($p < 0.01$) than the species reported by Longvah et al. (2017) in IFCT. Table 24 indicates the proximate composition of GC3 seeds.

The protein content of GC3 was found to be 26.16 ± 0.23 g/100 g, which lies within the acceptable nutritional range reported by Carvalho et al. (2012) and Gonçalves et al. (2016) (20.3 – 39.4 g/100 g), although the methionine content is generally low in Cowpea proteins. Notably, the protein content in GC3 is significantly higher ($p < 0.001$) than that reported in the IFCT, 2017 for cowpea brown species (20.36 ± 0.59 g/100 g) (Longvah et al., 2017), and is comparable to the highest values observed in Chinese cultivars such as JXCQJD (27.23 %) (Bai et al., 2020). These results highlight the potential of GC3 as a high-protein legume cultivar for nutritional applications.

Table 24: The proximate composition of GC3 seeds.

Proximate composition	**GC3 seeds**	**Cowpea species (IFCT,2017)**
	g/100g	
Moisture	9.97 ± 0.24[a]	9.42 ± 0.39[a]
Ash	3.36 ± 0.06[a]	2.90 ± 0.11[b]
Protein	26.16 ± 0.23[a]	20.36 ± 0.39[b]
Crude Fat	2.33 ± 0.08[a]	1.15 ± 0.06[b]
Dietary fiber (DF):		
Insoluble (IDF)	19.60 ± 1.28[a]	8.75 ± 0.09[b]
Soluble (SDF)	2.10 ± 0.12 [a]	2.80 ± 0.05 [b]
Total (TDF)	21.70 ± 1.28[a]	11.54 ± 1.28[b]
Carbohydrate	36.48 ± 1.33[a]	54.62 ± 0.49[b]

Results are the mean ± SD of a triplicate sample of GC3 seeds; a, b indicates significant difference ($p < 0.05$) between the values of GC3 and IFCT,2017.

The crude fat content of GC3 was 2.33 ± 0.08 g/100 g, aligning with the values reported by Bai et al. (2020) for other cultivars (1.88 – 3.14 g/100 g), but significantly higher ($p < 0.001$) than the IFCT, 2017 value (1.15 ± 0.06 g/100 g). While higher fat content may enhance energy density, it also raises concerns about oxidative rancidity, which can negatively affect

shelf life and sensory properties (Vieira et al., 2015). Nonetheless, lipids contribute to the overall energy value of legumes.

The total dietary fiber (TDF) content in GC3 seeds was estimated to be 21.7 ± 1.28 g /100 g, with insoluble dietary fiber (IDF) comprising the majority at 19.6 ± 1.28 g/100 g, and soluble dietary fiber (SDF) accounting for 2.10 ± 0.12 g / 100 g. The TDF content in GC3 was significantly higher ($p < 0.001$) than the value reported in IFCT (2017) (11.54 ± 0.13 g/100 g), indicating GC3 is rich in dietary fiber with possible benefits for gastrointestinal and metabolic health. Notably, the IDF fraction in GC3 was significantly higher ($p < 0.001$) than IFCT values, whereas the SDF fraction was significantly lower ($p < 0.05$).

Dietary fiber consists of a complex mixture of indigestible plant components, including resistant starch, non-starch polysaccharides such as pectin, cellulose, hemicellulose, and oligosaccharides, gums, waxes, and lignin (Rasika et al., 2021). The predominance of insoluble fiber in GC3 suggests enhanced benefits in promoting gastrointestinal motility and faecal bulk, potentially reducing the risk of constipation and colorectal disorders (Slavin, 2013). Although the soluble fiber content was lower compared to reference values, it remains important due to its fermentability and prebiotic effects, which support beneficial gut microbiota and contribute to metabolic health (Tomasik & Tomasik, 2020).

Moreover, the high fiber and moderate carbohydrate profile of GC3 seeds positions this cultivar as a valuable dietary option, particularly for individuals managing blood glucose levels or seeking to improve digestive health. Increased dietary fiber intake is associated with improved insulin sensitivity and weight management, highlighting the potential role of GC3 in dietary strategies targeting metabolic diseases (Qin et al., 2022). Future studies should emphasize *in vivo* studies to validate these health benefits and evaluate the effects of processing on fiber content and bioavailability.

The carbohydrate content of GC3 seeds was found to be 36.48 ± 1.33 g/100 g, which aligns with the general carbohydrate range reported for legumes (Gonçalves et al., 2016); however, it was significantly lower ($p < 0.001$) than that reported for known species (54.62 ± 0.49 g/100 g). This suggests a distinct macronutrient profile for GC3, characterized by reduced carbohydrate levels. The relatively lower carbohydrate content may be helpful for individuals controlling blood sugar or adhering to low-carbohydrate diets (Mysonhimer et al., 2022).

The low carbohydrate in our cultivar is consistent with the higher dietary fiber content observed in GC3, which contributes to the total carbohydrate by difference on calculation, thereby reducing available carbohydrates. The combination of lower digestible carbohydrates and higher fiber could translate into a reduced glycemic load, which is advantageous for metabolic health. Furthermore, the lower carbohydrate content may influence the energy density and functional properties of GC3 seeds when incorporated into food products. This nutrient profile enhances the value of GC3 as a functional food ingredient, potentially appealing to health-conscious consumers aiming for improved glycemic control and digestive wellness. However, future studies should include detailed carbohydrate profiling (e.g., sugar composition, starch types) and *in vivo* glycemic response assessments to better understand the nutritional implications of GC3 seed consumption.

The energy content of GC3 seeds was 271.26 ± 3.20 kcal/100 g, reflecting a balanced macronutrient profile. This energy arises from moderate carbohydrates (4 kcal/g), substantial protein, and lipids, each contributing to the overall caloric value. Proteins (4 kcal/g) enhance nutritional quality by supporting metabolic functions, while lipids (9 kcal/g) provide a dense energy source and aid nutrient absorption. This balanced composition supports energy needs and metabolic health, making GC3 a promising nutritious food source.

The significant differences in our cultivar and the species reported in IFCT, 2017, may be attributed to varietal and agro-climatic differences. Furthermore, one serving (30 g) of GC3 seeds is estimated to provide more than 25 % of the Recommended Dietary Allowance (RDA) for macronutrients such as protein, carbohydrate, and dietary fiber for adults, and over 50 % for children, supporting its role as a nutrient-dense functional food. Refer to Appendix IV for the detailed RDA, 2024 for Indians.

The nutrient profile of GC3 seeds indicates strong suitability for fermentation with halotolerant probiotics. The high total dietary fiber, especially the insoluble fraction (19.6 ± 1.28 g/100 g), can offer abundant fermentable substrates to support probiotic growth. Although soluble fiber is lower, it can contribute to prebiotic activity, enhancing microbial fermentation. The moderate carbohydrate content (36.48 ± 1.33 g/100 g) alongside balanced protein and lipid levels provides essential nutrients for microbial metabolism. Low moisture content (9.97 ± 0.24 g/100 g) ensures good substrate stability for fermentation processes. The energy content (271.26 ± 3.20 kcal/100 g) reflects a balanced composition favourable for

microbial activity. These characteristics suggest that GC3 seeds can sustain halotolerant probiotic growth and metabolite production. Consequently, GC3 is a promising substrate for developing fermented functional foods with enhanced nutritional and health benefits aimed at enhancing digestive and metabolic health.

4.4.2 Fatty acid profile of GC3

Linoleic acid (C18:2, ω-6) and α-linolenic acid (C18:3, ω-3) are essential Polyunsaturated fatty acids (PUFAs) in the human diet due to their roles in cell membrane integrity, immune modulation, and metabolic regulation (Hossain et al., 2016). In GC3 seeds, linoleic acid was quantified at 184.45 ± 9.50 mg/100 g and α-linolenic acid at 125.67 ± 6.45 mg/100 g, both significantly lower than the values reported for cowpea in IFCT, 2017 (343.00 ± 9.80 and 207.00 ± 16.00 mg/100 g, respectively), with $p < 0.001$ and $p < 0.01$, respectively. Despite this, GC3 maintained a favourable PUFA profile, potentially offering a distinct omega-6 to omega-3 ratio, which is relevant for dietary balance. Table 25 presents the fatty acid profile of GC3.

A notable finding was the presence of γ-linolenic acid (GLA, ω-6) at 139.98 ± 3.48 mg/100 g, below the detection limit in IFCT-reported cowpea. GLA, with its anti-inflammatory and therapeutic characteristics, is rarely reported in legumes, suggesting GC3 may serve as a novel source of this bioactive lipid. Similarly, eicosapentaenoic acid (EPA, ω-3), typically associated with marine oils, was identified at 22.95 ± 3.54 mg/100g in GC3 but was below detection in IFCT data, an unusual finding that may reflect cultivar-specific traits, environmental factors, or seed physiology.

Saturated fatty acids (SFAs) in GC3 also showed significant variation. Palmitic acid content (113.55 ± 7.45 mg/100 g) was significantly lower ($p < 0.01$) than the IFCT value (227.00 ± 6.60 mg/100 g), whereas stearic acid was markedly higher (101.84 ± 4.36 mg/100 g vs. 33.10 ± 1.30 mg/100 g, $p < 0.001$). Elevated levels of behenic and arachidic acids ($p < 0.01$) in GC3 further distinguish its SFA composition, indicating potential genotype or environmental influence. Among monounsaturated fatty acids (MUFAs), oleic acid levels in GC3 (62.81 ± 2.99 mg/100 g) were statistically similar to IFCT values (57.69 ± 6.88 mg/100 g, $p > 0.05$). However, myristoleic acid, a rare MUFA with possible physiological relevance, was detected at 123.34 ± 4.91 mg/100 g in GC3 but not reported in

IFCT 2017, indicating a potentially unique chemotypic marker for this cultivar. The overall fatty acid spectrum of GC3, encompassing SFAs, MUFAs, and PUFAs, underscores a diverse and nutritionally valuable lipid composition, with several components showing statistically significant differences from standard cowpea references.

These findings align with previous studies (Antova et al., 2014), which highlight the wide variability in fatty acid composition among legume cultivars. Moreover, the identification of bioactive lipids in GC3 supports its potential in the development of functional or therapeutic foods, particularly those aimed at cardiovascular, metabolic, and inflammatory health.

Table 25: Fatty acid profile of GC3.

Fatty acid	**GC3**	**Cowpea, IFCT,2017**
	(mg/100g)	
Myristoleic acid	123.34 ± 4.91	BDL
Palmitic acid	113.55 ± 7.45[a]	227.00 ± 6.6[b]
Stearic acid	101.84 ± 4.36[a]	33.10 ± 1.30[b]
Oleic acid	62.81 ± 2.99[a]	57.69 ± 6.88[a]
Linoleic acid	184.45 ± 9.50[a]	343.00 ± 9.80[b]
Arachidic acid	9.75 ± 1.55[a]	6.84 ± 0.76[b]
α-linolenic acid	125.67 ± 6.45[a]	207.00 ± 16.0[b]
γ-linolenic acid	139.98 ± 3.48	BDL
Behenic acid	30.79 ± 3.27[a]	11.52 ± 0.79[b]
Eicosapentaenoic acid	22.95 ± 3.54	BDL

Results are the mean ± SD value of triplicate analyses. a, b indicates significant difference ($p < 0.05$) between the values of GC3 and IFCT,2017of respective fatty acids

The GC3 cowpea cultivar exhibits a distinct fatty acid profile marked by the presence of rare and beneficial lipids, including GLA, EPA, and myristoleic acid, alongside favourable levels of essential PUFAs. The statistically significant differences from IFCT-reported cowpea values suggest that GC3 may serve as a nutritionally superior and functionally relevant seed variety, suitable for incorporation into health-focused dietary interventions or food formulations.

4.4.3 Profile of total amino acids

Amino acid composition of the GC3 cowpea cultivar was estimated and compared with the cowpea species data reported in the IFCT by Longvah et al. (2017) (Table 26). Overall, the amino acid profile of GC3 showed general consistency with previously reported cowpea cultivars (Anyango et al., 2011), indicating comparable nutritional quality.

Among essential amino acids, threonine, lysine, tyrosine, histidine, and arginine levels in GC3 were similar to IFCT values, suggesting retention of these key amino acids. However, valine, isoleucine, and leucine concentrations were significantly lower in GC3, highlighting a reduction in branched-chain amino acids (BCAAs) that are crucial for muscle metabolism and nitrogen balance (Wu et al., 2015). This suggests that while GC3 maintains baseline protein quality, complementary protein sources may be necessary to meet full dietary amino acid requirements when relying heavily on GC3.

Conversely, methionine (3.40 ± 0.14 g/100g) and cysteine (1.30 ± 0.04 g/100g) levels in GC3 were significantly higher ($p < 0.001$) than IFCT-reported values (0.38 ± 0.16 and 0.57 ± 0.09 g/100g, respectively). Since sulfur-containing amino acids often limit the nutritional advantage of legume proteins, the elevated levels in GC3 indicate an improved protein quality profile. This enhancement suggests potential for GC3 as a superior source of sulfur amino acids, important for human health and protein digestibility, thus representing an advantage in programs targeting nutritional improvement. A comparable trend, with minor variations, was reported by Bai et al. (2020) in the amino acid profiles of 24 cowpea cultivars collected from four provinces in China.

In the non-essential amino acid group, aspartic acid, alanine, and proline levels were largely comparable to IFCT data. However, glutamic acid, serine, and glycine were significantly lower in the GC3 cultivar. Given glutamic acid's role as a precursor for key nitrogen carriers like asparagine and glutamine (Kaur et al., 2014), these reductions may influence nitrogen metabolism and the overall efficiency of protein synthesis in the cultivar.

GC3 seeds are rich in protein and provide essential amino acids, enabling them as a valuable supplementary protein source alongside most cereals, as well as an important plant-based ingredient for the food industry. The bioavailability of these amino acids can be further improved through appropriate processing methods, such as fermentation (Li et al., 2023).

Table 26: Amino acid composition (g/100g protein) of GC3 seed cultivar in comparison with cowpea species as per IFCT,2017.

AMINO ACID	Cowpea species (IFCT, 2017)	GC3	p value
Essential	**g /100 g protein**		
Threonine	3.80 ± 0.61[a]	3.29 ± 0.31[a]	**NS**
Valine	4.87 ± 0.59[a]	0.41 ± 0.04[b]	($p < 0.005$) ↓
Isoleucine	4.10 ± 0.58 [a]	2.03 ± 0.31 [b]	($p < 0.01$) ↓
Leucine	7.49 ± 1.23 [a]	4.93 ± 0.76 [b]	($p < 0.05$) ↓
Lysine	6.67 ± 1.26[a]	5.70 ± 0.82[a]	**NS**
Phenylalanine	5.47 ± 0.49 [a]	3.60 ± 0.23 [b]	($p < 0.01$) ↓
Tyrosine	2.83 ± 0.26[a]	2.60 ± 0.18[a]	**NS**
Methionine	1.38 ± 0.16[a]	3.40 ± 0.14 [b]	($p < 0.001$) ↑
Cysteine	0.57 ± 0.09[a]	1.30 ± 0.04 [b]	($p < 0.001$) ↑
Tryptophan	1.05 ± 0.03[a]	0.38 ± 0.01 [b]	($p < 0.002$) ↓
Histidine	2.93 ± 0.42[a]	2.80 ± 0.36[a]	**NS**
Arginine	7.10 ± 1.36[a]	5.70 ± 0.58[a]	**NS**
Nonessential			
Aspartic acid	10.11 ± 1.15[a]	10.93 ± 1.17[a]	**NS**
Glutamic acid	19.02 ± 2.19 [a]	13.81 ± 1.18[b]	($p < 0.03$) ↓
Serine	4.44 ± 0.18 [a]	3.80 ± 0.12 [b]	($p < 0.001$) ↓
Glycine	4.69 ± 0.51 [a]	3.13 ± 0.20 [b]	($p < 0.02$) ↓
Alanine	4.24 ± 0.57[a]	3.67 ± 0.36[a]	**NS**
Proline	4.60 ± 0.26[a]	4.43 ± 0.26[a]	**NS**

NS=Not significant. Values are the mean ± SD value of triplicate analyses, and a, b indicates a significant difference between the values of GC3 and IFCT,2017

The observed variation in amino acid composition is likely influenced by a combination of genetic factors, environmental conditions, soil fertility, and agronomic practices (Bai et al., 2020). These factors highlight the need for integrated breeding and cultivation approaches to enhance both yield and nutritional quality. GC3 seeds are rich in protein and provide

essential amino acids, making them a valuable supplementary protein source alongside most cereals, as well as an important plant-based ingredient for the food industry. The bioavailability of these amino acids can be further improved through appropriate processing methods, such as fermentation(Li et al., 2023).

In conclusion, the GC3 cowpea cultivar exhibits a distinctive amino acid profile, featuring elevated levels of sulfur-containing amino acids but lower branched-chain amino acids (BCAAs). This unique composition indicates GC3's potential as a nutritionally valuable legume, especially when included in balanced diets that address its reduced BCAA content. These results emphasize the importance of further research on protein digestibility and amino acid bioavailability to fully harness GC3's dietary advantages and its application in functional food development.

4.4.4 Content of water-soluble and fat-soluble vitamins

Cowpea is a recognised source of B-complex vitamins, Riboflavin (Vit.B2), Niacin (Vit.B3), Pantothenic acid (Vit.B5), Folate (Vit.B9) (Affrifah et al. 2022). Table 27 presents the content of selected vitamins and the total carotenoid content of GC3 seeds.

Overall, the GC3 cultivar shows both nutritional advantages and limitations compared to standard cowpea species. The vitamin and carotenoid composition of the GC3 cultivar was compared with reference values reported for cowpea species in the IFCT by Longvah et al. (2017), as indicated in Table 27. Significant differences were noted between GC3 and the IFCT cowpea data in several vitamins. GC3 exhibited markedly lower levels of vitamins B3, B5, and B9, all showing statistically significant lower values ($p < 0.05$). Folate content in GC3 was 9.26 ± 1.02 µg/100g, compared to 231 ± 27.3 µg/100g in IFCT-reported cowpea, suggesting limited contribution of GC3 to dietary folate intake.

In contrast, vitamin K1 was significantly higher in GC3 (7.63 ± 0.30 µg/100g) than in the IFCT value (1.75 ± 0.14 µg/100g), highlighting GC3's potential for supporting physiological functions such as blood coagulation and bone metabolism. Vitamins B2 and B6 (pyridoxine) showed no significant differences ($p > 0.05$), indicating consistency in their levels between GC3 and IFCT reported cowpea varieties. Vitamin E, however, was below the detection limit in the GC3 cultivar.

In terms of carotenoids, GC3 seeds demonstrated a significantly higher total carotenoid content (367.77 ± 7.08 µg/100g) than the IFCT cowpea reference (218 ± 16.8 µg/100g). This elevation was primarily due to a six-fold increase in beta-carotene levels (46.08 ± 3.73 µg/100g in GC3.

Beta-carotene, a provitamin A compound, plays an important role in vision, immunity function, and antioxidant defense, thereby enhancing the nutritional appeal of GC3. While lutein content was lower in GC3, the difference was not statistically significant. Interestingly, zeaxanthin, which was below the detection limit in cowpea in the IFCT, was present in GC3 at 25.20 ± 2.69 µg/100g, indicating a potentially distinct carotenoid profile. Carotenoid concentrations are generally higher in leafy parts than in seeds (Jideani et al., 2021), and the presence of zeaxanthin and elevated beta-carotene in GC3 suggests favourable genetic or environmental influences.

Table 27: Vitamin and carotenoid content of GC3 seeds.

Vitamins	**Cowpea species (IFCT, 2017)**	**GC3**	**p-value**
	Content		
Vit.B2 (mg/100g)	0.09 ± 0.01[a]	0.07 ± 0.01[a]	NS
Vit. B3 (mg/100g)	1.64 ± 0.03[a]	0.57 ± 0.02[b]	p < 0.001↓
Vit. B5 (mg/100g)	1.47 ± 0.31 [a]	0.34 ± 0.01 [b]	p < 0.02↓
Vit. B6 (µg/100g)	0.30 ± 0.033[a]	0.43 ± 0.03[a]	NS
Vit. B9 (µg/100g)	231 ± 27.3[a]	9.26 ± 1.02[b]	p < 0.001↓
Vit. K1 (µg/100g)	1.75 ± 0.14[a]	**7.63 ± 0.30[b]**	p < 0.001↑
Vit. E (mg/100g)	0.70 ± 0.03	BDL	-
Carotenoids			
Lutein (µg/100g)	83.34 ± 26.30[a]	34.04 ± 3.53[a]	NS
Zeaxanthin (µg/100g)	BDL	**25.20 ± 2.69**	-
Beta carotene (µg/100g)	7.08 ± 1.58[a]	**46.08 ± 3.73[b]**	p < 0.001↑
Total carotenoids (µg/100g)	218 ± 16.8[a]	**367.77 ± 7.08[b]**	p < 0.001↑

NS=Non-significant; BDL-Below Detection Limit.

Results are mean ± SD value of triplicate analysis of GC3 seeds and a, b indicates significant difference (p< 0.05) between the values of GC3 and IFCT,2017.

The observed variations in both vitamin and carotenoid composition likely reflect a combination of genotypic differences, environmental conditions, agronomic practices, and post-harvest factors. While lower levels of certain B-vitamins may reduce GC3's micronutrient contribution, its significantly higher levels of beta-carotene and vitamin K1 suggest valuable applications in functional foods and dietary interventions aimed at addressing vitamin A and K deficiencies. Further studies on bioavailability and stability during processing are recommended to optimize its nutritional efficacy.

4.4.5 Mineral content

Legumes are a commonly consumed food group worldwide, loaded with carbohydrates and protein, with a good amount of micronutrient minerals(Affrifah et al., 2022). The mineral content of GC3 seeds was estimated to be considerably good. Table 28 below presents a comparison of mineral content (mg/100g) between the cowpea values reported in the IFCT, 2017, and those of the GC3 cultivar. In the present study, the content of some important minerals like calcium (Ca), phosphorus(P), iron (Fe) and zinc (Zn) in GC3 cultivar was estimated to be 157.91± 0.8 mg/100g, 277.26 ± 0.35 mg/100g, 12.49 ± 0.840 mg/100g and 6.62 ± 0.06 mg/100g respectively.

The iron content GC3 cultivar was significantly high (12.49 ± 0.84 mg/100g) compared to the IFCT cowpea, suggesting that GC3 can be used in biofortification programs aimed at reducing iron-deficiency anaemia. Calcium content in GC3 was also significantly higher (157.91 mg/100g) than in IFCT cowpea (81.73 mg/100g), indicating a plant-based source for improved bone health and reduced calcium-related deficiencies.

GC3 cultivar recorded a significantly lower phosphorus level (277.26 ± 0.35 mg/100g) compared to IFCT values, which could be due to genotype-specific differences in nutrient uptake or distribution. While phosphorus is essential, the decrease may not be detrimental if dietary intake from other sources is adequate. No significant difference was found in copper content between GC3 and cowpea species reported in IFCT. This suggests that copper levels remain stable across genotypes, and selection for other traits does not affect this mineral's concentration.

Zinc content in the GC3 cultivar (6.62 mg/100g) was nearly double that of IFCT cowpea, and this difference was highly significant. Zinc is vital for immune function, and the elevated levels in GC3 enhance its nutritional value, especially in zinc-deficient regions. GC3 exhibited a significantly higher potassium and manganese content than IFCT cowpea.

Table 28. Comparison of mineral content between the GC3 seed cultivar and the values reported for cowpea species by IFCT,2017.

Minerals	**Cowpea species (IFCT, 2017)**	**GC3 seeds**	**p-value/ significance**
	Content (mg/100g)		
Iron (Fe)	5.90 ± 1.26 [a]	12.49 ± 0.84 [b]	$p < 0.01$**↑
Calcium (Ca)	81.73 ± 7.63 [a]	157.91 ± 0.80 [b]	$p < 0.01$**↑
Phosphorous (P)	372.00 ± 32.9[a]	277.26 ± 0.35[b]	$p < 0.05$*↓
Copper (Cu)	0.81 ± 0.16[a]	0.88 ± 0.003[a]	$p > 0.05$ NS
Zinc (Zn)	3.41 ± 0.41 [a]	6.62 ± 0.06 [b]	$p < 0.01$**↑
Potassium (K)	1241.00 ± 116 [a]	1567.93 ± 25.59	$p < 0.05$*↑
Manganese (Mn)	1.51 ± 0.29 [a]	2.54 ± 0.35 [b]	$p < 0.05$*↑
Magnesium (Mg)	213.00 ± 20.3 [a]	167.68 ± 11.78 [b]	$p < 0.05$*↓

NS= not significant, *=Significant, **= Highly significant. Results are mean ± SD based on triplicate analysis (n=3).

In contrast, the magnesium content in GC3 was significantly lower than in the IFCT cowpea. Potassium is essential for cardiovascular health and fluid balance. Manganese is essential for bone formation, enzyme activation, and antioxidant function. indicating a potential health benefit from consuming GC3, which showed significantly higher content of both these minerals.

GC3 can serve as an important dietary source of calcium and iron, potentially meeting up to one-quarter of the RDA, 2024(ICMR-NIN, 2024), for various age groups, when consumed daily, an amount of 60–90 g (approximately 2 – 3 servings). Although phosphorus levels were lower and copper content remained unchanged compared to standard varieties, the overall mineral composition of GC3 suggests its valuable role in addressing micronutrient deficiencies through dietary diversification.

4.4.6 Oligosaccharides (stachyose, verbascose, raffinose) in GC3

The GC3 cultivar exhibited a significantly higher content of both raffinose (0.22 ± 0.01 g/100 g) and verbascose (0.27 ± 0.01 g/100 g) ($p < 0.001$) compared to the IFCT reference, as seen in Table 29. Notably, verbascose content in GC3 cultivar seeds was significantly higher than in the IFCT reference of cowpea species. In contrast, the IFCT cowpea species contained a significantly higher concentration of stachyose (1.24 ± 0.075 g/100 g) than the GC3 cultivar. Dhanasekar and Reddy. (2014) reported significant genotypic variation for total RFOs content in cowpea that ranged from 4.27 to 5.18 g/100 g with a mean value of 4.9 g/100g, which was higher than our cultivar.

Table 29: Oligosaccharide content of GC3 seeds and cowpea species as per IFCT,2017.

Oligosaccharides	**Cowpea species (IFCT, 2017)**	**GC3**	**Significance**
	Content (g/100g)		
Raffinose	0.14 ± 0.011	0.22 ± 0.01	$p< 0.001^{***}\uparrow$
Stachyose	1.24 ± 0.075	0.91± 0.08	$p < 0.01^{**}\downarrow$
Verbascose	0.01 ± 0.00	0.27 ± 0.01	$p< 0.001^{***}\uparrow$
Total RFOs	1.39 ± 0.076	1.41 ± 0.08	$P > 0.05^{NS}$

NS= non-significant, BDL-Below Detection Limit. Values are presented as mean ± SD, based on triplicate analysis (n=3). Significance is denoted as ** = Significant), *** = Highly significant.

This observed profile of RFOs is consistent with patterns reported in other legume species, though the specific proportions of raffinose, stachyose, and verbascose can vary significantly across species and even among cultivars. RFOs serve several physiological roles in plants: they act as carbohydrate storage in seeds and contribute to stress tolerance, particularly under drought and cold conditions (Langa et al., 2019). From a nutritional standpoint, RFOs are composed of galactose, glucose, and fructose and are abundantly found in legumes (Tomasik & Tomasik, 2020). Humans lack the enzyme α-galactosidase, required to hydrolyse RFOs in the small intestine (Hossain et al., 2016). As a result, RFOs reach the large intestine undigested, where they undergo microbial fermentation (Elango et al., 2022).

Although RFOs are commonly associated with flatulence, especially due to their fermentation, recent studies suggest that they may also act as prebiotics, facilitating the growth of beneficial gut microbes (Rasika et al., 2021). This dual effect makes their nutritional implications more nuanced.

In this context, the high stachyose content in both IFCT and GC3 suggests that cowpea consumption may cause digestive discomfort in some individuals. However, the lower levels of raffinose and verbascose in GC3, alongside its stachyose content, highlight a unique RFO profile that could have both nutritional and functional implications. This variation in oligosaccharide composition is likely influenced by genetic factors, environmental conditions, and seed maturity. Understanding these variations can inform breeding strategies and food processing methods aimed at reducing RFO content, thereby minimizing flatulence while preserving potential prebiotic benefits. Overall, these findings contribute valuable insights into the carbohydrate composition of cowpea and support its role as a functional food with potential applications in nutritional planning and gut health.

4.5 Physical and cooking properties of GC3 seeds

Physical and cooking properties govern the choice, preference, and economy of seeds (Appiah et al.,2011). Understanding the physicochemical and engineering properties of seeds is fundamentally important for addressing challenges in various agricultural and post-harvest operations. Physical properties of GC3 seeds are represented in Table 30.

4.5.1 Physical properties

4.5.1.1 Seed dimensions

Seed dimensions of dry GC3 seeds indicated an average length of 10 ± 0.50 mm, width of 6.02 ± 0.5 mm and a thickness of 5.4 ± 0.5 mm which significantly increased ($p < 0.01$) to 15.04 ± 0.4 mm, 9.01 ± 0.21 mm and 7.8 ± 0.6 mm respectively after 3 h of soaking, with no further increase in size seen on soaking up to 10 h. On cooking, the seed length, width, and thickness further increased ($p > 0.05$) to 15.43 ± 0.38 mm, 9.21 ± 0.19 mm, and 8.03 ± 0.4 mm, respectively, with no further increase in size seen on soaking up to 10 h. These dimensions were higher than those reported by Hamid et al. (2016), and such physical

properties and grain dimensions enable the study of product behaviour during various agricultural processes.

4.5.1.2 Geometric mean diameter

The Geometric mean diameter (as Dg) of dry GC3 seeds was 6.87 ± 0.51 mm, which significantly increased ($p < 0.05$) on soaking to 10.17 ± 0.40 mm. This increase is due to moisture absorption, causing the seed to swell in all three dimensions (length, width, and thickness). The highest increase was seen in the length and thickness. Soaking significantly increases seed volume due to hydration, which enhances seed softness and may affect processing properties like cooking time, grinding, or oil extraction (Sebastian et al., 2023).

Dg further slightly increased ($p > 0.05$) after cooking to 10.40 ± 0.34 mm, showing an increase of 0.23 mm. The dimensional expansion slows after cooking, suggesting the seed reaches near-saturation during soaking. Cooking may cause slight gelatinization of internal components and minor expansion. Thus, cooking does not dramatically increase size beyond soaking, indicating that most of the physical swelling happens during soaking, while cooking affects texture more than size (Hamid et al., 2016).

4.5.1.3 Sphericity

Sphericity is a critical physical parameter that reflects the shape characteristics of seeds and influences their behaviour during handling, processing, and planting operations. In this study, the GC3 seeds demonstrated a sphericity of 68.76 ± 2.26 %, which closely aligns with the value reported by Hamid et al. (2016), indicating consistency in morphological traits across similar genotypes. This value suggests that the GC3 seeds are moderately elongated rather than spherical, which may impact their flowability, packaging efficiency, and orientation in mechanical planting systems. The observed sphericity also implies that GC3 seeds may occupy more volume per unit mass, reducing bulk density and affecting storage design. Thus, understanding and optimizing seed sphericity not only supports efficient machinery design and handling practices but also contributes to enhanced crop establishment and resource use efficiency in the field.

According to Bhardwaj et al. (2014), sphericity significantly influences seed performance throughout the agricultural cycle from sowing to post-harvest processing. Seeds with lower

sphericity values (< 70 %) tend to have less predictable movement through hoppers and seed drills, potentially leading to irregular seed spacing and non-uniform emergence. Furthermore, such seeds may require customized equipment calibration to optimize flow and minimize mechanical damage during processing.

4.5.1.4 Length: Breadth ratio

The length-to-breadth (L: B) ratio is an important morphological indicator of seed shape. In this study, soaked GC3 seeds exhibited L: B ratios of 1.67 ± 0.07, indicating a consistently elongated form on soaking. The values above 1.0 suggest an oval to ellipsoidal geometry, typical of leguminous seeds (Huang et al., 2022). The results are better than those of Hamid et al. (2016), who have reported L: B ratios of red and black cowpea cultivars as 1.17 ± 0.11 and 1.42 ± 0.10, respectively.

4.5.1.5 Thousand seed weight

The 1000 seed weight of GC3 was recorded as 189.9 ± 7.9 g, with each seed weighing approximately 0.20 g, which is lower than the values reported by Bhardwaj et al. (2014). A lower 1000 seed weight may indicate smaller or less dense seeds, which can affect seed vigor and germination. According to AACC (2000), seeds with higher 1000 kernel weight are generally more robust and exhibit better yield potential. The observed variation may be attributed to genotypic differences and environmental factors such as soil fertility, water availability, and agronomic practices (Hamid et al., 2016). Despite this weight, our seed cultivar exhibits better germination performance, indicating that factors such as seed viability, maturity, and genetic potential may have played a more decisive role than seed size alone.

4.5.1.6 Seed bulk density

The loose bulk density of GC3 seeds was recorded as 0.76 ± 0.2 g/mL, representing the mass of seeds per unit volume when poured freely without compaction. This value aligns with the range of 0.72 – 0.80 g/mL reported by Hamid et al. (2016) and Verma et al. (2019). Bulk density is a critical parameter for seed quality assessment and is important in calculating

seeding rates, as well as in planning for handling, packaging, storage, and transportation (Appiah et al., 2011).

The moderate bulk density observed indicates the seeds are relatively light and loosely packed, which may result in higher porosity. While this is advantageous for aeration and drying efficiency, it may reduce storage efficiency due to increased void space. Bulk density can vary depending on factors such as seed size, shape, moisture content, and surface texture. In the context of seed flour production, particularly for weaning food formulations, a higher bulk density is generally not desirable, as reported by Kabeer et al. (2023), since lower-density flours tend to be more suitable for light, easily digestible preparations.

Table 30: Physical properties of GC3 cultivar.

Physical properties	**GC3**
Seed dimensions of dry seeds	
Length (mm)	10.00 ± 0.50
Width (mm)	6.02 ± 0.50
Thickness (mm)	5.40 ± 0.50
Geometric mean diameter(mm)	6.87 ± 0.51
Sphericity (%)	68.76 ± 2.26
Length: breadth ratio	1.67 ± 0.07
Thousand seed weight (g)	189.90 ± 7.90
Seed bulk density (g/mL)	0.76 ± 0.20
Colour: L	32.52 ± 0.34
a	10.47 ± 0.05
b	11.40 ± 0.08

Values are reported as mean ± SD.

4.5.1.7 Colour

GC3 seeds exhibited a brownish hue, with colour values of L = 32.52 ± 0.34 (darkness) and a =10.47±0.05(red-green component), indicating a relatively deep but warm tone. Seed colour is influenced by the existence of polyphenols in seed coat as well as inherent genetic factors (Verma et al., 2019). Visually, the unsoaked GC3 seeds appeared lighter than their soaked counterparts, suggesting that soaking induces perceptible darkening, likely due to water absorption and pigment concentration changes. Such colour variations in seeds and their flour have important implications for

industrial applications, as colour can affect consumer acceptance, product appearance, and suitability for specific food products. Maintaining consistent colour quality is therefore essential for optimizing the use of GC3 seeds in value-added food processing.

4.5.2 Cooking properties

Cooking and textural properties of the GC3 seeds were evaluated to assess their culinary quality and consumer acceptability.

4.5.2.1 Hydration capacity, Hydration index, Swelling capacity, and Swelling index

The weight of 1000 GC3 seeds was initially 189.9 ± 7.9 g, which significantly increased ($p < 0.05$) to 406.4 ± 4.6 g on soaking, with an average individual seed weight of 0.43 ± 0.04 g. Upon cooking, the weight further significantly increased ($p < 0.05$) to 493.6 ± 6.6 g. Notably, seeds that were cooked without prior soaking weighed 441 ± 6.2 g, indicating that soaking facilitates greater water absorption and weight gain during subsequent cooking.

The hydration capacity of the seeds was measured as 0.22 ± 0.01 g/seed, reflecting the average water uptake per seed during soaking. The hydration index of 1.05 ± 0.00 indicates a 5 % increase in seed weight relative to the dry seed. Hydration capacity measures the total amount of water absorbed by the seeds, and the index measures the efficiency of water absorption (Verma et al., 2019). Tresina & Mohan. (2012) reported the hydration capacity of 0.03 g/seed and hydration index of 0.9 for cowpea.

Similarly, the swelling capacity of our cultivar was found to be 0.62 ± 0.02 mL/seed, and the swelling index was 2.31 ± 0.14, indicating that seeds more than doubled their original volume upon hydration. These findings highlight the significant capacity of GC3 seeds to absorb water and swell during soaking and cooking, which is critical for optimizing processing parameters and improving texture in food applications.

4.5.2.2 Cooking time

The cooking time required to achieve a desirable texture in unsoaked GC3 seeds was recorded as 25 ± 0.35 min, whereas 3 h-soaked seeds required only 10 ± 0.00 min.

This significant reduction ($p<0.05$) in cooking time clearly demonstrates the effectiveness of pre-soaking, which softens seed texture and promotes water uptake, thereby accelerating the cooking process (Zia-Ul-Haq et al., 2010). Such a reduction in cooking time is beneficial for both domestic use and industrial applications, as it contributes to lower energy consumption, better nutrient retention, and improved processing efficiency. These findings are consistent with reports by Hamid et al. (2016) & Bhardwaj et al. (2014), who observed similar trends in red and black gram cultivars. The shorter cooking time in soaked seeds may be attributed to their higher hydration and swelling indices, which indicate greater water absorption and internal structural changes.

Soaking initiates physiochemical changes, including loosening of cell walls, partial gelatinization of starch, and increased permeability, all of which contribute to faster softening during cooking. Conversely, unsoaked seeds require longer exposure to heat and moisture to undergo these transformations (Zia-Ul-Haq et al., 2010).

Cooking time is a key quality parameter that influences nutrient availability, consumer acceptability, and even market preference (Mubaiwa et al., 2017). Prolonged storage may lead to physical and biochemical changes, such as protein matrix denaturation, reduced viability, and increased lignification, which can adversely affect cookability. Freshly harvested legumes, by contrast, often have more pliable cell walls and higher moisture content, making them inherently softer and quicker to cook (Hamid et al., 2016; Mubaiwa et al., 2017). While older legumes typically exhibit seed coat hardening and longer cooking times due to post-harvest aging and structural changes, the GC3 seeds in this study did not show signs of age-related hardening, suggesting they were relatively fresh and physiologically sound.

4.5.2.3 Cooked length–breadth ratio

The length-to-breadth (L: B) ratio of 3 h soaked and pressure-cooked GC3 seeds was recorded as 1.68 ± 0.06, indicating that the seeds retained an elongated shape even after hydration and cooking. A L:B ratio greater than 1.0 reflects an oval or ellipsoidal geometry, which remained largely unchanged during thermal processing. The consistency in seed shape after cooking suggests uniform expansion in both dimensions, maintaining overall structural integrity. The minimal difference between treatments implies that dimensional swelling

occurred uniformly, with proportionate increases in length and breadth. This shape stability is beneficial for mechanical handling, planting precision, and maintaining seed integrity during processing (Appiah et al., 2011; Sastry et al., 2019). The negligible outcome of cooking on elongation further supports the suitability of GC3 seeds for post-harvest and food applications where shape and texture are important quality attributes.

4.5.2.4 Water uptake ratio (WUR)

The WUR of GC3 seeds increased progressively with soaking and cooking treatments. Soaking for 3 h alone resulted in a WUR of 1.16 ± 0.10 g/g, indicating that the seeds absorbed slightly more than their dry weight in water, and on pressure cooking for 10 min, the WUR was 1.60 ± 0.10 g/g. When unsoaked seeds were directly pressure-cooked for 25 min, the WUR increased to 1.36 ± 0.06 g/g ($p > 0.05$), demonstrating that thermal treatment alone can enhance water absorption to some extent. This confirms that pre-soaking significantly improves the seed's ability to absorb water during cooking, likely due to increased permeability and softened seed structure. Hamid et al. (2016) have reported higher WUR in their red and black cowpea cultivar as 6.07 ± 0.14 and 4.86 ± 0.50 g/g, respectively.

These findings reflect typical legume behaviour, where hydration followed by thermal processing leads to maximum water absorption. Enhanced water uptake is advantageous for reducing cooking time, improving texture, and enhancing nutrient availability, making such treatments beneficial for both culinary and industrial applications(Huang et al., 2022).

4.5.2.5 Gruel solid loss

According to Appiah et al. (2011), gruel solid loss during cowpea cooking provides insight into seed quality post-cooking and cooking efficiency. In the present study, the gruel solid loss of GC3 seeds pressure-cooked for 10 min was recorded as 1.41 ± 0.24 % for unsoaked seeds and 1.60 ± 0.15 % for seeds pre-soaked for 3 h. These values are notably higher than the 0.27 to 0.78 % range reported for cowpea varieties by Oyeyinka et al. (2021). The elevated solid loss in GC3 seeds may be ascribed to differences in seed composition, seed coat permeability, or the specific cooking and soaking conditions applied.

The slightly higher loss in soaked seeds suggests that pre-soaking enhances water absorption, promoting greater leaching of soluble components such as starches, sugars, and proteins into the cooking medium (Hamid et al.,2016). Although this increase is modest, it indicates that pre-soaked seeds are more susceptible to nutrient migration, possibly due to cell wall softening and increased permeability (Sebastian et al., 2023). Nevertheless, the gruel solid loss values observed in this study remain relatively low, indicating good cooking stability and structural integrity under pressure cooking. Low solid loss is desirable as it minimizes nutrient loss and helps maintain the nutritional and sensory quality of cooked legumes.

4.5.2.6 Water absorption capacity (WAC)

The water absorption capacity (WAC) of GC3 whole seed powder was 1.47 ± 0.24 g/g, indicating that 1 g of powder absorbs approximately 1.47 g of water. This moderate WAC is within the typical range for legume powders such as cowpea and soybean, though slightly lower than values reported for dehulled or processed flours, and may be due to the presence of the seed coat limiting water-binding sites (Sabastian et al., 2023). Harouna et al. (2019) reported WAC in cowpea species ranging from 0.04 ± 0.00 to 1.10 ± 0.03 g/g.

Despite being moderate, GC3 powder exhibits good water-binding ability, making it suitable for applications requiring controlled hydration, such as doughs, extruded snacks, and baked products. For products needing higher moisture retention, such as soups or weaning foods, flours with higher WAC may be preferable. Functional properties are influenced by composition, particle fineness, and structural characteristics (Buta & Emire, 2015). Overall, with WAC values typical of cowpea flours (1.0–2.5 g/g; Hamid et al., 2016), GC3 powder demonstrates potential as an effective thickening and texture-enhancing ingredient in various food formulations.

4.5.2.7 Texture analysis

Texture profile analysis results are presented in Table 31 for soaked and pressure-cooked GC3 seeds, focusing on the key textural parameters: hardness, chewiness, cohesiveness, and adhesiveness. The hardness of GC3 seeds significantly decreased from 6.23 ± 2.24 kg in soaked seeds to 3.03 ± 0.35 kg after pressure cooking, indicating a substantial softening

effect due to thermal treatment. This reduction is desirable for enhancing mouthfeel and ease of mastication, particularly in cooked legumes.

Table 31: Cooking and textural properties of GC3 seeds.

Parameter	**GC3 seeds**
Hydration capacity (g/seed)	0.22 ± 0.01
Hydration index	1.05 ± 0.00
Swelling capacity (mL/seed)	0.62 ± 0.02
Swelling index	2.31 ± 0.14
Cooking time (min) with desirable texture– unsoaked Cooking time (min) with desirable texture – soaking 3 h	25 ± 0.35[a] 10 ± 0.00[b]
Cooked length-breadth ratio- soaked and cooked	1.68 ± 0.06
Gruel solid loss (%) – unsoaked and cooked	1.41 ± 0.24
Gruel solid loss (%) – soaked and cooked	1.60 ± 0.15
Water absorption capacity of GC3 whole seed powder (g/g)	1.47 ± 0.24
Hardness (kg) of soaked seeds Hardness (kg) of cooked seeds	6.23 ± 2.24[a] 3.03 ± 0.35[b]
Chewiness of soaked seeds Chewiness of cooked seeds	0.53 ± 0.16[a] 0.11 ± 0.08[b]
Cohesiveness of soaked seeds Cohesiveness of cooked seeds	0.11 ± 0.01[a] 0.11 ± 0.02[a]
Adhesiveness of soaked seeds (kg s) Adhesiveness of cooked seeds (kg s)	0.15 ± 0.03[a] 0.34 ± 0.02[b]

Values are reported as mean ± SD. a, b indicates a significant difference ($p < 0.05$) within the treatments of the respective parameters.

Similarly, chewiness dropped markedly from 0.53 ± 0.16 in soaked seeds to 0.11 ± 0.08 in cooked seeds, reflecting a softer, less resistant texture post-cooking. This aligns with the observed decrease in hardness and is indicative of improved palatability. Cohesiveness remained relatively unchanged between treatments, with values of 0.11 ± 0.01 (soaked) and 0.11 ± 0.02 (cooked), suggesting that the internal structural integrity of the seed matrix was maintained even after cooking. This stability can be beneficial for applications where the

seed needs to retain shape without becoming mushy. Interestingly, adhesiveness increased significantly from 0.15 ± 0.03 kg·s (soaked) to 0.34 ± 0.02 kg·s (cooked), indicating that cooked seeds were more likely to stick to surfaces (e.g., palate, packaging). This is commonly observed in legumes due to gelatinization of starches and hydration of proteins, which contribute to a stickier texture post-cooking.

Phase III

4.6 Fermentation of GC3 and its efficacy as a probiotic carrier

Fermentation trials using the GC3 cowpea cultivar demonstrated that it effectively supported the growth of probiotic strains, particularly *Bacillus licheniformis* PQ098138 and *Lactobacillus plantarum*.

The initial viable cell count of *B. licheniformis* PQ098138 in the slurry was approximately 4 log CFU/mL. This increased to over 6 log CFU/mL within 8 h and reached approximately 9 log CFU/mL by 12 h of fermentation ($p < 0.05$), as seen in Figure 9. In contrast, the standard control culture reached the recommended probiotic threshold (~6 log CFU/mL) only after 12 h, indicating a statistically significant difference in growth rate between the test and control cultures.

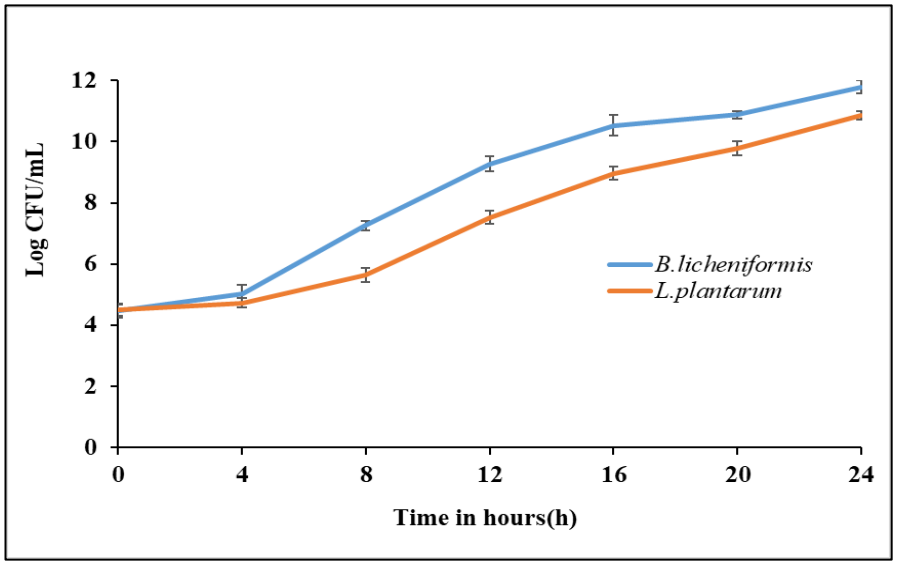


Figure 9: Viable cell counts in GC3 slurry during fermentation.

Similarly, *L. plantarum* showed an increase by ~ 6.0 log CFU/mL over the 24 h fermentation period in the GC3 substrate ($p < 0.05$), while *B. licheniformis* achieved a total 7.0 log CFU/mL increase. These results exceeded the minimum threshold required for probiotic efficacy (10^7 CFU/mL) and matched or surpassed the growth observed in common legume-based substrates like mung bean and maize.

These findings suggest that Goa cowpea 3 alone, without any additional supplementation, is an effective substrate for the rapid and robust growth of both *Bacillus* and *Lactobacillus* strains. Legumes are widely recognized for their nutritional density, particularly due to their high levels of protein, dietary fiber, and bioactive compounds. The presence of sugars and amino nitrogen in legumes serves as rapidly assimilable nutrients, which can support the growth of fermentative microorganisms during food processing (Affrifah et al., 2022).

Our study demonstrated, for the first time, that a cowpea cultivar, GC3 can serve as an efficient substrate for probiotic fermentation, comparable to well-established legumes like soybean and mung bean. The rapid and statistically significant increase in viable counts of *B. licheniformis* PQ098138 within 12 h reflects the substrate's high nutritional compatibility with this strain. This is particularly important, as probiotics must reach at least 10^6–10^7 CFU/mL to exert health benefits, including gut colonization, immune modulation, and metabolic regulation (Aziz et al., 2023).

The short fermentation time required to reach high microbial populations is advantageous from an industrial perspective, reducing the risk of contamination, improving shelf life, and contributing to cost-effective production (Rul et al., 2022). These benefits are further enhanced by the observation that both *Bacillus* and *Lactobacillus* species achieved high cell counts without the need for co-substrates or pre-treatment, indicating the natural fermentability of GC3.

The strong growth response of the probiotic cultures may be attributed to the unique RFO profile and TDF, previously characterized in GC3. Although RFOs can cause flatulence due to their fermentation in the large intestine, their prebiotic potential makes them valuable for developing synbiotic food combinations of probiotics and prebiotics designed to optimize gut health (Langa et al., 2019; Ashaolu et al., 2021).

Comparative literature supports our findings. For instance, *L. plantarum* grown in mung bean-based substrates showed similar log-scale increases (Wu et al., 2015), while *Bacillus*

subtilis growth in soybean fermentation has also been well documented (Jeon et al., 2018). The cell counts reported in our study (~9 log CFU/mL for *B. licheniformis*) are on par or superior to those achieved using other traditional cereals and legumes, such as maize, sorghum, and soybean (Pranoto et al., 2013).

The differences in microbial proliferation among substrates are likely due to variations in carbohydrate availability, protein composition, and antinutritional factors, all of which affect microbial metabolism and growth. The GC3 cowpea's oligosaccharide composition, along with its amino acid profile, may have resulted in supporting both *B. licheniformis* and *L. plantarum* during fermentation.

In summary, the findings underscore the potential of GC3 as a fermentation-friendly legume capable of supporting high probiotic viability. This opens up new possibilities in the development of cowpea-based functional foods, especially in regions where cowpea is a traditional staple. Future studies may explore optimization of fermentation parameters, co-culture strategies, and consumer acceptability to further enhance its application in synbiotic product development. These findings not only broaden the scope of cowpea utilization in functional food development but also pave the way for further research into legume-based synbiotic formulations, particularly in regions where cowpea is already a dietary staple.

4.6.1 Influence of *B. licheniformis* on the physicochemical properties of GC3 slurry during fermentation

Alongside monitoring the growth of *B. licheniformis* in the fermented GC3 slurry, the fermentation process was evaluated by measuring pH reduction, organic acid accumulation, free amino nitrogen (FAN) and sugar profiles, as well as changes in viscosity at different time points. Fermenting cowpea with probiotic bacteria may confer health benefits through the presence of live bacteria and/or the bioactive metabolites produced during fermentation (Li et al., 2023).

4.6.1.1 pH

During fermentation with *B. licheniformis*, the pH of the GC3 slurry showed a notable fluctuation over time. A gradual yet significant reduction ($p < 0.001$) in pH from an initial

value of 6.54 ± 0.03 to 5.08 ± 0.08 at 12 h of fermentation was observed (Figure 10 A). In contrast, fermentation with *L. plantarum* resulted in a faster pH drop, reaching below 5 within 4 h. This initial drop is characteristic of the robust growth and metabolite production. By the end of the 24 h fermentation period, the pH of the *B. licheniformis*-inoculated slurry started to increase to 5.25 ± 0.05 but was significantly lower ($p < 0.001$) than the initial pH, suggesting partial acidification. However, the pH began to increase thereafter, rising to 6.83 ± 0.05 at 36 h and further to 7.69 ± 0.13 at 48 h, with the change being statistically significant ($P < 0.01$ and $P < 0.001$, respectively).

This dramatic reversal is a clear indication that the proteolytic activity of *B. licheniformis* has become dominant. The breakdown of proteins and amino acids in the GC3 substrate may have released alkaline compounds (like ammonia), resulting in the medium's strong alkalinization. Along with this pH increase, a noticeable ammoniacal odor developed in the slurry, especially at 48 h, suggesting biochemical changes associated with microbial activity. The observed initial decrease in pH during the early stages of fermentation with *B. licheniformis* indicates organic acid production, likely as a result of carbohydrate metabolism. However, the later increase in pH beyond 36 h, accompanied by a strong ammoniacal smell, suggests the onset of alkaline fermentation, commonly associated with protein degradation.

This pattern aligns with previous findings by Fan et al. (2015) & Arbab et al. (2023), who reported that *Bacillus* spp. can utilize amino acids as carbon and energy sources through deaminase activity, leading to ammonia production. This shift from acidic to alkaline conditions is characteristic of the fermentation of protein-rich substrates, where ammonia and amines contribute to pH elevation (Aziz et al., 2023). In such systems, pH may reach values between 8 and 10, especially under extended fermentation durations. Though acidic fermentation ($pH \leq 4.6$) is traditionally preferred for its preservative effects and ability to improve flavor and safety (Buta & Emire, 2015; Behera et al., 2018), alkaline fermentations are also of increasing interest, particularly for functional food development. Alkaline conditions may facilitate the release of free amino acids and bioactive peptides, enhancing the nutritional value and sensory qualities of the final product.

Furthermore, earlier acidification observed with *L. plantarum* is consistent with studies like Min et al. (2017), who demonstrated rapid pH reduction in oat-based beverages fermented

with LAB strains, particularly in the presence of enzymes such as xylanase and α-amylase. The availability of fermentable sugars like glucose and maltose facilitates organic acid production, promoting quicker pH drops. In contrast, the slower acidification and later alkalinization in *B. licheniformis*-fermented GC3 slurries highlight differences in metabolic pathways between bacterial species. *Lactobacillus* strains are known for lactic acid production, while *Bacillus* strains often engage in proteolysis and alkaline shifts during fermentation.

Combining LAB with yeasts or *Bacillus* strains has been shown to enhance volatile compound production, improve sensory properties, and promote better shelf life (Sharma & Sharma, 2021). This suggests potential in developing multi-strain fermentations using GC3 to balance acidification and flavour profile, although this was beyond the scope of our study.

4.6.1.2 Total titratable acidity (TTA)

Total titratable acidity (TTA), expressed as percentage lactic acid (% LA), was used to monitor organic acid production during fermentation of the GC3 cowpea slurry with *Bacillus licheniformis* over a 72 h period (Figure 10 B). At baseline (0 h), the mean TTA was 0.25 ± 0.04 % LA. A statistically significant ($p < 0.05$) increase in TTA was observed as early as 4 h of fermentation (0.36 ± 0.02 % LA). The TTA continued to rise significantly, reaching 0.51 ± 0.03 % LA at 8 h ($p < 0.001$), and peaked at 12 h with a value of 0.63 ± 0.03 % LA, which was highly significant ($p < 0.0001$). Following the peak, a gradual decline in acidity was observed. At 16 h, TTA decreased slightly to 0.56 ± 0.02 % LA, yet remained significantly higher than baseline ($p < 0.001$). TTA values at 20 h and 24 h were 0.35 ± 0.01 % LA and 0.34 ± 0.03 % LA, respectively, still significantly above initial levels ($p < 0.05$), but lower than the 12 h peak.

By 48 h, the TTA had returned to baseline levels (0.24 ± 0.03 % LA), showing no significant difference ($p > 0.05$) from 0 h. After 72 h, a further decrease to 0.17 ± 0.02 % LA was observed, which was significantly lower than the initial value ($p < 0.05$), suggesting net acid depletion.

The TTA profile observed over the fermentation period provides valuable insights into the metabolic phases of *B. licheniformis* in the GC3 slurry. The initial rise in TTA from 0 to 12 h

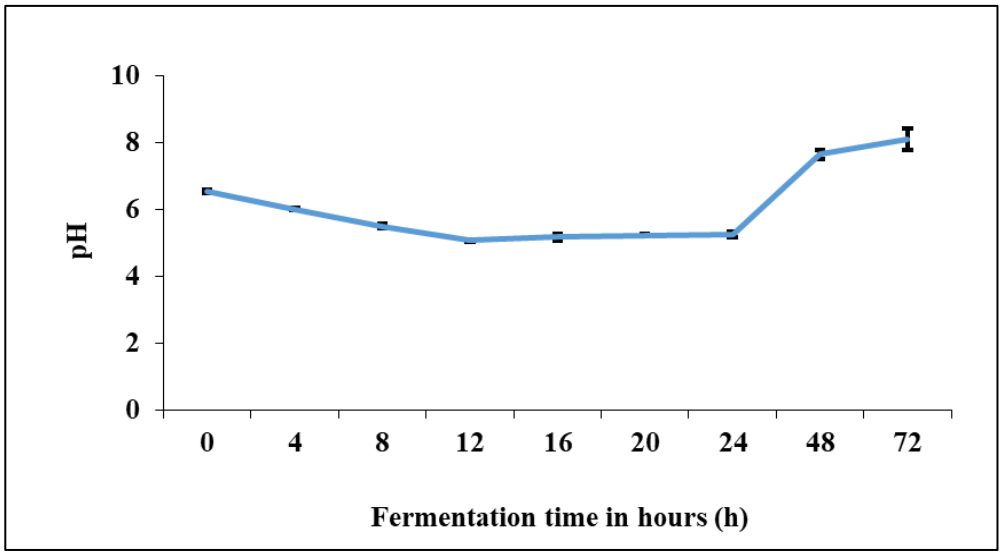
reflects the active acidogenic phase, where *B. licheniformis* metabolized available carbohydrates to produce organic acids, particularly lactic acid. Although *B. licheniformis* is not a classical lactic acid bacterium, its capacity to generate acidity in the early stages can be attributed to mixed acid fermentation pathways and production of SCFAs (Fan et al., 2015). This is consistent with earlier reports on non-LAB strains producing appreciable acidity in protein- and starch-rich substrates.

