

**Resistant starch encapsulated Lactoferrin for the effective
management of inflammation**

by

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Under the supervision of

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SYMBOLS

β	Beta
α	Alpha
μm	Micrometer
g	Gram
mg	Milligram
μg	Microgram
%	Percentage
$^{\circ}\text{C}$	Degree Celsius
mL	Milliliter
μL	Microliter
M	Molar
mM	Millimolar
w/v	Weight by volume
nm	Nanometre
V	Voltage
Da	Dalton
kDa	Kilo Dalton
min	Minutes
Kb	Kilobase
h	Hour
sec	Seconds
KPa	Kilopascal
Kcal	Kilocalorie
mol	Mole
$\text{\AA}$	Angstroms
mA	Milliampere

ABBREVIATIONS

aa	Amino acid
AA	Acetic acid
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AlCl ₃	Aluminum Chloride
AMG	Amyloglucosidase
APC	Antigen-presenting cells
ATR	Attenuated total reflection
BA	Butyric acid
bLF	Bovine lactoferrin
BSA	Bovine serum albumin
CFU	Colony-forming units
CH ₃ COOK	Potassium acetate
ESI	Electrospray Ionisation
CHO	Chinese hamster ovary
CID	Collision-induced dissociation
Cho-Imi-OS	Cholesterol/imidazole modified oxidised starch
CLA	Conjugated linoleic acids
CM	Carboxymethyl
CMV	Cytomegalovirus
COVID-19	Coronavirus disease 2019
COX-2	Cyclooxygenase-2
CSF-1	Colony-stimulating factor 1
CYBA	Cytochrome B alpha chain
DB	Debranching
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DS	Degree of substitution
DSC	Differential scanning calorimetry
EBC	Expanded bed chromatography

EE	Encapsulation efficiency
ERK	Extracellular signal-regulated kinase
FAO	<i>Food and Agriculture Organization</i>
FBS	Fetal bovine serum
FCV	Feline calicivirus
FGF2	Fibroblast growth factor 2
FTIR	Fourier transform infrared spectroscopy
GI	Gastrointestinal
gLF	goat lactoferrin
GLP-1	Glucagon-like peptide-1
GOD/POD	Glucose oxidase/peroxidase
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
hLF	Human lactoferrin
hLfRs	Human lactoferrin receptors
hMSCs	Human mesenchymal stem cells
HMT	Heat-moisture treatment
HPLC	High-performance liquid chromatography
HS	Heparan sulfate
HSV	Herpes simplex viruses
HUVECs	Human umbilical vein endothelial cells
I ₂	Iodine
IBD	Inflammatory bowel disease
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IV	Intravenous
JEV	Japanese encephalitis virus
KI	Potassium iodide
LAB	Lactic acid bacteria
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LF	Lactoferrin
LFc	Lactoferricin

LLPBNPs	LF -conjugated lipid polymer-based nanoparticles
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
LRM	Lactoferrin-Resistant starch based microencapsulates
LRMP	Lactoferrin-Resistant starch based microencapsulates postbiotics
MA	Malayannan
MAPK	Mitogen-activated protein kinase
MAYV	Mayaro virus
MD	Maltodextrin
ME	Mysore ethan
MES	Sodium 2-mercaptoethanesulfonate
MIC	Minimum inhibitory concentration
MKK	MAP kinase kinase
MON	Monthan
M ^{pro}	Main protease
MRM	Multiple Reaction-monitoring
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaB	Sodium butyrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaPA	Sodium phenylacetate
NaPB	Sodium phenylbutyrate
NCCS	National centre for cell sciences
NEN	Nendran
NF- κ B	Nuclear factor-kappa B
NK	Natural killer
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory medicines
OD	Optical density
OSA	Octenyl succinic anhydride
p38-MAPK	p38 mitogen-activated protein kinase
PA	Propionic acid

PALA	Palayankodan
PAMPs	Pathogen associated microbial patterns
PBMCs	Peripheral Blood Mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
p-ERK	Phosphorylated extracellular signal-regulated kinase
PEYA	Peyan
PGSS	Gas-saturated solutions
PIV	Parainfluenza virus
PMN	Polymorphonuclear
p-NF- κ B	phosphorylated nuclear factor-kappa B
PP	Potassium phosphate
p-p38-MAPK	Phosphorylated p38 mitogen-activated protein kinase
PPA	Porcine Pancreatic Amylase
p-SAPK/JNK	Phosphorylated stress-activated protein kinase/Jun N-terminal kinase
PVDF	Polyvinylidene difluoride
PYY	Peptide tyrosine tyrosine
RADD	Refrigerated adsorption dehumidified drier
RDS	Rapidly digestible starch
rhLF	Recombinant human lactoferrin
RIPA	Radioimmuno precipitation assay
ROB	Robusta
ROS	Reactive oxygen species
RS	Resistant starch
RSV	Respiratory syncytial virus
SAPK/JNK	Stress-activated protein kinase/Jun N-terminal kinase
SARS-CoV	Severe acute respiratory syndrome coronavirus
SC	Sodium citrate
sCD14	Soluble CD14
SCFAs	Short-chain fatty acids
SD	Standard deviation

SDS	Slowly digestible starch
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid
TBST	Tris-buffered saline with tween 20
T _c	conclusion temperature
TFC	Total flavonoid content
TGFβ1	Transforming growth factor beta 1
TLF	Talactoferrin
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
T _o	Onset temperature
T _p	Peak temperature
TPC	Total phenolic content
TSS	Total suspended solids
UHT	Ultra heat treatment
VEGF	Vascular endothelial growth factor
WAXD	Wide-angle X-ray diffraction
XDH	Xanthine dehydrogenase
XRD	X-Ray diffraction
YAN	Yangambi
ZAN	Zanzibar

PREFACE

Lactoferrin (LF), a multifunctional glycoprotein belonging to the transferrin family, is important in host defense systems because of its significant anti-inflammatory, immunomodulatory, and antibacterial properties. LF is mainly found in milk, saliva, and tears. Colostrum contains a higher amount of LF around 6-8 mg/mL. Its ability to regulate inflammation makes it a promising candidate for therapeutic applications in managing inflammatory disorders. However, the stability and bioavailability of LF in the gastrointestinal tract remain as a major challenge, necessitating the development of delivery systems that can protect LF. This thesis focuses on the fabrication of resistant starch (RS)-based lactoferrin microencapsulates (LRM) to improve the gastrointestinal stability, controlled release, and functioning of LF for effective control and management of inflammation by modulation of gut health. RS, a prebiotic dietary fiber with excellent encapsulating properties, acts as a protective matrix for LF, ensuring its continuous release while also improving gut health by promoting the growth of beneficial bacteria synergistically.

The study involves extraction of RS from selected cultivars of banana/plantain, optimisation of the conditions for encapsulation of LF with RS, characterisation of the encapsulates, and assessing their synergistic prebiotic benefits and *in vitro* release kinetics. Furthermore, the anti-inflammatory properties of postbiotics derived from LRM are investigated, which indicated that they can reduce pro-inflammatory cytokines and modulate key inflammatory pathways. These objectives have been thoroughly explored through the six chapters of this thesis

The introductory chapter, **Chapter 1** provides a complete introduction and literature review of lactoferrin (LF), including its production, health benefits, and applications. It also highlights physiological relevance of resistant starch (RS) and its role as a bioactive carrier.

The subject matter of **Chapter 2** is the evaluation of the nutritional composition of ten unripe (stage II) banana cultivars, with carbohydrates as the main component, mostly consisting of

starch. The isolated starch and RS were characterised in terms of zeta potential, FTIR, XRD, SEM, DSC, and drying curve analysis, suggesting RS as a suitable carrier for LF. Moreover, RS from the PALA cultivar showed the greatest potential as a matrix for encapsulation due to its good thermal properties, making it the best choice for the spray-drying.

Chapter 3A deals with the optimization of conditions of lactoferrin encapsulation by spray drying (LRM) using RS and maltodextrin as wall materials and was characterised in terms of particle size analysis, XRD, FTIR, SEM, and DSC. Characterisation studies showed that the LRM had a smaller particle size (2.296 ± 0.58), smooth surface morphology, better encapsulation efficiency, and effective LF encapsulation, suggesting that it could be used in functional food pharmaceutical applications.

In **Chapter 3B**, the prebiotic potential of LF is assessed and *in vitro* release kinetics from LRM under simulated gastrointestinal conditions are examined. When LRM was fermented with *Lactobacillus casei*, the synergistic effects of LF and RS were evident through enhanced SCFA synthesis and better prebiotic action.

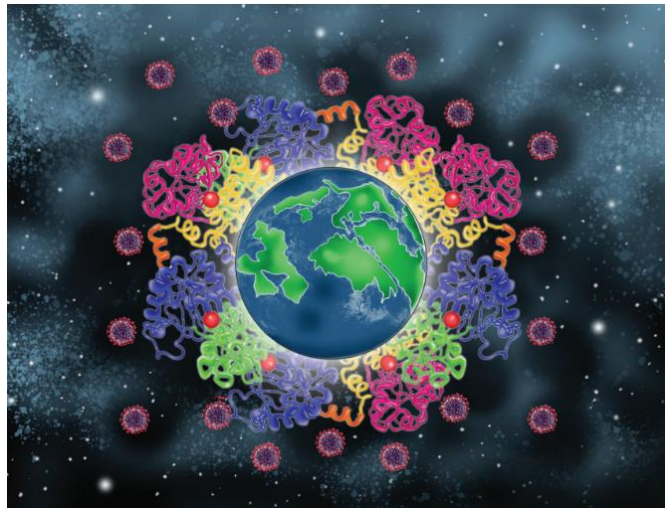
The anti-inflammatory potential of postbiotics derived from LRM is explored in **Chapter 4**. Postbiotics were shown to target the NF- κ B and MAPK signaling pathways in RAW 264.7 macrophage cells, thus downregulating inflammatory mediators and reducing the production of pro-inflammatory cytokines. These results highlight LRM-derived postbiotics as a potential therapeutic agent for inflammation management.

The primary findings of the study are covered in the summary and conclusion of **Chapter 6**. The study demonstrates the protective and functional benefits of RS-based encapsulation for LF, highlighting its applications in health-promoting formulations. The therapeutic and functional food potential of LRM may be further established by future research, including *in*

vivo validations, especially in enhancing immunity, gut health, and also managing viral susceptibility.

Chapter 1

Introduction and Review of Literature



1.1 Lactoferrin

Glycoproteins are molecules containing glycans (oligosaccharide chains) covalently linked to side chains of amino acids through a process called glycosylation. Glycosylation mainly occurs on proteins during the co-translational or post-translational modification. Mainly, there are two types of glycosylation; N-glycosylation, occurs when the glycans attached to nitrogen present in the side chain of arginine or asparagine, whereas O-glycosylation, occurs at the hydroxyl group of threonine, serine or tyrosine group of proteins. These glycoproteins have multiple physiological functions, acting as a transport molecule, hormone, enzyme, receptor, structural molecule, lubricant agent, immunological molecule, blood group determinants, and also a cell attachment site. In mammals, glycosylation mainly occurs in the cell membrane and most secreted proteins. Several glycoproteins are predominantly present in milk, and they show a vital role in the bio-modulatory effects of milk and eventually determine its nutritional quality (Gaunitz et al., 2017). Milk provides high nutritious value for infants and adult humans. Milk has a variety of biomolecules- proteins, oligosaccharides, and lipids that aid non-nutritional roles. Milk proteins are mainly divided into caseins, whey proteins, and mucins; the latter surrounds the milk fat globule membrane. Whey protein contains many proteins, such as immunoglobulin, lactoferrin (LF), α -lactalbumin, lactoperoxidase enzymes, β -lactoglobulin and glycomacropeptides. In human milk, the major part is constituted of whey proteins, while bovine milk is casein predominant.

In human milk, the major glycoproteins present are LF, secretory immunoglobulin, lactoperoxidase, mucin, lactadherin, leptin, adiponectin, α -Lactalbumin and lysozyme. Among these LF is a globular glycoprotein, which is the second most abundant whey protein in human milk. In 1939, LF was identified from bovine milk in its pure form (Sørensen & Sorensen, 1940). LF amount in milk differs between mammalian species during the lactation periods. In human milk, LF concentration is usually 2–4 mg/mL, but it is high in colostrum (6–8 mg/ml).

LF concentration in bovine milk differs between 0.02- 0.2 mg/mL, and in pig, mouse, and horse milk, it's between 0.2 - 2 mg/mL. It is also present in exocrine secretions such as tears, saliva, bile, semen, pancreas, vaginal fluids, gastrointestinal tract, nasal and bronchial secretions. In response to inflammatory reactions, secondary neutrophil granules, macrophages, and glandular epithelial cells produce significant amounts of LF. LF has multifunctional activities shown in Fig. 1.1, consisting of immunomodulation, antimicrobial, antifungal, antiviral, anti-inflammatory, antitumor, antioxidant, besides, it modulates the gut microbiota growth, iron metabolism and stimulates the osteoblast proliferation and differentiation (Giansanti et al., 2012).

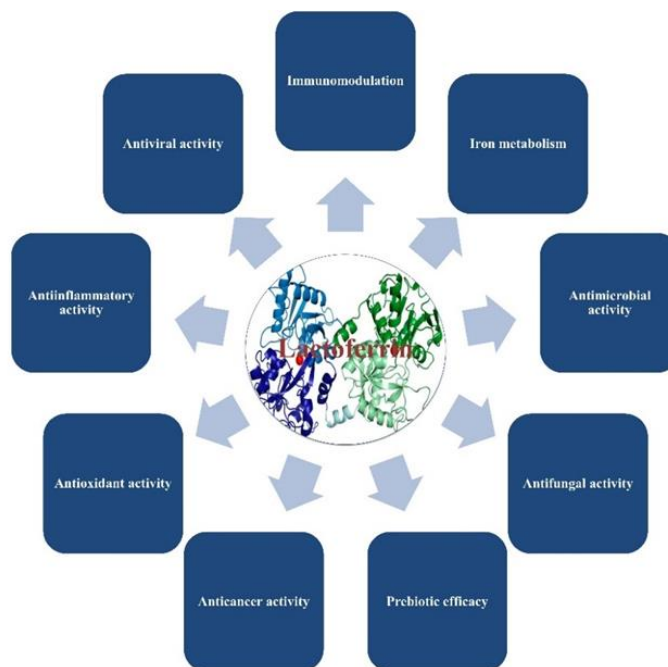


Figure 1.1. Physiological functions of Lactoferrin (LF)

In recent years, there has been a resumed interest in natural compounds. Hence, as an innate substance, LF is capable of resisting viral infections and simultaneously, it can maintain inflammatory homeostasis and rebalance iron. LF has a broad spectrum of antiviral activity against many viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), which is similar to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that caused the recent pandemic coronavirus disease 2019 (COVID-19) outbreak. The clinical

outcomes of individuals affected by COVID-19 are severe and may even result in mortality. At present, as a prevention measure, vaccinations are available, but COVID-19 treatments mainly include symptom alleviation methods and synthetic non-specific wide-spectrum antiviral drugs. LF can act as a potent nutraceutical aid for the treatment of SARS-CoV-2 infection by preventing the binding to the surface of the target cell, which could be specifically effective during the phase of viral replication in the salivary glands, throat, and upper respiratory tract (Redwan et al., 2014).

1.1.1 Structure of Lactoferrin

LF is an iron-binding globular glycoprotein, that belongs to the transferrin family, also known as lactotransferrin. The molecular weight is 80 kDa and is composed of about 796 amino acids (aa). LF has a great extent of homology between species. Human LF and bovine LF have 691 aa and 689 aa, respectively; they share approximately 70% sequence identity. LF tertiary structure has a single chain of polypeptide which is folded into two equal domains- N and C lobe. It consists of α - helices and β -sheets structure, and the domains are covalently linked by a short α -helix. The two domains are further halved into two sub-domains: N1 and N2; C1 and C2, which forms two clefts for each lobe, where the ferric ion is bound. Aspartic acid, histidine, and tyrosine are the most essential iron-binding amino acids in LF. Each ferric ion is bound tightly to the LF through a carbonate ion. When a ferric ion reversibly binds to LF, it can exist in a free iron form called apo-LF, a single iron bound form called mono-ferric LF, and a bound iron form called holo-LF. Also, LF can bind with other metals like Cu^{2+} , Zn^{2+} , Mn^{2+} and Al^{3+} ions. LF is a cationic protein, hence it can bind with anionic biomolecules like DNA, lipopolysaccharide, and heparin. LF is prominently a N-linked glycosylated protein; mannose and N-acetyl glucosamine are the main sugars coupled with it. The gene expression in individuals determines the types of sugar and the number of glycosylation sites in LF. The difference in glycosylation sites involved in LF, not only affects its susceptibility towards

thermal denaturation and proteolysis, but also alters its immunogenicity (E. N. Baker & Baker, 2009).

1.1.2 Synthesis and Digestion of Lactoferrin

The biosynthesis of LF is regulated by the type of cells producing it. The LF produced in the mammary gland is determined via prolactin, while the estrogens influence the protein production in reproductive tissues. In endometrium, LF synthesis is controlled by the estrogens and epidermal growth factor. LF is synthesized and secreted by exocrine glands in a continuous manner.

The LF production occurs in neutrophils during their differentiation from promyelocytes into myelocytes and it's stored in particular granules perhaps in the tertiary granules. LF is not produced as a large precursor and appears an unphosphorylated form. Depending on the acidification mechanisms the LF moves towards its storage granules and this takes place in the trans and medial cisternae of the Golgi body, hence it is treated like proteins designed for secretion. The LF present in neutrophil granules has two targets: it can be either released into the blood or nearby tissues, or it can fuse with phagosomes (Teng, 2002). The polymorphonuclear cell's secretion into blood circulation is determined by the degranulation factors, which in turn seem to be dependent on the cGMP, protein kinase C, and guanylate cyclase activation.

Digestion of food is essential because the body requires nutrients from drink and food to stay healthy and to work well. The digestive system breaks down nutrients like carbohydrates, fats, proteins, minerals, vitamins, and water into smaller subunits adequate for the body to uptake and use for energy, growth, and cell repair. The human digestive system consists of three digestive segments: salivary, gastric, and intestinal. In the salivary phase, the breakdown of carbohydrates begins, while the digestion of protein occurs in the gastric and intestinal phases.

LF is a glycoprotein, digested into peptides and essential amino acids, which are absorbed by the intestine and later transported into the blood. The *in vivo* digestion of LF in the gastrointestinal (GI) tract is not clearly understood. The degree of iron saturation determines the breakdown of LF. The holoLF is more resistant to digestion than the apoLF. Due to the digestion of orally supplemented LF, its biological activity diminishes in the intestine. However, in a recent study, the systemic activities of intravenous (IV) and orally administrated recombinant human LF (rhLF) were examined. It confirmed that the orally administrated rhLF showed systemic biological effects via stimulating signal transduction mechanisms similar to IV-administrated LF. In both cases, the upregulation genes (transforming growth factor beta 1 (TGFβ1), interleukin-1β (IL-1β), colony-stimulating factor 1 (CSF-1), xanthine dehydrogenase (XDH), cytochrome B Alpha Chain (CYBA), etc.) were specifically involved in the inflammatory response and oxidative stress (Kruzel et al., 2021). Additional genome analysis is required to understand signaling pathway crosstalk. However, some studies revealed that bioactive peptides derived from pepsin digestion of LF showed more antibacterial activity than intact ones (Tomita et al., 1991). Orally administrated LF improves gut health; to exert its effects in the human colon an intact LF is required (Y. Wen et al., 2017). So, the studies suggested that both intact as well as peptides derived from LF retain their biological activity after oral administration.

1.1.3 Production of Lactoferrin

1.1.3.1 Purification from milk

Industrial development in processing technologies and equipment leads to separating vital nutritional components from the milk of goats, cows, buffalo, sheep, and other species. In 1940, LF was discovered in its pure form and was easily identified by its pink color (Sørensen & Sorensen, 1940). Heat treatment leads to the denaturation of LF, so for the purification of bLF

pasteurized milk is unsuitable. Hence, LF is isolated and purified from cheese whey and skim milk on an industrial scale. According to the amino acid composition, LF exists as a cationic form; hence using a cation-exchange resin such as carboxymethyl (CM)-Sephadex, it can be readily adsorbed and later eluted using salt solutions, so, this method is mainly used for the purification of bLF. Whey cheese (pH 6.4) and skim milk (pH 6.7) are filtered and added to the column of cation-exchange chromatography by not changing its pH. Lactoperoxidase is eluted out from the column when a low concentration of NaCl solution is used for washing while using a high concentration of NaCl solution bLF is eluted. The eluted bLF is concentrated and desalted by ultrafiltration and diafiltration respectively, and then it is subjected to low heat treatment. The purified freeze-dried LF powder has 95% or higher purity obtained, otherwise, after sterile filtration bLF is spray-dried. Pasteurization of LF is very important to inactivate the microbes and viruses, it was observed that during heat treatment at acidic pH, LF is stable, but protein denaturation occurs under heat treatment at neutral pH (Abe et al., 1991). The suitable method for pasteurization of LF is by treating it to 90–100 °C, at pH $\approx$ 4 for 5–10 min besides, ultra-high temperature (UHT) treatment is used and it can be applied for the manufacturing of broad-spectrum products containing LF. By the treatment with porcine pepsin at acidic conditions LF pepsin hydrolysate is formed; after hydrolysis, inactivation of pepsin is done via heat treatment. Later the hydrolysate is filtered and concentrated through reverse osmosis, then, by pasteurization and freeze-drying the hydrolysate of LF is obtained, which is used in infant formula. Furthermore, from this hydrolysate of LF, lactoferricin (LFc) is purified through dual-step hydrophobic chromatography (Bellamy et al., 1992). In safety tests, the toxicity of purified LF was very low; studies showed that LF has no adverse effects in rats at 2000 mg/kg per day and didn't show any mutagenic potential (Yamauchi et al., 2000). The results suggested that the purified LF is regarded to remain safe as a food additive.

On laboratory scale, many chromatographic means have been used for LF purification. The heparin-sepharose affinity chromatography was used for the LF purification from bovine skim milk, human skim milk and human pancreatic juice, and the heparin-agarose affinity chromatography was used for bLF purification from the involution of mammary gland secretions (Rejman et al., 1989). bLF is also purified from bovine colostrum, milk, and whey using cation-exchange chromatography and affinity chromatography (Perraudin et al., 2020). Moreover, several new methods have been evaluated to increase the purity of LF. In 2020 an integrated method for LF separation and purification was proposed. LF is purified from sweet whey, through ultrafiltration and subsequently by cationic exchange expanded bed chromatography (EBC), resulting in 92.7% purity (Maciel et al., 2020). An aptamer affinity column combined with high-performance liquid chromatography could be used for purification and to analyze samples for LF in dairy factories and testing laboratories (N. Wang et al., 2021). A novel multimodal chitosan mini-spheres were used for the purification of LF from cheese whey without any pretreatment, got a yield of 68% purity. Thus, these developed processes and matrices revealed a potential use in industry (Hirsch et al., 2020). The commercially available method for the bLF manufacturing is in Figure 1.2. Skim milk is used as feedstock for the chromatographic separation of bLF. The unpasteurized skim milk is cooled below 8 °C to prevent the growth of microbes, passed through a clarifier, followed by a 1 µm filter to remove fat, any insoluble substances and to decrease the microbial load. The filtrate is subjected to ion exchange column; the column bound with the filtrate is washed first with demineralized water and then with sodium chloride solution to remove the weakly bound contaminants. The column is washed with concentrated sodium chloride to elute out the bound bLF, and desalted using ultrafiltration, pH is adjusted to 6.5, followed by pasteurization at 73.5 °C for 18 sec, followed by evaporation and spray drying. The manufacture bLF via freeze drying followed by

pulverization in the final step instead of spray drying is also being reported (*GRAS Notices*, n.d.).

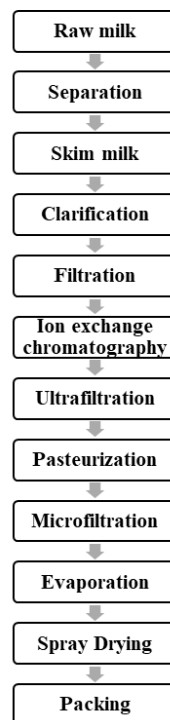


Figure 1.2. Process for the production of bovine milk-derived Lactoferrin

1.1.3.2 Recombinant Human Lactoferrin

LF production (hLF) from human milk has safety problems including human pathogens contamination and limitation of supply. Thus, for clinical and animal studies recombinant hLF (rhLF) has been developed mainly in transgenic cows, plants, and microbial species. In transgenic cows, the hLF gene is under the control of the bovine casein promoter, which produces rhLF; therefore, using Mono S chromatography, the transgenic cow milk which contain both bLF and rhLF was separated. A new cloning method is developed for the large-scale production of rhLF using a bovine mammary bioreactor. It has a large capacity for protein synthesis and an efficient production system with a 4.5-13.6 g/L yield (R) (M. Wang et al., 2017). However, the chances of potential contamination with infectious pathogens create public concerns about its biological safety. In rice, the rhLF amount is expressed as 25% of total soluble protein (Bethell & Huang, 2004). Aside from having plant pattern glycosylation, rhLF produced from rice has similar biochemical and physicochemical properties as that of

native hLF. Furthermore, a recent report revealed that transgenic tomatoes with hLF gene showed an increase in the resistance of plants to fungal and bacterial pathogens (Buziashvili et al., 2020). Using plants as a transgenic vector provides an efficient, safe, and cost-effective alternative, but the lack of research and yield change depending on various factors like genomic content, plant species, and growing conditions reduce its commercial application. It has been reported that rhLF is produced from *Aspergillus oryzae* and *Aspergillus niger* by fermentation. Apart from oligosaccharides variation, rhLF produced from *A.niger* possesses similar characteristics to hLF. Another yeast *Pichia pastoris* is used for expressing the LF gene from Tibetan sheep and by using macroporous microencapsulation a matrix-immobilized yeast used for the large-scale production of rhLF, the purified rhLF shows antibacterial, iron-binding activity, inhibitory activity against cancerous cells. It can rapidly reproduce up to 2 g/L yield; however, it has low secretion efficiency and is unsuitable for high-density protein production (Ho et al., 2015). Mammalian cells are also used to produce rhLF. Researchers examined the efficacy of Chinese hamster ovary (CHO) expressed rhLF to boost the BCG vaccine. The results showed that rhLF not only modulates its activity but also reduces hypersensitive response by the host immune system (Hwang et al., 2015). The CHO-derived rhLF is fully compatible with hLF; thus, it has the most promising therapeutic applications in humans in the future. In recent news, a U.S. and Singapore-based technology company, Turtle Tree has announced that it is ready to launch a cell-based LF for infant formula and also as a nutrient supplement (L. Kristiana, n.d.). This will be a stepping stone for both the food and pharmaceutical industry in this field of hLF commercialization.

1.1.4 Health Benefits of Lactoferrin

1.1.4.1 Immunomodulation

LF has the capability of altering innate and adaptive immune systems by either inducing or suppressing components of the immune response. It is the first-line defence that influence the development of adaptive immunity. The cytokines secretion is altered by iron sequestration of LF, that in turn decreases the oxidative stress. Human colostrum has a higher concentration (7 g/L) of LF, but comparatively, there is a low concentration of LF in breast milk (1 g/L). The modulation of the immune response of LF depends on the degree of glycosylation on a molecule. One of the main functions of colostrum is to supply infants with vital components for immune system development and provide protective functions. It provides systemic as well as localized protection in the intestine of infants by aiding in the development of immature intestines and sustaining postprandial pH. LF controls the immune reaction of lymphoid tissue related to the intestine and stimulates the small epithelial cells proliferation and differentiation, thus influencing the length, mass, and expression of digestive enzymes in the small intestine (Siqueiros-Cendón et al., 2014).

Many immune-modulatory activities of LF are associated with its potential to bind with numerous pro-inflammatory pathogen-associated microbial patterns (PAMPs), mainly identified by specific innate immune cell receptors, the toll-like receptors (TLRs). This interaction has been reported between unmethylated CpG-containing DNA and Gram-negative bacterial lipopolysaccharide (LPS). LF inhibits the TLR-4-related pathway, thereby interfering with the pulmonary inflammatory processes induced by LPS (H. Y. Li et al., 2021). The LF subunit LFc binds with LPS, which is a prototypical PAMP through the lipid-A moiety. In an in vitro study, such interaction was reported to block the signaling of LPS by receptors, like L-selectin and TLR-4, not only in immune cells such as neutrophils, lymphocytes, and

monocytes-macrophages, as well as in endothelial cells (Elass-Rochard et al., 1995). Moreover, LF has a high affinity towards the co-receptor of TLR-4 that is soluble CD14 (sCD14), only or combined with LPS. In the inflammatory regions, all these binds were shown to suppress the LPS-induced stimulation of immune cells, causing a lower secretion of proinflammatory cytokines, such as IL-6, IL-1, and tumor necrosis factor- α (TNF- α). This is similar to the IL-8 expression (an essential constituent in inflamed tissues) and adhesion molecules (E-selectin and intercellular adhesion molecule-1). As a result, polymorphonuclear leukocytes (PMN)-secreted LF could reduce responses and after that at the site of inflammation immune cells are recruited, thereby, decreasing further tissue damage. LF immune response in rats intoxicated by diazinon is evaluated, and found that LF treated group significantly decreased the number of WBCs and increased IL-10, total protein, globulin, and albumin level than other groups and this study proven that LF can be used as a prevention aid than as treatment drug (Sh et al., 2018).

LF interacts with antigen-presenting cells (APC), such as macrophages, dendritic cells, Langerhans cells, and B cells of the immune system. Macrophages are phagocytic cells; its primary role is to prevent microbial infection through the microorganism's destruction intracellularly or by inhibiting their replication by the cytokine's secretion or some mediators like nitric oxide. Also, they play a role in inflammation by the secretion of inflammatory mediators and pro inflammatory cytokines. When LF binds with its receptor macrophages are activated, thereby increasing their phagocytic ability and IL-12 synthesis; this recruits more macrophages to the site of inflammation and, thus, activates T- lymphocytes (CD⁴⁺). Dendritic cells are a heterogeneous cell population; they are specific in antigen detection and have an essential role in the immune system via regulating tolerance and immunity. When the dendritic cells interact with LF through specialized surface receptor, this binding promotes their maturation, differentiation and its functional activities. The rhLF, talactoferrin induces

dendritic cells maturation, this stimulates the secretion of IL-8 and CXCL10, with a significant decrease in the production of IL-10, IL-6, and CCL20 (Perdijk et al., 2018). Many review articles have discussed the immunomodulation and anti-inflammatory activities of LF. Recently the bioavailability and the role of different forms of LF in maintaining immune homeostasis and inflammatory processes and its recent clinical studies investigating LF application are discussed by researchers. Similarly, the vital role of LF in immune responses against infectious attacks and injury; and also the importance of LF in immunomodulation and anti-inflammatory mechanism to retain physiologic homeostasis were reviewed. Some researchers specifically reviewed the immunomodulatory action of LF on both innate and adaptive immune responses. All these articles support the role of LF in immunomodulation activity (Actor et al., 2009; Kruzel et al., 2017; Moreno-Expósito et al., 2018; Sienkiewicz et al., 2022).

1.1.4.2 Iron Metabolism

Iron act as a cofactor for key enzymes involved in several critical metabolic pathways and cell functions; its deficiency leads to reduced fatigue and immunity. LF has an excellent iron affinity with constant balance dissociation (KD) of approximately 10-20 and is required to maintain regular iron harmony and eliminate iron deficiency and iron overload. LF, being an iron-binding glycoprotein, has an excellent iron binding capacity and is essential to sustain an even iron balance. One molecule of LF can bind with two ferric ions in the presence of two molecules of carbonate ions, thus it can act as an iron transporter. Many studies have been done to discover the role of bLF in iron absorption; in a study, when pregnant women supplemented with oral bLF, they showed an increased level of hemoglobin, ferritin, and total serum levels and a decreased occurrence of iron deficient anemia (Paesano et al., 2010). Due to the occurrence of particular LF receptors, LF is assumed to have a vital role in the intestinal absorption of iron across the enterocyte brush border membrane. In a current meta-analysis

study, oral administration of bLF improved the iron supplies similar to that of ferrous sulfate with reduced gastrointestinal side effects in iron-deficient pregnant women (Rezk et al., 2016). In an *in vivo* study with Kenyan infants, the effects of holo-bLF, apo-bLF, and iron sulfate are compared, showing that both forms promote iron absorption (Mikulic et al., 2020). Moreover, the complexity of LF and iron interactions remains unknown, so iron-dependent pathologies in humans should be precisely evaluated

1.1.4.3 Antimicrobial Activity

LF structural features impart further functionality other than iron homeostasis which is familiar to the transferrin family. Mainly, LF shows a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts, fungi, and parasites, in addition, LF stimulates the selective growth of gut beneficial bacteria such as *Lactobacillus*, *Bifidobacterium*, etc. Being an iron-binding protein, LF has the iron sequestration ability; thus, it creates a nutrient shortage, which is essential for the growth and division of siderophores. Also, LF binds to the surface of bacteria and generates LPS release from the outer membrane, leading to cell damage and death. One of the significant factors involved in bacteriostatic LF properties is the disruption of the osmotic function of membranes. LF has a highly cationic N-terminal region that interacts with a negatively charged component of bacteria; lipoteichoic acid in Gram-positive bacterial membrane and LPS in Gram-negative bacteria, thereby exerts a strong bactericidal activity. This interaction destroys the lipid bilayer of microbial cell membranes, causing greater permeability, loss of cellular contents, and finally leads to death. However, the growth of some bacteria that have a lower iron requirement is promoted by LF, which is beneficial for human health. Several *in vitro* investigations have revealed that LF suppresses the pathogen adhesion to the epithelial cells and intestinal mucus. Furthermore, an *in vivo* study conducted by researchers discovered that rectal administration of LF has a dual effect on stopping *Escherichia. coli* O157:H7 colonization and improving the local immune

response to the infection of 6-month-old Holstein-Friesian calves with this organism (Rybarczyk et al., 2017). The antimicrobial activity of LF against *Streptococcus dysgalactiae* and *Streptococcus mutans* is by reducing its invasion into cells and bacteriostatic effect. It inhibits *Cronobacter sakazakii* adhesion and internalization and reduces the oxidative stress caused by these microbes. LF has been explored to treat *Helicobacter pylori* infection by inhibiting growth and reducing the expression of virulence factors. It has therapeutic activity against *Mycobacterium tuberculosis* and reduces the immune-mediated tissue damage in tuberculosis disease. LF also reduces the bacterial load of *Salmonella enterica* sv *Typhimurium* and decreases its infectious inflammation (Abad et al., 2021; O'Halloran et al., 2016; Velusamy et al., 2016; Welsh et al., 2011; Wu et al., 2016; Y. Yuan et al., 2015).

After the LF proteolytic digestion with pepsin, LFc, a bioactive peptide, is released from the N-terminal region of LF that shows higher antimicrobial action than LF (Bellamy et al., 1992). The LF and LFc antimicrobial activity has not been restricted to human pathogenic microbes. In a study evaluated the LFc activity in transgenic plants too. When tobacco plants are exposed to *Pseudomonas syringae* pv. *tabaci* and *Botrytis cinerea* infection; they showed that after 30 days of infection, transgenic plants survived, but due to pathogen damage, wild-type plants perished (Fukuta et al., 2012). A combination of milk proteins, like LF and lactoperoxidase had an inhibitory effect against multidrug-resistant *Acinetobacter baumannii* and showed a synergistic effect on their immunomodulatory activity against this organism (Mahdi et al., 2018). The LF nanoparticle produced through the electrostatic complexation to gellan gum showed intensified antimicrobial activity against *Staphylococcus aureus* and *E.coli* than LF in its pure state (L. G. R. Duarte et al., 2022).

1.1.4.4 Antifungal activity

LF and LF-derived peptides have a wide spectrum of antifungal activity due to their iron sequestration ability and binding with the fungal cell surface, which leads to cell membrane damage and leakage. Studies have shown that LF changes the cell membrane permeability in *Candida krusei*, *Candida albicans*, and *Cryptococcus neoformans*, leading to cell death. In *Candida* isolates, cell surface alterations are seen through scanning electron microscopy such as protein leakage, surface blebs formation, swelling, and cell collapse (Fernandes & Carter, 2017). In a recent study revealed that LF binding protein Pma1p, a proton pump that is inhibited by LF through interrupting the membrane ergosterol and sphingolipid-rich lipid rafts connected with Pma1p, thereby promoting the intracellular accumulation of ergosterol, and it also reduces the V-ATPase activity to stimulate antifungal activity (Santos-Pereira et al., 2021). Furthermore, hLF prevents the oral candidiasis caused by *C.albicans*, via the upregulation of anti-inflammatory cytokines and downregulation of virulent candida genes (Pawar et al., 2022). The antifungal drug like fluconazole combined with LF from various species significantly enhanced the inhibitory effect and decreased the minimum inhibitory concentration (MIC) against *Candida* species, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* (Lai et al., 2019).

1.1.4.5 Antiviral activity

The LF antiviral activity against enveloped or non-enveloped RNA and DNA viruses has always been well-known (Table 1.1). LF can prevent virus infections by blocking the viral attachment to host cells and thus inhibiting viral replication within the cell, and it also enhances systemic immune responses. Several mechanisms of action of LF have been proposed to prove its antiviral activity; the most commonly accepted one is based on its capacity to bind with viral receptors like glycosaminoglycans, chiefly heparan sulfate (HS). This interaction prevents the

viral attack by blocking the primary contact between target cells and the virus. Another widely accepted mechanism is by modulating the immune response; the phagocytosis action of macrophages is increased by binding with LF. It also improves the response of TH1 lymphocytes and Natural Killer (NK) cell activity, which produces cytokines that defend against viral infection (Redwan et al., 2014).

Table 1.1. Antiviral activity of Lactoferrin (LF)

References	Virus	Mode of action of Lactoferrin
I. Enveloped Viruses		
(Hara et al., 2002)	Hepatitis Viruses	Inhibition of viral entry, Inhibits intracellular replication
(Andersen et al., 2003)	Herpes Simplex Viruses (HSV)	Inhibition of viral adsorption, inhibits viral replication, Inhibition of cytopathic effect
(Beljaars et al., 2004)	Cytomegalovirus (CMV)	Inhibits viral replication
(Berkhout et al., 2002)	Human Immunodeficiency Virus (HIV)	Blocks viral entry, inhibits viral replication
(Mistry et al., 2007)	Human Papillomavirus	Blocks viral entry, Inhibition of cytopathic effect
(Taha et al., 2010)	Influenza A (H1N1)	Blocks viral entry, Inhibition of cytopathic effect
(Portelli et al., 1998)	Respiratory Syncytial Virus (RSV)	Inhibition of viral adsorption and growth
(Yamamoto et al., 2010)	Parainfluenza Virus (PIV)	Inhibition of viral adsorption
(Waarts et al., 2005)	Alphavirus	Blocks viral entry

(Chien et al., 2008)	Japanese Encephalitis Virus (JEV)	Inhibition of viral attachment
(Campione et al., 2021)	SARS-CoV	Inhibition of viral attachment
II.Non-enveloped Viruses		
(Superti et al., 2001)	Rotavirus	Inhibition of viral adsorption, Inhibits assembly of new viral particles, Inhibition of cytopathic effect
(Marchetti et al., 1999)	Poliovirus	Blocks viral entry, Inhibition of cytopathic effect
(Arnold et al., 2002)	Adenovirus	Inhibition of viral attachment, inhibits viral replication
(Oda et al., 2021)	Norovirus	Inhibition of cytotoxic damage
(McCann et al., 2003)	Feline Calicivirus (FCV)	Inhibition of viral attachment

Direct LF administration can prevent human RSV attacks at ten times lesser amounts than those present in human milk. LF also blocks the infection from non-enveloped viruses like enteroviruses and adenoviruses. The antiviral activity of LF is not only limited to viruses that affect humans but its activity is extended against animal viruses, like the Friend virus complex, which causes erythroleukemia in rodents, feline herpesvirus, feline immunodeficiency virus, and feline calicivirus (Kobayashi et al., 2008). It seems that the LF activity against viruses is exhibited in the early phase of infection. During the infection process, several pathogenic viruses, such as herpes simplex virus, have been shown to utilize the surface of host cells as a binding receptor. Results imply that a functional GAG-binding site of LF is formed by synergistic binding of GRRRRS sequence at the amino terminus of human LF with an RKVR sequence (Redwan et al., 2014).

A study in 2014 determined that when the target cell is infected, instead of hindering the replication of the virus, bLF blocks the host cell infection. When they monitored the bLF activity on the infection by Mayaro virus (MAYV), they found that it has strong antiviral activity, involving prevention of viral entry into the cell; this finding may help to develop a potent strategy in resistance to MAYV infection (Carvalho et al., 2014). Moreover, it is demonstrated that bLF prevents influenza A virus infection by interacting with fusion peptide hemagglutinin (Superti et al., 2019). However, *in vitro* studies depict that the efficacy of LF against HIV may be through its prevention of viral replication within the host cells. Thus, three LF antiviral mechanisms of action were identified. Firstly, it can inhibit the cellular virus internalization (including poliovirus type 1); Secondly, it can suppress the replication of virus within the cell (including HCV); and finally, it can block the glycosaminoglycan viral receptor heparan sulfate, thereby preventing the interaction between virus and host cell (Berkhout et al., 2002). SARS-CoV-2 is a highly contagious and easily replicating virus that causes the acute disease COVID-19, which is characterised by acute respiratory distress syndrome and pneumonia. SARS-CoV-2 is a spherical, enveloped virus containing positive-sense single-stranded RNA genome. This genome encodes 29 proteins including sixteen nonstructural, four structural and nine accessory proteins. The four structural proteins are nucleocapsid, envelope protein, matrix, and spike protein. The spike protein contains two subunits S1 and S2, which are located on the envelope. Through the S1 subunit, the virus attaches to the cell receptor ACE2 and S2 is involved in membrane fusion. SARS-CoV-2 also uses the HSPG proteins present on the cell membrane, as an attaching site, which has a vital role in the early phase binding of the virus. SARS-CoV-2 is an emerging virus that presently lacks curative treatments and the recent SARS-CoV-2 outbreak has made life come to a standstill all over the world. LF is a natural, non-toxic protein that has established its antiviral efficacy against a wide spectrum of viruses that cause human infection, including SARS-CoV-2 (Fig. 1.3). LF has many

mechanisms of action against SARS-CoV-2. LF directly binds with SARS-CoV-2 and thereby prevents viral entry and replication. Also, LF competes with the SARS-CoV-2 for the cell membrane receptors ACE2 and HSPGs which block the entry and replication of the virus. Moreover, LF is an immunomodulator that stimulates the immune cells like natural killer cells to fight against the virus. It promotes the antigen-presenting capacity of B cells and regulates T cell function thereby reducing inflammation. LF reduces the iron-containing ferritin protein level by iron chelation, thus limiting SARS-CoV-2 viral replication. LF's ability to bind with iron leads to a reduction in the formation of reactive oxygen species, and this antioxidant activity impairs SARS-CoV-2 replication. The invasion of SARS-CoV-2 stimulates the cytokine storm in a COVID-19 patient, and this may lead to acute respiratory distress and later death. LF reduces the level of cytokines like TNF- α , and IL-6, and acute phase reactants like ferritin. The SARS-CoV-2 virus decreases the production of interferons, that usually fight off viral infections. LF restores interferon action by increasing its production which leads to the reduction in the replication of the SARS-CoV-2 virus. LF also has the ability to reduce platelet aggregation by regulating the protein plasminogen, which is released during a SARS-CoV-2 viral invasion. SARS-CoV-2 viral replication starts at the respiratory system and continues its replication after reaching the gastrointestinal tract. So, LF protects the gastrointestinal tract by exerting antiinflammatory activity through maintaining a balance between good and bad gut microbes and reducing the intestinal damage caused by the SARS-CoV-2 virus (Rosa et al., 2023).

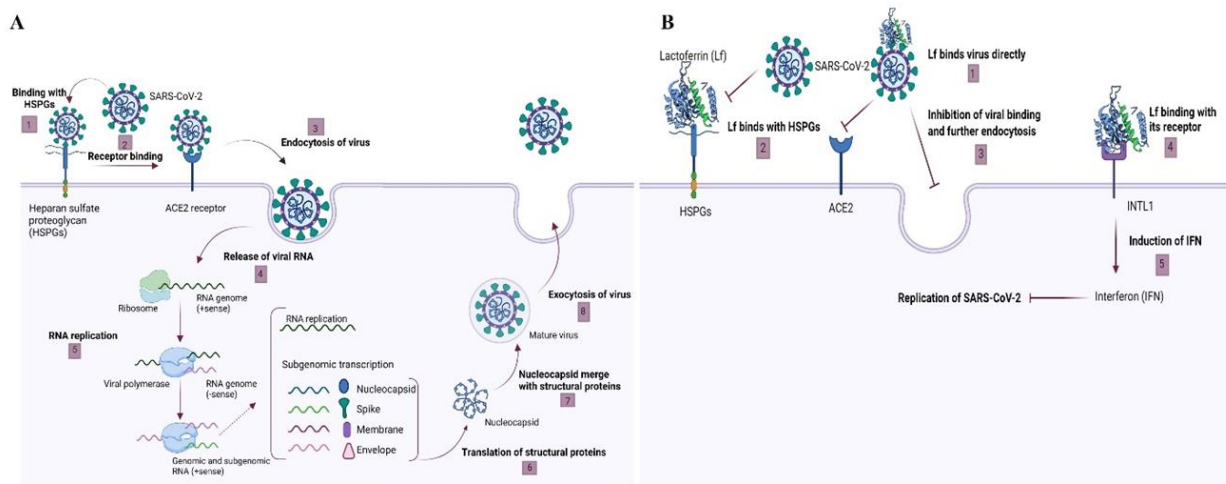


Figure 1.3. (A) SARS-CoV-2 attachment and replication in host cells. SARS-CoV-2 first binds to Heparan sulFate proteoglycans (HSPGs) (1) and then attaches with angiotensin-converting enzyme 2 (ACE2) receptor (2) that facilitates the fusion between host cell and SARS-CoV-2 and its endocytosis (3). After endocytosis, the viral genomic RNA is released into the cytoplasm (4). Replication occurs in the cytoplasm, where using the genomic RNA as a template, subgenomic RNA is formed (5). Using viral RNA genes, nucleocapsids, and viral structural proteins are transcribed and translated (6). The nucleocapsids combine with structural proteins and allow the release of mature new viruses (7) via exocytosis (8). Created with www.biorender.com. **(B) Anti-viral mechanism of lactoferrin (LF).** Direct binding of LF with SARS-CoV-2 (1); LF binds with heparan sulfate proteoglycans (HSPGs) and thus blocks the SARS-CoV-2 attachment with angiotensin-converting enzyme 2 (ACE2); hence prevents endocytosis and viral replication (2 and 3). LF might also inhibit the virus replication through the stimulation of intracellular cell signals like Interferon (IFN) activation by binding with its receptor Intelectin-1 receptor (INTL1) (4). Created with www.biorender.com

In a meta-analysis, it was revealed that LF reduces the risk of respiratory tract infections, so it can be used as an adjunct for the prevention and management of COVID-19 (Ali et al., 2021).

In vitro studies reported that due to the availability, safety, and *in vitro* efficacy, LF has protective effects against SARS-CoV-2 by modulating the antiviral immune response with a modest effect on infection (Salaris et al., 2021; Wotring et al., 2022). It also revealed that bLF binds to the heparan sulfate proteoglycans, thereby blocking the attachment of SARSCoV-2 to the host cell (Hu et al., 2021). Furthermore, LF enhances the antiviral activity of hypothiocyanite against SARS-CoV-2, when a combination of these compounds was given *in vitro* (Cegolon et al., 2021). Recently, a novel LF-derived peptide GSRY has been identified as a SARS-CoV-2 inhibitor; it has the potential to inhibit SARS-CoV-2 main protease (M^{pro}), which is involved in the coronavirus life cycle (W. Zhao et al., 2022).

1.1.4.6 Anti-inflammatory activity

LF acts as a natural host defense regulator with major anti-inflammatory activities by catalyzing the tissue-toxic hydroxyl radicals formation and scavenging free iron deposition in tissue, and this makes LF a potential therapeutic means in the treatment of inflammatory disorders. After exposure to pathogens, LF is one of the molecules released by neutrophils and promotes innate activation of adaptive responses through neutrophils release into the inflammation site. Studies have proven that the released LF has not only a response against pathogens but also against neutrophils released at the site of inflammation. Depending upon the iron saturation status it has an anti-apoptotic activity against neutrophils, which may lead to chronic inflammatory diseases (Francis et al., 2011). Thus, the iron saturation status of LF is important to exert its action against both pathogens and neutrophils. When the host encounters skin allergens LF can trigger the immune system against it, by the dendritic cell accumulation and prevention of Langerhans cell migration in lymph nodes. Furthermore, LF modulates the cytokine secretion from leukocytes; it promotes the anti-inflammatory cytokine production of IL-10; in addition, LF inhibits the synthesis of proinflammatory cytokines like IL-1 β , IL-6, TNF- α , and IL-8, and. Depends upon the signal type recognized by the immune system, the synthesis of these components occurs. LF amplifies the number of adaptive T cells (CD⁴⁺ and CD⁸⁺) and NK cells and increases PMN recruitment in the blood, which causes phagocytosis and regulates myelopoiesis. IL-12 is well known for its vital role in helper T-cell development. Hence, the LF role in controlling IL-12 and proinflammatory cytokines reveals communication among innate and adaptive immunity (Giansanti et al., 2016).

The wound healing ability of LF is well reported, a recombinant human LF, talactoferrin (TLF), aids in the secretion of proinflammatory cytokines TNF- α , IL-8, IL-6, and MIP-1 α . Also, it may up-regulate platelet-derived growth factor, stimulate the proliferation of keratinocytes, enhance hyaluronan expression needed for producing tissue granulation and migration necessary for wound re-epithelization, furthermore protect the cell from apoptosis

(Engelmayer et al., 2008).

1.1.4.7 Antioxidant activity

During cellular respiration reactive oxygen species (ROS), along with energy, excess amount of ROS production, and accumulation lead to metabolic disorders in humans. When the ROS production overcomes the capacity of the antioxidant system, oxidative stress occurs. Oxidative stress has a vital role in the disablement of endothelium-dependent vasodilation and the stimulation of hypertrophy, inflammation, and apoptosis by various signaling pathways activation. The excess formation of ROS causes endothelial cell damage via protein oxidation, lipid peroxidation, and DNA fragmentation. The LF antioxidant activity was associated with its iron binding ability, thereby reducing ROS production. LF has a lethal effect on pathogens by stimulating the ROS production by granulocytes; in contrast, at extracellular pH, the ROS production is inhibited by LF through the activation of iron binding by monocytes; this prevents the lipid peroxidative activity of neutrophils, thus preventing the oxidative tissue damage by ROS. However, LF has a direct effect on hydroxyl and oxygen scavenging capacity, which is independent of iron binding ability. It is shown that LF protects DNA from direct oxidative damage by acting as a ROS scavenger. LF has a cytoprotective effect against oxidative stress induced by hydrogen peroxide (H_2O_2). It reduced hydroperoxides concentration and increased FRAP value in the extra-and intra-cellular fluid. The pretreatment of human umbilical vein endothelial cells (HUVECs) with LF significantly reduced the intra-and extracellular hydro peroxides level assessed by FOX-1 assay. The results also showed a significant increase in total antioxidant capacity in intra-and extracellular fluids after pretreatment of HUVECs with LF (Safaeian et al., 2015).

In healthy individuals, the LF supplementation has been related to an increase in hydrophilic antioxidant activity. LF has also shown an antioxidant effect on erythrocytes through the inhibition of lipid peroxidation and hemolysis. It has been observed that subsequent treatment

of LF reduces the intracellular ROS levels induced by glucose oxidation, implying its capacity to decline oxidative stress-induced apoptosis. The study showed that LF pretreatment could guard human mesenchymal stem cells (hMSCs) against oxidative stress-induced senescence and apoptosis. It is revealed that LF inhibited peroxide-induced senescence by preventing the Akt and caspase-3 activation (Park et al., 2017).

In pregnant women, some pregnancy complications have also been associated with oxidative stress. LF administration to the vagina reduces the oxidative stress in pregnant women's amniotic fluid with mid-trimester genetic amniocentesis by reducing lipoperoxides formation through directly mediating Fe^{3+} elimination from Fenton reaction or ROS scavenging (Trentini et al., 2020).

1.1.4.8 Anticancer activity

LF has an antineoplastic effect against various types of cancer cells by different mechanisms of action, such as; immunomodulation, alteration of the cell membrane, apoptosis, cell cycle arrest, inhibition of metastasis, anti-angiogenic action, and necrosis of cells. LF has been recommended as a potent chemo-preventive agent against many cancerous cells. When LF is orally administrated, it cannot be absorbed or degraded by the small intestine, so it remains in the digestive system and thereby stimulates gastrointestinal immune response against carcinogenesis.

The chelating activity of LF against tumor formation is the most commonly studied mechanism of action. The free iron in the tissue causes oxidative stress on genomic material, which may lead to carcinogenesis, thus LF captures the free iron ions at the tissue level. Moreover, LF reduced the cytotoxicity induced by aflatoxin B1 and M1, oxidative stress, DNA damage, and levels of ERK1/2 and JNK in four cancerous cell lines- Caco-2, Hep-G2, HEK, and SK-N-SH (N. Zheng et al., 2018). The findings suggested that LF has a chemopreventive effect against diethylnitrosamine-induced hepatocarcinogenesis in rats by

decreasing the expression and activation of the PKB/Akt pathway (Hegazy et al., 2019). In addition, it is proven that a nano combination of LF and lactoperoxidase is more effective in apoptosis mediating anticancer activity than free forms in various human cancer cell lines (Abu-Serie & El-Fakharany, 2017). LF can produce cytokines in the cancer cell, arrest tumor growth, and induce apoptosis; it can also prevent the G1 to S phase transition in malignant tumors. Furthermore, the treatment with hLF inhibits tumor growth by increasing the anticarcinogenic cytokines levels like IL-18, and NK cells and T cells (CD⁸⁺) activation. In mice, oral administration of bLF inhibits tumor-induced angiogenesis, and hLF has a particular pro-angiogenic effect (Shimamura et al., 2004). Studies in animal models reported that bLF inhibits colon cancer, and clinical trials have proven that hLF reduces the risk of colorectal cancer, it is also revealed that goat LF (gLF) also showed anticancer activity (Abbas, 2015; Kozu et al., 2009). Furthermore, the antiproliferative function of colon cancer cells (SW480) is inhibited by the synergistic effect of the chlorogenic acid-LF stable complex; this complex promotes the cell cycle arrest at the G₀-G₁ transition phase (Y. Zhang, Chen, et al., 2022). The evidence suggested that for the treatment of cancers, the most promising approach may be the Akt signaling pathway inhibition. LF has been shown to reduce the growth of breast cancer cells, and it also inhibits the G1-S phase transition by the extrinsic LF addition into the culture media of MDA-MB-231 (breast cancer cell lines). Moreover, in HeLa cells, LF promotes cell cycle arrest, apoptosis, and Smad-2 nuclear accumulation (D. C. Duarte et al., 2011; Luzi et al., 2017).

In addition, it is commonly known that the LF receptors are overexpressed in cancerous cells to provide the increasing nutritional demands of these well-multiplying cells. Therefore, for the specific targeting of these cells, one of the excellent approaches is to develop LF nanocarriers. A LF -conjugated lipid polymer-based nanoparticles (LLPBNPs) are used for the targeted delivery of methotrexate into the cancerous cell (Kumar et al., 2023). A high drug

loading and stable LF dual drug (docetaxel and celastrol) nanoconjugate delivery system has been developed for synergistic breast cancer therapy (Abdelmoneem et al., 2021). Also, LF targeted-N-iso-propylacrylamide (NIPAM)-co-acrylic acid (AA) nanohydrogels developed for the delivery of bi-phenolic lignin, honokiol (HK) as a promising breast cancer therapy because it has a high affinity towards LDL and transferrin receptors present in various cancerous cells, also LF receptors (LRP1, LRP2) are overexpressed in breast cancer cells (Metawea et al., 2021). Zein-LF micelles showed tumor-specific targeted co-delivery of rapamycin/wogonin with low drug dose, maximized antitumor efficacy, and minimized side effects. Besides, a pH-dependent release of the drug is observed in LF nanocarriers. A faster release of drug is found at acidic pH; it might increase the release of drug at acidic sites like tumor tissue microenvironment, so this improves the therapeutic efficiency of hydrophobic bioactive encapsulated within nanocarriers (Sabra et al., 2018). Additionally, in the brain endothelial cells surface LF receptors are present; thus, when LF binds to its cell surface receptor, it can easily cross the blood-brain barrier by receptor-mediated transcytosis. Because of this ability, LF can be used to fabricate nanocarriers that specifically target brain tumor cells (Kumari et al., 2017).

1.1.4.9 Prebiotic efficacy

LF is a glycoprotein that stimulates the growth of specific probiotics. Milk is a rich source of LF, and its bioactive peptide LFc, is obtained by pepsin digestion of LF, that have an antimicrobial effect against pathogenic organisms (Bellamy et al., 1992). Many *in vitro* and *in vivo* studies have reported about the LF activity that promotes the growth of the probiotic. Both peptide LF and LFc enhance the growth of selected probiotics, including *Lactobacilli* sp. and *Bifidobacteria* sp (P.-W. Chen et al., 2017). These may be due to the physicochemical properties of surface components and enzyme secretion from probiotics that stimulate the growth-promoting activity of LF than antimicrobial activity. In an *in vivo* study, piglets were taken as a model to study the LF role in the growth and varieties of colon microbes and their

influence on the wellness of the neonatal intestine (Comstock et al., 2014). Through intestinal microbiota, LF can involve many processes, like providing energy for the growth of the intestine, intestinal cell maturation and differentiation, signaling of gut-brain-microbiota axis for nerve fibers maturation, and regulating inflammatory and immune responses for a beneficial effect on intestinal wellness.

Many studies have demonstrated the effects of human and bovine milk samples on the probiotic's growth. The growth of *Bifidobacterium* species like *B. bifidum*, *B. breve* and *B. infantis* was similarly enhanced by human and bovine milk (Oda et al., 2014). It is found that the collected one to six months postpartum human milk showed varying growth-stimulating activity on *B.breve*, *B.pennsylvanicus*, and *B.infantis*; however, in bovine milk, a stable bifidogenic activity was noted (Kim et al., 2005). These results may signify differences in bovine and human milk composition due to genetic variation, changes in bioactive components like LF present in milk in different postpartum periods, and the diverse fermentability of bifidobacteria strains. In a study, it was documented that unpasteurized human breast milk had inhibitory effects on neonatal pathogens like *Staphylococcus* and *E.coli*, as compared to pasteurized ones. This phenomenon was attributed to the higher amount of LF, in unpasteurized breast milk. Supplementation of hLF to the pasteurized human breast milk resulted in inhibition of pathogenic as well as probiotic cultures. And at higher dosages, bLF also showed antimicrobial activity against all tested species, including *B.breve* (Woodman et al., 2018). The findings implied that simultaneous administration of LF and probiotics might affect the efficacy of probiotic colonization. This result is contradictory to those found in other studies (P.-W. Chen et al., 2017).

Many studies have revealed that depending on the LF iron saturation shows various impacts on the growth of probiotic strains and inhibitory action against pathogens. The antimicrobial effect of LF and LFc B against *Bacteroides* species found on the human gastrointestinal tract

is investigated in an *in vitro* study; the results showed that the binding to laminin and biofilm formation were intensely inhibited by LF at low concentrations (de Sá Almeida et al., 2020). In probiotic culture, assays have shown that apobLF exhibited inhibitory activity against the growth of foodborne pathogenic organisms like *Enterococcus fecalis* and *Salmonella typhimurium*; in contrast, it inhibited or didn't involved in the growth of bifidobacteria and lactobacilli species (P.-W. Chen et al., 2013). A study with different forms of metal-bound LF like apobLF, Fe-bLF, and Cu-bLF showed an enhanced growth of bifidobacterial species. Besides, Zn-bLF and Cu-bLF have greater inhibitory activity on pathogenic growth, such as *Staphylococcus aureus* and *Escherichia coli*, than apobLF. These outcomes support that an iron-depleted form of bLF reduces the growth of pathogens without altering certain probiotics proliferation and provides an innate means for retaining a balance between gut homeostasis and microbiota (Vega-Bautista et al., 2019).

1.1.4.10 Other physiological functions of lactoferrin

1.1.4.10.1 Lactoferrin in bone metabolism

The primary bone-forming cell within the bone tissue is osteoblast which is essential for the remodeling of bone and homeostasis. Bone metabolism is regulated by a dynamic balance between osteoblasts and osteoclasts. Both bLF and native hLF increased the length of S and G₂/M phases and promoted the proliferation and nourishment of osteoblasts when compared with untreated osteoblasts (J. L. Zhang et al., 2018). A study shows that LF stimulated MC3T3-E1 osteoblast cell proliferation via c-Fos and c-Jun by activating JNK, ERK, and p38 signaling pathways (M. Liu et al., 2018). LF has been found to promote osteoblast proliferation while inhibiting apoptosis by mediating IGF-1R in an IGF-1R-independent mechanism, and it also activates the PI3K/RAS signaling pathway (Hou et al., 2015). It was demonstrated that bLF and its peptide could enhance osteoblast proliferation and differentiation and prevent osteoclast differentiation. LF has been shown to induce the secretion of some cytokines or growth factors

in osteoblasts and successively affect the activity and function of osteoblasts along with other skeletal component cells (An et al., 2019). Studies have shown that bLF is a potent osteogenic factor, that enhances osteoblast formation by the activation of p38 MAPK and Smad 2/3 signaling pathways (Inubushi et al., 2020). It also stimulates the production of angiogenic components, vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2) in osteoblasts, implying that LF can initiate the crosstalk between osteoblasts and endothelial cells via the paracrine activity of angiogenic factors. It has been discovered that LF increased the level of IL-6 and IL-11 mRNA in osteoblasts. LF treatment significantly upregulated the IL-6, THBS-2, Cyr61, FGF-basic, MCP-1, Serpin E1, and PDGF-AA levels, whereas the Serpin F1 level was down-regulated in osteoblasts. LF exerts a beneficial effect on osteoblasts by orchestrating growth factors and cytokines. Furthermore, the peptide LFP-C derived from bLF enhanced the MC3T3-E1 mouse cell line proliferation by increasing the osteoblasts proportion in the G₂ /M phases and it also increased the calcium deposition and alkaline phosphatase activity (Shi et al., 2020). Osteogenic effects of the P1 peptide derived by pepsin hydrolyzes of bLF were studied; it also promoted osteoblast proliferation and alkaline phosphatase activity (P. Wen et al., 2021). Moreover, a combination of LF and substance P incorporated within chitin-PLGA-calcium sulfate hydrogel showed more potent bone regeneration due to the chemotactic agent (SP) and pleiotropic growth protein (LF) (Amirthalingam et al., 2021). In a study, it was shown that bLF inhibits bone destruction during LPS-induced periodontitis, so it can be used as an adequate and safe preventive treatment for this (Y. Chen et al., 2021).

1.1.4.10.2 Lactoferrin in neural development and neuronal diseases

LF is a glycoprotein abundant in sialic acid, and because of this, it can contribute to neurodevelopment. Studies in piglets as an animal model have shown that the LF administration can promote cognitive functions, neural development, and memory. It has also

been implied that in piglets LF rich diet contributes to a reduction in anxiety and impulsivity (Jahan et al., 2017). Evidence showed that cerebral ischemic reperfusion is accompanied by inflammatory reactions, which are stimulated by the triggering of proinflammatory factors, chemokines, and adhesion molecules. TLR-4 is widely mediating inflammatory response during ischemic reperfusion. Many reports have proved the LF protective effect in cerebral ischemia injury. In a study, they compared the protective effects of LF, β -lactoglobulin, and α -lactalbumin in cerebral stroke; results indicated that TLR-4 targets these bioactive proteins to block cerebral ischemic reperfusion. In mouse models, LF is found to suppress the TLR-4 associated pathway in cerebral ischemic-reperfusion, anoxia, and reoxygenation cell; however, in a temperature-dependent manner, there is a decrease in LF activity (H.-Y. Li et al., 2020; Yang et al., 2020). Neurological diseases like Alzheimer's (AD) and Parkinson's diseases are related to iron metabolism. In Alzheimer's patients, iron and other metals accumulate in amyloid- β plaques, LF is a chelator molecule that can pass across the blood-brain barrier; hence, it is used as a potential therapy for this condition. LF has shown a protective effect in AD by its ability to reduce the cognitive decline and pathological cascade through the p-Akt/PTEN pathway modulation; this affects the vital players in oxidative stress and inflammation involved in the pathology of AD. In naturally aging C57/BL6J mice, LF was shown to improve cognitive ability; moreover, by the effects on inflammatory regulation and iron chelation, LF reduces the formation of ROS in the hippocampi of mice (J. Zheng et al., 2020). A high incidence of brain injury due to hypoxia-ischemia occurs in premature infants accompanied by subsequent neural development disabilities, so LF may have promising applications in protecting infants from neural damage. In addition to this, the specific delivery of drugs like doxorubicin hydrochloride towards glioma is mediated by nanogels coated with LF to penetrate the blood-brain barrier (M. Zhang et al., 2021). Being an innate substance that can cross the blood-brain barrier, LF can be directly used to treat

neurological disorders and also used as a delivery system for many neuro drugs without any side effects.

1.1.4.10.3 Lactoferrin in metabolic diseases

Recently, a new application of LF in clinical research has been revealed to be used as a potent aid in the treatment of metabolic disorders including diabetes, hypertension, and obesity. The LF mechanism of action against these metabolic diseases is yet unknown. A study in high-fat diet streptozotocin-induced diabetic mice showed that LF supplementation improved pancreatic dysfunction and hepatic insulin resistance by regulating the PI3K/AKT cell signaling pathway (Du et al., 2022). The interaction of mammalian receptors like Omentin-1 with LF is involved in type-2 diabetes, obesity, and LDL receptor-associated proteins, implicated in the metabolism of lipids. In high-fat-induced obese mice, the oral administration of bLF has displayed a positive modulation in gut microbiota, decreased inflammation, and regulated glucose, and lipid metabolism (J. Sun et al., 2016). The bLF administration in micro mini pigs also showed reduced LDL levels and fat accumulation. These findings indicated that the possible way for the prevention and treatment of metabolic disorders is the bLF administration in humans (Morishita et al., 2016).

1.1.5 Applications

1.1.5.1 Infant formula

LF is a milk protein most extensively used as a supplement due to its wide range of functional activities. WHO recommends breastfeeding throughout six months of age, and human milk is established as the gold standard for the nutrition of infants. However, a significant proportion of infants get complete or partial formula feeding. Many studies are going on to change the infant formula to match the dynamic characteristics of breast milk. LF is a well-studied bioactive constituent. After the development of freeze-drying and ultrafiltration in the dairy industry, pure and bioactive LF can be formed, which allows LF inclusion in infant formula.

Human LF shares approximately 70% sequence analogy and similar bioactivity with bLF, so it has been extensively used for its health benefits. The ingestion of bLF-fortified milk showed high total iron in the body and effective intestinal absorption of iron in breastfed infants. The dietary bLF was also demonstrated to reduce parasite colonization, invasive fungal infections in infants, and diarrhea and vomiting in older children (Manzoni, 2019). In infants, LF in unsaturated-iron form prevents the growth of the bacteria and lowers the incidence of diarrhea by iron scavenging from bacteria. A recent, study on the thermal stability of bLF with potassium phosphate (PP) and sodium citrate (SC) additives for application in infant formulas showed that PP and SC don't prevent the bLF denaturation, but they inhibit the aggregation of LF and maintain a relatively large amount of soluble LF after the heating treatment (Jiménez-Barrios et al., 2022). The recommended level of LF supplementation in powdered infant formula by the FDA is 100 mg/100 g.

1.1.5.2 Lactoferrin as a delivery system

There has been an increased interest in incorporating hydrophobic and hydrophilic bioactive into functional food products, like peptides, proteins, conjugated linoleic acids (CLA), carotenoids, polyphenols, and probiotics. However, due to the poor chemical stability, oral bioavailability, and water-dispersibility of these bioactive restricted their application. Because of these reasons, there has been a growing interest in developing delivery systems to protect, encapsulate, and release the bioactive compounds to improve their bioavailability and increase their food matrix compatibility. Several types of delivery systems have been developed, which include emulsions, solid lipid nanoparticles, liposomes, and biopolymer microgels.

LF has the capacity to bind with molecules and form covalent conjugates or physical complexes so that it can be used as a nutraceutical carrier in the field of medicine and food. Carboxymethyl tara gum- LF complex acts as a carrier for Vit D₃; this encapsulation increases

the stability and improves the controlled release of the vitamin (Santos et al., 2021). Many studies showed that the chemical stability and bioavailability of bioactive molecules like resveratrol and β -carotene are improved when it is encapsulated within LF-based emulsions; and carboxymethylcellulose-LF complex coacervates (El Ghazzaqui Barbosa et al., 2022). In a recent, a novel delivery system was designed using LF-functionalized hollow mesoporous manganese doped silica nanoparticles loaded with resveratrol for ischemic stroke treatment (Zou et al., 2022). Moreover, when polyphenols and polysaccharides form a covalent complex with LF, this can prevent the bioactive degradation in emulsions and increase their stability. The LF conjugation onto the surface of inorganic nanoparticles modifies their functional properties and stability.

Curcumin encapsulated within LF nanoparticles improved its cellular uptake, increased bioactivity, and enhanced its retention. The self-assembled nano micelles of LF peptides to deliver curcumin revealed more dilution, storage, and thermal stability with low cytotoxicity (Y. Wang et al., 2022). Simultaneously, the electrostatic complexation formed of LF and pectin and an oleogel-based Pickering emulsion stabilized by the transglutaminase- LF particle used to encapsulate curcumin improved its release profile, chemical stability, and antioxidant activity (Xia et al., 2022). The surface modification of LF nanoparticles with sodium 2-mercaptoethanesulfonate (MES) enhances its efficacy by specifically targeting the cells infected with HIV-1 through the controlled delivery and effective concentration of the drug at the infection area (Senapathi et al., 2020). A study reported that the LF nanoparticles (LFNPs) had been used as a delivery system for DNA. It is found to be stable, non-toxic, and resist digestion against nuclease. Additionally, in LF receptor-expressing cell lines, LFNPs can allow DNA uptake by receptor-mediated internalization (Kumari & Kondapi, 2018).

1.1.5.3 Lactoferrin as a dietary supplement

As an immunomodulator, LF can boost immune response. bLF is produced as a nutraceutical

that doesn't have any health risks. In *Caenorhabditis elegans*, LF-incorporated nutritional supplement extends lifespan and decreases amyloid β -peptide toxicity by stimulating the immune system (Martorell et al., 2017). Studies conducted on healthy volunteers have shown that the consumption of bovine milk-derived LF has immunomodulatory effects. This suggested that clinically bLF can be used to improve the immunity of patients by activating their immune response (Mulder et al., 2008). LF supplements are presently sold at a 96% purity level. To improve the purity of supplements, more research needs to be done to use hLF instead of bLF in nutrient supplements.

1.1.5.4 Lactoferrin in the cosmetic sector

LF has many applications in the cosmetic sector. LF promotes metabolic processes in the skin and anti-aging effect because of its antioxidant capacity, which scavenges free radicals. Due to its anti-bacterial activity, it's a highly required ingredient in cleansers. LF is also used in hairspray for smoothing and hydrating hair (Kazimierska & Kalinowska-Lis, 2021). Further research is needed to extend the use of LF in beauty products, including hair, face, make-up, and body care items.

1.2 Resistant starch

The main source of energy and a necessary nutrient for human life is starch (Alves et al., 2021). The two main components of a starch granule are usually linear amylose and highly branching amylopectin, with trace amounts of protein, fat, and minerals. Several functional properties, including viscosity, solubility, shear resistance, gelatinisation, and retrogradation, are determined by the amylopectin/amylose ratio (Chavan et al., 2021). According to the body's rate of glucose release and digestion, starches are divided into three groups: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Hedayati et al., 2018; H. Sun et al., 2022).

RS, which is readily degraded by digestive enzymes is metabolised by gut microbes,

producing short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate. RS has many physiological benefits including improving intestinal inflammation, avoiding type II diabetes, stabilising postprandial blood glucose levels, and controlling the phenotype of the gut microbiota (Birt et al., 2013; Englyst et al., 1992). According to Zhang, the body regularly consumes more meals with a high RS content to sustain health (S. Zhang, Yang, et al., 2022). However, developing RS-fortified starch sources and food items is necessary because the amounts of RS in the majority of native starches are limited (Z. Zhang & Bao, 2023). Therefore, to increase the use of starch, its structure must be changed. The production of RS currently employs physical, chemical, and enzyme modification manufacturing processes. Various methods, including chemical, physical, enzymatic, and intercalation complexes, can be used for modification. The insertion of different functional groups has resulted in the introduction of additional physical and chemical characteristics. The starch's molecular structure changes as chemical groups are added. The starch's digestibility is greatly reduced because the digestive enzymes α -amylase and α -glucosidase cannot recognise the chemical groups in the starch. Increasing the emulsifying properties of starch can be accomplished by enzymatic, chemical, physical, or a combination of these techniques. When amylopectin is allowed to retrograde, debranching can produce more short linear molecules and realign a new crystalline structure. Debranching is therefore frequently used in combination with heat-moisture treatment (HMT) or autoclaving-cooling to allow for the retrogradation of the short linear molecules and ultimately increase the RS yield. Using chemical modification, hydrophobic components in the form of octenyl groups are added to hydrophilic starch granules to give them amphiphilic characteristics and improve their emulsifying properties. Several processes, such as debranching (DB), octenyl succinic anhydride esterification (OSA), OSA modification following debranching (DBOS), and debranching following OSA modification (OSDB), can alter rice starch (Jain et al., 2019).

The altered starch can be used to deliver physiologically active substances, probiotics, and drugs. To create amphiphilic cholesterol/imidazole-modified oxidised starch (Cho-Imi-OS), it has been suggested that oxidised starch be synthesised and concurrently modified with imidazole and cholesterol. After that, Cho-Imi-OS was shaped into nanoparticles for the delivery of anticancer drugs to specific tumors. Curcumin was employed as a model drug encased in the hydrophobic core of nanoparticles. The nanoparticles release curcumin, which has an inhibitory effect on cancer cells (Xu et al., 2020). The structure of V-helical amylose provides a higher encapsulation affinity than other RS, particularly for hydrophobic or amphiphilic substances. The RS are more suitable as possible drug carriers because of their increased resistance to enzymatic hydrolysis (Prasher et al., 2021). With its balanced nutritional functioning and sensory impacts, RS is considered the ideal dietary fiber (Kaimal et al., 2021). As an emerging form of dietary fiber, RS can prevent colon cancer, regulate the gut microbiota, and prevent intestinal inflammation (Almeida-Junior et al., 2017; Hu et al., 2016; Lei et al., 2021; Ren et al., 2022).

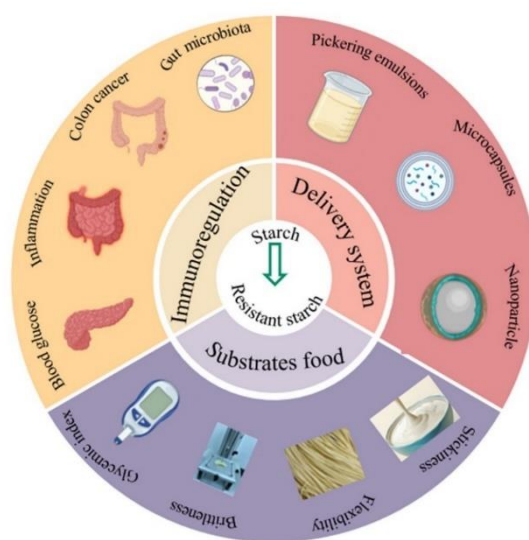


Figure 1.4. Schematic representation of RS functions and applications (Jiali et al., 2024)

1.2.1 Resistant starch classification

Resistant starch (RS) is classified based on its structural characteristics and sources, which

significantly influence its physiological parameters and health benefits. The classification includes various categories, each with distinct features (Raigond et al., 2015; Z. Wang et al., 2023). There are five subgroups of RS (RS1–RS5), some of which are naturally occurring and others of which can be passively generated under different processing conditions.

- Type 1 (RS1): Physically inaccessible starch present in whole grains and seeds, which is resistant to digestion due to its physical structure. When starch is cooked as whole grains, the proteins and cell walls of the legume seeds prevent water from penetrating the starch. As a result, the starch is unable to get enough water to swell and gelatinise, which makes it difficult to combine with amylase for hydrolysis (Jenkins et al., 1988). Modifications to the processing methods can impact its resistance and promote digestion (Bao et al., 2018).
- Type II (RS2): The Raw starch granules known as RS2 are inaccessible to amylase due to their compact structure that is crystal-embedded in either a B-type or C-type crystal (A. A. Baker et al., 2001). The primary sources of this kind of RS are raw rice, potatoes, and, bananas, as well as in high-amylose maize starch. Cooking and grinding are examples of processing techniques that can increase the activity of digestive enzymes and lower the amount of RS. After heating, the majority of starches undergo gelatinisation, losing their B-type or C-type crystalline structure and turning into fast-digesting starches, such as baked potatoes (Noor et al., 2021).
- Type III (RS3): Retrograded starch, is created when amylose regenerates when food is being processed. The long chains of amylose and amylopectin molecules recombine to form double helical polymers joined by hydrogen bonds when starch undergoes retrogradation under freezing conditions. This results in short linear crystals with a strong molecular structure that makes it difficult for amylase to break down in the gastrointestinal tract (Jane & Robyt, 1984). As an auxiliary raw material, it has

minimal impact on the food's color, flavor, and original nutritional components during heating and cooking, making it a novel kind of food ingredient with a broad range of applications (W. Li et al., 2021; Zeng et al., 2015).

- Type IV (RS4): Chemically modified starch, which is formed by adding new chemical groups to starch that already exists. These include phosphorylation, ethyl esterification, esterification, and γ -ray irradiation, which alter the molecular structure, and physical and chemical characteristics of starch that inhibit amylase adsorption and cross-link formation. Acetyl, phosphorylated, cross-linked, and heat-denatured starches are examples of common modified starches. These modified starches have textural qualities that make them suitable for a variety of food processing (Hsieh et al., 2020; Yaver & Bilgiçli, 2021).
- Type V (RS5): Amylose and fatty acids or fatty alcohols combine to form the amylose-lipid complex known as RS5. The distinctive crystal structure of RS5, which often has a V shape, decreases the area of interaction between the starch granules and digestive enzymes, making it indigestible. These complexes can be found in native starches like corn, soybeans, and grains. They can be made in the laboratory by the addition of lipids, and their content can be enhanced by heating them to high temperatures (Okumus et al., 2018; Putseys et al., 2010). RS5 is susceptible to retrogradation and possesses a high gelatinisation temperature and great thermal stability (Gelders et al., 2006).

1.2.2 Physiological Functions of Resistant Starch

1.2.2.1 Effects of RS on diabetes and blood glucose levels

Diabetes is a metabolic disorder characterised by abnormally high blood glucose levels. The prevalence of diabetes has risen dramatically in recent years, and both its prevention and treatment have emerged as global public health concerns. Dietary intervention is a key

component of treatment for type II diabetes, which affects 90–95% of diabetic patients. In diabetic patients, the basis of diabetes treatment is the diet, particularly the choice and management of staple foods, including the main source of carbohydrates (Hemmingsen et al., n.d.; Y. Zheng et al., 2018). Small intestine indigestion of RS lowers the glycemic index after consumption, and postprandial blood glucose fluctuation is low, thereby improving diabetic patient's glucose tolerance levels (Arias-Córdova et al., 2021; Maziarz et al., 2017). It has been demonstrated that RS2 effectively controls insulin resistance and fasting insulin levels in diabetic patients (Dainty et al., 2016). In type II diabetic rats, RS3-containing diet significantly decreased serum glucose, insulin levels, and the release of glucose-dependent insulinotropic polypeptides, improving glucose metabolism (Qadir et al., 2020).

1.2.2.2 Effects of RS on Gastrointestinal Health

Short-chain fatty acids like butyric, acetic, and propionic acids are the primary fermentation metabolites of RS. These compounds can provide intestinal mucosa energy, which makes them crucial for colonic mucosal nourishment. The azomethane-induced precancerous lesions of the rat colon were successfully prevented by RS5 (Cray et al., 2017; Y. Zhao et al., 2011). Metabolomics findings revealed that the various metabolites in the RS diet treatment group were primarily linked to the metabolism of lipids, vitamins, and amino acids, and intestinal inflammation, which helped to understand how RS promotes intestinal health (Qin et al., 2023). It was shown by this study that microbiomes have a significant impact on the results of RS supplementation. According to a randomised controlled trial, the RS diet does have an impact on intestinal flora as determined by quantitative PCR analysis and phylogenetic chip analysis; however, the diet's assessment of the microbiota's composition was significantly less extensive than individual differences (Salonen et al., 2014). According to study findings, individual microbial flora responds to the diet in rather distinct ways, which is a crucial consideration when assessing how RS affects intestinal integrity.

1.2.2.3 Effects of RS on obesity and body weight

Obesity is a global health concern that is defined by improper metabolism or excessive fat buildup from food consumption (Haslam, 2007). An efficient method of weight control is to reduce energy consumption, and there is increasing evidence that RS aids in weight management (H. Liu et al., 2020). RS produces low energy since it is difficult to break down into glucose, which is used directly by the human body (Higgins, 2014). By increasing the release of satiety-related hormones such as glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY), RS can reduce food intake (Zhou et al., 2008). According to Maziarz, muffins with a high RS content increased the feeling of fullness, slowed down the rate of digestion, and resulted in weight reduction (Maziarz et al., 2017). Postprandial fat oxidation increased significantly when RS was substituted for carbohydrates (Higgins et al., 2004), indicating that RS may prevent fat formation. Diets containing RS significantly reduced rats' body weight and calorie intake (Belobrajdic et al., 2012). Additionally, RS significantly lowered the body weight of obese rats (Higgins et al., 2011). Therefore, these studies show that RS improves satiety and decreases energy and fat buildup, which helps reduce body weight.

1.2.2.4 Effects of RS on the homeostasis of cholesterol

Maintaining homeostasis in the body is crucial since cholesterol is necessary for synthesizing steroid hormones, vitamin D, and cell membranes. Hyperlipidaemia, a major risk factor for atherosclerotic cardiovascular disease, is brought on by excessive consumption of meals high in calories, which raises plasma levels of triglycerides and cholesterol (Schade et al., 2020). Propionic acid is one among the several short-chain fatty acids generated by RS fermentation, and it has a significant impact on fatty acid synthesis, lipid absorption, and cholesterol reduction (Cheng & Lai, 2000). Numerous studies have demonstrated a positive correlation between the amount of propionic acid in the blood and the consumption of RS. Rats' serum and

liver levels of low-density lipoprotein and cholesterol are decreased by propionic acid (H. C. Yuan et al., 2018). In rodents, RS lowers plasma levels of triglycerides and total cholesterol while increasing SCFAs, suggesting that RS has a beneficial effect on cholesterol reduction (Le Thanh-Blicharz et al., 2014). Plasma triglyceride levels were considerably lowered in patients with metabolic syndrome fed a diet containing RS (Johnston et al., 2010). The lotus seed RS given to hyperlipidemia rats improves blood lipid levels by altering the structure of small intestine flora and increasing the conversion of cholesterol into bile acids (L. Liu et al., 2021).

1.2.3 Application of resistant starch as a delivery system

Starch has several advantageous characteristics including biodegradability, safety, biocompatibility, and abundance. Because of these features, it is frequently used for delivery. However, many native starch-based bio-actives are released suddenly in controlled-release systems due to the polyhydroxy structure of starch, which makes it highly hydrophilic. This causes the loss of embedded bioactives, probiotics, or drugs. As a result, native starch is inefficient as a delivery system. To provide higher-yield microencapsulation of substances while maintaining functionality and physicochemical characteristics, starch can be modified chemically, physically, or enzymatically (Hoyos-Leyva et al., 2018).

RS plays a crucial role in delivering bioactive compounds and drugs as functional dietary fiber. It improves the mechanical stability, encapsulation efficiency, and sustainability of the release of the delivery system (Ding et al., 2018). These features were significantly improved when RS was added to the nisin-loaded alginate particles (P. Wang et al., 2021). RS can be used in various ways as a delivery vehicle or as part of a carrier. RS is widely employed in combination with another compound as a microcapsule wall material for controlled release. RS-glucan matrices released caffeine at a slower and more regulated rate than beta-cyclodextrin, particularly in simulated intestinal fluid. Curcumin is efficiently encapsulated in a high-

amylose, stearic acid, and soy protein composite for long-term release in intestinal fluid (D. Zheng et al., 2021). The release of spray-dried mint flavor was moderately affected by the degree of substitution (DS) in octenyl succinic anhydride (OSA) -modified starch. Improved emulsification due to higher DS resulted in interfaces that slowed flavor release and improved retention (K. Wang et al., 2022). The viability of lyophilised probiotics encapsulated in RS was assessed, demonstrating RS's capacity to protect probiotic activity. FA-RSMCs are starch-based microcapsules that have been modified with folic acid for targeted delivery and controlled release of hydrophobic drugs. Coumarin 6 (C6) was encapsulated as a model drug, and C6-loaded FA-RSMCs demonstrated selective targeting of HeLa cells with controlled release features (Y. Sun et al., 2018). In addition to a microcapsule, RS can be used as a delivery system in various forms, such as nanoparticles, hydrogels, and Pickering emulsions (Jagtiani, 2022; Lu et al., 2020; Y. Zhang, Dong, et al., 2022).

1.3 Scope of work

Despite the growing recognition of lactoferrin's health benefits and its potential applications in functional foods and pharmaceuticals, there remains a need for further research to fully understand and optimize its bioavailability, stability during processing and storage, and efficacy in various health contexts. Additionally, challenges exist in developing cost-effective methods for incorporating lactoferrin into consumer products while maintaining its bioactive properties. Addressing these issues is crucial to fully exploit lactoferrin's therapeutic potential and effectively advance its integration into health management strategies. In light of the knowledge gained, this study aims to employ RS isolated from banana starch via the enzymatic method as a wall material. Moreover, this RS was employed as a delivery system for bioactive LF. To further investigate its potential, the spray-dried microencapsulates were analyzed for their gastrointestinal stability and synergistic impact on prebiotic efficacy. Additionally, the postbiotics derived from prebiotic studies were assessed for their anti-inflammatory potential

in the macrophage cell line RAW 264.7. In this context, the following are the aims and objectives of the current study.

1.4 Aim

The aim is to develop a resistant starch-based lactoferrin microencapsulate that can effectively regulate inflammation by improving lactoferrin stability, targeted release, and anti-inflammatory efficacy. This strategy seeks to protect LF from degradation, increase its bioavailability, and ensure its regulated release, thereby enhancing its therapeutic potential in reducing inflammatory responses.

1.5 Specific Objectives

1. Extraction and characterization of starch and resistant starch from different varieties of *Musa* species
2. Optimization and development of Lactoferrin-Resistant starch based microencapsulates (LRM)
3. *In vitro* release kinetics and prebiotic efficacy of LRM on *Lactobacillus casei*
4. Exploration of the anti-inflammatory potential of postbiotics from LRM on RAW 264.7 cell lines

1.6 Outline of chapters

Chapter 1: Introduction

This chapter gives an overview of LF, and RS physiological functions, and their application, specifically, it addresses the background and significance of employing LF, a bioactive protein, and RS as a carrier material. It also describes the aim, objectives, and the study's rationale.

Chapter 2: Extraction and characterization of starch and resistant starch from different varieties of *Musa* species

This working chapter discusses the extraction and characterisation of starch and resistant

starch (RS) from various *Musa* species. It explores the method for isolating starch from various banana varieties, followed by the conversion of starch into RS using enzymatic treatment. The chapter also covers the characteristics of isolated starches and RS, including their physicochemical properties, structural studies, and thermal properties.

Chapter 3A: Optimization and development of Lactoferrin-Resistant starch based microencapsulates (LRM)

The second working chapter focuses on the optimisation, development, and characterisation of lactoferrin-resistant starch based microencapsulates (LRM). The spray drying method is used for encapsulating LF with RS, optimising process parameters, and characterising microencapsulates in terms of particle size, encapsulation efficiency, and structural studies to improve targeted delivery and therapeutic efficacy.

Chapter 3B: *In vitro* release kinetics and prebiotic efficacy of LRM on *Lactobacillus casei*

This section investigates the *in vitro* release kinetics and prebiotic efficacy of lactoferrin-resistant starch-based microencapsulates (LRM) on *Lactobacillus casei*. It evaluates how LRM affects the controlled release of lactoferrin, as well as its effect on the growth and activity of *Lactobacillus casei*, to determine its prebiotic potential.

Chapter 4: Exploration of the anti-inflammatory potential of postbiotics from LRM on RAW 264.7 cell lines

This chapter explores the anti-inflammatory effects of postbiotics-derived LRM on RAW 264.7 macrophage cells. It assesses LRM-derived postbiotics ability to modify inflammatory markers, providing insights into their potential for inflammation management.

Chapter 5: Summary, Conclusions, and Future Prospects

The last chapter summarises the primary findings, conclusions, and implications of the

study. This section also highlights the potential future applications, as well as research directions to improve and broaden their applications.

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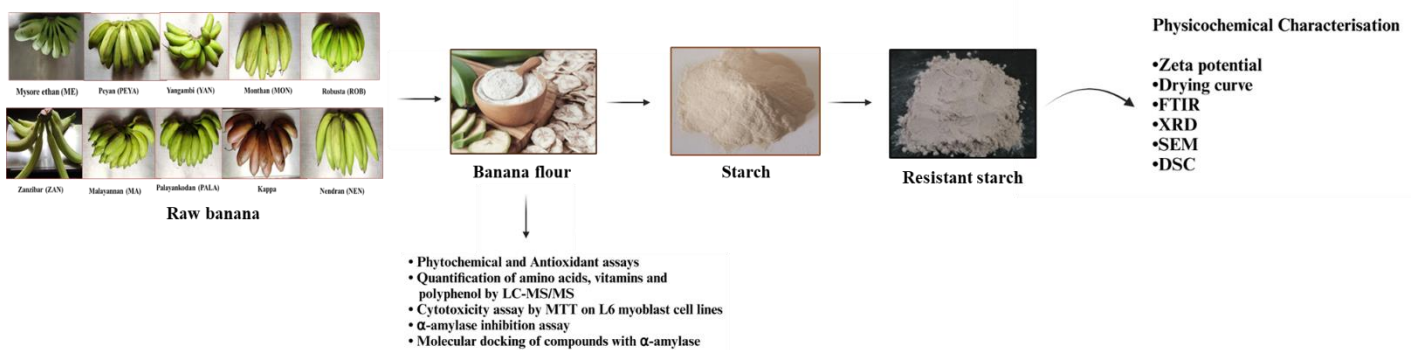
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Chapter 2

Extraction and characterisation of starch and resistant starch from different varieties of Musa species



2.1 Introduction

Starch is a well-studied second most abundant polymeric-carbohydrate, composed of glucose linked with α -D (1-4) and α -D (1-6) glycosidic linkage and consists of two structural components, linear amylose, and branched amylopectin polymer. The proportion of these polymers contributes to their physicochemical properties and it varies according to the species variety, degree of maturation, botanical source, and environmental conditions (Apriyanto et al., 2022). The interesting part of the biochemical and structural array of starchy-rich food is the difference in the arrangement of amylose and amylopectin within the starch granule, which plays a vital role in glucose release upon digestion. Starch resists the action of digestive enzymes in the small intestine, which are defined as RS. Due to the undigestible property of RS, it reaches the colon, where it will be used as a substrate for the growth of beneficial gut microbiota to produce short-chain fatty acids (SCFAs) (Liljeberg Elmståhl, 2002). Depending on its chemical modification, structural characteristics, lipids linkage, or cooking effect, RS is categorized into five types: RS I, RS II, RS III, RS IV, and RS V. Banana starch is classified under type II RS, a condensed and partially crystalline native starch resistant to enzymatic hydrolysis. However, human commonly consumes cooked starch than raw banana starch. This type of retrograded starch is classified under type III RS (Fuentes-Zaragoza et al., 2010; Sanz Taberner et al., 2009). The intake of RS has metabolic health benefits, its properties resemble dietary fiber. The consumption of RS not only increases the glycemic and postprandial insulinemic responses but also can help regulate the human system by improving insulin sensitivity, decreasing fat storage, elevating satiety, and reducing plasma cholesterol and triglyceride concentrations (Salih et al., 2017). These features raise the interest in RS as a potent dietary candidate for the prevention of type II diabetes, a dietary substitute for insulin resistance treatment, metabolic diseases related to weight loss diets, and dyslipidemia. In addition, the postbiotics can prevent colon cancer and Alzheimer's disease (Nugent, 2005).

Hence, RS has the potential to be used as an ingredient in functional foods and also as in nutraceuticals. The main sources of RS are white rice, whole grains, legumes, lentils, and raw bananas.

Banana, a member of the Musaceae family and belonging to the genus *Musa*, holds important global significance as one of the primary fruit crops. The banana ranks as the fourth major food crop globally, following wheat, rice, and maize (Dash & Rai, 2016). Bananas are widely harvested in tropical and semi-tropical regions (Aurore et al., 2011; Strosse et al., 2006). China, India, and Indonesia have consistently had the largest supply to their domestic markets since 2016 (*Bananas | FAO | Food and Agriculture Organization of the United Nations*, n.d.; *FAOSTAT*, n.d.). Among these, India is the largest producer of bananas, with a production of 32 million metric tonnes, which accounts for about 26.3% of the world's production in 2020. Bananas are cultivated in 150 nations in an estimated amount of 1,000 different varieties. These banana varieties are further classified into desserts and plantains. Dessert bananas are consumed raw in fully ripe form, and plantains are usually cooked to be consumed at different maturity stages (Gibert et al., 2009).

The banana is highly valued worldwide due to its delightful flavor, nutritional value, and consistent availability throughout the year. The chemical compositions of raw bananas vary depending on the climate, soil type, variety, and degree of maturity. In raw bananas, the main component is starch (70-80%), which undergoes vital changes during ripening. Due to the presence of RS, regular intake of raw banana flour is beneficial for human health (Maziarz et al., 2017). Studies report a hypoglycemic effect in humans following the intake of unripe plantains (Iroaganachi & Eleazu, 2015). Considering the importance of diet in the management of chronic diseases, raw plantains are gaining attention among consumers as a healthy functional food ingredient (Amini Khoozani et al., 2019; Arun et al., 2015). From this perspective, it is very important to explore the potential of underutilised and unexploited

traditional varieties of local cultivars. With this background, the objective of this chapter is the preliminary analysis of ten common but unexploited raw banana cultivars in terms of compositional analysis, free amino acids, vitamin content, polyphenols, phytochemical analysis, antioxidant potential, hypoglycemic effect, isolation of starch and RS, and its characterisation.

2.2 Materials and Methods

2.2.1 Materials

Sigma Aldrich Chemicals (St. Louis, MO, USA) provided 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), gallic acid, ascorbic acid, fetal bovine serum (FBS), dulbecco's modified eagle's media (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), aluminum chloride (AlCl_3), quercetin, α -amylase, amyloglucosidase, and protease. Folin–Ciocalteu reagent, methanol, sodium carbonate (Na_2CO_3), sodium phosphate monobasic, sodium phosphate dibasic, potassium acetate (CH_3COOK), and hydrochloric acid (HCl) were purchased from MERCK, Mumbai, India. Trypsin-EDTA, antibiotic-antimycotic, starch, potassium iodide (KI), iodine (I_2), and sodium hydroxide (NaOH) were obtained from Himedia Laboratories, Mumbai, India. Formic acid, ammonium formate, and acetonitrile (MS grade) were obtained from Biosolve Ultra International, Bangalore, India. Glucose oxidase/peroxidase (GOD/POD) kits were purchased from Biosystems Diagnostics, Pvt, Ltd. Minigen Nylon syringe filters (0.20 μm diameter) were procured from Genetix Biotech Asia Pvt. Ltd, New Delhi, India. All the chemicals used were of high-quality analytical grade.