Interestingly, while the TTA significantly increased, it did not correspond with a large drop in pH of the slurry, suggesting a high buffering capacity of the GC3 matrix. This buffering could be due to the presence of proteins, phosphate groups, and amino acids that resist pH change by neutralizing organic acids. Such buffering capacity is beneficial for probiotic delivery, as it protects microorganisms during gastrointestinal transit and promotes their colonization in the gut (Valero et al., 2020).

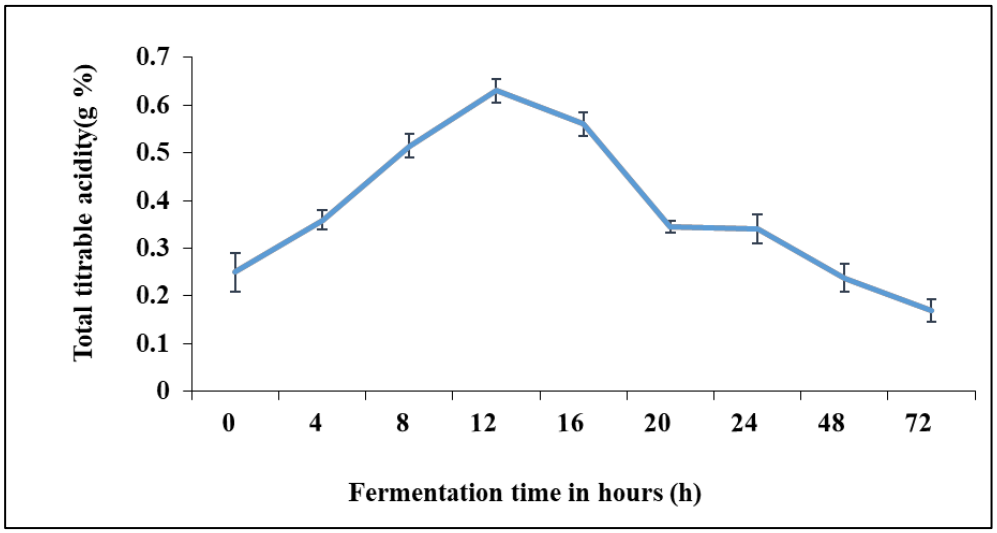
Following the peak at 12 h, the decline in TTA likely signals a shift in bacterial metabolism from carbohydrate fermentation to protein hydrolysis and amino acid utilization. This phase is characteristic of alkaline fermentation, where enzymatic breakdown of proteins releases ammonia and basic peptides, neutralizing existing acidity and reducing TTA (Aziz et al., 2023). Supporting this, the pH of the slurry increased during this period, and an ammoniacal odor developed, consistent with deaminase activity and amino acid catabolism by *Bacillus spp*. (Arbab et al., 2023).

By 48 to 72 h, the TTA dropped below the initial level, indicating that organic acids may have been either consumed or converted into non-acidic end-products. This aligns with late-stage fermentation patterns observed in traditional alkaline-fermented foods such as natto, where ammonia production and alkalinization dominate. These findings suggest that *B. licheniformis* exhibits a dual-phase metabolic profile in GC3 slurry, an early acidification phase (0 –12 h), favourable for suppressing spoilage microbes and enhancing food safety, and a later alkaline phase (after 16 h), which may promote bioactive peptide release and protein digestibility. This metabolic flexibility has functional implications in designing cowpea-based fermented products. Shorter fermentation times (up to 12 h) may be optimal for acid-preserved formulations, while extended fermentations (beyond 24 h) could be explored for protein-enriched or alkaline-fermented foods with unique organoleptic and nutritional profiles.

A



B



C

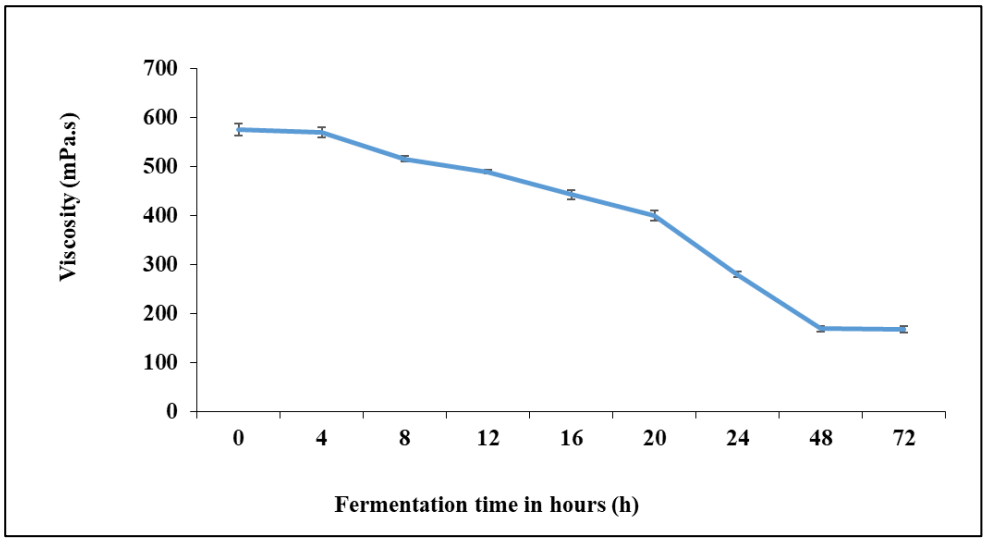


Figure 10 (A, B, C): Changes in pH, TTA, and viscosity of GC3 slurries during fermentation with *B. licheniformis.*

4.6.1.3 Organic acids and short-chain fatty acids (SCFAs)

The metabolic activity of *Bacillus licheniformis* during the fermentation of GC3 slurry led to significant changes in both the chemical profile of the medium and its acidity. As expected, *Bacillus* species utilize simple sugars for growth and typically produce organic acids, most notably lactic acid or acetic acid as major fermentation end-products (Sharma et al., 2020). Additional metabolites, including diacetyl, acetaldehyde, and various alcohols, may also be produced, contributing to the complexity of flavour and aroma in fermented foods (Naik & Kerkar, 2024).

In this study, fermentation resulted in a progressive decrease in pH from 6.56 ± 0.05 (0 h) to 5.08 ± 0.04 (12 h), which was consistent with the production of organic acids and SCFAs in the GC3 slurry, as shown in Table 32. The most substantial pH drop occurred within the first 8 h, likely reflecting the exponential growth phase of the microorganism and active metabolism of available carbohydrates.

The concentrations of both acetic acid and propionic acid increased continuously as the fermentation started. The most prominent organic acid produced was acetic acid, which increased significantly throughout the fermentation period up to 12 h, reaching 8.90 ± 0.04 g/L at 4 h,14.46 ± 0.02 g/L at 8 h, and 19.13 ± 0.01 g/L at 12 h. These results were supported by one-way ANOVA, which confirmed a statistically highly significant effect of fermentation time on acetic acid production ($p < 0.0001$) and a substantial effect on propionic acid ($p < 0.05$) up to 12 h.

Interestingly, lactic acid remained below detectable limits throughout the fermentation period, suggesting that *B. licheniformis* under these specific conditions favours acetic acid production. This observation corresponds well with previous findings of Liu et al. (2021), who reported acetic acid as the predominant organic acid in *pehtze*, a fermentation product from sufu (traditional Chinese cheese) involving rapid fermentation of soybean by *B. licheniformis*. Notably, a highly significant reduction in acetic acid concentration was observed after 12 h, with levels decreasing to 5.15 ± 0.02 g/L at 16 h ($p < 0.001$). This dramatic decline may be due to the re-utilization of acetic acid as a secondary substrate or a shift in microbial metabolism during the stationary phase.

Post-hoc Tukey's HSD analysis revealed that the mean acetic acid concentrations at all four time points were statistically distinct from each other, with a highly significant difference seen between 4 h and 12 h; the peak concentration was at 12 h and was significantly higher than all other points. The drop in acetic acid content from 12 h to 16 h was highly significant ($p < 0.001$), confirming a rapid shift from accumulation to degradation after 12 h. For propionic acid, only the change from 4 h to 12 h was statistically significant ($p < 0.05$), suggesting a slower or more variable production pathway.

The observed acidification of the fermentation medium is directly associated with the accumulation of organic and SCFAs, produced as by-products of carbohydrate metabolism (Omodara & Aderibigbe, 2018). Among these, acetic acid was found to play a dominant role in both the reduction of pH and metabolic activity. Beyond its preservative and antimicrobial effects, acetic acid is known to inhibit the growth of foodborne pathogens, enhancing food safety (Aziz et al., 2013; Shokryazdan et al., 2014); improve protein and carbohydrate digestion by promoting nutrient breakdown (Omodara & Aderibigbe, 2018); support beneficial gut microbiota and digestive health, potentially aid in appetite control and weight management by inducing satiety (Aziz et al., 2013), and alleviate gastrointestinal discomfort commonly associated with unfermented soybean products (Arbab et al., 2023).

Table 32: Concentration of acetic acid and propionic acid at different fermentation hours.

Fermentation hours(h)	**pH**	**Acetic acid**	**Propionic acid**
		Concentration in g/L	
0	6.56 ± 0.05	BDL	BDL
4	6.02 ± 0.03	8.90 ± 0.04^{a}	0.007 ± 0.001^{a}
8	5.51 ± 0.07	14.46 ± 0.02^{b}	0.010 ± 0.001^{b}
12	5.08 ± 0.04	19.13 ± 0.01^{c}	0.012 ± 0.003^{b}
16	5.18 ± 0.1	5.15 ± 0.02^{d}	0.009 ± 0.001$^{a, b}$

BDL: Below detection limits. Results are reported as mean ± SD (n = 3). Different superscript letters (a, b, c, d) within the same column indicate statistically significant differences between time points ($p < 0.05$) according to Tukey's HSD test.

In comparison, propionic acid, while produced in smaller amounts, also holds functional significance. It is recognized for supporting gut health as an SCFA, possesses preservative properties useful in food processing, reducing inflammation and improving metabolic outcomes, including reduced risk for insulin resistance and atherosclerosis, and inducing apoptosis in colon cancer cells (Aduol et al., 2020).

These health benefits, coupled with the sensory improvements imparted by organic acids and fermentation by-products, underscore the value of controlled fermentation using *B. licheniformis*. The present findings reaffirm its utility in enhancing the safety, nutritional quality, and functionality of cowpea-based fermented foods.

4.6.1.4 Viscosity

Changes in viscosity during fermentation are depicted in Figure 10C. A significant reduction in viscosity was observed in GC3 slurry with increased fermentation time ($p < 0.05$). The viscosity reduced significantly on fermentation from 576 ± 11.86 mPa · s (0 h) in unfermented slurry to 490 ± 3.74 after 12 h and 280.33 ± 6.34 after 24 h ($p < 0.05$), as indicated by a liquid consistency. Plate 20 indicates the change in viscosity after 8 h.



Plate 20: Changes in viscosity of GC3 slurry after 8 hours of fermentation.

Legume-based foods upon cooking exhibit high viscosities due to gelatinization, which reduces their energy density and sensory acceptability (Rodrigues et al., 2014). Fermentation with saltpan isolate may present an approach to address this concern. Some strains of bacillus exhibit amylolytic activity and are capable of producing starch-hydrolytic enzymes such as α-amylase and glucoamylase. These contribute to the saccharification and liquefaction of starch-based commodities. The starch within the commodity is hydrolysed to produce simpler

molecules like malto oligomers and glucose, thereby reducing the viscosity of the food (Pranoto et al., 2013; Ghosh et al., 2015), and our study strain used in fermentation is an amylase producer. This beneficial effect of probiotic fermentation on the viscosity of cooked legumes, coupled with their nutritional benefits, can be useful in the development of therapeutic foods and for infant and young child feeding requiring reduced viscosity.

No further reduction in viscosity in the GC3 slurry was observed beyond 48 h of fermentation, probably due to starch breakdown during the early hours of fermentation in our study. Forsido et al. (2020) have found a significant amount of starch hydrolysis in cassava and rice-based beverages fermented with a co-culture of *L. plantarum*, *Torulaspora delbrueckii,* and *L. acidophilus*. Kaur et al. (2020) reported that fortifying probiotic yogurt with chickpea flour using amylolytic strains, *L. acidophilus LA5* and *Bifidobacterium* BB12 led to a significant decrease in apparent viscosity. Overall, the GC3 substrate demonstrated its capability to support fermentation by *B. licheniformis* with cell counts above 8 log CFU/mL recorded after 24 hours of fermentation with desirable physicochemical changes.

4.6.1.5 Protein content

Figure 11 illustrates the dynamic changes in soluble protein content of GC3 during fermentation, as determined by the Lowry method. A statistically significant increase ($p < 0.001$) in protein content was observed during the initial 16 h of fermentation, reaching a peak of 15.2 g/L. However, by 24 h, the protein content significantly decreased to 11.58 ± 0.46 g/100g ($p < 0.05$).

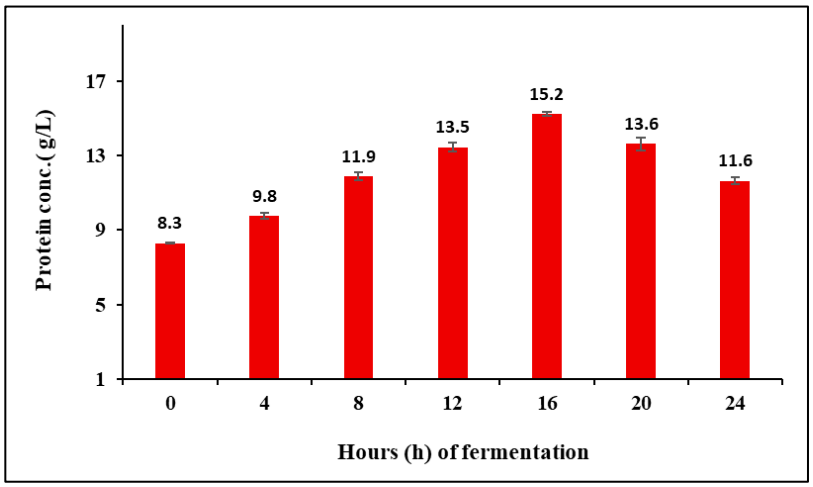


Figure 11: Effect of *Bacillus* fermentation on protein content of Goa cowpea 3.

The initial increase in soluble protein is likely attributable to the hydrolysis of complex cowpea proteins into free amino acids and smaller peptide fragments. *B. licheniformis*, known for its secretion of extracellular enzymes, particularly proteases and amylases, actively contributes to this proteolysis. This microbial activity increases protein solubility while also enhancing the nutritional and functional qualities of the fermented substrate (Zheng et al., 2017).

The complex matrix of cowpea, composed of proteins, carbohydrates, and other constituents, plays an integral role in modulating the rate and extent of protein breakdown and utilization. Moreover, the sample's dry weight decreases over time as fermentable carbohydrates are consumed by bacteria, which further affects protein concentration measurements.

The subsequent decline in protein content after 16 h can be attributed to the microbial utilization of released amino acids as nitrogen sources for growth and metabolism. As fermentation progresses, these amino acids may be depleted by *B. licheniformis*, resulting in a net reduction in soluble protein. Moreover, proteolytic enzymes themselves may undergo degradation over time, potentially reducing overall protease activity and limiting further release of amino acids (Li et al., 2023).

B. licheniformis also contributes directly to the protein pool through extracellular protein secretion. The protein contribution from microbial biomass was estimated at 0.35 ± 0.04 g/L at 0 h, increasing significantly to 3.85 ± 0.04 g/L by 16 h, further underscoring the organism's metabolic activity during fermentation. These findings suggest that the fermentation-induced changes in protein content can have a profound impact on the nutritional value of GC3, with potential implications for its use as a functional food or ingredient.

4.6.1.6 Free Amino Nitrogen (FAN)

During protein hydrolysis, the released individual amino acids and short peptides constitute free amino nitrogen (FAN), along with ammonia (Fan et al., 2015). Monitoring FAN provides insights into nitrogen metabolism, protein breakdown, and fermentation efficiency of *B. licheniformis*, with implications for nutritional quality, flavor, and texture of the fermented product. Changes in FAN concentration in GC3 slurry during 24 h fermentation

are shown in Figure 12. FAN significantly increased from 0.19 ± 0.01 g/L at 0 h to 0.57 ± 0.02 g/L at 12 h at $p < 0.001$, reaching 0.65 ± 0.04 g/L by 24 h, though no further statistically significant increases were observed beyond 12 h. One-way ANOVA confirmed significant variation over time ($p < 0.001$), with Tukey's post-hoc test showing the largest increases between 4 – 8 h ($p < 0.01$) and 8 –12 h ($p < 0.001$), with a subsequent plateau phase from 12–24 h.

FAN production exhibited a biphasic trend, with a non-significant rise during the initial lag phase (0–4 h), which likely reflects microbial adaptation and initial protease secretion. The exponential phase (4 –12 h) was marked by pronounced proteolysis, particularly between 8–12 h, indicating efficient enzymatic hydrolysis of cowpea proteins. During the plateau phase (12 – 24 h), FAN continued to rise slightly but non-significantly, suggesting reduced proteolytic activity and limited microbial uptake.

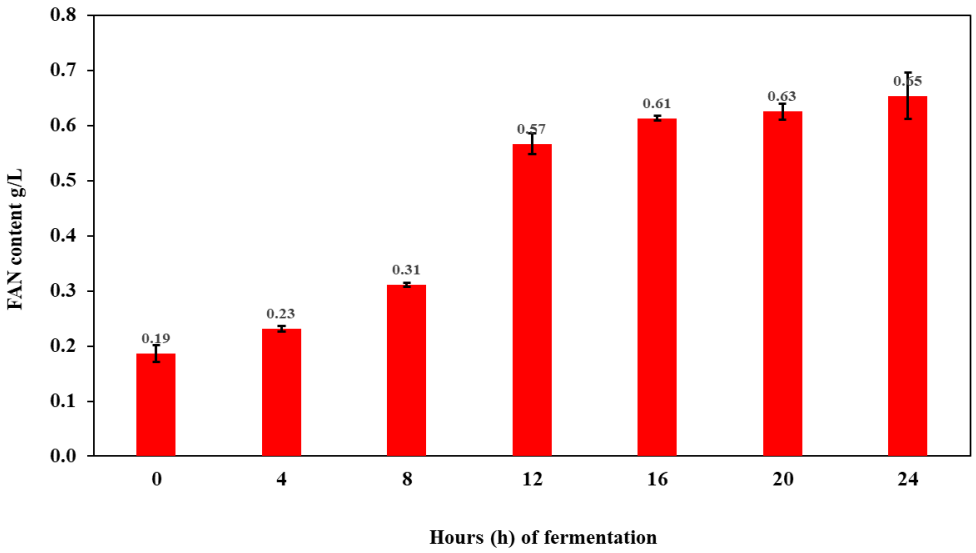


Figure 12: Effect of *Bacillus* fermentation on free amino nitrogen content of GC3 slurry.

Total protein, measured by the Lowry method, increased from 8.3 ± 0.30 g/L at 0 h to a peak of 15.2 g/L at 16 h, then declined, reflecting initial biomass accumulation followed by protein degradation or cell lysis. While total protein plateaued or decreased, FAN continued to rise, confirming ongoing proteolysis and release of nitrogenous compounds. These findings highlight the dual role of *B. licheniformis* in protein synthesis and degradation, supporting the nutritional enhancement of fermented cowpea. Proteolytic breakdown produces amino acids

and peptides with improved digestibility and bioavailability, consistent with previous studies on legume and cereal fermentations (Huang et al., 2022). Elevated FAN levels in fermented products have been linked to enhanced nutritional and sensory properties (Abebe & Alemayehu, 2022), confirming successful biotransformation of the substrate into a nutritionally enriched product.

4.6.1.7 Total carbohydrates (TC) and Total reducing sugars (TRS)

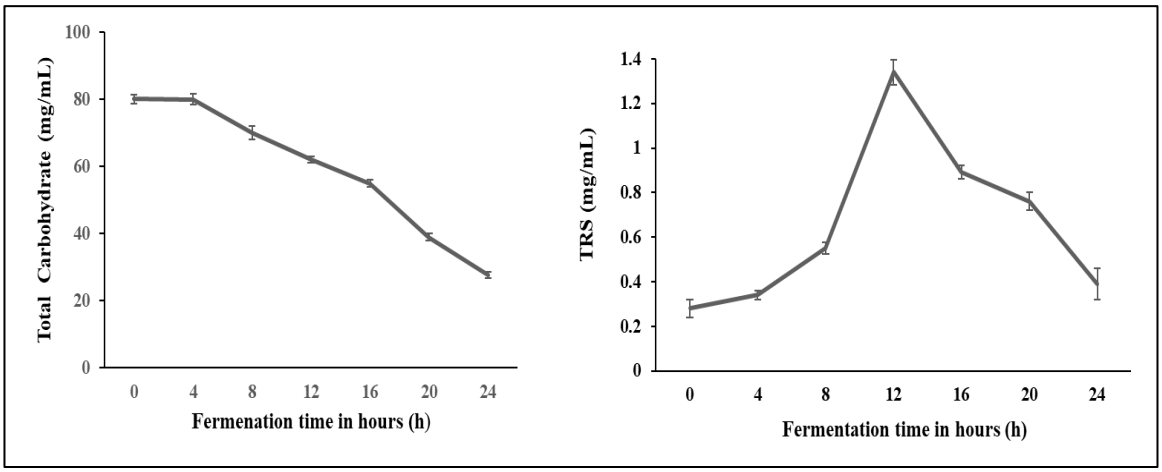
Carbohydrates in the substrate serve as primary energy and carbon sources for microbial growth. In fermentation systems, simpler sugars also act as prebiotics, promoting rapid proliferation of starter cultures. Microorganisms vary in their ability to utilize different carbohydrate types, necessitating monitoring of both TC and TRS, with TRS primarily indicating the presence of glucose, fructose, and maltose. Changes in TC and TRS during 24 h fermentation are shown in Figure 13.

A statistically significant decrease in TC was observed over the fermentation period ($p < 0.001$), indicating robust carbohydrate consumption by *B. licheniformis*. TC decreased from 80.14 ± 1.4 mg/mL at 0 h to 27.78 ± 0.97 mg/mL at 24 h. No significant change occurred during the initial 0 – 4 h phase ($p = 1.0$), reflecting microbial acclimation. From 4 h onward, each 4-h interval revealed a highly significant decline in TC ($p < 0.001$), demonstrating a sustained and accelerated carbohydrate utilization phase.

Total reducing sugars displayed a biphasic trend, with initial accumulation followed by rapid consumption. Overall, TRS changes were significant over 24 h ($p < 0.001$). TRS significantly increased from 0.28 mg/mL at 0 h to a peak of 1.34 mg/mL at 12 h, reflecting the enzymatic breakdown of complex carbohydrates by hydrolytic enzymes such as amylases. Tukey's HSD test confirmed significant increases between 4–8 h ($p < 0.001$) and 8–12 h ($p < 0.001$). Following the peak, TRS declined significantly during 12–16 h ($p < 0.001$), 16–20 h ($p < 0.05$), and 20 –24 h ($p < 0.001$). By 24 h, TRS (0.39 mg/mL) did not differ significantly from the initial value ($p = 0.0841$), indicating a net balance between hydrolysis and microbial sugar uptake. Our findings are in accordance with Rodrigues et al. (2024).

The overall reduction in carbohydrates reflects effective microbial metabolism. *B. licheniformis*, like other *Bacillus* species, catabolizes complex carbohydrates into simpler sugars via enzymes such as invertase, α-galactosidase, and amylases (Gopikrishna et al., 2021). These sugars are metabolized through glycolysis to generate ATP, supporting growth and biosynthesis. As microbial biomass increases, carbohydrate consumption intensifies, while metabolic end-products such as organic acids, alcohols, and CO_2 accumulate; these are not measured in total carbohydrate assays but indicate further substrate transformation (Li et al., 2023).

Carbohydrate dynamics also influence nutritional and sensory qualities. Enzymatic polysaccharide breakdown enhances the availability of simpler sugars, improving digestibility and nutrient bioavailability. Additionally, carbohydrate metabolism contributes to flavor-active compounds, positively affecting the taste and aroma of fermented cowpea products (Verni et al., 2022). Thus, observed trends in TC and TRS confirm the metabolic activity of *B. licheniformis* and highlight the functional benefits of fermentation in enhancing food quality.



(A) **(B)**

Figure 13: Profile of Total carbohydrates(A) and TRS(B) in GC3 slurry during fermentation.

4.6.2 Probiotic cell viability before and after lyophilization of Bacillus cell suspension and GC3 synbiotic formulation

Fermented GC3 slurry was lyophilized to obtain a suitable powder. Plate 21 depicts the lyophilized GC3 synbiotic powder, which was stored under refrigeration for further analysis. The effect of lyophilization on the viability of *B. licheniformis* was evaluated with and without the GC3 matrix and is indicated in Figure 14. Lyophilization of the bacterial suspension alone resulted in a survival rate of 50.37 ± 0.13 %, indicating a very significant loss in viability ($p < 0.001$).



Plate 21: Lyophilized GC3 synbiotic powder under refrigeration.

In contrast, the lyophilized synbiotic formulation containing the GC3 matrix exhibited a significantly higher survival rate of 88.38 ± 0.16 %. As shown in Figure 14, a major reduction in viable cell count occurred in the bacterial suspension post-lyophilization, whereas the synbiotic formulation showed only a non-significant reduction ($p > 0.05$), with viability decreasing by approximately 1 log cycle.

Maintaining the viability of probiotics during processing is critical for the efficacy of synbiotic formulations. Lyophilization, though widely used for preparing probiotic powders, can negatively impact cell survival due to membrane damage caused by ice crystal formation during the freezing step (Tripathi & Giri, 2014; Arenas et al., 2020). The sharp loss in viability observed in the bacterial suspension alone confirms the sensitivity of *B. licheniformis* to lyophilization stress, consistent with previous findings (Tripathi & Giri, 2014).

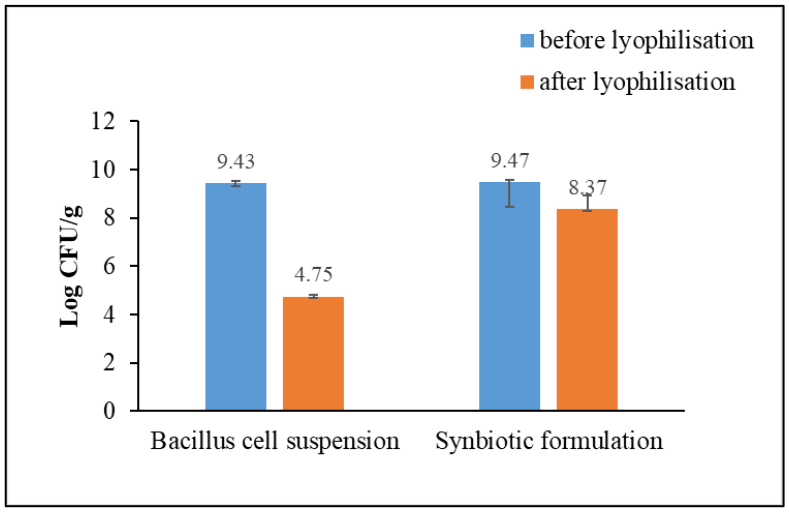


Figure 14: Viability of *Bacillus* in cell suspension and in GC3 synbiotic formulation before and after lyophilization.

However, the significantly higher survival rate in the GC3-based synbiotic formulation highlights the protective effect of the food matrix during freeze-drying. The GC3 likely acts as a cryoprotectant, mitigating ice crystal damage and reducing mechanical stress on the cells. The minimal reduction in viability by only 1 log cycle further supports the effectiveness of GC3 in preserving cell integrity. These observations reveal the potential of GC3 as a functional carrier matrix for stabilizing probiotic viability during lyophilization and subsequent storage.

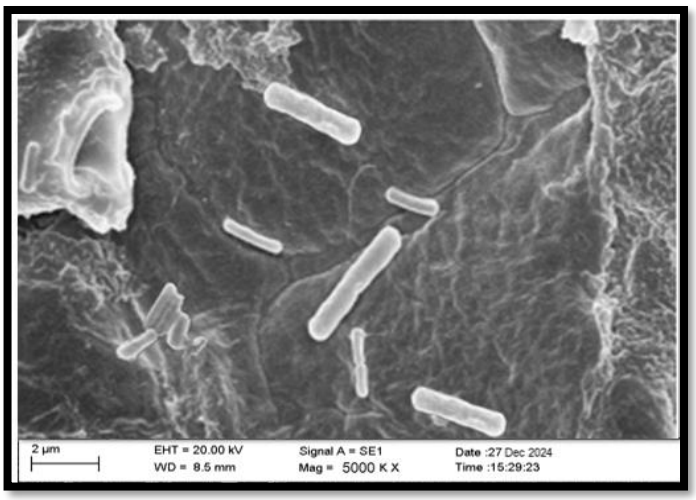


Plate 22: Scanning electron micrograph of *B. licheniformis* in GC3 matrix.

Presence of our saltpan culture *B. licheniformis* PQ098138 in the matrix of fermented GC3 upon lyophilisation was confirmed by Scanning Electron Microscopy (SEM) (Plate 22). The

organisms appear to be embedded or growing on a rough, textured surface, suggesting a solid-state fermentation or growth on a complex substrate/biofilm. The SEM shows rod-shaped cells (bacilli), which are relatively long and distinct with an approximate length of 2 to 5 μm.

4.6.3 Probiotic cell viability of the synbiotic formulation upon storage at low and room temperature

The viability of *B. licheniformis* in the lyophilized GC3 synbiotic formulation was evaluated under two storage conditions: 4 °C (refrigerated) and 28 ± 2 °C (room temperature) for a period of 12 months (Figure 15).

At 4 °C, the probiotic count declined gradually from an initial 9.06 ± 0.35 log CFU/g to 5.52 ± 0.28 log CFU/g by the end of 12 months. A significant ($p < 0.05$) reduction was first observed after 7 months, although the cell count remained above the minimum effective threshold of 7 log CFU/g for up to 10 months. After 11 and 12 months, the count significantly reduced ($p < 0.001$) to 5.73 ± 0.27 log CFU/g and 5.52 ± 0.28 log CFU/g, respectively, but no complete loss of viability was observed at any time point.

At 28 ± 2 °C, a more rapid decline in viability was recorded. While the initial counts were comparable to refrigerated storage, statistically significant losses ($p < 0.01$) began after 6 months, with the count reducing to 7.98 ± 0.35 log CFU/g after 6 months and 7.75 ± 0.32 log CFU/g after 7 months. The synbiotic powder after 8 months displayed signs of spoilage, including a slight putrid odour at room temperature. A nonsignificant reduction ($p > 0.05$) was recorded during the first 5 months at either storage temperature. A slight, non-significant increase in viable count was noted at room temperature compared to refrigerated storage at 5 months.

Maintaining probiotic viability during storage is crucial to ensure that synbiotic products deliver the intended health benefits. According to FAO/WHO and ISAPP guidelines, a minimum viable count of 6–7 log CFU/g is required at the time of consumption for a probiotic product to be considered effective. In this study, the lyophilized GC3 synbiotic formulation met or exceeded this benchmark for up to 10 months at 4 °C and 7 months at 28 ± 2 °C.

The superior stability under refrigerated conditions confirms that cold storage is essential for the long-term viability of *B. licheniformis* in the synbiotic matrix. Although some viability was retained at room temperature beyond 7 months showing no complete loss of viability signs of spoilage were observed thereafter, making room temperature storage unsuitable for shelf lives beyond this period. The protective effect of the GC3 matrix likely contributed to the sustained viability, especially under refrigeration. The matrix may act as a barrier against oxidative stress and moisture, and may also provide fermentable substrates or compatible solutes that help maintain membrane integrity during storage.

Interestingly, while a slight, non-significant increase in viable counts was observed at room temperature after 5 months, this was not sustained, and viability dropped rapidly after 7 months. This transient increase could be due to the release of nutrients or differential metabolic activity under storage stress, but it requires further investigation.

Previous studies have shown that food matrices significantly influence probiotic survival. For instance, Min et al. (2017) and Santivarangkna. (2016) have reported enhanced survival of probiotic bacteria in non-dairy matrices at ambient conditions. Similarly, Saarela et al. (2006) found that chocolate-coated breakfast cereals maintained higher probiotic counts than acidic apple juice during storage at 20 °C. Our findings demonstrate that GC3 is a promising matrix for delivering viable probiotic cultures and maintaining functionality over extended periods, especially under refrigerated conditions. To further improve shelf life at room temperature, formulations with higher initial cell density or inclusion of additional stabilizing agents may be considered.

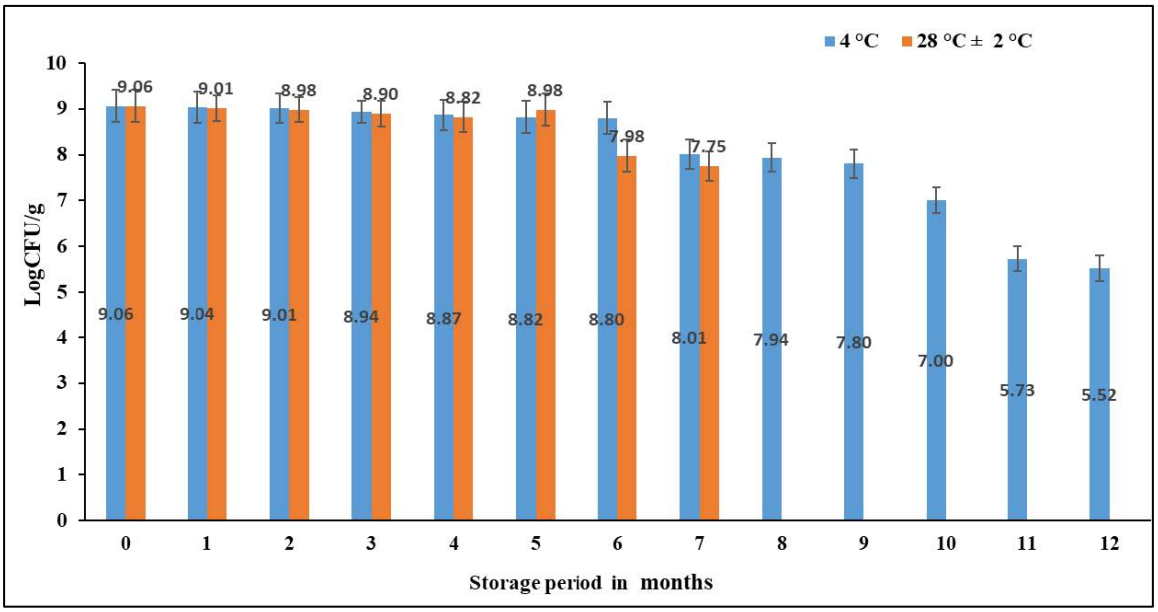


Figure 15: Viability of *B. licheniformis* in lyophilized GC3 formulation upon storage.

4.7 Effect of fermentation on proximate analysis, peptide formation, nutritional composition, phytate and oligosaccharide content of the synbiotic formulation

4.7.1 Proximate analysis

Functional foods should provide both nutritional and health benefits. Cowpea is a widely consumed legume in Goa; however, many consumers report intestinal gas or flatulence following its consumption. Fermentation, particularly with probiotic microorganisms, offers a strategy to enhance cowpea utilization by reducing the beany flavor and degrading anti-nutritional factors responsible for flatulence and increasing bioavailability of nutrients (Aduol et al., 2020). Table 33 presents the proximate composition of GC3 before and after *Bacillus* fermentation. The moisture content of GC3 remained statistically unchanged upon fermentation, showing a slight increase ($p > 0.05$). Total ash content, however, significantly increased ($p < 0.001$) from 3.36 ± 0.06 g/100 g to 4.57 ± 0.07 g/100 g, consistent with findings of Adebowale & Maliki (2011), who reported a ~ 20 % increase. This rise in ash content may result from the addition of salt during fermentation or the relative stability of mineral constituents under microbial processing. Overall, these changes align with numerous

studies indicating that *Bacillus* fermentation can markedly alter the proximate composition of legume-based foods.

Crude fat levels exhibited a slight, non-significant decrease ($p > 0.05$) following fermentation. Total dietary fiber exhibited a non-significant decrease ($p > 0.05$), declining from 21.7 ± 1.28 g/100 g to 18.45 ± 1.77 g/100 g. Insoluble dietary fiber showed a significant reduction ($p < 0.05$), decreasing from 19.6 ± 1.28 g/100 g to 15.05 ± 1.71 g/100 g, whereas soluble dietary fiber increased significantly ($p < 0.001$), rising from 2.1 ± 0.12 g/100 g to 3.40 ± 0.09 g/100 g.

These fiber modifications can be attributed to the enzymatic breakdown of complex insoluble structures into smaller, water-soluble components during fermentation, coupled with microbial synthesis of soluble exopolysaccharides. The balance between enzymatic degradation and microbial utilization of fiber depends on the specific microorganisms employed, fermentation conditions (time, temperature, pH), and the initial composition of the substrate.

Similar reductions in TDF and IDF (~10%) have been reported due to hydrolytic enzymes such as cellulase and α-galactosidase converting insoluble fibers into soluble forms (Garrido-Galand et al., 2021). Other studies have reported up to 74 % fiber reduction in fermented maize flour, attributed to lactic acid bacterial enzymatic activity (Dai et al., 2022;,Afoakwa et al., 2010). These findings are consistent with Adebowale & Maliki. (2011), who observed a significant decrease in TDF levels of pigeon pea following fermentation.

The minor change in fat content of fermented GC3 may be linked to lipolytic enzyme activity, which hydrolyses triglycerides into free fatty acids and glycerol (Danilova & Sharipova, 2020). Fermentation also resulted in a significant decrease in total protein levels ($p < 0.001$), suggesting nitrogen loss through ammonia volatilization or microbial assimilation. Although protein breakdown into lower peptides and amino acids during fermentation can improve digestibility, total nitrogen loss occurs when microorganisms utilize nitrogenous compounds for growth or when volatile nitrogenous metabolites are produced.

Fermented GC3 showed a carbohydrate content of 41.94 ± 0.32 g/100 g, slightly higher than unfermented GC3. As carbohydrates were estimated by difference, this apparent increase is

likely a calculation artifact resulting from relative decreases in other components rather than a true increase in carbohydrate mass.

Table 33: Proximate analysis of fermented GC3 synbiotic formulation.

GC3	Moisture	Ash	Crude fat	Protein	IDF	SDF	TDF	Carbohydrate
	g/100g							
Before fermentation	9.97 ± 0.24[a]	3.36 ± 0.06[a]	2.33 ± 0.08[a]	26.16 ± 0.23[a]	19.60 ± 1.28[a]	2.10 ± 0.12[a]	21.70 ± 1.28[a]	36.48 ± 1.22[a]
After fermentation	10.08 ± 0.21[a]	4.27 ± 0.07[b]	2.08 ± 0.03[a]	22.88 ± 0.15[b]	15.05 ± 1.71[b]	3.40 ± 0.09[b]	18.45 ± 1.77[b]	41.94 ± 0.32[b]

Results are reported as mean ± SD; a, b reveals a significant difference ($p < 0.05$) within the treatments of the respective parameters.

The observed modifications in proximate composition highlight the dual nutritional and functional benefits of fermentation. An increase in soluble fiber may improve gut health by supporting beneficial microbiota and enhancing stool consistency, while the reduction in insoluble fiber may improve digestibility and reduce flatulence associated with cowpea consumption. Although total protein decreased, the enzymatic hydrolysis into peptides and amino acids may enhance protein bioavailability. The slight increase in carbohydrate content (by difference) and stable fat content indicate that energy contribution remains largely unaltered. Collectively, these changes suggest that *Bacillus* fermentation not only improves the sensory and digestibility attributes of cowpea but also potentially enhances its health-promoting properties, making fermented GC3 a promising functional food ingredient.

4.7.2 Protein hydrolysis and peptide formation by SDS – PAGE

Bacillus fermentation brought about the proteolysis of GC3, as evidenced by the accumulation of lower molecular weight proteins (< 24 kDa) in lanes 2 and 3 in Plate 23. The extracellular proteases could have resulted in the proteolysis (Nkhata et al., 2018). These results correlate with earlier results on the production of FAN, which increased with fermentation times.

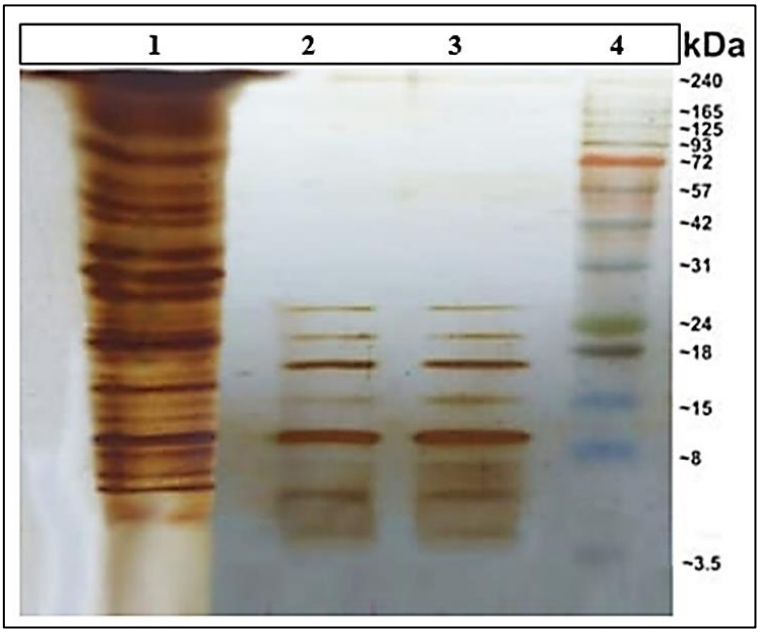


Plate 23: SDS-PAGE profile of GC3 before and after fermentation.

Lane 1: Unfermented GC3 powder, Lanes 2 and 3: Fermented GC3 powder
Lane 4: Prestained ladder marker

Microbial fermentation drives proteolysis, releasing short chains of amino acids known as food-derived peptides. While initial proteins provide essential amino acids, these peptides often exhibit additional biological activities and are referred to as bioactive peptides (Fan et al., 2015). Fermenting microorganisms produce diverse proteases responsible for this process, which contribute to the characteristic properties of fermented foods and enhance nutritional value (Li et al., 2023). Dai et al. (2022) reported degradation of soybean proteins into small peptides using *B. licheniformis* YYC4, while Li and Wang (2021) observed similar breakdown of chickpea proteins into low-molecular-weight peptides after 48 h fermentation with *B. subtilis* LWO.

Depending on the enzymes present, peptides may be further hydrolysed into free amino acids. These bioactive peptides exhibit multiple positive health effects, including antihypertensive, antioxidant, immunomodulatory, anti-inflammatory, and hypoglycaemic activities (Gopikrishna et al., 2021), with statin-like effects drawing particular interest.

Interest in plant-derived peptides has grown with rising demand for meat-free foods, motivated by health, climate, environmental, and ethical concerns. Functional foods from plant sources offer a sustainable alternative to animal proteins, and their consumption has spurred research into beneficial macro- and micronutrients. Plant-food-derived peptides have been shown to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), the enzyme central to cholesterol synthesis (Santos Sánchez et al., 2024). To date, peptides from several plant sources, *viz*. amaranth, cowpea, hemp, soy, lupin, chia, chickpea, mung bean, olive, and pigeon pea, demonstrate HMG-CoAR inhibition, highlighting their potential in cardiovascular health and functional food applications.

4.7.3 Comparative assessment of composition of free amino acids (FAAs)

There is growing attention on healthy, environmentally friendly, and sustainable consumption, leading to increased popularity of plant-based foods. Legume proteins are regarded as sustainable and renewable alternatives; however, their limited functional properties restrict their broader application. Fermentation with *Bacillus* offers a safer approach than chemical or physical modifications, enhancing the functional characteristics of legume proteins through biodegradation and structural modification, while also improving their digestibility (Liu et al., 2023).

In the present study only six FAAs (glutamic acid, aspartic acid, serine, arginine, histidine, and alanine) were detected in unfermented GC3, with a depletion of several essential amino acids (EAAs) whereas 12 h fermentation resulted in detection of 16 FAAs including most of EAAs (glutamic acid, aspartic acid, asparagine, threonine, arginine, serine, alanine, cysteine, tyrosine, valine, tryptophan, methionine, phenylalanine, isoleucine, leucine and lysine). This demonstrates that fermentation substantially increased the diversity and made free amino acids available. Table 34 presents the FFA profile of GC3 before and after fermentation.

A significant increase in aspartic acid, glutamic acid, and overall total FAAs was seen after 12 h of fermentation ($p < 0.001$). The concentrations (mg/g) of 16 FAAs in GC3 after

fermentation followed the order: glutamic acid (0.39 ± 0.04) > aspartic acid (0.07 ± 0.001) > methionine (0.04 ± 0.001) > alanine (0.03 ± 0.001) > phenylalanine (0.02 ± 0.001) > asparagine (0.02 ± 0.001) > isoleucine (0.02 ± 0.003) > tryptophan (0.02 ± 0.001), followed by tyrosine (0.01 ± 0.001), valine (0.01 ± 0.001) and serine (0.03 ± 0.001).

Table 34: Free amino acid profile of GC3 before and after fermentation.

Amino acid	Before fermentation	After fermentation	Significance
Essential **(mg/g)**			
Threonine	BDL	0.01 ± 0.001	-
Valine	BDL	0.01 ± 0.001	-
Isoleucine	BDL	0.02 ± 0.003	-
Leucine	BDL	BDL	-
Lysine	BDL	0.03 ± 0.001	-
Phenylalanine	BDL	0.02 ± 0.001	-
Tyrosine	BDL	0.01 ± 0.001	-
Methionine	BDL	0.04 ± 0.001	-
Cysteine	BDL	ND	-
Tryptophan	BDL	0.02 ± 0.002	-
Histidine	0.09 ± 0.001	ND	-
Arginine	0.03 ± 0.002	0.04 ± 0.001	$p < 0.01$ ↑
Nonessential **(mg/g)**			
Aspartic acid	0.03 ± 0.001	0.07 ± 0.001	$p < 0.001$ ↑
Glutamic acid	0.05 ± 0.001	0.39 ± 0.04	$p < 0.001$ ↑
Serine	0.03 ± 0.006	0.03 ± 0.003	-
Glycine	BDL	BDL	-
Alanine	0.03 ± 0.002	0.03 ± 0.002	-
Asparagine	BDL	0.02 ± 0.001	-
Total	0.26 ± 0.02	0.73 ± 0.06	$P < 0.001$ ↑

Values are reported as mean ± SD (n = 3). Significant differences after fermentation were considered at $p < 0.05$.

This detection of an increment in free amino acids after fermentation is a strong indication that the fermentation process was successful in breaking down proteins and releasing their constituent amino acids. This is a key aspect of how fermentation alters the biochemical profile of food, often contributing to changes in flavour, aroma, and digestibility (Sharma et al., 2020).

The protein quality, composition of amino acids, their digestibility, and absorption can be influenced by fermentation (Zheng et al., 2017; Arbab et al., 2023). It can increase the FAA contents in legume-based products, depending on the species and cultivars (Adebo et al., 2022; Arbab et al., 2023).

4.7.4 Water- and fat-soluble vitamins and carotenoids

The controlled fermentation of GC3 with potential probiotic *Bacillus* resulted in a mixed impact on its vitamin and carotenoid profile. Table 35 indicates the vitamin and carotenoid content. Controlled bacillus fermentation resulted in a significant increase in Vit. B2 and Vit. B3 content. However, a significant reduction was observed in several other vitamins, specifically Vit. B5, Vit. B6, and Vit. K1. Vitamin E was found to be BDL in both unfermented and fermented GC3. Increase in Vit. B2 and Vit. B3 is a common benefit of many microbial fermentations, particularly those involving *Bacillus* species (Li et al., 2023). The halotolerant Bacillus strain used may possess the necessary biosynthetic pathways to synthesize B-vitamins as essential cofactors for their energy and growth metabolism. In the controlled, nutrient-rich environment of the GC3 slurry, this microbial production rate may have resulted in enrichment of the formulation. Our findings are in accordance with those of Dai et al. (2017) and Huang et al. (2022), who reported an increase in vitamin content on bacillus fermentation of soyabean.

Vit.B5 and Vit.B6 are cofactors essential for microbial metabolism, such as in amino acid and fatty acid synthesis (Adebo et al., 2022). The *Bacillus* organism may likely have consumed the pre-existing vitamins in the GC3 as a nutrient source for rapid growth during the fermentation process, leading to a net decrease. As a lipophilic vitamin, Vit. K1 is susceptible to oxidative and enzymatic degradation.

Vitamin E was found to be BDL in both unfermented and fermented GC3, indicating its absence as an inherent property of GC3 or a consequence of the autoclaving pre-treatment, which can severely degrade this highly thermolabile and oxidation-sensitive vitamin.

Table 35: Vitamin and carotenoid content of GC3 before and after fermentation.

Nutrients	Before fermentation	After fermentation	Significance
Vitamin			
Vit.B2 (mg/100g)	0.07 ± 0.01	0.11 ± 0.01	$p < 0.01$ ↑
Vit.B3 (mg/100g)	0.57 ± 0.02	0.75 ± 0.02	$p < 0.001$ ↑
Vit.B5 (mg/100g)	0.34 ± 0.01	0.23 ± 0.01	$p < 0.001$ ↓
Vit.B6 (µg/100g)	0.43 ± 0.03	0.30 ± 0.01	$p < 0.05$ ↓
Vit.B9 (µg/100g)	9.26 ± 1.02	10.45 ± 1.04	$p > 0.05$, NS
Vit.K1 (µg/100g)	7.63 ± 0.30	5.49 ± 0.24	$p < 0.001$ ↓
Carotenoids (µg/100g)			
Lutein	34.04 ± 3.53	15.27 ± 1.39	$p < 0.001$ ↓
Zeaxanthin	25.20 ± 2.69	BDL	-
Beta Carotene	46.08 ± 3.73	12.36 ± 0.75	$p < 0.001$ ↓
Total carotenoids	367.77 ± 7.08	156.48 ± 2.41	$p < 0.001$ ↓

BDL-Below Detection Limit. Values are indicated as mean ± SD (n = 3). Significant differences after fermentation were considered at $p < 0.05$.

A marked reduction ($p < 0.001$) was observed in lutein, β-carotene, and total carotenoid content following fermentation, which is in accordance with the findings of Wu et al. (2015). This decline likely resulted from oxidative degradation under aerobic conditions. The halotolerant *Bacillus* culture, which is a lipase producer, may have generated free radicals and hydroperoxides that accelerated carotenoid breakdown into colourless products, causing peroxidative damage (Anaemene & Fadupin, 2022). Additionally, the lower pH arising from organic acid accumulation during fermentation may have promoted acid-catalysed degradation and isomerization of carotenoids, leading to the formation of undetectable isomeric or oxidized forms (zeaxanthin was below detection limit) and trans-to-cis

conversion, thereby reducing their extractable and bioactive levels. Overall, the results indicate that enzymatic and oxidative degradation processes, enhanced by aerobic and lipase-producing conditions, outweighed any potential retention, resulting in substantial depletion of these provitamin A and antioxidant compounds in the fermented GC3 formulation.

4.7.5 Mineral content upon fermentation

Table 36 below shows the composition of 07 important minerals in the fermented GC3 formulation. A marginally significant increase in iron and calcium content was seen after fermentation at ($p < 0.01$), with a non-significant ($p > 0.01$) increase in zinc. The results correspond with observations made by Ali et al. (2018), who reported an increase in calcium, iron, and zinc in Korean fermented soybean using *B. subtilis* from 1481.38 to 1667.32, 41.38 to 317.00, and 32.82 to 37.18 mg/kg, respectively. Pranoto et al. (2013) also reported similar findings after fermentation and attributed it to the degradation of phytates and oxalates, which bound the minerals. A significant reduction at $p < 0.05$ was seen in Mg levels after GC3 fermentation, possibly due to assimilation of Mg by the growing microbial population (N'zi et al., 2021). Scheers et al. (2016) investigated the impact of spontaneous fermentation and germination on kidney bean, soybean, and mung bean, reporting that such fermentation increased levels of iron across all legumes while significantly reducing tannin and phytic acid contents. Similarly, John et al. (2015) observed that mineral contents increased due to bacterial interactions, leading to the breakdown of complex metabolites. Obadina et al. (2013) also found higher mineral levels in fermented soymilk compared to its unfermented counterpart, suggesting the role of starter culture in facilitating the release of minerals bound in chelated complexes during fermentation. Collectively, these findings indicate that fermentation enhances both mineral content and bioavailability, particularly of iron, making it more accessible for intestinal absorption. Furthermore, lactic fermentation has been suggested to promote the formation of ferric (Fe^{3+}) species, which may contribute to improved bioavailability (Scheers et al., 2016).

The increase in iron, calcium, and zinc content observed in the present study, after Bacillus-mediated fermentation, can be attributed to several interrelated mechanisms. A key factor is the degradation of phytic acid by *Bacillus*-derived phytases and phosphatases, which hydrolyse strong mineral-phytate complexes and release bound divalent cations into more

soluble and bioavailable forms. Additionally, organic acid accumulation during fermentation lowers the pH, enhancing mineral solubility through acid-mediated chelation and dissolution of mineral salts (Wu et al., 2015).

Table 36: Mineral content of unfermented and fermented GC3.

Mineral content (mg/100g)	Unfermented	Fermented	Significance
Iron	12.49 ± 0.84^{a}	14.39 ± 0.31^{b}	↑
Zinc	6.62 ± 0.06^{a}	7.76 ± 0.98^{a}	↑ NS
Calcium	157.91 ± 0.80^{a}	161.54 ± 1.58^{b}	↑
Phosphorus	277.26 ± 0.35^{a}	282.46 ± 0.25^{b}	↑
Copper	0.88 ± 0.00^{a}	0.68 ± 0.03^{b}	↓
Potassium	1567.93 ± 25.59^{a}	1556.39 ± 261.31^{a}	↓
Magnesium	167.68 ± 11.78^{a}	103.97 ± 24.27^{b}	↓
Manganese	2.54 ± 0.35^{a}	2.39 ± 0.48^{a}	↓

NS=non-significant. Values are reported as mean ± SD. Significant differences after fermentation were considered at $p < 0.05$ and indicated by a and b.

Enzymes such as proteases and cellulases may further disrupt the cellular matrix and seed coat, releasing minerals otherwise entrapped within the structural components. Moreover, Bacillus species are known to secrete siderophores and small peptides that transiently bind and mobilize minerals, maintaining them in soluble forms detectable in assays (Vijayakumar, 2015). Together, these biochemical and structural modifications improve mineral extractability and likely explain the observed increases in measurable iron, calcium, and zinc in the fermented samples.

Minerals are the protective nutrients essential for the body to remain healthy. Calcium is essential for nerve function, blood clotting, immune system performance, and muscle contraction and relaxation. Magnesium supports protein synthesis, immune function, and helps prevent constipation, while potassium is crucial for nerve signaling, muscle contraction, and regulation of blood pressure. Iron, copper, manganese, and zinc serve as cofactors for numerous enzymes.

Iron, in particular, is present in red blood cells, facilitating oxygen transport and playing a key role in energy metabolism (Gharibzahedi & Jafari, 2017). Given these critical functions, minerals are indispensable for maintaining overall health. Fermented GC3 provides all these minerals and can be regarded as a functional and novel food with potential benefits in preventing conditions such as rickets, diarrhea, cardiac arrhythmias, and cognitive impairments.

4.7.6 Phytic acid content

A non-significant reduction in phytate content of GC3 was observed after 12 h fermentation ($p > 0.05$), from 326.22 ± 6.87 mg/100 g to 319.19 ± 3.6 mg/100 g. The limited reduction may be due to the short fermentation duration, but is likely mediated by endogenous and microbial phytases, which hydrolyze phytic acid into phosphoric acid and myoinositol.

Phytates typically form complexes with phosphorus, zinc, iron, and calcium, reducing their bioavailability. Microbial enzymes at specific pH can break these complexes, releasing soluble minerals (Tresina et al., 2012). In our study, total phosphorus increased slightly from 277.26 ± 0.35 to 282.46 ± 0.25 mg/100 g, indicating a modest enhancement in bioavailable phosphorus. Significant reductions in phytic acid and other antinutrients have been reported in similar studies, up to 44.4 % in phytic acid and 50.9 % in oxalates in African locust bean fermentation (Omodara et al., 2018), and decreases of 92 % phytic acid, 60 % saponins, 45 % trypsin inhibitors, and 65 % tannins after cowpea fermentation (Affrifah et al., 2022).

4.7.7 Oligosaccharide content after fermentation

Table 37 indicates quantitative analysis of RFOs in GC3 before and after fermentation. A significant ($p < 0.01$) reduction in total RFOs on GC3 fermentation from 1.41 ± 0.08 % to 1.05 ± 0.01 % was observed. A significant increase in raffinose content was observed at $p < 0.001$ after fermentation, and a significant reduction in stachyose ($p < 0.01$) and verbascose ($p < 0.0001$) content, indicating their utilization by the bacteria.