2.2.2 Sample preparation

Stage II raw bananas harvested from the College of Agriculture in Kerala were used for this study. Ten banana cultivars were selected: Yangambi, Mysore ethan, Zanzibar, Peyan,

Palayankodan, Malayannan, Nendran, Robusta, Kappa, and Monthan (Fig. 2.1). The cultivars were washed, their peel was removed, and the pulp was cut into slices and dried in a Refrigerated Adsorption Dehumidified Drier (RADD) at 45 ± 2 °C. The dried slices were ground, passed through a 50 mm mesh sieve to obtain banana flour, and stored at room temperature in an air-tight container until further analysis.

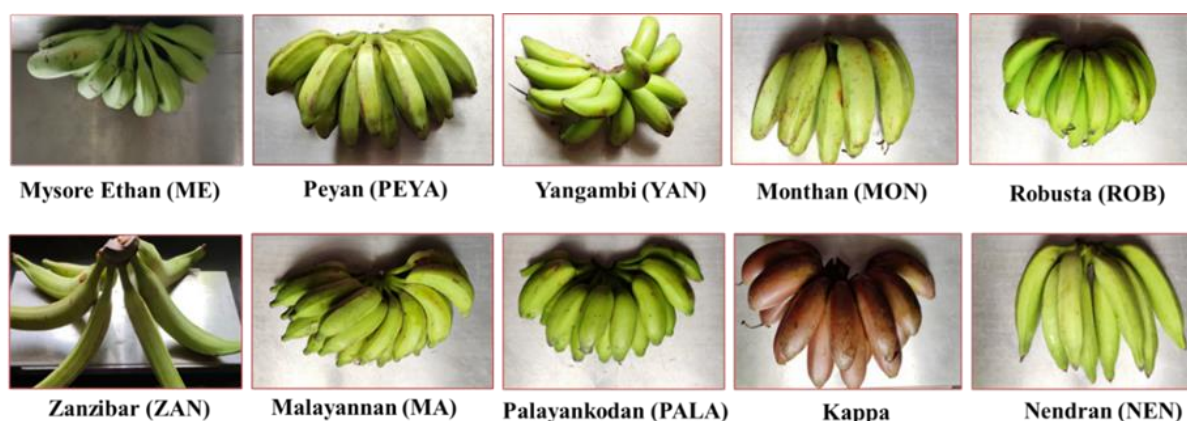


Figure 2.1. Ten banana cultivars

2.2.3 Methods

2.2.3.1 Proximate composition

The moisture, ash, crude fat, and protein contents of cultivars have been determined using the standard AOAC methods (930.15, 923.03, 983.23, 984.13). (*AOAC 923.03-1923, Ash of Flour: Direct Method - §14.15 : AOAC Official Method, n.d.; AOAC 930.15-1930(1999), Loss on Drying (Moisture) for Feeds (at - §14.15 : AOAC Official Method, n.d.; AOAC 983.23-1984, Fat in Foods - Chloroform-Methanol Extraction - §14.30 : AOAC Official Method, n.d.; AOAC 984.13-1994(1996), Protein (Crude) in Animal Feed and Pet f - §14.30 : AOAC Official Method, n.d.)* For determining the moisture content, triplicates of each sample were dried in an oven at 105 °C and then placed in a desiccator to cool to room temperature. The ash content was determined, by weighing the sample both before and after heat treatment in a muffle furnace at 550 °C for 12 h. The Soxhlet extraction method was employed to measure the fat

content, while protein levels were estimated using an automatic Kjeldahl distillation and titration system (KjelTRON™ Rapid Automatic Nitrogen Estimation System (Nitrogen Analyser), Tulin Equipments, Model: KDIG 4M, Chennai, India). The Nitrogen content was determined using the equation, wherein 14 is the atomic weight of Nitrogen

$$\text{Percentage Nitrogen} = \frac{(14 \times \text{Normality of HCl} \times \text{Volume of HCl} \times 100)}{(\text{Weight of the sample})} \times 1000 \quad (1)$$

Then, the protein content was calculated using the equation,

$$\text{Percentage Protein} = \text{Percentage Nitrogen} \times 6.25 \quad (2)$$

Carbohydrate content was determined using the difference method, as per the following formula:

$$\text{Carbohydrate content} = [100 - \{\text{moisture} + \text{ash} + \text{fat} + \text{protein}\}] \quad (3)$$

2.2.3.2 Phytochemical Characterisation

Raw banana flour (20 g) was weighed and extracted with 100 mL of 70% ethanol by continuous stirring for 1 h; the slurry was centrifuged at 10621 g for 10 min, and the supernatant was collected. The resulting extract was filtered and evaporated using a rotary evaporator (Heidolph, Germany) at 45 °C under low pressure. The hydro-alcoholic extracts were subsequently dissolved in corresponding solvents, made up to a specific volume, and stored at 4 °C until further analysis for determining phenolic compounds, free amino acids, and vitamins (Arun et al., 2020).

2.2.3.2.1 Determination of total phenolic content (TPC) and total flavonoid content (TFC)

Folin-Ciocalteu and aluminum chloride colorimetric methods were used to obtain the TPC and TFC of banana flour extract, respectively (Arun et al., 2015). Briefly, different concentrations of the extracts and gallic acid (standard) were made up to 750 µL with methanol

followed by the addition of Folin's reagent (80 μ L) and 7.5% of sodium carbonate solution (200 μ L) and kept at room temperature for 90 min in the dark. The absorbance was measured at 750 nm using a multimode plate reader (Synergy 4 BioTek, BioTek Instruments, Inc., Mumbai, India). The TPC was expressed as milligrams of gallic acid equivalent per gram of d/w (mg GAE/g).

The aluminum chloride colorimetric method was used to estimate the TFC of banana flour extract. In short, various concentrations of the samples and quercetin (standard) were combined with 10 μ L of 1M CH_3COOK , 10 μ L of 10% AlCl_3 , and 280 μ L of distilled water, incubated at room temperature for 40 min. The absorbance was measured at 415 nm using the multi-plate reader. The TFC was represented as a milligram of quercetin equivalent per gram of d/w (mg Q.E/ g).

2.2.3.2.2 DPPH and ABTS radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays were used to determine banana flour extract's free radical scavenging activity (Arun et al., 2015; Re et al., 1999). In brief, 500 μ L of 0.2 mM DPPH solution was added to 500 μ L of sample and gallic acid (standard) at different concentrations and incubated at room temperature for 30 min in the dark, and its absorbance was measured at 517 nm using a multimode reader. The percentage inhibition of DPPH was determined by the following equation. The IC_{50} values were calculated using linear regression analysis.

$$\text{Percentage inhibition of DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (4)$$

For ABTS radical scavenging activity, ABTS radicals were prepared by combining 50 mL 2 mM ABTS solution with 200 μ L 70 mM potassium persulfate. This mixture was allowed to stand in the darkroom for 12-18 h. Different concentrations of standard (ascorbic acid) and

samples were prepared and made up to 250 µL using distilled water followed by the addition of ABTS solution and 850 µL of phosphate buffer (pH 7.4) and incubated at room temperature for 7 min in the dark. The absorbance was read at 734 nm using a multimode reader. The percentage inhibition of ABTS was measured by the equation.

$$\text{Percentage inhibition of ABTS} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (5)$$

The IC₅₀ values were calculated using linear regression analysis.

2.2.3.3 Chemical profiling and quantification of raw banana flour extract using LC-MS/MS

Quantitative analysis of phenolic compounds, free amino acids, and vitamins present in the hydroalcoholic extracts (prepared as per 2.2.3.2) was carried out by LC-MS/MS. The extracts were filtered using a 0.20 µm syringe filter before the analysis.

2.2.3.3.1 Quantification of Polyphenols

Quantitative analysis was conducted on a total of 28 compounds, including catechol, quinine, tocopherol, gallic acid, naringenin, catechin, chlorogenic acid, epicatechin, syringic acid, vanillic acid, genistein, epigallocatechin, p-Coumaric acid, ferulic acid, myricetin, luteolin, apigenin, kaempferol, rutin, diadzein, quercetin, hesperetin, shikimic acid, morin, caffeic acid, cinnamic acid, and chrysin. (Abraham et al., 2020) Initial standardisation of the method was carried out using corresponding standard compounds. In brief, the analysis was done with LC-MS/MS (Nexera LC-30AD) with an autosampler (SIL30AC), temperature-controlled column oven (CTO-20AC), and prominence diode array detector (SPD-M20A), coupled to a triple quadrupole mass spectrometer. A Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d, 1.9 µm) was used with a mobile phase consisting of solvent A (water/formic acid, 100/0.1%) and solvent B (100% methanol). The following linear gradient system was used to elute the polyphenols: between 0.5 to 1.9 min 5% of solvent B, for 2.0 to 10.0 min; and 10.1–15 min

98% of solvent B, and 15.1–17 min 5% of solvent B, with 0.3 mL/min flow rate, 40 °C oven temperature and 10 µL of injection volume. During LC-MS/MS with electrospray ionisation (ESI), both positive and negative modes of the multiple reaction-monitoring (MRM) modes were operated. The Lab Solutions software (Shimadzu, Kyoto, Japan) was used to gather and process the LC-MS/MS data. Ionisation required an interface temperature of 400 °C, a desolvation line temperature of 300 °C, a heat block temperature of 400 °C, a drying gas flow rate of 10 L/min for nitrogen, and a nebulizing gas flow rate of 3 L/min for nitrogen. The average value of the data was taken as the representative for each point after every calibration solution was analysed in triplicate. The resulting data are expressed as polyphenols in µg/mL with SD (n=3).

2.2.3.3.2 Quantification of Amino acids and Vitamins

Free amino acids (tryptophan, serine, leucine, histidine, phenylalanine, asparagine, methionine, cystine, threonine, valine, tyrosine, lysine, glutamic acid, glycine, proline, aspartic acid, glutamine, isoleucine, arginine, hydroxyproline, cysteine, and alanine) and vitamins (thiamine, pyridoxine, nicotinic acid, folic acid, riboflavin, and ascorbic acid) present in the extracts were analysed by LC-MS/MS (Navami et al., 2023). In brief, an LC-MS/MS (Nexera LC-30AD) equipped with an autosampler (SIL30AC), temperature-controlled column oven (CTO-20AC), and prominence diode array detector (SPD-M20A), coupled to a triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) was used. The quantification was carried out with a Shimadzu Shim-pack GISS C18 column (150 X 2.1 mm i.d, 1.9 µm). The mobile phase used for amino acids consists of solvent A (water/formic acid, 100/0.1%) and solvent B (100% methanol). The following is the method of eluting amino acids using a linear gradient system: 0.5 – 4.9 min 5% of solvent B, for 5.0 –13 min and 13.1–15 min 85% and 5% of solvent B respectively, with a flow rate of 0.3 mL/min, and 40°C oven temperature. Both positive and negative multiple MRM modes were used during LC-MS/MS

with ESI. The ion spray voltage was 4 kV, the injection volume was 10 μ L, and 230 kPa of collision-induced dissociation (CID) gas. The average value of the results was taken as the representative for each point after each calibration solution was examined thrice. The acquired results are represented as amino acids μ g/mL with SD (n=3).

The mobile phase used for vitamins was 10 mM ammonium formate in water and 0.1% formic acid for solvent A and 10 mM ammonium formate in methanol and 0.1% formic acid for solvent B. A linear gradient system was employed as follows: 0.01-2.0 min 5% of solvent B, for 2.0-5.0 min 30% of solvent B, 5.0-8.0 min 45% of solvent B, 8.0-12.0 min 90% of solvent B, 12.0-16.0 min 5% of solvent B with a flow rate of 0.3 mL/min and 33 °C oven temperature. In both positive and negative MRM, the ESI was operated. For ascorbic acid only, the negative mode was employed. There was a 10 μ L injection volume, 4 kV ion spray voltage, the drying and heating gas flow was 10 L/min, whereas the nebulizing gas flow was 3.0 L/min. Each calibration solution was examined in triplicate, and the average of the findings was taken as the representative for each analysis. The results obtained are vitamins μ g/mL with SD (n=3). The analytical method was initially standardized using standard amino acids and vitamins. This standardisation ensured accurate quantification of the components in the extracts.

2.2.3.4 Cytotoxicity Assay

The cytotoxicity of extracts was quantified by MTT assay using L6 myoblast cells (Mosmann, 1983). L6-rat skeletal myoblast cells (NCCS, Pune, India) were cultured in DMEM with 10% FBS and a 0.5% antibiotic-antimycotic solution and kept in an incubator at 37 °C for 24-48 h provided with 5% carbon dioxide. Cells were trypsinized and seeded into a 96-well plate. After the cell attachment, it was treated with different concentrations of extracts and incubated for 24 h at 37 °C. The cytotoxicity of cells was quantified by the MTT assay, and cell viability was calculated by a subsequent equation.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (6)$$

2.2.3.5 α -Amylase Inhibition Assay

Extracts were tested for α -amylase inhibition with modifications to Xiao et al.'s method (Xiao et al., 2006). The test mixture containing 40 μ L of 0.02 M sodium phosphate buffer (pH 6.9), 0.02 units of porcine pancreatic amylase (PPA) solution, and varied concentrations of extracts, was incubated at 37 °C for 10 min. After adding 1% soluble starch, it was incubated for an additional 15 min. To halt the enzyme process, 20 μ L of 1 M HCl was added, followed by 100 μ L of iodine reagent (5 mM I₂ and 5 mM KI). Absorbance at 620 nm was measured, using acarbose as a positive control. The inhibition percentage was calculated based on the defined formula.

$$\text{Percentage inhibition of PPA} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (7)$$

2.2.3.5.1 Molecular docking studies of compounds with α -amylase

The human pancreatic α -amylase structure (PDB ID 4W93) from the Protein Data Bank was utilized for molecular docking, with hydrogens and Kollman charges being added post-structure refinement. The structures of LCMS/MS-identified compounds were obtained from PubChem and prepared as ligands in 3D .sdf format. Energy minimization and conversion to .pdbqt format were performed using OpenBabelGUI. Autodock 4.2.6 facilitated molecular docking with 100 runs and a population size of 150, assessing ligand interactions against α -amylase. PyMol was employed for the 3D visualisation of ligand interactions with α -amylase's amino acid residues. Binding energies (Kcal/mol) indicated the strength of affinity, with more negative values suggesting stronger interactions.

2.2.3.7 Isolation of starch

Starch was isolated from raw bananas using an alkaline extraction method with slight modifications (Noor, 2014). Initially, banana flour (5 g) was suspended in a 0.05 M NaOH solution (20 mL), and agitated continuously for 4 h. Subsequently, the mixture was centrifuged using a Kubota High-Speed Refrigerated Centrifuge (Model 7780, Kubota Corporation, Tokyo, Japan) at 956 g for 15 min at 4 °C. The same procedure was repeated once again and the resulting pellet was dispersed in distilled water (50 mL), and filtered. The residue was neutralised using a 1 M HCl solution and centrifuged. The pellet was washed thrice with distilled water, centrifuged, and the final pellet was dried overnight at 45 °C. The dried product was then ground and sieved through a 50 mm mesh to obtain a fine starch powder, which was stored in an airtight container for further analysis.

2.2.3.8 Isolation of resistant starch

The method used by Zhang et al. and Escarpa et al. (Escarpa et al., 1997; Y. Zhang et al., 2014) was slightly modified for the isolation and determination of RS from starch. Isolated starch (2 g) was mixed with 50 mL of 0.08 M phosphate buffer (pH 6) and α -amylase, incubated in a water bath shaker (RivotekTM water bath shaker TC544, Mumbai, India) at 95 °C for 15 min, cooled and the pH was adjusted to 7.5 using 1 M NaOH. To this, phosphate buffer (2.0 mL, pH 7.5) containing protease enzyme (50 mg/mL) was added, incubated at 60 °C for 30 min in a water bath shaker, and cooled to room temperature. The pH was adjusted to 4.5, followed by the addition of 2.5 mL of amyloglucosidase (AMG), and incubated in a water bath shaker at 60 °C for 30 min. It was allowed to cool after the incubation followed by the addition of ethanol (1:4 v/v) and allowed to stand overnight, centrifuged. The pellet was washed with distilled water, and freeze-dried using a lyophilizer (VirTis Genesis, USA). The dried material was then ground to a fine powder. Total glucose was analysed using the GOD-POD (glucose

oxidase/peroxidase) kit. The RS content was calculated by the equation; RS = glucose x 0.9 (correction factor).

2.2.3.9 Characterisation of Starch and Resistant starch

2.2.3.9.1 Zeta potential

The Malvern Zetasizer (Zeta Nano-ZS; Malvern Instruments, UK), which operates on the dynamic light scattering (DLS) principle, was used to evaluate the zeta potential of the starch and RS samples (Comunian et al., 2020).

2.2.3.9.2 Drying curve

The method of the slope of the drying curve is often used to determine the drying rate of samples (Karathanos et al., 1990). A known weight of the sample is initially recorded, followed by drying in an oven at 105 °C. The sample was weighed at regular intervals to determine the amount of weight loss. This process continues until the sample reaches a constant weight. Moisture content is then calculated from the initial and final weights. A drying curve is obtained by plotting moisture content against time (Eq. 4).

$$\text{Drying rate} = -\frac{dX}{dt} \quad (4)$$

Where, dX- rate of change of moisture content in g/g, dt- time in min

2.2.3.9.3 ATR-Fourier-Transform Infrared (FTIR) Spectroscopy

The Fourier transform infrared spectroscopy (FTIR) of starch and RS was performed using an ATR (attenuated total reflection)-FTIR spectrometer (Perkin Elmer Spectrum, USA) equipped with an ATR accessory containing a diamond crystal at a 45° incidence angle. Sample transmittance was measured at wave numbers ranging from 4000 to 400 cm⁻¹.

2.2.3.9.4 X-Ray Diffraction (XRD)

The XEUSS SAXS/WAXS system from Xenocs was used to analyse the wide-angle X-ray diffraction (WAXD) patterns of starch and RS. The system used Cu K α radiation wavelength of about 1.54 Å with current and voltage of 0.6 mA and 50 kV, respectively. The Mar345 plate was used to measure the 2D patterns of samples, and the data was analysed with Fit2D software.

2.2.3.9.5 Scanning electron microscopy (SEM)

The surface morphology of the starch and RS were examined using scanning electron microscopy (ZEISS; EVO 18, Germany). The samples were attached to an aluminum specimen holder with carbon tape. Then it was coated with a thin coating of gold using a gold/palladium sputter coater under vacuum (SC7620, Emitech, Quorum Technologies Ltd, Kent, UK). The microscopical evaluation of these materials was done using SEM under the accelerating voltage of 15 kV, and the micrographs were taken at 1200X magnification (da Silva Soares et al., 2021).

2.2.3.9.6 Differential Scanning Calorimetry (DSC)

The thermal properties of the starch and RS samples were evaluated using differential scanning calorimetry (DSC). The measurements were performed with a differential scanning calorimeter (DSC Q2000, TA Instruments, USA). Samples (3 mg) were weighed into aluminium sample pans and 6 μ L of distilled water was added. After sealing the pans, the samples were heated to temperatures ranging from 10 to 100 °C at a rate of 10 °C/min in a nitrogen atmosphere (Tao et al., 2016).

2.2.3.9.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.3.0, presenting results as mean $\pm$ standard deviation (SD). A one-way ANOVA and Dunnett's multiple comparison tests were employed, with significance considered at $p < 0.05$.

2.3 Result and Discussion

2.3.1 Proximate composition

Proximate analysis provides essential data on the major constituents of food products, including moisture, ash, fat, protein, and carbohydrates. This data is crucial for understanding the nutritional profile and potential applications of different food items. In the study, the proximate composition of ten banana cultivars is reported, showcasing the variance in composition that can impact both nutritional value and usability.

The proximate composition of ten varieties of banana cultivars is presented in Figure 2.2 a and b. The ash content, representing inorganic minerals, varied significantly across the cultivars, from $1.61 \pm 0.35\%$ in NEN to $3.52 \pm 0.19\%$ in Kappa. This variation suggests a considerable influence of soil mineral content and agricultural conditions on the mineral uptake by the bananas, as seen in other studies (Bangwaek, 2014; Oko et al., 2015; Onwuka & Onwuka, 2005). Elsewhere, Kumar et al. (Kumar et al., 2019) reported 2.5% ash content for Nendran, which was higher than the present study. This difference could be due to the variation in geographical and climatic changes. These cultivars varied in fat content, with PALA having $1.07 \pm 0.77\%$ and Kappa having $2.89 \pm 0.45\%$. This low fat content, as reported by Khoza et al. (Khoza et al., 2021) and Khoozani et al. (Amini Khoozani et al., 2020) is advantageous for reducing oxidative spoilage, thus extending the shelf life of banana flour. The sequence of fat content from lowest to highest (PALA, MON, MA, PEYA, ROB, YAN, ME, NEN, ZAN, Kappa) indicates that certain cultivars like Kappa might be more prone to oxidation than others like PALA, influencing storage and processing decisions. Protein content also showed variability, with NEN at the lowest ($3.47 \pm 0.63\%$) and ROB at the highest ($6.08 \pm 0.19\%$). These figures align closely with findings from Kumar et al. (Kumar et al., 2019) who reported protein contents for Monthan and Nendran that are similar to those observed in our study. Also, Khoza

et al. (Khoza et al., 2021) reported the raw banana flour protein content within the range of the current study. Given that protein content can affect the nutritional quality of food products, cultivars like ROB could be more suitable for protein-enrichment purposes, while NEN might be preferred in situations where lower protein content is beneficial.

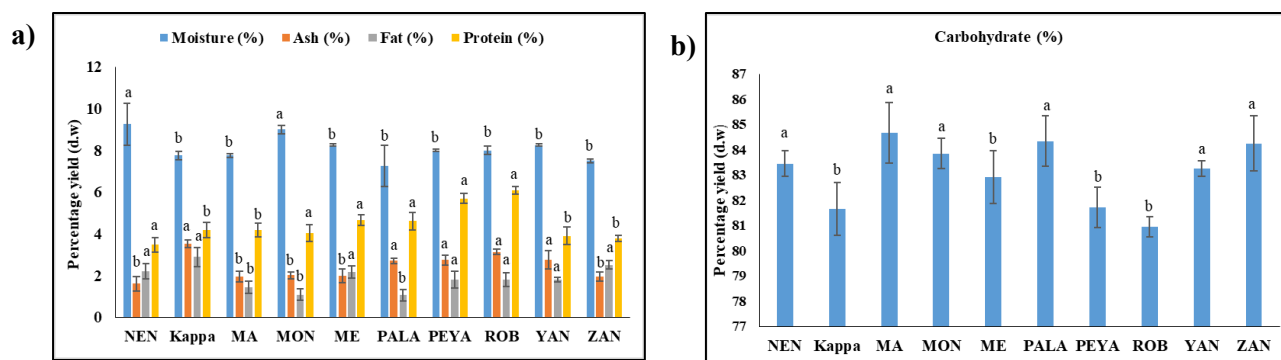


Figure 2.2. Proximate composition (moisture, ash, fat and protein) (a), carbohydrate (b) of banana cultivars (% d/w). Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

Proximate analysis of these banana cultivars reveals crucial differences in their chemical makeup that could inform both agricultural practices and food processing industries. For instance, selecting specific cultivars for cultivation based on soil mineral content could optimise mineral uptake (ash content), while the choice of cultivar for flour production could be influenced by desired characteristics such as longer shelf life (low fat) or specific nutritional needs (higher or lower protein content). This analysis aids in both understanding the inherent properties of these banana cultivars and utilising them for specialised food production and nutritional approaches.

2.3.2 Quantification of amino acids (aa) and vitamins using LC-MS/MS

Literature surveys reveal limited research on the amino acid profile of bananas, possibly due to the low protein content in green bananas. According to the US Department of Agriculture, bananas are rich in non-essential amino acids like aspartic acid and glutamic acid (*FoodData Central*, n.d.). Unripe bananas, serving as a natural source of phenylalanine, and other essential

and non-essential aa contribute to overall dietary intake (Lee et al., 2019). The study affirms bananas' richness in histidine compared to other fruits (Bezerra et al., 2013). The study examined 22 aa in the investigated banana cultivars (Table 2.1), revealing phenylalanine as the predominant amino acid in all the variants studied, except for Kappa and YAN.

Phenylalanine dominated in NEN (204.17 ± 0.025 $\mu\text{g/mL}$), followed by arginine (64.65 ± 0.005 $\mu\text{g/mL}$). In Kappa, histidine led was the prominent aa (98.26 ± 0.006 $\mu\text{g/mL}$), followed by phenylalanine (66.06 ± 0.014 $\mu\text{g/mL}$). MA featured the highest phenylalanine (366.17 ± 0.008 $\mu\text{g/mL}$) and lysine (64.04 ± 0.005 $\mu\text{g/mL}$). MON displayed amino acids in the order phenylalanine > glutamine > lysine > arginine > histidine, at 162.42 ± 0.004 $\mu\text{g/mL}$ phenylalanine. ME showed the highest phenylalanine (96.45 ± 0.055 $\mu\text{g/mL}$) and histidine (79.72 ± 0.008 $\mu\text{g/mL}$). PALA had phenylalanine at 241.16 ± 0.020 $\mu\text{g/mL}$ and histidine at 108.06 ± 0.044 $\mu\text{g/mL}$. PEYA topped with phenylalanine (495.67 ± 0.011 $\mu\text{g/mL}$), glutamine (345.94 ± 0.004 $\mu\text{g/mL}$), and lysine (246.72 ± 0.004 $\mu\text{g/mL}$). In ROB, histidine content (142.57 ± 0.003 $\mu\text{g/mL}$) exceeded others, while YAN had the highest histidine at 73.42 ± 0.021 $\mu\text{g/mL}$. Amino acids were ranked by ZAN as phenylalanine > arginine > glutamine > lysine > histidine. Amino acids play vital roles as biocatalysts, nutrients, cell constituents, and neurotransmitters (Mokbel & Hashinaga, 2005). Prior studies on vegetables like carrots, sweet potatoes, peas, cabbage, spinach, and komatsuna found higher levels of free amino acids, particularly asparagine and glutamine (Ito et al., 2019). Examining wild fruits in Assam revealed a high content of aspartic acid, arginine, glutamic acid, histidine, valine, and leucine, aligning with our findings (Islary et al., 2019). Studies indicate that phenylalanine, tryptophan, and tyrosine are three essential components in plant metabolism that act as precursors of production of aromatic secondary metabolites such as lignin, anthocyanins, flavonoids, and alkaloids. The amount of aromatic amino acids has significant influences on the production of phenolic compounds (Hu et al., 2020; Maeda & Dudareva, 2012).

Unripe bananas are reported to be a good source of vitamins B6, C, and provitamin A (Wall, 2006). Raw bananas selected under the present study were analysed for water-soluble vitamins (thiamine, pyridoxine, nicotinic acid, folic acid, riboflavin, and ascorbic acid) using LC-MS/MS (Table 2.2). Folic acid ($0.860 \pm 0.051 \mu\text{g/mL}$) and pyridoxine ($0.790 \pm 0.065 \mu\text{g/mL}$) were found to be abandoned in Kappa, while MA had higher nicotinic acid ($0.730 \pm 0.005 \mu\text{g/mL}$) and ascorbic acid ($0.770 \pm 0.067 \mu\text{g/mL}$). All cultivars showed significant amounts of Vit B6 and Vit C. Prior studies noted vitamin C and B complex vitamins (B1, B2, B3, B9, and B6) in raw bananas (Dakshinamurti et al., 2017; Qamar & Shaikh, 2018; Ranjha et al., 2022; Siji S & Nandini PV, 2016). It is observed from the available literature that the vitamin C content varies significantly throughout cultivars, with Cavendish and Dwarf Brazilian levels varying between 4.5 to 12.7 mg/100g (Wall, 2006). The varieties investigated in the current study displayed lower content than that of the reported one. This difference may be attributed to factors like variety, growing conditions, maturity, and drying conditions. A recent study showed that increased drying temperatures in ripe Nendran bananas resulted in decreased vitamin content (Kabeer et al., 2022). The present study highlights the nutritional richness of raw bananas, despite variations, offering essential vitamins in a well-balanced diet (Englberger et al., 2010).

Table 2.1. Amino acid quantification in ethanolic extracts of cultivars

Amino acids	Concentration in the samples (µg/mL)									
	NEN	Kappa	MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN
Tryptophan	3.10 ± 0.002 ^h	5.29 ± 0.001 ^f	6.98 ± 0.072 ^c	9.64 ± 0.005 ^d	3.31 ± 0.007 ^g	12.26 ± 0.003 ^c	12.97 ± 0.002 ^b	20.20 ± 0.001 ^a	1.40 ± 0.003 ⁱ	3.04 ± 0.006 ^h
Serine	6.89 ± 0.006 ^d	5.11 ± 0.007 ^f	7.10 ± 0.002 ^d	5.11 ± 0.003 ^f	4.77 ± 0.001 ^g	7.55 ± 0.008 ^c	15.02 ± 0.043 ^a	10.31 ± 0.002 ^b	5.75 ± 0.002 ^c	7.43 ± 0.004 ^c
Leucine	1.03 ± 0.001 ^f	2.19 ± 0.004 ^c	1.44 ± 0.009 ^c	1.08 ± 0.003 ^f	1.03 ± 0.006 ^f	2.74 ± 0.012 ^b	1.84 ± 0.007 ^d	6.37 ± 0.032 ^a	1.16 ± 0.056 ^f	0.98 ± 0.009 ^f
Histidine	36.46 ± 0.004 ⁱ	98.26 ± 0.006 ^c	37.89 ± 0.002 ^h	47.92 ± 0.011 ^g	79.72 ± 0.008 ^d	108.06 ± 0.044 ^b	52.88 ± 0.014 ^f	142.57 ± 0.003 ^a	73.42 ± 0.021 ^c	37.88 ± 0.023 ^h
Asparagine	5.44 ± 0.002 ^f	5.95 ± 0.005 ^c	6.29 ± 0.016 ^d	3.58 ± 0.003 ⁱ	4.61 ± 0.006 ^g	8.45 ± 0.018 ^b	6.91 ± 0.009 ^c	31.52 ± 0.001 ^a	2.32 ± 0.092 ^j	3.85 ± 0.007 ^h
Cystine	0.38 ± 0.011 ^e	0.41 ± 0.014 ^d	0.68 ± 0.004 ^b	0.24 ± 0.023 ^h	0.31 ± 0.006 ^g	0.45 ± 0.001 ^c	0.98 ± 0.002 ^a	0.20 ± 0.006 ⁱ	0.16 ± 0.004 ^j	0.36 ± 0.009 ^f
Threonine	2.25 ± 0.005 ^g	3.05 ± 0.003 ^d	2.45 ± 0.039 ^f	2.00 ± 0.036 ^h	1.96 ± 0.002 ^h	4.39 ± 0.017 ^c	5.19 ± 0.008 ^b	8.22 ± 0.023 ^a	1.64 ± 0.003 ⁱ	2.99 ± 0.002 ^c
Valine	1.93 ± 0.006 ^f	2.03 ± 0.053 ^c	2.54 ± 0.002 ^d	1.64 ± 0.007 ^h	1.73 ± 0.016 ^g	2.90 ± 0.004 ^c	4.30 ± 0.008 ^b	5.003 ± 0.033 ^a	1.63 ± 0.014 ^h	1.75 ± 0.005 ^g
Phenylalanine	204.17 ± 0.025 ^c	66.06 ± 0.014 ^j	366.17 ± 0.008 ^b	162.42 ± 0.004 ^g	96.45 ± 0.055 ^h	241.16 ± 0.020 ^d	495.67 ± 0.011 ^a	352.49 ± 0.006 ^c	72.65 ± 0.005 ⁱ	177.00 ± 0.003 ^f
Glutamic acid	1.002 ± 0.008 ^f	1.15 ± 0.001 ^c	2.06 ± 0.036 ^c	2.17 ± 0.006 ^b	1.12 ± 0.007 ^c	2.06 ± 0.023 ^c	7.24 ± 0.008 ^a	2.06 ± 0.014 ^c	1.04 ± 0.025 ^f	1.62 ± 0.006 ^d
Glycine	0.68 ± 0.002 ^c	0.69 ± 0.006 ^c	1.03 ± 0.016 ^d	0.704 ± 0.001 ^c	0.67 ± 0.088 ^c	1.32 ± 0.002 ^c	1.83 ± 0.004 ^b	2.41 ± 0.045 ^a	0.45 ± 0.019 ^f	0.93 ± 0.076 ^d
Proline	1.52 ± 0.036 ^g	1.11 ± 0.016 ^j	2.35 ± 0.005 ^d	2.15 ± 0.001 ^c	1.48 ± 0.005 ^h	3.59 ± 0.022 ^c	5.97 ± 0.025 ^a	5.46 ± 0.006 ^b	1.20 ± 0.004 ⁱ	1.64 ± 0.002 ^f
Aspartic acid	5.39 ± 0.008 ^h	4.95 ± 0.004 ⁱ	4.48 ± 0.003 ^j	13.52 ± 0.009 ^c	5.88 ± 0.016 ^g	8.53 ± 0.003 ^c	36.85 ± 0.064 ^a	15.70 ± 0.002 ^b	10.19 ± 0.013 ^d	7.39 ± 0.001 ^f
Glutamine	38.11 ± 0.005 ^h	41.61 ± 0.016 ^g	88.99 ± 0.032 ^c	99.01 ± 0.046 ^c	35.93 ± 0.025 ⁱ	95.510 ± 0.009 ^d	345.94 ± 0.004 ^a	118.83 ± 0.012 ^b	29.13 ± 0.003 ^j	54.45 ± 0.008 ^f
Lysine	27.74 ± 0.012 ^h	30.99 ± 0.002 ^g	64.04 ± 0.005 ^c	71.77 ± 0.017 ^c	25.96 ± 0.004 ⁱ	69.75 ± 0.077 ^d	246.72 ± 0.064 ^a	87.10 ± 0.002 ^b	21.22 ± 0.005 ^j	39.47 ± 0.003 ^f
Tyrosine	1.53 ± 0.021 ^g	2.07 ± 0.005 ^d	1.77 ± 0.001 ^f	1.97 ± 0.006 ^c	0.87 ± 0.071 ^j	3.00 ± 0.002 ^a	2.37 ± 0.007 ^b	2.31 ± 0.008 ^c	1.29 ± 0.009 ⁱ	1.37 ± 0.001 ^h
Isoleucine	1.12 ± 0.032 ^h	2.45 ± 0.004 ^c	1.59 ± 0.009 ^c	1.26 ± 0.006 ^f	1.17 ± 0.033 ^g	3.19 ± 0.002 ^b	2.28 ± 0.009 ^d	7.38 ± 0.003 ^a	1.25 ± 0.005 ^f	1.11 ± 0.029 ^h

Arginine	64.65 ± 0.005 ^g	33.48 ± 0.002 ⁱ	23.29 ± 0.032 ^j	70.68 ± 0.004 ^d	44.71 ± 0.015 ^h	75.64 ± 0.004 ^c	64.97 ± 0.007 ^f	144.18 ± 0.041 ^a	66.10 ± 0.037 ^e	116.91 ± 0.001 ^b
Hydroxy proline	1.00 ± 0.014 ^f	2.19 ± 0.064 ^c	1.44 ± 0.003 ^e	1.10 ± 0.052 ^f	1.07 ± 0.099 ^f	2.74 ± 0.001 ^b	1.95 ± 0.004 ^d	6.37 ± 0.009 ^a	1.12 ± 0.003 ^f	0.99 ± 0.002 ^f
Cysteine	1.200 ± 0.003 ^c	1.570 ± 0.024 ^d	1.630 ± 0.007 ^c	0.803 ± 0.008 ^g	1.180 ± 0.062 ^c	1.850 ± 0.017 ^a	1.650 ± 0.003 ^c	1.790 ± 0.002 ^b	1.20 ± 0.005 ^e	1.130 ± 0.001 ^f
Alanine	0.51 ± 0.005 ^c	0.14 ± 0.023 ^g	0.58 ± 0.006 ^c	0.46 ± 0.024 ^c	0.26 ± 0.006 ^f	0.86 ± 0.002 ^d	1.74 ± 0.081 ^b	2.05 ± 0.004 ^a	0.28 ± 0.008 ^f	1.07 ± 0.074 ^c

Note: Values are mean ± SD, followed by the different alphabets in each row are significantly different (p < 0.05). The letter ‘a’ represent the highest value.

Table 2.2. Vitamin quantification in ethanolic extracts of cultivars

Vitamins	Concentration in the samples (µg/mL)											
	NEN	Kappa		MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN	
Thiamine	0.007 ± 0.013 ^a	—	—	—	0.005 ± 0.007 ^a	—	—	—	—	—	0.002 ± 0.076 ^a	
Pyridoxine	0.060 ± 0.001 ^e	0.790 ± 0.065 ^a	±	0.220 ± 0.002 ^c	0.0610 ± 0.013 ^e	0.190 ± 0.009 ^d	0.180 ± 0.006 ^d	0.078 ± 0.024 ^e	0.307 ± 0.006 ^b	0.240 ± 0.005 ^c	±	0.164 ± 0.002 ^d
Folic acid	0.206 ± 0.011 ^c	0.860 ± 0.051 ^a	±	0.018 ± 0.007 ^h	0.140 ± 0.004 ^d	—	0.090 ± 0.081 ^e	0.480 ± 0.003 ^b	0.068 ± 0.007 ^f	—	—	0.054 ± 0.004 ^g
Nicotonic acid	0.440 ± 0.090 ^e	0.640 ± 0.007 ^c	±	0.730 ± 0.005 ^a	0.280 ± 0.009 ^h	0.340 ± 0.008 ^g	0.640 ± 0.016 ^c	0.430 ± 0.025 ^e	0.700 ± 0.005 ^b	0.601 ± 0.015 ^d	±	0.370 ± 0.017 ^f
Riboflavin	0.150 ± 0.006 ^a	—	—	—	—	—	—	—	—	—	—	0.130 ± 0.008 ^b

Ascorbic acid	0.280 ± 0.032 ^d	0.210 ± 0.009 ^f	0.770 ± 0.067 ^a	0.307 ± 0.005 ^c	0.240 ± 0.045 ^e	0.240 ± 0.076 ^e	0.360 ± 0.007 ^b	0.082 ± 0.009 ^h	0.360 ± 0.008 ^b	0.180 ± 0.014 ^g
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Note: Values are mean ± SD, followed by the different alphabets in each row are significantly different ($p < 0.05$). The letter 'a' represent the highest value.

2.3.3 Phytochemical Characterisation

2.3.3.1 Total phenolic and flavonoid content

Phenolic compounds, owing to their free radical-scavenging properties, act as excellent acting as antioxidants (Kamal et al., 2019). Hydro-alcoholic extracts of banana flour were analysed for total phenolic content and total flavonoid content. The phenolic content of raw banana flour followed the order MA>PALA>ZAN>Kappa>ME>ROB>PEYA>YAN>NEN>MON, ranging from 50.78 ± 0.15 – 96.14 ± 0.64 mg GAE/100g (Fig. 2.3a). Comparable TPC was observed in *Musa acuminata* by Menezes et al. (Menezes et al., 2011), while Kumar et al.(Kumar et al., 2019) reported 44.50 mg GAE/100g TPC in Monthan. Higher TPC in Nendran was recorded earlier, variations in TPC are attributed to genomic content, planting area, or geographical conditions affecting banana varieties (Aboul-Enein et al., 2016; Anyasi et al., 2015). The TFC in this study ranged from 0.92 ± 1.32 to 2.46 ± 1.88 mg Q.E./100 g, in the order Kappa>PEYA>YAN>ROB>PALA>ZAN>MON>MA>ME>NEN (Fig.2.3b). Siji and Nandini (Siji S & Nandini PV, 2016) reported TFC of 3.58 mg QE/100g for Kappa, aligning closely with our study. Suniati and Purnomo (Suniati & Purnomo, 2019), reported comparable TFC for raw Goroho (*Musa acuminata*, sp.). A study on Dwarf Cavendish also documented similar findings (1.69 ± 0.34 mg RE/g) in terms of TFC (Kim et al., 2021). Flavonoid synthesis, stimulated by sunlight, concentrates them in the outer portions of fruits (Tsao & McCallum, 2009). The most prevalent polyphenols in our regular diets are flavonoids, which exhibit antioxidant, antiviral,

antibacterial, antiallergenic, antithrombotic, anti-inflammatory, and hepatoprotective actions (J. Dai & Mumper, 2010; Xiang et al., 2019). In banana fruit, soluble flavonoids were shown to have a higher concentration and stronger antioxidant activity than bound flavonoids (Dong et al., 2016). Moreover, flavonoids have a complex structure, which makes them quite poor in bioavailability. Since small amounts of them can only be absorbed by small intestines, a large amount reaches the large intestine and interacts with intestinal microbes to promote the growth of beneficial microbiota (Tomás-Barberán et al., 2017). Gut microbiota, like *Bifidobacteriaceae*, *Lactobacillaceae*, *Lachnospiraceae*, and *Enterococcaceae*, contribute to flavonoid transformation (O-deglycosylation of flavonoids), whereas other species involved in the degradation of aglycones or flavonoid-C-glucosides cleaving (Braune & Blaut, 2016; Yan & Zhang, 2022). Dietary flavonoid intake can improve gut microbiota regulation, impacting host immunity, inflammation, and diversity of microbiota (H. Li et al., 2020). The regulation of gut microbes could be associated with regulating short-chain fatty acid metabolism and bile acid synthesis and uptake, potentially promoting bile acid homeostasis (Duan et al., 2021; L. Zhao et al., 2021). The phenolic and flavonoid composition established from the present study further unveils the potential application of raw banana flour in functional foods and health food applications.

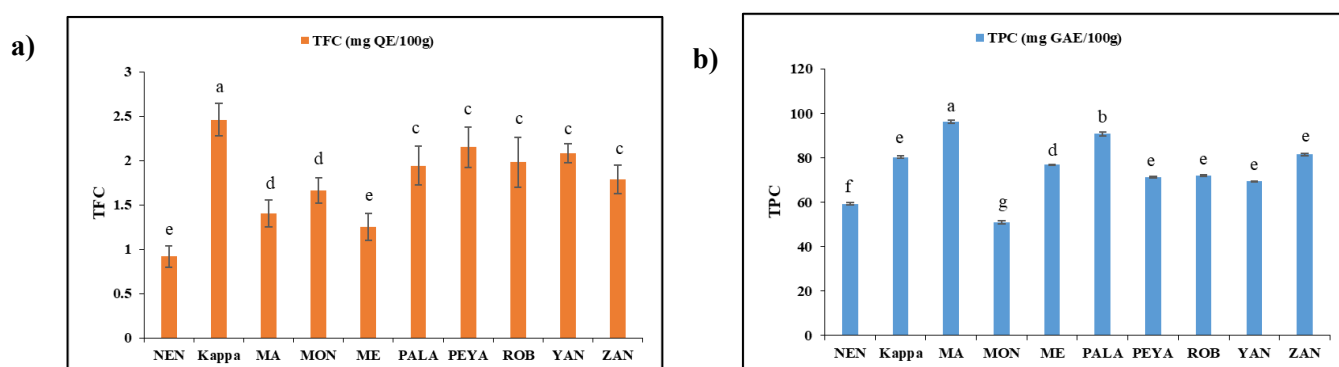


Figure 2.3. Phytochemical assays: a) TPC and b) TFC of different banana cultivars. Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

2.3.3.2 Antioxidant activity

The antioxidant activity of raw banana flour was studied by DPPH and ABTS free radical scavenging assays and is summarised in Figure 2.4 a & b. These assays are used to assess the free radical scavenging properties based on spectrophotometric measurements of the DPPH and ABTS concentration changes resulting from the reaction with an antioxidant. The results of the DPPH assay showed that the free radical scavenging potential was higher in ROB and PALA as compared to other varieties. The IC_{50} values for ROB and PALA were 35.27 ± 0.0062 and 35.39 ± 0.0063 $\mu\text{g/mL}$, respectively. The minimum DPPH activity shown by Kappa was 48.105 ± 0.0053 $\mu\text{g/mL}$. A lower IC_{50} value indicates strong free radical inhibitory activity. The DPPH activity of ethanolic extracts in this study was in agreement with that reported by Siji and Nandini, 2017 (S & P.V, 2017). Consistent with the DPPH activity, the ABTS free radical scavenging capacity of ROB (520.74 ± 0.0068 $\mu\text{g/mL}$) and PALA (832.53 ± 0.0021 $\mu\text{g/mL}$) was considerably higher than that of others; ME and Kappa had the lowest ABTS activity, 1680.68 ± 0.0024 $\mu\text{g/mL}$ and 1504.78 ± 0.0084 $\mu\text{g/mL}$, respectively. The ABTS activity of six Australian banana cultivars (Cavendish, Ladyfinger, Plantain, Ducasse, Monkey, and Red dacca) was reported in the range of 0.98 – 2.35 mg AAE/g, and these were close to NEN, MON, YAN, ZAN and PEYA cultivars of our study (Bashmil et al., 2021). Similar results are reported for crude ethanolic extracts of other plant species wherein the radical scavenging activity increased in dose dependent manner (Demirtas et al., 2013; Elmastaş et al., 2011; Erenler et al., 2016). It is also reported that TPC and antioxidant activity are positively correlated, and the variation in TPC and TFC is influenced by changes in climate, environmental conditions, and the cultivar (Alothman et al., 2009; Elmastaş et al., 2015; Savlak et al., 2016).

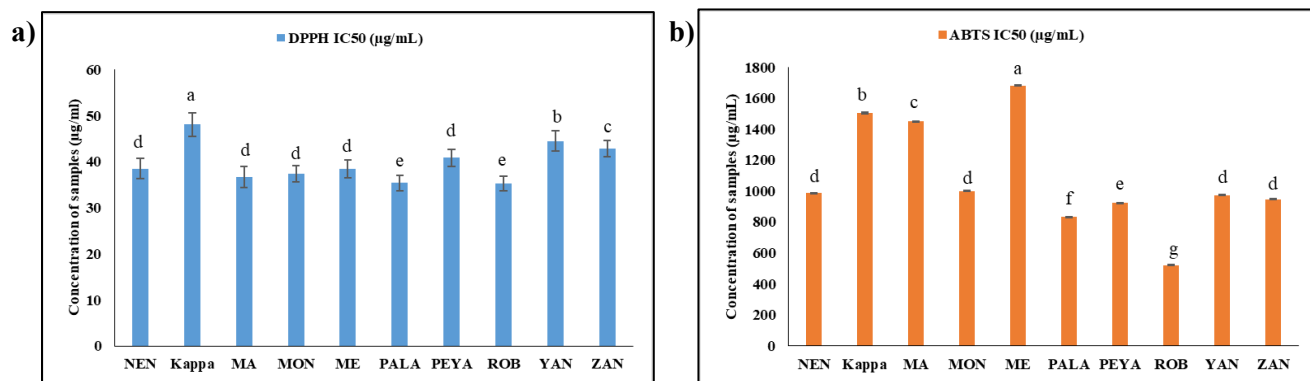


Figure 2.4. Antioxidant assays: a) DPPH and b) ABTS activity of different banana cultivars. Data presented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

2.3.4 Quantification of Polyphenols

TPC analysis highlighted raw bananas as excellent sources of phenolic compounds. LC-MS/MS is generally used for the quantitative estimation of phytochemicals in plant-based extracts (Atalar et al., 2023; Erenler et al., 2018, 2023). In the present study, we have employed the technique to analyse 28 polyphenolic compounds in raw banana flour extracts.