Raffinose, verbascose, and stachyose, which are present in relatively high amounts in cowpea, are often associated with flatulence (Elango et al., 2022). However, these oligosaccharides are also recognized for their role as functional prebiotics and enable the

action of beneficial microbes; fermenting microorganisms hydrolyse starch and non-digestible carbohydrates into readily digestible sugars and additionally influence the product's texture due to tissue softening (Ziar et al., 2014), Abebe & Alemayehu. (2022) reported a reduction in alpha-galactosides and raffinose components in millet, by 75 % and 83 %, respectively, on fermentation.

Table 37: Effect of fermentation on raffinose family oligosaccharide content of GC3.

Raffinose family oligosaccharide	**Fermentation (12 h)**	
(RFO)	**Before**	**After**
Raffinose	0.22 ± 0.01^{a}	0.32 ± 0.01^{b}
Stachyose	0.91 ± 0.08^{a}	0.67 ± 0.01^{b}
Verbascose	0.27 ± 0.01^{a}	0.05 ± 0.01^{b}
Total RFO	1.41 ± 0.08^{a}	1.05 ± 0.01^{b}

Findings are given as mean ± SD value of triplicate analysis of GC3 seeds, and a, b indicate a significant difference ($p < 0.05$).

4.8 *Bacillus* viability in the synbiotic food formulation in comparison to its cell suspension during exposure to simulated gastrointestinal conditions

Legumes offer an effective substrate for the production of synbiotic foods. Besides supporting good growth characteristics of probiotics, the matrix must also enhance their tolerance during gastrointestinal (GI) transit. The success of any newly developed formulation depends on the ability of the probiotic to maintain sufficient viable cell numbers capable of beneficially modulating the host gut microbiota. For probiotic bacteria to provide health benefits, they must first survive transit through the upper gastrointestinal tract and then persist in the gut (Palanivelu et al., 2022). Hence, the influence of the food matrix in protecting cell viability under simulated GI conditions was studied. The survivability of *B. licheniformis* PQ098138 in its free-cell suspension (BCS) was compared with its performance in the GC3 synbiotic formulation.

Table 38: Viability of *Bacillus* cell suspension in comparison to GC3 synbiotic formulation during exposure to simulated gastric juice.

	BCS	GC3 SF	BCS	GC3 SF	BCS	GC3SF	BCS	GC3SF
pH	1.2		2		3		4	
Time (h)	**Log CFU/mL**							
0h	8.88± 0.01[a]	9.65± 0.02[a]	8.88± 0.03[a]	9.67± 0.01[a]	8.89± 0.04[a]	9.83± 0.03[a]	8.88± 0.02[a]	9.75± 0.08[a]
1h	8.9± 0.02[a]	9.67± 0.03[a]	8.85± 0.01[a]	9.78± 0.02[a]	8.85± 0.04[a]	9.89± 0.04[a]	8.86± 0.04[a]	9.81± 0.03[a]
2h	8.83± 0.02[a]	9.66± 0.04[a]	8.75± 0.04[b]	9.47± 0.03[a]	8.78± 0.05[b]	9.82± 0.03[a]	8.79± 0.03[b]	9.85± 0.03[a]
3h	8.57± 0.02[b]	9.54± 0.12[a]	8.71± 0.08[b]	9.61± 0.05[a]	8.73± 0.06[b]	9.79± 0.01[b]	8.76± 0.02[b]	9.87± 0.02[b]

BCS=*Bacillus* cell suspension , GC3SF=GC3 synbiotic formulation

Results are mean ± SD value of triplicate analysis, and a, b indicates significant difference ($p< 0.05$) within the individual pH in respective columns at different time intervals.

In general, *B. licheniformis* PQ098138 exhibited lower viability in simulated gastric juice compared to the synbiotic GC3 formulation (Table 38). Significant decreases in viability were observed over time ($p < 0.01$) at acidic pH values (1.2 – 4). Specifically, at pH 1.2, viability remained stable up to 2 h but dropped sharply by 3 h. At pH 2–4, declines in viable counts appeared earlier (by 2 h), indicating the strain's sensitivity to acidic stress.

In contrast, in the GC3 formulation, no significant reduction in viability occurred up to 3 h at pH 1.2 and 2.0 ($p> 0.05$). At pH 3 and 4, a minor but significant reduction was evident only after 3 h ($p \approx 0.05$). These results demonstrate the highly significant protective effects of the food matrix in maintaining viability even under acidic and prolonged incubation conditions ($p< 0.001$). The protective advantage of the food matrix was most pronounced at low pH (1.2 – 2) and after longer exposure times (2–3 h). Tukey's post hoc analysis revealed that higher pH levels (3 – 4) maintained *Bacillus* viability better than lower pH levels (1.2 – 2), both with and without the GC3 matrix.

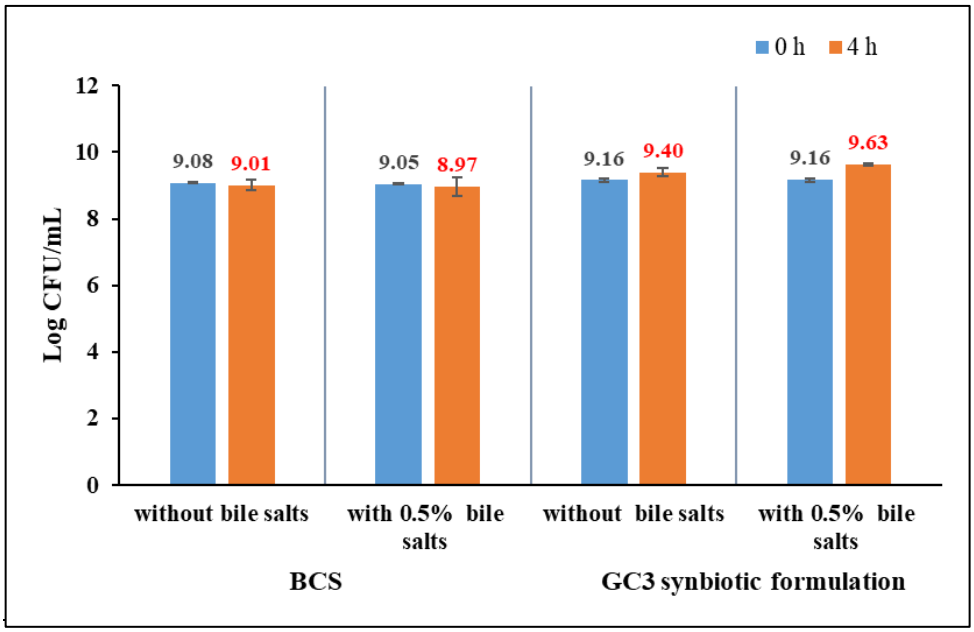


Figure 16: Viability of *Bacillus* cell suspension (BCS) in comparison to GC3 synbiotic formulation during exposure to simulated intestinal juice.

Figure 16 illustrates the survivability of *B. licheniformis* PQ098138 under simulated small intestinal conditions at pH 8.0, in the presence or absence of bile salts. The organisms retained desirable viability during simulated intestinal transit under both conditions. Free-cell BCS showed a slight, non-significant decrease in viability over 4 h with and without bile salts ($p > 0.05$). However, in the GC3 formulation, viability significantly increased in both no-bile ($p < 0.05$) and bile conditions ($p < 0.01$), likely due to cell recovery or proliferation within the protective food matrix.

After 4 h, viable counts in GC3 synbiotic formulation were significantly higher than in the bacillus cell suspension ($p < 0.05$), confirming the protective effect of the food matrix in improving bacterial tolerance to bile stress. A significant increase in viability ($p < 0.05$) was observed after 4 h exposure to bile salts, as well as without bile salts in the GC3 food matrix. The percentage survival of *Bacillus* cells after 4 h exposure without bile salts was 99.22 %, which increased to 102.62 % when incorporated in the GC3 food matrix. When exposed to bile salts, survival increased further to 105.02 %. Metabolizable sugars generally improve the survival of *Bacillus* probiotics under acidic conditions, as glucose provides ATP via glycolysis, enabling proton extrusion and improving acid tolerance (Jeon et al., 2018). Legume substrates provide soluble sugars that likely contributed to the enhanced stability

observed during GI passage. (Verni et al., 2022) reported that food products with pH between 3.5 and 4.5 and a high capacity to buffer elevate gastric pH, improving probiotic stability. In our fermented GC3 slurry, the pH ranged from 6.54 ± 0.03 to 5.20 ± 0.08 after 24 h, and although 12 h fermentation did not reach the optimal 3.5 – 4.5 range, the cells remained viable. The observed increase in reducing sugars during fermentation may have facilitated *Bacillus* survival under simulated GI conditions. Arena et al. (2014) similarly observed that *L. acidophilus*, *L. plantarum*, and *L. fermentum* survived simulated gastric stress better when delivered in β-glucan-enriched pasta or barley flour compared to saline suspension. The reduction in viable counts was greater at pH 2.0 than at pH 3.0. Stasiak-Różańska et al. (2021) reported that probiotic bacteria analyzed from commercial preparations with natural prebiotics were the most resistant to the simulated gastrointestinal conditions. Consistent with these reports, our results demonstrate that food matrices enhance probiotic tolerance to gastrointestinal passage. The potential probiotic *B. licheniformis* PQ098138 survived under *in vitro* conditions containing bile salts, indicating a strong probability of reaching the intestinal site when consumed as part of a fermented food. The spore-forming nature of the strain further contributes to its robustness, enabling survival in harsh bile environments, which is a positive indicator of its potential as an effective synbiotic component.

The results clearly show that the GC3 matrix markedly enhanced bacterial tolerance to bile salts. The observed stability and slight increase in viable counts after 4 h suggest that the matrix provides both physical protection and nutrient buffering, shielding cells from bile-induced membrane disruption. These findings agree with reports that polysaccharide–protein matrices and fermented food systems mitigate bile toxicity and preserve probiotic metabolic activity during simulated digestion (Arena et al., 2014). Overall, these findings indicate that incorporation of probiotics into food matrices is an effective strategy for enhancing survival under adverse gastrointestinal conditions. The marked improvement in viability in GC3 synbiotic formulation indicates its suitability as a reliable method for sustaining probiotic activity during processing.

4.9 Antibiotic susceptibility, antioxidant activity, and antimicrobial activity of the synbiotic food formulation

4.9.1 Antibiotic susceptibility of the synbiotic formulation

The complex biochemical environment of the fermented legume (pH changes, nutrients, presence of prebiotics, organic acids, polyphenols, anti-nutritional factors, and other microbial metabolites) can significantly alter the metabolic state, growth rate, and even gene expression of the *Bacillus* isolate (Tamang et al., 2020). These changes can lead to alterations in its intrinsic susceptibility to antibiotics. Thus, the antibiotic susceptibility of the synbiotic formulation was studied even though the susceptibility of the pure culture was already determined.

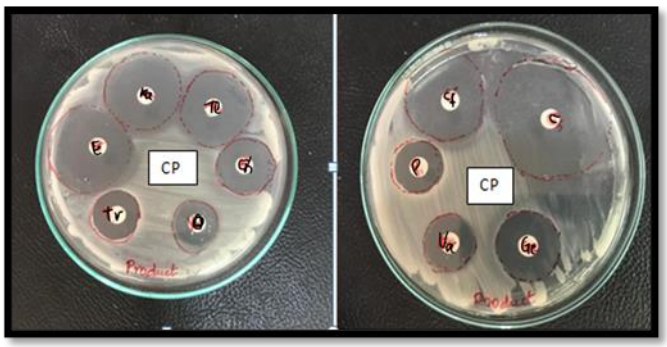


Plate 24: Antibiotic susceptibility of GC3 synbiotic formulation to Chloramphenicol (C), Kanamycin (K), Ciproflaxin (Cf), Tetracycline (Te), Erythromycin (Ery), Vancomycin (Va), Gentamycin (G), Cephalothin (Ch), Ofloxacin (O), Trimethoprim (Tr), and Ampicillin(A).

The synbiotic formulation, having a potential probiotic, was susceptible to most (9) of the commonly used antibiotics (Plate 37). Although the formulation showed resistance to Ampicillin, an increase in the zone diameter for Ampicillin was noted, as compared to the strain alone. Similarly, the other antibiotics, Vancomycin and Cephalothin, showed the same trend as Ampicillin, with a slight increase in the susceptibility. All the antibiotics belonging to the group of nucleic acid synthesis and inhibitors of protein synthesis exhibited an increase in susceptibility, as seen in Tables 19 and 39.

Table 39: Antibiotic susceptibility of the GC3 synbiotic formulation.

Antibiotic Name/ Dosage	***Bacillus* cell suspension**	**GC3 Formulation**
	Zone Diameter (mm) and interpretation *	
Inhibitors of cell wall synthesis		
Vancomycin [Va](30µg)	18^{S}	**19^{S}**
Ampicillin [A] (10 µg)	05^{R}	**13 ^{R}**
Cephalothin [Ch] (30 µg)	14^{R}	**19 ^{S}**
Inhibitors of protein synthesis		
Erythromycin [Ery] (15 µg)	30^{S}	**30^{S}**
Kanamycin [Ka] (30 µg)	25^{S}	**36^{S}**
Tetracycline [Te] (30 µg)	30^{S}	30^{S}
Chloramphenicol [C] (30 µg)	20^{S}	**48^{S}**
Gentamycin [G] (10 µg)	25^{S}	25^{S}
Penicillin [P](10 µg)	16^{MS}	**19^{MS}**
Streptomycin [St] (10 µg)	16^{S}	**18^{S}**
Inhibitors of nucleic acid synthesis		
Ciprofloxacin [Cf] (5µg)	29^{S}	**34^{S}**
Ofloxacin [O] (5µg)	16^{S}	**18^{S}**
Trimethoprim [Tr] (5µg)	13^{MS}	**14 ^{MS}**

*R (resistant), MS (moderately susceptible), or S (susceptible)

4.9.2 Antioxidant activity of the synbiotic formulation

Assessing the antioxidant capacity of synbiotic food formulation is vital to understand their potential health-promoting capabilities and to ensure their quality and efficacy as a functional food. For synbiotic food formulations to be marketed for their health benefits, quantifying antioxidant activity provides evidence of their potential efficacy. Synbiotics are combinations

of probiotics (beneficial live microorganisms) and prebiotics (non-digestible compounds that nourish the probiotics). Individually, both probiotics and prebiotics can exhibit antioxidant properties. However, when combined in a synbiotic, they can often produce synergistic and additional antioxidant effects that are superior to their individual components (Ali et al., 2018). Probiotics can convert prebiotics into more effective antioxidant compounds, and prebiotics can enhance the viability and activity of probiotic strains, leading to greater antioxidant production (Kim et al., 2020). Evaluating the antioxidant activity helps to confirm and quantify these enhanced effects. Antioxidant compounds may operate via diverse mechanisms against oxidising agents; therefore, no single method can comprehensively assess the antioxidant capacity of complex foods (Aziz et al., 2023). To address this, two complementary assays with distinct mechanisms, *viz.* DPPH and reducing power assays were employed to evaluate the antioxidant potential of fermented and unfermented GC3 extracts.

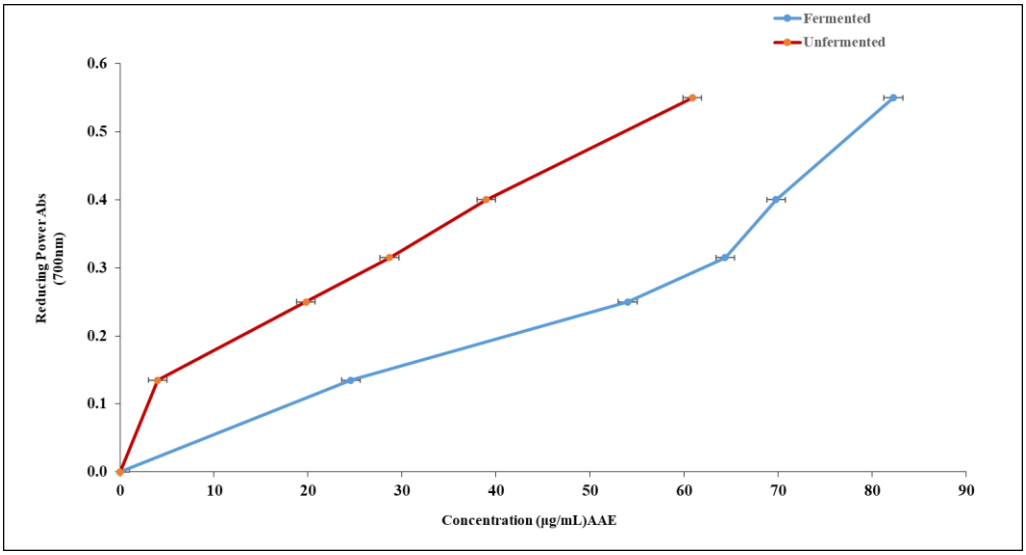


Figure 17: Reducing power of unfermented and fermented GC3 formulation

The fermented GC3 formulation exhibited the highest DPPH radical scavenging activity, with an inhibition of 66.48 ± 0.32%, which was significantly higher ($p < 0.01$) than that of the unfermented GC3 (44.01 ± 1.33%). The reducing power (RP) of the fermented GC3 extract was also significantly greater ($p < 0.05$) than that of the unfermented sample, with values ranging from 24.56 ± 2.91 µg/mL AAE to 82.23 ± 1.16 µg/mL AAE (Figure 17). In both extracts, the RP increased proportionally with concentration.

Reducing power is known to be closely associated to the antioxidant activity of polyphenolic compounds (Carvalho et al., 2012) and to the hydrogen-donating ability of reductones (Lee et al., 2008). In the present study, the enhanced RP of the fermented GC3 likely resulted from the generation of reductants during fermentation, capable of reacting with free radicals to terminate oxidative processes. Similar results were reported by Sanjukta et al. (2015), who observed a 3.1 to 4-fold enhancement in radical scavenging activity (DPPH and superoxide), total antioxidant capacity, and reducing power in fermented soybean extracts using Bacillus subtilis MTCC 5480 and MTCC 1747. These effects were attributed to increased levels of free phenolics, amino acids (proline, tryptophan, tyrosine, phenylalanine, histidine), and peptides generated during fermentation.

In agreement, fermentation of GC3 with *B. licheniformis* PQ098138 resulted in the formation of free amino acids and peptides, including aspartic acid, glutamic acid, asparagine, serine, threonine, arginine, alanine, tyrosine, cysteine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and lysine, which likely contributed to the enhanced antioxidant potential. Sanjukta et al. (2015) further reported that specific bioactive peptides produced during fermentation exhibit diverse health benefits depending on their amino acid sequence and composition. Besides antioxidant effects, such peptides may also possess antihypertensive, antimicrobial, antidiabetic, and anticancer properties.

Oxidative stress, resulting from the overproduction of free radicals, adversely affects lipids, proteins, and DNA, contributing to various human diseases. Natural antioxidants from fermented foods can mitigate these effects (Liu et al., 2022). Considering the potential risks associated with synthetic antioxidants (Kim et al., 2020), the identification of functional foods with natural antioxidant properties, such as fermented GC3, is of growing scientific and nutritional interest.

4.9.3 Antimicrobial activity of the synbiotic formulation

Numerous studies show synergistic antimicrobial effects in fermented foods (Monterio et al., 2019). Evaluation of the antimicrobial activity of GC3 synbiotic formulation can be of great therapeutic significance. The antimicrobial activity of the synbiotic GC3 formulation of different concentrations (25 µL, 50 µL, 75 µL, 100 µL) against previously grown *E. Coli*

(ATCC 700927), *S. Typhimurium* (MTCC 531), *S. Aureus* (ATCC 6538), and *S. enterica* (ATCC 35664) was evaluated. Probiotics mainly confer anti-pathogenicity to the formulation by producing organic acids as well as bacteriocins, hydrogen peroxide, or peptides (Dahiya and Nigam, 2022; Aziz et al., 2023).

The inhibition of the pathogenic microbes was observed in the synbiotic GC3 formulation, whereas GC3 without *Bacillus* displayed no zones of inhibition. Further, with an increase in the volume of extract, there was a respective increase in the inhibition zones, as seen in Plate 25. A zone of 15.12 ± 0.44 mm, 11.34 ± 0.44 mm, 12.27 ± 0.02 mm & 18 ± 0.4 mm was recorded with 100 µL of extract having 20 mg of powder for *S. enterica*, *E. coli*, *S. Typhimurium,* and *S. Aureus,* respectively. The inhibition of common pathogens could be attributed to its low pH and formation of acetic acid (19.13 ± 0.01 g/L).

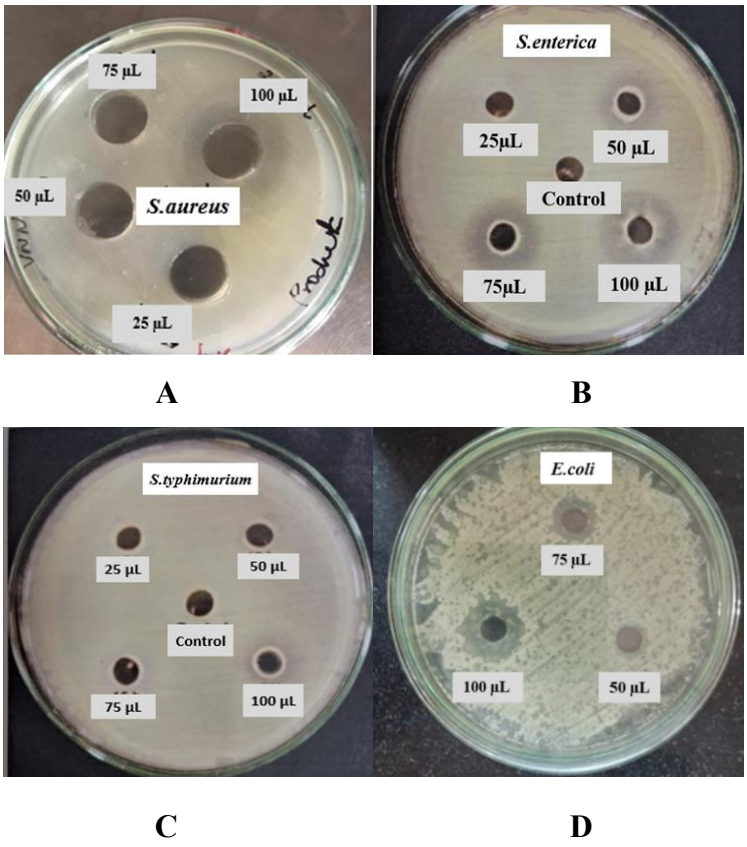


Plate 25: Antibacterial activity of GC3 synbiotic formulation against: (A) *S. aureus* ATCC 6538, (B) *S. enterica* ATCC 35664, (C) *S. typhimurium* MTCC 531, and (D) *E. coli* ATCC 700927.

Antimicrobial agents can also inhibit/inactivate the growth of unfavourable microbes, thereby acting as preservatives and prolonging the storage stability of food formulation (Behera et al., 2018). Results by Aziz et al. (2023) reported promising antibacterial properties of soya and oatmeal milk fermented with *L. plantarum* strains. Aziz et al. (2023) have reported antimicrobial activity of the bacillus-assisted products against the common gut pathogens attributed to their low pH and formation of organic acids. Our results show that GC3 synbiotic formulation can be used as a therapeutic food against these pathogens during an infection.

4.10 Functional properties of the synbiotic formulation for suitability in food products

Synbiotic foods work together to provide enhanced health benefits, particularly for the gut microbiome (Dahiya & Nigam, 2022). When probiotics and prebiotics are combined in a synbiotic, the prebiotic component helps the probiotic microorganisms survive the journey through the digestive tract and thrive once they reach the intestines. Cowpea seeds are a plant-based source of protein with abundant dietary fiber and carbohydrates. Beyond these major components, it contains smaller amounts of fats, polyphenols, and beneficial bioactive peptides. Given this nutritional profile, cowpea seeds are expected to remain a vital part of human diets for the foreseeable future. Successful incorporation of a new supplement into any food product requires assessment of its functional characteristics and consumer acceptance. Functional properties of food are an important area in creating successful recipes, and assessing them thus becomes essential. Components like water, carbohydrates, fats, and proteins are responsible for such behaviours and performance in food systems. Beyond just their nutritional value, things like starch in grains and proteins in legumes have functional properties that are crucial for how food is formulated and processed (Chandra et al., 2015).

Functional properties refer to the inherent physicochemical characteristics of a food ingredient that can influence the behaviour of food systems during processing, storage, and consumption, including solubility, foamability, gelation, and emulsification (Verni et al., 2022). Among these, protein solubility is a key functional attribute, as it strongly affects other properties such as emulsification, gel formation, and foaming capacity (Yang et al., 2022).

4.10.1 Emulsification capacity and stability

Figure 18 depicts the emulsification capacity and emulsification stability of unfermented and fermented GC3. The unfermented GC3 powder had the highest emulsification capacity ($p < 0.05$), which decreased upon fermentation. In our study, the possibility of enzymatic action on starch hydrolysis and proteolysis of the samples during fermentation could have resulted in lowering the emulsification capacity. Fermentation can reduce emulsification capacity due to several factors, including protein degradation and shifts in the overall structure of the food matrix (Yang et al., 2022). Specifically, the breakdown of proteins into smaller peptides (also observed in our study) can alter their ability to stabilize emulsions (Ozturk & Mc Clements, 2016). Although fermentation resulted in improvement of the quality of the GC3 slurry, leading to an improved fermentation profile, the functional properties were affected.

The emulsifying capacity of food powders dictates their versatility and utility in a vast array of food applications, directly impacting product stability, sensory quality, innovation, and consumer appeal. Starch and protein are widely exploited as emulsifiers in food. Starch is also being increasingly used to stabilize emulsions.

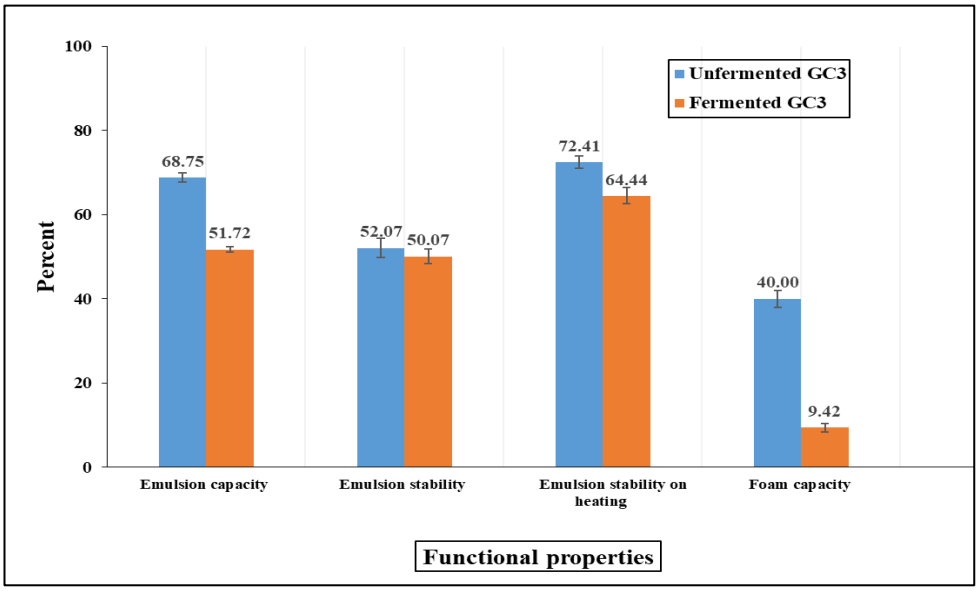


Figure 18: Functional properties of unfermented and fermented GC3.

The emulsion stability of unfermented GC3 powder before heating was estimated to be 52 % whereas on heating, it significantly increased ($p < 0.05$) to 74 % (Figure 18). A non-significant reduction ($p > 0.05$) in the emulsion stability of fermented GC3 formulation was observed before heating. Emulsion stability of the unfermented and fermented powders significantly increased on heating ($p< 0.05$). The increase in emulsion stability on heating of cowpea powder might be due to modification in the protein structure, enhancing its ability to stabilize the interface between oil and water (Appiah et al., 2011). Cowpea powder is a good source of protein, polysaccharides (complex carbohydrates), starch, and other contains several components that can act as emulsifiers (Chandra et al., 2015). These components help stabilize emulsions by reducing the surface tension between oil and water.

4.10.2 Foam capacity and stability

A foam consists of gas bubbles evenly distributed throughout a liquid or solid continuous matrix (Appiah et al., 2011). Understanding foam properties and behaviour is crucial for developing and improving various food products. Protein content and type, fat content, temperature, pH, and presence of stabilizers or destabilizers can affect the foaming properties. Foaming properties of fermented and unfermented GC3 powder were determined. Key characteristics of food foams include foam capacity and stability (Chandra et al., 2015). Foam capacity measures the volume of foam formed after stirring or whipping, whereas foam stability refers to the time; foam structure can be maintained without significant collapse or drainage (Appiah et al., 2011). Figures 18 and 19 present the foaming capacity and foaming stability of GC3, respectively. The foaming capacity was found to be the highest in the unfermented GC3 powder (40.00 ± 2.00 %), which significantly reduced upon fermentation to 9.42 ± 0.87 % ($p < 0.05$).

The foam stability of the unfermented GC3 was found to be significantly higher in comparison to fermented GC3 ($p < 0.05$). Fermentation brought about a change in the foam stability ($p > 0.05$). In the fermented formulation, a significant loss ($p > 0.05$) of foam was observed in 30 min (32.82 ± 1.91 %) as compared to unfermented GC3 powder (74.00 ± 2.65 %).

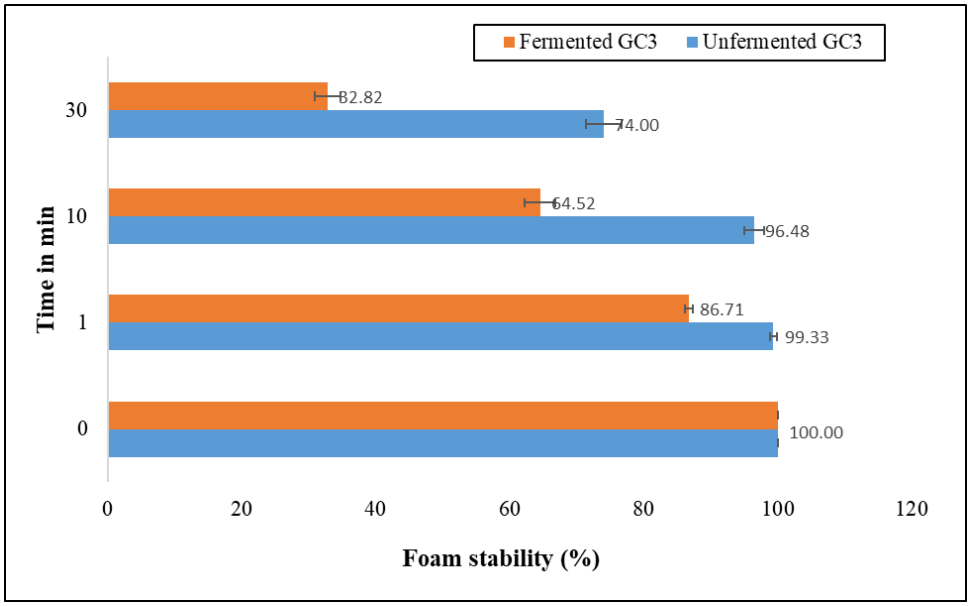


Figure 19: Foaming stability of unfermented and fermented GC3.

The uniform distribution of fine air cells in the structure of foods is brought about by proteins present in foams Khalid & Elharadallou, 2013). The foaming properties of food materials are mostly related to the molecular weight of the protein in the sample. Narayana & Rao (1984) & Verni et al. (2022 have reported improved foaming properties on partial hydrolysis of soy proteins upon fermentation due to the formation of more flexible and low molecular weight peptides. In our study, a decrease in foam capacity and stability of GC3 seed powder was observed despite a short duration of fermentation (16 h). Our findings are in accordance with Adebowale & Maliki (2011), who have reported a decrease in foam capacity and stability of pigeon pea seed flour upon 5 d of fermentation. The lower foaming properties could be related to the extensive protein hydrolysis brought about by the proteolytic enzymes of *B. Licheniformis,* which converted the protein into peptides of low molecular weight (< 24 kDa) (as depicted by the SDS PAGE profile of GC3 formulation on fermentation) and free amino acids. This indicates that the protein molecular weight is an important determinant of these properties. Extensive hydrolysis leads to loss in protein structure, leading to impaired protein film formation (Zheng et al., 2017; Yang et al., 2022).

4.10.3 Heat tolerance

Studying heat tolerance is fundamental to the successful development, manufacturing, and commercialization of effective probiotic products that deliver their promised health benefits to consumers. For probiotic-rich foods to be effective, they need to deliver a high number of live and active probiotic bacteria capable of withstanding passage through the upper GIT. This ensures enough of them reach and successfully colonize the gut, where they can provide their health benefits. Also, the food matrix is an active participant in protecting probiotic viability by providing physical shielding, chemical buffering, controlled environmental conditions (like water activity), and nutritional support, all of which contribute to delivering a higher number of live and effective probiotics to the consumer. Hence, the survivability of *Bacillus* under heat stress (40 – 70 °C) was evaluated for a synbiotic formulation and compared to that of cell suspension alone.

It was observed that the desirable levels of *B. licheniformis* (> 7 log cfu/ mL) were maintained in GC3 formulation upon heating up to 60 °C and up to 55 °C in cell suspension ($p > 0.05$) (Figure 10 A & 20 B). Above these temperatures, a progressive and significant reduction in cell counts was observed with increased duration of heating ($p < 0.05$). However, *B. licheniformis* in the GC3 matrix was found to survive better than the cell suspension alone at high temperatures. Viable counts of *B. licheniformis* in GC3 synbiotic formulation were found to be significantly higher than those of the cell suspension at temperatures of 60 - 65 °C ($p < 0.05$). In our study, no complete loss in cell viability was observed at higher temperature (70 °C) in GC3 and the cell suspension. Studies have indicated that EPSs secreted by microorganisms are one of the main components involved in the formation of extracellular biofilm matrix to protect microorganisms from adverse factors such as temperature, pH (Nguyen et al., 2020).

The potential probiotic selected in our study is an EPS producer and may have protected the cell viability. The food matrix components like proteins, carbohydrates (e.g., alginate, pectin, starch, gums), prebiotics, and fats can surround and embed the probiotic cells. These components may act as a natural or engineered barrier in protecting the cell survivability to harsh conditions (Yang et al., 2022). Prebiotics can act as a direct food source for probiotics, allowing them to thrive and potentially increase their numbers even within the food, and

importantly, can also enhance their survival and colonization once they reach the gut (Markowiak & Śliżewska, 2017).

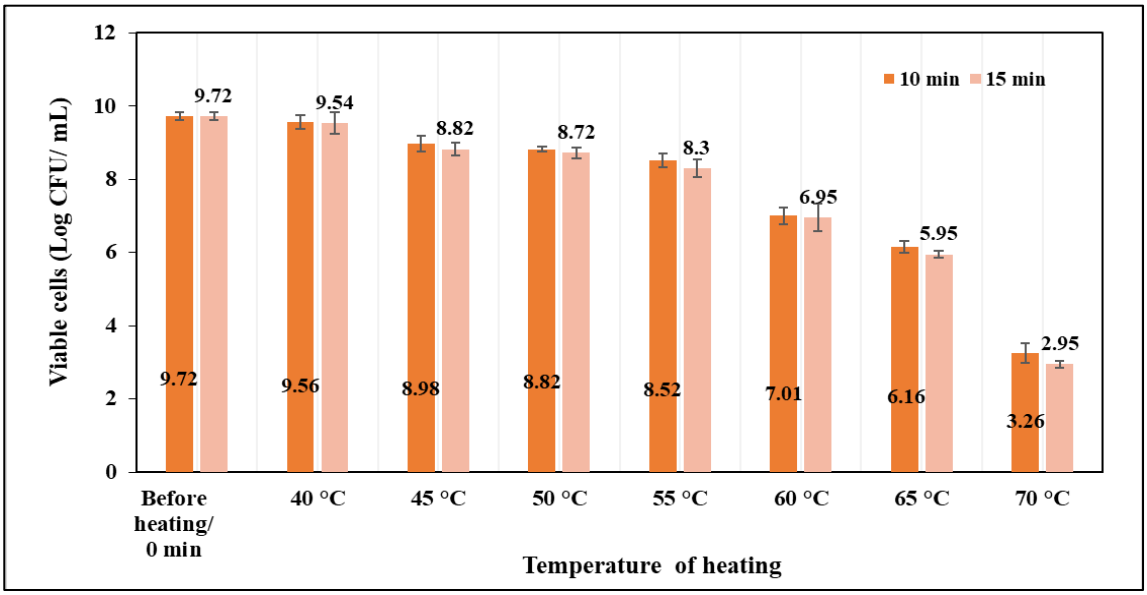


Figure 20 A.: Viable cell count of *B. licheniformis* cell suspension.

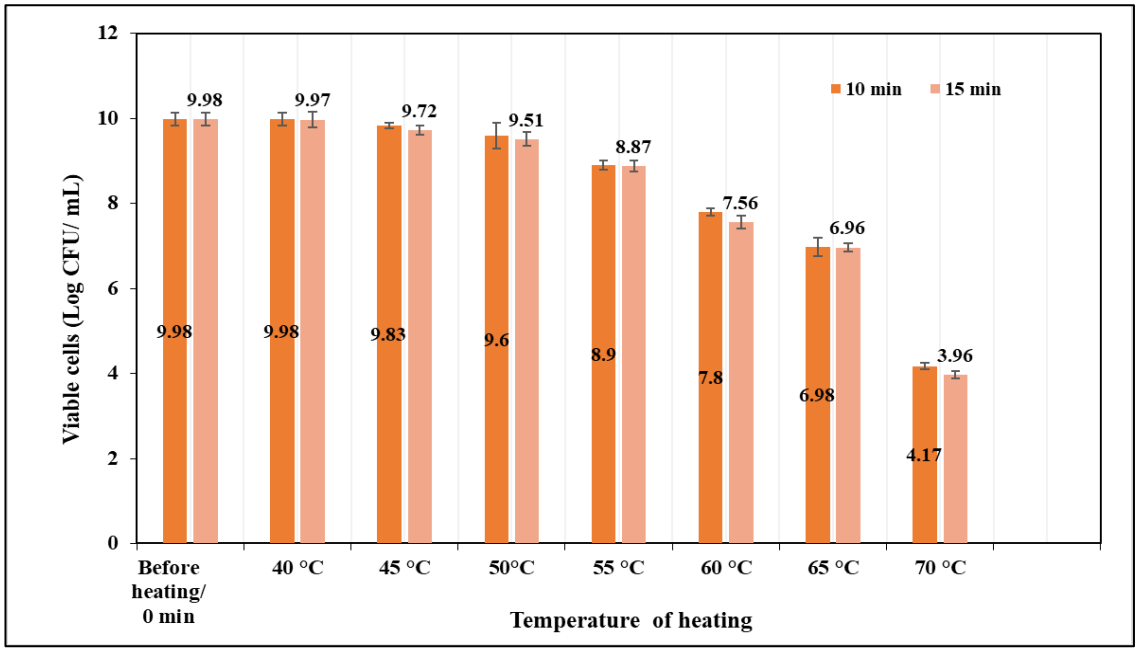


Figure 20 B: Viable cell count of *B. licheniformis* in GC3 formulation.

Thus, GC3 food matrix proved to be an effective carrier as well as a protectant in influencing the survivability of the probiotic cells. High temperature leads to protein denaturation in bacterial cells. Cell membranes and nucleic acids have also been recognized as targets of heat-induced cellular damage (Zanjani et al., 2014). Arenas et al. (2020) studied the survival of free and encapsulated cells of *Bifidobacterium* BB-12 upon exposure to heat treatment up to 70 °C. Greater loss in cell viability was noted in free cells as compared to encapsulated cells.

With the continuous growth of the global population, the demand for animal-derived proteins has significantly increased. However, the rising cost of these protein sources, coupled with environmental concerns and resource limitations, highlights the need for sustainable, affordable alternatives(Jahn et al., 2023). Climate-resilient legume crops, such as cowpea (Vigna unguiculata), offer promising solutions to address food security challenges and improve nutritional accessibility, particularly in developing regions (Abebe & Alemayehu et al., 2022).

As a multipurpose legume, cowpea is valued not only for its adaptability to harsh agro-climatic conditions but also for its nutritional richness, including proteins, complex carbohydrates, vitamins, minerals, including a variety of bioactive compounds (Abbas et al., 2018). In cereal-based traditional diets, cowpea and other pulses serve as a critical source of plant-based protein, complementing energy-rich staples and enhancing overall dietary quality.

To optimize the nutritional potential of cowpea cultivars, especially in terms of seed quality, targeted efforts are needed to enhance their nutrient bioavailability while minimizing the impact of anti-nutritional factors(Adebo et al., 2022). Various pre- and post-harvest processing methods have been shown to improve the functional and nutritional properties of legumes. Appropriate processing technologies, including soaking, germination, dehulling, crushing, malting, salt addition, extrusion, cooking, roasting, controlled fermentation, and irradiation, play a vital role in enhancing cowpea's nutritional profile and making it more acceptable for human consumption (Verni et al., 2022). Such methods can significantly reduce phytates, tannins, enzyme inhibitors, and other compounds that impair nutrient absorption(Nkhata et al., 2018). Gamma irradiation has been explored as a method to sterilize cowpea flour and pastes; however, while doses up to 5 kGy effectively control microbes,

higher doses may adversely affect nutrient integrity, for e.g., loss of vitamins and amino acids (Friday, 2020).

Among these techniques, controlled fermentation stands out as a cost-effective, household-level method that improves not only the nutritional quality but also the shelf life and safety of cowpea-based foods (Naik & Kerkar, 2024). Fermentation enhances digestibility by breaking down complex macronutrients into simpler, more bioavailable forms. Moreover, the use of safe and functional microbial strains in fermentation contributes to the degradation of toxic compounds (e.g., cyanogenic glucosides), production of beneficial metabolites and enzymes, and development of probiotic properties (Budhwar et al., 2020)

B. licheniformis, isolated from the Goan saltpans, demonstrated effective fermentation of a Goa cowpea cultivar. This strain exhibited desirable probiotic traits, facilitating the development of synbiotic formulations with potential health benefits, thereby opening new avenues for functional food innovation using cowpea.

SUMMARY
AND
CONCLUSION

Summary

Thesis on **"Assessment of nutritive value of Goa Cowpea 3 (*Vigna unguiculata*) cultivar and its synbiotic application"** explores the potential of GC3 in the development of a synbiotic formulation using a novel saltpan bacterium with probiotic properties.

The present study was conducted in 3 phases.

Phase I:

This phase screened the saltpan isolates and evaluated their probiotic attributes.

a. Thirty-nine of the 46 saltpan isolates screened for their characterisation and probiotic attributes were found to be Gram-positive bacilli with endospores in 25 isolates.
b. Twelve of the 25 isolates assessed for their fermentation potential exhibited a positive reaction for acid production, whereas 4 out of 25 isolates demonstrated acid and gas production. The incubation period varied for each isolate, from 1 to 5 days. All the isolates exhibited different patterns of sugar utilization.
c. Out of the 16 isolates tested qualitatively for the synthesis of extracellular enzymes,13 produced protease, 10 produced lipase, 13 produced amylase,14 produced cellulase, 8 produced phytase, and 9 produced β-galactosidases. Seven isolates produced all the extracellular enzymes.
d. The seven isolates were evaluated for their haemolytic activity. Three isolates- FSK 0.30, FSK 2, and FSK 3 were non-haemolytic. When further tested for their human safety in terms of the presence of endotoxin and enterotoxin production, they proved to be negative for the enterotoxin genes nheA, nheB, hblA, and entFM and endotoxin production.
e. FSK 2 and FSK 3 inhibited all the tested gastrointestinal tract as well as food pathogens (ATCC strains) on testing for antibacterial activity, indicating their antagonistic or inhibitory activity.
f. FSK 3 was susceptible to 9 antibiotics, FSK 2 to 7 antibiotics, and FSK 0.30 to 5 antibiotics. FSK 2 and FSK 3 were moderately susceptible to Penicillin and Trimethoprim. FSK 2 was also moderately susceptible to Kanamycin and Streptomycin. FSK 0.30 was found to be

resistant to most of the antibiotics (6). All the isolates were resistant to Ampicillin and Cephalothin, proving their tolerance to these antibiotics.

g. The 3 isolates could tolerate acid and bile in simulated conditions; however, FSK 2 and FSK 3 proved to be the most suitable. The 3 isolates could survive low pH for 3 h. The viable cell counts of FSK 0.30 decreased at pH 1.2 and 2 after 2 h of incubation, as compared to FSK 2 and FSK 3, indicating poor tolerance to acid. The viable count of FSK 0.30 was not affected at pH 4 and 7. All the isolates exhibited over 90 % survival at 0.5 % bile salt concentration, with FSK 3 and FSK 2 showing the highest survivability of 97.8 % and 96.37 %, respectively, inferring that they possess strong bile tolerance with an ability to withstand bile secretions in the human small intestine and maintain viability during intestinal transit.
h. The isolates were evaluated for bile salt hydrolase (BSH) activity; the plate method demonstrated the ability of FSK 2 and FSK 3 to hydrolyse bile salts due to BSH. This activity helps in regulating the concentration of bile, which indirectly helps maintain cholesterol synthesis in the body. The modified ninhydrin method revealed that FSK 3 yielded the best BSH activity towards glycocholate, followed by taurocholate at pH 6-9, followed by FSK 2.
i. Cell surface hydrophobicity was employed to assess the adhesion potential, which evaluates the capacity of the probiotic strains to adhere to the intestinal surface and subsequent colonization. The hydrophobicity of FSK 0.30, FSK 2, and FSK 3 was 26.5 %, 35.75 % and 40.75 % respectively, indicating their good hydrophobicity, with better results demonstrated by FSK 2 and FSK 3.
j. Presence of exopolysaccharides (EPS) enhances the adherence capacity of probiotics. FSK3 produced the highest EPS (1.42 mg/mL) with the highest mucus adhesion (86.92 ± 0.84 %).
k. A good antioxidant capacity of novel probiotic strains is crucial for enhancing the nutritional value, health benefits, and quality of fermented foods. The percent scavenging activity of FSK 2 and FSK 3 was found to be 60.2 ± 0.6 % and 66.8 ± 0.72 % respectively, with their reducing power being 12.23 ±1.32 µg/mL AAE and 14.78 ±1.56 µg /mL AAE, respectively.
l. FSK 2 and FSK 3 showed similarity to *Bacillus licheniformis* strain ATCC 14580 and DSM 13. Their Gen Bank accession numbers are FSK 2 -- PQ113773 and FSK 3-- PQ098138.
m. Shelf-life studies of *B. licheniformis* PQ098138 showed the cell viability at 4 °C and 28 ± 2 °C for 360 days, inferring their long-term stability. However, a desirable viable cell count

was maintained up to 60 days and 30 days at 4 °C and 28 °C ± 2 °C, respectively. The % survival of *B. licheniformis* stored at 4 °C and 28 ± 2 °C was 46.74 ± 2 % and 37.34 ± 1.62 % respectively, after 360 days, which was low, thus suggesting a cryoprotectant or a suitable food matrix to maintain the viability.

Phase II:

This phase involved evaluating the proximate analysis and selected nutrient analysis of the GC3 cultivar in addition to its physical and cooking properties. The values were compared to those of the Indian Food Composition database (IFCT), 2017, given by ICMR, NIN.

a. The protein (26.16 ± 0.23 g/100g) and fat (2. 33 ± 0.08 g/100g) content of the GC3 cultivar was significantly higher than the IFCT,2017 reported values of cowpea species.
b. The total dietary fiber content in GC3 seeds was determined to be 21.7 ± 1.28 g/100 g, with insoluble dietary fiber comprising the majority at 19.6 ± 1.28 g/100 g, and was significantly higher than the IFCT reference values. Dietary fiber is known to contribute to prebiotic properties.
c. The essential fatty acid, linoleic acid (omega-6) was estimated to be 184.45 ± 9.50 mg/100g, and α-linolenic acid (Omega-3) was 125.67 ± 6.45 mg/100g. Myristoleic acid, γ-linolenic acid (omega-6) (139.98 ± 3.48 mg/100g), and eicosapentaenoic acid were also detected in the GC3 cultivar, which were below the detection limit (BDL) in the cowpea species as per IFCT,2017.
d. The levels of essential amino acids, *viz.,* threonine, lysine, tyrosine, histidine, and arginine contents were comparable to the cowpea values in IFCT, 2017; however, valine, isoleucine, leucine, phenylalanine, and tryptophan contents were significantly lower than the IFCT values of cowpea species. Methionine (3.40 ± 0.14 g/100g) and cysteine content (1.30 ± 0.04) in the GC3 cultivar were significantly higher than IFCT reference values.
e. Vit.K_1 (7.63 ± 0.30 µg/100g) content was significantly higher than the IFCT, 2017 reported value. GC3 showed the presence of zeaxanthin (25.20 ± 2.69 µg/100g**),** which was BDL in cowpea species in IFCT,2017. A significantly higher content of beta carotene (46.08 ± 3.73 µg/100g) and total carotenoids (367.77 ± 7.08 µg/100g) was estimated in the GC3 cultivar.

f. The content of iron (12.49 ± 0.84 mg/100g), calcium (157.91 ± 0.80 mg/100g), zinc (6.62 ± 0.06 mg/100g), potassium (1567.93 ± 25.59 mg/100g) and manganese (2.54 ± 0.35 mg/100g) were significantly higher than IFCT, 2017 values of cowpea species.
g. GC3 was rich in raffinose family oligosaccharides like raffinose (0.22 ± 0.01 g/100g) and verbascose (0.27 ± 0.01g/100g), which are known to contribute to prebiotic properties.
h. GC3 demonstrated very good physical, hydration, cooking, and textural properties, confirming its suitability in food industry applications as an important plant-based nutritious source. The seed size and weight were adequate, with excellent water absorption and water holding capacity, and a short cooking time of soaked seeds.

Phase III:

This phase studied the efficacy of GC3 as a probiotic carrier, evaluated the fermentation ability of *B. licheniformis* PQ098138, and studied its effect on functional properties, nutritional composition, dietary fiber, and phytate content.

a. GC3 proved as an effective substrate in supporting the growth of saltpan isolate *B. licheniformis,* as evidenced by higher cell counts, pH drop, and a significantly high acetic acid accumulation followed by propionic acid within 12 h of controlled fermentation. Total titrable acidity peaked at 12 h.
b. A significant reduction in viscosity was observed in GC3 slurry with increased fermentation time from 580 ± 13 mPa.s (0 h) to 490 ± 12 mPa.s after 12 h.
c. Soluble protein content in the GC3 slurry significantly increased till 16 h of fermentation to 15.23 ± 0.12 g/100g but showed a reduction after 24 h of fermentation. A continuous increase in free amino nitrogen content was observed with a significant increase from 0.19 ± 0.01 g/L to 0.57 ± 0.02 g/L after 12 h of fermentation.
d. The levels of total carbohydrates decreased with fermentation time, with a significant reduction after 24 h to 24 ± 0.3 mg/mL, whereas the levels of total reducing sugar were higher upto 12 h of fermentation and thereafter significantly reduced.
e. Raffinose, verbascose, and stachyose, were present in GC3 in considerably large amounts, which generally are regarded to cause flatulence but also act as functional prebiotics.

Fermentation led to a significant reduction in total RFOs from 1.41 ± 0.08 g/100 g to 1.05 ± 0.01 g/100 g, with more reduction in verbascose content.

f. A synbiotic formulation was developed by lyophilising the fermented GC3 slurry. The % survival of *B. licheniformis* in the lyophilized synbiotic formulation was significantly higher than (88.38 ± 0.16 %) the bacillus cell suspension alone (50.37 ± 0.13 %), proving the protective effect of GC3 matrix in maintaining cell viability.
g. A viable cell count above the minimum recommended level of > 7 log CFU/ mL remained throughout 9 months and 7 months in the lyophilized product stored at 4 °C and 28 ± 2 °C, respectively.
h. Fermentation significantly decreased the crude total protein content of GC3; however, the process led to the release of 10 free amino acids, inclusive of most of the essential amino acids and low molecular weight peptides as confirmed by SDS-PAGE, thereby enhancing their accessibility. The total dietary fiber content significantly reduced from 21.7 ± 1.28 g/100 g to 18.45 ± 1.77 g/100 g, and the insoluble fiber content to 15.05 ± 1.71 g/100 g on fermentation.
i. Vitamins B2 and B3 content significantly increased on fermentation, with a nonsignificant increase in Vit.B9, whereas it led to a significant reduction in total carotenoid content.
j. An increase in iron, calcium, phosphorus, and zinc was seen after fermentation. The levels of phytate showed a non-significant reduction from 326.2 ± 6.6 mg/100 g to 318.19 ± 5.9 mg /100g.
k. *B. licheniformis* PQ098138 displayed reduced viability in simulated gastric juice at lower pH (1.2- 2.0) than at pH 3.0 - 4.0 in free as well as in GC3 matrix. However, the viability was significantly better in the GC3 matrix.
l. *B. licheniformis,* a potential halotolerant probiotic, could tolerate bile salts. GC3 matrix retained bacillus viability in simulated small intestinal conditions, both in the presence and absence of bile salts at pH 8.0, with a significant increase in viability after 4 h exposure to bile salts when in the food matrix. The synbiotic formulation exhibited significantly higher mean values than the free-cell suspension at all pH levels, 0.5 % bile salts, and time intervals in simulated gastrointestinal conditions.
m. Study findings indicate that incorporation of a potential probiotic into the GC3 food matrix markedly improved its resistance to acid stress and prolonged incubation, reflecting enhanced viability and stability during simulated gastrointestinal conditions. The GC3 food matrix also effectively protected the cell viability during exposure to high temperature.

n. The antibiotic susceptibility of *B. licheniformis* remained unaltered upon fermentation. Fermented GC3 formulation exhibited significantly higher DPPH scavenging activity (66.48 ± 0.32 %) and reducing power than the unfermented GC3. Moreover, fermented GC3 extract at concentrations of 50, 75, and 100 µL inhibited *S. enterica*, *E. coli*, and both *S. typhimurium* and *S. aureus*, respectively.

o. The unfermented GC3 powder displayed significantly higher emulsification capacity, emulsion stability, and foaming capacity. Further heating resulted in significantly higher emulsion stability of both unfermented and fermented formulations.

p. Hence, we have developed a tailored synbiotic formulation by integrating a nutrient-rich Goa Cowpea Cultivar 3, fermented with *B. licheniformis,* a salt-pan isolate, thereby creating a synbiotic food which may have future applications.

SUMMARY OF THE EFFECTIVE SYNBIOTIC FORMULATION	
Probiotic attributes of halotolerant *Bacillus licheniformis* PQ098138 ➢ Tolerant to acid and bile, which implies positive gastrointestinal colonization ➢ Antimicrobial activity against food and gut pathogens ➢ The EPS producer, thus, enhanced adhesion ➢ Adhered to mucus, thus likely to colonize the gut ➢ Positive bile salt hydrolase activity infers more substrate specificity to glycoconjugates ➢ Non-hemolytic; absence of enterotoxin genes; negative for endotoxins: confirms safety ➢ Susceptible to most of the antibiotics, less likely to transfer antibiotic resistance genes ➢ Remained viable for 12 months, with a desirable count for 9 months at refrigeration	
SUMMARY OF THE EFFECTIVE SYNBIOTIC FORMULATION	
Proximate analysis (g/100g)	
Moisture	10.08 ± 0.21
Ash	4.27 ± 0.07
Protein	22.88 ± 0.15
Fat	2.08 ± 0.03
Insoluble dietary fiber	15.05 ± 1.71
Soluble dietary fiber	3.40 ± 0.09
Total dietary fiber	18.45 ± 1.77
Carbohydrate	41.94 ± 0.32

SUMMARY OF THE EFFECTIVE SYNBIOTIC FORMULATION	
Nutritional composition	
Vitamins	
Vit.B2 (mg/100g)	0.11 ± 0.01
Vit. B3(mg/100g)	0.75 ± 0.02
Vit. B5(mg/100g)	0.23 ± 0.01
Vit. B6(µg/100g)	0.30 ± 0.01
Vit. B9(µg/100g)	10.45 ± 1.04
Vit. K1 (µg/100g)	5.49 ± 0.24
Lutein (µg/100g)	15.27 ± 1.39
Beta carotene (µg/100g)	12.36 ± 0.75
Total carotenoids (µg/100g)	156.48 ± 2.41
Free amino acids (mg/g)	
Threonine	0.01 ± 0.001
Valine	0.01 ± 0.001
Isoleucine	0.02 ± 0.003
Lysine	0.03 ± 0.001
Phenylalanine	0.02 ± 0.001
Tyrosine	0.01 ± 0.001
Methionine	0.03 ± 0.001
Tryptophan	0.02 ± 0.002
Arginine	0.03 ± 0.001
Aspartic acid	0.07 ± 0.001
Glutamic acid	0.39 ± 0.04
Serine	0.03 ± 0.003
Alanine	0.03 ± 0.002
Asparagine	0.02 ± 0.001
low-molecular-weight peptides:	Present
Acetic acid and propionic acid	19.13 ± 0.01 and 0.012 ± 0.003 g/L
DPPH radical scavenging activity	66.48 ± 0.32 %
Reducing power	82.23 ± 1.16 µg/mL AAE
Minerals (mg/100g)	
Iron	0.02 ± 0.003
Zinc	7.76 ± 0.98

Calcium	161.54 ± 1.58
Phosphorus	282.46 ± 0.25
Copper	0.68 ± 0.03
Potassium(K)	1556.39 ± 261.31
Magnesium (Mg)	103.97 ± 24.27
Manganese (Mn)	2.39 ± 0.48
Raffinose family oligosaccharide (RFO)	
Raffinose	0.32 ± 0.01
Stachyose	0.67 ± 0.01
Verbascose	0.05 ± 0.01
Total RFO	1.05 ± 0.01

The study findings indicate the promising *in vitro* attributes of the *Bacillus* strain from the Goan saltpan, fulfilling of a probiotic. The strain demonstrated antibacterial effects across a wide range of common food and intestinal pathogens, with a negative haemolytic and enterotoxin reaction, establishing its safety. Overall, the strain demonstrated antioxidant activity and was able to survive under simulated bile salt and acidic conditions. These probiotic properties were of paramount importance for their application in cowpea fermentation. *B. licheniformis* PQ098138 was thus used as a starter culture to initiate a controlled fermentation process and transform the cowpea cultivar into a “synbiotic food " that can offer health benefits beyond its basic nutrition.

Conclusion

The Goa cowpea 3 (GC3) cultivar is a nutritionally rich legume. *Bacillus licheniformis* PQ098138, isolated from a Goan saltpan, exhibited promising probiotic characteristics. This strain effectively served as a starter culture for the fermentation of GC3, improving its nutritional quality and thereby elevating GC3 to being a functional food, suggesting possible health-promoting effects for consumers. The GC3 seed powder demonstrated its suitability as a matrix in the development of a synbiotic formulation by supporting the growth of *B. licheniformis* PQ098138. The fermented cowpea product thus holds promise as a functional food and establishes the potential of the salt pan isolate as a probiont.

Bibliography

- AACC International. (2000). *Approved methods of the AACC*. AACC International Press.
- Abbas, Y., & Ahmad, A. (2018). Impact of processing on nutritional and antinutritional factors of legumes: A review. *Annals: Food Science & Technology, 19*(2), 200–210.
- Abdel-Aal, E. S. M. (2016). Processing pulses to enhance bioactive and anti-nutritional attributes: A mini-review. *Journal of Food Processing & Technology, 7*(5), 1–5.
- Abdulmawjood, A., Herrmann, J., Riede, S., Jimenez, G., Becker, A., & Breves, G. (2019). Evaluation of enterotoxin gene expression and enterotoxin production capacity of the probiotic strain *Bacillus toyonensis* BCT-7112T. *PLoS ONE, 14*(4), e0214536. https://doi.org/10.1371/journal.pone.0214536
- Abebe, B. K., & Alemayehu, M. T. (2022). A review of the nutritional use of cowpea (*Vigna unguiculata* L. Walp) for human and animal diets. *Journal of Agriculture and Food Research, 10*, 100383. https://doi.org/10.1016/j.jafr.2022.100383
- Adebo, J. A., Njobeh, P. B., Gbashi, S., Oyedeji, A. B., Ogundele, O. M., Oyeyinka, S. A., & Adebo, O. A. (2022). Fermentation of cereals and legumes: Impact on nutritional constituents and nutrient bioavailability. *Fermentation, 8*(2), 63. https://doi.org/10.3390/fermentation8020063
- Adebowale, O. J., & Maliki, K. (2011). Effect of fermentation period on the chemical composition and functional properties of pigeon pea (*Cajanus cajan*) seed flour. *International Food Research Journal, 18*(4), 1329–1333.
- Aduol, K. O., Onyango, A. N., & Imathiu, S. M. (2020). Proximate, microbial and sensory characteristics of cowpea milk fermented with probiotic starter cultures. *European Journal of Agriculture and Food Sciences, 2*(4). https://doi.org/10.24018/ejfood.2020.2.4.115
- Affrifah, N. S., Phillips, R. D., & Saalia, F. K. (2022). Cowpeas: Nutritional profile, processing methods and products—A review. *Legume Science, 4*(3), e131. https://doi.org/10.1002/leg3.131
- Akanni, G. B., De Kock, H. L., Naudé, Y., & Buys, E. M. (2018). Volatile compounds produced by *Bacillus* species alkaline fermentation of bambara groundnut (*Vigna subterranea* (L.) Verdc.) into a dawadawa-type African food condiment using headspace

solid-phase microextraction and GC×GC–TOFMS. *International Journal of Food Properties, 21*(1), 930–942. https://doi.org/10.1080/10942912.2018.1458602

- Akin, Z., & Ozcan, T. (2017). Functional properties of fermented milk produced with plant proteins. *LWT - Food Science and Technology, 86*, 25–30.
- Akweya, B. A., Mghalu, J. M., Yusuf, R. U. M., & Bitange, T. (2020). Probiotic potential of lactic acid bacteria isolated from traditionally fermented legume products of Western Kenya. *African Journal of Food Science, 14*(10), 373–377.
- Ali, K., Mehmood, M. H., Iqbal, M. A., Masud, T., Qazalbash, M., Saleem, S., … Shehzad, M. N. (2019). Isolation and characterization of exopolysaccharide-producing strains of *Lactobacillus bulgaricus* from curd. *Food Science & Nutrition, 7*(4), 1207–1213.
- Ali, M. W., Shahzad, R., Bilal, S., Adhikari, B., Kim, I. D., Lee, J. D., … Shin, D. H. (2018). Comparison of antioxidant potential, metabolites, and nutritional profiles of Korean fermented soybean (Cheonggukjang) with *Bacillus subtilis* KCTC 13241. *Journal of Food Science and Technology, 55*, 2871–2880.
- Ali, N. A., & Hussain, A. S. (2015). Detection of enterotoxin (hblA, hblC and hblD) genes of *Bacillus cereus* isolates from different food samples using traditional and molecular methods. *Iraqi Journal of Biotechnology, 14*(2).
- Amritha, G. K., & Venkateswaran, G. (2018). Use of lactobacilli in cereal-legume fermentation and as potential probiotics towards phytate hydrolysis. *Probiotics and Antimicrobial Proteins, 10*, 647–653.
- Amrutha, U., Sharon, C., Thomachan, P. S., Lakshmy, P., & Beena, A. (2023). Process optimization and evaluation of quality of the barnyard millet incorporated synbiotic yoghurt. *Journal of Research ANGRAU, 51*(3), 107–116.
- Anaemene, D., & Fadupin, G. (2022). Anti-nutrient reduction and nutrient retention capacity of fermentation, germination and combined germination-fermentation in legume processing. *Applied Food Research, 2*(1), 100059.
- Antova, G. A., Stoilova, T. D., & Ivanova, M. M. (2014). Proximate and lipid composition of cowpea (*Vigna unguiculata* L.) cultivated in Bulgaria. *Journal of Food Composition and Analysis, 33*(2), 146–152.
- Anyango, J. O., De Kock, H. L., & Taylor, J. R. N. (2011). Impact of cowpea addition on protein digestibility corrected amino acid score and other protein quality parameters of

traditional African foods made from non-tannin and tannin sorghum. *Food Chemistry, 124*(3), 775–780.

- AOAC. (2010). *Official methods of analysis of the Association of Official Analytical Chemists* (18th ed.). AOAC.
- AOAC. (2000). *Official methods of analysis of the Association of Official Analytical Chemists* (14th ed.). AOAC.
- Appiah, F., Asibuo, J. Y., & Kumah, P. (2011). Physicochemical and functional properties of bean flours of three cowpea (*Vigna unguiculata* L. Walp) varieties in Ghana. *African Journal of Food Science, 5*(2), 100–104.
- Arbab Sakandar, H., Chen, Y., Peng, C., Chen, X., Imran, M., & Zhang, H. (2023). Impact of fermentation on antinutritional factors and protein degradation of legume seeds: A review. *Food Reviews International, 39*(3), 1227–1249. https://doi.org/10.1080/87559129.2021.1931300
- Arena, M. P., Caggianiello, G., Fiocco, D., Russo, P., Torelli, M., Spano, G., & Capozzi, V.(2014). Barley β-glucans-containing food enhances probiotic performances of beneficial bacteria. *International Journal of Molecular Sciences, 15*(2), 3025–3039.
- Arenas-Jal, M., Suñé-Negre, J. M., & García-Montoya, E. (2020). An overview of microencapsulation in the food industry: Opportunities, challenges, and innovations. *European Food Research and Technology, 246*(7), 1371–1382.
- Arora, M., & Baldi, A. (2017). Comparative study of regulatory framework for probiotics: Current status and future recommendations. *Applied Clinical Research, Clinical Trials and Regulatory Affairs, 4*(2), 140–156.
- Asgher, M., Urooj, Y., Qamar, S. A., & Khalid, N. (2020). Improved exopolysaccharide production from *Bacillus licheniformis* MS3: Optimization and structural/functional characterization. *International Journal of Biological Macromolecules, 151*, 984–992.
- Ashaolu, T. J. (2020). Immune boosting functional foods and their mechanisms: A critical evaluation of probiotics and prebiotics. *Biomedicine & Pharmacotherapy, 130*, 110625.
- Ashaolu, T. J., Ashaolu, J. O., & Adeyeye, S. A. (2021). Fermentation of prebiotics by human colonic microbiota in vitro and short-chain fatty acids production: A critical review. *Journal of Applied Microbiology, 130*(3), 677–687.

- Athavale, A., Jirankalgikar, N., Nariya, P., & De, S. (2012). Evaluation of in vitro antioxidant activity of panchagavya: A traditional ayurvedic preparation. *International Journal of Pharmaceutical Sciences and Research, 3*(8), 2543.
- Awika, J. M., & Duodu, K. G. (2017). Bioactive polyphenols and peptides in cowpea (*Vigna unguiculata*) and their health-promoting properties: A review. *Journal of Functional Foods, 38*, 686–697
- Awolu, O. O., Olarewaju, O. A., & Akinade, A. O. (2017). Effect of the addition of pearl millet flour subjected to different processing on the antioxidants, nutritional, pasting characteristics and cookies quality of rice-based composite flour. *Journal of Nutritional Health & Food Engineering, 7*(2), 00232. https://doi.org/10.15406/jnhfe.2017.07.00232
- Aziz, T., Xingyu, H., Sarwar, A., Naveed, M., Shabbir, M. A., Khan, A. A., … Jalal, R. S. (2023). Assessing the probiotic potential, antioxidant, and antibacterial activities of oat and soy milk fermented with *Lactiplantibacillus plantarum* strains isolated from Tibetan kefir. *Frontiers in Microbiology, 14*, 1265188.
- Bagga, D., Reichert, J. L., Koschutnig, K., Aigner, C. S., Holzer, P., Koskinen, K., … Schöpf, V. (2018). Probiotics drive gut microbiome triggering emotional brain signatures. *Gut Microbes, 9*(6), 486–496.
- Bai, Z., Huang, X., Meng, J., Kan, L., & Nie, S. (2020). A comparative study on nutritive peculiarities of 24 Chinese cowpea cultivars. *Food and Chemical Toxicology, 146*, 111841.
- Ballav, S., Kerkar, S., Thomas, S., & Augustine, N. (2015). Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing antibacterial metabolites. *Journal of Bioscience and Bioengineering, 119*(3), 323–330.
- Banerjee, G., Nandi, A., & Ray, A. K. (2017). Assessment of hemolytic activity, enzyme production and bacteriocin characterization of *Bacillus subtilis* LR1 isolated from the gastrointestinal tract of fish. *Archives of Microbiology, 199*, 115–124.
- Bangar, S. P., Suri, S., Trif, M., & Ozogul, F. (2022). Organic acids production from lactic acid bacteria: A preservation approach. *Food Bioscience, 46*, 101615.
- Banwo, K., Olojede, A. O., Adesulu-Dahunsi, A. T., Verma, D. K., Thakur, M., Tripathy, S., … Utama, G. L. (2021). Functional importance of bioactive compounds of foods with potential health benefits: A review on recent trends. *Food Bioscience, 43*, 101320.
- Barat, A., & Ozcan, T. (2018). Growth of probiotic bacteria and characteristics of fermented milk containing fruit matrices. *International Journal of Dairy Technology, 71*, 120–129.

- Bascuñán, K. A., Araya, M., Roncoroni, L., Doneda, L., & Elli, L. (2020). Dietary gluten as a conditioning factor of the gut microbiota in celiac disease. *Advances in Nutrition, 11*(1), 160–174.
- Begley, M., Hill, C., & Gahan, C. G. (2006). Bile salt hydrolase activity in probiotics. *Applied and Environmental Microbiology, 72*(3), 1729–1738.
- Behera, S. S., Ray, R. C., & Zdolec, N. (2018). *Lactobacillus plantarum* with functional properties: An approach to increase safety and shelf-life of fermented foods. *BioMed Research International, 2018*, 9361614.
- Bell, V., Ferrão, J., Pimentel, L., Pintado, M., & Fernandes, T. (2018). One health, fermented foods, and gut microbiota. *Foods, 7*(12), 195.
- Berebon, D. P., Ofokansi, K. C., Attama, A. A., Eze, C. O., Onwusoba, R. C., & Ugwoke, I. C. (2019). Preliminary studies on isolation, bile tolerance and antibiogram of potential probiotics from locally fermented food products at Beach Market, Nsukka Metropolis, Enugu State, Nigeria. *Biotechnology Journal International, 22*(3), 1–10.
- Bezkorovainy, A. (2001). Probiotics: Determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition, 73*(2), 399S–405S.
- Bhardwaj, R., Singh, D. P., Rani, U., Tiwana, U. S., & Bajaj, R. K. (2014). Association studies in cowpea (*Vigna unguiculata* L.). *Journal of Food Legumes, 27*(1), 74–76.
- Biermann, R., & Beutel, S. (2023). Endospore production of *Bacillus* spp. for industrial use. *Engineering in Life Sciences, 23*(11), e2300013.
- Bilal, M., Ashraf, S., & Zhao, X. (2022). Dietary component-induced inflammation and its amelioration by prebiotics, probiotics, and synbiotics. *Frontiers in Nutrition, 9*, 931458.
- Bonvehí, J. S., Coll, F. V., & Rius, I. A. (2000). Liquid chromatographic determination of tocopherols and tocotrienols in vegetable oils, formulated preparations, and biscuits. *Journal of AOAC International, 83*(3), 627–634.
- Bora, P. (2014). Anti-nutritional factors in foods and their effects. *Journal of Academia and Industrial Research, 3*(6), 285–290.
- Brashears, M. M., Gilliland, S. E., & Buck, L. M. (1998). Bile salt deconjugation and cholesterol removal from media by *Lactobacillus casei*. *Journal of Dairy Science, 81*(8), 2103–2110
- Brown, L., Caligiuri, S. P., Brown, D., & Pierce, G. N. (2018). Clinical trials using functional foods provide unique challenges. *Journal of Functional Foods, 45*, 233–238.

- Budhwar, S., Sethi, K., & Chakraborty, M. (2020). Efficacy of germination and probiotic fermentation on underutilized cereal and millet grains. *Food Production, Processing and Nutrition, 2*(1), 12.
- Buta, M. B., & Emire, S. A. (2015). Effects of fermentation on the nutritional quality of QPM and soybean blends for the production of weaning food. *Journal of Food Processing & Technology, 6*, 507–517.
- Canesin, M. R., & Cazarin, C. B. B. (2021). Nutritional quality and nutrient bioaccessibility in sourdough bread. *Current Opinion in Food Science, 40*, 81–86.
- Carvalho, A. F. U., de Sousa, N. M., Farias, D. F., da Rocha-Bezerra, L. C. B., da Silva, R. M. P., Viana, M. P., … Freire Filho, F. R. (2012). Nutritional ranking of 30 Brazilian genotypes of cowpeas including determination of antioxidant capacity and vitamins. *Journal of Food Composition and Analysis, 26*(1–2), 81–88.
- Champagne, C. P., & Gardner, N. J. (2008). Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses. *Food Research International, 41*(5), 539–543.
- Chandra, S., Singh, S., & Kumari, D. (2015). Evaluation of functional properties of composite flours and sensorial attributes of composite flour biscuits. *Journal of Food Science and Technology, 52*(6), 3681–3688.
- Charteris, W. P., Kelly, P. M., Morelli, L., & Collins, J. K. (1998). Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *Journal of Food Protection, 61*(12), 1636–1643.
- Chevallet, M., Lescuyer, P., Diemer, H., Van Dorsselaer, A., Leize-Wagner, E., & Rabilloud, T. (2006). Alterations of the mitochondrial proteome caused by the absence of mitochondrial DNA: A proteomic view. *Electrophoresis, 27*(8), 1574–1583.
- Chua, J. Y., Koh, M. K. P., & Liu, S. Q. (2025). Gamma-aminobutyric acid: A bioactive compound in foods. In *Sprouted grains* (pp. 237–284).
- Chyne, D. A. L., Ananthan, R., & Longvah, T. (2019). Food compositional analysis of indigenous foods consumed by the Khasi of Meghalaya, North-East India. *Journal of Food Composition and Analysis, 77*, 91–100.
- Claus, D. (1986). Genus *Bacillus* Cohn 1872. In *Bergey's manual of systematic bacteriology* (Vol. 2, pp. 1105–1139).

- Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C., & Schmitt, P. (2004). Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating the human stomach. *Journal of Applied Microbiology, 97*(1), 214–219.
- Clemente, A., & Olias, R. (2017). Beneficial effects of legumes in gut health. *Current Opinion in Food Science, 14*, 32–36. https://doi.org/10.1016/j.cofs.2017.01.005
- Coda, R., Varis, J., Verni, M., Rizzello, C. G., & Katina, K. (2017). Improvement of the protein quality of wheat bread through faba bean sourdough addition. *LWT – Food Science and Technology, 82*, 296–302.
- Coffmann, C. W., & Garcia, V. V. (1977). Functional properties and amino acid content of a protein isolate from mung bean flour. *International Journal of Food Science & Technology, 12*(5), 473–484.
- Coico, R. (2006). Gram staining. *Current Protocols in Microbiology, 1*, A.3C.
- Collado, M. C., Meriluoto, J., & Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology, 226*(5), 1065–1073. https://doi.org/10.1007/S00217-007-0632-X
- Collins, C. H., & Lyne, P. M. (1984). *Microbiological methods* (5th ed.). Butterworths.
- Crowther, T. W., Rappuoli, R., Corinaldesi, C., Danovaro, R., Donohue, T. J., Huisman, J., … van Galen, L. G. (2024). Scientists' call to action: Microbes, planetary health, and the Sustainable Development Goals. *Cell, 187*(19), 5195–5216.
- Cuvas-Limon, R. B., Nobre, C., Cruz, M., Rodriguez-Jasso, R. M., Ruíz, H. A., Loredo-Treviño, A., … Belmares, R. (2021). Spontaneously fermented traditional beverages as a source of bioactive compounds: An overview. *Critical Reviews in Food Science and Nutrition, 61*(18), 2984–3006.
- Da Silva, A. C., da Costa Santos, D., Junior, D. L. T., da Silva, P. B., dos Santos, R. C., & Siviero, A. (2018). Cowpea: A strategic legume species for food security and health. In *Legume seed nutraceutical research*. IntechOpen.
- Dahiya, D., & Nigam, P. S. (2022). Probiotics, prebiotics, synbiotics, and fermented foods as potential biotics in nutrition improving health via microbiome–gut–brain axis. *Fermentation, 8*(7), 303.
- Dai, C., Ma, H., He, R., Huang, L., Zhu, S., Ding, Q., & Luo, L. (2017). Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with *Bacillus subtilis*. *LWT, 86*, 1–7.

- Dai, C., Hou, Y., Xu, H., Huang, L., Dabbour, M., Mintah, B. K., … Ma, H. (2022). Effect of solid-state fermentation by three different *Bacillus* species on composition and protein structure of soybean meal. *Journal of the Science of Food and Agriculture, 102*(2), 557–566.
- Danilova, I., & Sharipova, M. (2020). The practical potential of bacilli and their enzymes for industrial production. *Frontiers in Microbiology, 11*, 1782.
- Das, A. P., Kumar, P. S., & Swain, S. (2014). Recent advances in biosensor-based endotoxin detection. *Biosensors and Bioelectronics, 51*, 62–75.
- Das, D., Kalra, I., Mani, K., Salgaonkar, B. B., & Braganca, J. M. (2019). Characterization of extremely halophilic archaeal isolates from Indian salt pans and their screening for production of hydrolytic enzymes. *Environmental Sustainability, 2*, 227–239.
- Das, S., Surendran, P. K., & Thampuran, N. K. (2009). PCR-based detection of enterotoxigenic isolates of *Bacillus cereus* from tropical seafood. *Indian Journal of Medical Research, 129*(3), 316–320.
- Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., … Ghasemi, Y. (2019). Prebiotics: Definition, types, sources, mechanisms, and clinical applications. *Foods, 8*(3), 92.
- Davis, C. (2014). Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *Journal of Microbiological Methods, 103*, 9–17.
- De Brouwer, V., Storozhenko, S., Van De Steene, J. C., Wille, S. M., Stove, C. P., Van Der Straeten, D., & Lambert, W. E. (2008). Optimization and validation of a liquid chromatography–tandem mass spectrometry method for folates in rice. *Journal of Chromatography A, 1215*(1–2), 125–132.
- Delgado-García, M., Aguilar, C. N., Contreras-Esquivel, J. C., & Rodríguez-Herrera, R. (2014). Screening for extracellular hydrolytic enzymes production by different halophilic bacteria. *Mycopath, 12*(1), 17–23.
- Dev, S. S., Nisha, E. A., & Venu, A. (2016). Biochemical and molecular characterization of efficient phytase-producing bacterial isolates from soil samples. *International Journal of Current Microbiology and Applied Sciences, 5*(5), 218–226.

- Devi, C. B., Kushwaha, A., & Kumar, A. (2015). Sprouting characteristics and associated changes in nutritional composition of cowpea (*Vigna unguiculata*). *Journal of Food Science and Technology, 52*(10), 6821–6827.
- Di Stefano, E., White, J., Seney, S., Hekmat, S., McDowell, T., Sumarah, M., & Reid, G. (2017). A novel millet-based probiotic fermented food for the developing world. *Nutrients, 9*(5), 529.
- Diez-Gutiérrez, L., San Vicente, L., Barrón, L. J. R., del Carmen Villarán, M., & Chávarri, M. (2020). Gamma-aminobutyric acid and probiotics: Multiple health benefits and their future in the global functional food and nutraceuticals market. *Journal of Functional Foods, 64*, 103669.
- Dimidi, E., Cox, S. R., Rossi, M., & Whelan, K. (2019). Fermented foods: Definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. *Nutrients, 11*(8), 1806.
- DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.
- Duche, R. T., Singh, A., Wandhare, A. G., Sangwan, V., Sihag, M. K., Nwagu, T. N., … Ezeogu, L. I. (2023). Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. *BMC Microbiology, 23*(1), 142.
- Dueñas, M., Fernández, D., Hernandez, T., Estrella, I., & Muñoz, R. (2005). Bioactive phenolic compounds of cowpeas (*Vigna sinensis* L.): Modifications by fermentation with natural microflora and with *Lactobacillus plantarum* ATCC 14917. *Journal of the Science of Food and Agriculture, 85*(2), 297–304.
- Dullah, E. C., & Ongkudon, C. M. (2017). Current trends in endotoxin detection and analysis of endotoxin–protein interactions. *Critical Reviews in Biotechnology, 37*(2), 251–261.
- Eashwarage, I. S., Herath, H. M. T., & Gunathilake, K. G. T. (2017). Dietary fibre, resistant starch, and eleven commonly consumed legumes: Horse gram. *Research Journal of Chemistry, 7*, 1–7.
- Ehling-Schulz, M., Lereclus, D., & Koehler, T. M. (2019). The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. *Microbiology Spectrum, 7*(3), 10–1128.

- Elango, D., Rajendran, K., Van der Laan, L., Sebastiar, S., Raigne, J., Thaiparambil, N. A., … Singh, A. K. (2022). Raffinose family oligosaccharides: Friend or foe for human and plant health? *Frontiers in Plant Science, 13*, 829118.
- Elshaghabee, F. M., Rokana, N., Gulhane, R. D., Sharma, C., & Panwar, H. (2017). *Bacillus* as potential probiotics: Status, concerns, and future perspectives. *Frontiers in Microbiology, 8*, 1490.
- Ezeonu, C. S., Umaru, I. J., Sindama, A., & Onwurah, I. N. E. (2016). Estimation of total carbohydrate and sugar contents of fungi-treated rice husks. *FUW Trends in Science & Technology Journal, 1*(1), 45–49.
- Fabbri, A. D., & Crosby, G. A. (2016). A review of the impact of preparation and cooking on the nutritional quality of vegetables and legumes. *International Journal of Gastronomy and Food Science, 3*, 2–11.
- Fan, P., Li, L., Rezaei, A., Eslamfam, S., Che, D., & Ma, X. (2015). Metabolites of dietary protein and peptides by intestinal microbes and their impacts on gut. *Current Protein and Peptide Science, 16*(7), 646–654.
- Feng, K., Zhai, M. Y., Zhang, Y., Linhardt, R. J., Zong, M. H., Li, L., & Wu, H. (2018). Improved viability and thermal stability of probiotics encapsulated in a novel electrospun fiber mat. *Journal of Agricultural and Food Chemistry, 66*(41), 10890–10897.
- Fernandes, S., Kerkar, S., Leitão, J., & Mishra, A. (2019). Probiotic role of salt pan bacteria in enhancing the growth of whiteleg shrimp (*Litopenaeus vannamei*). *Probiotics and Antimicrobial Proteins, 11*, 1309–1323.
- Fernandes, C. G., Sonawane, S. K., & Arya, S. S. (2018). Cereal-based functional beverages: A review. *Journal of Microbiology, Biotechnology and Food Sciences, 8*(3).
- Fiske, C. H., & Subbarow, Y. (1925). The colorimetric determination of phosphorus. *Journal of Biological Chemistry, 66*(2), 375–400.
- Fontana, L., Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., & Gil, Á. (2013). Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition, 109*(S2), S35–S50.
- Forsido, S. F., Hordofa, A. A., Ayelign, A., Belachew, T., & Hensel, O. (2020). Effects of fermentation and malt addition on physicochemical properties of cereal-based complementary foods in Ethiopia. *Heliyon, 6*(7), e04606.

- Friday, D. (2020). Effect of gamma irradiation in food biopolymers. *Journal of Nutraceuticals and Food Science, 5*(4), 9.
- Funck, G. D., Marques, J. D. L., Cruxen, C. E. D. S., Sehn, C. P., Haubert, L., Dannenberg, G. D. S., & Fiorentini, Â. M. (2019). Probiotic potential of *Lactobacillus curvatus* P99 and viability in fermented oat dairy beverage. *Journal of Food Processing and Preservation, 43*(12), e14286.
- Ganguly, N. K., Bhattacharya, S. K., Sesikeran, B., Nair, G. B., Ramakrishna, B. S., Sachdev, H. P. S., … Hemalatha, R. (2011). ICMR-DBT guidelines for evaluation of probiotics in food. *Indian Journal of Medical Research, 134*(1), 22–25.
- Garrido-Galand, S., Asensio-Grau, A., Calvo-Lerma, J., Heredia, A., & Andrés, A. (2021). The potential of fermentation on nutritional and technological improvement of cereal and legume flours: A review. *Food Research International, 145*, 110398. https://doi.org/10.1016/j.foodres.2021.110398
- Garg, V., Velumani, D., Lin, Y. C., & Haye, A. (2024). A comprehensive review of probiotic claim regulations: Updates from Asia-Pacific, the United States, and Europe. *PharmaNutrition*, 100423.
- Gauthankar, M., Khandeparker, R., Shivaramu, M. S., Salkar, K., Sreepada, R. A., & Paingankar, M. (2021). Comparative assessment of amino acid composition in two types of marine fish silage. *Scientific Reports, 11*(1), 15235.
- Gawas, P., & Kerkar, S. (2023). Bacterial diversity and community structure of salt pans from Goa, India. *Frontiers in Microbiology, 14*, 1230929.
- Georgieva, R., Yocheva, L., Tserovska, L., Zhelezova, G., Stefanova, N., Atanasova, A., … Karaivanova, E. (2015). Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. *Biotechnology & Biotechnological Equipment, 29*(1), 84–91.
- Gharibzahedi, S. M. T., & Smith, B. (2021). Legume proteins as carriers to encapsulate bioactive compounds and probiotic bacteria: A review. *Comprehensive Reviews in Food Science and Food Safety, 20*(2), 1250–1279.
- Ghosh, K., Banerjee, S., Moon, U. M., Khan, H. A., & Dutta, D. (2017). Evaluation of gut-associated extracellular enzyme-producing and pathogen-inhibitory microbial communities as probiotics in Nile tilapia (*Oreochromis niloticus*). *International Journal of Aquaculture, 7*.

- Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology, 14*(8), 491–502. https://doi.org/10.1038/nrgastro.2017.75
- Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews, 17*(2), 259–275.
- Gobbetti, M., De Angelis, M., Di Cagno, R., Calasso, M., Archetti, G., & Rizzello, C. G. (2019). Novel insights on the functional and nutritional features of sourdough fermentation. *International Journal of Food Microbiology, 302*, 103–113.
- Golnari, M., Bahrami, N., Milanian, Z., Rabbani Khorasgani, M., Asadollahi, M. A., Shafiei, R., & Fatemi, S. S. A. (2024). Isolation and characterization of novel *Bacillus* strains with probiotic potential. *Scientific Reports, 14*(1), 1457.
- Gonçalves, A., Goufo, P., Barros, A., Domínguez-Perles, R., Trindade, H., Rosa, E., Ferreira, L., & Rodrigues, M. (2016). Cowpea (*Vigna unguiculata* L. Walp): Nutritional advantages and constraints. *Journal of the Science of Food and Agriculture, 96*(9), 2941–2951. https://doi.org/10.1002/jsfa.7644
- Gondwe, T. M., Alamu, E. O., Mdziniso, P., & Maziya-Dixon, B. (2019). Cowpea for food security: Evaluation of improved varieties in Swaziland. *Scientific Reports, 9*(1), 15991.
- Gopikrishna, T., Suresh Kumar, H. K., Perumal, K., & Elangovan, E. (2021). Impact of *Bacillus* in fermented soybean foods on human health. *Annals of Microbiology, 71*, 1–16.
- Goyal, K., Singh, N., Jindal, S., Kaur, R., Goyal, A., & Awasthi, R. (2022). Kjeldahl method. *Advanced Techniques in Analytical Chemistry, 1*, 105.
- Hamid, S., Muzaffar, S., Wani, I. A., Masoodi, F. A., & Bhat, M. M. (2016). Physical and cooking characteristics of cowpea cultivars grown in temperate Indian climate. *Journal of the Saudi Society of Agricultural Sciences, 15*(2), 127–134.
- Hernández-Gómez, J. G., López-Bonilla, A., Trejo-Tapia, G., Ávila-Reyes, S. V., Jiménez-Aparicio, A. R., & Hernández-Sánchez, H. (2021). In vitro bile salt hydrolase activity screening of probiotic microorganisms. *Foods, 10*(3), 674.
- Hill, A., & Stewart, G. (2019). Free amino nitrogen in brewing. *Fermentation, 5*(1), 22. https://doi.org/10.3390/fermentation5010022

- Homsuwan, N., Pruksasri, S., & Ngampanya, B. (2023). *Bacillus siamensis* FVP1 as a probiotic for soybean meal enhancement. *International Journal of Food Science and Technology, 58*(8), 4277–4287.
- Hossain, S., Ahmed, R., Bhowmick, S., Mamun, A. A., & Hashimoto, M. (2016). Proximate composition and fatty acid analysis of *Lablab purpureus*. *SpringerPlus, 5*, 1–10.
- Gottschalk, G. (1986). *Bacterial metabolism* (2nd ed.). Springer. *(Replaces incorrect URL entry)*
- Huang, Z., Liu, H., Zhao, L., He, W., Zhou, X., Chen, H., … Liu, Z. (2022). Effect of processing methods on fermented soybean whey-based tofu. *LWT - Food Science and Technology, 158*, 113139. https://doi.org/10.1016/j.lwt.2022.113139
- Ibrahim, O. O. (2018). Functional oligosaccharides: Structure, benefits, and applications. *Journal of Food Chemistry and Nanotechnology, 4*(4), 65–76.
- ICMR-NIN. (2020). *Recommended dietary allowances and estimated average requirements*. National Institute of Nutrition.
- Jahn, L. J., Rekdal, V. M., & Sommer, M. O. A. (2023). Microbial foods for improving human and planetary health. *Cell, 186*(3), 469–478.
- Jain, N., Mehta, A., & Bharti, V. (2017). Screening, characterization, and in vitro evaluation of probiotic properties of *Lactobacillus* strains. *Asian Journal of Pharmaceutical and Clinical Research, 10*(8), 288–293.
- Jakob, E., & Elmadfa, I. (1996). Determination of vitamin K1 in food items using HPLC. *Food Chemistry, 56*(1), 87–91.
- Jayathilake, C., Visvanathan, R., Deen, A., Bangamuwage, R., Jayawardana, B. C., Nammi, S., & Liyanage, R. (2018). Cowpea: Nutritional facts and health benefits. *Journal of the Science of Food and Agriculture, 98*(13), 4793–4806. https://doi.org/10.1002/jsfa.9074
- Jeon, H. L., Yang, S. J., Son, S. H., Kim, W. S., Lee, N. K., & Paik, H. D. (2018). Evaluation of probiotic *Bacillus subtilis* P229 in soybean fermentation. *LWT, 97*, 94–99.
- Jideani, A. I., Silungwe, H., Takalani, T., Omolola, A. O., Udeh, H. O., & Anyasi, T. A. (2021). Antioxidant-rich natural fruit and vegetable products and human health. *International Journal of Food Properties, 24*, 41–67.

- John, K. M., Enkhtaivan, G., Lee, J., Thiruvengadam, M., Keum, Y. S., & Kim, D. H. (2015). Spectroscopic determination of metabolic and mineral changes of soya chunk mediated by *Aspergillus sojae*. *Food Chemistry, 170*, 1–9.
- Kabeer, S., Govindarajan, N., Radhakrishnan, P., Alharbi, H. F., Essa, M. M., & Qoronfleh, M. W. (2023). Formulation of fortified instant weaning food from *Musa paradisiaca* and *Eleusine coracana*. *Frontiers in Nutrition, 10*, 1203955.
- Kamat, T., & Kerkar, S. (2011). Pharmaceutical potentials of bacteria from saltpans of Goa, India. *International Journal of Pharmaceutical Applications, 2*(1), 150–154.
- Kaplan, H., & Hutkins, R. W. (2000). Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. *Applied and Environmental Microbiology, 66*(6), 2682–2684.
- Kaur Sidhu, M., Lyu, F., Sharkie, T. P., Ajlouni, S., & Ranadheera, C. S. (2020). Probiotic yogurt fortified with chickpea flour: Physicochemical properties and probiotic survival. *Foods, 9*(9), 1144.
- Kaur, S., Kaur, S., Gupta, A. K., Kaur, N., & Javed, M. (2014). Biochemical and nutritional characterization of chickpea (*Cicer arietinum*) cultivars. *Indian Journal of Agricultural Sciences, 84*(4), 479–486. https://doi.org/10.56093/ijas.v84i4.3946
- Kavita, P., Suresh, N., & Babu, V. (2015). Probiotics, prebiotics, and synbiotics: A review. *Journal of Food Science and Technology, 52*(12), 7577–7587.
- Keller, D., Verbruggen, S., Cash, H., Farmer, S., & Venema, K. (2019). Spores of *Bacillus coagulans* GBI-30, 6086 show high germination, survival, and enzyme activity. *Beneficial Microbes, 10*(1), 77–88.
- Keulers, L., Dehghani, A., Knippels, L., Garssen, J., Papadopoulos, N., Folkerts, G., … van Bergenhenegouwen, J. (2022). Probiotics, prebiotics, and synbiotics in air pollution: The gut–lung axis. *Environmental Pollution, 302*, 119066.
- Khalid, I. I., & Elharadallou, S. B. (2013). Functional properties of cowpea and lupin flour and protein isolates. *Journal of Nutrition & Food Sciences, 3*(6), 1–6.
- Khan, M. N., Bashir, S., & Imran, M. (2023). Probiotic characterization of *Bacillus* species from fermented milk product dahi. *Folia Microbiologica, 68*(5), 757–769.
- Kim, H., Kim, J. S., Kim, Y., Jeong, Y., Kim, J. E., Paek, N. S., & Kang, C. H. (2020). Antioxidant and probiotic properties of *Lactobacilli* and *Bifidobacteria*. *Biotechnology and Bioprocess Engineering, 25*(3), 421–430.

- Kim, K. T., Yang, S. J., & Paik, H. D. (2021). Probiotic properties of *Levilactobacillus brevis* KU15147. *Food Science and Biotechnology, 30*, 257–265.
- Kook, S. Y., Chung, E. C., Lee, Y., Lee, D. W., & Kim, S. (2019). Isolation and characterization of novel probiotic strains. *PLoS ONE, 14*(10), e0223913.
- Kolaček, S., Hojsak, I., Berni Canani, R., Guarino, A., Indrio, F., Orel, R., … ESPGHAN Working Group. (2017). Commercial probiotic products: A call for improved quality control. *Journal of Pediatric Gastroenterology and Nutrition, 65*(1), 117–124.
- Korcz, E., & Varga, L. (2021). Exopolysaccharides from lactic acid bacteria: Applications. *Trends in Food Science & Technology, 110*, 375–384.
- Krausova, G., Hyrslova, I., & Hynstova, I. (2019). Evaluation of probiotic adhesion and aggregation. *Fermentation, 5*(4), 100.
- Kumar, D., Lal, M. K., Dutt, S., Raigond, P., Changan, S. S., Tiwari, R. K., … Singh, B. (2022). Functional fermented probiotics from non-dairy products. *Molecular Nutrition & Food Research, 66*(14), 2101059.
- Kumar, N., & Goel, N. (2019). Phenolic acids: Therapeutic applications. *Biotechnology Reports, 24*, e00370.
- Kumar, R., Grover, S., & Batish, V. K. (2012). Bile salt hydrolase activity screening of *Lactobacillus*. *Probiotics and Antimicrobial Proteins, 4*, 162–172.
- Laemmli, U. K. (1970). Cleavage of structural proteins during bacteriophage assembly. *Nature, 227*(5259), 680–685.
- Langa, S., Van Den Bulck, E., Peirotén, A., Gaya, P., Schols, H. A., & Arqués, J. L. (2019). Synbiotic semi-hard cheese development. *LWT – Food Science and Technology, 114*, 108361.
- Leeming, E. R., Johnson, A. J., Spector, T. D., & Le Roy, C. I. (2019). Effect of diet on gut microbiota. *Nutrients, 11*, 2862. https://doi.org/10.3390/nu11122862
- Li, W., & Wang, T. (2021). Solid-state fermentation effects on chickpeas. *International Journal of Food Microbiology, 338*, 108988.
- Li, Z., Zheng, M., Zheng, J., & Gänzle, M. G. (2023). *Bacillus* species in food fermentations. *Current Opinion in Food Science, 50*, 101007.
- Liang, X., Lv, Y., Zhang, Z., Yi, H., Liu, T., Li, R., … Zhang, L. (2020). Intestinal survival of probiotics. *LWT – Food Science and Technology, 124*, 109132.

- Lin, C. H., Wei, Y. T., & Chou, C. C. (2006). Antioxidative activity of soybean koji. *Food Microbiology, 23*(7), 628–633.
- Liu, W., Dun, M., Liu, X., Zhang, G., & Ling, J. (2022). Phenolic content and antioxidant activity of beans. *International Journal of Food Properties, 25*(1), 477–491.
- Liu, J., Chen, J., Li, S., Tian, W., Wu, H., & Han, B. (2021). Metabolites in fermented sufu. *International Journal of Food Properties, 24*(1), 553–563.
- Longvah, T., Ananthan, R., Bhaskarachary, K., & Venkaiah, K. (2017). *Indian food composition tables*. National Institute of Nutrition, ICMR.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.
- Lu, Y., Han, S., Zhang, S., Wang, K., Lv, L., McClements, D. J., … Li, L. (2022). Probiotic exopolysaccharides and mucin adhesion. *Current Research in Food Science, 5*, 581–589.
- Malik, W. A., & Javed, S. (2021). Cellulase characterization from *Bacillus subtilis*. *Frontiers in Bioengineering and Biotechnology, 9*, 800265.
- Malmo, C., Giordano, I., & Mauriello, G. (2021). Microencapsulation effects on probiotic survival. *Foods, 10*(2), 217.
- Mani, K., Salgaonkar, B. B., Das, D., & Bragança, J. M. (2012). Community solar salt production in Goa. *Aquatic Biosystems, 8*(1), 30.
- Manohara, K. K., Morajkar, S., Shanbhag, Y., & Patil, K. (2021). Cowpea genotypes under residual moisture conditions. *Legume Research, 44*(10), 1138–1143.
- Marco, M. L., Sanders, M. E., Gänzle, M., Arrieta, M. C., Cotter, P. D., De Vuyst, L., … Hutkins, R. (2021). ISAPP consensus on fermented foods. *Nature Reviews Gastroenterology & Hepatology, 18*(3), 196–208.
- Marco, M. L., Heeney, D., Binda, S., Cifelli, C. J., Cotter, P. D., Foligné, B., … Hutkins, R. (2017). Health benefits of fermented foods. *Current Opinion in Biotechnology, 44*, 94–102.
- Markowiak, P., & Śliżewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics. *Nutrients, 9*(9), 1021.
- Matemu, A., Nakamura, S., & Katayama, S. (2021). Antioxidative peptides from legumes. *Antioxidants, 10*(2), 316.
- Mathews, S. (2017). Microencapsulation of probiotics. *International Journal of Current Microbiology and Applied Sciences, 6*(4), 2080–2087.

- Mcmurray, C. H., Blanchflower, W. J., & Rice, D. A. (1980). α-Tocopherol determination. *Journal of AOAC, 63*(6), 1258–1261.
- Mejía-Caballero, A., Salas-Villagrán, V. A., Jiménez-Serna, A., & Farrés, A. (2021). Probiotics in cancer therapy. *Journal of Industrial Microbiology and Biotechnology, 48*(9–10), kuab052.
- Merenkova, S., & Zinina, O. (2021). Microbial fermentation of grain raw materials: Prospects for food technology—An analytical review. *Vestnik Yuzhno-Ural'skogo Gosudarstvennogo Universiteta. Seriya: Pishchevyye i Biotekhnologii, 9*(2), 5–15.
- Miller, G. L. (1959). Study of variables. *Analytical Chemistry, 31*(3), 426–428.
- Min, M., Bunt, C. R., Mason, S. L., Bennett, G. N., & Hussain, M. A. (2017). Effect of non-dairy food matrices on the survival of probiotic bacteria during storage. *Microorganisms, 5*(3), 43. https://doi.org/10.3390/microorganisms5030043
- Mohsenin, N. N. (1970). *Physical properties of plant and animal materials: Vol. 1. Structure, physical characteristics and mechanical properties.* Gordon and Breach Science Publishers.
- Monteiro, C. R., do Carmo, M. S., Melo, B. O., Alves, M. S., Dos Santos, C. I., Monteiro, S. G., & Monteiro-Neto, V. (2019). In vitro antimicrobial activity and probiotic potential of *Bifidobacterium* and *Lactobacillus* against species of *Clostridium*. *Nutrients, 11*(2), 448. https://doi.org/10.3390/nu11020448
- Mubaiwa, J., Fogliano, V., Chidewe, C., & Linnemann, A. R. (2017). Hard-to-cook phenomenon in bambara groundnut (*Vigna subterranea* (L.) Verdc.) processing: Options to improve its role in providing food security. *Food Reviews International, 33*(2), 167–194.
- Mukherjee, A., Gómez-Sala, B., O'Connor, E. M., Kenny, J. G., & Cotter, P. D. (2022). Global regulatory frameworks for fermented foods: A review. *Frontiers in Nutrition, 9*, 902642. https://doi.org/10.3389/fnut.2022.902642
- Muralidharan, J., Galiè, S., Hernández-Alonso, P., Bulló, M., & Salas-Salvadó, J. (2019). Plant-based fat, dietary patterns rich in vegetable fat and gut microbiota modulation. *Frontiers in Nutrition, 6*, 157. https://doi.org/10.3389/fnut.2019.00157
- Mysonhimer, A. R., & Holscher, H. D. (2022). Gastrointestinal effects and tolerance of nondigestible carbohydrate consumption. *Advances in Nutrition, 13*(6), 2237–2276. https://doi.org/10.1093/advances/nmac089

- Naik, V., & Kerkar, S. (2024). Fermentation assisted functional foods. In *Futuristic trends in biotechnology* (Vol. 3, Book 6, pp. 11–32). IIP Series. https://doi.org/10.58532/V3BJBT6P1CH2
- N'zi, K. P., Adingra, K. M. D., N'guessan, K. F., Attchelouwa, C. K., & Tano, K. (2021). Effect of spontaneous fermentation time on physicochemical, nutrient, anti-nutrient and microbiological composition of lima bean (*Phaseolus lunatus*) flour. *Journal of Applied Biosciences, 162*(1), 16707–16725.
- Nami, Y., Haghshenas, B., Bakhshayesh, R. V., Jalaly, H. M., Lotfi, H., Eslami, S., & Hejazi, M. A. (2018). Novel autochthonous lactobacilli with probiotic aptitudes as a main starter culture for probiotic fermented milk. *LWT, 98*, 85–93. https://doi.org/10.1016/j.lwt.2018.08.035
- Narayana, K., & Rao, M. N. (1984). Effect of partial proteolysis on the functional properties of winged bean (*Psophocarpus tetragonolobus*) flour. *Journal of Food Science, 49*(3), 944–947.
- Narli, M. B., & Ozcan, T. (2022). Assessment of bifidogenic potential of cowpea (*Vigna unguiculata* (L.) Walp.) extract in in vitro and milk fermentation models. *LWT, 157*, 113071. https://doi.org/10.1016/j.lwt.2022.113071
- Nguyen, P. T., Nguyen, T. T., Bui, D. C., Hong, P. T., Hoang, Q. K., & Nguyen, H. T. (2020). Exopolysaccharide production by lactic acid bacteria: The manipulation of environmental stresses for industrial applications. *AIMS Microbiology, 6*(4), 451–469. https://doi.org/10.3934/microbiol.2020028
- Niro, S., D'Agostino, A., Fratianni, A., Cinquanta, L., & Panfili, G. (2019). Gluten-free alternative grains: Nutritional evaluation and bioactive compounds. *Foods, 8*(6), 208. https://doi.org/10.3390/foods8060208
- Nithya, V., & Halami, P. M. (2013). Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources. *Annals of Microbiology, 63*, 129–137. https://doi.org/10.1007/s13213-012-0453-4
- Nithya, V., Muthukumar, S. P., & Halami, P. M. (2012). Safety assessment of *Bacillus licheniformis* Me1 isolated from milk for probiotic application. *International Journal of Toxicology, 31*(3), 228–237.
- Nkhata, S. G., Ayua, E., Kamau, E. H., & Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of

endogenous enzymes. *Food Science & Nutrition, 6*(8), 2446–2458. https://doi.org/10.1002/fsn3.846

- Nyati, H. (2000). An evaluation of the effect of storage and processing temperatures on the microbiological status of sous vide extended shelf-life products. *Food Control, 11*(6), 471–476.
- Obadina, A. O., Akinola, O. J., Shittu, T. A., & Bakare, H. A. (2013). Effect of natural fermentation on the chemical and nutritional composition of fermented soymilk nono. *Nigerian Food Journal, 31*(2), 91–97.
- Official Methods of Analysis. (2000). *AOAC International* (17th ed., Methods 950.46, 991.36). AOAC International.
- Oghbaei, M., & Prakash, J. (2016). Effect of primary processing of cereals and legumes on its nutritional quality: A comprehensive review. *Cogent Food & Agriculture, 2*(1), 1136015. https://doi.org/10.1080/23311932.2015.1136015
- Oluwatosin, O. B. (1998). Genetic and environmental variability in starch, fatty acids and mineral nutrients composition in cowpea (*Vigna unguiculata* (L.) Walp.). *Journal of the Science of Food and Agriculture, 78*(1), 1–11.
- Omodara, T. R., & Aderibigbe, E. Y. (2018). Microbiological, physicochemical and enzymatic changes in fermented African locust bean (*Parkia biglobosa*) seeds using *Bacillus subtilis* and additives. *Sustainable Food Production, 1*, 22–29.
- Onyenekwe, P. C., Njoku, G. C., & Ameh, D. A. (2000). Effect of cowpea (*Vigna unguiculata*) processing methods on flatus-causing oligosaccharides. *Nutrition Research, 20*(3), 349–358.
- Owusu-Kwarteng, J., Dominic, A., Fortune, A., Atuna, R. A., & Francis, K. A. (2022). Plant-based alkaline fermented foods as sustainable sources of nutrients and health-promoting bioactive compounds. *Frontiers in Sustainable Food Systems, 6*, 1–15. https://doi.org/10.3389/fsufs.2022.
- Oyeyinka, S. A., Kayitesi, E., Adebo, O. A., Oyedeji, A. B., Ogundele, O. M., Obilana, A. O., & Njobeh, P. B. (2021). A review on the physicochemical properties and potential food applications of cowpea (*Vigna unguiculata*) starch. *International Journal of Food Science & Technology, 56*(1), 52–60.

- Ozturk, B., & McClements, D. J. (2016). Progress in natural emulsifiers for utilization in food emulsions. *Current Opinion in Food Science, 7*, 1–6. https://doi.org/10.1016/j.cofs.2015.08.008
- Palanivelu, J., Thanigaivel, S., Vickram, S., Dey, N., Mihaylova, D., & Desseva, I. (2022). Probiotics in functional foods: Survival assessment and approaches for improved viability. *Applied Sciences, 12*(1), 455. https://doi.org/10.3390/app12010455
- Panghal, A., Janghu, S., Virkar, K., Gat, Y., Kumar, V., & Chhikara, N. (2018). Potential non-dairy probiotic products—A healthy approach. *Food Bioscience, 21*, 80–89. https://doi.org/10.1016/j.fbio.2017.12.003
- Patel, A., Lindström, C., Patel, A., Prajapati, J., & Holst, O. (2012). Probiotic properties of exopolysaccharide-producing lactic acid bacteria isolated from vegetables and traditional Indian fermented foods. *International Journal of Fermented Foods, 1*(1), 87–101.
- Patra, F., & Duary, R. K. (2022). Determination and safety aspects of probiotic cultures. In *Probiotics, prebiotics and synbiotics: Technological advancements towards safety and industrial applications* (pp. 122–160).
- Pawar, L., Singh, A., Chouhan, N., Amer, H. A., Likitha, K., Prasanthmadduluri, N., & Soltani, M. (2025). Physiology of spore formation for *Bacillus* probiotic production. In *Bacillus probiotics for sustainable aquaculture* (pp. 64–86). CRC Press.
- Pawaskar, M., Krishnan, K. P., & Kerkar, S. (2022). Salt-pan bacteria as potential plant growth promoters and their antagonistic activity to fungal pathogens of *Capsicum annuum* L. *Current Science, 123*(9).
- Peng, M., Tabashsum, Z., Anderson, M., Truong, A., Houser, A. K., Padilla, J., & Biswas, D. (2020). Effectiveness of probiotics, prebiotics, and prebiotic-like components in common functional foods. *Comprehensive Reviews in Food Science and Food Safety, 19*(4), 1908–1933. https://doi.org/10.1111/1541-4337.12565
- Phelps, R. J., & McKillip, J. L. (2002). Enterotoxin production in natural isolates of Bacillaceae outside the *Bacillus cereus* group. *Applied and Environmental Microbiology, 68*(6), 3147–3151.
- Phillips, R. D., McWatters, K. H., Chinnan, M. S., Hung, Y. C., Beuchat, L. R., Sefa-Dedeh, S., & Saalia, F. K. (2003). Utilization of cowpeas for human food. *Field Crops Research, 82*(2–3), 193–213.

- Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A. (2019). Mechanisms of action of probiotics. *Advances in Nutrition, 10*(Suppl. 1), S49–S66. https://doi.org/10.1093/advances/nmy063
- Pranoto, Y., Anggrahini, S., & Efendi, Z. (2013). Effect of natural and *Lactobacillus plantarum* fermentation on in vitro protein and starch digestibilities of sorghum flour. *Food Bioscience, 2*, 46–52. https://doi.org/10.1016/j.fbio.2013.04.001
- Prajapati, J. B. (2015). Probiotics—An Indian perspective. *International Journal of Probiotics & Prebiotics, 10*(1), 1.
- Qin, H., Wu, H., Shen, K., Liu, Y., Li, M., Wang, H., & Mu, Z. (2022). Fermented minor grain foods: Classification, functional components, and probiotic potential. *Foods, 11*(20), 3155. https://doi.org/10.3390/foods11203155
- Ramachandran, G. (2014). Gram-positive and gram-negative bacterial toxins in sepsis: A brief review. *Virulence, 5*(1), 213–218.
- Rasika, D. M. D., Vidanarachchi, J. K., Luiz, S. F., Azeredo, D. R. P., Cruz, A. G., & Ranadheera, C. S. (2021). Probiotic delivery through non-dairy plant-based food matrices. *Agriculture, 11*(7), 599. https://doi.org/10.3390/agriculture11070599
- Rathakrishnan, D., & Gopalan, A. K. (2022). Isolation and characterization of halophilic isolates from Indian salterns and their screening for production of hydrolytic enzymes. *Environmental Challenges, 6*, 100426.
- Rengadu, D., Gerrano, A. S., & Mellem, J. J. (2020). Prebiotic effect of resistant starch from *Vigna unguiculata* (L.) Walp. (cowpea) using an in vitro simulated digestion model. *International Journal of Food Science & Technology, 55*(1), 332–339.
- Rezac, S., Kok, C. R., Heermann, M., & Hutkins, R. (2018). Fermented foods as a dietary source of live organisms. *Frontiers in Microbiology, 9*, 1785. https://doi.org/10.3389/fmicb.2018.01785
- Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the healthy gut microbiota composition? *Microorganisms, 7*(1), 14. https://doi.org/10.3390/microorganisms7010014
- Ritter, A. C., Correa, A. P. F., Veras, F. F., & Brandelli, A. (2018). Characterization of *Bacillus subtilis* available as probiotics. *Journal of Microbiology Research, 8*(2), 23–32.

- Rodrigues, M., Mandalika, S., Jamdar, S. N., & Sharma, A. (2014). Evaluation of malted, gamma-irradiated and enzymatically processed finger millet–soybean blends in supporting growth of lactic acid bacteria. *LWT – Food Science and Technology, 59*(2), 908–914.
- Rodriguez-Amaya, D. B., & Kimura, M. (2004). *HarvestPlus handbook for carotenoid analysis.*
- Roy, F., Boye, J. I., & Simpson, B. K. (2010). Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Research International, 43*(2), 432–442.
- Rul, F., Béra-Maillet, C., Champomier-Vergès, M. C., El-Mecherfi, K. E., Foligné, B., Michalski, M. C., & Savary-Auzeloux, I. (2022). Underlying evidence for the health benefits of fermented foods in humans. *Food & Function, 13*(9), 4804–4824. https://doi.org/10.1039/D1FO03603G
- Saarela, M., Virkajärvi, I., Nohynek, L., Vaari, A., & Mättö, J. (2006). Fibres as carriers for *Lactobacillus rhamnosus* during freeze-drying and storage. *International Journal of Food Microbiology, 112*(2), 171–178.
- Samanta, S. (2022). Structural and catalytic features of different amylases and their potential applications. *Jordan Journal of Biological Sciences, 15*(2).
- Sánchez-Mata, M. C., Peñuela-Teruel, M. J., Cámara-Hurtado, M., Díez-Marqués, C., & Torija-Isasa, M. E. (1998). Determination of mono-, di-, and oligosaccharides in legumes by HPLC. *Journal of Agricultural and Food Chemistry, 46*(9), 3648–3652.
- Sanjukta, S., Rai, A. K., Muhammed, A., Jeyaram, K., & Talukdar, N. C. (2015). Enhancement of antioxidant properties of soybean varieties by *Bacillus subtilis* fermentation. *Journal of Functional Foods, 14*, 650–658.
- Santivarangkna, C. (2016). Storage stability of probiotic powder. In *Advances in probiotic technology*. CRC Press.
- Santos-Sánchez, G., Álvarez-López, A. I., Ponce-España, E., Lardone, P. J., Carrillo-Vico, A., & Cruz-Chamorro, I. (2024). Food-derived peptides with inhibitory capacity for HMG-CoA reductase activity. *Food Science and Human Wellness, 13*(6), 3083–3094.
- Saraniya, A., & Jeevaratnam, K. (2015). In vitro probiotic evaluation of phytase-producing *Lactobacillus* species. *Journal of Food Science and Technology, 2*, 5631–5640
- Sastry, D. V. S. S. R., Upadhyaya, H. D., & Srinivas, T. R. (2019). Variation for seed physical and hydration properties of chickpea (*Cicer arietinum* L.) mini core collection and their relevance to conservation and utilization. *Plant Genetic Resources, 17*(4), 311–324.