The LC-MS/MS analysis unveiled the polyphenol profile in various banana cultivars, with shikimic acid, epicatechin, ferulic acid, and rutin as predominant ones (Table 2.3). The phytochemical profile in YAN followed the order shikimic acid > ferulic acid > epicatechin, with a shikimic acid content of 107.730 ± 0.0012 µg/mL. NEN demonstrated significant polyphenols, with shikimic acid (78.820 ± 0.0021 µg/mL) and ferulic acid (3.490 ± 0.0076 µg/mL) as the major ones. Kappa was rich in shikimic acid (118.940 ± 0.0042 µg/mL) and ferulic acid (4.120 ± 0.0032 µg/mL). MA showcased shikimic acid (63.490 ± 0.0078 µg/mL) and gallic acid (3.630 ± 0.0031 µg/mL). MON led to shikimic acid (49.100 ± 0.0015 µg/mL). Shikimic acid (81.23 ± 0.0019 µg/mL) and ferulic acid (1.720 ± 0.0071 µg/mL) were the major phytochemicals in ME. PALA excelled with abundant shikimic acid (133.380 ± 0.0013 µg/mL) and notable ferulic acid (3.820 ± 0.008 µg/mL). Shikimic acid (65.130 ± 0.0042 µg/mL), and epicatechin (5.830 ± 0.0016) topped in PEYA. ROB featured the highest ferulic acid (19.430 ± 0.0023 µg/mL) among the cultivars and the highest shikimic acid (109.180 ± 0.0065 µg/mL).

Shikimic acid ($80.50 \pm 0.0017 \mu\text{g/mL}$) and ferulic acid ($2.8360 \pm 0.0033 \mu\text{g/mL}$) were the major compounds in ZAN.

When assessing the polyphenol component of all banana cultivars, it was noted that shikimic acid was the predominant compound, followed by ferulic acid and flavonoids including rutin, epicatechin, and catechin. Earlier studies reported that unripe fruit pulp contains shikimic acid (Hulme, 1958). In addition to the above-mentioned polyphenols, lower concentrations of compounds like naringenin, vanillic acid, myricetin, syringic acid, and gallic acid were also found in some banana cultivars. Rutin, myricetin, naringenin, gallic acid, and ellagic acid were reported earlier in bananas (Behiry et al., 2019). An earlier study of the phenolic and flavonoid contents in unripe and ripe Australian-grown banana types revealed that the presence of p-Hydroxybenzoic acid, quercetin, gallic acid, syringic acid, diosmin, chlorogenic acid, p-coumaric acid, protocatechuic acid, quercetin-3-rhamnoside, and kaempferol (Bashmil et al., 2021). In another study, the pulp and peel of banana cultivars were found to contain ferulic acid, ferulic acid-hexoside, epicatechin, caffeic acid-hexoside, sinapic acid, hydroxycinnamic acid, myricetin-deoxyhexose, rutin, quercetin-deoxyhexose, kaempferol-3-O-rutinoside, and other unidentified compounds (Passo Tsamo et al., 2015). The dried pulp powder of *Musa balbisiana* has been found to contain epicatechin, catechin, rutin, quercetin, chlorogenic acid, kaempferol 3-O-sophoroside, apigenin-6-C-glucoside-7-Oglucoside, and rhamnopyranoside (Kumari et al., 2020). Rutin, catechin, quinic acid, gallo catechin, propionic acid, caffeic acid-3-glucoside, kaempferol-O-rhamnosidehexoside, caffeic acid, L-Dopa, sennoside A, and dopamine have been identified in the hydro-alcoholic extract of *Musa Cavendish* with anti-cancerous activity (Barroso et al., 2019). These findings aligned with the current study on certain phenolics. Despite numerous studies conducted in this field, there is still a gap in the

quantification of phenolics due to their complex structures and the way environmental factors affect the secondary metabolite levels, which differ between cultivars.

Table 2.3. Polyphenol quantification in ethanolic extracts of cultivars

Compounds	Concentration in the samples (µg/mL)									
	NEN	Kappa	MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN
Catechin	1.220 ± 0.0011 ^c	2.280 ± 0.0022 ^b	1.040 ± 0.0065 ^f	1.040 ± 0.0054 ^f	0.510 ± 0.0033 ^g	1.520 ± 0.0012 ^c	3.470 ± 0.0021 ^a	1.380 ± 0.0064 ^d	0.260 ± 0.0070 ^h	1.530 ± 0.0052 ^c
Quinine	0.022 ± 0.0032 ^b	0.021 ± 0.0063 ^b	0.011 ± 0.0032 ^d	0.007 ± 0.0076 ^c	0.020 ± 0.0023 ^b	0.023 ± 0.0054 ^a	0.013 ± 0.0033 ^d	0.016 ± 0.0018 ^c	0.005 ± 0.0042 ^e	0.006 ± 0.0015 ^e
Naringenin	1.360 ± 0.0055 ^d	2.140 ± 0.0085 ^a	1.260 ± 0.0023 ^c	1.010 ± 0.0011 ^g	1.110 ± 0.0051 ^f	0.890 ± 0.0040 ^h	1.530 ± 0.0013 ^c	0.980 ± 0.0063 ^g	0.840 ± 0.0056 ⁱ	1.650 ± 0.0012 ^b
Tocopherol	0.088 ± 0.0057 ^c	0.130 ± 0.0044 ^b	0.160 ± 0.0034 ^a	0.097 ± 0.0066 ^c	0.095 ± 0.0076 ^c	0.120 ± 0.0011 ^b	0.120 ± 0.0025 ^b	0.100 ± 0.0098 ^c	0.110 ± 0.0084 ^c	0.091 ± 0.0067 ^c
Gallic acid	0.570 ± 0.0121 ^g	0.950 ± 0.0052 ^d	3.630 ± 0.0031 ^a	0.730 ± 0.0011 ^c	0.610 ± 0.0060 ^f	1.610 ± 0.0054 ^c	1.680 ± 0.0010 ^b	0.380 ± 0.0023 ⁱ	0.490 ± 0.0056 ^h	0.500 ± 0.0032 ^h
Chlorogenic acid	0.047 ± 0.0225 ^b	0.044 ± 0.0011 ^b	0.005 ± 0.0040 ^c	0.028 ± 0.0022 ^c	0.021 ± 0.0036 ^c	0.013 ± 0.0045 ^d	0.034 ± 0.0014 ^c	0.005 ± 0.0017 ^c	0.026 ± 0.0113 ^c	0.061 ± 0.0055 ^a
Epicatechin	1.750 ± 0.0064 ^g	2.590 ± 0.0017 ^d	3.200 ± 0.0134 ^b	2.160 ± 0.0012 ^f	1.290 ± 0.0046 ^h	3.100 ± 0.0020 ^c	5.830 ± 0.0016 ^a	2.400 ± 0.0018 ^c	1.120 ± 0.0021 ⁱ	1.730 ± 0.0042 ^g
Syringic acid	0.390 ± 0.0414 ^d	—	0.120 ± 0.0051 ^c	1.530 ± 0.0013 ^b	0.008 ± 0.0011 ^g	0.130 ± 0.0025 ^e	1.600 ± 0.0050 ^a	0.020 ± 0.0022 ^f	—	0.800 ± 0.0048 ^c
Vanillic acid	0.680 ± 0.0034 ^c	0.640 ± 0.0021 ^f	1.800 ± 0.0019 ^b	1.430 ± 0.0083 ^c	0.370 ± 0.0064 ^h	1.400 ± 0.0056 ^d	2.690 ± 0.0051 ^a	0.670 ± 0.0017 ^c	0.370 ± 0.0014 ^h	0.610 ± 0.0015 ^g
Caffeic acid	0.270 ± 0.0061 ^c	0.530 ± 0.0036 ^a	0.260 ± 0.0076 ^f	0.520 ± 0.0017 ^b	0.240 ± 0.0019 ^g	0.470 ± 0.0026 ^c	0.290 ± 0.0021 ^d	0.190 ± 0.0015 ⁱ	0.260 ± 0.0028 ^f	0.230 ± 0.0022 ^h
Epigallocatechin	0.012 ± 0.0052 ^c	0.018 ± 0.0020 ^b	0.021 ± 0.0114 ^a	0.013 ± 0.0066 ^c	0.013 ± 0.0026 ^c	0.018 ± 0.0020 ^b	0.018 ± 0.0097 ^b	0.019 ± 0.0037 ^b	0.021 ± 0.0017 ^a	0.014 ± 0.0022 ^c
Ferulic acid	3.490 ± 0.0076 ^d	4.120 ± 0.0032 ^b	1.890 ± 0.0086 ^h	2.180 ± 0.0014 ^g	1.720 ± 0.0071 ⁱ	3.820 ± 0.0087 ^c	1.520 ± 0.0021 ^j	19.430 ± 0.0023 ^a	2.500 ± 0.0045 ^f	2.860 ± 0.0033 ^c
Myricetin	0.370 ± 0.0016 ^g	0.600 ± 0.0019 ^e	1.260 ± 0.0010 ^a	0.240 ± 0.0044 ^h	0.170 ± 0.0035 ^j	0.780 ± 0.0012 ^d	0.790 ± 0.0073 ^c	0.950 ± 0.0017 ^b	0.180 ± 0.0034 ⁱ	0.530 ± 0.0028 ^f

p-Coumaric acid	0.460 ± 0.0081 ^d	0.210 ± 0.0023 ^h	0.920 ± 0.0011 ^a	0.490 ± 0.0034 ^c	0.170 ± 0.0072 ⁱ	0.340 ± 0.0081 ^c	0.750 ± 0.0024 ^b	0.240 ± 0.0055 ^g	0.290 ± 0.0012 ^f	0.350 ± 0.0016 ^c
Apigenin	0.013 ± 0.0049 ^b	0.018 ± 0.0032 ^a	0.017 ± 0.0065 ^a	0.015 ± 0.0006 ^b	0.012 ± 0.0016 ^b	0.018 ± 0.0046 ^a	0.017 ± 0.0010 ^a	0.017 ± 0.0023 ^a	0.019 ± 0.0042 ^a	0.019 ± 0.0016 ^a
Kaempferol	0.026 ± 0.0032 ^c	0.065 ± 0.010 ^a	0.040 ± 0.0020 ^c	0.052 ± 0.0050 ^b	0.025 ± 0.0031 ^c	0.037 ± 0.0003 ^c	0.030 ± 0.0038 ^d	0.050 ± 0.0007 ^b	0.018 ± 0.0011 ^f	0.052 ± 0.0018 ^b
Rutin	2.590 ± 0.0011 ^c	2.650 ± 0.0040 ^b	1.720 ± 0.0056 ^d	1.170 ± 0.0077 ^g	0.550 ± 0.001 ⁱ	1.540 ± 0.0010 ^e	4.350 ± 0.021 ^a	1.250 ± 0.0027 ^f	0.790 ± 0.0047 ^h	2.590 ± 0.0073 ^c
Hesperetin	0.016 ± 0.0010 ^b	0.025 ± 0.0015 ^a	0.025 ± 0.0044 ^a	0.016 ± 0.0025 ^b	—	0.024 ± 0.0036 ^a	0.026 ± 0.0021 ^a	0.027 ± 0.0014 ^a	0.024 ± 0.0087 ^a	0.018 ± 0.0022 ^b
Shikimic acid	78.820 ± 0.0021 ^g	118.940 ± 0.0042 ^b	63.490 ± 0.0078 ⁱ	49.100 ± 0.0015 ^j	81.230 ± 0.0019 ^e	133.380 ± 0.0013 ^a	65.130 ± 0.0042 ^h	109.180 ± 0.0065 ^c	107.730 ± 0.0012 ^d	80.500 ± 0.0017 ^f
Ellagic acid	—	0.410 ± 0.0112 ^b	0.220 ± 0.0064 ^c	0.042 ± 0.0045 ^c	0.160 ± 0.0014 ^d	—	—	0.600 ± 0.0031 ^a	—	—
Genistein	0.011 ± 0.0024 ^b	0.018 ± 0.0025 ^a	0.019 ± 0.0076 ^a	0.012 ± 0.0010 ^b	0.011 ± 0.0086 ^b	0.019 ± 0.0043 ^a	0.017 ± 0.0032 ^a	0.020 ± 0.0015 ^a	0.020 ± 0.0054 ^a	0.011 ± 0.0044 ^b
Cinnamic acid	0.410 ± 0.0132 ^d	0.390 ± 0.0045 ^c	0.850 ± 0.0025 ^a	0.420 ± 0.0016 ^d	0.140 ± 0.0063 ^g	0.140 ± 0.0059 ^g	0.310 ± 0.0047 ^f	0.640 ± 0.0043 ^b	0.480 ± 0.0033 ^c	0.160 ± 0.0034 ^f

Note: Values are mean ± SD, followed by the different alphabets in each row are significantly different ($p < 0.05$). The letter ‘a’ represent the highest value.

2.3.5 Cytotoxicity Assay

The MTT assay investigated the cytotoxic effects of raw banana flour extracts on L6 myoblasts, revealing no impact on cell viability up to 500 µg/mL. The cell lines treated with different concentrations showed above 70% cell viability. When compared among the flour extracts, PALA (81.78%) and PEYA (79.62%) exhibited higher viability, while YAN (69.83%) showed lower viability compared to other cultivars at 500 µg/mL (Fig. 2.5). Many previous studies have reported the poor cytotoxicity of banana extracts against normal cell lines. Dahham et al. (Dahham et al., 2015) tested the cytotoxic activity of *Musa sapientum* fruit extract against the normal human umbilical vein endothelial cell line (HUVEC) and

the human colorectal carcinoma cell line HCT-116. The result revealed that the banana extracts showed lower cytotoxicity against normal cell lines than cancer cells. Extracts from 20 different fruit extracts were analysed for cytotoxicity against leukemic cell lines and normal human peripheral blood mononuclear cells (PBMCs). Some of these extracts exhibited cytotoxicity against both normal and leukemic cell lines, but some extracts specifically showed toxicity against leukemic cell lines, and others did not show any toxicity. Among these, the *Musa sapientum* L. extracts presented no cytotoxic effect on normal PBMC cell lines (Ampasavate et al., 2010). The non-toxic nature of the extracts indicates that they could be used as an ingredient or additive without causing harm to normal cells. The lower cytotoxicity implies that bananas could be considered safe for a wide range of applications, particularly in the food and pharmaceutical industries.

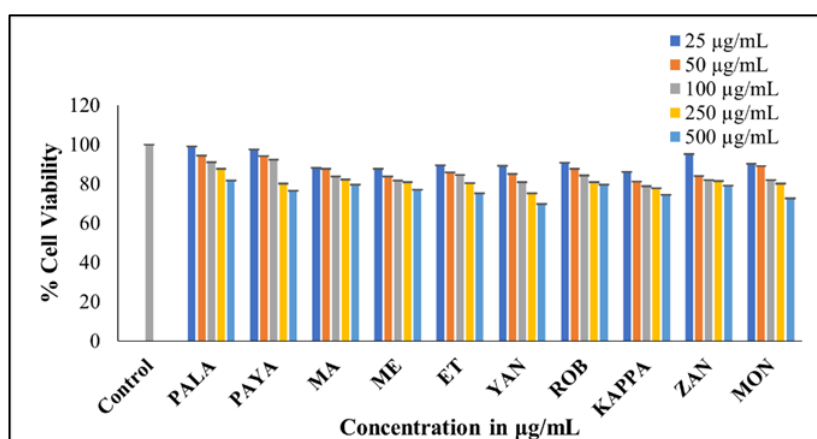


Figure 2.5. MTT assay showing cytotoxicity of ethanolic extract of ten cultivars against L6 cells. Data presented are mean value $\pm$ SD.

2.3.6 α -Amylase Inhibition Assay

Hyperglycemia results from the rapid breakdown of starchy meals by carbohydrate-hydrolyzing enzymes such as α -amylase and α -glucosidase. The therapeutic approach for diabetes involves synthetic inhibitors of α -amylase to impede glucose absorption, which is reported to cause undesirable side effects (Oboh et al., 2015). Many dietary bioactive components are reported to inhibit these enzymes. Banana extracts (200 μ g/mL) and acarbose

(100 µg/mL) were evaluated against PPA. The findings demonstrate that the α -amylase inhibitory activity of flour extracts varied across samples, with PALA and ROB with higher inhibitory potential (66.96% and 50.50%) (Fig. 2.6). The standard acarbose showed 76.84% inhibition. The results are in agreement with previous reports on *Musa paradisiaca*'s antidiabetic potential (Patel et al., 2015). α -amylase inhibitory may be correlated with the bioactive phytochemicals, in line with previous reports (Adedayo et al., 2016; Ademiluyi & Oboh, 2013). The results could have implications for managing postprandial blood sugar levels, making them potentially useful in the dietary management of diabetes.

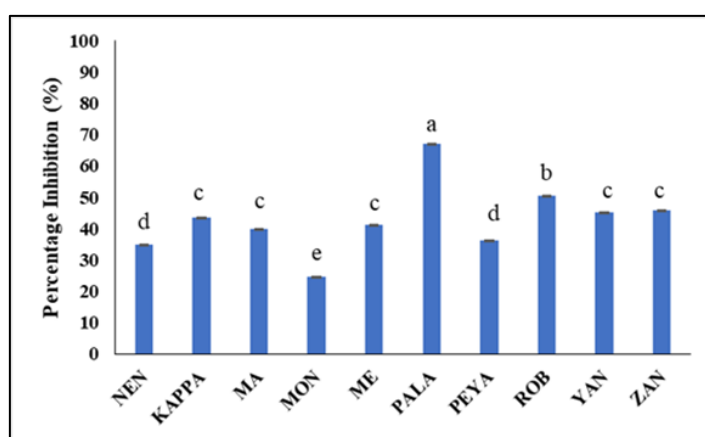


Figure 2.6. The percentage inhibition of PPA by different banana cultivars. Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

2.3.7 Molecular docking studies

According to LCMS/MS analysis, shikimic acid and ferulic acid were identified as the major phytochemicals in the raw banana flour. Both shikimic acid and ferulic acid are reported to have hypoglycemic activity due to their inhibition potential of α -amylase (Cao et al., 2023; Zheng et al., 2020). We adopted molecular docking to correlate the same. The docking analysis identified the optimal poses for both, with the lowest energies at -5.45 and -5.48 Kcal/mol respectively, for shikimic and ferulic, suggesting high binding affinity. Table 2.4 and Fig.2.7

illustrate the hydrogen bond residues involved in their interactions with α -amylase. Earlier studies reported a binding energy of -5.3 Kcal/mol for the ferulic acid- α -amylase complex (Zheng et al., 2020). However, the binding energy was lower compared to acarbose (-8.8 Kcal/mol) (Okechukwu et al., 2020). PALA and ROB were found to have higher estimated amounts of ferulic acid and shikimic acid in comparison to the other variants. Thus, the α -amylase inhibitory activity of the banana extracts can be correlated with the presence of phytochemicals such as shikimic and ferulic acid, which was confirmed by docking studies.

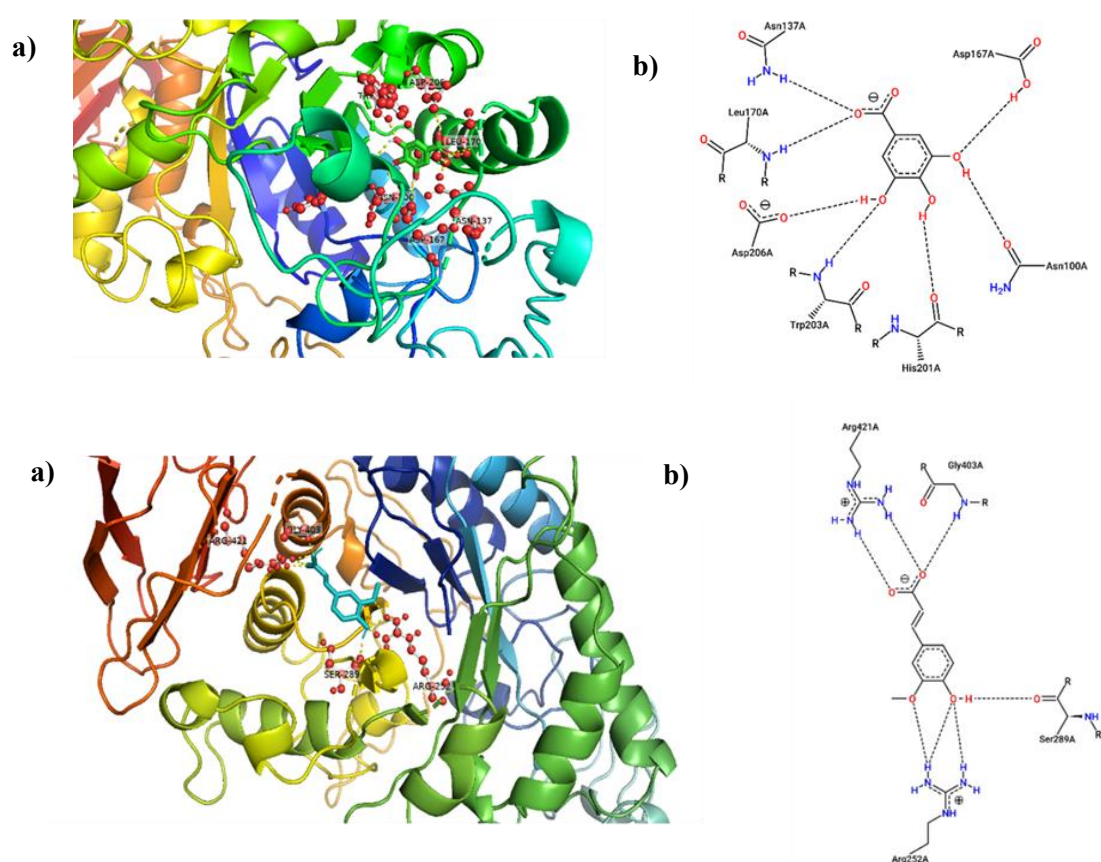
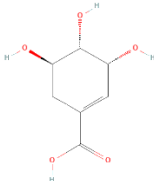
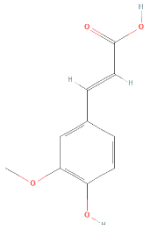


Figure 2.7. Molecular docking for shikimic and ferulic acid with α -amylase amino acid residues; a) & b) 3D & 2D interaction image of shikimic acid c) & d) 3D & 2D interaction image of ferulic acid.

Table 2.4. Molecular docking results of banana cultivars phenolics towards α -amylase (PDB ID: 4W93)

Ligands	PubChem CID	Structure	H-bonded residues	Binding Energy (Kcal/mol)
Shikimic acid	8742		Asn137 Leu170 Asp206 Trp203 His201 Asn167 Asn100	-5.45
Ferulic acid	445858		Arg421 Gly403 Arg252 Ser289	-5.48

2.3.8 Isolation of starch and resistant starch

The percentage yield of starch ranged between 84.30 ± 1.25 - $95.8 \pm 1.32\%$ dry weight (d/w) (Fig. 2.8). It can be seen that NEN variety had the highest and PEYA had the lowest content of starch. A previous study by Babu et al. (Babu et al., 2014) reported 92.62% starch content in the Monthan variety, which is similar to the present study (91.60%). Furthermore, the plantain banana harvested from Mexico is reported to have 82.3% starch content (Carlos-Amaya et al., 2011). Many studies have also reported that raw banana pulp contains up to 60-80% starch (JUAREZ-GARCIA et al., 2006; M.-C. Li et al., 2020; Z. Li et al., 2018). The variation in the starch content of the reported values from the current study may be associated with the differences in the isolation process, variety of the cultivars, maturity, and geographical origin.

The inclusion of banana starch can impact the physicochemical and pasting properties of the substance through many mechanisms, including structural interactions, interactions with proteins and lipids, water retention, and flavor (Marta et al., 2019; Vatanasuchart et al., 2012a). In addition to this, the electrostatic interactions within banana flour have several effects on its physicochemical properties, like viscosity, gelation, water absorption, texture, and elasticity. These interactions play a crucial role in the development of food products, such as bread, pasta, or gluten-free baking (Amini Khoozani et al., 2020; Mohamed et al., 2010). The unripe banana contains a significant amount of starch that breaks down into sugars during ripening with the aid of ethylene-mediated regulation of starch-degrading enzymes (Cordenunsi et al., 2019).

Unripe bananas are reported to have health benefits due to the high amount of indigestible polysaccharides, mainly RS. Even though many studies have discussed RS from bananas, there are no previous reports on the RS of some of the varieties (YAN, PEYA, ZAN, and ME) that have been discussed in the present study (Fig. 2.8). The RS yield varied between $41.78 \pm 1.29\%$ and $26.43 \pm 1.27\%$ d/w, with MA having the highest and ZAN the lowest yields. Unripe Taiwanese bananas reported a similar yield of RS (M.-C. Li et al., 2020). Prior studies also compared the RS content of peeled and unpeeled stage II cavendish bananas, and the results showed that the RS content was higher for peeled ones (40.16%) than unpeeled ones (33.86%) (Bezerra et al., 2013), this result supports the current study.

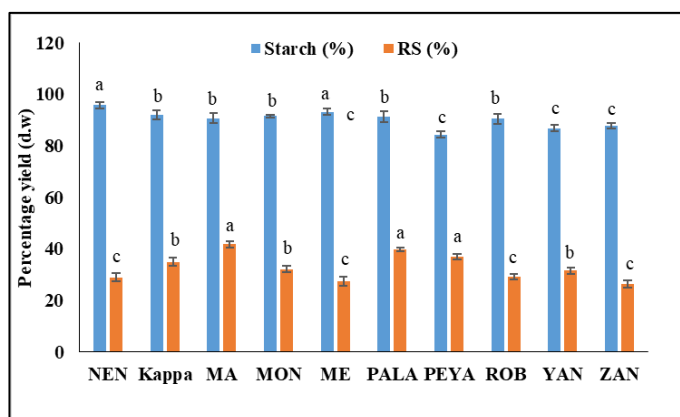


Figure 2.8. Starch and resistant starch yield of banana cultivars (% d/w). Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

A recent investigation by Thuy et al. (Nguyen Minh et al., 2022), compared the RS content of different starchy food sources like sweet potato, black beans, red kidney beans, mung beans, corn starch, potato starch, and green banana starch and found that 48.29% RS in green banana. RS resists the action of digestive enzymes in the small intestine and serves as a substrate for beneficial gut microbiota. Furthermore, RS aids in the lowering of blood cholesterol, postprandial glucose due to the low glycemic index, and in the prevention of colon cancer (G. Zhang & Hamaker, 2009). Foods with high RS exhibit functional as well as physiological health benefits. Thus, raw banana flour could be exploited ingredient for the formulation of various functional foods.

2.3.9 Characterisation of Starch and Resistant starch

2.3.9.1 Zeta potential

The zeta potential of RS isolated from native starch using enzymatic methods gives valuable insights into the surface charge and stability of starch granules, which varies significantly depending on the source and processing conditions. The zeta potential is an index usually used to quantify surface charges in a biopolymer, which had a vital role when it was combined with molecules as a coating material (Mao & McClements, 2013). The zeta potential of starch and RS is presented in Table 2.5. Native starch and RS granules showed a negative zeta potential ranging from -8.07 to -18.55 mV and -34.45 to -57.65 mV respectively, due to the presence of hydroxyl groups on the glucose units in amylose and amylopectin. This surface charge maintains a degree of electrostatic repulsion, which helps the granules stay in suspension (Espinosa-Solís et al., 2021).

After enzymatic treatment, the exposure of more functional groups occurs. These alterations significantly result in a greater negative zeta potential, which enhances electrostatic repulsion between granules and improves dispersion stability in aqueous solutions. These results are in

line with earlier studies that found that starch, starch nanoparticles, and modified starch have shown negative zeta potential values (Ahmad et al., 2020; Sathyan & Nisha, 2022). Moreover, previous reports have proven that a higher negative zeta potential reduces Van der Waals force through electrostatic repulsion between particles (Ahmad et al., 2020; Schäfer et al., 2010). This reduces particle agglomeration and improves particle stability (L. Dai et al., 2018). Enzymatic treatment of native starch significantly alters its zeta potential, resulting in a more negative surface charge and enhanced dispersion stability. This makes RS a promising ingredient in food and pharmaceutical applications, particularly in drug delivery. The negative zeta potential of RS makes it more suitable for encapsulating cationic proteins because it promotes better stability and particle formation.

Table 2.5. Zeta potential of starch and resistant starch from ten banana cultivars

Samples	Starch (mV)	Resistant starch (mV)
NEN	-9.66 ± 0.14 ^d	-44.50 ± 0.13 ^b
Kappa	-18.55 ± 0.54 ^a	-46.75 ± 0.33 ^b
MA	-12.60 ± 0.36 ^c	-34.45 ± 0.20 ^c
MON	-15.35 ± 0.66 ^b	-45.50 ± 0.43 ^b
ME	-11.80 ± 0.23 ^c	-36.8 ± 0.22 ^c
PALA	-11.20 ± 0.78 ^c	-47.35 ± 0.54 ^b
PEYA	-17.00 ± 0.65 ^a	-52.20 ± 0.50 ^a
ROB	-8.075 ± 0.38 ^d	-44.95 ± 0.22 ^b
YAN	-14.70 ± 0.87 ^b	-57.65 ± 0.67 ^a
ZAN	-18.10 ± 0.44 ^a	-38.35 ± 0.45 ^c

Note: Values are mean ± SD, followed by the different alphabets in each column are significantly different ($p < 0.05$). The letter 'a' represents the highest value.

2.3.9.2 Drying curve

The drying curve of starch provides key insights into understanding its drying kinetics, as it can be modified by temperature, air velocity, and moisture content. The drying process is often divided into two phases: a constant rate period and a falling rate period. The properties of these curves can provide information about the specific surface area and moisture diffusivity of starch, which are critical for optimising drying conditions (Caparanga et al., 2017). The constant phase is often not observed in starch drying consistent with present findings, in the falling phase moisture content decreases, and the drying rate is regulated by the internal migration of moisture within starch particles (Benali, 2012). The drying curve of RS in terms of coat material for spray drying is determined by several parameters, including the type of starch used, its composition, and the spray drying conditions. Different RS have distinct thermal and structural properties that influence microencapsulation efficiency and drying characteristics. Among the different banana cultivars, the RS isolated from the PALA variety showed a higher drying rate (Fig.2.9), indicating that moisture removal occurs more rapidly in this cultivar compared to others. For spray drying applications where RS serves as a coating material, a higher drying rate is beneficial, as it facilitates faster and more efficient moisture evaporation, resulting in a stable, high-quality coating. Previous research has demonstrated that maize, potato, and jinicuil seed RS have superior thermal resistance and high microencapsulation efficiency, making them ideal for encapsulating probiotics (Aparicio-Saguilán et al., 2024; Muhammad et al., 2021).

RS isolated by enzymatic methods often differs from native starch in its drying behavior, owing to the modified crystalline structure that binds water more tightly, because of that RS has a slower initial drying rate, implying that more energy is required to remove bound moisture, most likely due to higher hydrogen bonding inside its crystalline regions (Zięba et al., 2011). The drying curve is particularly significant when using RS as a coating material since it affects

physical properties such as moisture content and powder stability, both of which are crucial for encapsulation efficiency in food and pharmaceutical applications (Shang et al., 2023). Therefore, optimal drying parameters and higher drying rate coating material can form a uniform coating layer around bioactive, which is critical for encapsulation and controlled release applications (Bilancetti et al., 2010). Hence, understanding the drying curve of RS produced by the enzymatic method and optimising drying conditions might result in RS with enhanced functionality, supporting its role as an efficient coating material in various formulations. Based on these findings, starch, and RS from the PALA cultivar were selected for further characterisation to compare structural and thermal changes caused by enzymatic treatment.

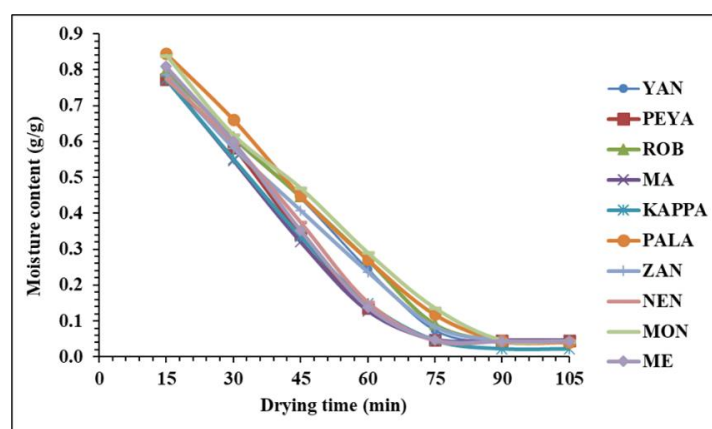


Figure 2.9. Drying curve of resistant starch from different banana cultivars

2.3.9.3 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of RS isolated from native starch by enzymatic methods provides insight into the molecular structure and functional group changes that occur during enzymatic conversion. Enzymatic treatment hydrolyses amorphous regions of starch producing a more ordered, crystalline structure that is less digestible. Fig. 2.10 displays the FTIR spectrogram of the starch and RS of PALA. The spectra show that the characteristic absorption peaks detected were similar for both starch and RS, indicating that no chemical modification occurred after enzymatic treatment (Sathyan & Nisha, 2022; B. Zhang et al., 2012). There were four

significant characteristic bands observed, which comprise absorption peaks between 3000–3500 cm^{-1} indicating the vibrational stretching of free and hydrogen-bonded hydroxyl groups present in the polysaccharide. The absorption band near 2800-3000 cm^{-1} is associated with C-H stretching; the intensity of peaks between 800 cm^{-1} and 1600 cm^{-1} reflected the vibrational stretching of C-H, C-C, and C-OH; and absorption peaks at 1643, 1150, and 1077 cm^{-1} represented the presence of C=O, C-O-C, and C-O bonds, respectively. The FTIR absorption bands at 930, 860, and 764 cm^{-1} are attributed to D-glucopyranose (Wang et al., 2023).

Following enzymatic treatment, the FTIR spectra of RS show a significant difference in the intensity near 1000 cm^{-1} , where peaks for amylose and amylopectin, indicate less amorphous regions and higher crystallinity. This change indicates a more ordered structure, as enzymatic hydrolysis removes easily digestible regions while retaining the resistant, crystalline regions (Liang et al., 2021). Previous studies that used starch from various sources and employed different methods to form RS support the current study findings, indicating that enzymatic treatment does not alter the molecular structure of resistant starch (Khatoon et al., 2009; Shrestha et al., 2010). Therefore, the FTIR spectra demonstrate that enzymatic treatment successfully converts native starch into RS by selectively targeting amorphous regions, enhancing the crystalline structure, and contributing to its indigestibility. This structural modification is useful for applications where resistance to enzymatic digestion, such as delivery systems and prebiotic supplements.

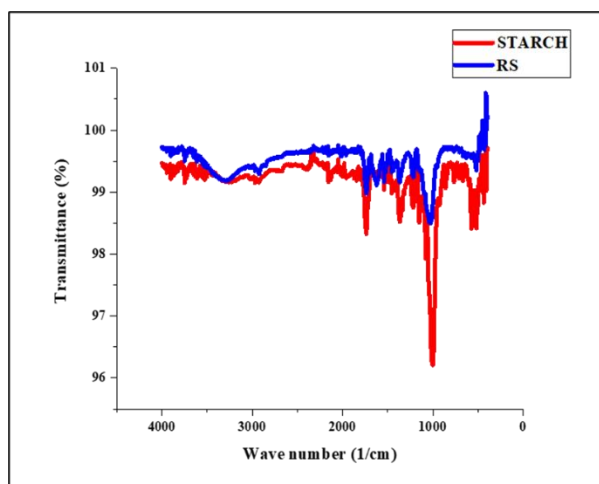


Figure 2.10. FTIR images of starch and resistant starch

2.3.9.4 X-ray diffraction (XRD)

X-ray diffraction (XRD) is an important technique for determining the structural properties of RS isolated using enzymatic techniques. This approach gives information about the crystalline forms and physicochemical properties of RS, which is essential for understanding its digestibility and potential applications in food science. XRD determines the crystalline pattern of starch owing to the ordered array of double helices of the amylopectin side chain, on this basis A, B, and C-type patterns are present. In type B and C patterns, the amylopectin forms highly branched long chains connected with amylose molecules, whereas the A-type pattern presents a high proportion of short-chain amylopectin (Izidoro et al., 2011; Vatanasuchart et al., 2012b). Starch obtained from banana cultivar showed prominent peaks located at approximately 5°, 15°, 17°, and 23° diffraction angles in 2θ , which were the characteristics peaks of a B-type crystallinity pattern. Previous reports have observed a similar B-type crystalline pattern in plantain starch granules (Agama-Acevedo et al., 2015; de Barros Mesquita et al., 2016). However, some researchers have reported variations, including A-type, C-type, and A and B-type mixtures, in bananas. These variations are influenced by factors such as plant variety, environmental conditions, and other variables. Furthermore, the method used

to isolate starch might affect the internal organisation of starch granules, which contributes to structural differences (Tester et al., 2004).

XRD diffractograms of RS isolated by enzymatic treatment show enhanced intensity peaks than native starch, indicating a higher crystallinity. The ratio of crystalline to amorphous regions is often higher in RS because the enzymatic process selectively hydrolyses the amorphous regions, which may lead to macromolecular rearrangement and recrystallization, resulting in a more crystalline structure. This is particularly evident in stronger peaks at 17° and 23° for B-type starch, confirming that the essential crystalline structure remains intact but becomes more prominent in the RS. These findings align with prior research, which identified B-type crystalline patterns in RS derived from chickpeas and maize (Mun & Shin, 2006; Polesi et al., 2018). Moreover, a study showed that microwave and ultrasound-assisted enzymatic hydrolysis has been found to increase RS content and change the crystalline structure (Wang et al., 2023). The increase in crystallinity of RS makes it less available to digestive enzymes (H. Zhang & Jin, 2011). This characteristic makes RS a promising element for functional food products as well as a potential carrier for drug delivery. The XRD diffractograms of the PALA starch and RS are shown in Figure 2.11.

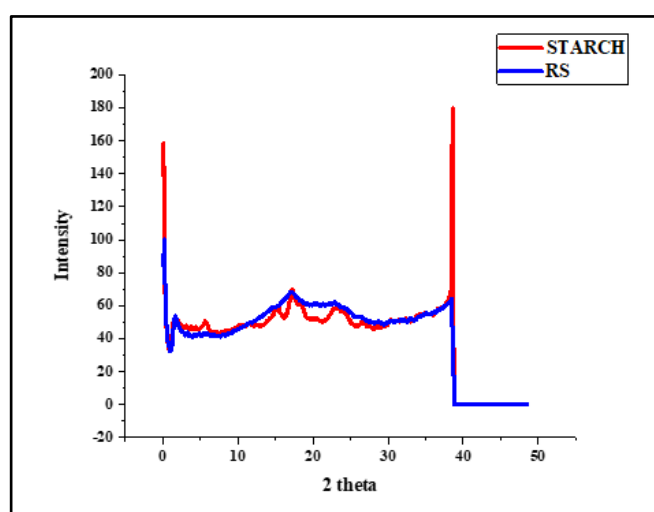


Figure 2.11. XRD images of starch and resistant starch

2.3.9.5 Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) is used to visualise the morphology of starch granules, allowing for a comparison of the structural changes that occur in RS when it is isolated from native starch using the enzymatic method (Fig. 2.12). The surface of native starch consists of smooth, well-defined granules with rounded, or oval shapes with intact surfaces, this indicates the isolation process was efficient and didn't cause any damage to the array of the starch components in the granule. This structure encloses starchy biomass, which prevents the partial or complete action of digestive enzymes. SEM images of raw banana starch observed in the present study were similar to the previous study (Chávez-Salazar et al., 2022; de Barros Mesquita et al., 2016)

In contrast, the SEM image of RS demonstrated a bigger granular size, rigid irregular rougher surfaces, and compact block-like structures observed in enzymatically modified starches. These properties point to the selective hydrolysis of amorphous areas, the present study aligns with previous reports (Wang et al., 2023). The results of various treatments, such as enzymatic and enzymatic-assisted physical methods applied to bananas and other starches, are also consistent with the current observations (Das et al., 2022; Surendra Babu et al., 2018; Zhou et al., 2019). Furthermore, a prior study found that dual enzymatic modification leads to the formation of pores and fissures in the starch granules, indicating structural changes that enhance RS content (Liu et al., 2022). While enzymatic methods increase RS content and change the structural properties of starch, it is important to note that the efficiency of these methods varies depending on the source of starch and the enzymes used. This variability may have an impact on the functional properties of RS in food applications as well as its health benefits.

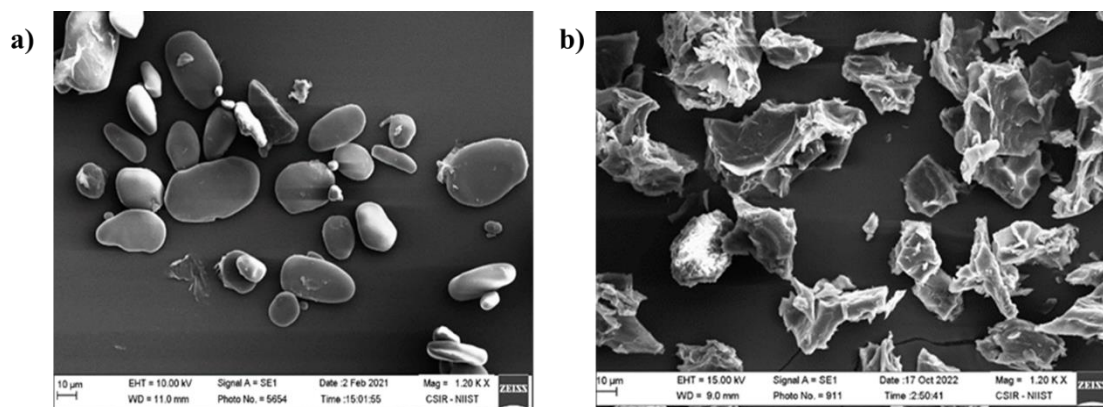


Figure 2.12. SEM images of starch and resistant starch

2.3.9.6 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is an important method for analysing thermal properties of starch, revealing information about its gelatinisation, retrogradation, and structural changes. DSC measures the thermal transitions of starch, such as gelatinisation and melting temperatures, which represent the crystalline structure and thermal durability (Fuentes et al., 2019). In the context of RS, DSC data are critical for understanding how enzymatic modification affects thermal stability (Fig.2.13), which is crucial for performance in high-temperature applications like spray drying, particularly for encapsulation.

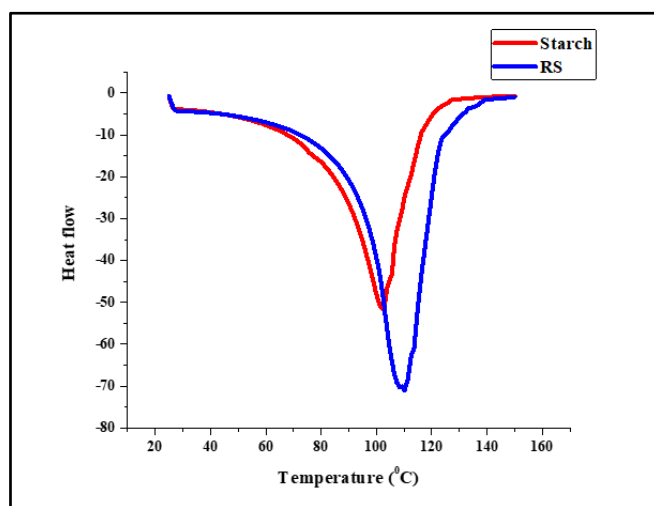


Figure 2.13. DSC thermogram of starch and resistant starch

The onset temperature (To), peak temperature (Tp), and conclusion temperature (Tc) are given in Table 2.6.

Table 2.6. DSC analysis of starch and resistant starch

Samples	To (°C)	Tp (°C)	Tc (°C)
Starch	40.17± 0.60	79.65± 0.49	116.57± 0.91
RS	78.21± 0.85	109.29± 0.64	128.23± 0.42

In native starch, the gelatinisation temperature is relatively low due to its less ordered structure. However, RS, especially when isolated using enzymatic methods, has a higher gelatinisation temperature and enthalpy values, indicating a more crystalline and thermally stable structure. This stability results from the enzymatic treatment, which selectively destroys the amorphous regions of the starch granule, thereby enriching the more organised crystalline regions. Gelatinisation is the process of breakdown of starch's intramolecular and intermolecular hydrogen bonds caused by an excess of heat and water. Consequently, water is irreversibly absorbed, causing the starch granules to swell. Amorphous regions allow water to enter easily, but crystalline regions only allow for increased temperature and excess water (A.-Q. Zhao et al., 2018). Thus, the proportion of crystalline areas is directly linked to the gelatinisation of starch granules, with higher crystallinity resulting in higher gelatinisation temperature (Zięba et al., 2011). The findings of DSC analysis correlate with earlier research, which found that enzymatically treated modified starch has better thermal properties than native starch (Sathyan & Nisha, 2022). Moreover, these findings have been supported by XRD studies, which show that enzyme hydrolysis increases the crystalline regions of RS, making it more resistant to gelatinisation. As a result, the RS obtained through enzymatic hydrolysis exhibited higher thermal resistance. The DSC results reveal that enzymatic treatment of starch yields resistant starch with improved thermal resistance, making it appropriate as a coating material for spray

drying, particularly in applications requiring high thermal stability and fast encapsulation. Thus, the DSC results reveal that enzymatic treatment of starch yields RS with improved thermal resistance, making it suitable as a wall material for spray drying, particularly in applications requiring high thermal stability and efficient encapsulation.

2.4 Conclusion

This study delved into the nutritional composition, starch, RS, phytochemicals, antioxidants, and cytotoxicity of ten raw banana cultivars at stage II maturity. While proximate composition remained comparable across varieties, distinctions in phenolic, flavonoid, and antioxidant potential were noted, reflecting variations in genomic and chemical constituents. LC-MS/MS analysis quantified significant polyphenols (shikimic acid, ferulic acid, epicatechin, and rutin), amino acids (phenylalanine, histidine, glutamine, lysine, and arginine), and vitamins (pyridoxine, ascorbic acid, and nicotinic acid) among cultivars. Unripe banana flour emerged as a notable source of RS, which can play an important role in contributing to gut health. Among the cultivars, PEYA, MA, and PALA showed the highest yield of RS. With the presence of bioactive phytochemicals and hypoglycemic properties enriched with RS, there is a great scope for the value addition of unripe bananas. The isolation and characterisation of starch and RS from distinct *Musa* species provide essential insights into selecting the best RS variant to be used as a wall material in spray drying applications. This study found considerable changes in the physical and functional features of RS between starch by comparing its thermal, structural, and morphological characteristics. RS isolated from the PALA cultivar exhibited a higher drying rate, higher thermal stability, increased crystalline content, and favorable surface morphology, making it suitable for encapsulating bioactive during spray drying. The enzymatic treatment of native starch significantly increased the RS crystalline regions, resulting in improved thermal resistance and a higher gelatinisation temperature. These characteristics are critical for maintaining encapsulation stability under the high temperatures associated with

spray drying. The zeta potential investigation also found that RS possess negative surface charge, making them more compatible with core material such as cationic compounds, particularly used in the food and pharmaceutical industries. The study further emphasises the potential of banana-derived RS as a natural and effective encapsulating material. Future studies could expand on these findings by investigating encapsulation trials with isolated PALA RS to better understand their encapsulation efficiency, and release characteristics, thereby optimising their use for a wide range of applications in food, pharmaceutical, and nutraceuticals.