- Schaeffer, A. B., & Fulton, M. D. (1933). A simplified method of staining endospores. *Science, 77*(1990), 194.
- Scheers, N., Rossander-Hulthén, L., Torsdottir, I., & Sandberg, A. S. (2016). Increased iron bioavailability from lactic-fermented vegetables is likely an effect of promoting the formation of ferric iron (Fe^{3+}). *European Journal of Nutrition, 55*, 373–382.
- Schillinger, U., Guigas, C., & Holzapfel, W. H. (2005). In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. *International Dairy Journal, 15*, 1289–1297.
- Sebastian, J., Longvah, T., Subhash, K., Chary, P. M., Loukrakpam, B., Aaliya, B., & Ananthan, R. (2023). Morphology, comprehensive physicochemical and cooking characteristics of bamboo rice (*Bambusa arundinacea* Wild.). *Measurement: Food, 10*, 100089.
- Sedláčková, P., Horáčková, Š., Tiange, S., Kosová, M., & Plocková, M. (2015). Two different methods for screening of bile salt hydrolase activity in *Lactobacillus* strains. *Czech Journal of Food Sciences, 33*(1).
- Selvarajan, R., Sibanda, T., Tekere, M., Nyoni, H., & Meddows-Taylor, S. (2017). Diversity analysis and bioresource characterization of halophilic bacteria isolated from a South African saltpan. *Molecules, 22*(4), 657. https://doi.org/10.3390/molecules22040657
- Selvin, J., Maity, D., Sajayan, A., & Kiran, G. S. (2020). Revealing antibiotic resistance in therapeutic and dietary probiotic supplements. *Journal of Global Antimicrobial Resistance, 22*, 202–205.
- Sell, M., Steinhart, H., & Paschke, A. (2005). Influence of maturation on the alteration of allergenicity of green pea (*Pisum sativum* L.). *Journal of Agricultural and Food Chemistry, 53*(5), 1717–1722.
- Shanahan, F., Ghosh, T. S., & O'Toole, P. W. (2021). The healthy microbiome—What is the definition of a healthy gut microbiome? *Gastroenterology, 160*(2), 483–494.
- Sharma, S., & Sharma, N. (2021). Preparation of probiotic enriched functional beverage of kodo millet (*Paspalum scrobiculatum*): A nutritionally enriched new product for commercialization. *Journal of Pharmacognosy and Phytochemistry, 10*(1), 752–758.

- Sharma, R., Garg, P., Kumar, P., Bhatia, S. K., & Kulshrestha, S. (2020). Microbial fermentation and its role in quality improvement of fermented foods. *Fermentation, 6*(4), 106. https://doi.org/10.3390/fermentation6040106
- Shehata, M. G., El-Sohaimy, S. A., El-Sahn, M. A., & Youssef, M. M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol-lowering property and bile salt hydrolase activity. *Annals of Agricultural Sciences, 61*(1), 65–75. https://doi.org/10.1016/j.aoas.2016.03.001
- Shiferaw Terefe, N., & Augustin, M. A. (2020). Fermentation for tailoring the technological and health-related functionality of food products. *Critical Reviews in Food Science and Nutrition, 60*(17), 2887–2913.
- Shokryazdan, P., Sieo, C. C., Kalavathy, R., Liang, J. B., Alitheen, N. B., Faseleh Jahromi, M., & Ho, Y. W. (2014). Probiotic potential of *Lactobacillus* strains with antimicrobial activity against human pathogenic strains. *BioMed Research International, 2014*, 927268. https://doi.org/10.1155/2014/927268
- Shu, L., Zhang, X., Zhou, J., Zhu, Q., & Si, C. (2023). Ultra-processed food consumption and increased risk of metabolic syndrome: A systematic review and meta-analysis of observational studies. *Frontiers in Nutrition, 10*, 1211797. https://doi.org/10.3389/fnut.2023.1211797
- Sikkandar, S., Murugan, K., Al-Sohaibani, S., Rayappan, F., & Nair, A. (2013). Halophilic bacteria—A potent source of carotenoids. *Journal of Pure and Applied Microbiology, 7*(4), 2825–2830.
- Singh, N. (2017). Pulses: An overview. *Journal of Food Science and Technology, 54*, 853–857.
- Siró, I., Kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food: Product development, marketing and consumer acceptance—A review. *Appetite, 51*(3), 456–467.
- Sivakumaran, K., Herath, T., & Wansapala, M. A. J. (2017). Comparison of phytates and saponins and the effect of processing in selected edible beans in Sri Lanka. *International Journal of Food Science and Nutrition, 2*(2), 95–100.

- Slavin, J. (2013). Fibre and prebiotics: Mechanisms and health benefits. *Nutrients, 5*, 1417–1435. https://doi.org/10.3390/nu5041417
- Somplang, P., & Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: A review. *Journal of Animal Science and Technology, 58*(26), 1–11. https://doi.org/10.1186/s40781-016-0108-2
- Song, Z., Song, R., Liu, Y., Wu, Z., & Zhang, X. (2023). Effects of ultra-processed foods on the microbiota–gut–brain axis: The bread-and-butter issue. *Food Research International, 167*, 112730. https://doi.org/10.1016/j.foodres.2023.112730
- Sorokulova, I. B., Pinchuk, I. V., Denayrolles, M., Osipova, I. G., Huang, J. M., Cutting, S. M., & Urdaci, M. C. (2008). The safety of two *Bacillus* probiotic strains for human use. *Digestive Diseases and Sciences, 53*, 954–963.
- Sridevi, N., & Prabhune, A. A. (2009). *Brevibacillus* sp.: A novel thermophilic source for the production of bile salt hydrolase. *Applied Biochemistry and Biotechnology, 157*, 254–262.
- Stasiak-Różańska, L., Berthold-Pluta, A., Pluta, A. S., Dasiewicz, K., & Garbowska, M. (2021). Effect of simulated gastrointestinal tract conditions on survivability of probiotic bacteria present in commercial preparations. *International Journal of Environmental Research and Public Health, 18*(3), 1108. https://doi.org/10.3390/ijerph18031108
- Šušković, J., Klarić, B., Beganović, J., Pavunc, A. L., Habjanič, K., & Matošić, S. (2010). Antimicrobial activity—The most important property of probiotic and starter lactic acid bacteria. *Food Technology and Biotechnology, 48*(3), 296–307.
- Taj, R., Masud, T., Sohail, A., Sammi, S., Naz, R., Sharma Khanal, B. K., & Nawaz, M. A. (2022). In vitro screening of EPS-producing *Streptococcus thermophilus* strains for probiotic potential from dahi. *Food Science & Nutrition, 10*(7), 2347–2359. https://doi.org/10.1002/fsn3.2856
- Tamang, J. P., Cotter, P. D., Endo, A., Han, N. S., Kort, R., Liu, S. Q., & Hutkins, R. (2020). Fermented foods in a global age: East meets West. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 184–217. https://doi.org/10.1111/1541-4337.12520

- Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*, 3022–3027. https://doi.org/10.1093/molbev/msab120
- Tanaka, H., Hashiba, H., Kok, J., & Mierau, I. (2000). Bile salt hydrolase of *Bifidobacterium longum*: Biochemical and genetic characterization. *Applied and Environmental Microbiology, 66*(6), 2502–2512.
- Tomasik, P., & Tomasik, P. (2020). Probiotics, non-dairy prebiotics and postbiotics in nutrition. *Applied Sciences, 10*(4), 1470. https://doi.org/10.3390/app10041470
- Tresina, P. S., & Mohan, V. R. (2012). Physicochemical and antinutritional attributes of gamma-irradiated *Vigna unguiculata* (L.) Walp. seeds. *International Food Research Journal, 19*(2).
- Tripathi, M. K., & Giri, S. K. (2014). Probiotic functional foods: Survival of probiotics during processing and storage. *Journal of Functional Foods, 9*, 225–241. https://doi.org/10.1016/j.jff.2014.04.030
- U.S. Department of Agriculture. (2021). *FoodData Central*. https://fdc.nal.usda.gov/
- Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Role of the gut microbiota in nutrition and health. *BMJ, 361*, k2179. https://doi.org/10.1136/bmj.k2179
- Valero-Cases, E., Cerdá-Bernad, D., Pastor, J. J., & Frutos, M. J. (2020). Non-dairy fermented beverages as potential carriers of probiotics, prebiotics, and bioactive compounds. *Nutrients, 12*(6), 1666. https://doi.org/10.3390/nu12061666
- Valls, F., Sancho, M. T., Fernández-Muiño, M. A., & Checa, M. A. (2001). Determination of vitamin B6 in cooked sausages. *Journal of Agricultural and Food Chemistry, 49*(1), 38–41.
- Verma, A., Kushwaha, A., Kumar, A., & Baghel, S. S. (2019). Physicochemical properties and nutritional composition of improved varieties of grain cowpea. *Journal of Pharmacognosy and Phytochemistry, 8*, 334–338.
- Verni, M., Pontonio, E., Montemurro, M., & Rizzello, C. G. (2022). Fermentation as a strategy for improving nutritional and functional properties of legumes. In *Legumes research* (Vol. 2).

- Vieira, S. A., McClements, D. J., & Decker, E. A. (2015). Challenges of utilizing healthy fats in foods. *Advances in Nutrition, 6*(3), 309S–317S. https://doi.org/10.3945/an.114.006940
- Vijayakumar, M., Ilavenil, S., Kim, D. H., Arasu, M. V., Priya, K., & Choi, K. C. (2015). In vitro assessment of probiotic potential of *Lactobacillus plantarum* KCC-24. *Anaerobe, 32*, 90–97.
- Vinderola, G., Cotter, P. D., Freitas, M., Gueimonde, M., Holscher, H. D., Ruas-Madiedo, P., & Cifelli, C. J. (2023). Fermented foods: A perspective on their role in delivering biotics. *Frontiers in Microbiology, 14*, 1196239. https://doi.org/10.3389/fmicb.2023.1196239
- Vinderola, G., Sanders, M. E., & Salminen, S. (2022). The concept of postbiotics. *Foods, 11*(8), 1077. https://doi.org/10.3390/foods11081077
- Wang, J., Wang, J., Yang, K., Liu, M., Zhang, J., Wei, X., & Fan, M. (2018). Screening for potential probiotics from fermented non-dairy foods. *Annals of Microbiology, 68*(12), 803–813.
- Wang, K., Niu, M., Song, D., Song, X., Zhao, J., Wu, Y., & Niu, G. (2020). Preparation and characterization of exopolysaccharides from *Lactobacillus fermentum* S1. *Journal of Bioscience and Bioengineering, 129*(2), 206–214.
- Watson, D., O'Connell Motherway, M., Schoterman, M. H. C., van Neerven, R. J., Nauta, A., & Van Sinderen, D. (2013). Selective carbohydrate utilization by lactobacilli and bifidobacteria. *Journal of Applied Microbiology, 114*(4), 1132–1146.
- Woollard, D. C., Indyk, H. E., & Christiansen, S. K. (2000). Analysis of pantothenic acid in milk and infant formulas by HPLC. *Food Chemistry, 69*(2), 201–208.
- Wu, H., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2015). Mung bean (*Vigna radiata*) as probiotic food through fermentation. *LWT – Food Science and Technology, 63*(1), 445–451.
- Xiang, H., Sun-Waterhouse, D., Waterhouse, G. I. N., Cui, C., & Ruan, Z. (2019). Fermentation-enabled wellness foods: A fresh perspective. *Food Science and Human Wellness, 8*(3), 203–243.

- Xiong, H. H., Lin, S. Y., Chen, L. L., Ouyang, K. H., & Wang, W. J. (2023). Interaction between flavonoids and intestinal microbes: A review. *Foods, 12*(2), 320. https://doi.org/10.3390/foods12020320
- Xu, Z., Chen, G., Xue, L., Zhang, H., Wang, J., Xiang, H., & Zheng, K. (2019). Structural characterization and bioactivities of exopolysaccharides from *Bacillus licheniformis*. *International Journal of Biological Macromolecules, 141*, 298–306.
- Yang, Q., Liang, Q., Balakrishnan, B., Belobrajdic, D. P., Feng, Q. J., & Zhang, W. (2020). Role of dietary nutrients in gut microbiota modulation: A narrative review. *Nutrients, 12*(2), 381. https://doi.org/10.3390/nu12020381
- Yang, R., Zhu, L., Meng, D., Wang, Q., Zhou, K., Wang, Z., & Zhou, Z. (2022). Proteins from leguminous plants: Structure and functional roles. *Critical Reviews in Food Science and Nutrition, 62*(19), 5203–5223.
- Yao, Z. D., Cao, Y. N., Peng, L. X., Yan, Z. Y., & Zhao, G. (2020). Coarse cereals and legumes and gut microbiota interactions. *Journal of Agricultural and Food Chemistry, 69*(3), 861–877.
- Yasmin, I., Saeed, M., Khan, W. A., Khaliq, A., Chughtai, M. F. J., Iqbal, R., & Tanweer, S. (2020). In vitro probiotic potential and safety evaluation of *Bifidobacterium* strains. *Microorganisms, 8*(3), 354. https://doi.org/10.3390/microorganisms8030354
- Yasumatsu, K., Sawada, K., Moritaka, S., Misaki, M., Toda, J., Wada, T., & Ishii, K. (1972). Whipping and emulsifying properties of soybean products. *Agricultural and Biological Chemistry, 36*(5), 719–727.
- Yoha, K. S., Nida, S., Dutta, S., Moses, J. A., & Anandharamakrishnan, C. (2022). Targeted delivery of probiotics: Research and commercialization perspectives. *Probiotics and Antimicrobial Proteins, 14*(1), 15–48.
- Zanjani, M. A. K., Tarzi, B. G., Sharifan, A., & Mohammadi, N. (2014). Microencapsulation of probiotics and survival in simulated gastrointestinal conditions. *Iranian Journal of Pharmaceutical Research, 13*(3), 843.
- Zhang, C., Fan, L., & Zhao, H. (2020). Rapid detection of short-chain fatty acids in biological samples. *Chromatographia, 83*, 305–310.

- Zheng, L., Li, D., Li, Z. L., Kang, L. N., Jiang, Y. Y., Liu, X. Y., & Wang, J. H. (2017). Effects of *Bacillus* fermentation on soybean meal. *Letters in Applied Microbiology, 65*(6), 520–526. https://doi.org/10.1111/lam.12806
- Ziar, H., Gérard, P., & Riazi, A. (2014). Effect of prebiotic carbohydrates on probiotic bacteria. *Journal of the Science of Food and Agriculture, 94*(6), 1184–1190.
- Ziarno, M. (2008). In vitro cholesterol uptake by *Lactobacillus acidophilus*. *Acta Scientiarum Polonorum Technologia Alimentaria, 7*(3), 65–73.
- Zia-Ul-Haq, M., Ahmad, S., Chiavaro, E., & Ahmed, S. (2010). Studies of oil from cowpea (*Vigna unguiculata* (L.) Walp.) cultivars. *Pakistan Journal of Botany, 42*(2), 1333.

APPENDIX I

MEDIA COMPOSITION

A solution of sodium hydroxide or hydrochloric acid was used to adjust the pH of the media.

If otherwise not specified, all the media were sterilized at 121˚C, 15 psi for 20 minutes.

1. NUTRIENT AGAR (NA) HiMedia M001

Ingredients	**g/L**
Peptone	10.00
NaCl	5.00
Beef extract	3.00
Agar	15.00
Distilled water	1000 mL
pH	7.0

2. NUTRIENT BROTH HiMedia M002

Ingredients	**g/L**
Peptone	10.00
NaCl	5.00
Beef extract	3.00
Distilled water	1000 mL
pH	7.2

3. LURIA BERTANI BROTH (LB) HiMedia M1245

Ingredients	**g /L**
Casein hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Distilled water	1000 mL
pH	7.5 ± 0.2

4. MUELLER-HINTON AGAR (MHA) HiMedia M173

Ingredients	**g/L**
HM infusion B	300.00
Acid Hydrolysate of casein	17.50
Starch	1.50
Agar	17.00
Distilled water	1000 mL
Final pH (at 25°C)	7.3 ± 0.1

5. PHYTASE SPECIFIC MEDIUM HiMedia GRM6226

Ingredients	**g/L**
Glucose	15.00
Ammonium nitrate	5.00
Calcium phytate	5.00
Magnesium sulfate	0.50
Potassium chloride	0.50
Calcium chloride	0.50
Iron sulfate	0.01
Manganese sulfate	0.01
Agar	20.00
Distilled water	1000mL

6. TRYPTONE BROTH (INDOLE TEST)

Ingredients	**g/L**
Tryptone	10.000
Sodium chloride	5.000
Distilled water	1000 mL
pH	7.5 ± 0.2

7. STARCH AGAR

Ingredients	**g/ L**
Disodium hydrogen Phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.50
Ammonium chloride	1.00
Starch, soluble	10.00
Agar	15.00
Distilled water	1000 mL
Final pH (at 25 °C)	7.6 ± 0.2

8. SOFT MANN ROGOSA SHARPE (MRS) AGAR HiMedia M641

Ingredients	**g/L**
Peptone	10.00
Yeast extract	5 .00
Dextrose	20.00
Tween 80	1.00
Ammonium citrate	2.00
Sodium acetate	5.00
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium hydrogen phosphate	2.00
Distilled water	1000 mL
Agar	12.00
pH	6.5 ± 0.2

9. TRIBUTYRIN AGAR MEDIUM

Ingredients	**g /L**
Disodium hydrogen phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.50
Ammonium chloride	1.00
Distilled water	1000 mL
Agar	15.00
Tributyrin	10.00
Final pH (at 25°C)	7.6 ± 0.2

10. CARBOXY-METHYL CELLULOSE AGAR

Ingredients	**g/L**
Disodium hydrogen phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.50
Ammonium chloride	1.00
Agar	15 .00
Distilled water	1000 mL
Carboxy-methyl cellulose	10.00
Final pH (at 25°C)	7.6 ± 0.2

11. SKIMMED MILK AGAR

Ingredients	**g/L**
Disodium hydrogen phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.5
Ammonium chloride	0.1
Skimmed milk powder	10.00
Distilled water	1000 mL
Agar	15.00
Final pH (at 25°C)	7.6 ± 0.2

12. BLOOD BASED AGAR

Ingredients	**g/L**
Peptone	10.00
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Sterile human blood	6 % (v/v)
Final pH (at 25°C)	7.3 ± 0.2

13. Methyl Red-Voges Proskauer Broth (Glucose Phosphate Broth) Hi Media M070

Ingredients	**g/L**
Buffered Peptone	7.00
Dextrose (Glucose)	5.00
Dipotassium phosphate	5.00
Distilled water	1000 mL
Final pH (at 25°C)	6.9 ± 0.2

14. MINIMAL AGAR

Ingredients	g/L
Disodium hydrogen phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.50
Agar	20.00
Distilled water	1000 mL
Ammonium chloride	1.00
Final pH (at 25°C)	7.6 ± 0.2

15. MINIMAL BROTH

Ingredients	g/L
Disodium hydrogen phosphate	6.00
Potassium dihydrogen phosphate	3.00
Sodium chloride	0.50
Distilled water	1000 mL
Ammonium chloride	1.00
Final pH (at 25°C)	7.6 ± 0.2

16. SIMMON CITRATE AGAR HiMedia M099

Ingredients	g/L
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Distilled water	1000 mL
Bromothymol blue	0.08
Agar	15.0
Final pH (at 25°C)	6.8 ± 0.2

17. CARBOHYDRATE FERMENTATION BROTH

Ingredients	g/L
Peptone	10.00
Sodium chloride	5.00
Phenol red	0.018
Distilled water	1000 mL
Specific carbohydrate (e.g., glucose, lactose, sucrose)	10.0
pH	7.4 ± 0.2

APPENDIX II

REAGENTS, BUFFERS, PRIMERS AND ANTIMICROBIAL AGENTS

1. Coomassie brilliant blue G-250 solution

0.2 g of dye was dissolved in 100 mL distilled water (DW), warmed and cooled. 100 mL 2N H_2S0_4 was added to the solution and incubated overnight at room temperature for 3 h, filtered and used.

2. Congo red dye (0.08%)

Congo red (0.08 g) was dissolved in 100 mL of distilled water.

3. 3,5-dinitrosalicylic acid reagent (DNSA)

3,5-dinitrosalicylic acid (1.0 g) was weighed and dissolved in 50 mL of DW followed by addition of 30.0 g sodium potassium tartrate tetrahydrate slowly. 2 N NaOH (20 mL) was further added and diluted to a final volume of 100 mL with DW and stored in amber coloured bottle.

4. Trichloroacetic acid (TCA) (20%)

TCA (100 g) accurately weighed was added to 45.4 mL of DW. 10 mL of this solution was further diluted to 50 mL with DW. To 25 mL of 20% TCA stock solution, 25 mL of DW was added and mixed well to make 10% TCA.

5. Ninhydrin reagent (For BSH activity)

Ninhydrin	2 g
Acetone	100 mL

6. Methyl red indicator Hi Media 1007 (For MR test)

Methyl red	0.2 g
Ethyl alcohol	60 mL
Distilled water	40 mL

7. Barrit's reagent A HiMedia R029 (Alpha-naphthol, 5% color intensifier)

Alpha-naphthol	5.0 g
Absolute ethyl alcohol	100 mL

8. Barrit's reagent B HiMedia R030 (Potassium Hydroxide, 40%, oxidizing agent)

Potassium hydroxide	40 g
Distilled water	100 mL

9. Kovac's Reagent HiMedia R008 (for Indole test)

p-dimethylamino benzaldehyde	5 g
Amyl alcohol	75 mL
Hydrochloric acid	25 mL

10. Lowry reagents

- Reagent A: 2 % sodium carbonate (Na_2CO_3) in 0.1N sodium hydroxide (NaOH).
- Reagent B: 0.5 % copper sulfate ($CuSO_4$) solution.
- Reagent C: Sodium potassium tartrate solution (1 %).
- Folin-Ciocalteu reagent: Diluted (usually 1:1 with DW).

11. Bovine Serum Albumin (BSA)

1 g powdered molecular biology grade BSA was dissolved in 10 mL of DW and gently mixed to avoid clumping to obtain a 10 % (100 mg/mL) stock solution of BSA.

12. 1 M Acetate Buffer

Sodium acetate	13.6 g
Glacial acetic acid	6.0 mL

The ingredients were added to 794 mL of DW, pH was adjusted as desired, and volume was made to 1L using DW.

13. Lugol's iodine solution

To 20 mL DW, 10 g Potassium iodide was added, followed by 5 g of Iodine. the solution was heated gently with constant stirring until it dissolved completely. The volume was made up to 100 mL and stored in amber coloured bottle.

14. Crystal Violet (0.1 %)

2 g crystal violet was dissolved in 20 mL of 95 % ethyl alcohol. 0.8 g ammonium oxalate monohydrate was dissolved in 80 mL of deionized water. The crystal violet and ammonium oxalate monohydrate solutions were mixed to make the crystal violet stain and filtered.

15. Gram's Decolourizer

Acetone	50 mL
Ethyl alcohol (95 %)	50 mL

16. Counterstain Basic Fuchsin

Basic fuchsin (0.3 g) was dissolved in 10 mL 95 % ethyl alcohol. 5 mL melted phenol crystals were added to 95 mL of distilled water. 5 % phenol solution was added to the fuchsin solution and allowed to stand overnight. This was filtered through coarse filter paper.

17. Gram's staining method:

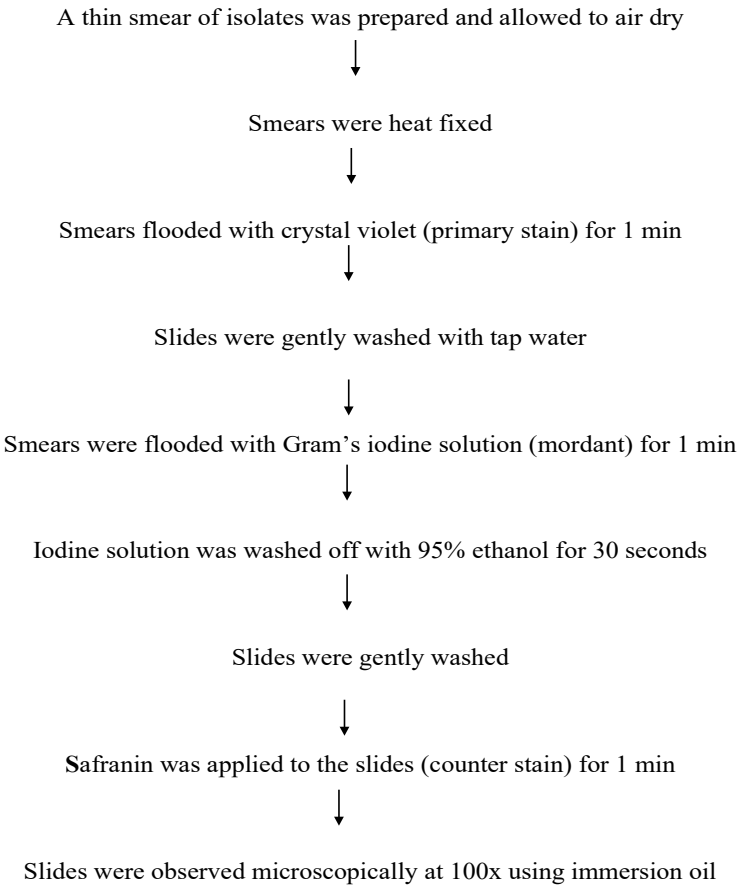


Figure 1: Gram staining process

18. 1X Phosphate Buffer Saline

Ingredients	**g/L**
Sodium chloride	8.00
Potassium chloride	0.20
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.24
Distilled water	1000 mL
pH	7.4 ± 0.2

19. 0.5 M Sodium Citrate buffer (pH 4.5)

Sodium Citrate dehydrate (11.975 g) was accurately weighed and added to 800 mL of DW followed by addition of Citric Acid (11.385 g) to the solution. pH was adjusted using HCl and/or NaOH. The volume was then made up to 1 L using DW.

20. Sodium Phosphate buffer

Ingredients	**0.1 M**
Disodium hydrogen phosphate	13.4 g
Sodium dihydrogen phosphate	6.9 g
pH	pH 6.7
Add the ingredients to 800 mL of distilled water, adjust the pH and make up the volume to 1 L with DW	

21. Tris-HCl buffer (0.05 M, pH 8.5)

0.786 g of Tris-base was solubilized in 80 mL of distilled water. pH was adjusted to 8.5 using 1 M HCl and final volume was adjusted to 100 mL using DW.

22. 1,1-diphenyl-2-picrylhydrazil (DPPH) reagent

1.2 mg of DPPH powder was dissolved in 15 mL of ethanol in an ultrasonic bath. It was then diluted to the desired concentration (3 mL of the stock solution was diluted to 50 mL with ethanol in a volumetric flask) and stored in the dark.

23. Colour reagent

Solution A was made using ascorbic acid (10 % w/v) in Sulphuric acid (1 M). Solution B contained ammonium molybdate (5 % w/v). 1 part solution B was mixed with 5 parts solution A.

24. Schaeffer & Fulton's method (Endospore staining method)

5 % Malachite green **(**5 g Malachite green in 100 mL DW)

0.5 % Safranin **(**0.5 g Safranin in 100 mL DW)

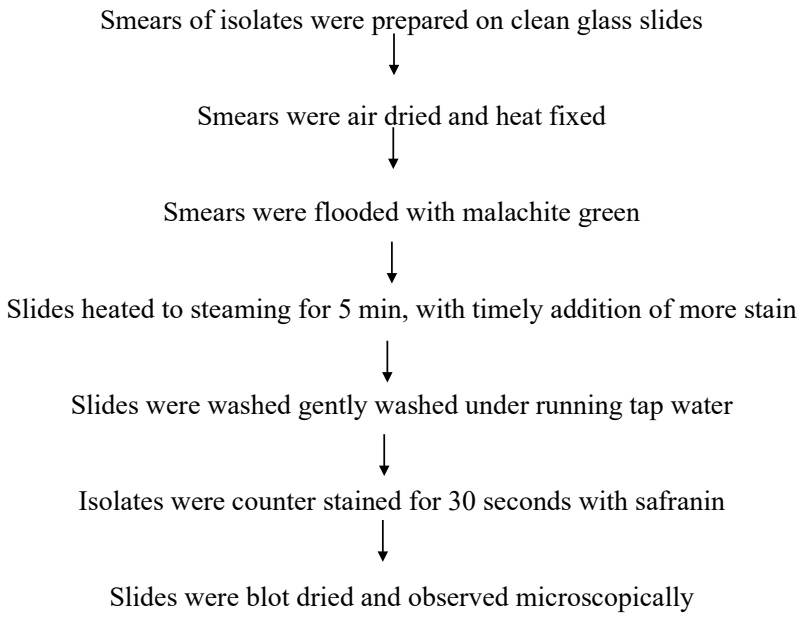


Figure 2: Process of **e**ndospore staining

APPENDIX III

Antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing.

Group /Antibiotic Name/ Dosage	R	MS	S
Inhibitors of cell wall synthesis			
Vancomycin [Va](30µg)	≤ 14	15-16	≥ 17
Ampicillin [A] (10 µg)	≤ 12	13-15	16
Cephalothin [Ch] (30 µg)	≤ 14	15-17	≥ 18
Inhibitors of protein synthesis			
Erythromycin [Ery] (15 µg)	≤ 13	14-17	≥ 18
Kanamycin [Ka] (30 µg)	≤13	14 - 17	≥18
Tetracycline [Te] (30 µg)	≤ 14	15-18	≥ 19
Chloramphenicol [C] (30 µg)	≤ 13	14-17	≥ 18
Gentamycin [G] (10 µg)	≤ 12	13-14	≥ 15
Penicillin [P](10 µg)	≤ 11	12-21	≥22
Streptomycin [St] (10 µg)	≤ 11	12-14	≥15
Inhibitors of nucleic acid synthesis			
Ciprofloxacin [Cf] (5µg)	≤ 13	14-18	≥ 19
Ofloxacin [O] (5µg)	≤ 12	13-14	≥ 15
Trimethoprim [Tr] (5µg)	≤ 11	12-14	≥ 15

R (resistant), MS (moderately susceptible), or S (susceptible) (Charteris et al, 1998)

APPENDIX IV A

Summary of the RDA of Nutrients for Indians, 2024

Category / Age group	Physical activity level	Body Wt	Protein		Dietary Fiber	Ca	Mg	Fe	Zn	I	Vit. B1	Vit. B2	Vit B3	Vit B6	Vit B9	Vit. B12	Vit. C	Vit.A	Vit. D
		Kg	g/d	g/kg/d	g/d	mg/d	mg/d	mg/d	mg/d	µg/d	mg/d	mg/d	mg/d	mg/d	µg/d	µg/d	µg/d	µg/d	IU/d
Adult Men >18 y	Sedentary	65	54.0	0.83	30	1000	440	19	17	140	1.4	2.0	14	1.9	300	2.2	80	1000	600
	Moderate				40						1.8	2.5	18	2.4					
	Heavy				50						2.3	2.2	23	3.1					
Adult women >18 y	Sedentary	55	46.0	0.83	25	1000	370	29	13.2	140	1.4	1.9	11	1.9	220	2.2	65	840	600
	Moderate				30						1.7	2.4	14	1.9					
	Heavy				40						2.2	3.1	18	2.4					
Pregnant woman**	--	55 + 10 GWG	55.5 68.0	0.83 (2nd Trimester, addl. 9.5g /day) 0.83 (3rd Trimester, addl. 22g / day)	--	1000	440	27	14.5	220	2.0	2.7	13	2.3	570	2.45	80	900	600
Lactation 0-6 m	--	55	63.0	0.83 (addl.17g/day)	--	1200	400	23	14.1	280	2.1	3.0	16	2.16	330	3.2	115	950	600
7-12 m			59.0	0.53 (addl. 13g/day)								2.9		2.07					

APPENDIX IV B

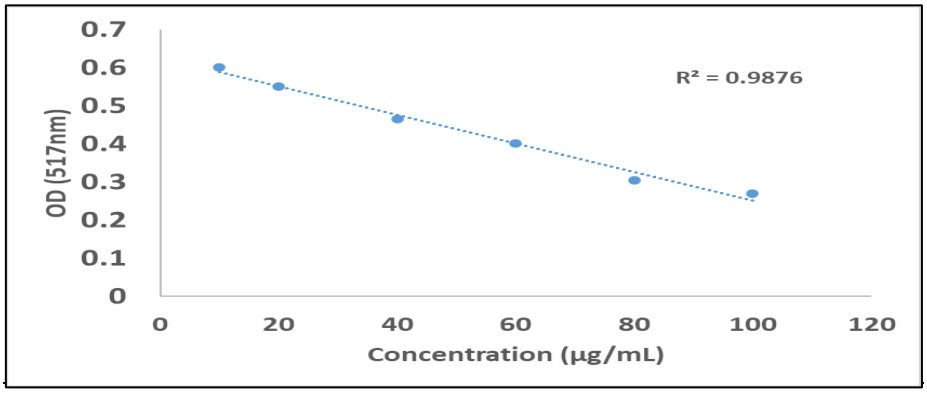
Summary of the RDA of Nutrients for Indians, 2024

Category/ Age group	Physical activity level	Body Wt	Protein		Dietary Fiber	Ca	Mg	Fe	Zn	I	Vit. B1	Vit. B2	Vit B3	Vit B6	Vit B9	Vit. B12	Vit. C	Vit.A	Vit. D
		Kg	g/d	g/kg/d	g/d	mg/d	mg/d	mg/d	mg/d	µg/d	mg/d	mg/d	mg/d	mg/d	µg/d	µg/d	µg/d	µg/d	IU/d
Infants 0-6 m	--	5.8	8.0	1.40	--	300	30	--	--	100	0.2	0.4	2	0.1	25	1.2	20	350	400
6-12 m		8.5	10.5	1.23		300	75	06	2.5	130	0.4	0.6	5	0.6	85	1.2	30	350	400
Child ren 1-3y	--	12.9	12.5	0.97	15	500	90	08	3.3	90	0.7	1.1	07	0.9	120	1.2	30	390	600
4-6 y		18.3	16.0	0.87	20	550	125	11	4.5	90	0.9	1.3	09	1.2	135	2.2	35	510	
7-9 y		25.3	23.0	0.92	26	650	175	15	5.9	90	1.1	1.6	11	1.5	170	2.2	45	630	
Boys 10-12 y	--	34.9	32.0	0.91	33	850	240	16	8.5	100	1.5	2.1	15	2.0	220	2.2	55	770	600
Girls 10-12y	--	36.4	33.0	0.90	30	850	250	23	8.5	100	1.4	1.9	14	1.9	225	2.2	50	790	600
Boys13-15y	--	50.5	45.0	0.89	43	1000	345	22	14.3	140	1.9	2.7	19	2.6	285	2.2	70	930	600
Girls 13-15y	--	49.6	43.0	0.87	36	1000	340	30	12.8	140	1.6	2.2	16	2.2	245	2.2	65	890	600
Boys 16-18y	--	64.4	55.0	0.86	50	1050	440	26	17.6	140	2.2	3.1	22	3.0	340	2.2	85	1000	600
Girls 16-18y	--	55.7	46.0	0.83	38	1050	380	32	14.2	140	1.7	2.3	17	2.3	270	2.2	70	860	600

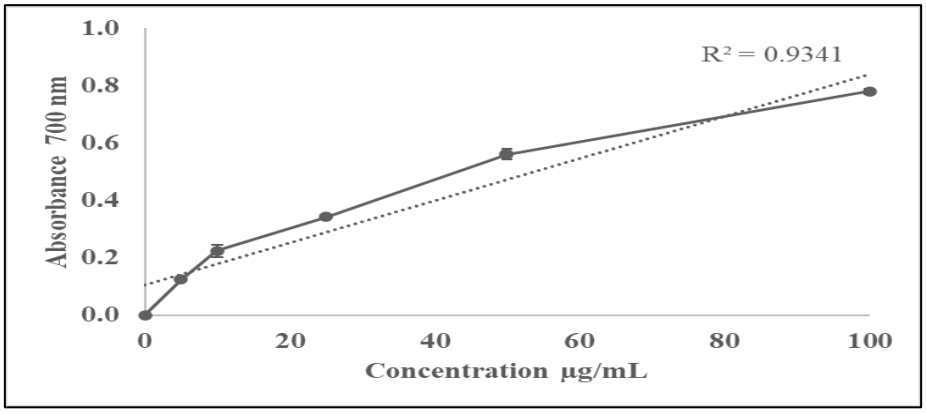
APPENDIX V

STANDARD GRAPHS

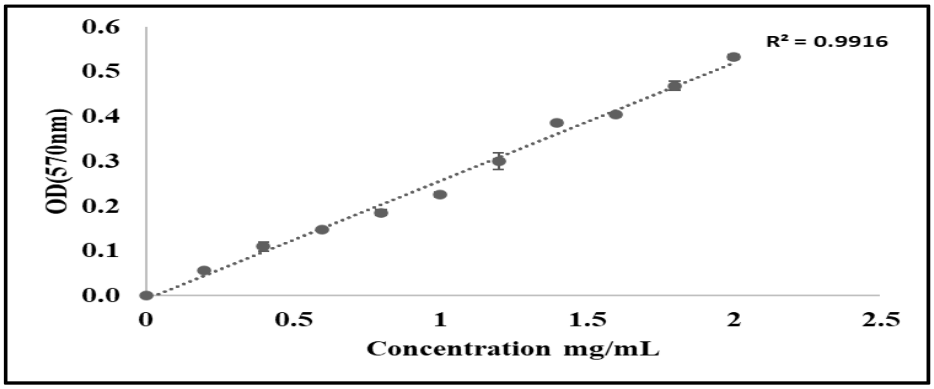
Standard graph for DPPH assay



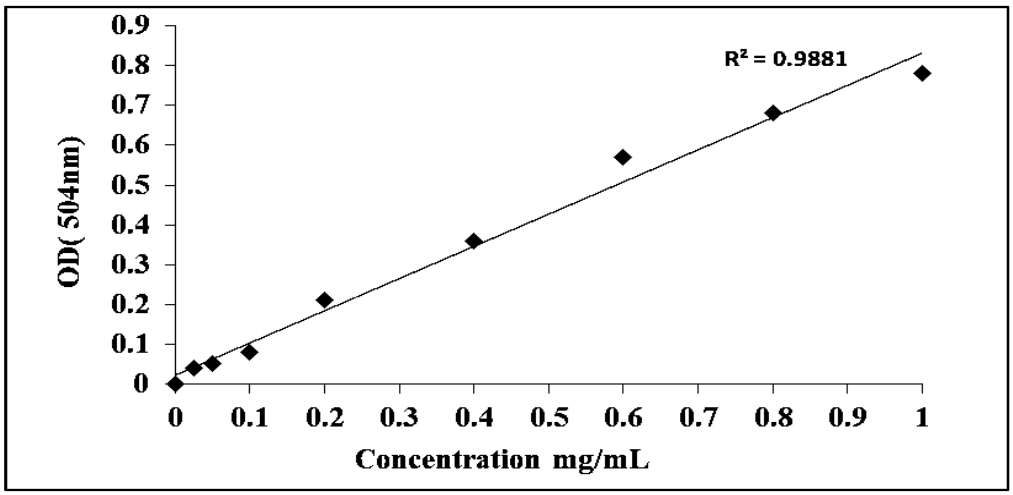
Standard graph for ascorbic acid for reducing power



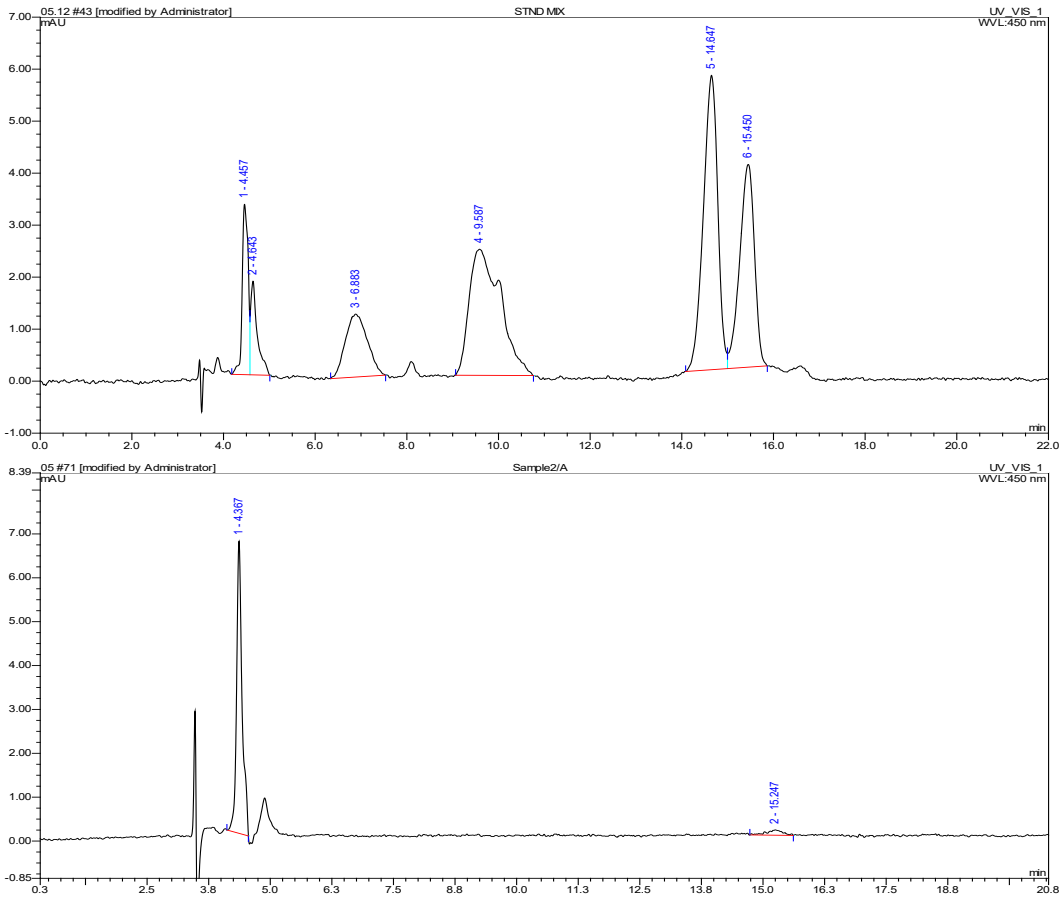
Standard graph for Taurine -BSH assay



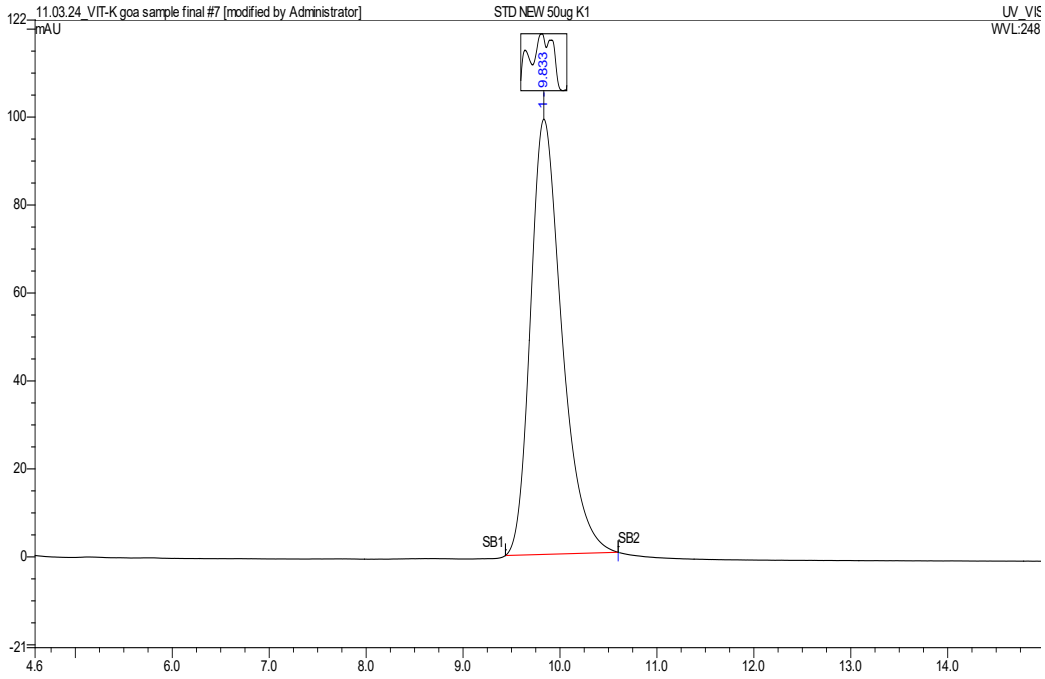
Standard graph for Glycine for FAN estimation

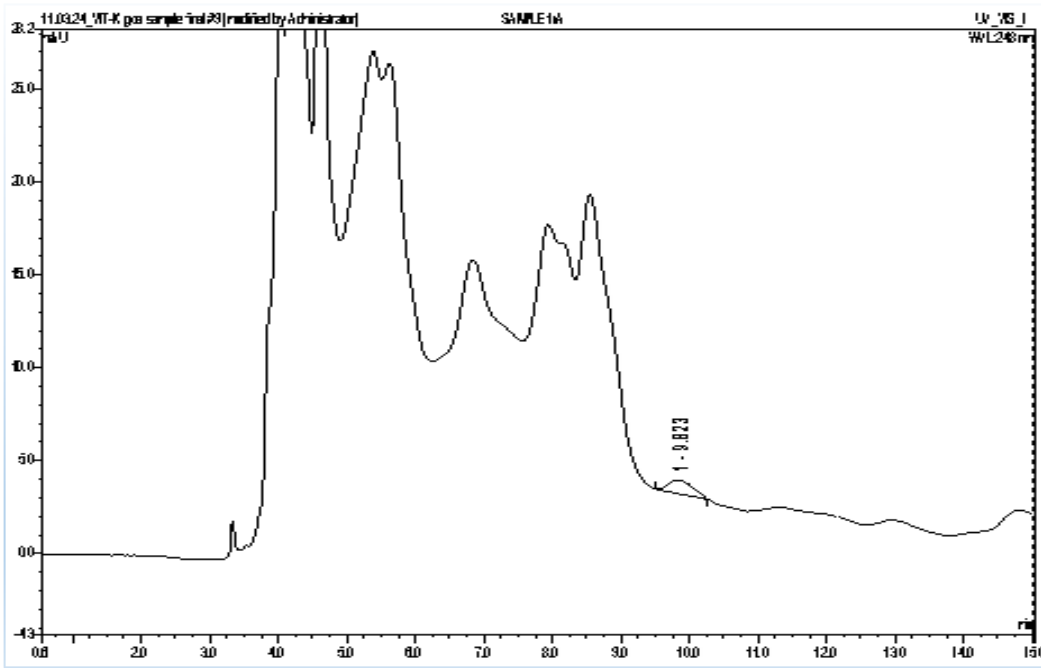


Chromatogram of Carotenoids: Standard and GC3

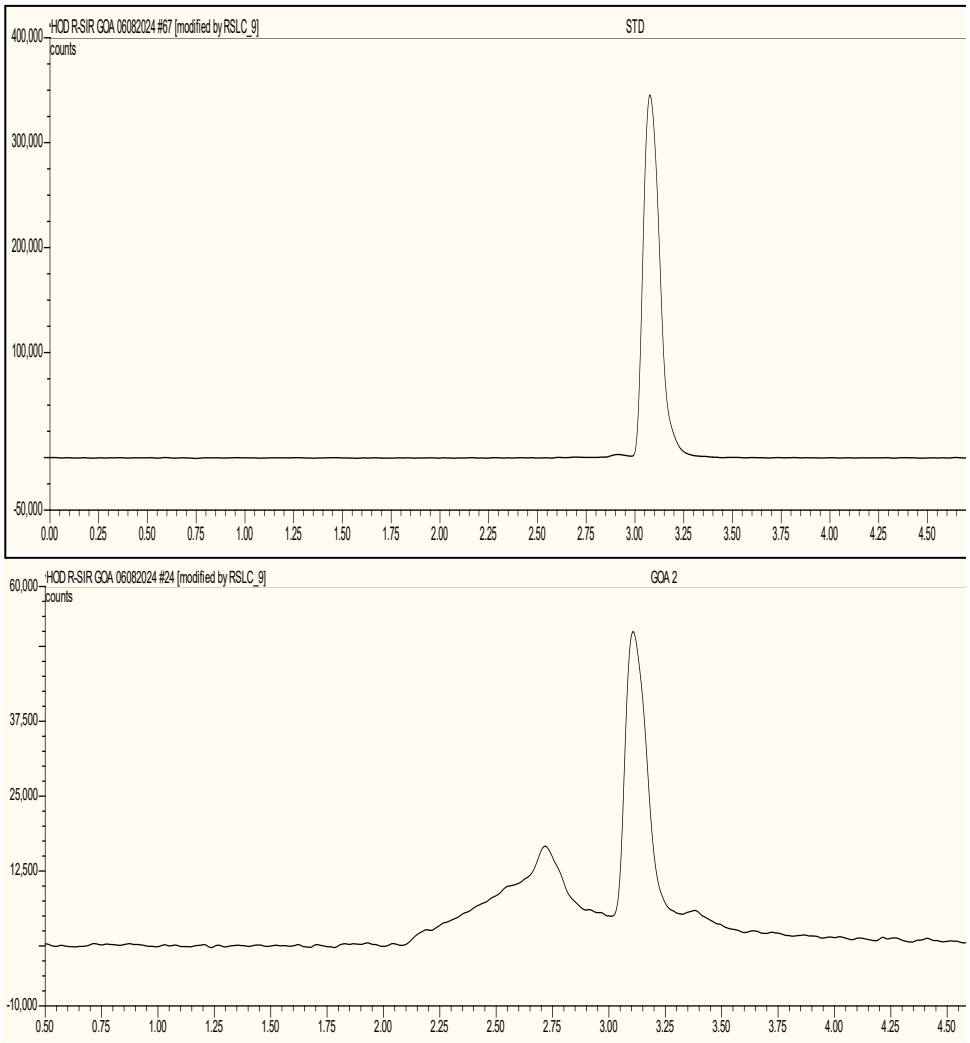


Chromatogram of Vitamin K: Standard and GC3

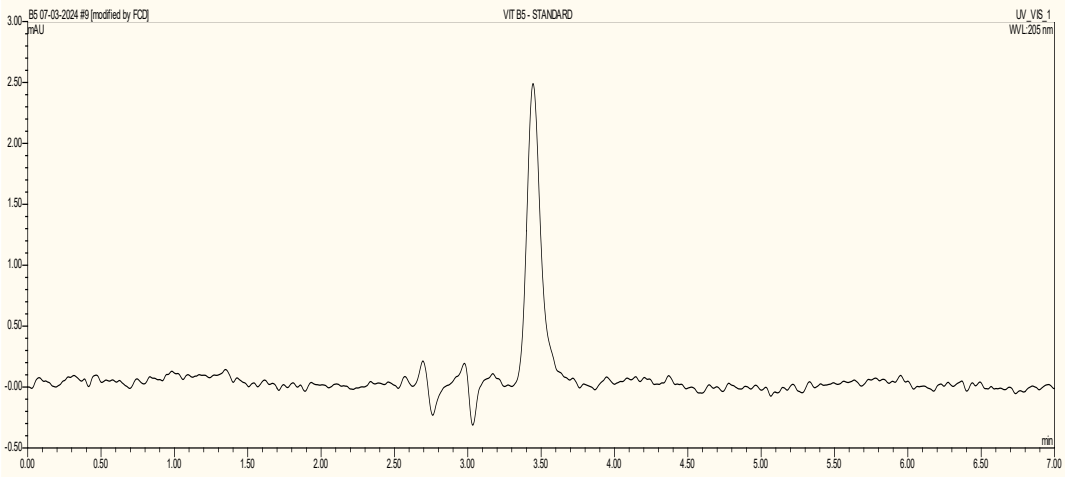


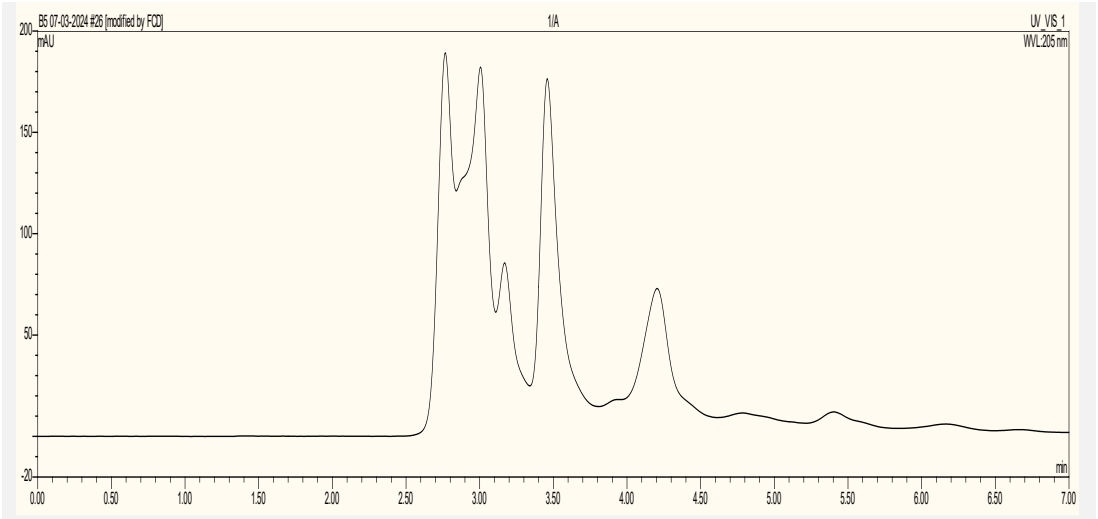


Chromatogram of Vitamin B2: Standard and GC3

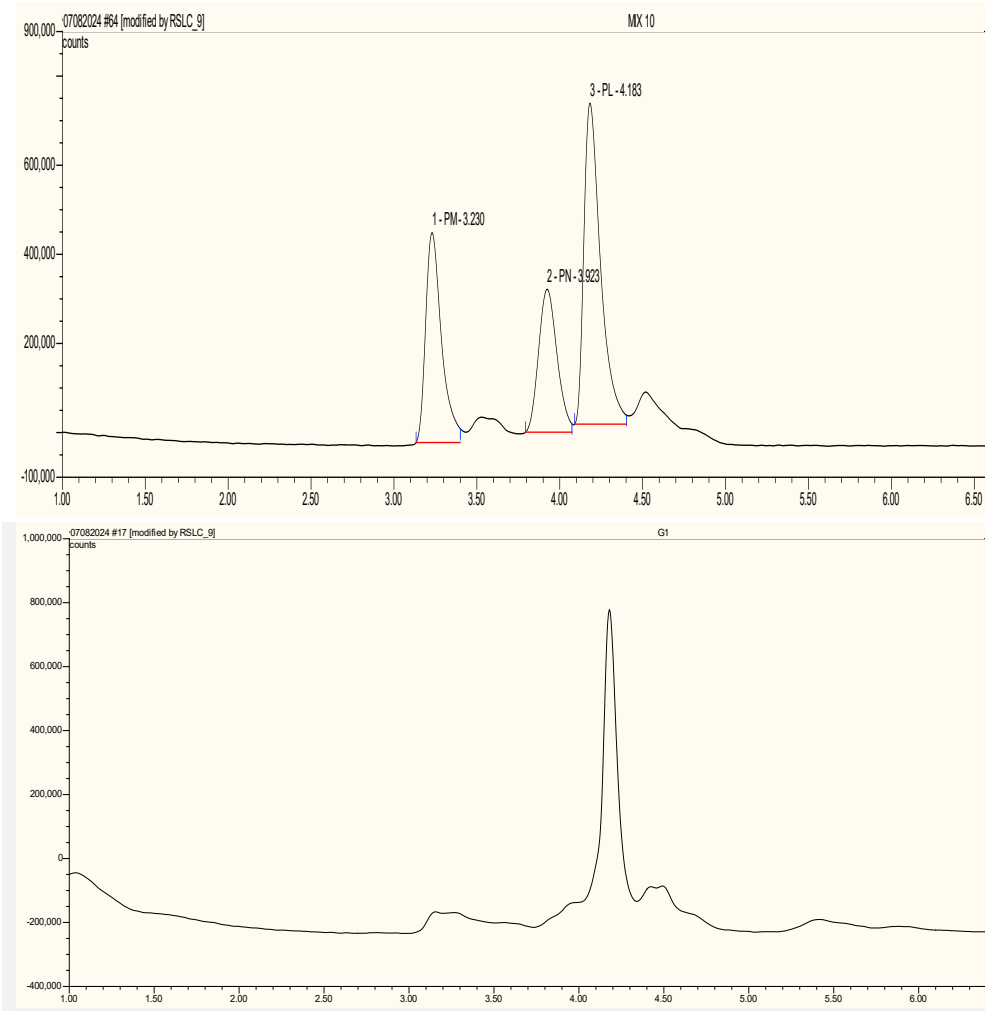


Chromatogram of Vitamin B5: Standard and GC3

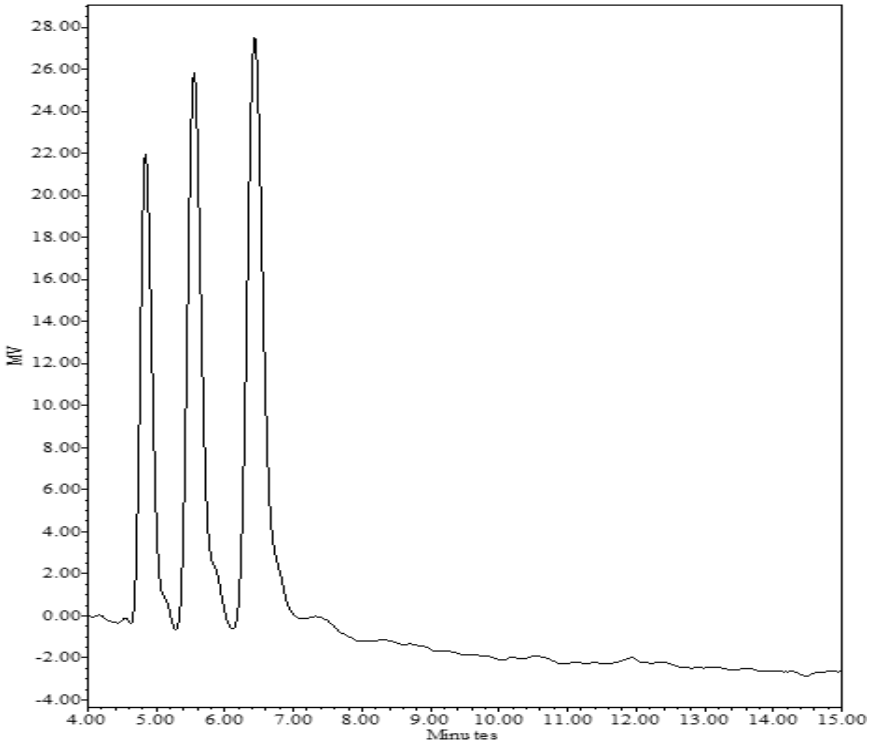


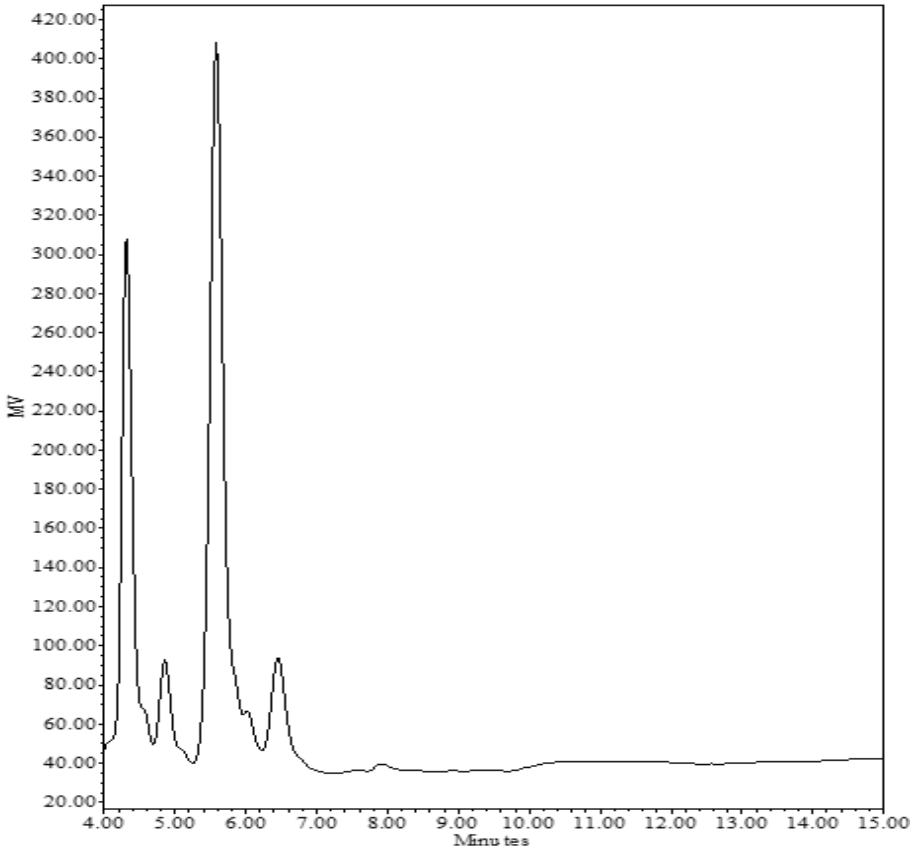


Chromatogram of Vitamin B6: Standard and GC3

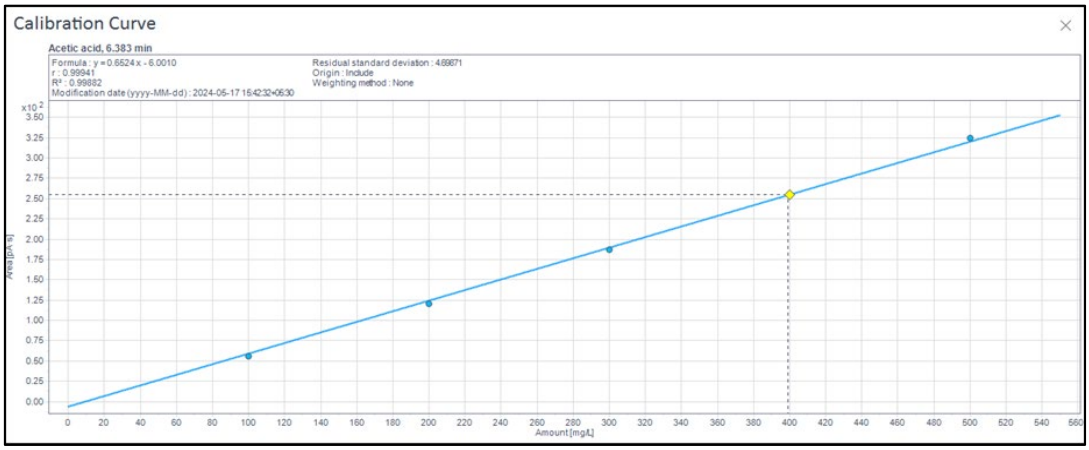


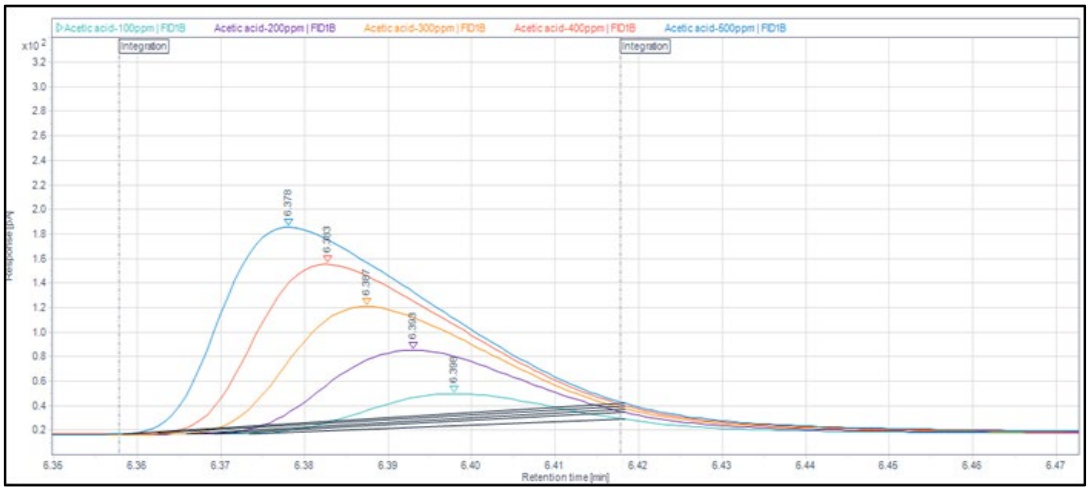
Chromatogram of Oligosaccharide: Standard mix and GC3



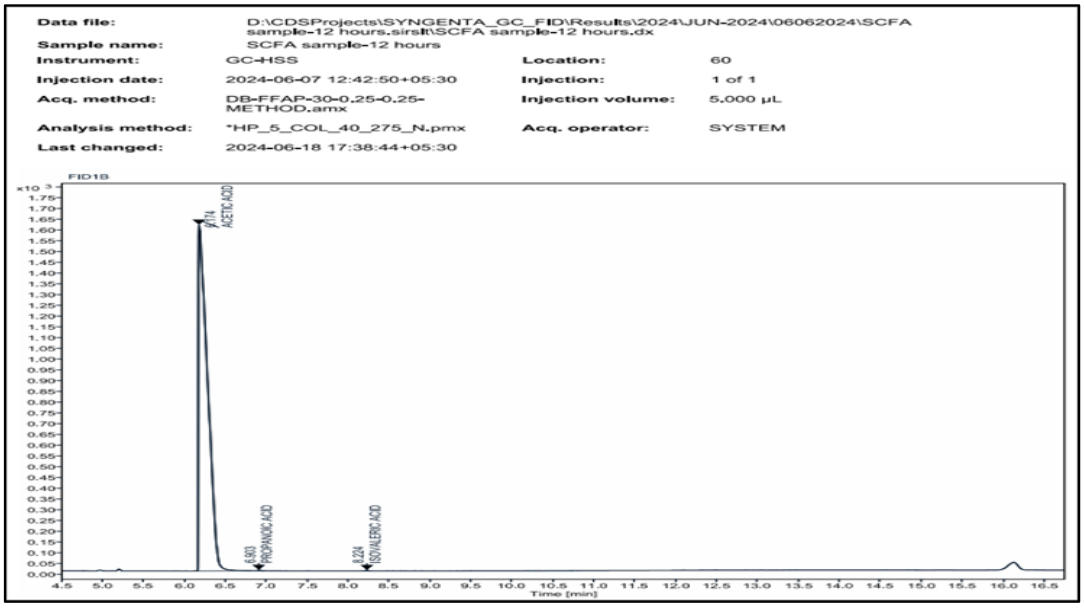


Calibration curve for acetic acid standard





Chromatogram for acetic acid and propionic acids in GC3 on fermentation



Publications

Manuscripts published:

1. **Varsha, N**., Savita, K., Ananthan, R., and Manohara, K. K. (2024). **Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp]: Physico-chemical Characteristics, Nutritional Composition, and Its Efficacy as Probiotic Carrier.** The Journal of Plant Science Research, 40(3), 482-494. doi:10.32381/JPSR.2024.40.03.13.

2. **V. Naik** and S. Kerkar (2024). **Study on Bacterial Growth on the Medium Containing Millet – Legume Mixture**.BIOINFOLET 21 (3), a Quarterly Journal of Life Sciences: 397 – 398. doi:10.5958/0976-4755.2024.00119.8 (Short communication).

Manuscript accepted:

1. **Varsha Naik** and Savita Kerkar (2023). **Saltpan *Bacillus licheniformis* for the development of a fermented functional cowpea powder**. (Short Communication, National Academy of Science Letters, Springer. Revisions completed, Editor assigned).

Manuscript under processing:

1. Plant-Based **Millet and Legume-Derived Biotics: Path to Nourish Gut**. (Review article, Indian Journal of Nutrition and Dietetics). **Varsha Naik** and Savita Kerkar (2023).

2. **गॅस्ट्रोइंटेस्टीनल ट्रॅक्टाच्या भलायकेर कड्डणांतल्यां प्रीबायोटिक क्षमतेचो प्रभाव.** (Mini-Review with original findings in Ananya journal, Goa Konkani Academy, Govt.of Goa). वर्षा नायक आनी सविता केरकार (2025).

Book chapter published:

1. **Varsha Naik** and Savita Kerkar (2024). **Fermentation Assisted Functional Foods**. In: Futuristic Trends in Biotechnology, e-ISBN: 978-93-6252-180-4, IIP Series, Volume 3, Book 6, Part 1, Chapter 2. doi/Link: https://www.doi.org/10.58532/V3BJBT6P1CH2.

Abstracts published/ poster /paper presentations in National and International conferences:

1. **Prospects of saltpan bacteria as potential probiotics with cholesterol lowering effect** by **Varsha Naik** and Savita Kerkar at the International Conference on Environmental Sustainability and Biotechnology: Opportunities and Challenges, by department of Microbiology, Ponda Education Society's, Ravi S. Naik college of Arts and Science, Goa India Department of Microbiology Government College of Arts, Science and Commerce Khandola, Goa, India, Society for Environmental Sustainability (Registered under Societies registration act 1860), Lucknow, Uttar Pradesh, India in collaboration with National Institute of Disaster Management (NIDM), New Delhi and Springer Nature, Goa,16th -19th November 2022.
2. **Cowpea (*Vigna unguiculata*) as a plant-based carrier for potential probiotic bacteria in health promotion** by **Varsha Naik** and Savita Kerkar at PaushtiCon 2023, National E-Conference for oral presentation under the theme- Good Health, Well Being and Nutrition organised by Nutrition Society of India, Mumbai Chapter in collaboration with College of Home Science, Nirmala Niketan, Mumbai; MGM, School of Biomedical Sciences, MGMIHS, Navi Mumbai; Lifeness Science Institute, Mumbai, 27th – 28th January, 2023.
3. **Probiotic delivery through gluten free grain matrix** by **Varsha Naik** and Savita Kerkar at the International Conference on Innovative Advances with Indigenous Knowledge in Family and Community Sciences (Home Science) (Virtual Mode) under the theme-Foods and Nutrition organised by Home Science Association of India, Gujarat Chapter in association with Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, 29th November 2023.
4. ***Bacillus licheniformis* from hypersaline environment as a potential probiotic for enhancing functional aspects of cowpea** by **Varsha Naik** and Savita Kerkar at the 3rd International Conference on Innovations in Biotechnology Research for Sustainable Bioresources and Bioeconomy: Challenges and Practices under the theme Food Biotechnology organised by Sanjivani Arts, Commerce and Science College, Kopargaon (Shirdi), Maharashtra (India), in association with Microbiologists Society, India (MBSI), Ural Federal University, Russia, and Vijnana Bharati (VIBHA), 28th -29th March 2025 (Won **best oral presentation** award).

National Academy Science Letters
https://doi.org/10.1007/s40009-025-01858-1

SHORT COMMUNICATION

Saltpan *Bacillus licheniformis* for Development of a Fermented Cowpea Powder

Varsha Naik[1,2] · **Savita Kerkar**[1]

Received: 12 February 2024 / Revised: 3 September 2025 / Accepted: 26 September 2025
© The Author(s), under exclusive licence to The National Academy of Sciences, India 2025

Abstract

Saltpans are a reservoir to microorganisms with proven biotechnological applications. *Bacillus licheniformis* has been reported in food fermentation and this bacterial isolate from Goan saltpan, fulfilling safety and probiotic attributes was used as a starter culture to develop a fermented powder using a cowpea cultivar *var* Goa Cowpea (GC3) with enhanced nutritional properties. The potential probiotic was tested negative for enterotoxin genesand endotoxins, thus confirming its safety. This study was conducted to assess the fermentation ability of *B. licheniformis* PQ098138 in GC3 and to assess its effect on protein content, anti-nutritional factors like phytate and non-dietary polysaccharides. *B. licheniformis* resulted in effective fermentation of GC3 with a desirable viable count of GC3 slurry (11 log cycle), change in pH, TTA and viscosity. This fermentation resulted in an increase in the protein content from 12.06±0.01% to 25.32±0.01% at 16 h with the detection of 16 free amino acids. There was also a corresponding increase in free amino nitrogen on 16 h fermentation. The fermented GC3 slurry was developed into lyophilized powder which displayed viable count at a desirable level (>7 log cfu/ g). A considerable reduction in phytate, raffinose family oligosaccharides and dietary fiber of fermented GC3 was observed. The use of starter culture facilitated controlled fermentation, eliminating the chances of contamination of powder with pathogens and spoilage organisms.

Keywords *Bacillus licheniformis* · Fermentation · Goa Cowpea · Phytates · Polysaccharides · Protease activity

Abbreviations

GC3	Goa Cowpea
TDF	Total dietary fiber
SDF and IDF	Soluble / Insoluble dietary fibers
ANFs	Anti-nutritional factors
NDPs	Non-dietary polysaccharides
FAN	Free amino nitrogen
RFO	Raffinose family oligosaccharides
TTA	Total titratable acidity
FAAs	Free amino acids

Introduction

Legumes constitute an important part of nutrition and their processing can further enhance its nutritional significance. [1, 2]. Cowpea (*Vigna unguiculata (L.)* Walp) contains 50–60% carbohydrates and 18–35% protein; is rich in tryptophan and lysine but less cysteine and methionine amino acids [3, 4]. Cowpea contains an appreciable amount of vitamin A, iron and calcium [5]. The nutritive value, however can vary depending on the cultivar [4] Additionally, Cowpea is gluten free and has important fermentable components as non-dietary polysaccharides (NDPs) like total dietary fiber (TDF), soluble or insoluble dietary fibers (SDF and IDF) and RFOs (raffinose, stachyose and verbascose); considered as prebiotic substrates for gut bacteria and fermenting microbes [7]. Excess intake of NDPs may cause abdominal discomfort or flatulence [8, 9]. Cowpea contain anti nutritional factors (ANFs) such as tannins, lectins, trypsin inhibitors, phytates, that can affect the nutrient bioavailability [1, 4]. Various methods to degrade ANFs, NDPs and enhance bioavailability of nutrients such as soaking,

✉ Savita Kerkar
drsavitakerkar@gmail.com

Varsha Naik
biotech.varsha@unigoa.ac.in

[1] Biotechnology Discipline, School of Biological Sciences and Biotechnology, Goa University, Taleigao Plateau, Goa 403206, India

[2] Department of Food, Nutrition and Dietetics, Goa College of Home Science, Campal, Panaji, Goa 403001, India

Published online: 01 November 2025

cooking, and germination have been adopted in addition to enzymatic treatment, germination, and fermentation as biological methods [3]. These methods can alter food constituents, increase palatability, organoleptic properties, reduce cooking time and extend shelf life by virtue of microbial assimilation, enzymatic activities and production of metabolites [10]. As heat may result in reducing the nutritive value [2] alternative processing methods are desirable. Controlled fermentation of legumes using safe fermenting bacteria particularly probiotics, can help enhancing its nutritional quality with improved functional and health attributes [10]. Saltpans are a reservoir to numerous bacteria with biotechnological applications [11]. The aim of this study was to develop a fermented Cowpea cultivar 3(GC3) powder with enhanced nutritional and functional properties using a saltpan *Bacillus* sp. The objectives were to determine the effect of *Bacillus* sp. fermentation of GC3 on RFO, TDF, SDF, IDF, phytate, protein content and on the physicochemical parameters.

Saltpan isolate from Ribander, Goa, *B. licheniformis* PQ098138 was studied for its safety by studying for presence of enterotoxin genes; nhe A, nhe B, hblA A and Ent FM using polymerase chain reaction (PCR) [12, 13] and for the presence of endotoxins using Bacterial endotoxin test (BET) [14]. Standard culture of enterotoxin producing *Bacillus cereus* NCIM 2106 and standard endotoxin from *E. coli* UKT-B (G-96 USA) were used as a positive control respectively. Seeds of GC3, a released cultivar from ICAR, Old Goa were ground in a hammer mill. GC3 flour slurry of 10% (w/v) was autoclaved (15psi,15–20 min, 121 °C), cooled and inoculated with *B. licheniformis* showing probiotic attributes; with an initial cell count of approx. 5 log cfu/mL slurry and incubated at 37 °C for up to 80 h at 150 rpm to facilitate fermentation. Viable cell count (log cfu/mL) of *Bacillus* sp. was enumerated at different fermentation times by spread plating along with the physicochemical parameters like pH (pH meter, Eutech instruments, India), total titrable acidity (TTA) as % lactic acid and viscosity (Brookfield Viscometer) in millipascal second (mPa.s) using standard protocols. The fermented GC3 with favorable attributes was subjected to lyophilization without any protective medium to develop in to powder and its viable cell count was enumerated.

Table 1 Mean values of pH, TTA and viscosity during fermentation of GC3

Fermentation Period (hours)	Fermentation parameters		
	pH	TTA (% acetic acid)	Viscosity (mPa.s)
0	6.54±0.02	*0.26±0.04	*580±13
12	5.20±0.01	0.58±0.03	498±09
16	5.80±0.01	*0.63±0.03	*401±09
32	6.03±0.01	0.28±0.03	298±09
48	7.69±0.03	*0.23±0.05	*177±06
64	8.10±0.2	0.22±0.05	171±06
80	7.41±0.02	0.23±0.01	169±09

GC3-Goa Cowpea 3, *Mean levels are significantly different

Protein of GC3 seeds and free amino nitrogen (FAN) was analysed before and after fermentation at different time intervals using Lowry's method. Free amino acids (FAAs) were determined after 16 h and 80 h of fermentation using HPLC with diode array detector using Agilent ZORBAX Eclipse plus C18 Column (4.6 × 250 mm) and modified ninhydrin colorimetric method (absorbance 504 nm) [2]. Simple sugars and easily fermentable oligosaccharides provide easily metabolizable substrates for rapid microbial growth and are utilized by the beneficial microbes as prebiotics [8, 15]. Since the ability of any culture to metabolize different sugars widely varies, the profile of dietary fiber (enzymatic kit method, Megazyme International) and RFO of unprocessed and fermented lyophilized GC3 powder by HPLC, Waters 2625 module, Mitford, Massachusetts, USA with a Supelco NH2 polymer column (5 µm, 25 cm x 46 mm) equipped with refractive index (RI) detector was studied (Xiaoli et al. 2008). Analysis was conducted in triplicate and the data expressed as the mean ± standard deviation with significant differences at $P < 0.05$.

The prospective probiotic did not produce any endotoxins and did not show presence of enterotoxin genes, indicating its safety for use in GC3 fermentation. Fermented legumes can serve as carriers of probiotics, and may generate desirable metabolites which can support the gut health without causing undesirable sensory changes in products on prolonged storage [15]. Spore forming *Bacillus* sp. probiotic strains have better survival rates and stability to processing conditions such as heat, cold, moisture, dehydration and gastric conditions [16]. The probiotic addition to nutrient rich food matrix may bring additional health benefits [17, 18]. The 16 h fermentation resulted in increased viable cell count of *Bacillus* sp. from 5.58 log cfu/mL to 11 log cfu/mL slurry, proving GC3 as an effective substrate. The lyophilized powder although showed a significant reduction ($p < 0.05$) in the viable count of bacillus on lyophilization to approximately 9 log cfu/g, that could be attributed to freeze stress, however, it indicated the natural cryoprotectant ability of GC3 matrix in maintaining the cell count to appreciable number. The potential to increase the population of beneficial bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp. by dietary fibers and NDPs has been recognized [19].

The TTA and pH are important during fermentation. The pH decreased from 6.54±0.02 to 5.8±0.01 after 16 h fermentation with a significant increase in TTA of 0.63±0.03 ($p < 0.05$) and the pH reached up to 8.1±0.01 at 64 h with ammoniacal smell of GC3 slurry (Table 1). The drop in pH could be attributed to the production of organic acids from

utilization of carbohydrate in GC3 [20]. The increased pH could be due to ammonia production on protein hydrolysis, and deaminase activity of the fermenting bacteria following utilization of amino acids as energy and carbon sources [2]). In alkaline-fermentation the substrate pH (usually protein-rich foods) increases to 8–10, mostly due to protein degradation/hydrolysis to peptides and essential amino acids (EAAs) and its further degradation to ammonia by dominant *Bacillus* spp contributing to a strong ammoniacal smell [21]. Viscosity is an important parameter for any cereal legume-based formulations [16, 17, 20], viscosity of GC3 significantly reduced with increased fermentation times, with no significant change after 48 h. Viscosity changes could be attributed to physicochemical changes during fermentation and its reduction is a valuable strategy in optimizing fermentation processes, improving product quality, and increasing efficiency across various applications [19, 20].

A significant increase in the protein content of GC3 slurry more than twofold, was observed at 16 h fermentation ($p<0.05$), with a further reduction from 64 h and above ($p>0.05$) (Fig. 1a). This increase could probably be due to partial solubilization of insoluble protein or due to microbial metabolic activities, or may partly be due to release of peptides and amino acids on microbial degradation of complex proteins [20, 21, 23]. A significant increase in protein content by 15.32% was reported in pearl millet over 16 h of fermentation [19, 20, 22]. Several studies have reported an increase in protein and FAAs content of legumes after fermentation [19, 22, 23]. However, studies also report that fermenting microorganisms could utilize amino acids thus lowering the protein content and quality of the fermented food [20, 23, 24]. In the present study only 6 FAAs (aspartic acid, glutamic acid, serine, histidine, arginine and alanine) were detected on heat treatment of GC3 with a depletion of several essential amino acids (EAAs) whereas 16 FAAs inclusive of most of EAAs (aspartic acid, glutamic acid, asparagine, serine, threonine, arginine, alanine, tyrosine, cysteine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine and lysine) were estimated at 16 h *Bacillus* sp. fermentation, with a significant overall increase from 0.26 ± 0.02 mg/mL to 0.68 ± 0.02 mg/mL ($p<0.05$). Fermentation can improve the nutritional value of foods by the action of proteases and amylases on protein and insoluble carbohydrates respectively [1, 6, 19]. Increased levels of

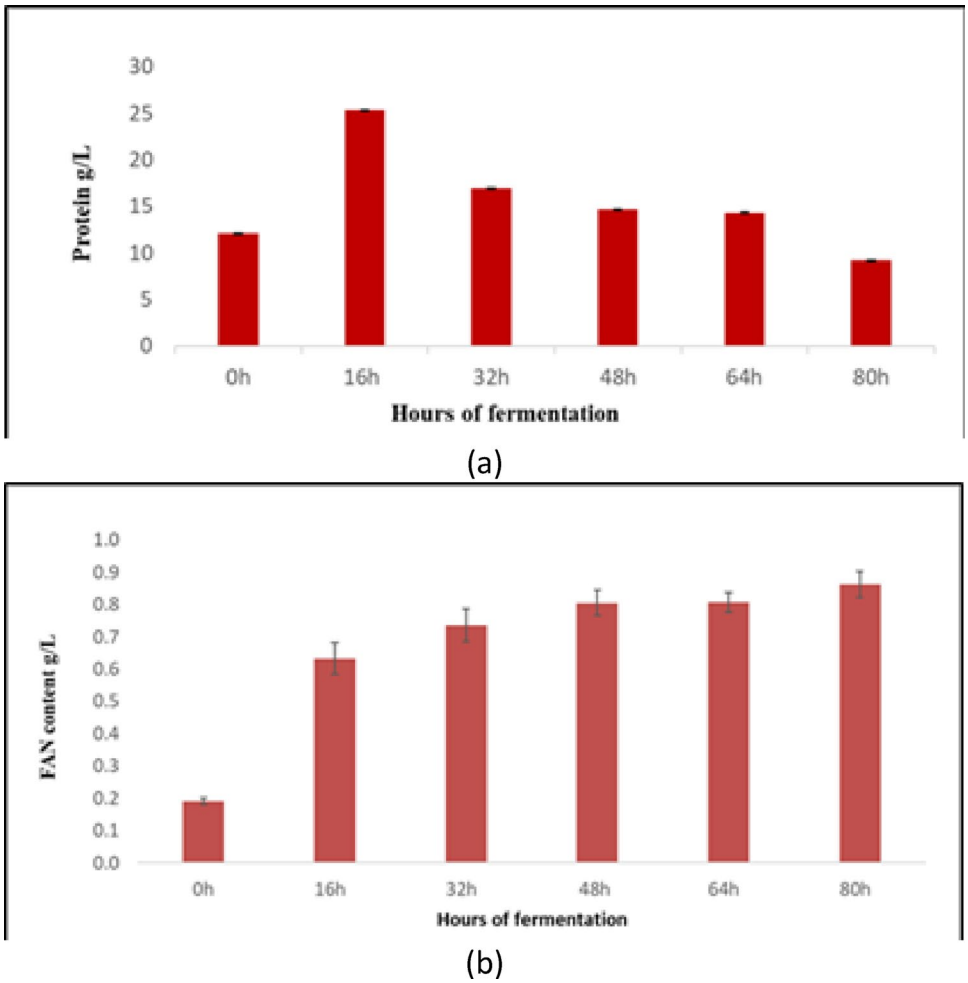


Fig. 1 **a** Effect of fermentation on protein content of Goa Cowpea 3, **b** Effect of fermentation on free amino nitrogen of Goa Cowpea 3

Table 2 Effect of fermentation on dietary fiber and raffinose oligosaccharide content of GC3

Percent (%)	Fermentation (16 h)	
Dietary fiber	Before	After
SDF	4.43±0.2	3.12±0.05
IDF	19.44±0.36	17.21±0.2
TDF	23.87±0.59	20.33±0.2
Percent (%)	Fermentation (16 h)	
Raffinose oligosaccharide (RFO)	Before	After
Raffinose	0.22±0.01	0.31±0.01
Stachyose	0.91±0.08	0.69±0.01
Verbascose	0.27±0.01	0.06±0.01
Total RFO	1.41±0.08	1.07±0.01

*SDF-Soluble dietary fiber, IDF-Insoluble dietary fiber, TDF-Total dietary fiber

non-protein, soluble nitrogen, FAAs with increased concentration of methionine, cysteine isoleucine, phenylalanine, leucine, tyrosine and lysine (limiting amino acid in plant sources) are reported on fermentation [20]. Degradation of native proteins is crucial to enhance the FAA bioavailability and to improve the digestibility of polypeptides and release of potential bioactive peptides with enhanced taste and aroma [7, 21, 22]. GC3 showed an increase in FAN (sum of the individual amino acids and small peptides) as the fermentation progressed with time, with highest amount at 80 h fermentation (Fig. 1b) indicating high proteolytic activity.

Table 2 indicates quantitative analysis of dietary fiber and RFOs in GC3 before and after fermentation; *Bacillus* fermentation reduced TDF to 20.33±0.2%. About 10% reduction in the TDF and IDF on cereal fermentation is reported [1, 7, 24] due to increased activity of hydrolyzing enzymes such as cellulase, α-galactosidase, etc., that cause rapid hydrolysis of the IDF and its conversion into SDF or partial solubilization of cellulose and hemicellulose type of materials by microbial enzymes [2]. Other authors suggested the fiber reduction in fermented maize flour (74%) due to enzymatic breakdown by lactic acid bacteria, that utilized the fiber as a carbon source [5, 7, 8]. RFOs act as functional prebiotics and enable the action of beneficial microbes; fermenting microorganisms hydrolyze starch and non-digestible carbohydrates into readily digestible sugars and additionally influences product's texture due to tissues softening [8, 17]. A non-significant ($p>0.05$) reduction in total RFOs on GC3 fermentation was observed with more reduction in stachyose and verbascose content indicating their utilization by the bacteria.

Phytates, usually forms complexes with zinc, iron and calcium; affecting their bioavailability. Microbial enzymes at specific pH can degrade these complexes by unfolding the bonding between the phosphorus of phytic acid and the minerals and release soluble minerals [16, 24]. A reduction in phytate was seen in fermented GC3 powder from 326.22%±6.87 to 319.19%±8.6 with no change being observed on heat processing. Reduced levels of phytic acid and oxalates by up to 44.4 and 50.9% each is reported by a study on fermentation of accessions of African locust bean [25]. Another study reported a significant decrease in phytic acid (92%), saponin (60%), trypsin inhibitors (45%), and tannin contents (65%) after cowpea fermentation [2, 16].

The study thus reveals that the effective fermentation of GC3 with *B. licheniformis* results in enhanced protein and FAN content. This fermentation further reduced the levels of TDF, RFO and phytate, and can enhance the bioavailability of GC3 nutrients. GC3 proved to be an effective substrate resulting in desirable viability of *B. licheniformis*. The controlled fermentation eliminated the chances of contamination of the powder with pathogens and spoilage organisms. Fermented GC3 powder inclusive of a prospective *Bacillus* probiotic has a potential to be used as a dietary supplement owing to its enhanced quality attributes, and gluten free property subject to further clinical trials, and sensory evaluation. Novel safe fermenting microorganisms with more systematic research approach on indigenous plant-based products are of great demand that can provide good quality and inexpensive protein with valuable benefits.

Acknowledgements We are wholeheartedly thankful to National Institute of Oceanography, Goa for providing with laboratory facilities.

Declarations

Conflict of interest The authors have no conflict of interest.

References

1. Adebo JA, Njobeh PB, Gbashi S, Oyedeji AB, Ogundele OM, Oyeyinka SA, Adebo OA (2022) Fermentation of cereals and legumes: impact on nutritional constituents and nutrient bioavailability. Fermentation 8(2):63. https://doi.org/10.3390/fermentation8020063
2. Arbab Sakandar H, Chen Y, Peng C, Chen X, Imran M, Zhang H (2023) Impact of fermentation on antinutritional factors and protein degradation of legume seeds: a review. Food Rev Int 39(3):1227–1249. https://doi.org/10.1080/87559129.2021.1931300
3. Huang Z, Liu H, Zhao L, He W, Zhou X, Chen H, Zhou J, Liu Z (2022) Evaluating the effect of different processing methods on fermented soybean whey-based tofu quality, nutrition, and flavour. LWT 158:113139. https://doi.org/10.1016/j.lwt.2022.113139
4. Nkhata SG, Ayua E, Kamau EH, Shingiro JB (2018) Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. Food Sci Nutr 6(8):2446–2458. https://doi.org/10.1002/fsn3.846
5. Jayathilake C, Visvanathan R, Deen A, Bangamuwage R, Jayawardana BC, Nammi S, Liyanage R (2018) Cowpea: an

overview on its nutritional facts and health benefits. J Sci Food Agric 98(13):4793–4806. https://doi.org/10.1002/jsfa.9074
6. Kaur S, Kaur S, Gupta AK, Kaur N, Javed M (2014) Biochemical and nutritional characterization of chickpea (Cicer arietinum) cultivars. Indian J Agri Sci 84(4):479–486. https://doi.org/10.56093/ijas.v84i4.3946
7. Clemente A, Olias R (2017) Beneficial effects of legumes in gut health. Curr Opin Food Sci 14:32–36. https://doi.org/10.1016/j.cofs.2017.01.005
8. Elango D, Rajendran K, Van der Laan L, Sebastiar S, Raigne J, Thaiparambil NA, Singh AK (2022) Raffinose family oligosaccharides: friend or foe for human and plant health? Front Plant Sci 13:829118. https://doi.org/10.3389/fpls.2022.829118
9. Mysonhimer AR, Holscher HD (2022) Gastrointestinal effects and tolerance of nondigestible carbohydrate consumption. Adv Nutr 13(6):2237–2276. https://doi.org/10.1093/advances/nmac094
10. Marco ML, Heeney D, Binda S, Cifelli CJ, Cotter PD, Foligné B, Hutkins R (2017) Health benefits of fermented foods: microbiota and beyond. Curr Opin Biotechnol 44:94–102. https://doi.org/10.1016/j.copbio.2016.11.010
11. Selvarajan R, Sibanda T, Tekere M, Nyoni H, Meddows-Taylor S (2017) Diversity analysis and bioresource characterization of halophilic bacteria isolated from a South African saltpan. Molecules 22(4):657. https://doi.org/10.3390/molecules22040657
12. Ali NA, & Hussain AS (2015) Detection of Enterotoxin (hblA, hblC and hblD) Genes of Bacillus cereus Isolates from Different Food Samples Using Traditional and Molecular Methods. Iraqi J Biotechnol 14(2).
13. Bhutia MO, Thapa N, Tamang JP (2021) Prevalence of enterotoxin genes and antibacterial susceptibility pattern of pathogenic bacteria isolated from traditionally preserved fish products of Sikkim, India. Food Control 125:108009
14. Sandle T (2011) A practical approach to depyrogenation studies using bacterial endotoxin. J GXP Compliance 15(4):90
15. Tomasik P, Tomasik P (2020) Probiotics, non-dairy prebiotics and postbiotics in nutrition. Appl Sci 10(4):1470. https://doi.org/10.3390/app10041470
16. Zheng L, Li D, Li ZL, Kang LN, Jiang YY, Liu XY, Chi Y-P, Li Y-Q, Wang JH (2017) Effects of *Bacillus* fermentation on the protein microstructure and anti-nutritional factors of soybean meal. Lett Appl Microbiol 65(6):520–526. https://doi.org/10.1111/lam.12806
17. Fan P, Li L, Rezaei A, Eslamfam S, Che D, Ma X (2015) Metabolites of dietary protein and peptides by intestinal microbes and their impacts on gut. Curr Protein Pept Sci 16(7):646–654. https://doi.org/10.2174/1389203716666150630133657
18. Valero-Cases E, Cerdá-Bernad D, Pastor JJ, Frutos MJ (2020) Non-dairy fermented beverages as potential carriers to ensure probiotics, prebiotics, and bioactive compounds arrival to the gut and their health benefits. Nutrients 12(6):1666. https://doi.org/10.3390/nu12061666
19. Sharma R, Garg P, Kumar P, Bhatia SK, Kulshrestha S (2020) Microbial fermentation and its role in quality improvement of fermented foods. Fermentation 6(4):106. https://doi.org/10.3390/fermentation6040106
20. Pranoto Y, Anggrahini S, Efendi Z (2013) Effect of natural and *Lactobacillus plantarum* fermentation on in-vitro protein and starch digestibilities of sorghum flour. Food Biosci 2:46–52. https://doi.org/10.1016/j.fbio.2013.04.001
21. Roy F, Boye JI, Simpson BK (2010) Bioactive proteins and peptides in pulse crops: pea, chickpea and lentil. Food Res Int 43(2):432–442. https://doi.org/10.1016/j.foodres.2009.09.002
22. Nour AAM, Mohamed AR, Adiamo OQ, Babiker EE (2018) Changes in protein nutritional quality as affected by processing of millet supplemented with Moringa seed flour. J Saudi Soc Agric Sci 17(3):275–281. https://doi.org/10.1016/j.jssas.2016.05.006
23. Awolu OO, Olarewaju OA, Akinade AO (2017) Effect of the addition of pearl millet flour subjected to different processing on the antioxidants, nutritional, pasting characteristics and cookies quality of rice based composite flour. J Nutritional Health Food Eng 7(2):00232. https://doi.org/10.15406/jnhfe.2017.07.00232
24. Garrido-Galand S, Asensio-Grau A, Calvo-Lerma J, Heredia A, Andrés A (2021) The potential of fermentation on nutritional and technological improvement of cereal and legume flours: A review. Food Res Int 145:110398. https://doi.org/10.1016/j.foodres.2021.110398
25. Omodara TR, Aderibigbe EY (2018) Microbiological, physicochemical and enzymatic changes in fermented African locust bean (*Parkia biglobosa*) seeds using *Bacillus subtilis* and additives. Sustainable Food Production 1:22–29. https://doi.org/10.18052/www.scipress.com/SFP.1.22

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

The Journal of Plant Science Research

2024 Volume 40 Number 3

CONTENTS

Editorial (i-ii)
Ashwani Kumar

Phytochemical and Pharmacological Review of *Commiphora berryi* (Arn.) Engl. (Burseraceae): An Endemic Medicinal Plant 357-364
Gopal T.C., Sidanand V. Kambhar and M. Ajmal Ali

Plants used as Herbal Remedies to Cure Diabetes in Gorakhpur, Uttar Pradesh, India 365-376
Harshita Pathak, Kshama Tripathi, Preeti Prakash, Vikas Singh, Shashank Kumar Singh,S. Dominic Rajkumar and Alok Ranjan Rai

Screening of Antibacterial Activity and Phytochemical Analysis of *Acalypha Indica* L.: A Study with Clinical Aspect 377-381
M. Guru Sekhar, K.R. Shanmugam, K. Siva Prasad and Ch. Ramakrishna

Cytotoxic Potentiality of Leaflet with Rachis Extract of *Simarouba glauca* DC. against MDA-MB-231- A Triple-negative Breast Cancer (TNBC) Cell Lines 382-391
M.B. Manoj and M.H. Niranjan

Soil Bacteria Isolated from the Paddy Field of T. Athikarai, Sivagangai District, Tamil Nadu 392-398
O. Muthurakku and B. Sadhana

Responses of Aromatic Rice Cultivars to Induced Drought Stress at Seedling Stages using PEG-6000 399-410
Sheikh Benazir, Rozina Yendrembam and Kananbala Sarangthem

Assessment of Lead Bioaccumulation in soybean at Different Growth Stages 411-417
Siddhi Gupta and Manoj Kumar Meena

***In vitro* Plant Development of *Aerides Multiflora* Roxb. and Genetic Fidelity Assessment through RAPD Marker** 418-425
Purnima Paramanik, Subrata Raha and Dipak Kumar Kar

Floristic Study of Garudeshwar and Nandod Taluka of Narmada District, Gujarat, India 426-451
Mayuri C. Rathod, Dhale D. A. and Henixa N. Patel

Systematic Enumeration of Algal Taxa in Relation to Water Quality of Murguma dam, Purulia District, West Bengal 452-457
Pinaki Dey and Latika Sharan

Micropropagation of Vetiver: A Sustainable Approach to Production of Essential Oil 458-463
Roshita Shrivastava, Ankan Singh, Shailja Verma and Rajneesh K. Agnihotri

Infrageneric Variability of Seed Coat Morphology in Some Members of *Blyxa* spp. Thou. (Hydrocharitaceae) from Eastern India and the Taxonomic Utility of Seed Characters 464-481
Debasis Bhunia

Continued on inside back cover

2024 THE JOURNAL OF PLANT SCIENCE RESEARCH VOLUME 40 (3)

ISSN : 0970-2539
E-ISSN : 0976-3880

THE JOURNAL OF PLANT SCIENCE RESEARCH

VOLUME 40 NUMBER 3, 2024

EDITOR-IN-CHIEF
ASHWANI KUMAR

PRESIDENT
P. C. TRIVEDI

PRINTS PUBLICATIONS PVT LTD
NEW DELHI

FORUM FOR THE PROMOTION OF PLANT SCIENCE RESEARCH

Forum for the Promotion of Plant Science Research

President : **Dr. P. C. Trivedi, Former Vice Chancellor, Jai Narain Vyas University, Jodhpur, M.D.S. University, Ajmer, DDU Gorakhpur University, Gorakhpur, Dr. R.M.L. Avadh University, Faizabad, Maharaja Ganga Singh University, Bikaner, Raj. Former Head, Botany and Biotechnology, University of Rajasthan, Jaipur, Email: pctrivedi33@gmail.com**

Vice Presidents : **Dr Rakesh Pandey, CSIR-Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow. Email: rakeshpandey66@gmail.com**

Prof. N K Dubey, FNASc, FNAAS, Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi-221005. Email: nkdubeybhu@gmail.com

Founder Chief Editor : **Late Prof. C P Malik, FNA, Formerly at School of Life Sciences, Jaipur National University, Jaipur.**

Editor-in-Chief : **Prof. Ashwani Kumar, University of Rajasthan, Jaipur-302004. Email: kumar.ashwani104@gmail.com**

Editorial Board : **Dr. Krishnendu Mukherjee, Senior Scientist, Muenster 48149, Germany. Email: mukherjee.krishnendu@gmail.com**

Prof. Shinjiro Ogita, Prefectural, University of Hiroshima, Hiroshima, Japan. Email: ogita@puhiroshima.ac.jp

Prof. Yuan-Yeu Yau, Northeastern State University, Broken Arrow, Oklahoma, USA. Email: academicfy1@gmail.com

Advisor : **Prof. H N Verma, Pro-Chancellor, Jaipur National University, Jaipur. Email: vermalko@yahoo.co.uk**

Submitting Articles to the Journal of Plant Science Research

Contribution/manuscripts should be sent to Prof. Ashwani Kumar, Editor-in-chief, H. No. 2, Kha 14, Sector-2 Jawaharnagar, Jaipur-302004, India or through e-mail on ashwanikumar214@gmail.com.

SUBSCRIPTIONS :

Annual Subscription Price	
₹ 3000.00 (National)	US$ 300.00 (International)

Online version free with print subscription

All remittance must be paid in favour of **Prints Publications Pvt Ltd** payable at **New Delhi.**

Periodicity : This journal is published Tri-annually.

Electronic Version : This Journal can be accessed electronically at http://www.printspublications.com

© Prints Publications Pvt Ltd

DOI No. : 10.32381/JPSR

This journal is Indexed in
UGC, Proquest (USA), EBSCO Publishing (USA), CABI, Genamics (Journal Seek)

Note : All views expressed in the articles are that of the authors. The Publishers are not responsible for the contents of the authors.

Published by :

Prints Publications Pvt Ltd
Viraj Tower 2, 4259/3,
Ansari Road, Darya Ganj,
New Delhi-110 002. India

Phone : +91-11-45355555
E-mail : **contact@printspublications.com**
Website : **www.printspublications.com**

Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp]: Physico-chemical Characteristics, Nutritional Composition and Its Efficacy as Probiotic Carrier **482-494**
Naik Varsha, Kerkar Savita, Rajendran Ananthan and KK Manohara

Assessment of *In Vitro* Antioxidant Efficacy and Phytochemical Profiling in Different Solvent Extracts of Cannonball tree (*Couroupita guianensis* Aubl.) **495-503**
Saran Kumar Gupta and Suchisree Jha

Multivariate Analysis of Seed Germination and Early Seedling Growth in Fenugreek (*Trigonella foenum-graecum* L.) Varieties under Salt Stress **504-517**
Pramod V. Shelar, Ganesh D. Mankar, Om P. Sontakke, Uttam R. Wayase and Rajkumar B. Barmukh

Observation of Phyto-geological Relationship in Forest of Ramgarh Vishdhari Wildlife Sanctuary, Rajasthan **518-522**
O. P. Sharma

Diversity Assessment, Utility, Ethno Medicinal Uses and Conservation Status of Medicinal Plants in Tehri District, Uttarakhand, India **523-536**
Pradeep Kumar, G. K. Dhingra and L. R. Dangwal

Conservation, Phytochemical investigations and Medicinal Attributes of *Gentiana kurroo* Royle: A Review **537-546**
Deepanshu Kumawat, Pooja Mangal, Papita Choudhary, Jai Singh, Shikha Gupta and Amit Kotiya

The Journal of Plant Science Research

2024 Volume 40 Number 3

CONTENTS

Editorial (*i-ii*)
Ashwani Kumar

Phytochemical and Pharmacological Review of *Commiphora berryi* (Arn.) Engl. (Burseraceae): An Endemic Medicinal Plant **357-364**
Gopal T.C., Sidanand V. Kambhar and M. Ajmal Ali

Plants used as Herbal Remedies to Cure Diabetes in Gorakhpur, Uttar Pradesh, India **365-376**
Harshita Pathak, Kshama Tripathi, Preeti Prakash, Vikas Singh, Shashank Kumar Singh, S. Dominic Rajkumar and Alok Ranjan Rai

Screening of Antibacterial Activity and Phytochemical Analysis of *Acalypha Indica* L.: A Study with Clinical Aspect **377-381**
M. Guru Sekhar, K.R. Shanmugam, K. Siva Prasad and Ch. Ramakrishna

Cytotoxic Potentiality of Leaflet with Rachis Extract of *Simarouba glauca* DC. against MDA-MB-231- A Triple-negative Breast Cancer (TNBC) Cell Lines **382-391**
M.B. Manoj and M.H. Niranjan

Soil Bacteria Isolated from the Paddy Field of T. Athikarai, Sivagangai District, Tamil Nadu **392-398**
O. Muthurakku and B. Sadhana

Responses of Aromatic Rice Cultivars to Induced Drought Stress at Seedling Stages using PEG-6000 **399-410**
Sheikh Benazir, Rozina Yendrembam and Kananbala Sarangthem

Assessment of Lead Bioaccumulation in soybean at Different Growth Stages **411-417**
Siddhi Gupta and Manoj Kumar Meena

***In vitro* Plant Development of *Aerides Multiflora* Roxb. and Genetic Fidelity Assessment through RAPD Marker** **418-425**
Purnima Paramanik, Subrata Raha and Dipak Kumar Kar

Floristic Study of Garudeshwar and Nandod Taluka of Narmada District, Gujarat, India **426-451**
Mayuri C. Rathod, Dhale D. A. and Henixa N. Patel

Systematic Enumeration of Algal Taxa in Relation to Water Quality of Murguma dam, Purulia District, West Bengal **452-457**
Pinaki Dey and Latika Sharan

Micropropagation of Vetiver: A Sustainable Approach to Production of Essential Oil **458-463**
Roshita Shrivastava, Ankan Singh, Shailja Verma and Rajneesh K. Agnihotri

Infrageneric Variability of Seed Coat Morphology in Some Members of *Blyxa* spp. Thou. (Hydrocharitaceae) from Eastern India and the Taxonomic Utility of Seed Characters **464-481**
Debasis Bhunia

Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp]: Physico-chemical Characteristics, Nutritional Composition and Its Efficacy as Probiotic Carrier **482-494**
Naik Varsha, Kerkar Savita, Rajendran Ananthan and KK Manohara

Assessment of *In Vitro* Antioxidant Efficacy and Phytochemical Profiling in Different Solvent Extracts of Cannonball tree (*Couroupita guianensis* Aubl.) **495-503**
Saran Kumar Gupta and Suchisree Jha

Multivariate Analysis of Seed Germination and Early Seedling Growth in Fenugreek (*Trigonella foenum-graecum* L.) Varieties under Salt Stress **504-517**
Pramod V. Shelar, Ganesh D. Mankar, Om P. Sontakke, Uttam R. Wayase and Rajkumar B. Barmukh

Observation of Phyto-geological Relationship in Forest of Ramgarh Vishdhari Wildlife Sanctuary, Rajasthan **518-522**
O. P. Sharma

Diversity Assessment, Utility, Ethno Medicinal Uses and Conservation Status of Medicinal Plants in Tehri District, Uttarakhand, India **523-536**
Pradeep Kumar, G. K. Dhingra and L. R. Dangwal

Conservation, Phytochemical investigations and Medicinal Attributes of *Gentiana kurroo* Royle: A Review **537-546**
Deepanshu Kumawat, Pooja Mangal, Papita Choudhary, Jai Singh, Shikha Gupta and Amit Kotiya

Editorial

The September to December 2024 issue 40 (3) of The Journal of Plant Science Research (JPSR), started by the Late Professor C. P. Malik, is a UGC care Journal. We have been receiving a large number of valuable articles from different states of the country. Our team of reviewers, assisted by Ms Shyaloo and Ms Jasbeer at Prints, helps us make final selections of the articles included in this issue.

Floristic research plays a crucial role in evaluating a given area's botanical richness and potential. A floristic study of Garudeshwar and Nandod taluka of the Narmada district of Gujarat has been presented. (Mayuri et al. this volume). These studies will contribute to informed decision-making regarding land use, biodiversity conservation, and sustainable development in the area. Ramgarh Vishdhari Wildlife Sanctuary of Bundi District, Rajasthan, is a single compact and large forest ecosystem in the Haroti region. The study attempts to analyse the Phyto-geological relationship with the forest flora of Ramgarh Vishdhari Wildlife Sanctuary of Rajasthan. (Sharma, this volume). Seed coat analysis is a significant diagnostic characteristic that can be utilised for taxonomic identification and helps construct an identification key. The genus *Blyxa* is a dominant aquatic plant genus of the family Hydrocharitaceae. The taxonomic utility of seed characters has been studied using seed coat morphology in some members of *Blyxa* spp—thou (Bhunia this volume). Maintenance of freshwater resources and quality of their water is a big challenge in sustainable development. Many algal taxa are regarded as bioindicators. They are pollution tolerant and integral to low-cost biological purification systems used for polluted water, especially those with organic pollutants. Studies have been reported on algal taxa and water quality from Murgumadam Purulia District, West Bengal. Algal tools in water quality monitoring and phytoremediation can be of great help. (Dey and Sharan, this volume).

Herbal medicines are used to cure different diseases. Pathak et al. (this volume) presented a comprehensive documentation of approximately 31 plant species utilised by local communities as herbal remedies for diabetes from Gorakhpur, Uttar Pradesh. Manoj and Niranjan (this volume) reported cytotoxic potentiality of the leaflet with rachis extract of Simarouba glauca DC. against MDA-MB-231-Triple-negative Breast Cancer (TNBC) Cell Lines. However, the authors suggested "further investigation before their utilisation". Some other phytochemical, pharmacological and antibacterial studies have been reported on *Acalypha indica* L (Guru Sekhar et al., this volume). In India, the bark and leaf of *Commiphora berryi* (Arn.) Engl in Karnataka is used to cure colds, fever, wound healing and as an anti-inflammatory agent (Gopal et al. this volume). Kumar et al. (this volume) carried out studies in the Chamba, Narendra Nagar, Jakhnidhar, Thauldar, Pratapnagar, Hindolakhal, Bhilangna, Kirtinagar and Jaunpur blocks of Tehri District, Uttarakhand. The study aimed to assess plant diversity, utilisation patterns, and ethno-medicinal uses by residents. *Gentiana kurroo* Royle, a well-known Himalayan medicinal herb, possesses many bioactive compounds. These phytochemicals can potentially treat various ailments like Alzheimer's, inflammation, immune diseases, urinary infections, bronchial asthma, anorexia, leukoderma, skin disorders etc. The species is currently endangered in India's native habitats. Because of the plant's therapeutic value, conservation studies are required to ensure its survival (Kumawat et al., this volume).

Muthurakku and Sadhana (this volume) isolated soil bacteria in paddy field soil of Sivagangai District, Tamil Nadu, and suggested that these bacteria can enhance nutrient solubilisation. Benazir et al. (this volume) studied the influence of drought stress at seedling stages of rice cultivars R005, R004 and R001. Screening for salt tolerance can be achieved through a rapid, cost-effective, and reliable approach using seed germination and early seedling growth attributes. Different varieties of *Trigonella foenum-graecum* L have been tested for

salt stress (Shelar et al., this volume). This research provides a valuable framework for understanding fenugreek's physiological responses to salinity. It offers key insights for breeding programs to enhance salt tolerance in fenugreek and potentially other crops. Various external and internal factors influence the availability of lead in soil and its uptake by plants. Gupta and Meena (this volume) reported the bioavailability of lead in soybean plants, highlighting the need for effective strategies to mitigate potential risks to human health. *In vitro* plant regeneration of *Vetiveria zizanioides* (L.) Nash, which is synonymously known as *Chrysopogon zizanioides* (L.) Roberty has been successfully achieved using a callus-mediated indirect organogenesis method. Such advancements will benefit commercial cultivation and essential oil production and support sustainable environmental management practices (Shrivastava et al. this volume). *Aerides multiflora* Roxb. is popularly known as the Fox Tail Orchid. Paramanik et al. (this volume) studied *in vitro* micropropagation and establishment of genetic fidelity by using RAPD marker. This study can also be successfully applied to large-scale production in the pharmaceutical and floriculture industries.

Cowpea is a viable gluten-free option for patients with celiac disease. This nutritionally rich Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp] could effectively be used as a food substrate for probiotic growth. The cannonball tree (*Couroupita guianensis* Aubl.) is a tropical plant belonging to the Lecythidaceae family. It has a great potential source of bioactive compounds due to its diverse ethnobotanical uses. *Couroupita guianensis* is a promising natural antioxidant source for nutraceutical and functional food applications (Gupta and Jha this volume).

As editor-in-chief, I acknowledge the support of our team of reviewers along with Ms. Shyaloo and Ms. Jasbeer, and the entire team at Prints Publications for bringing out the issue excellently. We acknowledge Mr Pranav Gupta's unwavering support for maintaining this publication's high quality. Mr Pranav Gupta holds several positions in the publication world and this year represented us at the Frankfurt Book Fair.

Ashwani Kumar
Editor-in-chief

Jour Pl Sci Res **40 (3)** 482–494 2024

Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp]: Physico-chemical Characteristics, Nutritional Composition and Its Efficacy as Probiotic Carrier

Naik Varsha[1,2], Kerkar Savita[1], Rajendran Ananthan[3] and KK Manohara[4]

[1]*Discipline of Biotechnology, School of Biotechnology and Biological Sciences (SBSB), Goa University, Taleigao Plateau, Goa, India.*

[2]*Discipline of Food, Nutrition and Dietetics, Goa College of Home Science, affiliated to Goa University, Campal, Panaji, Goa, India.*

[3]*Food Chemistry Division, ICMR-National Institute of Nutrition, Hyderabad, Telangana, India.*

[4]*Crop Sciences Section, ICAR - Central Coastal Agricultural Research Institute, Ela, Old Goa, Goa, India.*

**Corresponding author email: drsavitakerkar@gmail.com*

Goa Cowpea Cultivar 3 [*Vigna unguiculata* (L.) Walp] seeds of Leguminosae family, indigenously known as Alsando, is not explored for its nutritional, physicochemical properties and as a matrix for probiotic delivery. This study was undertaken to evaluate the selected nutritional components of Goa cowpea cultivar (GC3) and assess its efficacy in supporting the growth of probiotic bacteria. Seed nutritive value was comparable to that of ICMR, NIN values with protein, carbohydrate and total dietary fiber content being 26.88±1.28 g/100g, 59.41±1.22 g/100g and 21.7±1.28 g/100g respectively. The mean value of calcium and phosphorus concentrations were found to be higher (157.91±00.80 mg/100g and 277.26±00.35 mg/100g respectively). Total carotenoid content was 367.77±7.08 µg/100g. GC3 seeds displayed favorable physical and cooking properties, with doubling of seed size within 3h of soaking and 10 ± 0.0 min to pressure cook with lower gruel solid loss (7.45 ± 0.28 per cent) as compared to other cultivars reported. GC3 substrate effectively resulted in a six-log increase in probiotic cell count within 14 h-16 h fermentation with reduction in pH, viscosity and accumulation of organic acids with corresponding changes in total reducing sugars and amino nitrogen in the course of fermentation. Heat exposure of fermented GC3 revealed a probiotic count of ≥ 5 log cfu/mL up to 65°C. *Lactobacillus* fermentation inhibited the common gut and food pathogens and displayed susceptibility to some of the broad-spectrum antibiotics tested. This results on nutritional value of GC3, faster cooking and as a supportive growth matrix for probionts can be exploited by nutritionists and utilized for applications in food and health science.

Keywords: Goa Cowpea Cultivar, Physico-chemical, Probiotics, *Lactobacillus*, Antibiotic Susceptibility, Anti-pathogenic Activity.

INTRODUCTION

Plant foods belonging to the family Leguminosae are one among the key dietary contributors vital for humans (Da Silva et al., 2018). Cowpea is primarily important for their supply of energy in diets and are important sources of fat, protein, carbohydrate, reasonable quantity of vitamins and nutritionally important minerals like calcium and iron (Gonçalves et al., 2016). Cowpea has exclusive taste in addition to its unique nutrient composition (Affrifah et al., 2022). Studies have reported different nutritional characteristics and properties in cowpea cultivar and regional diversity (Bai et al., 2020). The presence of various phytochemicals including prebiotics makes legumes effective candidates for functional food development (Behera et al., 2018). Indigenous cowpea, grown mainly by subsistent farmers is preferred owing to its color, bold seeds yielding high biomass with fleshy pods and can serve as a major contributor to food security, nutrition and health.