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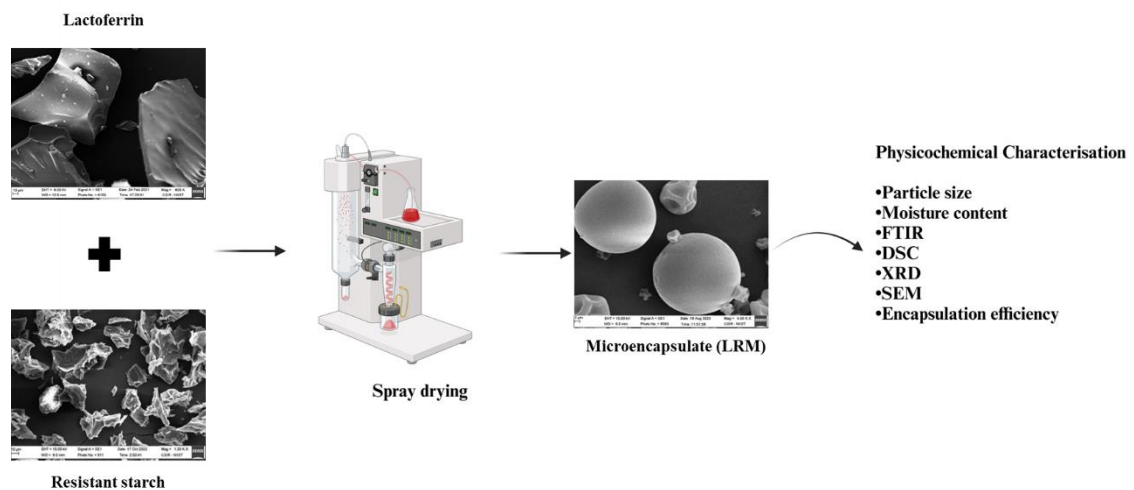
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Chapter 3A

Optimization and development of Lactoferrin-Resistant starch based microencapsulates (LRM)



3A.1 Introduction

Lactoferrin (LF), is an iron-binding glycoprotein present mainly in milk and also in secreted mammal fluids. LF has anti-inflammatory, immunomodulatory properties, antibacterial, antiviral, and anticancerous activity (Giansanti et al., 2016; Park & Nam, 2015). LF is frequently used as an oral supplement due to its low availability during development and aging. Since the neonatal gastrointestinal system is still developing, LF survives gastric digestion in newborns. However, in the adult stomach, enzymatic hydrolysis causes LF to be rapidly digested and does not reach the small intestine. Thus, it is essential to protect LF from the acidic environment of the stomach and the presence of proteolytic enzymes, make sure it reaches the targeted site (Onishi, 2011; Troost et al., 2002). Encapsulation of bioactive compounds has gained significant attention in recent years in the fields of food and pharmaceutical industry because it allows a variety of substances to be protected by embedding them in a protective matrix. This delivery system can generally improve the stability, bioavailability, and controlled release of sensitive molecules by protecting them during their passage through the digestive system and under unfavorable processing and storage circumstances (light, temperature, moisture, and oxygen) (Timilsena et al., 2020).

In the food, cosmetic, and pharmaceutical industries, microencapsulation is one of the most promising methods for protecting functional components. Several encapsulation techniques have been developed, including emulsion, ionic gelation, liposome, freeze drying, spray chilling and cooling, coacervation, and fluidized bed coating. However, there are several disadvantages to these techniques, including the effect of high temperatures on thermolabile compounds, the variability in particle size and shape that restricts bioavailability, and the high operating costs (Timilsena et al., 2020). Spray drying is preferred above all other approaches due to its cost-effectiveness, scalability, and ability to produce uniformly small particles, the final product is a dry formulation, and also suitable for heat-sensitive molecules appropriate

for applications requiring controlled release (F. Gibbs, 1999). The spray drying process begins with the atomisation of a solution into droplets via spraying, followed by the quick evaporation of the sprayed droplets into solid powder by hot air at a specific temperature and pressure, and finally particle collection at the cyclone. The selection of compatible biopolymers and appropriate techniques are crucial for obtaining high encapsulation efficiency and stability (Gharsallaoui et al., 2007). Commonly used agents include proteins (whey protein, casein), polysaccharides (maltodextrin, gum arabic, starch, and resistant starch), and lipids. These materials help to provide a protective barrier around the bioactive and reduce its sensitivity to external harsh environmental conditions (Cano-Chauca et al., 2005; Goula & Adamopoulos, 2005).

Resistant starch (RS), a modified form of starch can be exploited as an encapsulating material due to its low cost, high availability, and diverse functionality. Also, it is an effective encapsulation matrix because of its structural stability and resistance to enzymatic digestion in the upper gastrointestinal tract (Topping & Clifton, 2001). This resistance permits RS to enter the colon mainly intact, where it is fermented by the colonic microbiota to create short-chain fatty acids (SCFAs) including acetate, propionate, and butyrate, which are essential for gut health (Slavin, 2013). Utilising RS as an encapsulating agent prevents bioactive from premature degradation and creates a dual-function system that releases bioactive in the colon while promoting prebiotic effects. Therefore, RS which was isolated from the PALA cultivar was utilised as a wall material for the encapsulation of LF, as mentioned in the preceding chapter.

In this chapter, we aim to explore the optimisation, development, and characterisation of resistant starch-based microencapsulated lactoferrin for enhanced functionality and stability in the gastrointestinal system. The focus is on optimising encapsulation parameters and evaluating

key properties such as particle size, encapsulation efficiency, as well as structural and thermal properties of encapsulates to confirm the successful encapsulation of LF within wall material.

3A.2 Materials and Methods

3A.2.1 Materials

Himedia Pvt. Ltd. (Mumbai, India) supplied maltodextrin and pectin. Hydrochloric acid, acetonitrile, sodium citrate, and formic acid were procured from Merck Limited, Mumbai, India. Lactoferrin was gifted by MILEI GmbH, Morinaga Milk Group, Germany. All other reagents utilised in this study were of analytical grade.

3A.2.2 Methods

3A.2.2.1 Formulations of Wall Material Composites for Spray-Drying

Lactoferrin was taken as core, which is soluble in distilled water. Resistant starch (RS) maltodextrin (MD), and pectin at different proportions were used as the wall material for trials. Preliminary trials were conducted to determine the efficient LF, RS, and MD combination that produced the highest product yield. The optimally proportioned wall material components including RS and MD were prepared. The resistant starch-containing formulations were heated separately for 10 min at 75 °C. Using a magnetic stirrer, the resistant starch-based suspensions were agitated throughout the procedure and allowed to cool to 25 °C. Following that, the MD and LF mixtures were blended for 5 min at room temperature. A fixed core-to-wall ratio of 1:4 was chosen. The mixture was homogenised at room temperature (28 ± 2 °C) for 5 min at 10000 rpm using a rotor-stator homogeniser (T25 ULTRA-TURRAX digital; dispersion tool: S 25 N-8G; IKA India Private Limited, Bengaluru). The resultant suspension was immediately taken for spray drying.

3A.2.2.2 Spray-Dried Microencapsulation Process

A mini spray dryer (LABULTIMA, Process Technologies Pvt Ltd., Mumbai, India, Model LU 228 ADVANCED) was used to convert the suspension into encapsulated powder immediately as it was prepared. It was operating in a co-current setup. For atomisation, a two-fluid nozzle with a 1 mm opening diameter was employed. Spray drying was carried out at various inlet (110 and 120 °C) and outlet (55 and 60 °C) air temperatures. Trails were conducted at two distinct feed flow rates and aspirator rates and a constant air pressure of 1.5 kg/cm² was maintained. The spray dryer was operated under the following optimised conditions; constant air pressure (1.5 kg/cm²), feeding rate (1 mL/min), drying air flow rate (73 m³/h), and inlet and outlet temperatures (120 ± 2 °C and 60 ± 2 °C), respectively. Using a magnetic stirrer, all compositions were continuously stirred before being pumped into the spray drying chamber. The microcapsule powder was collected from the dryer's cyclone. The samples were stored in Duran bottles until further analysis.

3A.2.2.3 Characterization of spray-dried encapsulates

3A.2.2.3.1 Production yield

By comparing the mass percentage of the final product to the mass of all the solids measured in the feed solution, the production yield of the spray-drying process was calculated using the following formula (Eq. 1) and expressed as a percentage.

$$Yield (\%) = \frac{\text{Powder after spray drying (g)}}{\text{Solids in the feed solutions (g)}} \times 100 \quad (1)$$

3A.2.2.3.2 Microencapsulation efficiency

The encapsulation efficiency (EE) of LF encapsulated within RS-maltodextrin was determined using high-performance liquid chromatography (HPLC). EE is the percentage of the protein content that is retained within the resistant starch matrix after encapsulation, indicating the effectiveness of the encapsulation process. Briefly, the encapsulated sample was accurately weighed, then sodium citrate (1% w/v) was added. The sample was then stirred at 200 rpm for 1 h until complete dissolution of encapsulates. Add 0.02 M HCl followed by centrifugation at 10000 rpm for 5 min at 4 °C, the filtrate was then passed through a 0.22 µm membrane filter (Hosseini et al., 2014).

Using HPLC analysis, the amount of LF present in the supernatant was determined. It was performed on a Prominence HPLC system (Shimadzu, Japan). The UFLC system consists of an LC-20AD system controller, a C18 column (dimensions: 250 x 4.6 mm, 5 µm; Phenomenex Gemini), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µL volume, and a photodiode array (PDA) detector (SPD-M20A) (Arun et al., 2017). The HPLC analysis was carried out using an earlier methodology with some modifications (Yao et al., 2013). The mobile phase is an isocratic system containing 90% (v/v) acetonitrile with a pH 2 adjusted using formic acid in HPLC-grade distilled water. The sample injection volume was 10 µL, and the flow rate was set at 0.6 mL/min. The column was kept at 30 °C, and the eluted fractions were monitored at 236 nm. The peak corresponding to LF present in LRM was identified by comparing it with the retention time of the standard LF solutions. Data was acquired and analysed using the LC LabSolutions software. EE is evaluated from the following equation (Eq. 2).

$$EE (\%) = \frac{\text{Total amount of LF detected by HPLC}}{\text{Initial amount of LF}} \times 100 \quad (2)$$

3A.2.2.3.3 Moisture content

A moisture analyser (HC 103 Mettler Toledo, India) was used to determine the moisture content of LRM. The analyser was adjusted to 105 °C and 1 g LRM powder was added until the weight remained constant. The digital display of the moisture analyzer was used to record the moisture content value in percentage.

3A.2.2.3.4 Particle size

The Malvern Zetasizer (Zeta Nano-ZS; Malvern Instruments, UK), which operates based on dynamic light scattering (DLS), was used to determine the size of LRM (Comunian et al., 2020). The values are presented as an average of multiple scans.

3A.2.2.3.5 ATR-Fourier-Transform Infrared (FTIR) Spectroscopy

An FTIR-ATR (attenuated total reflection) spectrometer (Perkin Elmer, USA) equipped with an ATR accessory with a diamond crystal at a 45° incidence angle was used to record the Fourier-transform infrared (FTIR) spectroscopy of LRM, LF, and wall materials. Transmissions were measured at wave numbers ranging from 4000 to 400 cm⁻¹, which represent the average of multiple scans.

3A.2.2.3.6 X-Ray Diffraction (XRD)

Wide-angle X-ray diffraction (WAXD) measurements were recorded in the transmission mode using the XEUSS SAXS/WAXS systems from Xenocs. The analysis was performed using the powdered LRM, LF, and wall material samples. The Cu K α radiation had a wavelength of approximately 1.54 Å, the working voltage was 50 kV and the current was 0.6 mA. A Mar345 image plate was used to record the 2D patterns, and Fit2D software was used to analyse the data.

3A.2.2.3.7 Scanning Electron Microscopy (SEM)

A scanning electron microscope (Carl Zeiss EVO-18, Germany) and software called Digimizer Version 4.6.1 (Digimizer Version 4.6.1, Copyright © 2005-2016, MedCalc Software, Belgium) at 15 kV voltage accelerating beam was used to determine the morphology of the LRM and LF. Carbon tape was used to mount the prepared samples to an aluminum specimen holder. Before the microscopical analysis, a thin layer of gold was then vacuum-coated onto the sample using a gold/palladium sputter coater (SC7620, Emitech, Quorum Technologies Ltd, Kent, UK). The micrographs were taken with an 800–4000X magnification.

3A.2.2.3.8 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was used to assess the thermal behavior of LRM, LF, and wall materials. A differential scanning calorimeter (DSC Q2000, TA Instruments, USA) was used for DSC measurements following earlier research with slight modification (Tao et al., 2016). Briefly, aluminum sample pans that had been previously weighed were filled with 2 mg of samples after distilled water (6 µL) had been added. With a heating rate of 10 °C/min and in a nitrogen atmosphere, the samples were scanned between 10 and 100 °C. The temperature values that have been determined were the temperature at the onset (To), peak (Tp), and conclusion (Tc).

3A.2.2.3.9 Statistical analysis

GraphPad Prism 9.3.0 was used for statistical analysis, and the data were displayed as mean ± standard deviation (SD). Dunnett's multiple comparison test and a one-way ANOVA were used; significance was determined at $p < 0.05$.

3A.3 Results and Discussion

3A.3.1 Optimization and Production yield of encapsulate

Optimization of the spray drying process of LRM, varying core (LF) compositions and various wall material compositions (RS, MD, and pectin) were employed at a core-to-wall ratio (1:4). The total suspended solids (TSS) were set at 20% and 30%, with homogenization carried out at a constant speed of 10000 rpm for durations ranging from 5 to 15 min. The spray drying parameters included inlet temperatures of 110 °C and 120 °C, outlet temperatures of 55 °C and 60 °C, and flow rates of 1 mL/min and 1.2 mL/min (Table 3A.1).

The product yield of spray-dried LRM is an important factor in determining the efficiency and scalability of the encapsulating process. Product yield is often defined as the ratio of the actual amount of dried powder collected at the cyclone to the initial quantity of particles used for spray drying. A high yield is desirable since it indicates minimal component loss during processing and fast recovery of the encapsulated product. However, yield can be controlled by various factors, including inlet and outlet temperatures, feed rate, air flow rate, and the composition and viscosity of the feed (S. M. Jafari et al., 2008). Depending on process optimisation, a higher percentage of 40.23% cyclone yield was obtained when RS and MD were combined with a ratio of 1.25: 2.75 having a total suspended solid (TSS) content of 20% (Table 3A.1). Yield is also greatly impacted by feed viscosity and concentration. Higher RS concentrations in the feed can increase viscosity which can cause uneven atomisation and nozzle clogging, which lowers product yield. A typical feed concentration of 10% to 20% solids is often optimal for formulations because it provides enough viscosity for stable droplet formation without clogging problems (Desai & Jin Park, 2005).

Table 3A.1. Optimization trails for LRM fabrication

Trials	LF (gm)	RS (gm)	MD (gm)	Pectin (gm)	TSS (%)	Homogenization (rpm and min)	Inlet (°C)	Outlet (°C)	Flow rate (mL/min)	Cyclone yield (%)
1	2.1	2.4	1.2	0.3	30	10,000, 15	120	60	1	-
2	1.2	2.4	2.1	0.3	30	10,000, 15	120	60	1	-
3	1.5	1.2	3.1	0.2	30	10,000, 15	120	60	1	6.945
4	1.2	1.0	3.5	0.1	30	10,000, 15	120	60	1	4.425
5	0.8	2.4	0.8	-	20	10,000, 10	110	55	1.2	6.99
6	0.8	1.6	0.8	0.8	20	10,000, 10	110	55	1.2	13.23
7	0.8	0.8	1.7	0.5	20	10,000, 10	110	55	1.2	14.55
8	0.8	1.7	1.5	0.4	20	10,000, 10	110	55	1.2	4.155
9	1.2	1.4	1.2	0.2	20	10,000, 10	110	55	1.2	7.95
10	1.2	1.4	1.0	0.4	20	10,000, 10	110	55	1.2	18.63
11	0.8	1.0	2.1	0.1	20	10,000, 15	120	60	1	16.92
12	0.8	0.8	2.2	0.2	20	10,000, 10	120	60	1	10.34
13	0.8	1.0	2	0.2	20	10,000, 10	120	60	1	6.99
14	1.0	1.0	1.8	0.2	20	10,000, 7	120	60	1	4.10
15	0.8	1.0	2.2	-	20	10,000, 7	120	60	1	29.45
16	1.5	1.0	0.5	-	20	10,000, 7	120	60	1	10.88
17	1.0	2.0	1.0	-	20	10,000, 7	120	60	1	18.05
18	1.0	1.2	1.8	-	20	10,000, 5	120	60	1	16.70
19	0.8	1.2	2.0	-	20	10,000, 5	120	60	1	15.18
20	0.8	1.0	2.2	-	20	10,000, 5	120	60	1	40.23

Furthermore, using RS and MD in the encapsulating matrix increases yield by creating a strong matrix that is less prone to stickiness and agglomeration in the spray drying chamber. MD, in particular, functions as a drying aid, increasing the glass transition temperature of the particles and decreasing their tackiness while drying. This reduces wall deposition, contributing to increased yield. Studies have shown that the blend of starch and maltodextrin generally provides good results, by improving both encapsulation efficiency and yield of bioactive and probiotics (Costa et al., 2023; Esquivel-González et al., 2022; Kaul et al., 2022). When raising the inlet temperature typically promotes greater moisture removal and enhances yield by forming a stable outer crust on the particles, which helps prevent stickiness and adherence to

the dryer walls. However, higher temperatures have the potential to denature proteins, therefore the coating material can shield LF from the effects of high temperatures by withstanding the thermal destruction of LF in the feed solution (Murali et al., 2015). Thus, safe temperature control is essential to balance maximum yield with good product quality.

3A.3.2 Microencapsulation efficiency

Microencapsulation efficiency indicates how much of the bioactive component is successfully retained within the encapsulating matrix. LF quantification by HPLC is a reliable method, as the distinct peak corresponding to LF indicates its presence and quantity after encapsulation. The EE of the LRM produced by spray drying in the current experiment was determined to be around $73.14 \pm 2.54\%$; the chromatogram of LF and LRM is shown in Figure 3A.1. This higher EE indicates that the combination of RS and MD creates an effective barrier around LF, which reduces losses during the spray drying process and maintain the integrity of LF. MD's hydrophilic nature helps in encapsulation by creating a cohesive film around the LF, while RS provides additional stability because of its semi-crystalline structure, which promotes entrapment and prevents rapid diffusion of LF out of the wall matrix. Studies on the encapsulation of bioactive found that using polysaccharide blends increased EE, with hydrogen bonding identified as a crucial mechanism that increases the stability of encapsulation (Augustin et al., 2006; Díaz-Montes, 2023; Ruengdech & Siripatrawan, 2022).

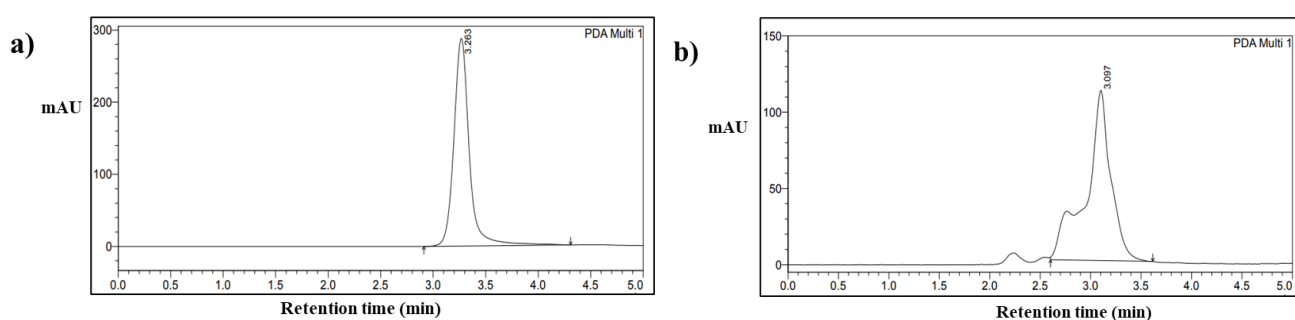


Figure 3A.1. HPLC chromatogram of (a) LF and (b) LRM

The EE of LF varies significantly depending on the encapsulation method and wall materials employed. Several studies have reported efficiencies ranging from 44.93% to 85%, showing the benefits of different polymers and approaches in preventing LF degradation in the gastrointestinal tract. The EE of LF that was observed is aligned with previous studies, gas-saturated solutions (PGSS) achieved an EE of $71 \pm 2\%$ with shellac as a coating medium (Ono et al., 2020). In another study, chitosan biopolymer achieved a 75% EE by using an ion gelation technique (Hedyeloo et al., 2023). Using bovine serum albumin and tannic acid multilayer microcapsules, for various shell configurations, EE of $76 \pm 6\%$ and $85 \pm 2\%$ were obtained, efficiently protecting LF from gastric digestion (Kilic et al., 2017). Moreover, lower EE of LF was observed in a recent study, alginate-based hydrogel bead used sodium alginate and gellan gum composites to achieve an EE of 44.93% to 56.59% (Cao et al., 2024). The increase or decrease in EE observed in studies could be linked to variations in matrix, processing conditions, and encapsulation techniques. The EE of LRM is consistent with earlier studies and supports the ability of RS as an effective encapsulating agent for bioactive substances. This high EE demonstrates the potential of LRM in prebiotic applications, where it enhances stability and sustained release of LF in targeted sites.

3A.3.3 Moisture content

Moisture content in encapsulated microcapsules affects drying efficiency, powder flowability, stickiness, and storage durability through glass transition and crystallisation. The glass transition temperature is lowered by maintaining a low moisture content because it prevents water from acting as a plasticiser (Nayak & Rastogi, 2010). Maintaining an optimal moisture level is particularly important for protecting the bioactivity of sensitive molecules like LF since higher moisture can lead to protein degradation, microbial growth, and reduced encapsulation efficacy gradually (S. M. Jafari et al., 2008). The moisture content of the spray-dried powder varies depending on the spray drying conditions and the composition of the encapsulating

matrix. The microencapsulated LF using RS and MD showed a lower moisture content of around $5.98 \pm 1.53\%$. Previous studies have shown that the moisture content of microparticles generated during spray-drying ranged between 6.06% to 6.60% (Papoutsis et al., 2018; Ramírez et al., 2015); the present outcomes are similar to these values. The moisture content of the microcapsule was significantly influenced by the outlet temperature as well as the cubic effects of the outlet temperature, showing that temperature has a significant impact on drying (Felix et al., 2017). Thus, a low-moisture content powder was created by raising the outlet temperature and the inlet temperature. Moreover, a protective matrix that promotes effective drying and results in a powder with lower moisture content is produced when RS is combined with MD, a well-known drying aid. Since MD is less hygroscopic than starch, it improves stability and decreases water absorption (Ozcelik & Kulozik, 2023; Tonon et al., 2008). Therefore, low moisture content improves the physical stability of the LRM by reducing clumping and increasing flowability, which is essential for effective handling (Gharsallaoui et al., 2007). However, very low moisture levels may cause powder brittleness, potentially affecting encapsulation stability during storage. Thus, moisture levels must be kept within an optimal range to maintain both stability and bioactivity.

3A.3.4 Particle size

In spray-dried encapsulates, particle size is a crucial factor that affects the product stability, solubility, and release profile of bioactive. The particle size of the LF microencapsulate was smaller, ranging about $2.296 \pm 0.58 \mu\text{m}$. In previous studies, a comparison of spray-drying microencapsulation of polyphenol bioactive using various natural fiber polymers as encapsulants revealed a size distribution between 0.5 and 15 microns; in another one, spray-dried microencapsulates with particle sizes below $5 \mu\text{m}$ were produced, which is consistent with the present findings (Mardani et al., 2024; Sun-Waterhouse et al., 2013). Also, numerous investigations found that encapsulates with larger particle sizes (Raj et al., 2024; Younesi et al.,

2023). The size of spray-dried particles is primarily determined by processing parameters such as atomisation pressure, size of the nozzle, inlet, and outlet temperatures, feed concentration, and flow rate. Higher atomisation pressure often produces smaller droplets, resulting in finer particles upon drying (Xie et al., 2010). Furthermore, the type of encapsulation matrix, reduced viscosity of the feed solution, and the nature core material, all have an impact on particle size. Smaller particles have a greater surface area-to-volume ratio, which can improve the solubility and bioavailability of the encapsulated bioactive in the digestive system (Gharsallaoui et al., 2007). This smaller-sized particle is enough to prevent early degradation and protect the encapsulated bioactive until it reaches the site of action (Díaz-Montes, 2023). Therefore, this RS-MD matrix ensures better functionality of the encapsulated product by enhanced particle size control and uniformity. Hence, fine, uniform particle sizes increase product stability and shelf life, making them ideal for commercial prebiotic or functional food formulations. In pharmaceutical applications, smaller particle size encapsulates are often excellent for controlled, sustained release, making this product beneficial for nutraceutical development.

3A.3.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared (FTIR) spectroscopy provides critical insight into molecular interactions, bonding, and structural integrity within the RS-MD matrix, especially in encapsulation systems involving LF. FTIR analysis is instrumental in identifying functional groups and bonding patterns, thus revealing whether the encapsulation process impacts the structural stability of LF and the encapsulating agents. Furthermore, it evaluates the compatibility and efficacy of the matrix components in protecting bioactive compounds (Pérez et al., 2002).

Figure 3A.2 illustrates the FTIR spectra of LRM, LF, and wall materials. For LF, the broad absorption band near 3285 cm^{-1} corresponds to O-H stretching vibration, highlighting its

hydrophilic nature. The characteristic amide I band at 1632 cm^{-1} , primarily due to C=O stretching vibrations in the protein backbone, and the amide II band at 1531 cm^{-1} , attributed to N-H bending and C-N stretching, are indicative of the protein's secondary structure. RS and MD display distinct peaks at approximately 3300 cm^{-1} (O-H stretching), 2900 cm^{-1} (C-H stretching), and $1000\text{--}1200\text{ cm}^{-1}$ (C-O-C stretching and C-O-H bending), demonstrating the characteristic bonds of the wall materials.

The LRM spectrum reveals a noticeable reduction in the intensity of LF's amide bands in the fingerprint region, indicating that LF is effectively encapsulated within the wall material. Additionally, the microcapsule spectra show similarities to the coating materials, confirming that the encapsulation matrix successfully protects the bioactive LF. The observed changes in the amide bands suggest the formation of hydrogen bonds and other molecular interactions between LF and the wall matrix, enhancing the integrity and stability of the encapsulate (Aprodu et al., 2020; Chen et al., 2022). These interactions provide a robust yet non-invasive matrix that preserves LF bioactivity and ensures controlled release, making it suitable for food and pharmaceutical applications.

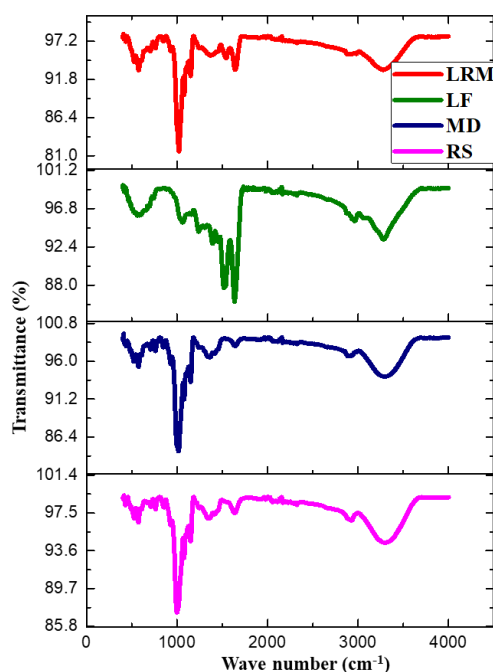


Figure 3A.2. FTIR analysis of LRM, LF, MD, and RS

3A.3.6 X-ray diffraction (XRD)

The X-ray diffraction (XRD) analysis of LRM and matrix components provides critical insights into the crystallinity, structural integrity, and molecular interactions within the encapsulation system. XRD is a valuable tool for assessing whether the encapsulation process influences the crystalline or amorphous forms of the materials, directly impacting the stability, solubility, and release profile of the encapsulated protein (Todica et al., 2016).

Figure 3A.3 illustrates the XRD diffractograms of LRM, LF, and wall materials (RS and MD). The LF diffractogram exhibits sharp peaks at 2θ values of 20.1° and 30.2° , which are indicative of its crystalline structure. In contrast, RS and MD show broad diffraction patterns between 15° and 25° , representing their predominantly amorphous nature. Upon encapsulation, the LRM diffractogram reveals significant reductions in the intensity of LF's characteristic peaks at 20.1° and 30.2° , with intensities decreasing by approximately 50%. These changes suggest that LF disrupts the crystalline structure of RS and transitions to a more amorphous state. The formation of an amorphous halo in the LRM matrix further supports this observation, indicating successful molecular interactions between LF and the encapsulating agents. The reduction in crystallinity enhances LF's bioavailability and improves its stability within the encapsulation system (Muhammad et al., 2021). Additionally, the interactions between LF and RS, as observed in the XRD patterns, imply the formation of hydrogen bonds or other molecular interactions that reinforce the matrix structure.

The amorphous nature of LRM correlates with better protection and thermal stability of LF, as encapsulated LF resists degradation more effectively than its free form. This aligns with findings from earlier studies. For instance, the incorporation of atorvastatin into starch cryoaerogels resulted in an amorphous structure with improved solubility (S. Jafari et al., 2023), while the encapsulation of aspirin in RS microspheres showed successful incorporation

with minimal alteration of the starch crystalline structure (Zhou et al., 2023). Overall, the findings confirm that LF encapsulated in the RS-MD matrix forms an amorphous structure with reduced crystallinity, offering enhanced stability, protection, and a controlled release profile. This low-crystallinity matrix is particularly suited for bioactive delivery in food and pharmaceutical applications.

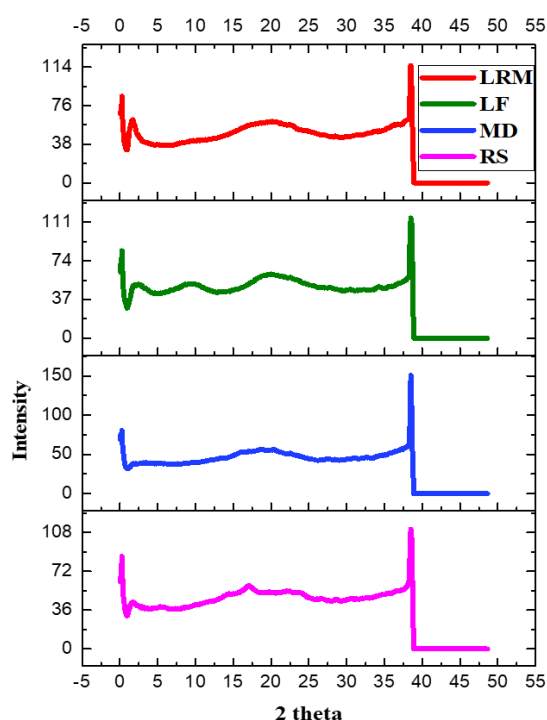


Figure 3A.3. XRD analysis of LRM, LF, MD, and RS

3A.3.7 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) gives a visual representation of the microcapsules, allowing for the assessment of their morphology, porosity, and encapsulation efficacy. Analysing these features is essential to understanding how well the encapsulation process has formed a protective barrier around the core material. SEM images of microcapsules and LF are shown in Figure 3A.4. The SEM analysis reveals a rhomboidal structure for LF, which is consistent with the prior report (Ono et al., 2020). SEM images of LRM show spherical particles, a characteristic morphology in spray-dried powders, which indicate effective atomisation and encapsulation during spray drying. These particles have smooth surfaces,

although some may display minor indentations or surface fissures caused by the droplet's rapid evaporation and shrinkage during spray drying (Rosenberg et al., 1985). The smoothness and sphericity of particles are crucial for increasing flowability and lowering surface area exposure, which can increase stability and reduce moisture absorption. Previous work has shown that the SEM image of LF encapsulated with chitosan revealed uniform, cubic forms, suggesting stability and efficient encapsulation against digestive enzymes (Hedylloo et al., 2023). Similar morphology has been seen in various spray-dried encapsulates containing bioactives, where sphericity is associated with maximum encapsulation efficiency (Lourengo et al., 2017; Ordoñez & Herrera, 2014; Raj et al., 2024). The current wall material produces smoother, uniform particles. Maltodextrin is known to help generate smoother surfaces by providing a more cohesive structure upon drying. Therefore, the morphology of LF microcapsules indicates a uniform and spherical shape required for stability and bioavailability. It is reported that the wall components, such as dextrin and octenylsuccinate starch, significantly impact microcapsule structure, affecting size, shape, and surface area (Porte et al., 2011). The present study demonstrated that this matrix provides smooth, spherical encapsulate, highlighting the effective encapsulation efficiency of LF when using RS-MD as the wall material.

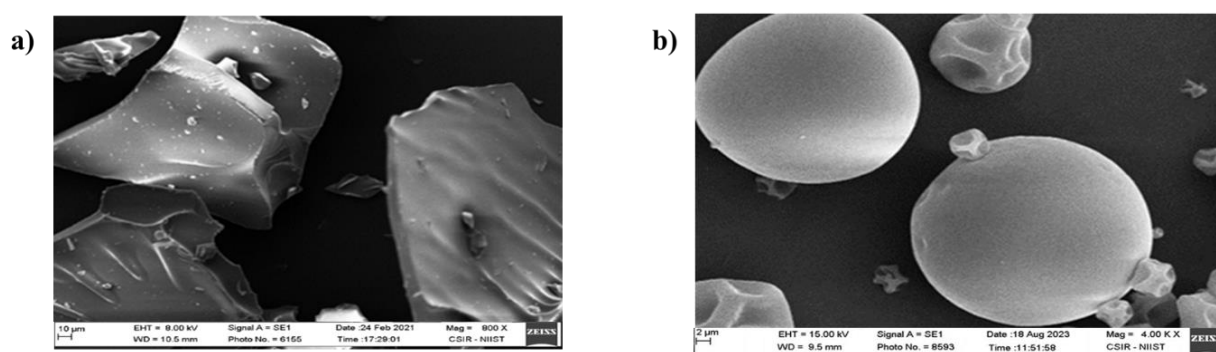


Figure 3A.4. SEM images of (a) LF and (b) LRM

3A.3.8 Differential Scanning Calorimetry (DSC)

The thermal stability and interactions between the core and wall components are shown by Differential Scanning Calorimetry (DSC) analysis. In DSC analysis the thermal behavior and

stability of encapsulates are interpreted using the three essential thermal parameters, onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c). Table 3A.2 provides the T_o , T_p , and T_c of LRM, LF, and RS.

Table 3A.2. DSC analysis of LRM, LF and RS

Samples	T_o (°C)	T_p (°C)	T_c (°C)
LRM	21.61± 0.51	46.23± 0.07	70.02± 0.40
LF	15.05± 0.08	36.49± 0.76	66.26± 0.09
RS	39.13± 0.62	55.30± 0.58	68.22± 0.06

The T_o denotes the initial detectable change in heat flow, which usually results from melting, and indicates the beginning of the transition of encapsulating material. The point of maximum heat absorption is known as the T_p , which correlates with the material's highest thermal transition rate. At the T_c , the thermal transition comes to an end, indicating that melting or degradation is complete. As shown in Figure 3A.5 the peak of LRM shows shifts and broadening in comparison to LF, showing the interaction between the wall and core materials that confirmed its successful encapsulation. Since RS is more crystalline, it has higher T_o , T_p , and T_c . DSC study demonstrates that the encapsulation procedure changes the thermal behavior of the drugs, indicating an effective interaction between the drug and RS (Ema et al., 2023). For instance, the gelatinisation temperature of RS falls when it is combined with drugs, indicating enhanced release characteristics of bioactive in the intestinal environment (Zhou et al., 2023).

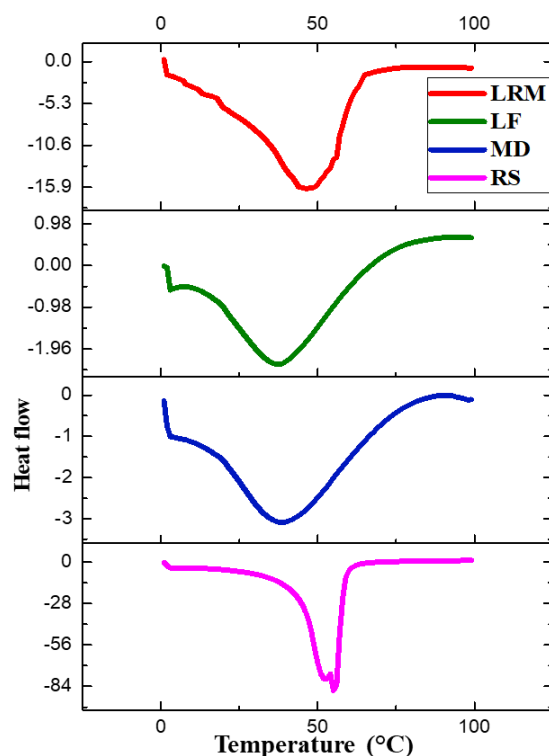


Figure 3A.5. DSC thermogram of LRM, LF, MD and RS

In the current study, LRM DSC thermograms show higher thermal values (21.61 ± 0.51 to 70.02 ± 0.40 °C) when compared to LF (15.05 ± 0.08 to 66.26 ± 0.09 °C), indicating good thermal protection and encapsulation. Prior studies on DSC analysis show that RS microcapsules have higher thermal stability, which is critical for maintaining the integrity of bioactive during processing and storage (Ashwar et al., 2018). Also, maize-resistant starch microcapsules demonstrated a glass transition temperature (T_g) range of 38.77-93.13 °C, showing excellent thermal stability (Muhammad et al., 2021). Combining RS with other polymers, such as pectin, and MD further enhances the efficiency of the drug delivery method (Sewnarain et al., 2024). Compared to alternative encapsulation matrices, the combination of RS and MD creates a thermally stable system that successfully protects lactoferrin's biological activity. These findings are consistent with prior research, which has shown that modified starches and MD matrices provide superior thermal protection, making them appropriate for

encapsulating sensitive bioactives such as proteins and flavor compounds under variable thermal conditions (Kang et al., 2019).

3A.4 Conclusion

In conclusion, the optimisation and development of LRM demonstrate an innovative method for protecting and delivering LF, a valuable bioactive protein with antibacterial, anti-inflammatory, and immunomodulatory properties. The current study developed an efficient, stable microencapsulate of LF by optimising the condition of the encapsulation process, such as spray-drying viz. temperatures, feed concentration, and atomization pressure. The resulting LRM system has a high encapsulation efficiency, which is crucial for ensuring that the maximum amount of LF reaches the gut intact. This encapsulate also makes use of RS's inherent prebiotic properties, which may synergistically support the growth and activity of beneficial gut microbiota, thereby potentially enhancing the overall therapeutic effect of LF.

The findings of this study set the way for future research, which will focus on *in vitro* and *in vivo* validation to demonstrate the prebiotic efficacy and bioactivity. Additionally, further exploration into the release profiles and interactions of encapsulated LF with the gut microbiota will be required to improve this system for optimal therapeutic uses. The development of LRM has great potential for enhancing the delivery of LF and other bioactive compounds, hence improving their health benefits in areas such as immunity, gut health, and beyond. With additional validation, LRM could become a highly important ingredient in the nutraceutical and functional food industries, providing increased bioavailability, targeted health advantages, and multifunctionality to promote overall wellness.

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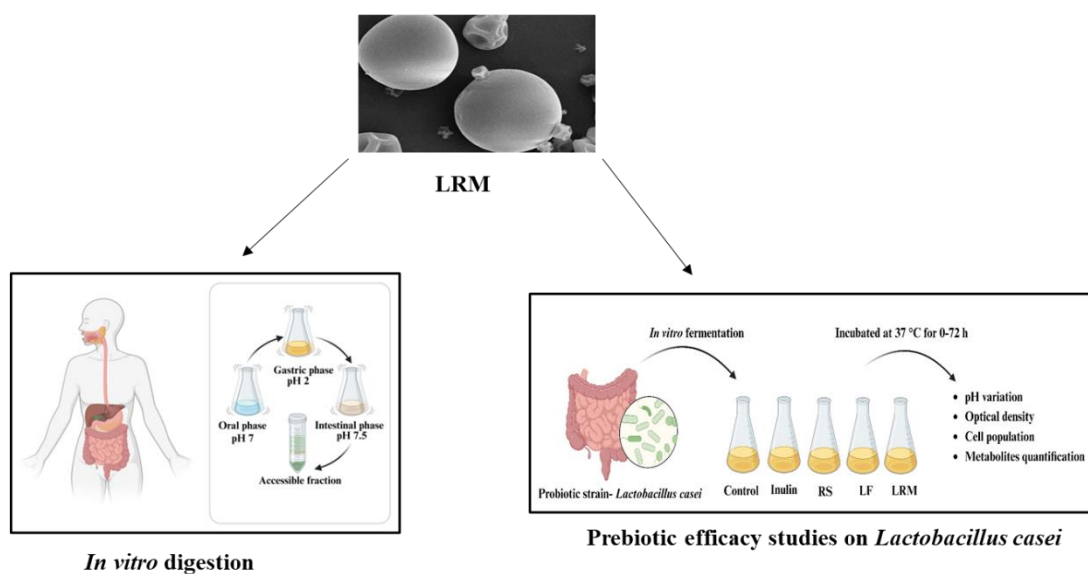
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Chapter 3B

*In vitro release kinetics and prebiotic efficacy of LRM on *Lactobacillus casei**



3B.1 Introduction

The term "gut microbiota" describes the diverse community of microorganisms that live in the gastrointestinal tracts of both humans and animals, including bacteria, viruses, and fungi (Dai et al., 2023; Ley et al., 2008). This microbial ecosystem is essential for maintaining host health, aiding in digestion, and regulating the immune system (Clemente et al., 2012). *Lactobacillus* and *Bifidobacterium* are common probiotic strains that are well-known for their functions in supporting intestinal homeostasis and boosting immunological responses (Gómez-Gallego & Salminen, 2017). These beneficial bacteria contribute to gut health by producing short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (Koh et al., 2016; Louis & Flint, 2017). Butyrate possesses anti-inflammatory activities by regulating the production of pro-inflammatory cytokines, maintains the integrity of the gut barrier, and is a key energy source for colonocytes (Canani et al., 2011). While absorbed into the bloodstream, propionate and acetate promote gluconeogenesis and lipogenesis in the liver, and support metabolic health by regulating appetite (Morrison & Preston, 2016).

Having a well-balanced gut microbiota is crucial for overall health because dysbiosis, or disruptions in its composition, has been linked to many diseases, such as type 2 diabetes, obesity, inflammatory bowel disease (IBD), and even neurological disorders (Birkeland et al., 2020; Meijer et al., 2022). Recent research suggests that the gut microbiota affects not only digestive health but also mental and cognitive function by communicating with distant organs through the gut-brain axis (Cryan et al., 2020). Dietary habits and lifestyle factors significantly influence gut microbiota composition and function; beneficial effects are often associated with diets rich in fiber (legumes, fruits, vegetables, and whole grains) and fermented foods (sauerkraut, tempeh, kimchi, and kefir) (Zmora et al., 2019).

Resistant starch (RS) is known to provide a prebiotic role in promoting gut health. The fermentation of RS by colonic microbiota results in the production of SCFAs. These SCFAs

are essential for retaining the integrity of the gut and regulating inflammation (Bindels et al., 2015). Numerous studies have shown that RS significantly increases the population of *Lactobacillus* and *Bifidobacterium* species, among other beneficial bacteria (Slavin, 2013). In particular, it has been demonstrated that RS promotes the growth of *Lactobacillus casei*, a probiotic strain with well-established benefits for immune modulation and gut barrier function (Fuentes-Zaragoza et al., 2011).

The glycoprotein LF is known for its anti-inflammatory, immunomodulatory, and antibacterial properties, it also has prebiotic potential. LF supplementation enhanced the gut microbiota diversity and reduced the risk of complications associated with microbiome dysbiosis. Furthermore, it has shown potential in reducing intestinal dysfunction caused by mycotoxins, confirming LF's function in maintaining gut homeostasis (D'Amico et al., 2022; Hu et al., 2022). However, its efficiency is limited due to degradation by enzymes in the stomach and small intestine (B. Li et al., 2024). As discussed in the previous chapter, LF is protected from degradation and its targeted release in the colon is facilitated by its encapsulation with RS. Previous research has demonstrated that the encapsulation with RS improves the stability of sensitive compounds, like curcumin, vitamins, and polyphenols by providing a controlled release that ensures bioactivity in the colon (Pires et al., 2022; Shi et al., 2021; Yao et al., 2023). Therefore, chapter 3B explores the *in vitro* release kinetics of LRM and assesses its prebiotic potential on the growth of *Lactobacillus casei*. These findings will provide valuable insights for the development of functional foods aimed at enhancing gut health and modulating immune responses.

3B.2 Materials and Method

3B.2.1 Materials

Lactobacillus casei (NCDC 17) was obtained as a freeze-dried culture from the National Dairy Research Institute (ICAR) in Karnal, Haryana, India. Himedia Laboratories Pvt. Ltd. (Mumbai, India) supplied the *Lactobacillus* MRS broth and agar powder. Sigma-Aldrich (St. Louis, Missouri, United States) provided the bile salt, α -amylase from *Aspergillus oryzae*, pepsin from the porcine gastric mucosa, and pancreatin from the porcine pancreas. Sodium chloride and sodium hydroxide were procured from Sisco Research Laboratories Pvt. Ltd. in Mumbai, India. Hydrochloric acid from Avantor™ Performance Material India Ltd. (Haryana, India) and calcium chloride from Spectrochem Pvt. Ltd. (Mumbai, India) were utilized. All other chemicals used were analytical grade.

3B.2.2 Methods

3B.2.2.1 *In vitro* release studies of resistant starch encapsulated lactoferrin

Three phases of *in vitro* release studies were conducted: simulated oral, gastric, and intestinal phases. The preparation of each phase was based on prior studies with slight modifications (Zhang et al., 2019). 25 μ l of 0.3 M NaCl and α -amylase (0.2 mg/mL) were mixed to prepare the oral phase. The ratio of the sample to the simulated salivary phase was 1:4. In 1000 mL of water, 2 g of NaCl and 7 mL of HCl were combined to produce the gastric phase. A 20 mL sample from the oral phase was combined with an equivalent quantity from the stomach phase. To this mixture, 0.064 g of pepsin was added and the pH was adjusted to 2.5. The mixture was incubated at 37 °C for 2 h at a speed of 130 rpm on a shaker. After incubation, 5 mL of the gastric phase mixture was transferred to a separate tube for examination. Equal amounts of intestinal phase (bile salts: 1.8 g, 1.5 mL of CaCl₂- 36.7 mg/mL, and NaCl-219.1 mg/mL) were

combined with 30 mL of gastric phase. This was followed by adding 1.8 g of pancreatin, adjusting the pH to 7, and then incubating the mixture for 2 h at 37 °C and 130 rpm in a shaker.

3B.2.2.2 Prebiotic potential of LRM

Lactobacillus casei, a probiotic strain, was cultivated in MRS broth for 24 h at 37 °C to investigate the prebiotic potential of LRM (Lactoferrin-Resistant starch microencapsulate). To attain an 80% transmittance at 600 nm, the culture was diluted, and 1 mL of the diluted culture was then inoculated into the media. Five treatment groups contained in the MRS broth were inoculated with the *Lactobacillus casei* culture: (i) Control, (ii) Positive Control-Inulin, (iii) RS: Resistant starch (iv) LF: Lactoferrin, and (v) LRM (Fig 3B.1). The control group had only media with the selected organisms, while groups ii, iii, iv, and v were supplemented with 1% inulin, RS, LF, and LRM, respectively. When the treatment groups were incubated at 37 °C, postbiotics containing short-chain fatty acids (SCFA) were produced (B et al., 2019). To assess the prebiotic potential of an LRM, postbiotics were collected, and centrifuged, and the supernatant was monitored for variations in OD, pH, colony count, and SCFA production at each time intervals (24 h, 48 h, and 72 h).

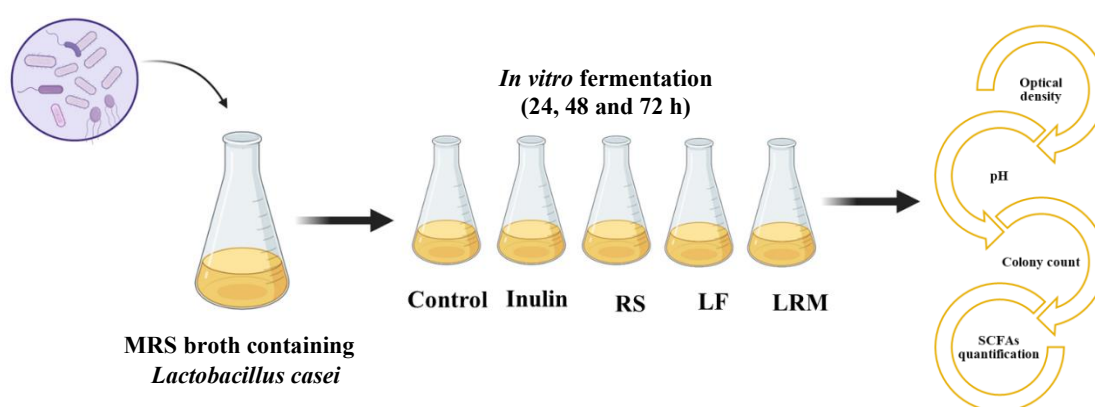


Figure 3B.1. *In vitro* fermentation method to analyse prebiotic efficacy of LRM

3B.2.2.2.1 Estimation of optical density of postbiotics

The optical density (OD) of the samples was measured at 600 nm using a UV spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) at particular time intervals of 24, 48, and 72 h.