Plant origin fermented foods are popular vehicles for delivering probiotic microorganisms during gastro-intestinal digestion, showing synergistic impact on human health (Rul et al., 2022). The desire for enhanced health has led to the interest in probiotics to facilitate proliferation of gut microflora

DOI:- https://doi.org/10.32381/JPSR.2024.40.03.13

(Dahiya and Nigam, 2022). With increase in lactose intolerance, milk allergies and gluten sensitivity; there are renewed interests in non-dairy and gluten free sources among health-conscious people (Rasika et al., 2021). Cowpea is a gluten free viable option for patients with celiac diseases. Cooking involves starch gelatinization, denaturation of proteins, solubilization of some of the polysaccharides, and softening of the seeds and is essential for achieving the desired texture and confirming their adequate sensory properties (Fabbri et al., 2016). Controlled fermentation can enable formulation of legume based functional foods with enhanced nutrition and health benefits (Verni et al., 2022). Fermentation can eliminate or destroy some of the anti-nutritional compounds (Di Stefano et al.,2017). Controlled fermentation also averts the chances of contamination and growth of pathogenic bacteria due to the formation of antimicrobial compounds and decrease in pH of product (Forsido et al., 2020). Additionally, accretion of organic acids with decrease in pH are also vital towards food safety (Verni et al., 2022). Although Soybean and Green gram are extensively studied; data on use of Cowpea for probiotic delivery are very scarce. Their utilization in the production of functional foods is attributed to the presence of fermentable prebiotic constituents like oligosaccharides, amylose, glucose, maltose and non-dietary components (Dahiya and Nigam, 2022). These constituents in addition to facilitating the growth of probiotic microorganisms, can also enhance their stability (Tomasik and Tomasik, 2020). *Lactobacillus* probiotic are reported to improve protein and starch digestibility of the product, decrease their viscosity (Forsido et al., 2020; Behera et al., 2018) and contribute to improved organoleptic properties due to the formation of postbiotics (Tomasik and Tomasik, 2020). Further, an imperative criterion for production of probiotic food is the strain's ability to grow and achieve high cell populations (>10^7 cfu/mL) in minimum time (Palanivelu et al., 2022). With this in view, Goa cowpea cultivar was evaluated for its physical properties, selected nutritional composition and its efficacy in supporting growth of *Lactobacillus plantarum* MTCC2621.

MATERIALS AND METHODS

Clean and a month-old harvested dry seeds of Cowpea (*Vigna unigulata*) cultivar (GC3) were procured from the Indian Council of Agricultural Research (ICAR, Goa), were milled into a fine powder using a commercial cyclone mill (UDY Corporation, USA) and refrigerated at 5°C in airtight containers until further analysis.

Lactobacillus plantarum MTCC 2621(Microbial Type Culture Collection, Haryana, India) was the bacterium used for study. Antibiotic discs of HiMedia were used. Clinical pathogens *Escherichia coli* ATCC 25922 and *Salmonella enterica* ATCC 35664 were obtained from Goa Medical College. Chemicals of analytical grade and authentic standards of Sigma-Aldrich® and Merck (Merck India), other standard suppliers and suitable media of HiMedia were used. High-performance liquid chromatography (HPLC) grade solvents were used for extraction and in the mobile phase of liquid chromatography.

Analysis of Physico Chemical Properties of GC3 Seeds

Study of Physical Properties of Seeds

GC3 seeds were studied for physical properties. The main grain dimensions viz. length (L), width (W) and thickness (T), were studied using a SSEA stainless iron nickel vernier caliper to an accuracy of 0.05 mm. Sphericity (ϕ) was calculated as a function of the three principal dimensions using the below formula following Mohsenin (1986).

$$\phi = \frac{(LxWxT)^{1/3}}{L}$$

Thousand seed weight was estimated by manually counting and weighing 1000 seeds, length/breadth ratio of 10 randomly selected seeds (raw and cooked seeds) was ascertained by dividing the cumulative length by breadth (AACC, 2000). Average of ten determinations was reported. The seed bulk density (Pb) as g/mL was measured following (Hamid et al., 2016).

The surface color of seeds was analysed by CIE color scales in the values of Hunter L (lightness), a (redness to greenness) and b (yellowness to blueness) using a portable Hunter Lab Spectro colorimeter (Miniscan XETM, Hunter Associates Laboratory Inc., Reston, Virginia, USA) according to AACC, 2000. Auto measurement mode was followed at 30 s, 60 s and 120 s intervals and the average value given.

Determination of Chemical Properties: Cooking and Textural Properties of Seeds

To assess the cooking properties, GC3 seeds (soaked and unsoaked) were cooked and cooking time was checked as suggested by Hamid et al. (2016). Water absorption after soaking and cooking was measured by the increase in weight of GC3 seeds. Gruel solid loss (per cent) was estimated by pressure cooking 100 seeds in 100 mL of double distilled water for 10 min. The gruel was then transferred to beakers, evaporated in hot air oven at 90°C till fully dried solids formed, which were weighed. Hydration capacity, hydration index, swelling capacity and swelling index of GC3 seeds were determined (Hamid et al., 2016). Textural properties viz. hardness, chewiness, cohesiveness, and adhesiveness of soaked and cooked seeds were analysed by method of AACC, 2000 using texture analyzer (IMADA, Model FRTS-5N).

Analysis of Nutritional Composition of Seeds

Analysis of Proximate Composition of Seeds

The proximate composition; moisture (AOAC 950.46), fat (AOAC 991.36), protein (AOAC 928.08), total dietary fiber (TDF), insoluble and soluble dietary fiber (IDF and SDF) (AOAC 985.29), ash content (AOAC 920.153) was determined as per the standard methods of AOAC, 2010 and Raffinose oligosaccharide (RFO) by (Sanchez-Mata et al., 1998) Carbohydrate content was estimated by difference (Carbohydrate = 100 – (moisture +fat + protein+ TDF + ash), Atwater factors as Energy value (kcal) = % Protein × 4 + % Carbohydrate × 4 + % Fat × 9.0 was used to calculate total energy (Atwater and Bryant, 1906).

Estimation of Minerals, Vitamins and Carotenoid Content

Phosphorus (Fiske and Subbarow,1925), Calcium, Iron, Zinc, Potassium, Magnesium, Manganese, Copper, Vit.B2, Vit.B5, Vit.B9, Vitamin K1, and Carotenoid contents were evaluated using the Association of Official Analytical Chemists (AOAC, 2010) standard methods.

Evaluation of GC3 Flour as a Probiotic Carrier

Slurry Preparation and Inoculation of The Starter Culture

L. plantarum MTCC 2621 was inoculated in standard MRS broth media (De Man, Rogosa, & Sharpe), incubated for 24 h at 35 ± 2°C, and centrifuged at 7000 rpm at 10°C for 8 min. The cells were resuspended in the same after washing with saline (0.85 per cent). GC3 flour (5 per cent w/v) slurry was prepared using distilled water, autoclaved at 15 psi at 121°C for 15 min, cooled and inoculated with the starter culture (~ 4 log cfu/mL slurry).

Controlled Fermentation and Enumeration of Viable Cell Count

To initiate fermentation, the culture was inoculated in the GC3 slurry after suitable dilutions to obtain a cell count of ~ 4 log cfu/mL slurry and incubated in an orbital shaker incubator at 120 rpm at 35 ± 2 °C for 24 h. The viable cell count of *L. plantarum* as log cfu/mL fermented slurry was enumerated by spread plating on nutrient agar after incubating at 35 ± 2°C for 24 h.

pH Measurement and Estimation of Organic Acids

The slurry pH was checked at different fermentation intervals by means of a digital pH meter (EUTECH, Singapore). Assay kits of Megazyme International, Ireland was used to quantify lactic acid and acetic acid in GC3 slurry according to the manufacturer's instructions.

Analysis of Total Reducing Sugars (TRS) and Free Amino Nitrogen (FAN)

The dinitrosalicylic acid method (DNS) was used to estimate TRS of the samples during fermentation (Miller, 1959) by measuring the absorbance at 560 nm. FAN concentration was analysed by modified ninhydrin colorimetric method (Hill and Stewart, 2019) at different fermentation times (0-36 h) by spectrophotometer (Abs 540_{nm}) (UV-1800, Shimadzu, Japan).

Viscosity

A Brookfield Viscometer (DV-I + Viscometer, Brookfield Engineering Laboratories, MA, USA) with 7 number spindle set at 50 rpm at 30± 2°C was used to measure viscosity of GC3 slurries at different fermentation times (Rodrigues et al., 2014).

Heat Tolerance Assay

Comparison of the viable cell counts of 1mL of 16 h fermented GC3 with that of free cell suspension (FCS), dispensed in 2 mL capacity microfuge tubes was carried out after 10 min and 15 min of heat exposure by spread plate method. The tubes were placed in water bath set to different temperatures from 40°C to 70°C.

Evaluation of Antibiotic Susceptibility and Antibacterial Activity of Fermented Extract

Antibiotic susceptibility of GC3 assisted *L.plantarum* was studied by disc diffusion assay (Charteris et al., 1998) on MRS agar plates. The inhibition zones were recorded in mm to classify the strain as R (resistant), MS (moderately susceptible) or S (susceptible).

The antimicrobial activity of fermented GC3 extract was tested against the previously grown gut pathogen, *E. coli* and *S. enterica* in Luria Bertani broth by well diffusion assay on Mueller Hilton agar plates incubated at 28°C by observing the inhibition zones in mm.

Statistical Analysis

Statistical analysis was performed using SPSS, Version 22.0. (Chicago, SPSS Inc.). Comparison of the differences in mean between the samples was performed by One-way ANOVA and Students t-test. Level of significance was determined at p value of 0.05.

RESULT AND DISCUSSION

GC3 seeds were analysed for physical and cooking properties, proximate composition and vitamins and minerals. GC3 slurry subjected to fermentation by *L. plantarum* was ascertained for enumeration of cell count and the cell abundance, as well as for changes in pH, lactic and acetic acids, FAN, TRS and viscosity at 4 h intervals from 0 h to 36 h.

Physical Chemical Properties: Cooking and Textural Properties of Seeds

All the values of physical and chemical properties were of 10 replications. Seed dimensions of GC3 seeds indicated an average length of 10 ± 0.50 mm, width of 6.02± 0.5mm and a thickness of 5.4± 0.5mm which increased to 15.04 ± 0.4mm, 9.01 ± 0.21mm and 7.8± 0.6mm respectively after 3 h of soaking, and on cooking further increased to 15.43 ± 0.38mm, 9.21 ± 0.19 mm and 8.03± 0.4mm respectively with no further increase seen on soaking up to 10h. These dimensions were higher than those reported by Hamid et al., 2016) and such physical properties and grain dimensions enable in studying of product behavior during various agricultural processes (Hamid et al., 2016).

Length: breadth ratio of soaked seeds and cooked seeds was estimated to be 1.67± 0.07 and 1.68±0.06 respectively. GC3 seeds displayed a sphericity value of 68.76 per cent which was comparable to that reported by Hamid et al. (2016). Seed sphericity determines seeds performance in various stages from handling to planting (Bhardwaj et al., 2014). Understanding and optimizing sphericity facilitates improved crop production and more effective seed management.

Thousand seed weight helps assess seed quality and uniformity, 1000 GC3 seed weight was found to be 189.9±7.9 g with each seed approximately weighing 0.20g; lower than that reported by Bhardwaj et al., (2014). Seeds with a higher 1000 kernel weight are usually more robust and may display better germination potential and yield (AACC, 2000). The 1000 kernel weight can vary significantly among cultivars owing to the environmental conditions viz soil fertility, water availability, and growing practices that influence the final seed weight of different cowpea varieties (Hamid et al., 2016).

The bulk density of GC3 seeds was 0.76± 0.2 g/mL and is in accordance with the values (0.72–0.80 g/mL) reported by Hamid et al., (2016) and Astuti et al., (2019). Bulk density measurement determines seed quality assessment, calculating seeding rates,

and planning storage and transportation (Appiah et al., 2011). With regard to seed flour, higher bulk density is not desirable for the weaning foods formulation of (Kabeer et al., 2023).

GC3 seeds displayed a brownish color with values of '*L*' (72.52 ± 0.31) and '*a*' (0.47 ± 0.05) for color property. Color of seeds are due to presence of polyphenols in the seed coat as well as genetic factors (Astuti et al., 2019). When observed through unaided eye, unsoaked GC3 seeds appeared light in color as compared to the soaked seeds. Color of seeds and its flour could influence the suitability in industrial applications.

Physicochemical and cooking properties govern the preference, choice, and the economy of seeds (Appiah et al., 2011). Pressure cooking time of month old harvested 100 unsoaked seeds varied from 25±5 min whereas, 3 h soaked seeds cooked with desirable texture in 10± 0.01 min of pressure cooking ($p<0.05$).

The weight of 1000 seeds increased significantly on soaking to 406.4 ±4.6 g with each seed weighing 0.43±0.04 g and to 493.6 ± 6.6 g on cooking and was significantly higher than that of cooked unsoaked seeds (441± 6.2 g). This indicates the water absorption after soaking and cooking of GC3 seeds. Lower cooking time could be due to its higher hydration index and swelling index on soaking. Significant reduction in cooking time of Red and Black gram cultivars after soaking has been reported (Hamid et al., 2016; Bhardwaj et al., 2014). Older legumes exhibit toughness and extended time to cook compared to fresher ones, the age of GC3 seeds did not affect the cooking time. Cooking time influences the nutrients, determines the cooking quality and influences the purchase of seeds and grains (Mubaiwa et al., 2017).

Legumes soften through a process of hydration and cooking. Cooking causes break down of cell walls, starch absorb water, thus softening the seeds. During storage, the non-starch constituents may undergo physical changes and factors like denaturation of the protein matrix and changes in viability play a major role in cooking time. Moreover, freshly harvested legumes typically have more fragile and pliable cell walls due to their higher moisture content and less lignification, making them softer and often quicker to cook compared to their dried counterparts (Hamid et al., 2016; Mubaiwa et al., 2017).

According to Appiah et al. (2011), gruel solid loss during the cooking of cowpea helps in understanding the seed quality after cooking and the efficiency of the process, this loss was measured to be 7.45 ± 0.28 per cent.

Hydration capacity of GC3 seeds was 0.22± 0.01 g/seed and swelling capacity was measured as 0.62± 0.02 mL/seed. Table 1 presents the various properties of GC3. Hydration capacity measures the total amount of water absorbed by the seeds and index measures the efficiency of water absorption (Astuti et al.,2019). Tresina and Mohan (2012) reported the hydration capacity of 0.03 g/seed and hydration index of 0.9 for cowpea.

Table 1: Hydration, Cooking and Textural Properties of GC3 Seeds (n = 10)

Parameter	Value
Hardness (kg) of soaked seeds	6.23 ± 2.24 [a]
Hardness (kg) of cooked seeds	3.03 ± 0.35 [b]
Chewiness of soaked seeds	0.53 ± 0.16 [a]
Chewiness of cooked seeds	0.11 ± 0.08[b]
Cohesiveness of soaked seeds	0.11 ± 0.01
Cohesiveness of cooked seeds	0.11 ± 0.02
Adhesiveness of soaked seeds (kg s)	0.15 ± 0.03[a]
Adhesiveness of cooked seeds (kg s)	0.34 ± 0.02[b]
Hydration capacity (g/seed)	0.22± 0.01
Hydration index	1.05± 0.00
Swelling capacity (mL/seed)	0.62± 0.02

Parameter	Value
Swelling index	2.31± 0.14
Cooking time with desirable texture (min) – unsoaked Cooking time (min) with desirable texture – soaking 3 h	25± 0.35[a] 10± 0.00[b]
Gruel solid loss (%) – unsoaked Gruel solid loss (%) – soaked	1.41 ± 0.24 1.60 ± 0.15
Cooked length breadth ratio-soaked and cooked	1.68 ± 0.06
Water uptake ratio on soaking of seeds (g/g) Water uptake ratio of cooked seeds at 25min – unsoaked(g/g) Water uptake ratio of cooked seeds at 10 min – soaked(g/g)	2.14± 0.10 2.52± 0.06 2.6± 0.10

Values reported are mean ± standard deviation.[a, b] indicates significant difference ($p< 0.05$) within the treatments of the respective parameters

Source: Author's Own

Proximate Composition of the GC3 Seeds

The moisture content of GC3 seeds was estimated to be 9.97±0.24 per cent, suggesting an extended shelf-life quality of the legume (Hossain et al., 2016). The protein content of GC3 was 26.16 ± 0.23 g/100g. Carvalho et al. (2012) and Gonçalves et al. (2016) reported nutritionally good quality of protein content in legumes as 20.3-39.4 g/100g, nonetheless low in methionine. The moisture content of 24 cultivars of cowpea ranged from 10.24 g/100g - 12.15 g/100g and the protein content ranged from 17.30–27.23 g/100g (Bai et al., 2020). GC3 cultivar with its higher mean concentration of protein than the Cowpea species reported in Indian Food Composition Table (IFCT), 2017 (20.26± 0.59 g/100g) can be an important source (Longvah et al., 2017). The crude fat content of GC3 was 2.33±0.08 g/100g and support the findings of Bai et al., (2020) as 1.88 g/100g to 3.14 g/100g but more than that of IFCT, 2017 (1.15 ± 0.06 g/100g). The values are also in accordance with (Carvalho et al., 2012) who reported low fat in Cowpea (3 g/100g). Presence of fat in food in high amount could be unfavourable as it can facilitate rancidity, thus affecting the shelf life of food products and, causing disagreeable odour and compounds (Vieira et al., 2015). However, fat is also associated to the amount of energy the Cowpea supplies. The energy value of GC3 was determined to be 271.31±1.20 Kcal.

The carbohydrate content was 59.41±1.22 g/100g, and the TDF content of GC3 was found to be 21.7±1.28 g/100g, with IDF (19.60±1.28 g/100g) being more than the SDF (02.10±0.12 g/100g), that followed the general pattern reported for pulses (Gonçalves et al., 2016). Dietary fibre includes the complex mixture of indigestible resistant starch, non-starch polysaccharides (cellulose, pectin, hemicellulose, oligosaccharides, gums), waxes and lignin (Rasika et al., 2021). SDF and IDF are the fractions of dietary fibre; is fermentable and can act as a prebiotic in supporting the growth of probiotic (Tomasik and Tomasik, 2020). The ash content determines the presence of total amount of minerals in food, GC3 had an ash content of 3.36±0.06 g/ 100g and verbascose oligosaccharides, are abundantly found in legumes (Tomasik and Tomasik, 2020). Humans lack α-galactosidase; thus, oligosaccharides cannot be digested and are available for bacterial fermentation (Hossain et al., 2016). Although oligosaccharides are flatulence causing, there is some indication that they may stimulate probiotic activity (Rasika et al., 2021). Stachyose was estimated to be more than (0.91 ± 0.08 g/100g) Raffinose (0.22 ± 0.01 0.06 g/100g) and Verbascose (0.27 ± 0.01 g/100g).

Mineral, Vitamin and Carotenoid Composition of GC3 Cultivar Seeds

Cowpea is desirable source of B-complex vitamins (niacin, thiamine, pantothenic acid,) and minerals like calcium (38.3-1062 mg/100g), phosphorus (345-673 mg/100g), iron (6.0-8.1 mg/100g), zinc

(2.7-4.4 mg/100g), potassium (957-1225 mg/100g), magnesium (130-160 mg/100g), and manganese (1.7-2.9 mg/100g (Gonçalves et al., 2016; Carvalho et al., 2012). Table 2 presents the content of selected minerals and vitamins of GC3 seeds. An amount of 157.91±0.80 mg/100g of calcium and phosphorus content of 277.349±0.35 mg/100g was estimated. Genotypic and environmental impact; and the degree of processing can influence the mineral absorption in grains (Verni et al., 2022). Further differences in agronomic conditions, soil nutrient composition and the bioaccumulation of nutrients by the respective plants during grain formation may impact the composition (Bai et al., 2020). As compared to the IFCT,2017 value of brown Cowpea (231± 27.3 µg/100g), GC3 cultivar had very less quantity of Vit. B9 (9.26 ± 1.02 µg/100g).

The consumption of foods rich in phenolic compounds impact good health and may guard against numerous chronic diseases (Durazzo et al.,2019). Carotenoids are largely present in higher amounts in leaves than the seeds (Jideani et al., 2021). Table 2 indicates the total carotenoids content of the GC3 seed cultivar. Beta-carotene was the most abundant carotenoid in GC3 (46.08±3.73 µg/100g) whereas Zeaxanthin content was 25.20±2.69 µg/100g.

Table 2: Mineral, Vitamin and Carotenoid Estimation of GC3 Seeds

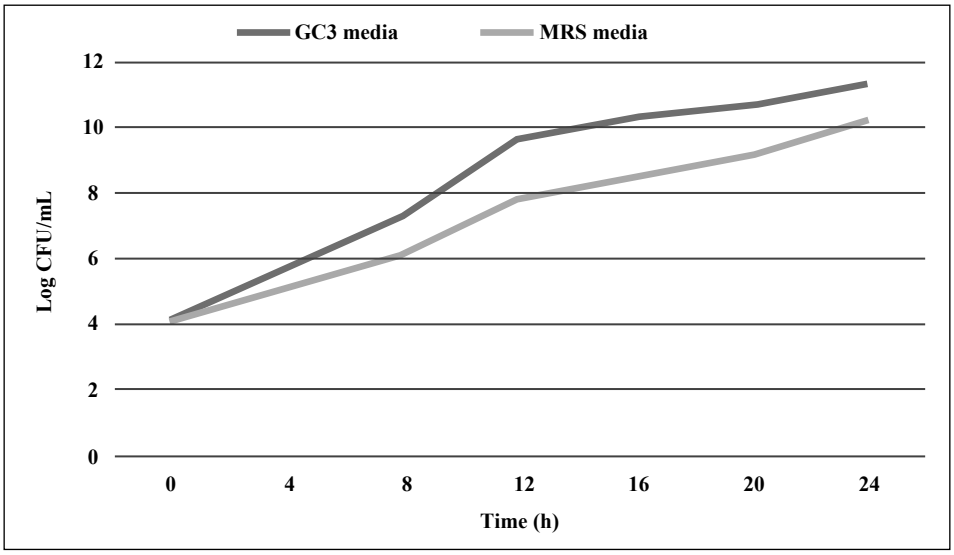
Minerals	**Content (mg/100g)**
Fe	012.49±0.84
Ca	157.91±0.80
P	277.26±0.35
Cu	00.88±0.01
Zn	06.62±0.06
K	1567.93±25.59
Mn	02.54±0.35
Mg	167.68±11.78
Vitamins	**Content**
Vit.B2(mg/100g)	00.07±0.00
Vit.B5(mg/100g)	00.34±0.01
Vit.B9(µg/100g)	09.26 ±1.02
Vit.K1(µg/100g)	07.63±0.30
Carotenoids	**Content (µg/100g)**
Lutein	34.04±3.53
Zeaxanthin	25.20±2.69
Beta-carotene	46.08±3.73
Total carotenoids	367.77±7.08

Source: Author's Own

Efficacy of GC3 as a Probiotic Carrier

Enumeration of Viable Cell Counts on Fermentation

The cell counts in GC3 and MRS media during fermentation is presented in Fig 1. Achieving a high probiotic biomass production requires optimizing nutritional medium with cost-effective production techniques. MRS media is the most frequently used for the probiotic enumeration, the cost of which is very high, which in turn makes the production costly. An increase by 3 log cycle in cell count was seen on 8 h of GC3 fermentation ($p < 0.05$), with a 9 log cfu/mL count observed in 12-14h of GC3 fermentation. A short fermentation period is important in decreasing the contamination, thus guaranteeing safety and improved shelf life of the formulation (Rul et al., 2022). A rise in cell count by 6-log cycle was seen in the GC3 slurry ($p \leq 0.05$) at 16 h, with a total cell count of 11 log cfu/mL at the end of 24 h. Further, the growth profile of the *L. plantarum* in GC3 was higher by 2 log cycle than that in standard MRS media at 16h. The maximum cell counts of 11 log cfu/mL attained in GC3 slurry within a span of 24 h are higher than the reported counts (Rodrigues et al., 2014). The use of GC3 cultivar as substrate for growth of lactic acid bacteria in this study is reported for the first time.



The values represent mean ± S.E. of three independent experiments.

Source: Author's Own

Fig. 1: Viable Cell Count of *L. plantarum* on Cowpea Cultivar (GC3) and De Man Rosa (MRS) Media at Different Hours of Fermentation

pH and Organic Acids

The pH reduced from 6.54 ± 0.3 to 5.17± 0.1 in GC3 slurries after 6 h of fermentation as seen in Table 3. A drop in the pH to ≤ 4.6 in the formulation of fermented product is vital, for the lactic acid formed to show preservative effect and enhance its organoleptic properties (Behera et al., 2018; Rodrigues et al., 2014). A decrease in pH to 4.71± 0.02 was observed in the GC3 slurry at the end of 14 h fermentation and corresponded to the production of acetic and lactic acids in the slurry. Lactic acid formation was almost 5-fold higher (2.53±0.02 g/L) than that of acetic acid (0.51±0.02 g/L) signifying it to be a major fermentation product, possibly due to the microbial degradation of complex substrate into easily metabolizable form. The results are in accordance with Verni et al., (2022), who reported increased generation of lactic acid with the fermentation time.

Total reducing sugars and Free amino nitrogen

The levels of TRS during the entire process of fermentation were observed to be higher due to the breakdown of starch and other metabolizable sugars. As the fermentation progressed, TRS levels increased

till 4–8 h and decreased subsequently as seen in Table 3.

The FAN content increased with fermentation times with a higher content seen at 20-24 h of fermentation. Table 2 indicates the profile of FAN content of GC3 slurry at different fermentation times. A 4-fold increase in levels of FAN ($p<0.05$) was seen at 16-20 h fermentation that might be attributed to proteolytic action of *L. plantarum* on proteins and implying an increase in the rate of protein hydrolysis (Gharibzahedi and Smith, 2021). This implies the association of the probiotic with GC3 seed flour in enabling enhanced microbial activity and release of enzymes for optimum fermentation activity. The FAN attained its peak at 36 h.

Viscosity

Viscosity is a vital property for products prepared using cereal, millets, and legumes. Usually, such foods are highly viscous at increased dry matter content and can increase the dietary bulk (Rodrigues et al., 2014). Further, the bulky nature of foods may not be suitable in weaning foods Table 3 indicates the changes in viscosity over a period of 36 h. The initial viscosity of Cowpea slurry changed through fermentation times, indicating the physicochemical changes responsible for this change in viscosity. Slurries prepared from GC3 were highly viscous (528.67 ± 3.06 mPa.s) at 0 h which decreased to 489.67 ± 2.08 in 8 h with a significant reduction to 246.33 ± 8.50 in 24 h of fermentation ($p < 0.05$) which could be due to the amylolytic activity of the *Bacillus* species that acts upon starch degradation bringing about a change in viscosity (Behera et al., 2018; Forsido et al., 2020). Starch degradation may also result in gradual decrease in the molecular weight of amylopectin and amylose, thus decreasing the viscosity (Forsido et al., 2020). The viscosity decreased with increase in fermentation time thus proving the potential to improve the dietary bulk of complementary foods.

Table 3: Effect of Fermentation on pH, Organic Acids, TRS, FAN and Viscosity of GC3

Time (h)	pH	Lactic acid g/L	Acetic acid g/L	TRS g/L	FAN g/L	Viscosity mPa.s
0	6.54± 0.05	BDL*	BDL*	2.62±0.05	0.00	528.67±3.06
4	6.46± 0.02	BDL*	BDL*	3.61±0.08	0.04±0.00	506.67±2.08
8	6.03± 0.11	0.06±0.01	0.01±0.01	3.52±0.05	0.13±0.01	489.67±2.08
12	5.07± 0.02	0.68±0.03	0.15±0.01	3.33±0.28	0.15±0.01	402.00±4.00
16	4.71± 0.05	1.52±0.01	0.34±0.06	1.21±0.05	0.19±0.02	389.00±8.00
20	4.56± 0.05	2.53±0.02	0.51±0.02	0.86±0.06	0.25±0.01	348.00±10.00
24	4.17± 0.03	3.01±0.01	0.616±0.01	0.71±0.04	0.36±0.01	246.33±8.50
28	4.03± 0.02	3.11±0.02	0.641±0.01	0.24±0.02	0.38±0.01	193.00±8.00
32	3.97± 0.01	3.46±0.02	0.702±0.01	0.18±0.02	0.45±0.03	182.00±3.00
36	3.84± 0.02	3.72±0.04	0.761±0.01	0.08±0.01	0.53±0.03	156.33±9.29

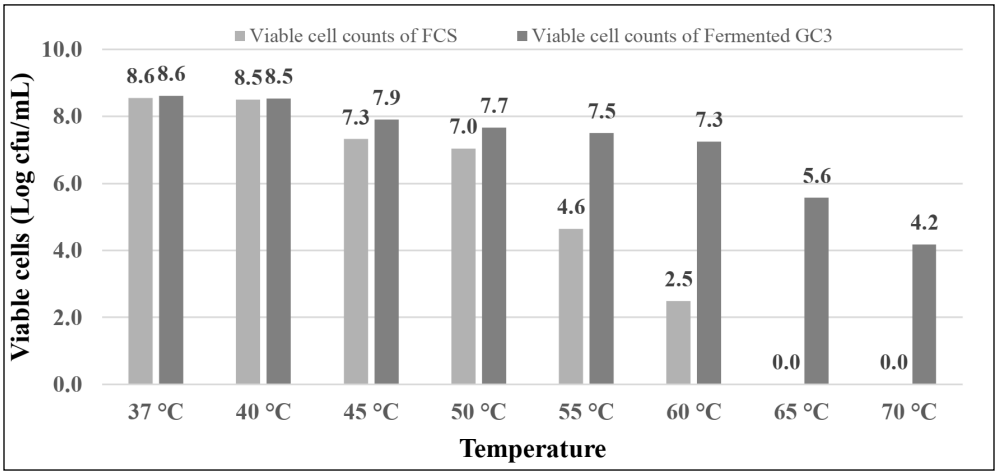
** BDL-Below detectable limits*

Source: Author's Own

Heat Tolerance Assay

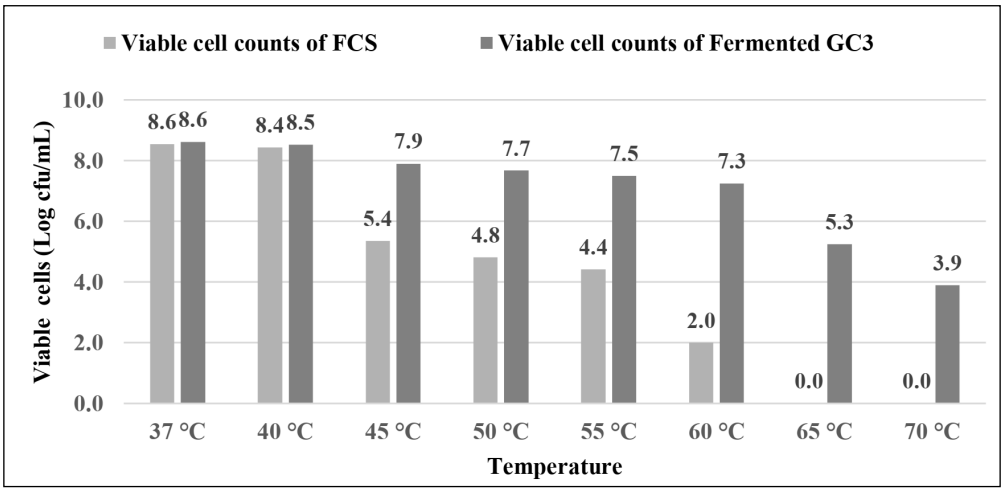
Food processing that involves heat application may impact the survival of probiotics and may further affect their successful colonization in the gut. The GC3 medium enhanced the survivability of *L. plantarum* under heat stress as compared to FCS ($p<0.05$) as seen in Fig 2a and 2b. The desirable probiotic count (>7log cfu/mL) was maintained ($p<0.05$) up to 60°C of fermented GC3 as compared to FCS at 10 and 15min of heat stress. Starch and prebiotics may reduce the thermal conductivity and thereby act as

a physical barrier to confer protection for probiotics and thus enable cell survival (Palanivelu et al., 2022). It was observed that the desirable count was maintained only up to 50°C in FCS with a significant reduction from 55°C onwards at 10 mins of heat stress ($p<0.05$). The viable count of fermented GC3 significantly reduced above 60°C ($p<0.05$) whereas a complete loss was seen on 10min of heat stress at 65°C in FCS. Studies have indicated improved survival rates of microencapsulated probionts than free probiotic bacteria ($p<0.05$) (Palanivelu et al., 2022; Dahiya and Nigam, 2022).



Source: Author's Own

Fig. 2a: Viable Cell Counts in Log cfu/mL at 10 min of Heat Stress of FCS and Fermented GC3



Source: Author's Own

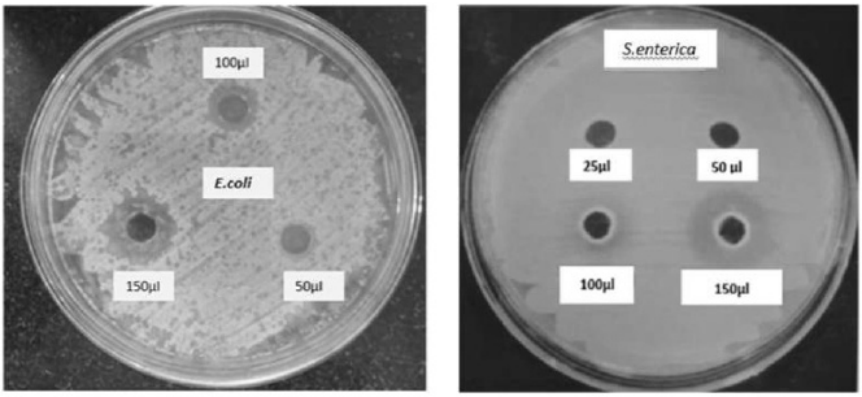
Fig. 2b: Viable Cell Counts in Log cfu/mL at 15 min of Heat Stress of FCS and Fermented GC3

Antibiotic Susceptibility and Anti-pathogenic Activity of Fermented Extract

There is a possibility that food associated *lactobacillus*, although given GRAS (generally recognized as safe) status as probiotics, they may be sources for transferable antibiotic resistance (Selvin et al., 2020). This is of great concern towards food safety and human health. *L. plantarum* 2621 was found to be susceptible to most of the antibiotics such as gentamycin, tetracycline, chloramphenicol, ampicillin, kanamycin, ciproflaxin and moderately susceptible to erythromycin. It showed resistance to vancomycin and cephalothin. The antibiotic

susceptibility pattern was found to be similar to the pattern reported for other commonly used probiotics (Duche et al., 2023) and thus its safety was established.

Anti-pathogenic activity is one of the desirable attributes of probiotics against pathogens for maintaining a healthy microbial balance in the gastro intestinal tract. Certain *bacillus* strains can produce specific inhibitory substances and have shown *in vitro* activity against pathogens like *Enterococcus, Listeria, S. aureus,* and *E. coli*. (Selvin et al., 2020). The evaluation of antimicrobial activity of fermented GC3 can be of therapeutic significance. Probiotics mainly confer anti pathogenicity to the formulation by producing organic acids in addition to the production of bacteriocins, hydrogen peroxide or peptides (Aziz et al , 2023; Dahiya and Nigam, 2022). The fermented extract demonstrated significant inhibition of common gut pathogens which could be attributed to its low pH and formation of lactic acid (1.52±0.01g/L) and acetic acid (0.34 ±0.06g/L). The inhibition of the pathogenic microbes was observed in the presence of *lactobacillus*, whereas GC3 without LAB displayed no zones of inhibition. Further, with an increase in the volume of inhibitory extract, there was a respective increase in the inhibition zones, as seen in Fig 3. A zone of 11.27±1.01mm and 7.12±0.41mm was recorded with 150µL of extract for *S. enterica* and *E. coli* respectively. Antimicrobial agents can also inhibit/inactivate the growth of unfavourable microbes, thereby acting as preservatives and extending the shelf life of food formulation (Behera et al., 2018). Results by (Aziz et al., 2023) reported promising antibacterial properties of soya and oat meal milk fermented with *L. plantarum* strains.



Source: Author's Own

Fig. 3: Antibacterial Activity of Fermented GC3 against *E. coli* and *S. enterica*

CONCLUSION

This study reports for the first time the nutritional composition of the GC3 cultivar and its use as a novel matrix for the growth of *L. plantarum*. GC3 seeds are very good source of protein, carbohydrates and mineral elements, with a substantial amount of dietary fiber. This nutritionally rich Goa cowpea cultivar could effectively be used as a food substrate for the probiotic growth. Nutritional quality and functional characteristics imparted to the final product eventually determines the use of cowpea as a food ingredient. Cowpea in the form of meal and flour could be used as an effective functional ingredient in various food systems and can be processed into value added products owing to its nutrient content. The use of fermented GC3 as fortifiers is strongly recommended in food formulations especially as a gluten free substitute and can be a valuable functional food.

References

Da Silva AC, da Costa Santos D, Junior DLT, da Silva PB, dos Santos RC, Siviero A. (2018). Cowpea: A strategic legume species for food security and health. In Legume seed nutraceutical research. Intech Open.

Gonçalves, A., Goufo, P., Barros, A., Domínguez-Perles, R., Trindade, H., Rosa, E., Ferreira, L., & Rodrigues, M. (2016). Cowpea (Vigna unguiculata L. Walp) a renewed multipurpose crop for a more sustainable agri-

food system: nutritional advantages and constraints. *Journal of the Science of Food and Agriculture,* 96(9), 2941-2951.

Affrifah NS, Phillips RD, Saalia FK. (2022). Cowpeas: Nutritional profile, processing methods and products—A review. Legume Science, 4(3), e131.https://doi.org/10.1002/leg3.131

Bai Z, Huang X, Meng J, Kan L, Nie S. (2020). A comparative study on nutritive peculiarities of 24 Chinese cowpea cultivars. *Food and Chemical Toxicology,* (146), 111841

Behera SS, Ray R C, Zdolec N. (2018). Lactobacillus plantarum with functional properties: an approach to increase safety and shelf-life of fermented foods. BioMed research international, 2018.

Rul F, Béra-Maillet C, Champomier-Vergès M C, El-Mecherfi KE, Foligné B, Michalski M C, Savary-Auzeloux I. (2022). Underlying evidence for the health benefits of fermented foods in humans. *Food & Function,* 13(9), 4804-4824.

Dahiya D, Nigam PS (2022). Probiotics, prebiotics, synbiotics, and fermented foods as potential biotics in nutrition improving health via microbiome-gut-brain axis. *Fermentation,* 8(7), 303.

Rasika DM D, Vidanarachchi J K, Luiz S F, Azeredo DRP, Cruz AG , Ranadheera CS. (2021). Probiotic delivery through non-dairy plant-based food matrices. *Agriculture,* 11(7), 599.

Fabbri AD, Crosby GA (2016). A review of the impact of preparation and cooking on the nutritional quality of vegetables and legumes. *International Journal of Gastronomy and Food Science,* (3), 2-11.

Verni M, Pontonio E, Montemurro M, Rizzello C G. (2022). Fermentation as strategy for improving nutritional, functional, technological, and sensory properties of legumes. *In Legumes Research*-Volume *2*. IntechOpen.

Di Stefano E, White J, Seney S, Hekmat S, McDowell T, Sumarah M, Reid G. (2017). A novel millet-based probiotic fermented food for the developing world. *Nutrients,* 9(5), 529.

Forsido SF, Hordofa AA, Ayelign A, Belachew T, Hensel O. (2020). Effects of fermentation and malt addition on the physicochemical properties of cereal based complementary foods in Ethiopia. *Heliyon,* 6(7).

Tomasik P, Tomasik P. (2020). Probiotics, non-dairy prebiotics and postbiotics in nutrition. *Applied Sciences,* 10(4), 1470.

Palanivelu J, Thanigaivel S, Vickram S, Dey N, Mihaylova D, Desseva I. (2022). Probiotics in functional foods: survival assessment and approaches for improved viability. *Appl Sci.* 12(1), 455.

Mohsenin NN. (1986). Physical properties of plant and animal Materials, 2nd edition. Gordon and Breach Science Publishers, New York.

Hamid S, Muzaffar S, Wani IA, Masoodi FA, Bhat MM. (2016). Physical and cooking characteristics of two cowpea cultivars grown in temperate Indian climate. *Journal of the Saudi Society of Agricultural Sciences*, 15(2), 127-134.

AACC International. (2000). Approved Methods of the AACC. Method*s*.

Official Methods of Analysis (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method 950.46,991.36

Sánchez-Mata MC, Peñuela-Teruel MJ, Cámara-Hurtado M, Díez-Marqués C, Torija-Isasa ME. (1998). Determination of mono-, di-, and oligosaccharides in legumes by high-performance liquid chromatography using an amino-bonded silica column. *Journal of Agricultural and Food Chemistry*, 46(9), 3648-3652.

Atwater WO, Woods CD, Bryant AP. (1906). The chemical composition of American food materials (No. 28). US government printing office.

Fiske CH, Subbarow Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem*, 66(2), 375-400.

AOAC (2010). Official Methods of Analysis. International 18th edn. Association of Official Analytical Chemists, Washington D.C., U.S.A.

Miller GL. (1959). *Analytical chemistry,* 31(3), 426-428.

Hill AE, Stewart GG. (2019). Free amino nitrogen in brewing. *Fermentation*, 5(1), 22.

Rodrigues M, Mandalika S, Jamdar SN, Sharma A. (2014). Evaluation of the efficacy of malted, gamma irradiated and enzymatically processed finger millet–soybean blends in supporting growth of a mixed culture of lactic acid bacteria. *LWT-Food Science and Technology*, 59(2), 908-914.

Charteris WP, Kelly PM, Morelli L, Collins JK. (1998). Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *Journal of food protection*, 61(12), 1636-1643.

Bhardwaj R, Singh DP, Rani, U, Tiwana U S, Bajaj, R K. (2014). Association studies in cowpea (*Vigna unguiculata* L.). *Journal of Food Legumes*, 27(1), 74-76.

Astuti V, Archana K, Anil K and Suman SB (2019). Physico-chemical properties and nutritional composition of improved varieties of grain cowpea grown in Pantnagar. *J Pharmacogn Phytochem*; 8(5S), 334-338.

Appiah F, Asibuo JY, Kumah P. (2011). Physicochemical and functional properties of bean flours of three cowpea (*Vigna unguiculata* L. Walp) varieties in Ghana.

Kabeer S, Govindarajan N, Radhakrishnan P, Alharbi HF, Essa MM, Qoronfleh, MW. (2023). Formulation of fortified instant weaning food from Musa paradisiaca (banana) and Eleusine coracana. *Frontiers in Nutrition,* (10) 1203955.

Mubaiwa J, Fogliano V, Chidewe C, Linnemann AR. (2017). Hard-to-cook phenomenon in bambara groundnut (*Vigna subterranea* (L.) Verdc.) processing: Options to improve its role in providing food security. *Food Reviews International*, 33(2), 167-194

Tresina PS, Mohan VR. (2012). Physico-chemical and antinutritional attributes of gamma irradiated *Vigna unguiculata* (L.) Walp. *subsp. unguiculata seeds. International Food Research Journal*, 19(2).

Hossain S, Ahmed R, Bhowmick S, Mamun AA, Hashimoto M. (2016). Proximate composition and fatty acid analysis of *Lablab purpureus* (L.) legume seed: implicates to both protein and essential fatty acid supplementation. *Springerplus*, *5*, 1-10.

Carvalho AFU, Sousa NM, Farias DF, Rocha-Bezerra LCB, Silva RMP, Viana MP, Gouveia ST, Sampaio SS, Sousa M B, Lima GPGL, Morais SMM, Barros CC, Freire R Fo. (2012). Nutritional ranking of 30 Brazilian genotypes of cowpeas including determination of antioxidant capacity and vitamins. *Journal of Food Composition and Analysis*, 26(1-2), 81-88

Longvah T, Ananthan R, Bhaskaracharya K, Venkaiah K. (2017). Indian food Composition Tables. National Institute of Nutrition.

Vieira SA, McClements DJ, Decker EA. (2015). Challenges of utilizing healthy fats in foods. *Advances in Nutrition,* 6(3), 309S-317S.

Durazzo A, Lucarini M, Souto EB, Cicala C, Caiazzo E, Izzo AA, Santini A. (2019). Polyphenols: A concise overview on the chemistry, occurrence, and human health. *Phytotherapy Research*, 33(9), 2221-2243.

Jideani AI, Silungwe H, Takalani T, Omolola AO, Udeh HO, Anyasi TA. (2021). Antioxidant-rich natural fruit and vegetable products and human health. *International Journal of Food Properties*, (24) 41–67.

Selvin J, Maity D, Sajayan A, Kiran GS. (2020). Revealing antibiotic resistance in therapeutic and dietary probiotic supplements. *Journal of global antimicrobial resistance*, (22) 202-205.

Duche RT, Singh A, Wandhare AG, Sangwan V, Sihag MK, Nwagu TN, Ezeogu LI. (2023). Antibiotic resistance in potential probiotic lactic acid bacteria of fermented foods and human origin from Nigeria. *BMC microbiology,* 23(1), 142.

Gharibzahedi SMT, Smith B. (2021). Legume proteins are smart carriers to encapsulate hydrophilic and hydrophobic bioactive compounds and probiotic bacteria: A review. *Compr. Rev. Food Sci. Food Saf*. 20(2), 1250-1279.

Aziz T, Xingyu H, Sarwar A, Naveed M, Shabbir MA, Khan AA, Jalal RS. (2023). Assessing the probiotic potential, antioxidant, and antibacterial activities of oat and soy milk fermented with *Lactiplantibacillus plantarum* strains isolated from Tibetan Kefir. *Frontiers in Microbiology*, (14) 1265188.

Received: 14.08.24

Accepted: 19.09.24

DOI:10.5958/0976-4755.2024.00119.8
BIOINFOLET 21 (3) : 397 - 398, 2024

STUDY ON BACTERIAL GROWTH ON THE MEDIUM CONTAINING MILLET – LEGUME MIXTURE

Varsha Naik and Savita Kerkar*

Discipline of Food, Nutrition and Dietetics, Goa College of Home Science (affiliated to SBSB, Goa University), Campal, Panaji, Goa, 403001, India.
*Discipline of Biotechnology, School of Biotechnology and Biological Sciences (SBSB), Goa University, Taleigao Plateau, Goa, 403206, India.
* Email drsavitakerkar@gmail.com

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 164.100.214.50 on dated 23-Jun-2025

ABSTRACT

Cowpea and Proso-millet were assessed for their utilization as substrates for the growth of salt pan *Bacillus* sp.Enhanced growth of the bacteria was observed on this millet-legume combination. Lowering of pH, total titratable acidity, viscosity and reducing sugars in the medium was observed indicating its fermentation.Survival of the bacterial isolate at 45°C in the Cowpea-Proso-millet blend was experienced.

Key words: *Bacillus;* Fermentation; Prosomillet; Cowpea

Introduction:

Fermented food products of plant origin are popular among Indians. Although cereals and legumes are widely used to produce the fermented product, the use of legumes - millet combination is not common. Cowpea (*Vigna unguiculata* (L.)Walp) and Proso millet (*Panicum miliaceum*) are the two important dietary sources(Huang, et. al.,2022), however they may contain anti-nutritional factors too, which can be removed by fermenting their mixture (Pragyani et. al, 2019) with suitable micro-organism.Present study was undertaken to find out the efficacy of Cowpea and Proso-millet blend in supporting growth of *Bacillus* sp.isolated from saltpan.

Material and Methods:

The seeds of Cowpea and Proso-millet grains were procured from ICAR, old Goa and from a government approved millet store respectively, mixed in 2: 3 proportion,ground to flour in a hammer mill, sieved and a slurry (5% w/v) was made from it by dispensing the powder in water. It was then autoclaved at 15 psi for 20min. at, 121°C and cooled.

Previously isolated culture from Ribander saltpan, Goa was screened for its potential probiotic characteristics and identified as *Bacillus* sp. using VITEK and 16Sr DNA sequencing was used to carry out fermentation. This culture grown overnight in 25% of standard Nutrient broth media (Hi Media MM 244), beef extract, 0.25 g peptone and, 0.125 g NaCl in 100 ml was inoculatedin the cooled cowpea and proso-millet slurry. It was incubated at room temperature for 24h in orbital shaker rotating at 150 rpm to facilitate fermentation.

Viable count of *Bacillus* sp. was measured as log cfu/mL slurry at different stages of fermentation by spread plating method (Gupta and Bajaj, 2017)**.** The pH of the media kept for fermentation was measured with the help of pH meter (EUTECH, Singapore) and titratableacidity was determined following Melia et al (2022). Viscosity was measured using a Brookfield Viscometer (DV-I + Viscometer, Brookfield Engineering Laboratories, MA, USA).Reducing sugars content was estimated using dinitrosalicylic acid method (DNS) as outlined by Miller (1959). The viability of

bacterial strain was assessed after exposing it at 45°-65°C,for 15 and 30 min. (Lapsiri and Wanchaitanawong, 2011).All the analysis were carried out in triplicate.The data were expressed as mean ± standard deviation. It was subjected to statistical analysis as described by Panse and Sukhatme (1985).

Results and Discussion

Fermentation for 24 h resulted in increased viable cell countfrom 4 to 11 log cfu/ml of the slurry, while the pH decreased from 6.20 ± 0.1 to 5.67± 0.2 ($p>0.05$). Viscosity of the product significantly reduced with the increase in fermentation time due to physico-chemical changes occurred during fermentation.This reduction in viscosity may be attributed to the amylolytic activity exhibited by *Bacillus* sp. (Xu et. al, 2020).The amounts of reducing sugars were found to be higher during the entire fermentation period.It initially increased from 5.08 to 10.78g/L till 4-12 h and there after significantly decreased to 5.86 g/l after 24 h ($p< 0.05$) of fermentation, suggesting that these sugars were utilized by the bacteria during fermentation(Elsanhoty et al.,2017).The blend showed a significant reduction ($p<0.05$) in the percentage survival of *Bacillus* from 67.19% at 45°C to 4.22% on 15 min of heat stress at 55°C with a further loss to 0.06% survival at 65°C. The percentage survival was much lower after 30 min of heat stress in blend.

Thus Cowpea-Proso-millet blend proved to be an effective plant-based combination in achieving highest *Bacillus* cell counts with positive impact on fermentation and may serve as a plant based functional food.

References

Gupta, M., and Bajaj, B. K. (2017).*Food Bioscience*, **20 :**104.

Huang, Z., Liu, H., Zhao, L., He, W., Zhou, X., Chen, H., and Liu, Z. (2022).*LWT*, **158**, 113139

Lapsiri, W., Nitisinprasert, S. and Wanchaitanawong, P. (2011). *Agriculture and Natural Resources,* **45(6)**, 1071.

Melia, S., Juliyarsi, I., Kurnia, Y. F., Pratama, Y. E., and Azahra, H. (2022).*Adv. Anim. Vet. Sci,***10(1):** 114.

Miller, G. L. (1959). *Analytical chemistry*, **31(3)**; 426.

Panse, V.G. and P. V. Sukhatme (1985) "*Statistical methods for Agricultural workers* ", ICAR, New Delhi.

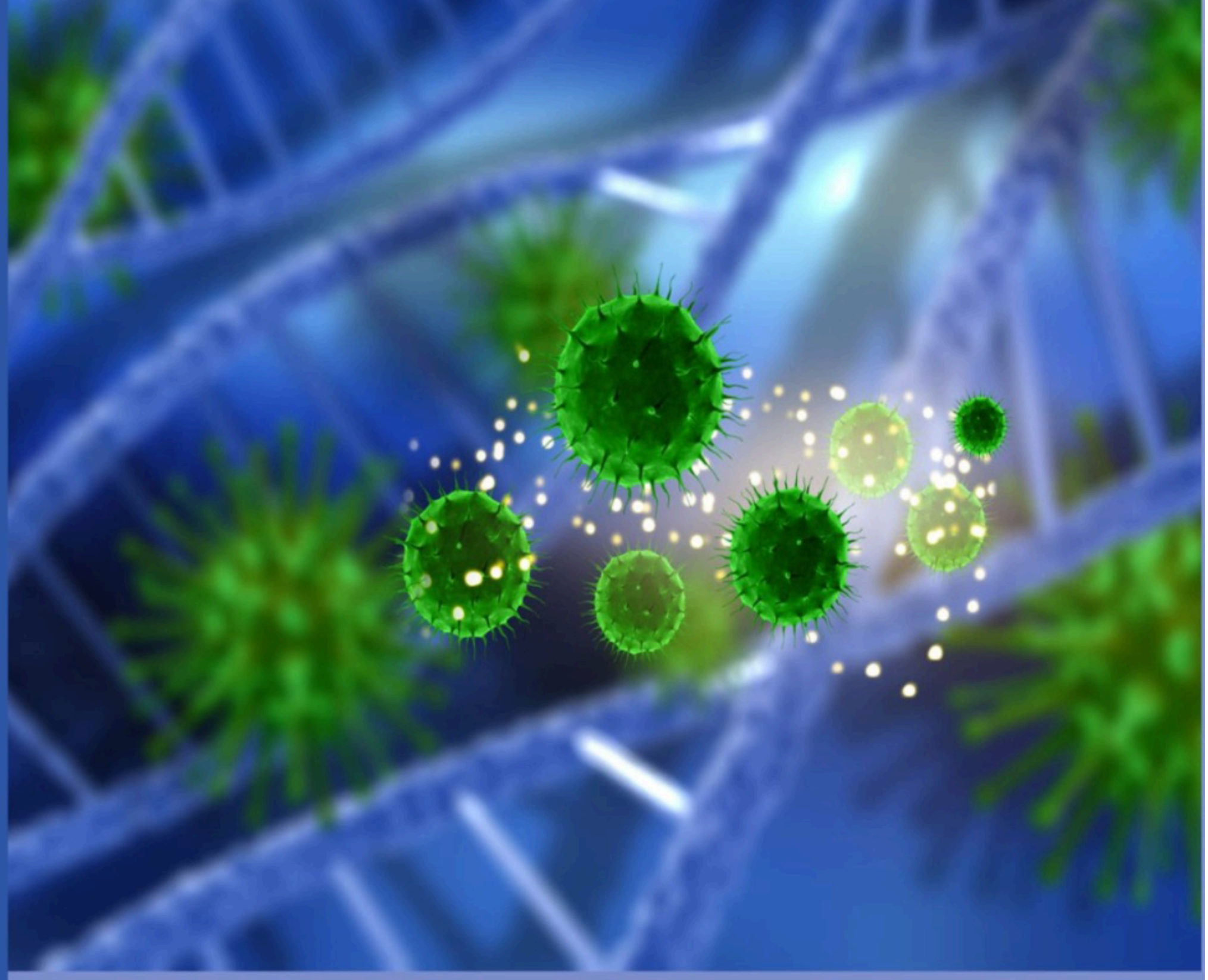
Pragyani, B., Sanaa, R., and Massimo, M. (2019).*Intern.Journal of Food Sciences.and Nutrition*, **70(6)**, 714.

Xu, Y., Ding, J., Gong, S., Li, M., Yang, T. and Zhang, J. (2020). *International journal of biological macromolecules*, **158**, 656.

www.IndianJournals.com
Members Copy, Not for Commercial Sale
Downloaded From IP - 164.100.214.50 on dated 23-Jun-2025

Futuristic Trends in

Biotechnology



Volume 3, Book 6, 2024, IIP Series

Iterative International Publishers
IIP

FERMENTATION ASSISTED FUNCTIONAL FOODS

Abstract

Human gut can be exposed to live, and potentially beneficial, microbes through fermented foods made via favourable microbial growth and enzymatic conversions of food components. Functional fermented foods (FFFs) with probiotics contribute to health and nutrition owing to their beneficial effects. Fermentation enhances nutritive value, quality and organoleptic properties of foods. Prebiotic constituents of food and of microbes used augment the properties of probiotic cultures during the manufacture of probiotic fermented foods. Prebiotics can escalate the nutraceutical properties of foods, facilitate the intestinal growth of beneficial bacteria and protect the viability of probiotics in gastrointestinal tract. Individual outcome of the probiotics and prebiotics can be enriched by using them collectively as synbiotic. Microbial action during fermentation results in the production of metabolites known as postbiotics with bioactive properties. Fermented food products can contribute to enhanced dietary diversity and offer sustainable food security. Manufacturers and consumers need to be guided on regulatory advances in FFFs on safe production, storage, transport and distribution with improved regulatory clarity, consistency and harmonization, in addition to composition and recommended intake.

Keywords: Prebiotics, Food, Microbiome Food Fermentation

Authors

Varsha Naik
Assistant Professor
Department of Food, Nutrition and Dietetics
Goa College of Home Science
School of Biological Science and Biotechnology(SBSB)
Goa University
Goa, India.
varsh18877@gmail.com

Savita Kerkar
Professor
Discipline of Biotechnology
SBSB, Goa University
Goa, India.
drsavitakerkar@gmail.com

Copyright © 2024 Authors

I. INTRODUCTION

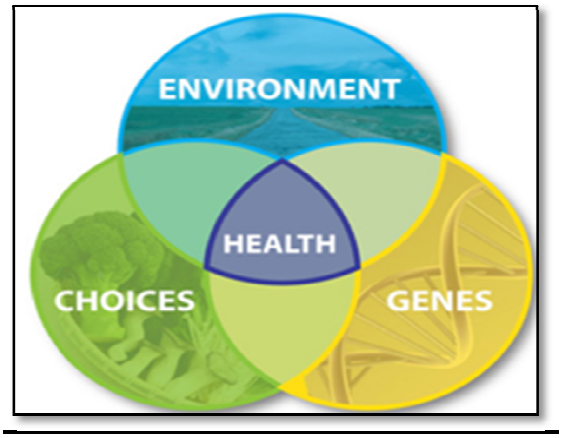
Food is the utmost basic need of life that meets the nutritional demand of an individual. Fats, carbohydrates, proteins, vitamins and minerals are the nutrients needed for growth and maintenance. Dietary fiber, phytochemicals, antioxidants, probiotics, prebiotics, etc. are considered as non-nutrient factors that boost human health with their positive influence on the physiology of host and global epigenetic imprints. Nutraceuticals or functional foods or foodiceuticals or medifoods are the food categories that may act as prophylactics or therapeutics and whose dietary inclusion may regulate the disease controlling mechanisms .

Food ,particularly the traditional ,can optimize human health and environmental sustainability if provided in right quantity and quality. It is a great challenge to provide healthy diets using indigenous food processing techniques. Since time immemorial, fermented foods have been traditionally valued and are usually categorized as “functional foods” owing to their health benefits. Functional foods are reported to support basic nutrition, affirming good health and longevity with natural ingredients as influential drivers . Fermentation brings about desirable changes in food constituents by enhancing the palatability, organoleptic properties and bioavailability of nutrients and altering the functional properties of food and also facilitating the viability of beneficial probiotic bacteria. Fermented foods comprising live microorganisms is now considered as a paramount dietary approach to improve human health. Bacteria, yeast and fungi generally facilitate food fermentations. A considerable confusion exists on what type of fermented foods actually include live microorganisms and also the understanding their impact on the gut microbiome. Introducing the prospective advantageous microbes to the gastrointestinal tract by intake of microbe-having foods seem to be an easiest and suitable option.

Most microbes related to fermented foods may withstand digestion, reach intact to the gastrointestinal tract and eventually deliver health benefits. The vast benefits of such fermented foods has paved way in the development of synbiotic foods comprising of prebiotics and probiotic bacteria. Further novel functional foods with promising health effects are in huge demand among health conscious public and in the population influenced by changing eating patterns induced by urbanization.

II. DIET , GUT MICROBIOME AND MICROBIOTA

Copyright © 2024 Authors



Sedentary lifestyle or various morbidities can impair the composition of human gut microbiota(HGM) , eventually contributing to metabolic disorders ,gut diseases and neurological diseases. A diversified healthy diet with restricted refined grains, highly processed foods and sugars should augment health and provide ideal nutrient intake. Although global consumption of fruits, vegetables, nuts and legumes has increased with a decreased consumption of red meat and sugar among health conscious consumers, processed foods still continue to be a part of diet. Dietary modification can prevent, treat, or ease some of the obesity, celiac disease or gastrointestinal(GI) disorders associated symptoms and improve general health.

Microbiome implies to the sum total microbial genome in a particular ecology, whereas microbiota is the community of microbes. A microbiome originating from a normal healthy gut is complex and consists a highly diverse collection of microorganisms (approximately 100 trillion) consisting of bacteria, viruses, fungi, parasites, viruses, protozoa, bacteriophages, archaea and other tiny organisms. The number of other bacterial cells are indicated by “human microbiome project” as approximately 10 times the number of human cells in the body, with more than 1000 different metabolically active bacteria (*Bacteroides* and *Firmicutes* being the main dominant flora) .This flora ably works with the host immune system , resisting stress associated changes and thus strongly impacting control of energy metabolism, metabolic functions , immune system , nutrition and intestinal functions of the host . Additionally, microbial residues of exopolysaccharides , building blocks of microbes , proteins, or metabolites, may influence human health with direct or indirect interaction with human physiology and gut microbiome. There is an ever-growing research with commercial concern in several aspects of prebiotic and probiotic functional foods to neutralize “dysbiosis” by substituting unfavourable microflora with favorable ones .

Various conditions like obesity, autoimmune diseases, and infections may cause predominance of harmful bacteria. Probiotics with its direct interaction with immune cells may maintain the immune balance in the GI tract, thus can be regarded as potential adjuvant therapy. There has been recent advancements in significant treatment of neurodegenerative illnesses owing to microbiome gut–brain axis. Quick gut lumen communication with brain after meals and the contribution of microbiota in homeostasis regulation with suitable signaling has been evidenced by research with the finding of “neuropod cells”. An encouraging association has been reported in mothers consuming fermented foods and sleep period among year-old infants, attributed to variation in mother’s gut microflora. The conversion of dietary flavonoids is determined by gut microbiota and its interaction with

Copyright © 2024 Authors

intestinal microbiota may not only regulate the HGM composition and functional enhancement of probiotics but it significantly increases the bioavailability and bioactivity of flavonoids thus regulating the intestinal diseases.

Primary and secondary metabolites, considered as bioactive compounds are formed during fermentation process, which include antibiotics, antimicrobial peptides, alcohol, carbon dioxide, vitamins like folates and organic acids such as short chain fatty acids (SCFAs) (acetate, butyrate, propionate). Bioactive molecules with its therapeutic potential, are reported to be advantageous to the gut by improving the intestinal barrier, inhibiting production and subsequent development of pathogens, toxic elements and help prevent metabolic disorders. Dairy and non-dairy fermented foods may act as carriers of probiotics, prebiotics, and/or bioactive compounds (phenolic compounds, antioxidants, bioactive peptides, etc.) and significantly impact the gut microbiota. **Biotics** that include **probiotics, prebiotics, postbiotics, synbiotics, paraprobiotics, oncobiotics, pharmabiotics** and **psychobiotics** are emerging in recent years gaining much attention.

III. FOOD FERMENTATION: BENEFITS, CHANGES BROUGHT ABOUT IN FOOD AND TYPES OF FOOD FERMENTATION

Food fermentation is an ancient practice known to human kind around the globe. Derived from the Latin term “fermentum”, fermentation when applied to foods has a much broader implication as against its biochemical description as “an ATP-generating process with organic compounds acting together as electron donors and acceptors”. Foods made by preferred microbial growth along with enzymatic alterations of food components are referred as fermented foods. Approximately over 5,000 types of fermented foods and beverages are globally produced and consumed from diverse raw materials like roots and tubers, vegetables, cereals, legumes, dairy products, fish, meat and fruits. Fermentation is much used in the making of probiotics, prebiotics and synbiotics containing great microbial viability and functionality.

1. **Benefits of Food Fermentation:** Fermented foods and ingredients has appealed interest as they can support HGM, thus encouraging human well-being. Meeting the expectations of consumers to make available functional foods with added benefits, is a big challenge to food science and technology. Food fermentation, is considered as a good method for augmenting nutritional and sensory qualities. Consumption of fermented foods is promoted in Indian food pyramid affirming their significance in actual human nutrition framework.

   Fermentation provide an assured guarantee to improve quality, organoleptic properties, nutritional, biological value of perishable foods with breakdown of large organic molecules into simpler ones via microbial action. The characteristic taste, flavor, color, consistency, appearance, texture, improved vitamins, essential amino acids, proteins, reduced anti-nutrients, extended shelf life and functional properties and health benefits due to microbial assimilation, actions of enzymes and of released metabolites. Fermented foods can be considered as a dietary form of living organisms.

   Generally lactic acid bacteria (LAB) like *Lactobacillus, Streptococcus, Lactococcus Enterococcus* and *Bifidobacterium*) are used in processing of fermented food

Copyright © 2024 Authors

products in addition to molds (*Aspergillus oryzae, Aspergillus sojae, Penicillium roqueforti* and *Penicillium chrysogenum*) and yeasts (e.g. *Saccharomyces cerevisiae, Andida krusei* and *Candida humilis*). Fermentation is brought about by mono or mixed cultures of bacteria, fungi and yeasts, which act parallel or sequentially altering dominant microbiota. Yeast enzymes convert sugars and starches to alcohol with proteins converting to peptides/amino acids. The actions by microbes or enzymes tend to ferment food and causing desirable biochemical changes that significantly modifies the food. For eg; degradation of complex phytochemical compounds to smaller ones, release of more bioactive polyphenols prove advantageous for the microbiome, reduce inflammatory reactions and cytokine production. The plausible mechanisms for additional benefits of fermentation include reduction of free radicals or its neutralized effects and subsequent reduction in oxidative stress, controlling antioxidant enzyme activity and increase in activity of immune system. Reduced occurrence of asthma and atopic dermatitis, improvement in fasting blood glucose and other symptoms of metabolic syndrome were observed in overweight and obese Korean adults consuming kimchi and other fermented vegetables. Similarly, intake of phytoestrogens and bioactive peptides rich fermented soybean foods showed reduced dangers of Type 2 diabetes, hypertension with improved serum triglyceride levels in Japanese adults. Strong evidence in improvement of lactose tolerance with yogurt, owing to microbial release of β-galactosidase has been documented. The nature of substrate (legumes, cereal and pseudo cereals) utilized in fermentation is of significant importance in the ideal delivery of bioactive substances in human nutrition.

2. **Changes on Fermentation in Food: Microbiological Activities, Biochemical Modifications and Health Impact:** Making informed food choices and the acceptance of novel foods is influenced by sensory properties of food, thus the prospects of fermentation in creating edible and pleasant flavors and textures are of great relevance in changing eating habits. Fermentation is a natural way of improving nutritional quality and organoleptic food properties. Changes happening during the fermentation that includes chemical transformation of primary compounds to new secondary metabolites can be categorized into 5 stages (Fig: 1). **Stage 1** results in transformation of sugars to end products like carbon dioxide, simpler acids and alcohols that further enhances the nutrient bioavailability. **Second stage**, leads to hydrolysis of polymers with enzymatic synthesis of bioactive compounds like antioxidants. In **third stage**, there is enhanced bioavailability of phytochemicals and minerals with higher amounts of vitamins and amino acids. **Stage 4** results in degradation of the toxic and anti-nutritional factors. Eventually, promoters get synthesized for improving absorption and uptake of compounds.

Copyright © 2024 Authors

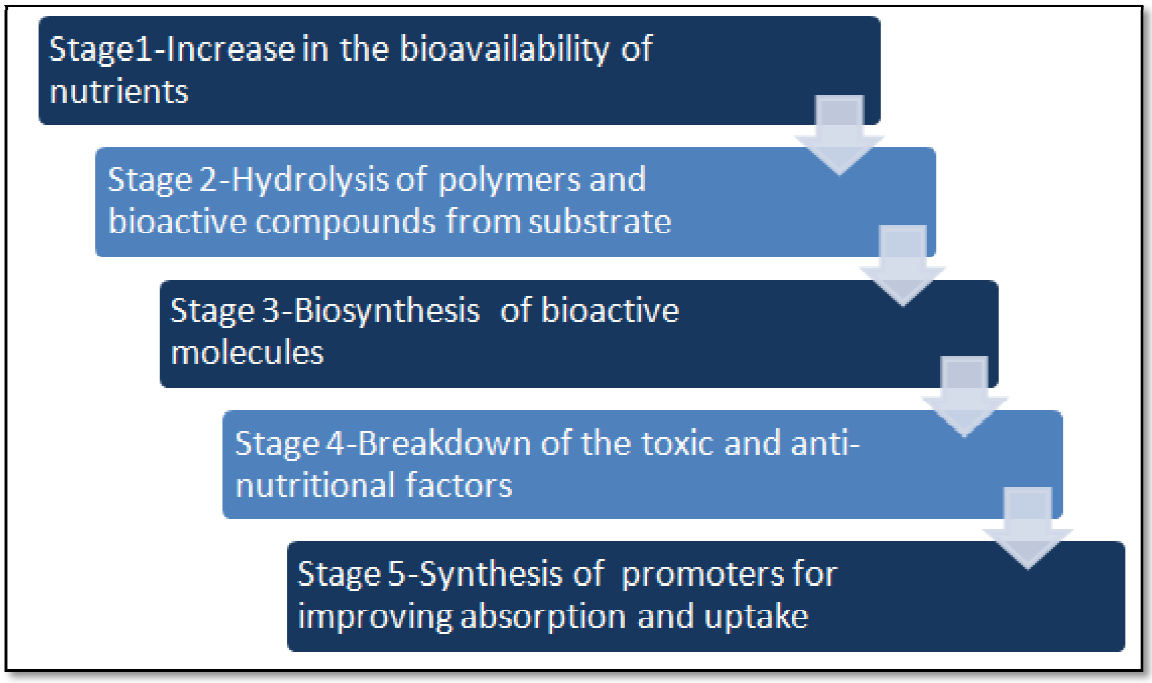


Figure 1: Five Stages Indicating Changes During Food Fermentation.
Source: R. B. Cuvas-Limon *et al*, (2021)

Changes on Fermentation in Food that Occur are as Follows

1. Food containing high fiber , toxins, anti-nutritional factors(ANFs) such as lectins, phytic , oxalic acids, tannins, raffinose, alkaloids, pyrimidine glycosides (e.g. vicine and convicine), trypsin and protease inhibitors ; that chelate metal ions , decrease digestibility and interfere with nutrient bioavailability, are likely to be changed to nutritionally enhanced and palatable foods via fermentation.

2. Substantial enhancement in protein digestibility can be brought about by fermentation.

3. Reduction in cyanide levels in leaves and the roots of staple cassava plant to acceptable levels and the removal of flatulence factors in legumes on its fermentation has been reported.

4. Fermentation may result in production of biotransforming phytochemicals from dietary polyphenols with a potential to trigger favorable changes to stimulate microbial development.

5. The Short chain fatty acids (SCFAs) produced on fermentation, especially butyrate, are responsible to maintain integrity of the intestinal mucosa, decreasing development of colon cancer risk by decreasing cell multiplication, encouraging cell differentiation and maturation of the enteric neurons. They are also recognized as important immune system modulators.

6. *De novo* synthesis of pleasant textures and flavors can be obtained by microbial fermentation eg: diverse cheese textures, chocolate flavor, soy sauce, beverages,

Copyright © 2024 Authors

condiments, etc. Fermentation synthesizes and buildup volatile and non-volatile aroma-active compounds, associated with tastes like sweet, bitter, sour, umami and salty. The attributing factors for changes in flavor may be due to the production of amines, alcohol, aldehydes, esters, fatty acids (mainly volatile species), phenols, ketones, lactones, terpenes, organic acids, thiophene, dactyl, sulfur compounds, pyrazines and other nitrogenous complexes.

7. Fermentation conditions along with microbial activities either with changes in pH, nutrient competition or by releasing preservatives having anti-fungal or anti-microbial properties in fermented foods may lessen the microbial count of pathogenic organisms.

8. Fermentation increases the release of bioactive substances (Table 1) from numerous raw material and functional biomolecules, that constitute LAB/yeast metabolism.

9. Yogurt and fermented milk has high in vitro antioxidant characteristics, owing to formation of biopeptides on milk proteins proteolysis (usually of α-lactalbumin, β-lactoglobulin and α-casein)

10. Microbial fermentation has also enabled synthesis of GABA (g- Aminobutyric acid), principal inhibiting neurotransmitter of the central nervous system with a role to play in induction of antihypertension, prevention of diabetes, diuresis and tranquilizer effects.
11. As a food-based option, fermented foods can help meet folic acid demand of at-risk populations in dealing with global deficiency problem.

12. Fermentation reduces the energy required for cooking

13. As compared to its raw equivalents, fermented products are usually safer for consumption.

14. Extended shelf life of most fermented foods leads to the reduced food loss thereby improving efficiency and resource use of food production.

Table 1: Functional Properties Related to Bioactive Substances in Fermented Foods.

Functional Attributes	**Bioactive substances**	**Microbes**	**Fermented foods released in**	**Health aspects**
Antimicrobial Properties	Antimicrobial compounds Bacteriocin Pediocin Nisin Z	LAB *Lactococcus lactis* BH5, Leuconostoc *citreum* GJ7, *Pediococcus pentosaceus.* *Lactococcus lactis.*	Vegetables Milk Kimchi Dahi (India)	GI tract protection, Anticancer effects Antiviral effects

Copyright © 2024 Authors

		Weisella cibaria.	Fermented cabbage product	
Antioxidant Properties	Antioxidants compounds, Peptides, Enzymes.	*Bacillus, Lactobacillus, Bifidobacterium*	Asian fermented soybean foods Soybean food of India and Nepal	Modulation of circulatory oxidative stress Protection of cells against carcinogen-induced damage. Anti-inflammatory Anticancerous
Immunomodulatory Properties	Amino acids ,Bioactive peptides	Proteolytic microorganisms (*Bacillus*), LAB.	Fermented beverages	Energy balance maintained Contribute to antioxidant effects , Anti-inflammatory effects
Inhibitory properties	Amino acids, Bioactive peptides	Microbial enzyme (ACE)	Kefir Koumiss Yogurt	Minimize the risk of developing chronic or degenerative diseases.
Enhancement of metabolic functions	Lipases, Proteases, Esterases, Amylases b-galactosidase, Lactase	LAB, *Bifidobacterium.*	Fermented beverages	Help to improve the metabolism ,Improve nutrient absorption ,Mitigate lactose intolerance
Production of SCFAs	Propionate, Acetate, Butyrate.	LAB	Fermented beverages	Provides 60–70% of the energy needed by the colon, muscle and brain cells. Therapeutic effects: Anti-inflammatory bowel , Anti-colon cancer

Copyright © 2024 Authors

				,aids in prevention of heart diseases ,management of obesity and diabetes.
Production of vitamins	Vitamin B12, Vitamin K, Vitamin B6, Cobalamin, Folate, Thiamin.	*Bifidobacterium, Lactobacillus.*	Fermented beverages	Enables DNA synthesis , Fights cardiovascular disease

Source: R. B. Cuvas-Limon *et al*, (2021).

3. **Types of Food Fermentation:** Fermented foods are normally referred to as those processed by different types of fermentation methods. It results in enzymatic changes of primary and secondary food components with cost-efficient bioprocess. Food fermentation is impacted by many factors, like substrate composition, fermenting microorganisms, food treatment and duration of fermentation whilst processing. Depending on the type of the matrices (substrate) exposed to fermentation, there exists **nine main** categories of fermented foods: (1) fermented cereals; (2) fermented legumes; (3) fermented vegetables and bamboo shoots ; (4) fermented roots/tubers; (5) fermented milk products; (6) fermented and preserved meat products; (7) fermented, dried and smoked fish products; (8) alcoholic beverages; and (9) miscellaneous fermented products. To initiate any microbial metabolism, the raw material used must be rich in chemical composition, together with high concentration of mono and di-saccharides and minerals with no inhibitors like toxins that may inhibit the microbial growth. Environmental conditions also impact the fermentation process.

   Food fermentations are classified either based on added or naturally present starter cultures as **controlled** or **uncontrolled** (**Natural/ spontaneous**) fermentation respectively and based on the product produced. Based on the dominant metabolic process, three fermentation processes include: **acidic, alkaline, and alcoholic fermentation**, each contributing in a unique way. Acid fermented products or alcoholic beverages are widely documented for its nutritional and health benefits as compared to alkaline fermented foods (AFFs), thus the focus here will be on AFFs. Active research on "**alkaline food fermentation**" commenced in the late 1980s, while lactic acid fermentation was popular with detailed scientific research way back in 1950.

   - **Acidic Fermentation:** is primarily done by LAB, acetic acid bacteria (AAB) and yeast with lactic acid as main metabolite along with different types of organic acids, thus called as LAB fermentation. Curd/ Yogurt and sourdoughs are produced by lactic acid and yeast fermentation respectively. AAB (mainly *Acetobacter*) oxidize carbohydrates, alcohols and sugar alcohols (polyhydric alcohols or polyols) to their equivalent primary product acetic acid, organic acids, ketones and aldehydes in oxidative fermentation.

Copyright © 2024 Authors

- **Alkaline Fermentation:** often occurs on seed fermentation and carried out by diverse species of *Bacillus* and fungi Eg. AFFs such as natto, dawadawa, bikalga, ugba,,kinema, iru ogiri and thua-nao.

- **Alcoholic Fermentation:** with AAB and yeasts as predominating organisms primarily yields ethanol and CO_2. Eg. alcoholic beverages such as beer, wine, cider.

❖ **Alkaline-Fermented Foods**: ***Nutritional significance of AFFs:*** Alkaline-fermented foods made from various raw ingredients, are a lesser known food product in developing and a few developed countries. In alkaline-fermentation the pH of the substrate (usually protein-rich foods) increases to alkaline values (pH 8–10). This fermentation is either due to alkali-treatment during production or due to protein degradation/hydrolysis (proteolysis) to peptides, amino acids (essential) and further degradation of amino acids to ammonia by dominant *Bacillus spp*. The increase in pH contributes to a strong ammoniacal smell which can be controlled by restricting the bacterial growth and metabolism without hindering the action of flavor producing proteolytic enzymes. Holding of fermented beans at appropriately low temperature during maturation can also enable this restriction and is also possible by adding glycerol as humectant to partially fermented beans or by limiting oxygen supply. Proteolysis is considered as the main metabolic activity in alkaline fermentation.

Traditionally, AFFs are usually produced using spontaneous fermentation (dependent on existing autochthonous or native microorganism of raw substrate and of immediate environment) but can also be developed by controlled fermentation. Predominant microorganisms in AFFs include *Bacillus subtilis, B. subtilis var. natto, B. cereus, B. megaterium, B. thuringiensis, B. endophyticus, B. licheniformis, B. borstelensis, B. pumilus, B. coagulans, B. circulans, B. amyloliquefaciens, B. firmus, B. megaterium, Paenibacillus polymyxa, Lysinibacillus sphaericus* and *Lysinibacillus fusiformis*, in addition to the involvement of secondary microorganisms (LAB, *staphylococci* and *micrococci*). *Bacillus spp.* are used as probiotic in humans.

❖ **AFFs as functional foods:** ***Nutritional significance and health benefits of AFFs:*** Plant-based substrates are usually used with soybean being the most common in AFFs. Textural and color changes of AFFs on softening are their important features. Softening of plant based foods leading to the desired texture occur due to hydrolysis of pectin and protein by pectic and proteolytic enzymes. Browning reactions (non-enzymic) which involves amino groups of amino acids and the non-reducing sugar components and microbial activity may contribute to the color development. Widespread production of whitish sticky mucilaginous polymers assumed to be polysaccharides are the characteristics of many alkaline fermentations. AFFs can immensely achieve dietary needs in communities producing and consuming them. Alkaline fermentations are known for two important features as follows:

  - **Hydrolysis of macronutrient molecules** i.e. of complex carbohydrates like oligosaccharides, proteins and fats by the enzymes produced from predominant bacteria, thereby augmenting nutrient bioavailability and digestibility as compared to unfermented substrate. For example, fermentation with B. *subtilis* can effectively hydrolyze soybean proteins and polysaccharides into low-molecular-weight, water-soluble products requiring minimal subsequent degradation by

Copyright © 2024 Authors

enzymes from GIT. Increased levels of non-protein, soluble nitrogen, free amino acids with increased concentration of methionine, cysteine isoleucine, phenylalaline, leucine, tyrosine and even lysine which is a limiting amino acids in plant sources are reported in alkaline fermentations.

Most plant based sources used for AFFs are abundant in carbohydrates with non-digestible oligosaccharides in large quantity such as galactamannan, stachyose, raffinose, verbascose, sucrose and arabinogalactan. They act as functional prebiotics and enable the action of beneficial microbes. Some of these non-digestible carbohydrates in humans cause flatulence and abdominal distension. Fermenting microorganisms hydrolyze starch and non-digestible carbohydrates into readily digestible sugars (melibiose, fructose, galactose) and additionally positively influencing product's texture due to tissue softening. Enzymes like galactosidase, amylase, galactanase, fructofuranosidase and glucosidase produced on alkaline fermentation by *Bacillus spp.* degrade carbohydrates.

Alkaline fermentation also results in the change of crude fat and fatty acid profiles attributed to the lipolytic activity of dominating species of microbes. Most studies have reported increase in free fatty acid levels with very few reporting contradicting results. The composition of the free fatty acids like monounsaturated fatty acids, saturated fatty acids and polyunsaturated fatty acids varies. The presence of lignoceric, stearic, palmitic arachidic, behenic, linolenic, myristic acids, palmitoleic,and gadoleic acids along with linoleic and oleic acids in high amounts have been reported in AFFs.

- **Enzymatic degradation:** This results in substantial reduction of allergens, ANFs and toxic components that occur naturally in the raw food substrate, thus improving the palatability of toxic raw material, inedible or difficult to digest foods. Fermentation brings about improvement in the nutritional value of foods by the action of protease and amylase enzymes on protein and insoluble sugar.

The most popular alkaline-fermented soybean products is Natto that has distinct odor, flavor and stringy material (fructan and glutamic acid based polypeptide). Nattokinase which is a polypeptide of 27 amino acid residues is present in Natto. During natto production lipids, protein and minerals like calcium iron and zinc and tend to increase with decreased lipid content. Another remarkable compositional change in natto is the significant rise in vitamin K content, correlated to synthesis of vitamin K2 (menaquinone-7 or MK-7) by *B. subtilis natto* . A positive correlation has been reported on bone density with increased consumption of natto in Japanese females with increase in ϒ-carboxylated osteocalcin and serum vitamin K which are bone mineralization facilitators. Fermentation also revealed 3-fold increase in riboflavin and thiamine content with five times increase in vitamin B12 levels in comparison to the non-fermented substrate. Fermented soybeans are abundant in an amino acid polymer gamma-polyglutamic acid (PGA), which is responsible for imparting sticky texture to the product, acting as dietary fiber responsible for reduced levels of serum cholesterol and increase in absorption of calcium. Isoflavone genestein, a

Copyright © 2024 Authors

chemo preventive agent in cancer is also present in high amounts in fermented soyabeans .

- **Formation of Aroma Compounds in AFFs:** Flavor of traditional AFFs has been mainly attributed to various volatile compounds viz: Pyrazines (acetoin, tetramethylpyrazine, 2,5–dimethylpyrazine,, 2-decanone, 3-methylbutanal, ethyl linoleate, , 3,5-dimethylphenylmethanol, , chlorobenzene), aldehydes, , esters, ketones, alcohols, alkanes, acids, alkenes, sulphurs, benzenes, amines, phenols, furans groups and pyridines formed during fermentation by the metabolic activities of *Bacillus* spp. The amino acid, particularly glutamate as well as peptides may lead to flavor enhancement. Free fatty acids are positively known to influence the development of characteristic flavors in food, but rancidity may be caused due to their their high levels. Pyrazines formed on heat treatment due to reactions between amino acids and sugars during the alkaline fermentation contributes to the flavor. However, sun-drying of AFF products may cause loss of some volatile aroma compounds. The data on volatile compounds produced in legumes by controlled microbial alkaline fermentation may help in deciding for the future choice of starter cultures.

- **Food Safety of Products by Alkaline Fermentation:** Alkaline-fermentation are generally known to produce safe products. For most AFFs, raw materials take a long time to cook before fermentation (up to 40 h), thus contributing in removal of pathogenic bacteria, usually non-spore–forming. In addition, the AFFs are well-preserved and stable as result of dominant microorganism's antimicrobial effects on harmful bacteria and molds. Isolation of *B. subtilis* and *B. pumilus* from AFF showed inhibition and inactivation of Gram-positive as well as Gram-negative bacteria like *Micrococcus luteus Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes Enterococus facium, , Salmonella typhimurium, Escherichia coli, Shigella dysenteriae, Yersinia enterocolitica*, and *Aspergillus* mold. *Bacillus* strains isolated from some AFF is found to release antifungals (iturin A, surfactin). Further the free ammonia and high pH of AFFs does allow the growth of spoilage microorganisms.

Although AFFs may be generally thought as safe, they can at times be risky for food borne disease, a usual problem in all types of traditionally fermented foods. Further with household production using basic equipment under unhygienic conditions and hardly any training on Good Manufacturing Practices (GMP), can contribute to unsafe AFFs, persistently recontaminating the fermented seeds. Thus positive actions like adequate GMP guideline based training on hygienic conditions and hazard analysis critical control point (HACCP), better constructed equipment like improved fermenters easy to maintain, sanitation and cost effective operating cost and production of desirable starter cultures for controlled fermentation can improve the safety of traditional AFFs.

IV. SPONTANEOUS VERSUS CONTROLLED FERMENTATION

Fermentation can be spontaneous with uninoculated cultures (**Natural /Uncontrolled fermentation**) or can be facilitated by addition of starter cultures (**Controlled fermentation**).

Copyright © 2024 Authors

1. **Spontaneous Fermentation:** Natural fermentation which is a spontaneous phenomenon occurs with natural presence of a microbial consortium (autochthonous) in either animal or vegetable substrates to initiate fermentation. The microorganisms may also be present in the equipment or manufacturing environment.

   During the progress of fermentation, microorganisms may act in a sequential manner or parallel in tune with the fluctuating dominant flora. *Leuconostoc, Streptococcus, Micrococcus, Lactobacillus, Pediococcus* and Bacillus are the usual fermenting bacterial species.

   Aspergillus, Cladosporium, Paecilomyces,Penicillium, Fusarium and *Trichothecium* are the common genera of fungi leading to fermentation. Fermentation of various cereal and legume based substrates is by yeasts, lactic acid bacteria (LAB) and/or fungi to produce variety of food by natural fermentation. They classically result in products with comparable microbes (with same species as well), irrespective of their origin. For instance, fermentations of cabbage and other green leafy vegetable commence with *Leuconostoc mesenteroides* with subsequent action by species of *Lactiplantibacillus* and *Levilactobacillus brevis*, irrespective of the product name as sauerkraut in North America and Europe, sinki of Nepal, Korean kimchi or Chinese suan-cai. Such a fermentation microbiota which is highly reproducible in spontaneous plant based fermentations reveals a steady association between organisms with raw materials. With such consistency in the classic techniques, environments, raw materials and **factors** such as pH, concentrations of salt, atmosphere and other controllable measures if in place during food fermentation, any erratic events likely to cause fermentation failure, are relatively rare. Spontaneous fermentations can also create a potential for strain isolation having industrially friendly attributes, like unique flavor production. Integration of such strains can enhance the products flavour in mixed fermentations apart from achieving better control on the process. With the attractiveness of spontaneous fermentations, it becomes vital to characterize and know the fermentation dynamics for regulation on the process for achieving improved quality attributes.

   Since a complex microbial consortium influence spontaneous fermentations, their individual genotypic and phenotypic nature is dependent on the community assembly, intensely influenced by abiotic and biotic factors. Despite spontaneous fermentations great utility in safeguarding plant based foods, chances of fermentation failure, with respect to nutrient composition, incorrect inhibition of spoilage organisms or pathogens and the dangerous risks associated with their implantation/domination in humans and unfavorable sensory traits are significant. With heterogeneous microbiota involved in spontaneous fermentation, there are chances of finding toxic by-products (ethyl carbamate, mycotoxins, biogenic amines) releasing microbial pathogens and/or strains that can impact the fermented products safety. Thus spontaneous uncontrolled fermentations is a big challenge to ensure food safety and quality.

2. **Controlled Fermentation:** Safety of fermented food associated with microbes in spontaneous fermentation can be conciliated using personalized specific starter cultures to mimic associated protechnological microbial diversity. Selected starter cultures are used to bring about fermentation, even the naturally existing cultures regarded as probiotics that are isolated from fermented products. They may speed up the fermentation process,

Copyright © 2024 Authors

warranting increased shelf life of the fermented foods with enhanced functional and organoleptic features, provide uniform quality products and render health benefits.

The selection of starter culture(s) for use in production of functional foods are based on key criteria like antimicrobial properties, probiotic properties, antioxidant, fibrinolytic activity, production of peptides, poly-glutamic acid, destruction of ANFs, etc. Appropriate starter cultures may enhance product quality by their accelerated metabolic activities with appropriate organoleptic properties, more likely improved and safe fermentations with reduced hygienic risks. keystone towards safety and quality assurance of fermentation is the use of starter culture technology as it may reduce food-borne disease risks, offer personalized nutrition and help achieve new health targets. This technology may sustainably extend shelf life, especially of traditional, artisanal, typical, biodynamic and organic productions, yield increase, and food security.

V. FUNCTIONAL FOODS: PREBIOTICS, PROBIOTICS, SYNBIOTICS AND POSTBIOTICS IN FOOD FERMENTATION

There is a rapid and continuous interest in functional foods globally. Synbiotic foods, owing to their health benefits are playing a significant vital role in nutrition. There is great demand for the food creations having dietary fibers, fatty acids, vitamins, proteins, minerals and flavonoids, either naturally present or added.

1. **Definitions:** International Scientific Association for Probiotics and Prebiotics (ISAPP),2010 defines prebiotics as “selectively fermented ingredients resulting in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health”. The definition of prebiotic was quite recently revised as “*a substrate that is selectively utilized by host microorganisms conferring a health benefit*”

2. **Probiotics** can be defined as “*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.*”

3. **Synbiotics** are a combination of prebiotics and probiotics, having a synergistic effect by inhibiting the growth of pathogenic bacteria and enhancing the growth of beneficial organisms

4. **Post-Biotics** are bioactive soluble by-products released either by the metabolic processes of probiotics or through their lysis. A technical definition of post-biotics, by ISAPP is “*preparation of inanimate microorganisms and/or their components that confer a health benefit on the host*”

5. **Prebiotics:** Prebiotics are categorically functional foods defined as the non-digestible food ingredients. Their consumption as dietary ingredients, may positively influence the human gut microbiota (HGM) composition and metabolic functions in small intestine and colon. Different bacteria may be modified by diverse prebiotics at strain or species level.

Copyright © 2024 Authors

Since prebiotics cannot be degraded by gut digestive enzymes, it reaches intact to the large intestine . Therein, thus serving as a substrate and allowing gut probiotic bacteria to grow by acting as a source of energy or carbon, thus resulting in increased probiotic count. Prebiotics act synergistically with probiotics with symbiotic action resulting in optimal repopulation of the flora and suppressing the pathogens, which subsequently augment the immune system and gastrointestinal functions. Intestinal optimal pH is essential for the probiotics existence and is maintained by prebiotics. Peristaltic stimulation and reduction in gas formation are also prebiotic functions in addition to their role in increasing calcium and magnesium absorption and improving levels of blood glucose and plasma lipids. Diets with prebiotics, besides increasing the HGM diversity, influence microbial metabolic activities and the synthesis of end products like SCFA's, branched-chain fatty acids, peptides, organic acids, ammonia, phenolic compounds ,amines and gases . Prebiotics are also extensively studied as protectants for encapsulation of probiotic strains to enhance the probiotic viability

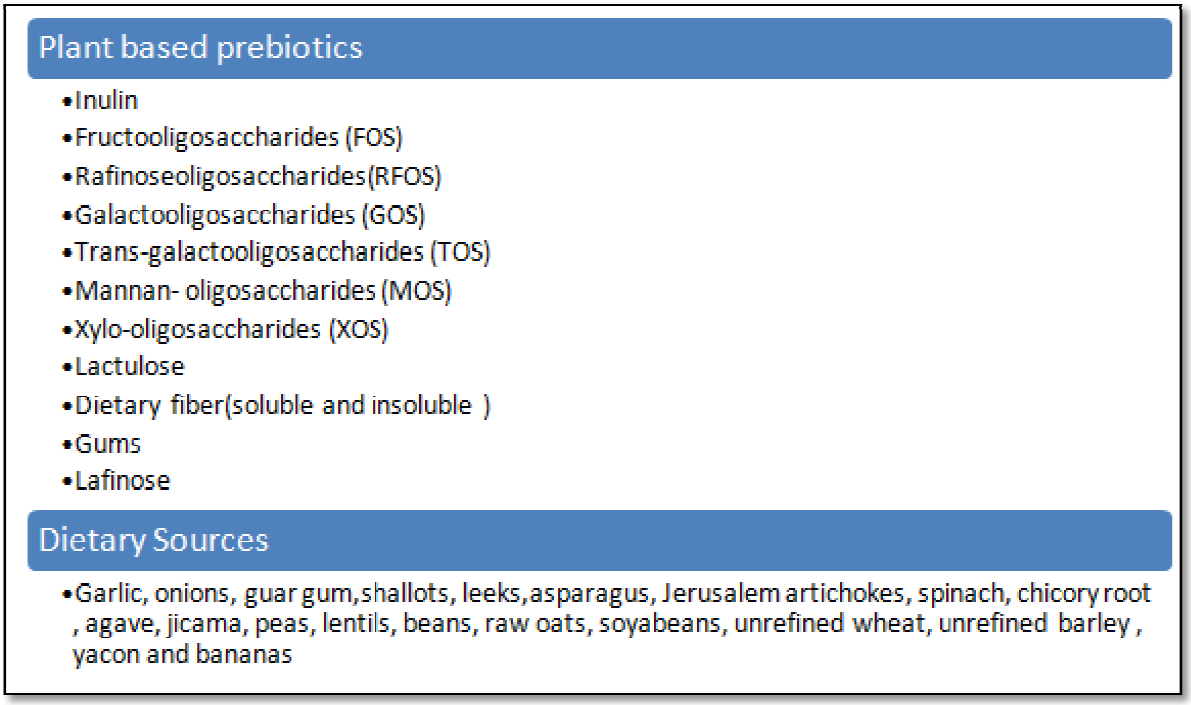


Figure 2: Sources of Prebiotics

Most of the prebiotics are plant products, well recognized being carbohydrates (examples in fig.2) whereas certain lipids, some proteins and peptides are possible prebiotics . The effect of prebiotic at specific site in the GI tract and its benefits to host is determined by chain length of the chemical short chain, middle chain or the long-chain). Breast milk oligosaccharides are reported as original prebiotic and dietary sources like plant cell wall polysaccharides and gentio-oligosaccharides have also gained attention as novel prebiotics in addition to marine poly-/oligo-saccharides, sugar alcohols (xylitol, erythritol,sorbitol, isomalt, mannitol, lactitol, polyglycitols, maltitol) .Algae and microorganisms are extensively studied for their prebiotic potential ,the most familiar algal polysaccharides of brown algae being laminarins, alginates and fucoidans , carrageenans and agar (from red algae) and ulvans (from green algae) .

Copyright © 2024 Authors

Food-grade bacteria are prospective sources of non-digestible carbohydrates and bifidogenic prebiotics. Species of *Lactobacillus, Streptococcus,* and *Leuconostoc* are known to produce oligosaccharides like dextran, levan and mucan and hetero or homo-polysaccharides.

A **dietary substance** to fulfill prebiotic definition must meet specific criterias by *in vitro* and *in vivo* tests of non-digestibility (resisting enzymatic digestion, intestinal absorption and low gastric acid pH); undergo fermentation using intestinal microbiota and stimulate the growth and activity of intestinal bacteria selectively without getting affected by conditions of food processing. Prebiotics should be accessible in the intestine for bacterial action without chemically altered, and non-degraded. **Fructooligosaccharides, lactulose, galactooligosaccharides** and **non-digestible** carbohydrates are able to meet these criteria *as* prebiotics.

6. **Probiotics:** The science of probiotics covers features in microbiology field to food processing. Intake of probiotics in sufficient amounts may provide health benefits to the host. They control the functioning of intestinal epithelial cells, lessen gastrointestinal infections, improve lactose metabolism and show antimutagenic and anti-carcinogenic properties.

Supplementation of Probiotic has a potential to diminish the intensity or reduce the period of the infections with its stimulating effects on immune system. Right type and levels of exogenous dietary supplementation of probiotics species/strain can briefly colonize the GIT with stability in the composition of native microflora, thereby reestablishing dynamic physiological function of a symbiotic flora. The multitude of health benefits of probiotics, are through their biological mechanisms in the body.

Food based probiotic functional foods can be divided into **dairy** (cheeses, creams, yogurts, ice cream, milk, acidified milks) and **non-dairy products** (meat and meat products, cereal grain based bread or other fiber snacks, chocolates, fruit juices, other fruit preparations). It is vital to preserve the efficacy of probiotic bacteria and enhance its viability during the development of functional foods. As per International standards, probiotic functional products claiming health benefits should have a minimum count of **10^6 -10^7 CFU/g** viable probiotic bacteria, when sold. To protect probiotic survival and viability and help deliver bioactive ingredients both to the food matrix itself and to the GI tract several approaches have been undertaken like immobilization technologies , composite carrier matrix systems, synbiotic development, adding osmo, thermo, or cryo-protectants to the drying matrix or growth media, lyophilization , microencapsulation(ME) through food matrix modifications and manipulation of the living cells adaptive mechanisms for survival under stressful conditions .

Lactobacillus, Lactococcus, Bacillus, Streptococcus, Bifidobacterium, Pediococcus, and *Propionibacterium* are familiar probiotic species with *Lactobacillus* and *Bifidobacterium* being the most common. Yeast probiotics include *Saccharomyces carisbergensis* ,*S. cerevisiae* and *S. boulardii* and *Aspergillus niger* and *A. oryzae* are regarded as fungal probiotics. Probiotics are commercially available as functional foods, dietary supplements and medicinal probiotics and are usually orally administered. Probiotics have been linked with nutrients in competing with

Copyright © 2024 Authors

pathogens, inhibit/block pathogenic bacterial adhesion in the colonic lumen, and improve mucus production, thus enhancing the intestinal epithelial barrier for immune system stimulation .As a prophylactic regime , probiotic supplementation has proven advantageous in antibiotic-associated diarrhea (AAD) by enabling replenishment of the gut microflora , probiotics has been found to reduce allergies in children , decline *Helicobacter pylori* colonization in the stomach , manage relapse of some inflammatory bowel conditions , reduce the risk of certain cancers by secreting specific antioxidant and anticarcinogenic metabolites , proven useful in dealing with periodontitis and oral *Candida* infections. Probiotics with its bile salt hydrolase and other enzymatic activities are reported to decrease toxins and increase the bioavailability of nutrients in the body.

Probiotics-linked signaling of nerve functions facilitated by specific mechanisms in the central nervous system, can stimulate possible therapeutic actions on neuropsychiatric disorders and stress-induced diseases. Probiotics also support brain functioning, overcomes irritable bowel syndrome, reduce l serum low-density lipoprotein levels, manage yeast/ bacterial vaginal and urinary tract infections, prevent pancreatitis, advance pancreatic health, facilitate respiratory tract health, inhibit tumorigenesis and contribute to metabolic homeostasis to help in dealing with metabolic diseases like diabetes cardiovascular diseases and non-alcoholic fatty liver disease. Apart from its physiologic effects, the bioavailability of micronutrients can be enhanced by probiotics, probiotics may reduce ferric to ferrous ions thus increasing duodenal absorption of iron as well as calcium uptake and absorption. Probiotics are also involved in synthesis of vitamin D synthesis and its absorption.

Probiotic through their metabolic activity produce soluble factors, called **postbiotics** such as bioactive metabolites through non-specific mechanism such as SCFA's, vitamins (Vit-B_2 and Vit-K_2) , enzymes, lactic acid, bacteriocins , ketones, methyl acetates, carbon disulfides and secrete pathogenic bacteria destroying antimicrobial peptides. **Postbiotics**, directly or indirectly exert a favorable effect on the host, and the risks associated with their intake being minimal as they are devoid of live microorganisms.

Table 2: Probiotic Products Available Commercially

Brand	**Description**	**Producer**
Actimel	Drinking yogurt with *L. casei Imunitass* as probiotic	Danone, France
Activia	Creamy yogurt having *Bifidus actiregularis*	Danone, France
Gefilus	An extensive range of LGG products	Valio, Finland
Hellus	Dairy products containing *L. fermentum* ME-3	Tallinna Piimatoööstuse AS, Estonia
Jovita Probiotisch	Cereals, fruit and probiotic yogurt blend	H&J Bruggen, Germany

Copyright © 2024 Authors

Pohadka	PohadkaYogurt milk with probiotic cultures	Valašské Meziříčí Dairy, CzechRepublic
ProViva	Refreshing natural fruit drink and yogurt in many different flavors containing *L. plantarum*	Skåne mejerier, Sweden
Rela	Yogurts, cultured milks and juices with *L. reuteri*	Ingman Foods, Finland
Revital Active	Yogurt and drink yogurt with probiotics	Olma, Czech Republic
Snack Fibra	Snacks and bars having natural fibers , extra vitamins and minerals	Celigüeta, Spain
SOYosa	Soy and oat based variety products like refreshing drink and a probiotic soy oat yogurt	Bioferme, Finland
Soytreat	Kefir like product with six probiotics	Lifeway, USA
Yakult	Milk drink containing	Yakult, Japan
Yosa	Yogurt-like oat product flavored with natural fruits and berries containing probiotic bacteria (L. acidophilus, B. lactis)	Bioferme, Finland
Vitality	Yogurt with pre- and probiotics and omega-3	Müller, Germany
Vifit	Vifit Drink yogurts with LGG, vitamins, and minerals	Campina, the Netherlands

Source: Adapted from Siró, I. 2008. Appetite, 51, 456–467.

Table 3: Differences between Prebiotics and Probiotic

Categories	Prebiotics	Probiotic
Content	Non-digestible but selectively fermentable ingredients	Live microorganisms
Functions	Provide food for probiotics; increase number and improve activity of probiotics	Enhances the digestive tract of their host organisms' by its health enhancing properties
Health Benefits	Supports probiotics	Decrease the amount of pathogenic bacteria in the GIT thus improves GIT function; improves immune functions; prevents cellular damage from oxidative stress
Sources	Oatmeal, asparagus, Jerusalem artichokes, , onion, garlic , inulin, guar gum , bananas	Isolated microbial cultures, yogurt, sauerkraut, yakult, miso soup, snack bars and fermented breakfast cereal, sourdough, soft cheeses, kombucha

Copyright © 2024 Authors

	,pulses, etc.	and kimchi
Side Effects	Increased fermentation resulting in increased gas production, bloatedness or bowel movement	Chances of sepsis on administering to immune compromised patients

VI. SYNBIOTIC APPROACH TOWARDS FUNCTIONAL FOODS

Synbiotics with prebiotics and probiotics combination, inhibits pathogenic bacterial growth whereas augments the growth of beneficial organisms and thus displays a synergistic effect. A good synbiotic diet can strongly influence the gut microbiome. A well designed synbiotic formula should have probiotic encouraging prebiotics, predominantly facilitate their growth. Synbiotics are formulated to beneficially affect the host with improved survival of health-promoting probiotics and its implantation in colon by selectively stimulating the growth therein or their metabolism in addition to maintenance of the intestinal biostructure. Synbiotics should improve the gut microbial composition with production of metabolites and bioactive compounds with the concomitant inhibition of prospective pathogens residing in the GI tract. Synbiotics are reported to decrease the number of infections in postoperative patients, improve the outcome of irritable bowel disease(IBD), and present anticancer effects. Patients affected by small intestinal bacterial overgrowth (SIBO) showed better results on treatment with antibiotics and synbiotic supplementation (with *B. coagulans* and prebiotics) than antibiotics alone. Synbiotic are also reported to lower the quantity of unwanted metabolites, lessen the inactivation of nitrosamines and carcinogenic substances. Determining the best pre-probiotic combination warrants for a structured approach in individual and specific disease setting.

VII. DIFFERENCE BETWEEN FERMENTED FOODS AND PROBIOTICS

The practice of traditional food fermentation dates back to human history. The International Scientific Association for Probiotics and Prebiotics (ISAPP) redefined fermented foods and beverages in 2019 as foods made through desired microbial growth and enzymatic conversions of food components.

Fermented foods and beverages are at times labelled as “probiotic foods” or “contain probiotics” by the manufacturers in an attempt to communicate to consumers about the presence of health stimulating live microorganisms in the product. Term ‘probiotic’ should only be used when there is established health benefit offered by characterized live microorganisms, extending beyond any nutritional advantage of the food substrate. ‘**Fermented food**’ and ‘**probiotics**’ are the terms not to be used interchangeably. Some jurisdictions recognize several common species for which the term ‘probiotic’ can be used in foods, such as Health Canada recognizes more than 20 species of the *Lactobacillus* genus complex and *Bifidobacterium* provided they are delivered at a minimum of 10^9 CFU per serving. European Food and Safety Authority approves health claims of live yoghurt cultures, particularly their improved lactose digestion, essentially owing to the presence of the lactase enzyme in yoghurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*). However, the label “contains probiotics” in fermented foods should only be used when the strains (regardless of one or more used) are defined to the strain level with

Copyright © 2024 Authors

known genome sequences and are present at in desirable numbers throughout the shelf-life of product.

In recent times, maximum of commercially sold fermented foods do not belong to the class of “probiotic fermented food”. Undefined microbial consortia are often found in fermented foods and beverages although at variable levels, with no demonstration of their potential health benefits. It is suggested that manufacturers indicate their product contains “live and active cultures” with assurance that food is not treated to remove or destroy the fermentation microorganisms. Further these microorganisms should be present at expected levels depending on the foods. Pasteurized fermented foods having no live microorganisms can be labelled as “foods made by fermentation”. Although microbial cultures organisms used for fermentations are characterized and identified at strain level, they are generally chosen based on their performance characteristics, like rapid acidification, substrate conversion, flavor and texture properties and not only on health associated functions.

VIII. REGULATORY CONSIDERATIONS FOR FERMENTED FOODS

Food fermentation guidelines mainly concerned with food safety are covered in International regulations. Irrespective of strains developed by recombinant DNA technology or genetic modification, their safety needs to be established and use be regulated. There exist designations like ‘Generally Recognized as Safe’(GRAS) in the USA or the ‘Qualified Presumption of Safety’(QPS) designation assigned by the European Food and Safety Authority to groups of microorganisms. Further identification of vital microbial components in fermented foods has prospects to lead to new food labelling regulations. Regulations with regard to fermentation warrant that minimum prerequisites are met concerning to the contribution of specific microbial taxa. A few standards exist, commonly for cultured dairy foods. As per Codex Alimentarius, yoghurt manufacturing should use a combination of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and kefir (fermented milk) should include *Lentilactobacillus kefiri* , lactose fermenting yeasts (*Kluyveromyces marxianus*) ,non-lactose fermenting yeasts (*Saccharomyces unisporus*, *S. cerevisiae* and *S.exiguus*) and species of *Leuconostoc*, *Lactococcus* and *Acetobacter*. With identification of newer fermenting microbes with beneficial effects similar standards could emerge like that for kombucha and water kefir.

IX. INTEGRATING FERMENTED FOODS INTO DIETARY GUIDELINES

Fermented foods would contribute to meet important macronutrients as well as supply large count of prospective beneficial microbes to the gastrointestinal tract. The concept of “shared core benefits” explains how and why phylogenetically related organisms could deliver similar health benefits. Regular intake of fermented foods having live microorganisms should be promoted around the globe by health care professionals and should be included in dietary guidelines for all the age groups. The Indian food pyramid clearly encourages the intake of fermented foods testifying their significance in actual human nutrition. Many of the microbes in fermented foods are largely related to probiotic organisms as they often share similar molecular mechanisms, accountable for health supporting properties; even though by definition they cannot be considered probiotic.

X. SUMMARY

Copyright © 2024 Authors

1. There is a rapid interest in functional fermented foods and probiotic foods for their immense contribution to nutrition owing to their beneficial health effects. Nutritive value, quality and organoleptic properties of foods are enhanced by fermentation.

2. Fermentation, a biotransformation process involves the added starter culture (in controlled fermentation) or indigenous microorganisms (in natural or uncontrolled fermentation) and the substrate as two prime factors in addition to inherent factors like temperature and the pH conditions. The presence of starter cultures or native microorganisms contribute to the probiotic functions of several fermented foods.

3. Fermented food products contribute to an enriched indigenous dietary diversity offering sustainable food security. Depending on the type of microbes, fermentations can be categorized as bacterial or fungal. Based on the food used, it is referred to as fermented cereals, vegetables, fruits, legumes, roots/tubers, milk, meat, and fish products.

4. Fermentation results in the production of metabolites (primary and secondary) by microbial action such as antibiotics, antimicrobial peptides (ACE inhibiting peptides}, carbon dioxide, alcohol, vitamins, folates, SCFAs, intracellular and extracellular polysaccharides of microbes, antioxidants compounds, complex compound degrading enzymes resulting in simple ones, bacteriocins, and cell wall components, all collectively known as postbiotics and are considered bioactive compounds. These components directly or indirectly can benefit the host.

5. The properties of probiotic cultures are augmented by prebiotic constituents during the manufacture of probiotic foods. Prebiotics can escalate the nutraceutical properties of foods, facilitate the intestinal growth of beneficial bacteria and protect the probiotics in gastrointestinal tract. Individual outcome of the probiotics and prebiotics can be enriched by using them collectively as synbiotic.

REFERENCES

[1] Borresen, E.C., Henderson, A.J., Kumar, A., Weir, T.L., Ryan, E.P. (2012). Fermented foods: patented approaches and formulations for nutritional supplementation and health promotion. *Recent Pat Food Nutr Agric.* (2012) 4:134–40. 10.2174/2212798411204020134

[2] Capozzi, V., Fragasso, M., Romaniello, R., Berbegal, C., Russo, P., Spano, G. (2017). Spontaneous Food Fermentations and Potential Risks for Human Health. *Fermentation*. 3(4):49. https://doi.org/10.3390/fermentation3040049

[3] Carding, S., Verbeke, K., Vipond, D.T, Corfe, B.M., Owen, L.J. (2015). Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis.* 26:26191. 10.3402/mehd.v26.26191 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[4] Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot,B., et al.(2014). The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastro Hepat.* 11:506–14. 10.1038/nrgastro.2014.66

[5] Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486:207–14. [PMC free article] [PubMed] [Google Scholar]

[6] Kailasapathy, K. (2013). Commercial sources of probiotic strains and their validated and potential health benefits-a review. *International Journal of Fermented Foods, 2*(1), 1-17.

[7] Leech ,J., Cabrera-Rubio, R., Walsh ,A.M., Macori, G., Walsh, C.J., Barton, W., et al.(2020). Fermented-food metagenomics reveals substrate-associated differences in taxonomy and health-associated and antibiotic resistance determinants. *mSystems.* 5:e522–520. 10.1128/mSystems.00522-20 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

Copyright © 2024 Authors

[8] Leeming, E.R., Johnson, A.J., Spector, T.D., Le ,Roy C.I. (2019).Effect of diet on the gut microbiota: rethinking intervention duration. *Nutrients.* 11:2862.10.3390/nu11122862 [PMCfree article] [PubMed] [CrossRef] [Google Scholar]

[9] Marco, M.L., Heeney, D., Binda, S., Cifelli, C.J., Cotter, P.D., Foligne, B., et al. (2017).Health benefits of fermented foods: microbiota and beyond. *Curr Opin Biotechnol.* 44:94–102. 10.1016/j.copbio.2016.11.010 [PubMed] [CrossRef] [Google Scholar]

[10] Marco, M.L., Sanders ,M.E., Gänzle, M., Arrieta, M.C., Cotter, P.D., De Vuyst ,L., et al. (2021).The international scientific association for probiotics and prebiotics (ISAPP) consensus statement on fermented foods. *Nat Rev Gastroenterol Hepatol.* 18:196–208. https://doi.org/10.1038/s41575-020-00390-5. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[11] Mudoor, S. M., Willing, B.P., Bourrie ,B.C.T.(2023). Opportunities and Challenges of Understanding Community Assembly in Spontaneous Food Fermentation. *Foods*.12(3):673. https://doi.org/10.3390/foods12030673

[12] Palanivelu, J., Thanigaivel, S., Vickram, S., Dey, N., Mihaylova ,D., Desseva I. (2022).Probiotics in Functional Foods: Survival Assessment and Approaches for Improved Viability. *Applied Sciences*. 12(1):455. https://doi.org/10.3390/app12010455.

[13] R. B. Cuvas-Limon, Clarisse, N., Mario Cruz., Rosa, M. Rodriguez.,Jasso, Héctor. A., Ruíz, Araceli, Loredo-Treviño., J. A. Texeira & Ruth Belmares (2021). Spontaneously fermented traditional beverages as a source of bioactive compounds: an overview, Critical Reviews in Food Science and Nutrition.

[14] Rezac, S., Kok, C.R., Heermann, M., Hutkins R. (2018).Fermented foods as a dietary source of live organisms. *Front Microbiol.* 9:1785. 10.3389/fmicb.2018.01785 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[15] Sanlier, N., Gokcen, B. B. & Sezgin, A. C. Health benefits of fermented foods. *Crit. Rev. Food Sci. Nutr.* **59**, 506–527 (2019).

[16] Sharma, R,, Garg, P., Kumar ,P., Bhatia, S.K., Kulshrestha ,S.(2020). Microbial Fermentation and Its Role in Quality Improvement of Fermented Foods. *Fermentation.* 6(4):106. https://doi.org/10.3390/fermentation6040106

[17] Shiferaw Terefe, N. & Augustin, M. A. (2020).Fermentation for tailoring the technological and health related functionality of food products. *Crit. Rev. Food Sci. Nutr.* **60**, 2887–2913 .

[18] Staudacher, H. M. & Nevin, A. N.(2019). Fermented foods: fad or favourable addition to the diet? *Lancet Gastroenterol. Hepatol.* **4**, 19 .

[19] Steinkraus, K.H.(1997). Classification of fermented foods: worldwide review of household fermentation techniques. *Food Control.* 8:311–7. 10.1016/s0956-7135(97)00050-9 [CrossRef] [Google Scholar]

[20] Tamang, J.P., Watanabe, K,, Holzapfel, W.H. (2016).Review: diversity of microorganisms in global fermented foods and beverages. *Front Microbiol.* (2016) 7:377. 10.3389/fmicb.2016.00377 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[21] Tsilingiri, K., Rescigno, M. (2013).Postbiotics: What else? *Benef. Microbes* , *4*, 101–107. [Google Scholar] [CrossRef]

[22] Verbeke, K.A,, Boobis, A.,R, Chiodini, A., Edwards, C.A., Franck, A., Kleerebezem, M., (2015).Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr Res Rev.* 28:42–66. Doi:10.1017/S0954422415000037 [PMC free article] [PubMed] [CrossRef] [Google Scholar]

[23] Xiong, H. H., Lin, S. Y., Chen, L. L., Ouyang, K. H., & Wang, W. J. (2023). The Interaction between Flavonoids and Intestinal Microbes: A Review. *Foods (Basel, Switzerland)*, *12*(2), 320. https://doi.org/10.3390/foods12020320

[24] Yao, Z.D., Cao, Y.N., Peng ,L.X., Yan, Z.Y., Zhao G. (2020).Coarse cereals and legume grains exert beneficial effects through their interaction with gut microbiota: a review. J. Agric. Food Chem. 69:861–877. doi: 10.1021/acs.jafc.0c05691. [PubMed] [CrossRef] [Google Scholar]

[25] Yoha, K. S., Nida, S., Dutta, S., Moses, J. A., & Anandharamakrishnan, C. (2022). Targeted Delivery of Probiotics: Perspectives on Research and Commercialization. *Probiotics and antimicrobial proteins*, *14*(1), 15–48. https://doi.org/10.1007/s12602-021-09791-7)

Copyright © 2024 Authors