3B.2.2.2.2 Estimation of pH of postbiotics

Using a pH meter (Oakton, pH 700, Benchtop meter, Oakton Instruments, USA), the pH of the samples was determined at certain intervals of 24, 48, and 72 h.

3B.2.2.2.3 Colony count

Colony counts were performed at time intervals of (24, 48, and 72 h) and represented as log colony formation units (log CFU/mL). Postbiotics from each group were serially diluted (1 mL) to achieve concentrations ranging from 10^{-1} to 10^{-10} . The pour plate method was then used to estimate the colony count using 1 mL from each sample. After the plates were incubated for 72 h at 37 °C, the colonies were counted (Clark, 1965).

3B.2.2.2.4 Assessment of short-chain fatty acid production of postbiotics

High-performance Liquid Chromatography (HPLC) was used to examine and quantify short-chain fatty acid (SCFA) synthesis at different time intervals (B et al., 2019). After centrifuging the fermentation medium for 10 min at 5000 rpm, the supernatant containing postbiotics was used to analyze SCFA. SCFAs, acetic acid, propionic acid, and butyric acid (standard), along with treatment groups (supernatant), were filtered through a 0.45 µm filter and 10 µL were injected into the system. A Prominence UFLC (Shimadzu, Japan) with an LC 20 AD system controller, Rezex RHM-Monosaccharide H⁺ (300×7.8 mm) column, column oven (CTO-20A), and diode array detector (SPD-M20A) was utilized for the analysis. Milli Q water was used as the mobile phase. The column was kept at a temperature of 50 °C, a flow rate 0.8 ml/min, and

with an injection volume of 10 μ L. SCFA was measured at 210 nm. The sample peaks were identified by comparing their retention times to those of the standard peaks. The software LC Lab Solution was employed for data collection and analysis.

3B.2.2.3 Statistical analysis

The data is shown as the mean $\pm$ standard deviations of triplicate experiments. Graph Pad Prism 9.3.0 software was used to analyse the results using a one-way ANOVA, and $P < 0.05$ was considered significant.

3B.3 Results and discussion

3B.3.1 *In vitro* release studies

LF encapsulated within RS was evaluated for its *in vitro* release in three distinct simulated fluids: Simulated Intestinal Fluid (SIF), Simulated Gastric Fluid (SGF), and Simulated Salivary Fluid (SSF). The findings revealed a gradual increase in the percentage of LF released when the encapsulated product passed through different environments, mimicking the gastrointestinal tract. In SSF, the release of LF was $10.94 \pm 0.019\%$, indicating minimal release in the salivary environment. The protection of the encapsulated LF during the oral phase depends on this limited release. The slow breakdown of RS in this neutral environment is consistent with prior studies, which have shown its stability in the mouth and early phases of digestion (Situ et al., 2014; Teshome et al., 2024). The moderate LF release in SGF is $31.35\% \pm 0.052\%$, suggesting that RS begins to break down in the stomach's acidic environment. As a result, some LF can become accessible while the rest remains protected until it reaches the intestine. In SIF, LF release was highest at $52.78\% \pm 0.239\%$, where it can help in the growth of gut microbiota that can further support associated health benefits. It was reported that liposomes derived from the rapeseed phospholipids system which showed 67–80% LF retention after the gastric phase, while intestinal digestion retained 16–35% (Vergara et al.,

2020). The particles from gas-saturated solutions showed good stability at low pH values and a significantly higher LF release over time, of about 70% indicating efficient stomach protection (Ono et al., 2020). In the present study, LRM exhibited better gastrointestinal stability.

Earlier studies have shown that RS nanoparticles work as a colon-specific delivery system for bioactive compounds, preventing digestion in the upper gastrointestinal tract while promoting prebiotic effects in the colon (C. Wang et al., 2022). Also, RS has been used as a delivery system for probiotics, which will enhance its encapsulation yield and survivability under simulated gastrointestinal conditions (Gani et al., 2023). This finding shows the potential of RS as a reliable encapsulating material for bioactive delivery systems. The release pattern seen in each phase demonstrates that it is suitable for applications requiring targeted and delayed releases. LF exhibits potential for usage in pharmaceuticals, nutraceuticals, and functional foods when it is encapsulated with RS, particularly in conditions where site-specific intestinal release is needed.

3B.3.2 Prebiotic efficacy of postbiotics

Prebiotics are indigestible food components, primarily dietary fibers, that support the growth and activity of probiotics, or beneficial gut microbiota, whereas postbiotics are bioactive compounds produced by probiotics during their growth. Each has a unique function in the symbiotic relationship with the human host and significantly contributes to the homeostasis of the body. The probiotic *Lactobacillus casei* was used to produce the fermentation metabolites. To assess the prebiotic potential of the LRM, the postbiotics were collected and evaluated for changes in OD, pH, colony count, and SCFA production at each time interval (24, 48, and 72 h).

3B.3.2.1 Change in optical density

At each time interval (0, 24, 48, and 72 h), media was collected and analysed for OD; if the sample has potential prebiotic activity, the growth of organisms and OD values will be higher, which is measured at 600 nm. The OD directly correlates with the bacterial mass in growth media (Arun et al., 2019). Figure 3B.2 shows the OD measurements for the different treatment groups.

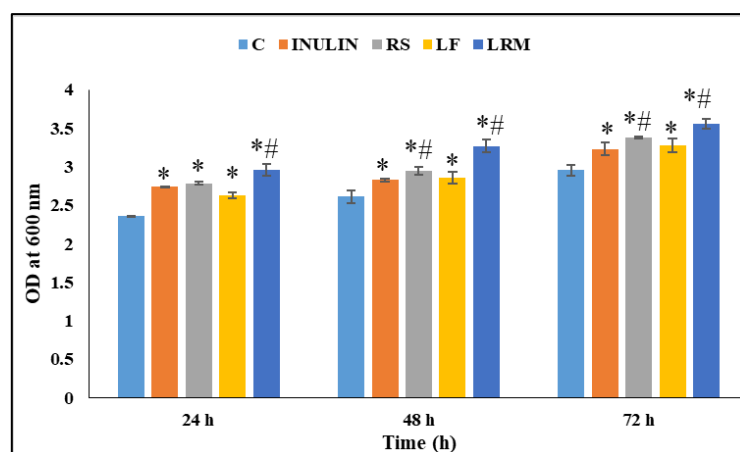


Figure 3B.2. Prebiotic potential of LRM determined by change in optical density for *Lactobacillus casei*. * $p \leq 0.05$ versus control. # $p \leq 0.05$ versus inulin. RS: Resistant starch; LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate.

As can be seen, as the incubation time increased, the OD value also increased for all groups. It is evident from the data that the RS encapsulated LF promotes the growth of probiotics, which raises the turbidity of the medium. Compared to the control and positive control groups, the OD of the RS, LF, and LRM-treated groups increased significantly. Among RS and LF, RS showed a higher OD value, ranging from 2.786 ± 0.017 to 3.375 ± 0.013 , whereas LF has shown 2.631 ± 0.038 to 3.282 ± 0.088 . Therefore, as the incubation period increased from 24 to 72 h, a synergistic effect was shown by LRM, which exhibited higher OD values ranging between 2.961 ± 0.02 and 3.557 ± 0.06 as compared to inulin, which varied from 2.74 ± 0.004 to 3.23 ± 0.08 . Studies show that LF significantly increases the growth of probiotics like *Lactobacillus* and *Bifidobacterium* when their growth is retarded, particularly at lower

temperatures (P.-W. Chen et al., 2017). As a prebiotic, LF improves gut health by modulating the gut microbiota, preventing harmful bacteria, while promoting beneficial strains, which can be indirectly assessed through increased optical density (Zhao et al., 2023).

RS is resistant to digestion in the small intestine, allowing it to reach the colon, where gut microbiota ferment it. When RS is fermented, SCFAs are produced. These SCFAs are good for gut health and can increase the optical density of microbial cultures (Khan et al., 2022; Polnaya et al., 2024). The studies show that modified arrowroot starch and resistant potato starch have prebiotic potential as they promote the growth of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium*, and *Roseburia*, indicated by an increase in optical density; further lowering the growth of *Shigella* and *E.coli* (McLaren & McLaren, 2021; Tarazi-Riess et al., 2024). Previous studies show that RS nanoparticles enhance the growth of *Lactiplantibacillus plantarum* resulting in increased viable cell counts (C. Wang et al., 2022). Also, the OD was significantly increased by RS from purple yam, indicating the enhanced proliferation of *Bifidobacterium adolescentis* (T. Li et al., 2018). Earlier studies have shown that certain starch and protein combinations can promote the growth of beneficial microbes while inhibiting the growth of non-beneficial ones, suggesting a potential for dietary manipulation to promote gut health. In addition to microbial diversity, protein-starch interactions influence metabolic processes like lipid metabolism (K. Wang et al., 2022). According to the study, RS altered the colonic environment by increasing the production of SCFAs and reducing the generation of toxic protein fermentation products, which may synergistically influence microbial growth and reduce the risk of colon cancer (Le Leu et al., 2007). Consistent with previous studies, the current findings confirmed that LRM exhibited a synergistic effect, promoting the growth of probiotics than RS or LF individually, as evidenced by the change in their OD. An increase in OD indicates that LRM functions as a potent prebiotic, stimulating the growth of beneficial

bacteria. This displays the combined potential activity of LRM to improve gut health by promoting the proliferation and viability of probiotics.

3B.3.2.2 Determination of colony count

The colony count method was the most reliable and preferred method for the enumeration of microorganisms. Bacteria can be quantified by growing them on an agar plate following serial dilution and calculating the number of colony-forming units (CFU) (Davis, 2014). The pour plate method was used to culture microorganisms and analysed colony forming units.. Figure 3B.3 shows the colony counts of the various treatment groups, displayed as log values.

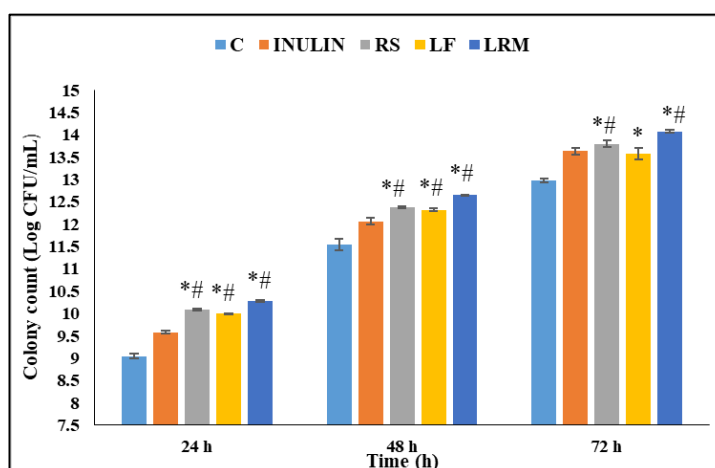


Figure 3B.3. Prebiotic potential of LRM determined by colony count by pour plate method for *Lactobacillus casei*. * $p \leq 0.05$ versus control. # $p \leq 0.05$ versus inulin. RS: Resistant starch; LF: Lactoferrin; LRM: Lactoferrin-resistant starch microencapsulate.

As the incubation period increased from 24 to 72 h, the number of colonies increased in each experimental group. After 72 h of incubation, when compared to inulin (9.57 ± 0.032 to 13.63 ± 0.071 log CFU/mL), RS, LF, and LRM treatment groups displayed a significant increase in the number of colonies. Comparing RS and LF, RS (10.08 ± 0.017 to 13.79 ± 0.074 log CFU/mL) showed a higher colony count than LF (9.99 ± 0.015 to 13.57 ± 0.124 log CFU/mL). LRM exhibited the highest colony counts among all treatment groups, ranging from $10.28 \pm$

0.02 to 14.07 ± 0.03 log CFU/mL, indicating its strong prebiotic potential. The higher CFU counts observed with LRM compared to LF alone suggest that the synergistic effect of both RS and LF plays a key role in supporting probiotic growth. The RS in LRM served as a stable carbohydrate source, promoting fermentation and the production of SCFAs, which stimulate probiotic development. These results are in line with other research that shows RS can enhance probiotic development by serving as a fermentable substrate (Ríos-Covián et al., 2016). The LRM group showed significantly more colonies than the control group at the end of the incubation period. This is due to the synergistic effect of LF and RS. LF supports the growth of probiotics by increasing the availability of iron, a nutrient required for microbial growth (Vega-Bautista et al., 2019). Moreover, certain probiotic strains, such as *B.bifidum* and *L. acidophilus*, have LF-binding proteins that facilitate their proliferation when LF is present (Kim et al., 2004). In addition to being prebiotic, LF has antibacterial activity that inhibits pathogenic species while favoring the growth of beneficial bacteria such as *Lactobacillus casei* (H.-L. Chen et al., 2010; Inay et al., 2012). Studies have shown that such dual-action encapsulates containing prebiotic and antibacterial components can effectively enhance probiotic count (Q. Chen et al., 2017). It has also been demonstrated that RS protects probiotics from harsh gastric conditions, promoting their survival and growth in the gut (Ashwar et al., 2018; Gani et al., 2023). Previous reports suggest that the presence of starch-protein complexes in the culture media can affect microbial growth. Moreover, modified starch-protein interactions may enhance microbial habitat stability and improve nutrient availability which may lead to increased colony counts (Soukoulis et al., 2016; Ying et al., 2013). The findings of the current study align with previous results, as the increased colony counts indicate that the combination of LF and RS in LRM effectively stimulates probiotic growth, suggesting its potential as a valuable prebiotic for enhancing gut health.

3B.3.2.3 Change in pH

Short-chain fatty acids are produced when samples are fermented by the probiotic strain *Lactobacillus casei*. During the fermentation process, a gradual reduction in pH was observed, signifying the synthesis of SCFAs such as butyric, lactic, acetic, and propionic acids. Probiotic fermentation produces these acids as metabolic byproducts, which help create an acidic environment that supports the growth of beneficial gut microbiota (Vinelli et al., 2022). Thus, as shown in Fig. 3B.4, we calculated pH at various time intervals (24, 48, and 72 h). The graph makes it evident that as the incubation period increases, the pH value falls for all treatment groups. As the incubation period increased from 24 to 72 h, the LRM groups within the treatment groups displayed a lower pH of 4.13 ± 0.01 to 3.43 ± 0.007 . When comparing the positive control and control groups, RS and LF exhibited almost similar pH values, whereas the pH of the LRM treatment groups dropped significantly, showing the combined effects of both compounds.

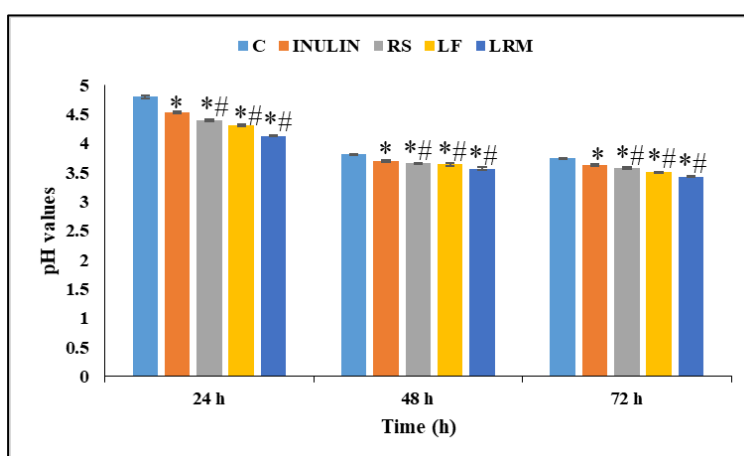


Figure 3B.4. Prebiotic potential of LRM determined by change in pH for *Lactobacillus casei*. * $p \leq 0.05$ versus control. # $p \leq 0.05$ versus inulin. RS: Resistant starch; LF: Lactoferrin; LRM: Lactoferrin-resistant starch microencapsulate.

The findings show that the samples considerably increase the proliferation of probiotic bacteria, which is linked to the synthesis of SCFA. The prior studies suggest that LF increases the production of SCFA, which may reduce the pH of the gut, potentially contributing to its

prebiotic effect and modulating the gut microbiota (Connell et al., 2021; Zhao et al., 2023). Similar research has shown that when gut microbiota ferment RS, the pH drops as a result of the generation of SCFA, promoting the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* while inhibiting pathogenic species (Brouns et al., 2002; Slavin, 2013). The prebiotic potential of LRM is confirmed by the observed pH drop that occurs during probiotic fermentation. The production of SCFA, which reduces pH, and promotes the growth of beneficial microbes. Thus, this study indicates that LRM has a promising prebiotic potential as it effectively supports a healthy gut environment.

3B.3.2.4 Quantification of short-chain fatty acid production

The quantification of short-chain fatty acids (SCFAs) produced by prebiotics is critical to understanding their impact in gut health and metabolism. Various methods have been developed to accurately quantify SCFAs in biological samples, where they are formed predominantly through the fermentation of dietary fibers and prebiotics by gut bacteria (Morrison & Preston, 2016). Acetate, propionate, and butyrate are the three primary SCFAs, accounting for 90% of SCFAs produced by gut bacteria, which play a unique role in gut health. The action of SCFAs is not limited to the GI tract; it also contributes significantly to the bidirectional communication between the GI tract and the central nervous system, which led to a concept of the microbiota-gut-brain axis (Szentirmai et al., 2019; van de Wouw et al., 2018). Probiotics produce SCFAs through the metabolism of undigested carbohydrates, which was analyzed by HPLC. Figure 3B.5 displays the HPLC chromatogram of the standard SCFA, which includes acetic acid (AA), propionic acid (PA), and butyric acid (BA). SCFA retention times for AA, PA, and BA were determined to be 10.593, 12.995, and 14.826 min, respectively. The production of SCFAs by various treatment groups at different time intervals was detected

and quantified. The results obtained show that when the incubation period extended from 24 to 72 h, the production of SCFA increased in all treatment groups.

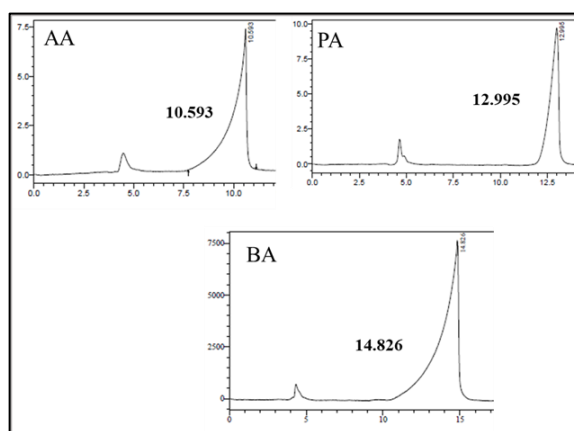


Figure 3B.5. HPLC chromatograms of standard short-chain fatty acids and their retention time. AA: Acetic acid, PA: Propionic acid, BA: Butyric acid.

The postbiotics produced by a LRM incubated for 72 h at 37 °C contain more SCFA. The graphical representation of the SCFA, acetic acid, propionic acid, and butyric acid produced by incubating various groups with *Lactobacillus casei* for 24 to 72 h is shown in Fig. 3B.6. All treatment groups produced higher levels of SCFAs compared to the control. Between LF, inulin, and RS, RS generated more SCFAs than others. The synergistic effect of both LF and RS was evident in LRM, when incubated at 37 °C which exhibited the highest concentrations of acetic acid (22.47 mg/mL), followed by propionic acid (19.03 mg/mL), and butyric acid (1.14 mg/mL) was used for further study. Quantification of SCFAs shows that acetate production is higher in all the treatment groups, followed by propionic and butyric acid. This SCFA distribution pattern is supported by research, which shows that acetate is the most prevalent SCFA in a variety of dietary fiber fermentation studies. Studies found that acetate was consistently the predominant SCFA generated involving RS and other fermentable fibers. This distribution is primarily due to gut microbe's metabolic pathways, in which many species, including *Bacteroidetes* predominantly produce acetate while fewer species specialise in

propionate and butyrate production (Besten et al., 2013; Macfarlane & Macfarlane, 2012). These findings validated the LRM prebiotic efficacy.

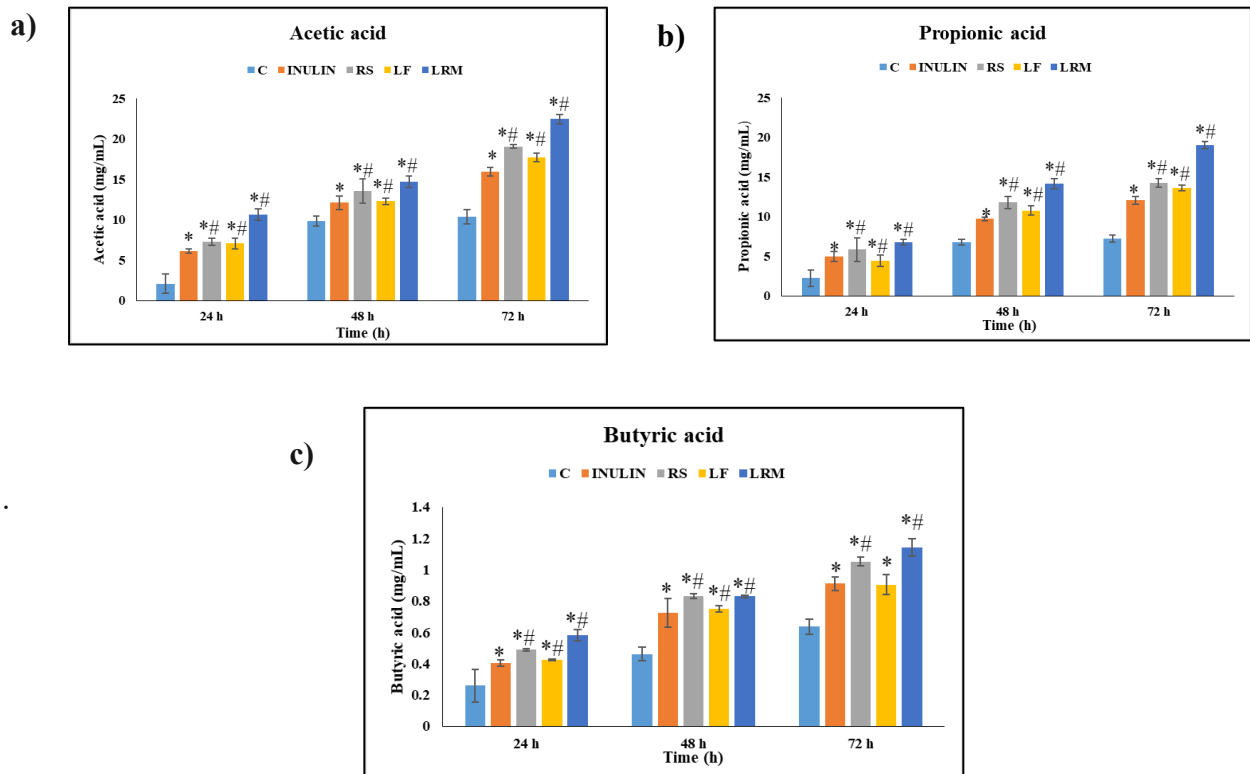


Figure 3B.6. Estimation of short-chain fatty acids (a) Acetic acid (b) Propionic acid (c) butyric acid by HPLC. * $p \leq 0.05$ versus control. # $p \leq 0.05$ versus inulin. RS: Resistant starch; LF: Lactoferrin; LRM: Lactoferrin-resistant starch microencapsulate.

Previous research has reported the prebiotic potential of RS and LF on gut microbiota. RS acts as a prebiotic, that resists digestion in the upper gastrointestinal system, instead reaching the colon where bacteria such as *Bacteroides*, *Bifidobacterium*, and *Lactobacillus* species ferment it. This microbe degrades the complex carbohydrate structure of RS, producing SCFAs. These fatty acids are essential for colonic health because they keep the colon's pH acidic, which prevents the growth of harmful microbes and creates a favorable environment for good bacteria. The synthesis of SCFAs contributes to gut health and metabolic processes (Bird et al., 2000; Topping & Clifton, 2001). Prior research has revealed that different RS types, including those made from sago starch and high-amylose maize starch, exhibit varying fermentation rates and SCFA yields (Polnaya et al., 2024). When compared to other starch varieties, RS from

starch-ferulic acid complexes produced the highest levels of SCFA (167.4 mM) (Liu et al., 2023). It has also been reported that RS supplementation alters the balance of the gut microbiota by increasing beneficial bacterial populations and reducing the Firmicutes/Bacteroidetes ratio (Sobh et al., 2022).

LF plays a significant role in the generation of SCFAs through its modulation of the gut microbiota, particularly Firmicutes, and Bacteroidetes that produce butyrate and acetate, respectively (Wangko, 2020). Specific strains of lactic acid bacteria (LAB) metabolize oligosaccharides into SCFAs during fermentation, with LF potentially enhancing this process (Maryati et al., 2021). Research indicates that LF enhances the formation of SCFA, is associated with reduced inflammatory cytokines, and induces regulatory immune cells, which contributes to overall health (Connell et al., 2021). These findings thus reinforce that LRM ensures the formation of SCFA in the colon by acting as a prebiotic substrate. Its unique combination of LF and RS takes on the benefits of both components, LF's prebiotic activity, and immunomodulatory and anti-inflammatory properties, while RS's prebiotic efficacy. These synergistic effects improve intestinal health and maintain a healthy microbiome.

3B.4 Conclusion

Lactoferrin-Resistant Starch Microencapsulate's (LRM) *in vitro* digestion and prebiotic efficacy study provides valuable insights into its potential as a functional food ingredient. When lactoferrin is encapsulated in RS, a novel protective matrix is created that maintains LF bioactivity while allowing the RS component to reach the colon intact, where it can undergo microbial fermentation. The *in vitro* digestion results showed that LRM remained stable under simulated gastric and small intestinal conditions. It also ensured that the LRM reached the large intestine, demonstrating its suitability for controlled delivery of bioactive to the lower gastrointestinal tract. The prebiotic potential of LRM was further validated in terms of

increased optical density, colony count, and decreased pH; the HPLC quantification of SCFA further confirmed its potential.

When LRM entered the colon, the gut microbiota digested it, producing a significant amount of acetate along with moderate amounts of propionate and butyrate. The study confirmed the prebiotic potential of LRM by SCFA production which is associated with the formation of a favorable gut environment, that reduces colonic pH thereby promoting the growth of beneficial bacteria such as *Lactobacillus casei*, which contributes to a balanced microbiota and promotes many health benefits. In conclusion, LRM has significant potential for supporting gut health due to its unique encapsulation of LF within RS. The stability of LRM during stomach and intestinal transit, combined with excellent fermentation by gut microbiota, makes it a promising candidate for functional food applications. The production of SCFAs, together with the controlled release of LF, indicates the role of LRM in promoting the growth of beneficial microbiota, maintaining a balanced microbiome, and supporting immune health. In light of this, we have decided to conduct further investigations to assess the potential of the postbiotics for the effective management of inflammation. The next chapter, chapter 4, will address this topic.

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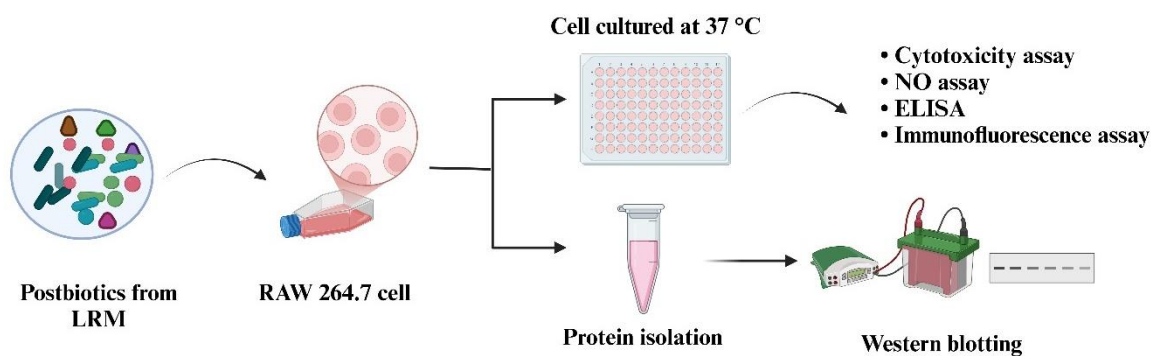
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Chapter 4

Exploration of anti-inflammatory potential of postbiotics from LRM on RAW 264.7 cell lines



4.1 Introduction

Inflammation is the response of the immune system present in the body to harmful stimuli such as pathogens, damaged cells, or irritants. It serves as a protective mechanism and initiates the healing process. While inflammation is a normal and helpful reaction, uncontrolled or chronic inflammation can be harmful, contributing to the development of a wide range of diseases such as cardiovascular disorders, diabetes, renal diseases, cancer, and autoimmune diseases (Chen et al., 2017). Excessive inflammation causes overexpression of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β), increased nitric oxide (NO) production, and changes in cellular morphology and blood markers (Hotamisligil, 2017). Antibiotics, immunosuppressants, and non-steroidal anti-inflammatory medicines (NSAIDs) are currently the most commonly used medications to treat inflammation. However, these drugs have severe side effects such as nephrotoxicity, digestive tract damage, and decreased immune cells, which restricts their broad application for prophylactic or chronic purposes (Artym & Zimecki, 2023; Bindu et al., 2020). As a result, the development of safe and easily absorbed natural anti-inflammatory drugs, and the prevention and management of inflammatory responses are gaining more attention as a strategic approach, this area has become a key focus of research, as using antibiotics to treat inflammation caused by bacterial infections can lead to antibiotic resistance, making the infection more difficult to treat (Lin et al., 2024).

Proteins from various food sources are vital for a wide range of functions and meet the essential nutritional needs for human growth and development. Peptides derived from both animal and plant proteins have the potential to provide beneficial biological effects. Lactoferrin (LF), a bioactive glycoprotein present in various body secretions including milk, saliva, and tears, has been shown to have broad-spectrum immunomodulatory, anti-inflammatory, and antibacterial effects (González-Chávez et al., 2009). Research reports have shown that lactoferrin regulates the host immune system to reduce the generation of pro-inflammatory cytokines such as tumor

necrosis factor-alpha (TNF- α) and interleukins (Cutone et al., 2020; Pan et al., 2021; Togawa et al., 2002). However, lactoferrin's direct application faces challenges such as instability in gastrointestinal conditions and susceptibility to degradation by gastric enzymes (Troost et al., 2001; Yao et al., 2023). The stability, bioavailability, and targeted delivery of lactoferrin have been improved using microencapsulation techniques that use certain wall materials to overcome these limitations. Microencapsulation is one of the most promising methods for protecting functional components in pharmaceutical, cosmetic, and culinary applications. LF can be encapsulated using a variety of encapsulation strategies, such as multilayer microcapsules made by layer-by-layer assembly of tannic acid, bovine serum albumin, or calcium-alginate nanospheres made by emulsification and shellac microcapsules using particles from gas-saturated solutions (Kilic et al., 2017; Ono et al., 2020; Raei et al., 2015). Although there are certain limitations, including premature release into the stomach, considerably reduced LF absorption. Thus, for optimal delivery throughout the gastrointestinal tract, a more efficient encapsulation method must be developed that not only prevents enzymatic degradation of LF but also allows sustained release to the targeted site.

Researchers and manufacturers in the food, cosmetics, and pharmaceutical sectors are focusing on the application and development of biopolymer-based particle systems aiming to create safer and healthier nutritious foods and other commodities (Bengoechea et al., 2011; Cohen Benshitrit et al., 2012; Matalanis et al., 2011). There has been an increased interest in using dietary proteins and carbohydrates because all of the elements are usually regarded as safe and provide an approach to benefit human health while providing bioactive substances like nutraceuticals (Chen et al., 2006; Jones & McClements, 2010; Matalanis et al., 2011). Resistant starch (RS), a non-digestible carbohydrate that does not digest in the upper gastrointestinal tract, is fermented by gut microbiota in the colon, producing functional bioactive compounds termed postbiotics. Postbiotics therefore contain a wide range of

constituents, including short-chain fatty acids (SCFAs), metabolites, microbial cell fractions, extracellular polysaccharides (EPS), functional proteins, teichoic acid, cell lysates, and peptidoglycan-derived muropeptides. These compounds either directly or indirectly mediate favorable biological actions (Gurunathan et al., 2024; Wegh et al., 2019). A synbiotic, which is generally thought to be a synergistic combination of probiotics and prebiotics, benefits the host by enhancing the colonisation and survival of living beneficial microbes in the gastrointestinal system, which further promotes the formation of postbiotics (Gurry, 2017). Postbiotics are non-viable microbial cells, their metabolites, and cellular components that provide health benefits to the host (Cicenia et al., 2014). Among the postbiotics, SCFAs such as butyrate, acetate, and propionate are important in terms of their beneficial activities (Zaman & Sarbini, 2016). These fatty acid levels in the blood and GI tract may prevent diseases like cancer, diabetes, and IBD. SCFAs have been extensively investigated for their function in modulating immune responses, demonstrating their anti-inflammatory benefits by inhibiting nuclear factor kappa-light chain-enhancer of activated B cells (NF- κ B) signaling pathway, a vital pathway in inflammation (Vinolo, Rodrigues, Nachbar, et al., 2011).

The global rise in chronic inflammatory diseases has led to interest in the prevention and management strategies that can reduce inflammation through natural and biologically active compounds (Zhang et al., 2023). In this context, postbiotics have emerged as promising agents for their potential health benefits, especially in immune modulation and anti-inflammatory actions (Aguilar-Toalá et al., 2018). The preceding chapters demonstrated that LRM has promising prebiotic potential and creates significant SCFAs. The current study aims to investigate the anti-inflammatory potential of postbiotics produced from lactoferrin-resistant starch microencapsulate (LRM) on RAW 264.7 murine macrophage cells, a well-established model for assessing inflammatory responses *in vitro*.

4.2 Materials and Method

4.2.1 Materials

Himedia Pvt. Ltd. (Mumbai, India) supplied Dulbecco's Modified Eagle Medium (DMEM), American Type Culture Collection (ATCC), Trypsin-EDTA, Antibiotic-Antimycotic, Foetal Bovine Serum (FBS), bovine serum albumin (BSA), and skimmed milk. Sigma-Aldrich (USA) provided the 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), lipopolysaccharide (LPS), Radioimmuno Precipitation Assay buffer (RIPA buffer), TRIzol phosphate-buffered saline (PBS), protease inhibitor, griess reagent, indomethacin, and methanol for this study. TNF- α , IL-6, and IL-10 ELISA kits were purchased from BD Biosciences (USA). Thermo Scientific (Rockford, USA) provided the BCA protein assay kit and ECL substrate kit. The PVDF membrane was purchased from Immobilon P™ (Millipore 154 R, USA). Monoclonal antibodies of β -actin, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF- κ B), phosphorylated nuclear factor-kappa B (p-NF- κ B), stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), phosphorylated stress-activated protein kinase/Jun N-terminal kinase (p-SAPK/JNK), p38 mitogen-activated protein kinase (p38-MAPK), phosphorylated p38 mitogen-activated protein kinase (p-p38-MAPK), extracellular signal-regulated kinase (ERK), phosphorylated extracellular signal-regulated kinase (p-ERK) and HRP-linked anti-rabbit IgG were from Cell Signalling Technology (Denver, U.S.A). All chemicals utilised were analytical grade and all solvents used were HPLC-grade.

4.2.2 Methods

4.2.2.1 Cell culture

RAW-264.7 cells, a BALB/c-derived murine macrophage cell line, were obtained from the National Centre for Cell Sciences (NCCS) in Pune, India. The cells were grown in DMEM

with 10% FBS and 100 units/mL of penicillin-streptomycin mix in a humid incubator with 5% CO₂ at 37 °C. Cells were pretreated with postbiotics from LRM for 1 h, then stimulated with LPS (1 µg/mL) for 24 h.

4.2.2.2 MTT Assay

The noncytotoxic concentration in RAW 264.7 was determined by a 24 h MTT assay (Masters, 2000). RAW 264.7 cells were cultivated in a 96-well plate at a density of 1×10^4 cells/well. After reaching 70% confluence, the cells were treated with different concentrations of postbiotics (2.5 µL/mL, 5 µL/mL, 7.5 µL/mL, 10 µL/mL, and 20 µL/mL). 1 mL of LRM postbiotics (LRMP) contains 22.47 mg/mL of acetic acid, 19.03 mg/mL of propionic acid, and 1.14 mg/mL of butyric acid. As a control, cells treated with a medium devoid of postbiotics were used. After 24 h of pretreatment, the medium was decanted, washed, and then, 100 µL of MTT (0.5 mg/mL) suspended in plain media was added and incubated for 4 h at 37 °C in a CO₂ incubator. The supernatant was decanted, and formazan crystals formed in each well were dissolved with DMSO. After 30 min shaking in the plate shaker (Orbit plate shaker, Labnet International, USA), the absorbance at 570 nm was measured using a microplate reader (Biotek Synergy 4, USA). The percentage viability was determined using the following equation.

$$\text{Percentage of viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

4.2.2.3 Determination of Nitric Oxide (NO) Production

Using the Griess method, the quantity of nitrite in the culture medium (Green et al., 1982), a marker of the formation of nitric oxide was measured after a 1 h pretreatment with two different concentrations of LRMP (5 µL/mL, and 10 µL/mL), 10 µL/mL of LFP, and indomethacin, and a 23 h incubation with lipopolysaccharide (LPS-1 µg/mL). After pretreatment, mix 50 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5%

phosphoric acid) with an equivalent volume of cell supernatant. Incubate for 10 min at room temperature. The absorbance was measured at 540 nm using a Biotek Synergy microplate reader (USA). Nitrite concentration was measured using a standard curve for sodium nitrite. As a positive control, indomethacin has been used.

4.2.2.4 Determination of Cytokines

RAW 264.7 cells were seeded in six-well plates (1×10^6 cells/well) and treated for 24 h with postbiotics at a concentration of 5 $\mu\text{L/mL}$, 10 $\mu\text{L/mL}$, and LPS (1 $\mu\text{g/mL}$). Cells cultured in media without postbiotics were employed as LPS treated group. After collecting the pretreated cell-free culture supernatant, following the manufacturer's instructions, ELISA kits were used to measure the amounts of the cytokines (TNF- α , IL-6, and IL-10) in the culture medium. The use of indomethacin served as the positive control.

4.2.2.5 Immunofluorescence Assay

In a 96-black-well plate with a clear bottom, RAW 264.7 cells were seeded at a density of 2×10^5 cells. Before being incubated with LPS (1 $\mu\text{g/mL}$) for 24 h, the cells were pretreated with postbiotics from LRM (5 $\mu\text{L/mL}$, and 10 $\mu\text{L/mL}$) and the positive control indomethacin (20 μM) for 2 h. Following incubation, 200 μL of PBS was used to wash the cells twice, and they were then fixed for 15 min with 4% paraformaldehyde. The cells were blocked with 5% BSA in PBS for 1 h at room temperature after being permeabilized with 0.5% Triton x-100 for 10 min. After that, the cells were treated at 4 $^{\circ}\text{C}$ overnight with a primary antibody specific to NF- κB p65 (1:250). Following a phosphate buffered saline with tween 20 (PBST) wash, the cells were incubated for 2 h at 37 $^{\circ}\text{C}$ with a secondary antibody labeled with Alexa fluor 594 diluted to 1:400 in PBST. The nuclei were stained after washing using 4-diamidino-2-phenylindole (DAPI), and PBS was used to wash the wells. Images were captured using a fluorescent

microscope (OLYMPUS, Japan) with excitation/emission wavelengths of 360/450 nm for DAPI and 590/617 nm for Alexa Fluor-594.

4.2.2.6 Western Blotting

RAW 264.7 cells were seeded at a density of 4×10^6 cells per well in a six-well plate. The cells were treated with postbiotics from LF and LRM (10 μ L/mL) and the positive control indomethacin (20 μ M) for 2 h before they were incubated with LPS (1 μ g/mL) for 24 h. Following their harvest, the cells were lysed using 200 μ L of RIPA cell lysis solution and then incubated for 30 min at 4 °C. After collecting the cell lysate and centrifuging it for 5 min at 4°C, a BCA protein assay kit was used to determine the amount of total protein present. Protein samples are electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes after being resolved using 8–10% SDS-polyacrylamide gel electrophoresis. The membranes were blocked for 1 h at room temperature using tris-buffered saline that included 5% skim milk and 0.1% tris-buffered saline with tween 20 (TBST). The particular primary antibody against iNOS, COX-2, NF- κ B, p-NF- κ B, SAPK/JNK, p-SAPK/JNK, p38-MAPK, p-p38-MAPK, ERK, p-ERK, and β -actin (1:1000) was incubated on each blotted membrane for overnight at 4 °C. Following three TBST washes, the membranes were incubated for 2 h at room temperature with HRP-conjugated secondary antibody (1:2000). The ECL substrate was then used to probe the membranes in Chemidoc MP Imaging systems (BioRad, USA). Image Lab software version 6.1 (BioRad, USA) was used to do a densitometric analysis of the bands that were obtained.

4.2.2.7 Statistical analysis

The data is shown as the mean $\pm$ standard deviations of triplicate experiments. Graph Pad Prism 9.3.0 software was used to analyse the results using a one-way ANOVA, and $P < 0.001$ was considered significant.

4.3 Results and Discussion

The postbiotics reported in the preceding chapter were discovered to contain potential SCFA, which was further investigated for their ability to prevent and management of inflammation using RAW 264.7 cell lines.

4.3.1 Determination of cytotoxicity of postbiotics by MTT Assay

The cytotoxic effect of postbiotics from LRM was studied using the MTT assay to determine the optimal dose for an inflammatory response. Cell viability is indicated by MTT, which assesses cellular metabolic activity. MTT, a yellow dye, transforms into purple formazan in living cells, and this color change was detected using colorimetry. Following pretreatment with the different concentrations of postbiotics, the cytotoxicity assay demonstrated a dose-dependent reduction in cell viability in RAW 264.7 cell lines. Figure 4.1 shows that none of the postbiotics treated at 5, and 10 $\mu\text{L/mL}$ doses for 24 h exhibited any evident cytotoxic effects on RAW 264.7 cell lines. Both doses exhibited the least toxicity when compared to control cells. Therefore, these doses were selected to assess their capacity to reduce inflammation. For the following experiments, the cells were initially pretreated with LRMP (5 and 10 $\mu\text{L/mL}$), LFP (10 $\mu\text{L/mL}$), and positive control (indomethacin- 20 μM) and then exposed to LPS to trigger inflammation.

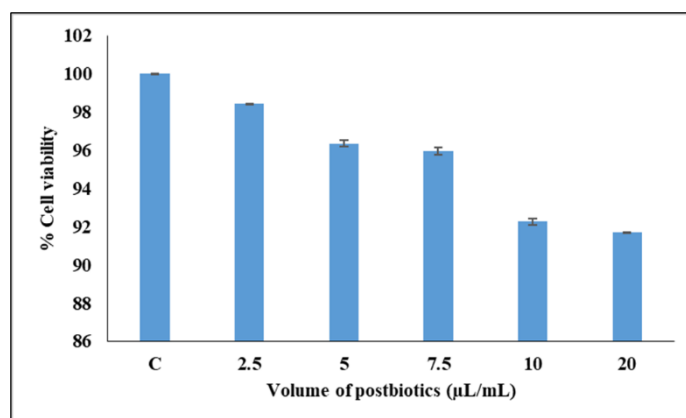


Figure 4.1: MTT cytotoxicity assay of postbiotics. RAW 264.7 cells were treated with various concentrations of postbiotics (2.5, 5, 7.5, 10, and 20 $\mu\text{L/mL}$) for 24 h and postbiotic cytotoxicity was determined by MTT assay.

Data are expressed as the mean $\pm$ SD of triplicate experiments. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate.

4.3.2 Inhibitory Effect of Postbiotics on NO Production

Lipopolysaccharide (LPS) binds to the TLR-4 receptor to produce NO, a membrane-permeable signaling molecule that raises a crucial defense against foreign infiltration. On the other hand, unregulated NO generation accelerates several chronic inflammatory diseases (Bengmark, 2006). Therefore, it is essential to maintain the NO level using various anti-inflammatory mediators (Kim & Park, 2016). By degrading L-arginine, LPS-induced macrophages promote the uncontrolled release of NO (Van Amersfoort et al., 2003). Based on the MTT assay, 5 and 10 μ L/mL of LRMP, and 10 μ L/mL of LFP were used for further studies. Indomethacin, an anti-inflammatory drug, was employed as a positive control. The Griess test was used to determine nitrite concentration as an indication of NO generation after 24 h of stimulation.

From the graph, it is clear that all the treatment groups exhibited a considerable reduction in NO generation when compared with the LPS-treated group. LF 10 and LRM 5 produced higher levels of NO compared to indomethacin, whereas LRM 10 displayed an approximate reduction in NO level similar to that of positive control. Comparing LF to both LRM concentrations, LRMP showed lower NO production than LFP. Furthermore, LRM reduced NO production in a dose-dependent manner. In murine macrophages, due to the synergistic effect of postbiotics, LRMP has shown greater anti-inflammatory activity than the LFP alone, exhibited by the decrease in NO production in the LRMP-treated groups (Figure 4.2). Therefore, the LRMP and LFP that showed significant NO inhibition were chosen for further assessment of their anti-inflammatory potential.

Postbiotics containing SCFAs have been widely examined for their inhibitory influence on NO production, which has revealed strong anti-inflammatory characteristics. SCFAs, including acetate, propionate, and butyrate, block the NF- κ B pathway, reducing NO generation and

modulating macrophage activity. Consistent with current research, earlier studies show that SCFAs reduce LPS-induced NO generation by blocking NF- κ B activation in RAW264.7 cells, resulting in reduced iNOS expression and proinflammatory cytokines (T. Liu et al., 2012). Butyrate and propionate effectively inhibit NO generation in response to *Staphylococcus aureus* lipoproteins, whereas acetate has minimal effects (J. W. Park et al., 2019). The capacity of SCFAs to regulate NO production raises the possibility of therapeutic uses in the management of metabolic and inflammatory illnesses (Mishra et al., 2024).

Earlier research has validated the current findings by confirming the anti-inflammatory effects of fermentation metabolites from RS and LF individually. The fermentation of RS by probiotics leads to the production of SCFAs, which have been shown to regulate various physiological processes, including inflammation and cell proliferation. iNOS expression can be inhibited by SCFAs produced from RS, which lowers NO production, which is frequently increased in inflammatory conditions (Haenen et al., 2013). As previously stated, butyrate, propionate, and acetate are the main products of RS fermentation and are essential for gut health and have anti-inflammatory properties (Bird et al., 2010). Butyrate, one of the main SCFA stimulates regulatory T cell populations, which further suppresses excessive NO generation and promotes a balanced immunological response (Trachsel et al., 2018). The inhibitory effect of LF on NO production is supported by several studies. LPS-stimulated NO production in macrophages was reduced by LF, indicating a regulatory function in inflammatory reactions (Choe & Lee, 2000). In LPS-treated mice, bovine lactoferrin (bLF) markedly reduced plasma NO and prostaglandin E2 levels, suggesting that it may regulate inflammatory pathways that contain NO generation (Talukder & Harada, 2007). Moreover, it has been demonstrated that *Lactobacillus* strains and LF together suppress the expression of iNOS, further lowering NO levels in inflammatory conditions (Shi et al., 2022).

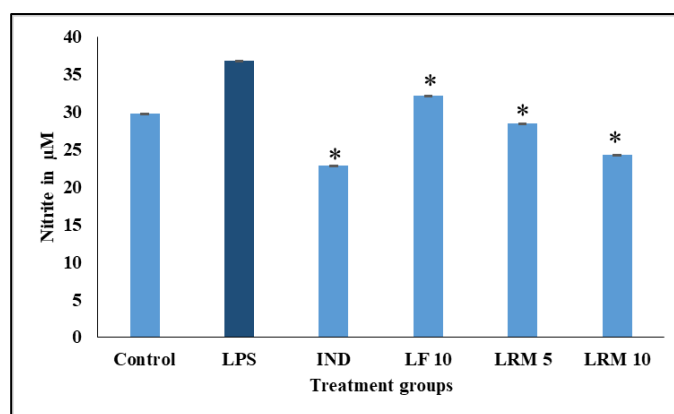


Figure 4.2. NO assay of postbiotics. RAW 264.7 cell lines were pretreated with 5, and 10 μL postbiotic concentrations for 1 h before treatment with 1 $\mu\text{g/mL}$ LPS. After 24 h, NO production was determined by the Griess method. Data are presented as the mean $\pm$ SD ($n = 3$) * $p < 0.001$ vs LPS group. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate.

4.3.3 Effect of Postbiotics on Pro and Anti-inflammatory Cytokines Production

Numerous pro-inflammatory cytokines, including $\text{TNF-}\alpha$, IL-6, and IL-1 α , are secreted in excess by LPS-stimulated cells. In cellular models, elevated levels of these cytokines indicate the effects of inflammation (Muniandy et al., 2018). In fact, IL-10 is a potential cytokine that reduces inflammation, and concurrently increases the synthesis and expression of anti-inflammatory molecules (Riley et al., 1999). Thus, using the enzyme-linked immunosorbent assay (ELISA), we investigated the efficacy of LFP (10 $\mu\text{L/mL}$) and LRMP (5 and 10 $\mu\text{L/mL}$) on the levels of $\text{TNF-}\alpha$, IL-6, and IL-10 in the supernatant of LPS-stimulated RAW 264.7 cell lines. The graph indicates that when compared to control cells, LPS-treated groups showed a significant rise in IL-6, and $\text{TNF-}\alpha$ cytokine concentrations, while a notable reduction in IL-10 concentration in RAW 264.7 cells, indicating that the cellular inflammatory model in murine macrophages was successfully established. All treatment groups restored the LPS-induced upsurges and decline by significantly lowering the $\text{TNF-}\alpha$ and IL-6; and increasing the IL-10 cytokine levels. In comparison to the positive control, LRM 5 AND 10 caused a greater reduction in IL-6 and $\text{TNF-}\alpha$ cytokines than indomethacin, while LF showed a lesser reduction. When LF was compared to LRM, LRM reduced cytokine levels in a dose-dependent manner. IL-10 concentrations were significantly higher in all treated groups compared to the LPS group.

In comparison with indomethacin, LF 10 showed lower IL-10 production, while LRM led to a higher IL-10 production than the positive control. Furthermore, both LRM groups exhibited a greater increase in IL-10 concentration than LF (Fig 4.3).

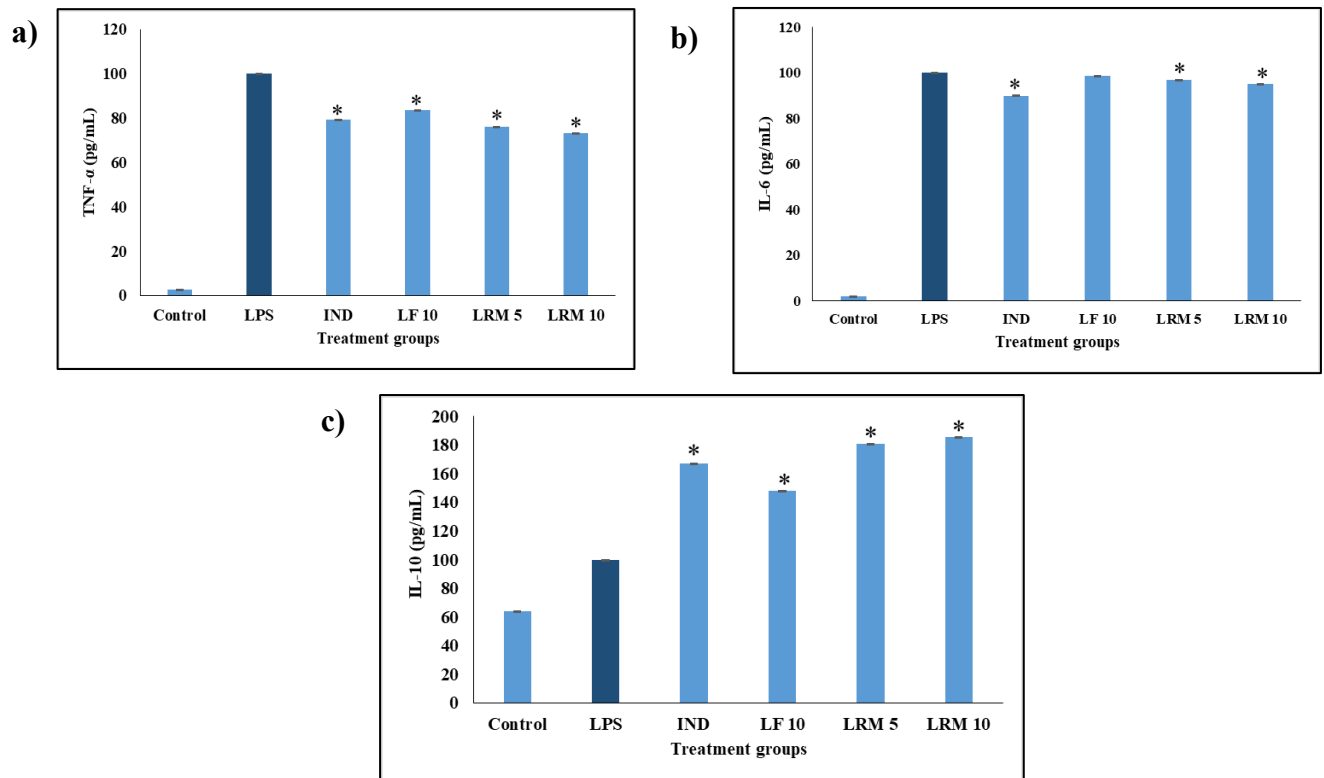


Figure 4.3. LPS induced cytokines production of postbiotics: (a) TNF- α , (b) IL-6, and (c) IL-10 in RAW 264.7 cell lines. Cells were pretreated with 5, and 10 μ L/mL postbiotics concentrations for 1 h, before treatment with 1 μ g/mL LPS. After 24 h of incubation, cytokine production was determined by ELISA. The mean $\pm$ SD is used to express the data (n = 3). *p < 0.001 vs LPS group. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate.

Based on the findings, LRMP showed the strongest anti-inflammatory potential by preventing the generation of pro-inflammatory and promoting the formation of anti-inflammatory cytokines in cellular models. When compared to the decline in TNF- α production, the decrease in IL-6 production was lesser. Moreover, LRMP produced more anti-inflammatory cytokines than the LFP and positive control, indomethacin, in a dose-dependent manner. Thus, this study confirms that the combined effects of LRMP have superior anti-inflammatory activities compared to postbiotics produced only from LF.

SCFAs containing postbiotics derived from beneficial bacteria demonstrate immunomodulatory properties that can impact inflammatory responses in various biological systems. Postbiotics decrease pro-inflammatory cytokines like IL-1 β and TNF- α while increasing anti-inflammatory cytokines like TGF- β , which aligns with our findings (J. K. & Ravi, 2024; Z. Yu et al., 2023). Postbiotics from *Lactobacillus* and *Bifidobacterium* species reduced IL-1 β release in macrophage models (Du et al., 2022). Additionally, formulations including postbiotics have shown anti-inflammatory effects in ocular models by reducing cytokine production in response to LPS stimulation (Layús et al., 2022). The variation in the inflammatory response is based on the biological condition and postbiotic type that depends on specific probiotic-prebiotic interaction.

Several studies focussing on the modulation of inflammatory responses show the inhibitory effect of postbiotics produced by *Lactobacillus casei* from RS on pro- and anti-inflammatory cytokines, which could lead to therapeutic uses in inflammatory diseases. *Lactobacillus casei* has been proven to reduce pro-inflammatory cytokines like TNF- α and IL-1 β in various animal models, including acute liver failure and obesity (Novotny Núñez et al., 2015; Y. Wang et al., 2016). RS supplementation has been associated with lower inflammatory marker levels in patients with chronic renal disease, suggesting that it modulates immune responses (de Paiva et al., 2020). Also, it promotes beneficial microbiota by increasing the production of SCFAs such as butyrate, which are known to exhibit anti-inflammatory effects (Qin et al., 2022). Moreover, the combination of *Lactobacillus casei* and RS can synergistically improve gut health and reduce inflammation by supporting a healthy microbiota (Jiang et al., 2022). All these studies about RS validate our findings.

Like RS, LF has shown the ability to modulate inflammatory responses through, pro-inflammatory cytokine suppression and immunological system regulation. In studies, including Crohn's disease and skin inflammation induced by *Propionibacterium acnes*, LF has been

shown to reduce the levels of pro-inflammatory cytokines including TNF- α , IL-6, and IL-8 (Bertuccini et al., 2014; Su et al., 2020). A healthy gut microbiota is required for LF immunomodulatory effects, as dysbiosis can alter cytokine profiles and reduce LF efficacy (Wen et al., 2017). SCFAs produced by gut microbiota may enhance the effects of LF by modulating immune responses and promoting gut health (Q. He et al., 2023).

4.3.4 Effect of Postbiotics on NF- κ B translocation on LPS-stimulated RAW 264.7 Cells by Immunofluorescence Analysis

The efficacy of the postbiotics to prevent the NF- κ B from translocating in LPS-stimulated RAW 264.7 was evaluated to investigate their method of action. Previous research has shown that the anti-inflammatory drug can reduce the synthesis of pro-inflammatory molecules by preventing NF- κ B from translocating (Aggarwal, 2004; Krakauer, 2004; Murakami & Ohigashi, 2007). In the cell cytoplasm, NF- κ B and I κ B coexist as an inactive dimer. However, when NF- κ B is activated by external stimuli, it phosphorylates and moves into the nuclei, where it stimulates the production of several pro-inflammatory mediators. Therefore, one of the most efficient methods for reducing inflammation is to downregulate the expression of p-NF- κ B. Using immunofluorescence assays, the effect of LFP (10 μ L/mL) and LRMP (5 and 10 μ L/mL) on the NF- κ B translocation was evaluated to determine its anti-inflammatory process. The positive control was indomethacin. The cells were examined under a fluorescence microscope, where the Alexa Fluor 594 emits red fluorescence when excited by the appropriate wavelength of light allowing the detection of NF- κ B translocation from cytoplasm to nucleus. The intensity of red fluorescence inside the nucleus increased significantly in LPS-treated group, indicating that it increased the translocation of NF- κ B into the nucleus. The immunofluorescence image reveals a reduction in red fluorescence within the nucleus for all treatment groups. Compared to indomethacin, LRM 5 and LRM 10 treatments resulted in lower fluorescence intensity, while LF exhibited higher intensity than the positive control. When

comparing LRM with LF, LRM exhibited a dose-dependent decrease in fluorescence intensity, whereas LF showed more intense red fluorescence.

LRMP significantly reduced the distribution of NF- κ B in the nucleus, as illustrated in Figure 4.4a. This indicates that phosphorylation and transport of NF- κ B from the cytoplasm to the nucleus are suppressed. The fluorescence intensity histogram obtained using cellSens software (OLYMPUS, Japan) showed a significant increase in nuclear p65 in the LPS-treated cells. However, nuclear p65 was significantly reduced in the cells treated with postbiotics from LRM in a dose-dependent manner, which was similar to that of the positive control, indomethacin (Figure 4.4b). A higher concentration of LRM postbiotics (10 μ L/mL) was used for further anti-inflammatory investigation.

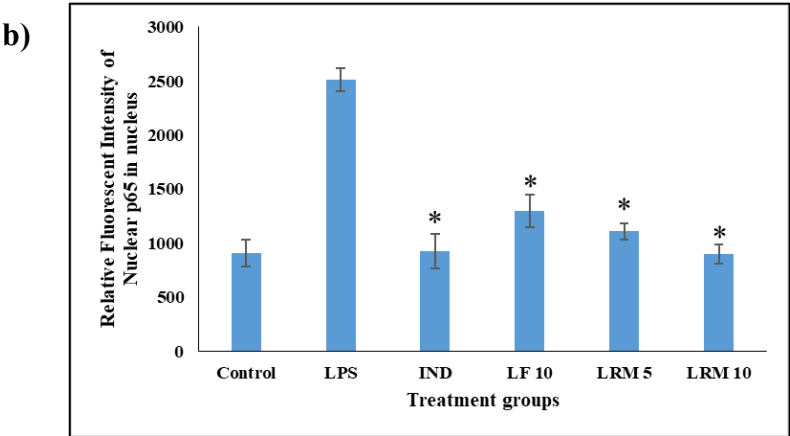
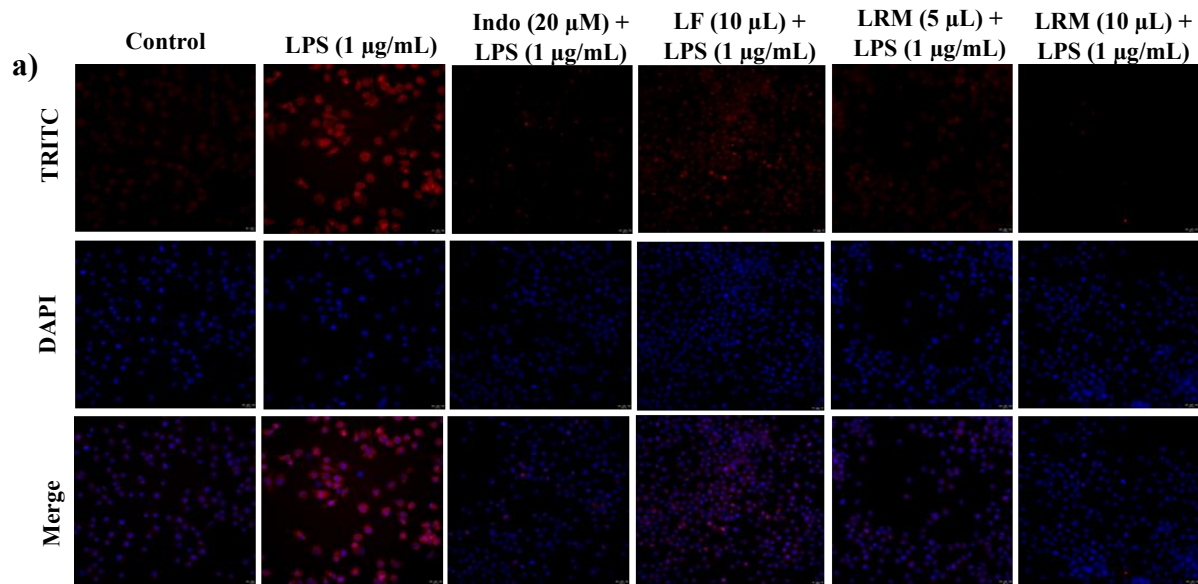


Figure 4.4. (a) Immunofluorescence staining detected the distribution of NF- κ B-p65 in both the nuclei and the cytoplasm of postbiotics-treated RAW 264.7 cells by some colocalization of receptors (p65, Alexa Fluor 594, red) with the nuclei (DAPI, blue). Using a fluorescent microscope (OLYMPUS, Japan), images were captured. Scale = 50 μ M. (b) Intensity histogram of NF- κ B translocation. The cellSens program (OLYMPUS, Japan) was used to quantify the fluorescence intensity. Data are represented as the mean $\pm$ SD (n = 3). *p < 0.001 vs LPS group. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate.

4.3.5 Western Blot Analysis

An investigation into the expression levels of key transcription factors that are essential to the potential anti-inflammatory effects of anti-inflammatory agents such as postbiotics has been conducted to gain a better understanding of the molecular basis of the inhibitory effects of postbiotics on inflammation. Western blotting was used to evaluate the expression of some of the key proteins involved in the inflammation pathways.

4.3.5.1 Effect of Postbiotics on NF- κ B Signaling Pathway and Pro-inflammatory enzymes on LPS-stimulated RAW 264.7 Cells

The p50 and p65 monomers together form the heterodimers that makeup NF- κ B proteins, the activity is prevented by I κ B α . When phosphorylation of I κ B α occurs, NF- κ B is phosphorylated and translocated from the cytoplasm into the nucleus to regulate particular transcription factors. Many studies have demonstrated that the nuclear transcription factor NF- κ B transcriptionally mediates the expression of proinflammatory cytokines and enzymes like COX-2 and inducible nitric oxide synthase (iNOS) (Christian et al., 2016; Ghosh & Hayden, 2008; Xue et al., 2018). NO, which is generated from the amino acid L-arginine by the iNOS, is crucial in a variety of inflammatory diseases. Proinflammatory cytokines have the ability to regulate NO in tissues. (Ibrahim Abdelwahab et al., 2012; Wimalawansa, 2008). Many triggers, including LPS, TNF- α , and IFN- γ , can cause activated macrophages to produce a significant amount of NO $\cdot$, which can contribute to the pathogenic processes in several acute and chronic inflammatory conditions (Verma et al., 2010). The inflammatory process and tissue damage are facilitated by prostaglandins, which are produced by inducible enzymes cyclooxygenases. High NO levels

have also been shown to activate COX-2, which can lead to more severe inflammatory reactions, as observed in many chronic inflammatory illnesses (Ibrahim Abdelwahab et al., 2012).

Western blotting was used to study the effect of postbiotics on the NF- κ B signaling pathway and pro-inflammatory enzymes such as iNOS and COX-2 to determine its anti-inflammatory mechanism. Indomethacin was used as the positive control. Figure 4.5a & b shows that on LPS-stimulated RAW 264.7 cells, p-NF- κ B expression elevated in response to LPS stimulation. In contrast, indomethacin, LRMP, and LFP led to a reduction in NF- κ B phosphorylation. When compared to the positive control, both LF and LRM exhibited a greater reduction in p-NF- κ B expression. Between LF and LRM, LRM showed a more significant reduction in p-NF- κ B expression. It was earlier observed the anti-inflammatory mechanism of LRMP by immunofluorescence assays was further confirmed, which works by inhibiting the NF- κ B signaling pathway, was validated through the observation of its effect on NF- κ B nuclear translocation.

The proinflammatory enzymes iNOS and COX-2, these mediator genes are crucial for the onset and maintenance of inflammation because they are highly activated during this period (Bonizzi & Karin, 2004; Verma et al., 2010). The enzymes iNOS and COX-2 regulate the amount of NO and PGE2 production respectively. Therefore, the effect of LRMP and LFP on the elevated levels of iNOS and COX-2 expression caused by LPS was also assessed. The graph clearly shows that all treated groups exhibited a reduction in both iNOS and COX-2 compared to the LPS group. When compared to the indomethacin, both LRMP and LF displayed a significant reduction in the expression of COX-2. However, LF showed an increased iNOS expression. In both cases, the synergistic effects of LRMP demonstrated a more substantial decrease in the expression of proinflammatory enzymes than in other treated groups. LRMP, LFP, and

indomethacin treatment markedly reduced the elevated expression of COX-2 and iNOS (Fig. 4.5a, b, c, & d). This outcome validated its anti-inflammatory properties.

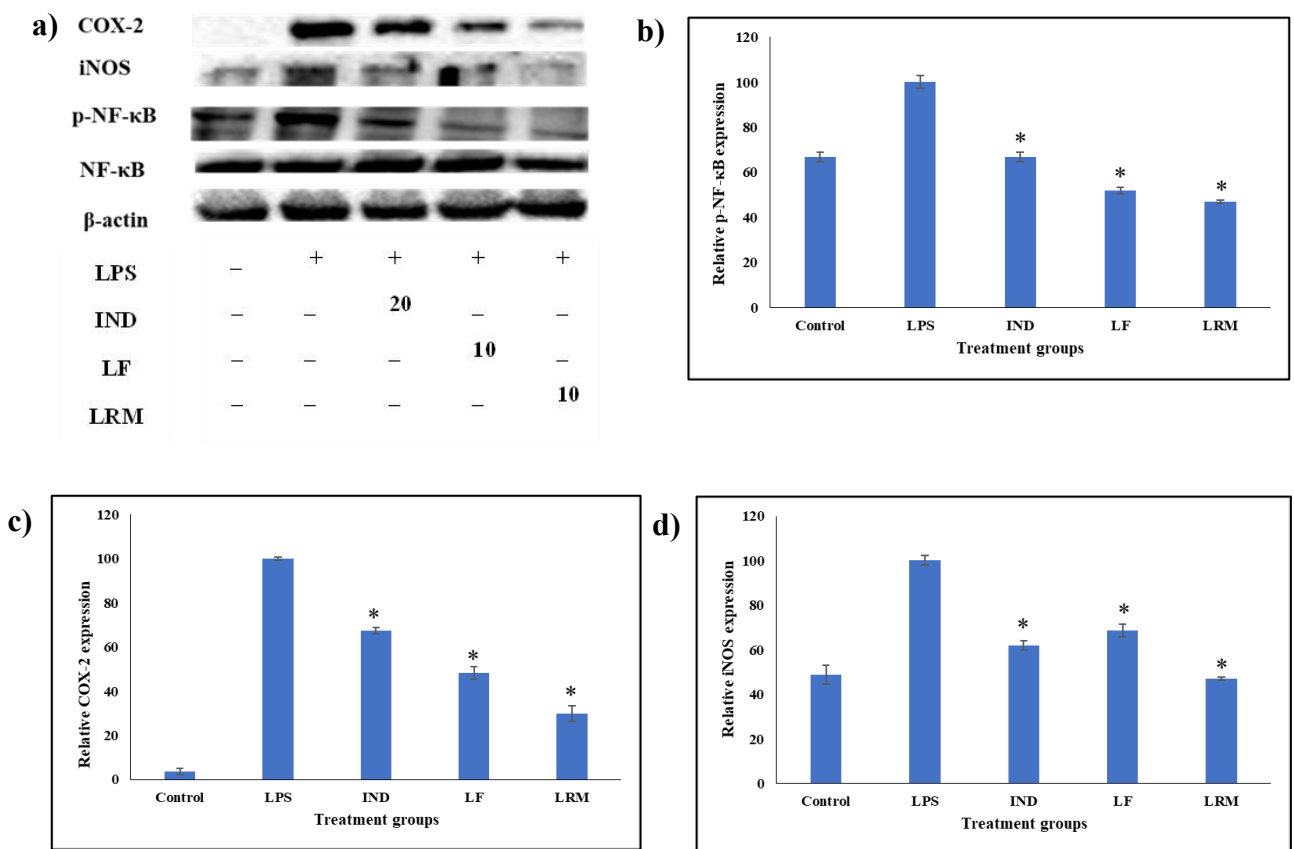


Figure 4.5: Effect of postbiotics on the b) NF-κB, c) COX-2, and d) iNOS expression in LPS-stimulated RAW264.7 cells. Cells were pretreated with postbiotics from LF and LRM (10 μL/mL); and indomethacin (20 μM) for 1 h and then stimulated with or without 1 μg/mL of LPS for 24 h. Protein expression was detected by Western blotting (a). Data are represented as the mean ± SD (n = 3) *p < 0.001 vs LPS group. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin- resistant starch microencapsulate

Various studies indicated that postbiotics, including fermented *Codonopsis lanceolata* extracts and hydrogenated isoflavones, effectively inhibit NF-κB signaling by reducing p65 nuclear translocation and IκB phosphorylation (Choi et al., 2023; K. Li et al., 2023). Thereby reduce TNF-α, IL-6, iNOS, and COX-2 levels in LPS-stimulated RAW 264.7 cells, indicating reduced inflammatory responses (M. Y. Park et al., 2022). Studies proved that *Lactobacilli* strains can inhibit TLR4-mediated NF-κB activation further supporting their potential as anti-inflammatory agents (Shi et al., 2022).

Research has already demonstrated that RS and LF regulate NF- κ B signaling, which is consistent with the findings of this study. LF has significant immunomodulatory properties, particularly in decreasing inflammation by targeting key signaling pathways. Pretreatment with LF significantly reduces phosphorylation of I κ B and NF- κ B (p65), resulting in decreased activation of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Nemati et al., 2021; Yami et al., 2023). It also downregulates the production of COX-2 and iNOS, two enzymes essential to the inflammatory response (Hu et al., 2020). RS supplementation has been associated with decreased NF- κ B activation, which is important for the generation of pro-inflammatory cytokines like TNF- α and IL-6 in LPS-stimulated cells, indicating a direct anti-inflammatory activity (Qin et al., 2022). RS primarily alters the composition of the gut microbiota, increasing beneficial microbiota that generates SCFAs, which further reduce inflammation. RS-derived butyrate promotes histone methylation, suppressing NF- κ B1 and downregulating inflammatory genes (Y. Liu et al., 2016). Thus, RS can improve overall health by regulating the NF- κ B pathway and lowering pro-inflammatory cytokines, leading to a more balanced immune response (Tian et al., 2003). Hence, these previous reports provide evidence that the synergistic effect of LRMP refers to the enhanced anti-inflammatory activity achieved when its components LF and RS, work together rather than individually. LF and RS contribute to modulating immune responses and inhibiting proinflammatory mediators, through SCFAs produced during prebiotic fermentation by gut microbiota. In LRMP, postbiotics derived from RS and LF amplify each other's effect, leading to greater suppression of the NF- κ B pathway, and stronger reduction in pro-inflammatory markers like iNOS and COX-2. This synergistic impact results in more effective inflammation management compared to using LF or RS alone.

4.3.5.2 Effect of Postbiotics on MAPK Signaling Pathway on LPS-stimulated RAW 264.7

Cells

Intracellular signaling pathways that transfer the signal required to initiate the synthesis of inflammatory mediators are triggered in response to inflammatory stimuli that activate macrophages. Cytokines such as IL-1 α and TNF- α , as well as primary inflammatory stimuli (microbial products), act via the Toll receptors, IL-1 receptor (TIR) family, or TNF receptor family, respectively. Bacterial wall component lipopolysaccharide (LPS), a commonly used inducer of the monocyte/macrophage cell lineage, acts through Toll-like receptor 4 (TLR4) to activate mitogen-activated protein (MAP) kinase cascades and the pathway that stimulates NF- κ B. Both the NF- κ B and MAPK pathways are key intracellular signaling pathways that are triggered by receptor activation (Kyriakis & Avruch, 2001; Lee et al., 1994; Tak & Firestein, 2001). Human Extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAP kinase are the three main categories of uniquely regulated MAP kinase cascades that result in changes in the expression of genes. MAP kinase kinase (MKK) and MKK2 activate ERK, MKK4, and MKK7 activate JNK, and MKK3, MKK4, and MKK6 activate p38 MAP kinase (Pearson et al., 2001; Raingeaud et al., 1996). Transcription factors in the cytoplasm or nucleus are phosphorylated and activated upon MAP kinase activation, which causes target gene expression and a biological response.

LPS activation stimulates the MAPK pathway in RAW 264.7 macrophages, resulting in the production of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β . ERK, JNK, and p38 MAPKs are the three main MAPK cascades that primarily initiate this activation. Each of these cascades has a particular role in the transcription of inflammatory genes (Jeong et al., 2019; Kyriakis & Avruch, 2001). Based on recent research, postbiotics, which are metabolic metabolites of probiotics or non-viable bacterial products, may influence immune cell's inflammatory reactions by altering the MAPK pathway. It has been demonstrated that

postbiotics generated from *Lactobacillus* and *Bifidobacterium* species decrease the phosphorylation of ERK, JNK, and p38 MAPKs in macrophages stimulated by LPS, which in turn downregulate the subsequent generation of inflammatory cytokines (Castro-Bravo et al., 2019; Kwon et al., 2020; Magryś & Pawlik, 2023). These findings show that postbiotics have the potential to be safer, more metabolically stable substitutes for live probiotics in anti-inflammatory treatments.

In LPS-stimulated RAW 264.7 macrophages, the impact of postbiotic supplementation on the markers of the MAPK signaling pathway was investigated using western blotting, to find the mechanism by which it reduces inflammation. Indomethacin was used as the positive control. It was shown that p-JNK, p38 MAPKs, and p-ERK expression drastically increased in response to LPS stimulation on RAW 264.7 cells (Fig 4.6 a, b, c, and d). However, the indomethacin, LRMP, and LF treatment group displayed a reduction in phosphorylated proteins (p-JNK, p38 MAPKs, and p-ERK) expressions. Compared to the positive control, LF only reduced the p-ERK levels but showed increased expression of p-JNK and p-p38. When comparing LF and LRM, LRM demonstrated a significant reduction in all three phosphorylated proteins. The phosphorylation was downregulated by LRMP more effectively than by the positive control, indomethacin. This demonstrated the synergistic impact of LRMP over free LF. When compared with p-JNK, and p-ERK proteins, the western blot of p-p38 MAPK did not show any evident suppression of the protein expression between the LRM, LF, and indomethacin treated group. Additionally, p-ERK expression was downregulated at a higher percentage in all treated groups when compared to p-JNK.

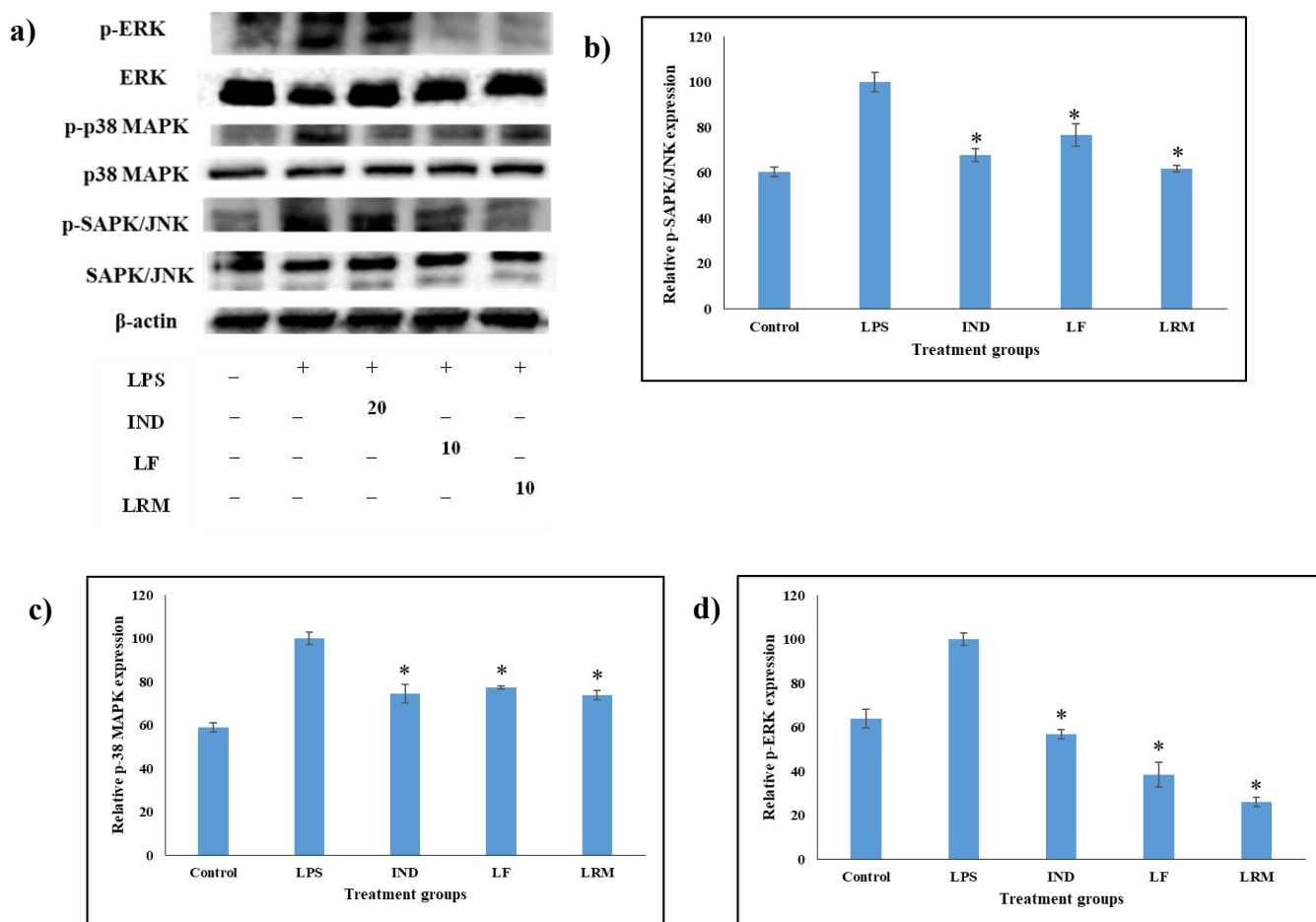


Figure 4.6. Effect of postbiotics on the MAPK signaling pathway b) p-JNK, c) p38 MAPK, and d) p-ERK in LPS-stimulated RAW 264.7 cells. Cells were pretreated with postbiotics from LF and LRM (10 μ l); and indomethacin (20 μ M) for 1 h and then stimulated with or without 1 μ g/mL of LPS for 24 h. Protein expression was detected by Western blotting (a). Data are represented as the mean $\pm$ SD (n = 3) *p < 0.001 vs LPS group. LPS: Lipopolysaccharide; IND: Indomethacin LF: Lactoferrin; LRM: Lactoferrin-resistant starch microencapsulate.

The findings of the current study align with previous research that has shown the effectiveness of postbiotics. Postbiotics like *Weissella cibaria* JW15 and surface-layer proteins from *Lactobacillus acidophilus* significantly reduce the expression of cytokines such as TNF- α , IL-1 β , and IL-6 in LPS-stimulated cells (H. Wang et al., 2018; H.-S. Yu et al., 2019). These postbiotics reduce the phosphorylation of key MAPK proteins, including ERK, JNK, and p38, which are necessary for the inflammatory response. Additionally, they have been found to decrease NO and prostaglandin E2 levels by downregulating iNOS and COX-2 expression (Shi

et al., 2022; H.-S. Yu et al., 2019). The results demonstrate the beneficial effects of postbiotics, but it's essential to consider that the efficacy may differ depending on the specific strains and prebiotics used.

Research suggests that LF modulates major inflammatory mediators and signaling pathways, including NF- κ B and MAPK, which play a significant role in the inflammatory response. As evidence of its function in reducing inflammation, LF lowers the phosphorylation of MAPK components, such as JNK, ERK, and p38, which are involved in the inflammatory response. That, in turn, significantly decreases pro-inflammatory cytokines like TNF- α , IL-1 β , and IL-6 (Hu et al., 2020; H. Y. Li et al., 2021; Zong et al., 2015). As mentioned earlier, the fermentation of RS by probiotic microorganisms, such as *Lactobacillus casei*, can produce SCFAs that alter the MAPK (Mitogen-Activated Protein Kinase) signaling pathway (J. He et al., 2020; Metzler-Zebeli et al., 2019; Zeng et al., 2017). Therefore, RS indirectly regulates inflammation through RS-derived SCFAs by regulating the MAPK pathway, which may reduce the production of pro-inflammatory cytokines and enhance systemic and gut immunological function (Vinolo, Rodrigues, Hatanaka, et al., 2011; Xiao et al., 2018). In alignment with the present findings, the study found that in IFN- γ -stimulated RAW 264.7 cells, three SCFAs sodium butyrate (NaB), sodium phenylbutyrate (NaPB), and sodium phenylacetate (NaPA) have anti-inflammatory effects by raising IL-10 expression and decreasing iNOS, TNF- α , and IL-6 levels (J.-S. Park et al., 2007). These findings indicate that RS fermentation by probiotics could be a promising way to modulate inflammation via a gut-mediated mechanism. Consequently, the inflammatory response is improved by the combined action of LF and RS. Schematic representation of the anti-inflammatory potential of postbiotics from LRM on NF- κ B and MAPK signaling pathway (Fig 4.7).

Anti-inflammatory effect of LRM Postbiotics on NF- κ B and MAPK signaling pathway

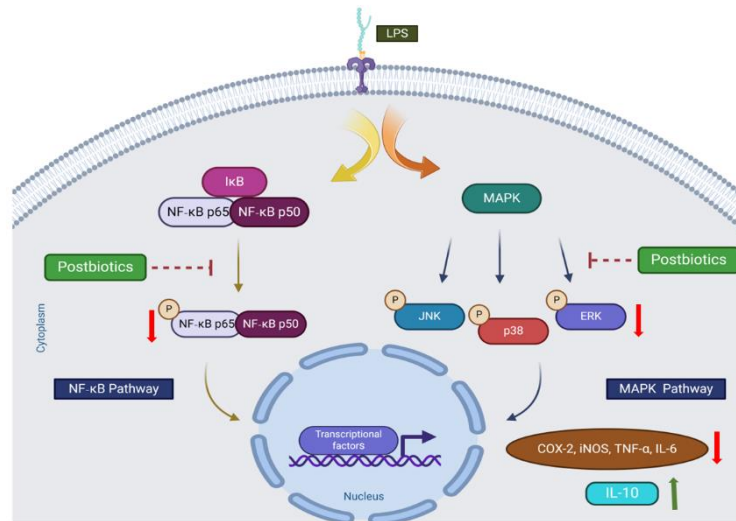


Figure 4.7. Schematic representation of the anti-inflammatory potential of postbiotics from LRM

4.4 Conclusion

The RAW 264.7 cell line was used to investigate the anti-inflammatory properties of postbiotics derived from LRM, highlighting their ability to modulate inflammatory responses at the cellular level. This study shows that in LPS-stimulated RAW 264.7 cells, the combined action of LRM-derived postbiotics significantly elevates the anti-inflammatory cytokine IL-10 and reduces the production of pro-inflammatory cytokines including TNF- α and IL-6 as well as important inflammatory mediators like nitric oxide (NO). These decreases were attributed to the downregulation of important inflammatory signaling pathways, namely NF- κ B and MAPK (ERK, JNK, and p38), as well as the inhibition of the production of inflammatory enzymes such as iNOS and COX-2. Furthermore, LRM postbiotics appear to have a similar or even better anti-inflammatory effect than traditional anti-inflammatory medications by efficiently modifying macrophage activity. The observed effects suggest that LRM postbiotics-specific composition, which includes bioactive peptides from LF and complex polysaccharides from RS, may play a synergistic role in enhancing anti-inflammatory activity by downregulating cytokine production and inhibiting inflammation-promoting enzymes. This study contributes

to the extending evidence to support the therapeutic potential of postbiotics as a stable, effective alternative to probiotics in inflammatory disease treatment. These findings pave the path for future studies to investigate the synergistic potential of LRM postbiotic effects *in vivo* and clinical settings. The ability of LRM postbiotics to target key inflammation pathways in immune cells holds potential for novel application in nutraceutical and pharmaceutical formulations, particularly for the management of chronic inflammatory conditions.

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Chapter 5

Summary, Conclusion, and Future Prospectives

Summary

The thesis is organised into six chapters. A comprehensive introduction and literature review of lactoferrin (LF), including its synthesis, health advantages, and uses, are provided in **Chapter 1**. Moreover, the physiological implications of resistant starch (RS) and its function as a carrier material for bioactives are discussed.

Chapter 2

Chapter 2 investigates the evaluation of the nutritional composition of ten unripe (stage II) banana cultivars. Proximate analysis revealed that carbohydrates were the most abundant nutrient, followed by protein and ash, with carbohydrates primarily composed of starch, accounting for more than 80% of the composition. Resistant starch (RS) was isolated from the starch of these banana varieties. All unripe cultivars were found to be good sources of RS, with cultivar MA having the highest RS content ($41.78 \pm 1.29\%$ d/w), followed by PEYA and PALA. Starch and RS were characterised using zeta potential, FTIR, XRD, SEM, DSC, and drying curve analysis. XRD examination revealed a B-type crystallinity pattern in both starch and RS, whereas FTIR revealed similar molecular structures, implying no alteration during enzymatic treatment. SEM analysis revealed smooth, oval-ellipsoidal granules in starch and rough, compact block structures in RS. Among the cultivars, RS from PALA was most suitable for LF encapsulation by spray drying, since its thermal properties, including higher gelatinisation temperatures, indicated greater temperature stability and a faster drying rate.

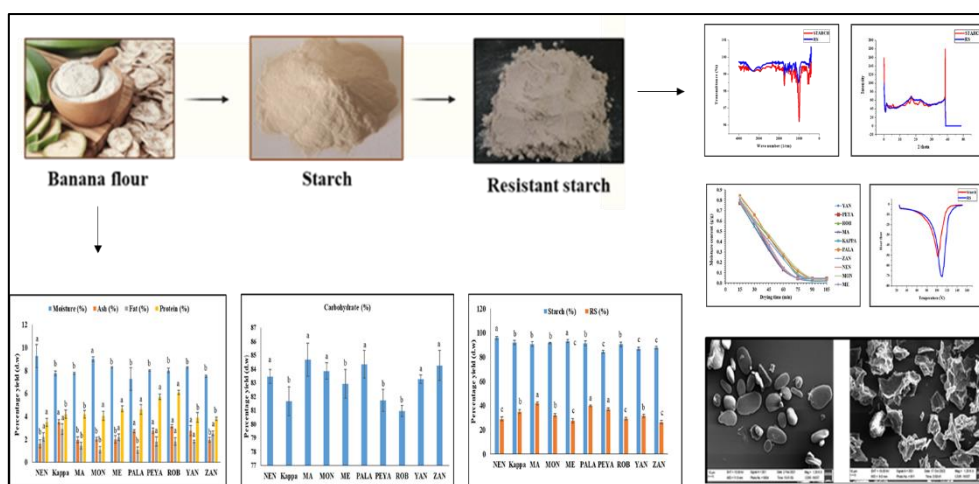


Figure 5.1. Overview of Chapter 2 results

Chapter 3 A

Chapter 3A deals with the optimisation and development of spray-dried lactoferrin-resistant starch microencapsulates (LRM). A stable LRM was achieved by using RS and maltodextrin as wall materials. The LRM was characterised using particle size analysis, XRD, FTIR, SEM, and DSC. Microencapsulate showed a particle size of $2.296 \pm 0.58 \mu\text{m}$. FTIR analysis revealed no chemical interactions between the components, confirming efficient LF encapsulation. SEM images revealed a smooth surface morphology, with some minor surface fissures caused by rapid evaporation and shrinkage of droplets during spray drying. The LRM displayed better LF encapsulation efficiency ($73.13 \pm 2.43 \%$). These data indicate that RS can be an effective matrix for LF encapsulation for use in functional food and pharmaceutical applications.

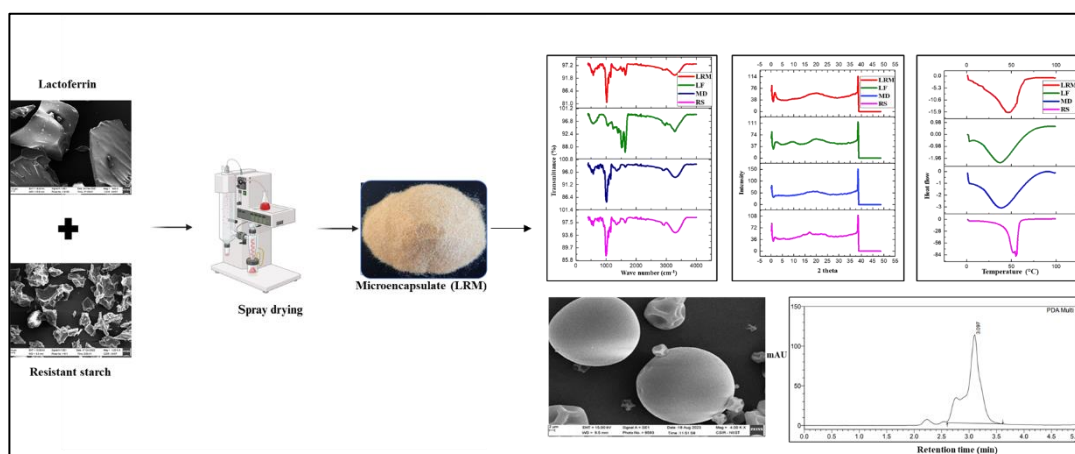


Figure 5.2. Overview of Chapter 3A results

Chapter 3 B

The *in vitro* release of LF from LRM under simulated gastrointestinal conditions, as well as an assessment of LRM's prebiotic potential, was discussed in Chapter 3B. The core release studies revealed that LF release was limited under simulated gastrointestinal conditions. In prebiotic efficacy studies, *Lactobacillus casei* was used to ferment the LRM and LF as a means to assess the prebiotic properties of LRM and the synergistic effect of LF and RS. The prebiotic potential was assessed by monitoring pH, optical density, colony count, and production of postbiotics in terms of short-chain fatty acids (SCFA) viz., acetate, propionate, and butyrate. The outcomes validated the synergistic impact, with LRM exhibiting better prebiotic potential than LF alone.

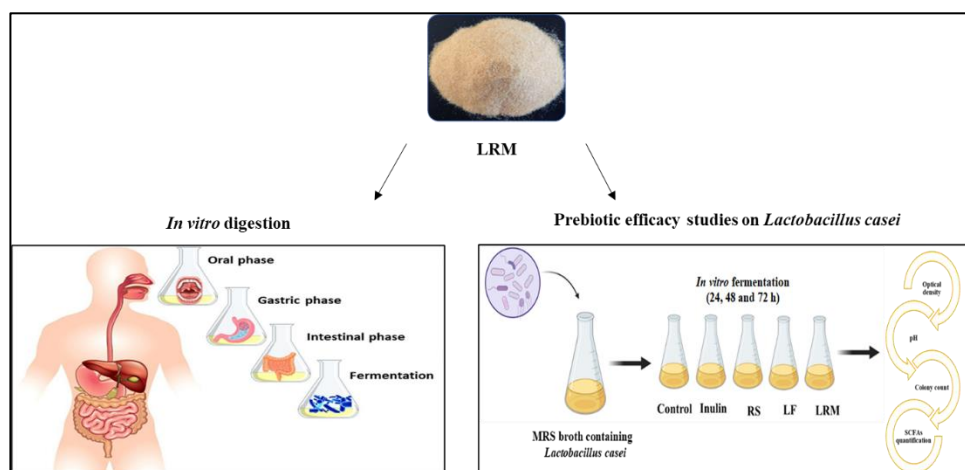


Figure 5.3. Overview of Chapter 3B results

Chapter 4

Chapter 4 explored the anti-inflammatory properties of postbiotics containing SCFAs using the RAW 264.7 macrophage cell line. The cell viability was determined using the MTT assay. A comprehensive *in vitro* study evaluated the anti-inflammatory potential of fermentation metabolites (postbiotics) by assessing their effects on inflammatory mediators like nitric oxide, tumor necrosis factor- α , interleukin- 6, and 10 in bacterial lipopolysaccharide-stimulated macrophages. Postbiotics significantly increased IL-10 levels and decreased NO, TNF- α , and IL-6 levels generated by LPS, compared to the positive control (indomethacin). Molecular

mechanistic investigations demonstrated that the postbiotics downregulate the expression of pro-inflammatory enzymes such as COX-2 and iNOS; and proinflammatory cytokine release by targeting the NF- κ B and MAPK signaling pathways. These findings indicate that LRM-derived postbiotics can significantly reduce the production of pro-inflammatory cytokines and enzymes, highlighting their potential for effective inflammation management.

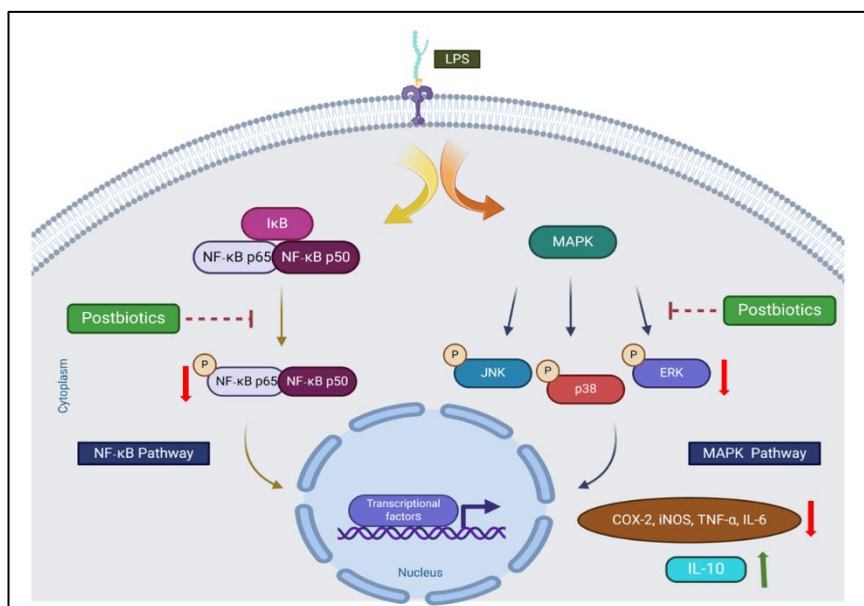


Figure 5.4. Overview of Chapter 4 results

Conclusion

In conclusion, RS, a prebiotic dietary fiber that is resistant to digestion, serves as a protective coating for LF during its transport through the digestive system. Spray drying produces LRM that efficiently protects LF and provides controlled release properties. Characterisation studies demonstrated the suitability of RS from the PALA cultivar, which had high thermal stability and encapsulation efficiency for LF. The study explored the *in vitro* release profiles of LF from microencapsulates under simulated gastrointestinal conditions. The study further investigated the prebiotic potential of LRM. The findings showed promising synergistic prebiotic effects, by promoting beneficial gut microbiota and enhancing SCFA synthesis. Moreover, LRM-derived postbiotics effectively reduce inflammation by modulating the NF- κ B and MAPK

signaling pathways. Overall, this research emphasizes the potential of LF-RS microencapsulates as a novel delivery system, paving the way for future applications in functional foods and pharmaceutical formulations aimed at promoting human health and well-being.

Future Prospectives

Further validation of LRM's anti-inflammatory activities *in vivo* studies is essential to confirm its therapeutic potential. Animal model studies would assist validate efficacy and safety in a complex biological system, which is crucial for advancing LRM's usage as a therapeutic anti-inflammatory agent. Furthermore, LRM can serve as a functional food ingredient to improve immunity in vulnerable groups such as the elderly and infants by promoting beneficial gut microbiota and enhancing immune responses. LF, known for its antiviral properties, may also benefit from being combined with prebiotics such as RS to improve gut homeostasis. This synergy has the potential for developing dietary anti-viral applications, particularly functional foods aimed at reducing viral susceptibility through enhanced gut health.

APPENDIX

Sl. No	Instruments	Manufacturer
1	Overhead stirrer	Remi, RQ126/D, with 40V, Mumbai, India
2	Spectrophotometer	Shimadzu UV-2600, Japan
3	Hot air oven	Globe Tex, Digital Laboratory Hot Air Oven, Ghaziabad, India
4	Microplate reader	Synergy 4 Biotek, USA
5	Homogenizer	T25 ULTRA-TURRAX digital; dispersing tool: S 25 N -8G: IKA, Private Limited, China
6	Gel electrophoresis system	Bio-Rad Laboratories, Germany
7	Differential scanning calorimetry	DSC Q2000, TA Instruments, USA
8	HPLC	Shimadzu, Japan
9	LC-MS/MS	Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan
10	Scanning Electron Microscope	Carl Zeiss EVO-18, Germany
11	Oven	Bajaj OTG, 4500 TMCSS, Mumbai, India
12	Confocal Microscopy	Pathway 855, BD Bioscience, USA
13	Malvem Zeta sizer	Zeta Nano-ZS; Malvern Instruments, UK
14	Gel Doc	Bio-Rad Laboratories, Germany
15	Oakton pH 700	Benchtop Meter, Oakton Instruments, USA
16	FTIR-ATR spectrometer	Perkin Elmer, Spectrum Two, US
17	Kjeldahl distillation and titration system	KjelTRON™ Rapid Automatic Nitrogen Estimation System, Chennai, India
18	Rotary evaporator	Heidolph, Germany
19	Fluorescence microscope	Olympus IX83, Olympus Corporation of the Americas, Center Vally, PA, USA
20	Refrigerated Centrifuge	Beckman Coulter, Pasadena, CA, USA
21	Mini spray dryer	LABULTIMA, Process Technologies Pvt Ltd., Mumbai, India, Model LU 228 ADVANCED

ABSTRACT

Name of the student: Shini V S	Registration No: 10BB18A39034
Faculty of study: Biological Sciences	Year of submission: 2024
CSIR Lab: CSIR NIIST	Name of the supervisor: Dr. P Nisha
Title of the thesis: Resistant starch encapsulated Lactoferrin for the effective management of inflammation	

Lactoferrin (LF), a glycoprotein presents in milk and other fluids with immunomodulatory, anti-inflammatory, antioxidant, antibacterial, antiviral properties and prebiotic potential. However, oral administration faces challenges due to gastrointestinal proteolysis. Encapsulation, especially using resistant starch (RS), protects LF from degradation and facilitates targeted intestinal release. RS, a prebiotic fiber resistant to digestion in the small intestine, promotes beneficial gut microbiota, and synergistically enhances the health benefits of LF. This study developed resistant starch-based lactoferrin microencapsulates (LRM) to improve gut homeostasis and provide immunomodulatory benefits. Nutritional analysis of ten banana cultivars identified carbohydrates, primarily starch, as the main component. RS was isolated and characterized using FTIR, XRD, SEM, DSC, and zeta potential analysis, confirming its structural integrity. RS from PALA was chosen for LF encapsulation due to its superior thermal stability and drying rate. Spray drying conditions were optimized, producing spherical LRM encapsulate with smooth surfaces and high encapsulation efficiency. *In vitro* digestion studies showed controlled LF release under simulated gastrointestinal conditions. The prebiotic studies of LRM with *Lactobacillus casei* showed increased bacterial growth and postbiotic production, including SCFAs like acetate, propionate, and butyrate. LRM-derived postbiotics significantly reduced inflammatory mediators (NO, TNF- α , IL-6) and increased IL-10 levels, modulating NF- κ B and MAPK pathways. These findings confirm RS as an effective carrier for LF, enabling controlled release, prebiotic activity, and anti-inflammatory effects. LF-RS microencapsulates hold promise as a novel system for improving gut health, immunity, and inflammation management, with potential applications in functional foods.

LIST OF PUBLICATIONS

List of publications emanating from the thesis work

1. Shini, V. S., Billu, A., Suvachan, A., & Nisha, P. (2024). Exploring the nutritional, physicochemical and hypoglycemic properties of green banana flours from unexploited banana cultivars of southern India. *Sustainable Food Technology*, 2(4), 1113-1127 (<https://doi.org/10.1039/D4FB00066H>).
2. Shini, V. S., Udayarajan, C. T., & Nisha, P. (2022). A comprehensive review on lactoferrin: A natural multifunctional glycoprotein. *Food & Function*, 13(23), 11954-11972 (<https://doi.org/10.1039/D2FO02371G>).
3. Shini, V. S., & Nisha, P. Microencapsulated lactoferrin-resistant starch: physicochemical characterization and prebiotic efficacy (under preparation).
4. Shini, V. S., & Nisha, P. Lactoferrin-resistant starch microencapsulate-derived postbiotics' anti-inflammatory effects on RAW 264.7 cell lines (under preparation).

Cover art

5. Featured inside front cover art on *Food Funct.*, 2022,13, 11932-11932 (<https://doi.org/10.1039/D2FO90088B>)

List of publications not related to thesis work

Book chapter

1. Reshmitha, T.R., Shini, V.S., Nisha, P. (2021). Nanotechnology Approaches for Colorectal Cancer Diagnosis and Therapy. In: Vishvakarma, N.K., Nagaraju, G.P., Shukla, D. (eds) *Colon Cancer Diagnosis and Therapy* (pp.171-186). Springer, Cham (https://doi.org/10.1007/978-3-030-64668-4_8).

LIST OF CONFERENCES PRESENTED/PARTICIPATED

- Shini V S and P Nisha. “Comparison of physicochemical characterization of starch and resistant starch from different varieties of dessert and plantain bananas”. Proceedings of 29th ICFoST organized by AFSTI from 5th -7th January 2023 (poster)

“Comparison of physicochemical characterization of starch and resistant starch from different varieties of dessert and plantain bananas”

Shini V S^{1,2} and P Nisha^{1,2}

To evaluate the physicochemical characterization of starch and resistant starch (RS) isolated from ten different varieties of raw bananas that are not exploited much. Raw banana flour was used to prepare starch, and from this RS was isolated by utilizing enzymatic treatment. Furthermore, the starch and RS were subjected to physicochemical characterization such as Zeta potential, Scanning electronic microscope (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and Differential scanning calorimetry (DSC).

Among the ten cultivars, peyan, malayannan, and palayankodan showed a high yield of resistant starch. Zeta potential of starch was higher for malayannan cultivar, and for RS it was higher for kappa variety. SEM of starch and RS displayed a smooth granule mixture of oval-ellipsoidal shape structures and compact block structures, respectively. XRD images showed a B-type crystallinity pattern for both starch and RS, but due to enzymatic degradation of amylopectin crystalline structure, a diminished peak intensity was observed in RS. FTIR analysis demonstrated similar bands for starch and RS, showing that molecular structure was not altered during enzymatic treatment. DSC values were shown higher by RS due to the molecular rearrangement taking place in starch.



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2024, 2, 1113

Exploring the nutritional, physicochemical and hypoglycemic properties of green banana flours from unexploited banana cultivars of southern India†

V. S. Shini,^{ab} Abraham Billu,^{abc} Abhijith Suvachan^a and P. Nisha^{ab}

The present study investigates the nutritional, phytochemical, and antioxidant potential of ten banana cultivars, in unripe form, popular in southern India, viz. Yangambi (YAN), Mysore Ethan (ME), Zanzibar (ZAN), Peyan (PEYA), Palayankodan (PALA), Malayannan (MA), Nendran (NEN), Robusta (ROB), Kappa and Monthan (MON). All cultivars in the unripe form were found to be good sources of resistant starch, with MA being the richest source ($41.78 \pm 1.29\%$ d/w). The phenolic compounds, free vitamins, and amino acids were estimated and quantified using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The antioxidant activity was assessed in terms of DPPH and ABTS free radical scavenging abilities. Among the varieties, MA showed higher phenolic content, whereas flavonoid content was highest for Kappa. LC-MS/MS characterisation revealed the presence of significant amounts of phenolic compounds such as shikimic acid, epicatechin, ferulic acid, and rutin; free amino acids like phenylalanine, histidine, glutamine, lysine, and arginine; and free vitamins such as pyridoxine, nicotinic acid, and ascorbic acid among the varieties. ROB and PALA demonstrated potential DPPH and ABTS radical scavenging activity, and α -amylase inhibition when compared to other cultivars. Molecular docking of shikimic and ferulic acid with α -amylase amino acid residues resulted in high binding energies of -5.45 and -5.48 kcal mol⁻¹, respectively, confirming the hypoglycemic potential. The bioactive and nutritional potential of these cultivars, as detailed in the present study, positions them as excellent sources for food, functional food, and nutraceutical applications.

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Sustainability spotlight

Research on the physicochemical and nutritional characteristics of banana flours made from underutilized southern Indian banana cultivars of stage II maturation opens up a new path for the development of sustainable food resources. By highlighting the significant amount of starch & resistant starch with appreciable presence of amino acids, vitamins, and polyphenols found in these flours, it presents their potential as a useful functional food component that can improve the nutritional value of food items, which supports United Nations Sustainable Development Goal (SDG) 2: zero hunger. This helps to ensure that everyone has access to adequate, nutritious, and secure food. Furthermore, the study aligns with SDG 3: good health and well-being, and the presence of a significant amount of resistant starch can potentially alleviate metabolic disorders such as diabetes, which would help in achieving overall health. Moreover, it also supports SDG 12 (responsible consumption and production) by promoting the efficient use of unexploited banana varieties by the food industry. In short, this study contributes to the broader mission of the UN's SDGs to achieve a more sustainable future.

1 Introduction

The banana, a member of the Musaceae family and belonging to the genus *Musa*, holds important global significance as one of the primary fruit crops. The banana ranks as the fourth major

food crop globally, following wheat, rice, and maize.¹ Bananas are widely harvested in tropical and semi-tropical regions.^{2,3} China, India, and Indonesia have consistently had the largest supply to their domestic markets since 2016.^{4,5} Among these, India is the largest producer of bananas, with a production of 32 million metric tonnes, which accounts for about 26.3% of the world's production in 2020. Bananas are cultivated in 150 nations in an estimated amount of 1000 different varieties. These banana varieties are further classified into desserts and plantains. Dessert bananas are consumed raw in fully ripe form, and plantains are usually cooked to be consumed at different maturity stages.⁶

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The banana is highly valued worldwide due to its delightful flavor, nutritional value, and consistent availability throughout the year. The chemical compositions of raw bananas vary depending on the climate, soil type, variety, and degree of maturity. In raw bananas, the main component is starch, which undergoes vital changes during ripening. Starch accounts for about 70–80% of their dry weight (d/w), mostly resistant starch (RS), which is indigestible in the small intestine.⁷ Due to the presence of these functional ingredients, regular intake of raw banana flour is beneficial for human health.⁸ The higher content of RS in the diet reduces insulin responses and post-prandial glucose levels in humans. Studies report a hypoglycemic effect in humans following the intake of unripe plantains.⁹ In unripe banana peel, the main lipophilic components present are stearyl esters and free sterols, while the pulp contains free sterols and fatty acids. Thus, the evaluation of bioactive compounds and antioxidant activity in raw bananas is of great interest. Other health benefits of bananas include aiding in improving digestion, as well as gut, heart, and kidney health, boosting immunity, helping in weight loss, and helping to fight against anemia.^{10,11}

Considering the importance of diet in the management of chronic diseases, raw plantains are gaining attention among consumers as a healthy functional food ingredient.^{12,13} From this perspective, it is very important to explore the potential of underutilised and unexploited traditional varieties of local cultivars. However, there is no consolidated report on the nutritional composition of different plantain varieties. With this background, the objective of this study is the preliminary analysis of ten common but unexploited raw banana cultivars in terms of proximate composition, RS, free amino acids, vitamin content, phytochemical characterisation, antioxidant potential, and hypoglycemic effect.

2 Materials and methods

2.1 Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), gallic acid,

ascorbic acid, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), aluminum chloride (AlCl₃), quercetin, α -amylase, amyloglucosidase, and protease were procured from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Folin-Ciocalteu reagent, methanol, sodium carbonate (Na₂CO₃), sodium phosphate monobasic, sodium phosphate dibasic, and potassium acetate (CH₃COOK) were purchased from MERCK, Mumbai, India. Trypsin-EDTA, antibiotic-antimycotic, starch, potassium iodide (KI), iodine (I₂), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were obtained from Himedia Laboratories, Mumbai, India. Formic acid, ammonium formate, and acetonitrile (MS grade) were obtained from Biosolve Ultra International, Bangalore, India. Glucose oxidase/peroxidase (GOD/POD) kits were purchased from Biosystems Diagnostics, Pvt. Ltd. Minigen Nylon syringe filters (0.20 μ m diameter) were procured from Genetix Biotech Asia Pvt. Ltd, New Delhi, India. All the chemicals used were of high-quality analytical grade.

2.2 Sample preparation

Stage II raw bananas harvested from the College of Agriculture in Kerala were used for this study. Ten banana cultivars were selected: Yangambi, Mysore Ethan, Zanzibar, Peyan, Palayankodan, Malayannan, Nendran, Robusta, Kappa, and Monthan (Fig. 1); their features are summarised in ESI Table 1.† The cultivars were washed, their peel was removed, and the pulp was cut into slices and dried in a Refrigerated Adsorption Dehumidified Drier (RADD) at 45 $\pm$ 2 °C. The dried slices were ground, passed through a 50 mm mesh sieve to obtain banana flour, and stored at room temperature in an air-tight container until further analysis.

2.3 Proximate composition

The moisture, ash, crude fat, and protein contents of cultivars have been determined using the standard AOAC methods (930.15, 923.03, 983.23, and 984.13).^{14–17} For determining the moisture content, triplicates of each sample were dried in an

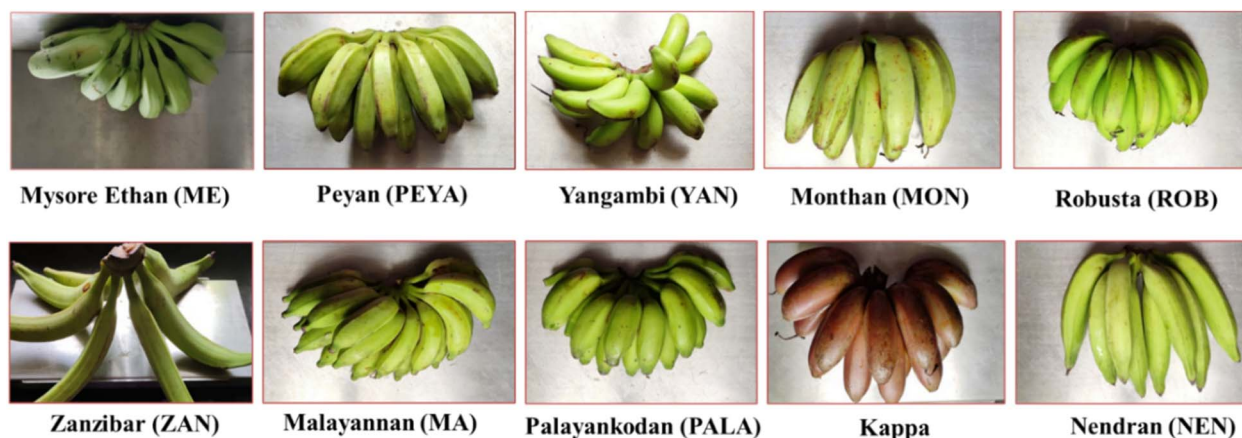


Fig. 1 Ten banana cultivars.



oven at 105 °C and then placed in a desiccator to cool to room temperature. The ash content was determined by weighing the sample both before and after heat treatment in a muffle furnace at 550 °C for 12 h. The Soxhlet extraction method was employed to measure the fat content, while protein levels were estimated using an automatic Kjeldahl distillation and titration system (KjelTRON™ Rapid Automatic Nitrogen Estimation System (Nitrogen Analyser), Tulin Equipments, model: KDIG 4M, Chennai, India). The nitrogen content was determined using the following equation, where 14 is the atomic weight of nitrogen

Percentage nitrogen =

$$\frac{(14 \times \text{normality of HCl} \times \text{volume of HCl} \times 100)}{(\text{weight of the sample})} \times 1000 \quad (1)$$

Then, the protein content was calculated using the equation,

$$\text{Percentage protein} = \text{percentage nitrogen} \times 6.25 \quad (2)$$

Carbohydrate content was determined using the difference method, as per the following formula:

$$\text{Carbohydrate content} = [100 - \{\text{moisture} + \text{ash} + \text{fat} + \text{protein}\}] \quad (3)$$

2.4 Isolation of starch

Starch was isolated from raw bananas using an alkaline extraction method¹⁸ with slight modifications. Initially, banana flour (5 g) was suspended in a 0.05 M NaOH solution (20 mL), and agitated continuously for 4 h. Subsequently, the mixture was centrifuged using a Kubota High-Speed Refrigerated Centrifuge (Model 7780, Kubota Corporation, Tokyo, Japan) at 956g for 15 min at 4 °C. The same procedure was repeated once again and the resulting pellet was dispersed in distilled water (50 mL), and filtered. The residue was neutralised using a 1 M HCl solution and centrifuged. The pellet was washed thrice with distilled water, centrifuged, and the final pellet was dried overnight at 45 °C. The dried product was then ground and sieved through a 50 mm mesh to obtain a fine starch powder, which was stored in an airtight container for further analysis.

2.5 Isolation of resistant starch

The method used by Zhang *et al.* and Escarpa *et al.*^{19,20} was slightly modified for the isolation and determination of RS from starch. Isolated starch (2 g) was mixed with 50 mL of 0.08 M phosphate buffer (pH 6) and α -amylase, incubated in a water bath shaker (Rivotek™ water bath shaker TC544, Mumbai, India) at 95 °C for 15 min, cooled and the pH was adjusted to 7.5 using 1 M NaOH. To this, phosphate buffer (2.0 mL, pH 7.5) containing protease enzyme (50 mg mL⁻¹) was added, incubated at 60 °C for 30 min in a water bath shaker, and cooled to room temperature. The pH was adjusted to 4.5, followed by the addition of 2.5 mL of amyloglucosidase (AMG), and incubated

in a water bath shaker at 60 °C for 30 min. It was allowed to cool after the incubation followed by the addition of ethanol (1 : 4 v/v) and allowed to stand overnight and centrifuged. The pellet was washed with distilled water, and freeze-dried using a lyophilizer (VirTis genesis, USA). The dried material was then ground to a fine powder. Total glucose was analysed using the GOD-POD (glucose oxidase/peroxidase) kit. The RS content was calculated using the equation RS = glucose $\times$ 0.9 (correction factor).

2.6 Phytochemical characterisation

Raw banana flour (20 g) was weighed and extracted with 100 mL of 70% ethanol by continuous stirring for 1 h; the slurry was centrifuged at 10 621g for 10 min, and the supernatant was collected. The resulting extract was filtered and evaporated using a rotary evaporator (Heidolph, Germany) at 45 °C under low pressure. The hydro-alcoholic extracts were subsequently dissolved in corresponding solvents, made up to a specific volume, and stored at 4 °C until further analysis for determining phenolic compounds, free amino acids, and vitamins.²¹

2.6.1 Determination of total phenolic content (TPC) and total flavonoid content (TFC). Folin-Ciocalteu and aluminum chloride colorimetric methods were used to obtain the TPC and TFC of banana flour extract, respectively.¹³ Briefly, different concentrations of the extracts and gallic acid (standard) were made up to 750 μ L with methanol followed by the addition of Folin's reagent (80 μ L) and 7.5% of sodium carbonate solution (200 μ L) and kept at room temperature for 90 min in the dark. The absorbance was measured at 750 nm using a multimode plate reader (Synergy 4 BioTek, BioTek Instruments, Inc., Mumbai, India). The TPC was expressed as milligrams of gallic acid equivalent per gram of d/w (mg GAE per g).

The aluminum chloride colorimetric method was used to estimate the TFC of banana flour extract. In short, various concentrations of the samples and quercetin (standard) were combined with 10 μ L of 1 M CH₃COOK, 10 μ L of 10% AlCl₃, and 280 μ L of distilled water, and incubated at room temperature for 40 min. The absorbance was measured at 415 nm using the multi-plate reader. The TFC was represented as a milligram of quercetin equivalent per gram of d/w (mg QE per g).

2.6.2 DPPH and ABTS radical scavenging activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays were used to determine banana flour extract's free radical scavenging activity.^{13,22} In brief, 500 μ L of 0.2 mM DPPH solution was added to 500 μ L of sample and gallic acid (standard) at different concentrations and incubated at room temperature for 30 min in the dark, and its absorbance was measured at 517 nm using a multimode reader. The percentage inhibition of DPPH was determined using the following equation. The IC₅₀ values were calculated using linear regression analysis.

Percentage inhibition of DPPH =

$$\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (4)$$



For ABTS radical scavenging activity, ABTS radicals were prepared by combining 50 mL of 2 mM ABTS solution with 200 μ L of 70 mM potassium persulfate. This mixture was allowed to stand in a darkroom for 12–18 h. Different concentrations of standard (ascorbic acid) and samples were prepared and made up to 250 μ L using distilled water followed by the addition of ABTS solution and 850 μ L of phosphate buffer (pH 7.4) and incubated at room temperature for 7 min in the dark. The absorbance was read at 734 nm using a multimode reader. The percentage inhibition of ABTS was measured using the following equation.

Percentage inhibition of ABTS =

$$\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (5)$$

The IC₅₀ values were calculated using linear regression analysis.

2.7 Chemical profiling and quantification of raw banana flour extract using LC-MS/MS

Quantitative analysis of phenolic compounds, free amino acids, and vitamins present in the hydroalcoholic extracts (prepared as per Section 2.6) was carried out by LC-MS/MS. The extracts were filtered using a 0.20 μ m syringe filter before the analysis.

2.7.1 Quantification of polyphenols. Quantitative analysis was conducted on a total of 28 compounds, including catechol, quinine, tocopherol, gallic acid, naringenin, catechin, chlorogenic acid, epicatechin, syringic acid, vanillic acid, genistein, epigallocatechin, *p*-coumaric acid, ferulic acid, myricetin, luteolin, apigenin, kaempferol, rutin, daidzein, quercetin, hesperetin, shikimic acid, morin, caffeic acid, cinnamic acid, and chrysin.²³ Initial standardisation of the method was carried out using corresponding standard compounds. In brief, the analysis was done with an LC-MS/MS (Nexera LC-30AD) with an autosampler (SIL30AC), temperature-controlled column oven (CTO-20AC), and Prominence diode array detector (SPD-M20A), coupled to a triple quadrupole mass spectrometer. A Shimadzu Shim-pack GISS C18 column (150 $\times$ 2.1 mm i.d., 1.9 μ m) was used with a mobile phase consisting of solvent A (water/formic acid, 100/0.1%) and solvent B (100% methanol). The following linear gradient system was used to elute the polyphenols: for 0.5 to 1.9 min 5% of solvent B, for 2.0 to 10.0 min and 10.1–15 min 98% of solvent B, and for 15.1–17 min 5% of solvent B, with a 0.3 mL min⁻¹ flow rate, 40 °C oven temperature and 10 μ L injection volume. During LC-MS/MS with electrospray ionisation (ESI), both positive and negative modes of the multiple reaction-monitoring (MRM) modes were operated. The Lab Solutions software (Shimadzu, Kyoto, Japan) was used to gather and process the LC-MS/MS data. Ionisation required an interface temperature of 400 °C, a desolvation line temperature of 300 °C, a heat block temperature of 400 °C, a drying gas flow rate of 10 L min⁻¹ for nitrogen, and a nebulizing gas flow rate of 3 L min⁻¹ for nitrogen. The average value of the data was taken as the representative for each point after every calibration solution was

analysed in triplicate. The resulting data are expressed as polyphenols in μ g mL⁻¹ with SD ($n = 3$).

2.7.2 Quantification of amino acids and vitamins. Free amino acids (tryptophan, serine, leucine, histidine, phenylalanine, asparagine, methionine, cystine, threonine, valine, tyrosine, lysine, glutamic acid, glycine, proline, aspartic acid, glutamine, isoleucine, arginine, hydroxyproline, cysteine, and alanine) and vitamins (thiamine, pyridoxine, nicotinic acid, folic acid, riboflavin, and ascorbic acid) present in the extracts were analysed by LC-MS/MS.²⁴ In brief, an LC-MS/MS (Nexera LC-30AD) equipped with an autosampler (SIL30AC), temperature-controlled column oven (CTO-20AC), and Prominence diode array detector (SPD-M20A), coupled to a triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) was used. The quantification was carried out with a Shimadzu Shim-pack GISS C18 column (150 $\times$ 2.1 mm i.d., 1.9 μ m). The mobile phase used for amino acids consists of solvent A (water/formic acid, 100/0.1%) and solvent B (100% methanol). The following is the method of eluting amino acids using a linear gradient system: for 0.5–4.9 min 5% of solvent B, for 5.0–13 min and 13.1–15 min 85% and 5% of solvent B respectively, with a flow rate of 0.3 mL min⁻¹, and 40 °C oven temperature. Both positive and negative MRM modes were used during LC-MS/MS with ESI. The ion spray voltage was 4 kV, the injection volume was 10 μ L, and the collision-induced dissociation (CID) gas pressure was 230 kPa. The average value of the results was taken as the representative for each point after each calibration solution was examined thrice. The concentration of amino acids are represented as μ g per mL with SD ($n = 3$).

The mobile phase used for vitamins was 10 mM ammonium formate in water and 0.1% formic acid for solvent A and 10 mM ammonium formate in methanol and 0.1% formic acid for solvent B. A linear gradient system was employed as follows: for 0.01–2.0 min 5% of solvent B, for 2.0–5.0 min 30% of solvent B, for 5.0–8.0 min 45% of solvent B, for 8.0–12.0 min 90% of solvent B, for 12.0–16.0 min 5% of solvent B with a flow rate of 0.3 mL min⁻¹ and 33 °C oven temperature. In both positive and negative MRM modes, ESI was performed. For ascorbic acid only, the negative mode was employed. There was a 10 μ L injection volume, 4 kV ion spray voltage, and the drying and heating gas flow rate was 10 L min⁻¹, whereas the nebulizing gas flow was 3.0 L min⁻¹. Each calibration solution was examined in triplicate, and the average of the findings was taken as the representative for each analysis. The vitamin content is expressed as μ g per mL with SD ($n = 3$). The analytical method was initially standardized using standard amino acids and vitamins. This standardisation ensured accurate quantification of the components in the extracts.

2.8 Cytotoxicity assay

The cytotoxicity of extracts was quantified by MTT assay using L6 myoblast cells.²⁵ L6-rat skeletal myoblast cells (NCCS, Pune, India) were cultured in DMEM with 10% FBS and a 0.5% antibiotic-antimycotic solution and kept in an incubator at 37 °C for 24–48 h provided with 5% carbon dioxide. Cells were



trypsinized and seeded into a 96-well plate. After the cell attachment, it was treated with different concentrations of extracts and incubated for 24 h at 37 °C. The cytotoxicity of cells was quantified by the MTT assay, and cell viability was calculated using a subsequent equation.

$$\text{Percentage Cell viability} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (6)$$

2.9 α -Amylase inhibition assay

Extracts were tested for α -amylase inhibition with modifications to Xiao *et al.*'s method.²⁶ The test mixture containing 40 μ L of 0.02 M sodium phosphate buffer (pH 6.9), 0.02 units of porcine pancreatic amylase (PPA) solution, and varied concentrations of extracts, was incubated at 37 °C for 10 min. After adding 1% soluble starch, it was incubated for an additional 15 min. To halt the enzyme process, 20 μ L of 1 M HCl was added, followed by 100 μ L of iodine reagent (5 mM I₂ and 5 mM KI). Absorbance at 620 nm was measured, using acarbose as a positive control. The inhibition percentage was calculated based on the defined formula.

$$\text{Percentage inhibition of PPA} =$$

$$\frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100 \quad (7)$$

2.10 Molecular docking studies of compounds with α -amylase

The human pancreatic α -amylase structure (PDB ID 4W93) from the Protein Data Bank was utilized for molecular docking, with hydrogens and Kollman charges being added post-structure refinement. The structures of the LCMS/MS-identified compound were obtained from PubChem and prepared as ligands in 3D .sdf format. Energy minimization and conversion to .pdbqt format were performed using OpenBabelGUI. Auto-dock 4.2.6 facilitated molecular docking with 100 runs and a population size of 150, assessing ligand interactions against α -

amylase. PyMol was employed for 3D visualisation of ligand interactions with α -amylase's amino acid residues. Binding energies (kcal mol⁻¹) indicated the strength of affinity, with more negative values suggesting stronger interactions.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.3.0, presenting results as mean $\pm$ standard deviation (SD). A one-way ANOVA and Dunnett's multiple comparison test were employed, with significance considered at $p < 0.05$.

3 Results and discussion

3.1 Proximate composition

Proximate analysis provides essential data on the major constituents of food products, including moisture, ash, fat, protein, and carbohydrates. This data is crucial for understanding the nutritional profile and potential applications of different food items. In the study, the proximate composition of ten banana cultivars is reported, showcasing the variance in composition that can impact both nutritional value and usability.

The proximate composition of ten varieties of banana cultivars is presented in Fig. 2a. The ash content, representing inorganic minerals, varied significantly across the cultivars, from $1.61 \pm 0.35\%$ in NEN to $3.52 \pm 0.19\%$ in Kappa. This variation suggests a considerable influence of soil mineral content and agricultural conditions on the mineral uptake by the bananas, as seen in other studies.^{27–29} Elsewhere, Kumar *et al.*³⁰ reported 2.5% ash content for Nendran, which was higher than that in the present study. This difference could be due to the variation in geographical and climatic changes. These cultivars varied in fat content, with PALA having $1.07 \pm 0.77\%$ and Kappa having $2.89 \pm 0.45\%$. This low fat content, as reported by Khoza *et al.*³¹ and Amini Khoozani *et al.*,³² is advantageous for reducing oxidative spoilage, thus extending the shelf life of banana flour. The sequence of fat content from lowest to highest (PALA, MON, MA, PEYA, ROB, YAN, ME, NEN, ZAN, Kappa) indicates that certain cultivars like Kappa might be

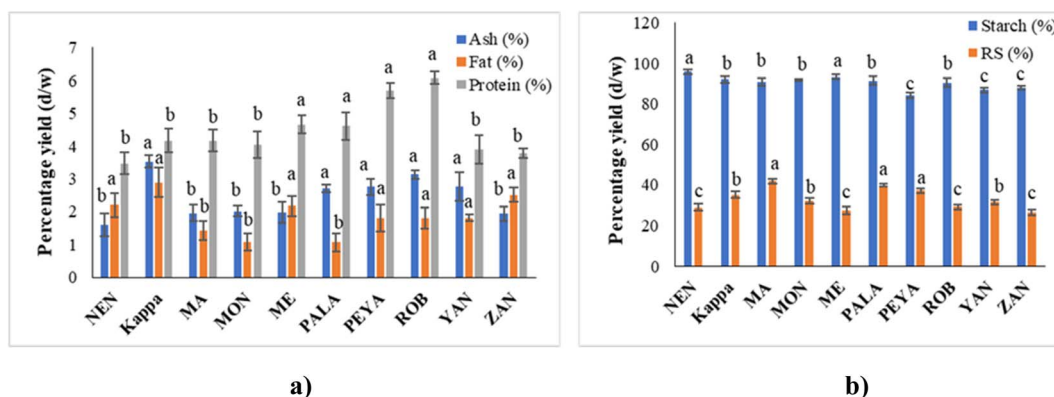


Fig. 2 Proximate composition (a), and starch and RS yield (b) of banana cultivars (% d/w). Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).



more prone to oxidation than others like PALA, influencing storage and processing decisions.

Protein content also showed variability, with NEN at the lowest ($3.47 \pm 0.63\%$) and ROB at the highest ($6.08 \pm 0.19\%$). These figures align closely with findings from Kumar *et al.*³⁰ who reported protein contents for Monthan and Nendran that are similar to those observed in our study. Also, Khoza *et al.*³¹ reported the raw banana flour protein content within the range of the current study. Given that protein content can affect the nutritional quality of food products, cultivars like ROB could be more suitable for protein-enrichment purposes, while NEN might be preferred in situations where lower protein content is beneficial.

Proximate analysis of these banana cultivars reveals crucial differences in their chemical makeup that could inform both agricultural practices and food processing industries. For instance, selecting specific cultivars for cultivation based on soil mineral content could optimise mineral uptake (ash content), while the choice of cultivar for flour production could be influenced by desired characteristics such as longer shelf life (low fat) or specific nutritional needs (higher or lower protein content). This analysis aids in both understanding the inherent properties of these banana cultivars and utilising them for specialised food production and nutritional approaches.

3.2 Isolation of starch and resistant starch

The percentage yield of starch ranged between 84.30 ± 1.25 and $95.8 \pm 1.32\%$ dry weight (d/w) (Fig. 2b). It can be seen that the NEN variety had the highest and PEYA had the lowest content of starch. A previous study by Babu *et al.*³³ reported 92.62% starch content in the Monthan variety, which is similar to that in the present study (91.60%). Furthermore, the plantain banana harvested from Mexico is reported to have 82.3% starch content.³⁴ Many studies have also reported that raw banana pulp contains up to 60–80% starch.^{35–37} The variation in the starch content of the reported values from those in the current study may be associated with the differences in the isolation process, variety of the cultivars, maturity, and geographical origin.

The inclusion of banana starch can impact the physicochemical and pasting properties of the substance through many mechanisms, including structural interactions, interactions with proteins and lipids, water retention, and flavor.^{38,39} In addition to this, the electrostatic interactions within banana flour have several effects on its physicochemical properties, like viscosity, gelation, water absorption, texture, and elasticity. These interactions play a crucial role in the development of food products, such as bread, pasta, or gluten-free foods.^{32,40} The unripe banana contains a significant amount of starch that breaks down into sugars during ripening with the aid of ethylene-mediated regulation of starch-degrading enzymes.⁴¹

Unripe bananas are reported to have health benefits due to the high amount of indigestible polysaccharides, mainly RS. Even though many studies have discussed RS from bananas, there are no previous reports on the RS of some of the varieties (YAN, PEYA, ZAN, and ME) that have been discussed in the present study (Fig. 2b). The RS yield varied between $41.78 \pm 1.29\%$ and $26.43 \pm 1.27\%$ d/w, with MA having the highest and

ZAN the lowest yields. Unripe Taiwanese bananas reported a similar yield of RS.³⁵ Prior studies also compared the RS content of peeled and unpeeled stage II cavendish bananas, and the results showed that the RS content was higher for peeled ones (40.16%) than unpeeled ones (33.86%);⁴² this result supports the current study.

A recent investigation by Nguyen Minh *et al.*⁴³ compared the RS content of different starchy food sources like sweet potato, black beans, red kidney beans, mung beans, corn starch, potato starch, and green banana starch and found 48.29% RS in green banana. RS resists the action of digestive enzymes in the small intestine and serves as a substrate for beneficial gut microbiota. Furthermore, RS aids in the lowering of blood cholesterol, postprandial glucose due to the low glycemic index, and in the prevention of colon cancer.⁴⁴ Foods with high RS exhibit functional as well as physiological health benefits. Thus, raw banana flour could be exploited as an ingredient for the formulation of various functional foods.

3.3 Quantification of amino acids (aa) and vitamins using LC-MS/MS

Literature surveys reveal limited research on the amino acid profile of bananas, possibly due to the low protein content in green bananas. According to the US Department of Agriculture, bananas are rich in non-essential amino acids like aspartic acid and glutamic acid.⁴⁵ Unripe bananas, serving as a natural source of phenylalanine, and other essential and non-essential aa contribute to overall dietary intake.⁴⁶ The study confirms bananas' richness in histidine compared to other fruits.⁴² The study examined 22 aa in the investigated banana cultivars (Table 1), revealing phenylalanine as the predominant amino acid in all the variants studied, except for Kappa and YAN.

Phenylalanine dominated in NEN ($204.17 \pm 0.025 \mu\text{g mL}^{-1}$), followed by arginine ($64.65 \pm 0.005 \mu\text{g mL}^{-1}$). In Kappa, histidine was the prominent aa ($98.26 \pm 0.006 \mu\text{g mL}^{-1}$), followed by phenylalanine ($66.06 \pm 0.014 \mu\text{g mL}^{-1}$). MA featured the highest phenylalanine ($366.17 \pm 0.008 \mu\text{g mL}^{-1}$) and lysine content ($64.04 \pm 0.005 \mu\text{g mL}^{-1}$). MON displayed amino acids in the order phenylalanine > glutamine > lysine > arginine > histidine, with $162.42 \pm 0.004 \mu\text{g mL}^{-1}$ phenylalanine. ME showed the highest phenylalanine ($96.45 \pm 0.055 \mu\text{g mL}^{-1}$) and histidine content ($79.72 \pm 0.008 \mu\text{g mL}^{-1}$). PALA had phenylalanine at $241.16 \pm 0.020 \mu\text{g mL}^{-1}$ and histidine at $108.06 \pm 0.044 \mu\text{g mL}^{-1}$. PEYA topped with phenylalanine ($495.67 \pm 0.011 \mu\text{g mL}^{-1}$), glutamine ($345.94 \pm 0.004 \mu\text{g mL}^{-1}$), and lysine ($246.72 \pm 0.004 \mu\text{g mL}^{-1}$). In ROB, histidine content ($142.57 \pm 0.003 \mu\text{g mL}^{-1}$) exceeded others, while YAN had the highest histidine content at $73.42 \pm 0.021 \mu\text{g mL}^{-1}$. In ZAN, amino acids were ranked as phenylalanine > arginine > glutamine > lysine > histidine. Representative LC-MS/MS chromatograms of the standard and sample are shown in ESI Fig. 1a and b,† and ion chromatograms in ESI Fig. 2A and B.† Amino acids play vital roles as biocatalysts, nutrients, cell constituents, and neurotransmitters.⁴⁷ Prior studies on vegetables like carrots, sweet potatoes, peas, cabbage, spinach, and komatsuna found higher levels of free amino acids, particularly asparagine and





Table 1 Amino acid quantification in ethanolic extracts of cultivars^a

Amino acids	Concentration in the samples (µg mL ⁻¹)									
	NEN	Kappa	MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN
Tryptophan	3.10 ± 0.002 ^h	5.29 ± 0.001 ^f	6.98 ± 0.072 ^e	9.64 ± 0.005 ^d	3.31 ± 0.007 ^g	12.26 ± 0.003 ^c	12.97 ± 0.002 ^b	20.20 ± 0.001 ^a	1.40 ± 0.003 ⁱ	3.04 ± 0.006 ^h
Serine	6.89 ± 0.006 ^d	5.11 ± 0.007 ^f	7.10 ± 0.002 ^d	5.11 ± 0.003 ^f	4.77 ± 0.001 ^g	7.55 ± 0.008 ^c	15.02 ± 0.043 ^a	10.31 ± 0.002 ^b	5.75 ± 0.002 ^e	7.43 ± 0.004 ^e
Leucine	1.03 ± 0.001 ^f	2.19 ± 0.004 ^c	1.44 ± 0.009 ^e	1.08 ± 0.003 ^f	1.03 ± 0.006 ^f	2.74 ± 0.012 ^b	1.84 ± 0.007 ^d	6.37 ± 0.032 ^a	1.16 ± 0.036 ^f	0.98 ± 0.009 ^f
Histidine	36.46 ± 0.004 ⁱ	98.26 ± 0.006 ^c	37.89 ± 0.002 ^h	47.92 ± 0.011 ^g	79.72 ± 0.008 ^d	108.06 ± 0.044 ^b	52.88 ± 0.014 ^f	142.57 ± 0.003 ^a	73.42 ± 0.021 ^e	37.88 ± 0.023 ^h
Asparagine	5.44 ± 0.002 ^f	5.95 ± 0.005 ^e	6.29 ± 0.016 ^d	3.58 ± 0.003 ⁱ	4.61 ± 0.006 ^g	8.45 ± 0.018 ^b	6.91 ± 0.009 ^c	31.52 ± 0.001 ^a	2.32 ± 0.092 ^j	3.85 ± 0.007 ^h
Cystine	0.38 ± 0.011 ^e	0.41 ± 0.014 ^d	0.68 ± 0.004 ^b	0.24 ± 0.023 ^h	0.31 ± 0.006 ^g	0.45 ± 0.001 ^c	0.98 ± 0.002 ^a	0.20 ± 0.006 ⁱ	0.16 ± 0.004 ^j	0.36 ± 0.009 ^f
Threonine	2.25 ± 0.005 ^g	3.05 ± 0.003 ^d	2.45 ± 0.039 ^f	2.00 ± 0.036 ^h	1.96 ± 0.002 ^h	4.39 ± 0.017 ^c	5.19 ± 0.008 ^b	8.22 ± 0.023 ^a	1.64 ± 0.003 ⁱ	2.99 ± 0.002 ^e
Valine	1.93 ± 0.006 ^f	2.03 ± 0.053 ^e	2.54 ± 0.002 ^d	1.64 ± 0.007 ^h	1.73 ± 0.016 ^g	2.90 ± 0.004 ^c	4.30 ± 0.008 ^b	5.003 ± 0.033 ^a	1.63 ± 0.014 ^h	1.75 ± 0.005 ^g
Phenylalanine	204.17 ± 0.025 ^e	66.06 ± 0.014 ^j	366.17 ± 0.008 ^b	162.42 ± 0.004 ^g	96.45 ± 0.055 ^h	241.16 ± 0.020 ^d	495.67 ± 0.011 ^a	352.49 ± 0.006 ^c	72.65 ± 0.005 ⁱ	177.00 ± 0.003 ^f
Glutamic acid	1.002 ± 0.008 ^f	1.15 ± 0.001 ^e	2.06 ± 0.036 ^c	2.17 ± 0.006 ^b	1.12 ± 0.007 ^e	2.06 ± 0.023 ^c	7.24 ± 0.008 ^a	2.06 ± 0.014 ^c	1.04 ± 0.025 ^f	1.62 ± 0.006 ^d
Glycine	0.68 ± 0.002 ^e	0.69 ± 0.006 ^e	1.03 ± 0.016 ^d	0.704 ± 0.001 ^e	0.67 ± 0.088 ^e	1.32 ± 0.002 ^c	1.83 ± 0.004 ^b	2.41 ± 0.045 ^a	0.45 ± 0.019 ^f	0.93 ± 0.076 ^d
Proline	1.52 ± 0.036 ^g	1.11 ± 0.016 ^j	2.35 ± 0.005 ^d	2.15 ± 0.001 ^e	1.48 ± 0.005 ^h	3.59 ± 0.022 ^c	5.97 ± 0.025 ^a	5.46 ± 0.006 ^b	1.20 ± 0.004 ⁱ	1.64 ± 0.002 ^f
Aspartic acid	5.39 ± 0.008 ^h	4.95 ± 0.004 ⁱ	4.48 ± 0.003 ^j	13.52 ± 0.009 ^c	5.88 ± 0.016 ^g	8.53 ± 0.003 ^e	36.85 ± 0.064 ^a	15.70 ± 0.002 ^b	10.19 ± 0.013 ^d	7.39 ± 0.001 ^f
Glutamine	38.11 ± 0.005 ^h	41.61 ± 0.016 ^g	88.99 ± 0.032 ^e	99.01 ± 0.046 ^c	35.93 ± 0.025 ⁱ	95.510 ± 0.009 ^d	345.94 ± 0.004 ^a	118.83 ± 0.012 ^b	29.13 ± 0.003 ^j	54.45 ± 0.008 ^f
Lysine	27.74 ± 0.012 ^h	30.99 ± 0.002 ^g	64.04 ± 0.005 ^e	71.77 ± 0.017 ^c	25.96 ± 0.004 ⁱ	69.75 ± 0.077 ^d	246.72 ± 0.064 ^a	87.10 ± 0.002 ^b	21.22 ± 0.005 ^j	39.47 ± 0.003 ^f
Tyrosine	1.53 ± 0.021 ^g	2.07 ± 0.005 ^d	1.77 ± 0.001 ^f	1.97 ± 0.006 ^e	0.87 ± 0.071 ^j	3.00 ± 0.002 ^a	2.37 ± 0.007 ^b	2.31 ± 0.008 ^c	1.29 ± 0.009 ⁱ	1.37 ± 0.001 ^h
Isoleucine	1.12 ± 0.032 ^h	2.45 ± 0.004 ^c	1.59 ± 0.009 ^e	1.26 ± 0.006 ^f	1.17 ± 0.033 ^g	3.19 ± 0.002 ^b	2.28 ± 0.009 ^d	7.38 ± 0.003 ^a	1.25 ± 0.005 ^f	1.11 ± 0.029 ^h
Arginine	64.65 ± 0.005 ^g	33.48 ± 0.002 ⁱ	23.29 ± 0.032 ^j	70.68 ± 0.004 ^d	44.71 ± 0.015 ^h	75.64 ± 0.004 ^c	64.97 ± 0.007 ^f	144.18 ± 0.041 ^a	66.10 ± 0.037 ^e	116.91 ± 0.001 ^b
Hydroxyproline	1.00 ± 0.014 ^f	2.19 ± 0.064 ^c	1.44 ± 0.003 ^e	1.10 ± 0.052 ^f	1.07 ± 0.099 ^f	2.74 ± 0.001 ^b	1.95 ± 0.004 ^d	6.37 ± 0.009 ^a	1.12 ± 0.003 ^f	0.99 ± 0.002 ^f
Cysteine	1.200 ± 0.003 ^e	1.570 ± 0.024 ^d	1.630 ± 0.007 ^c	0.803 ± 0.008 ^g	1.180 ± 0.062 ^e	1.850 ± 0.017 ^a	1.650 ± 0.003 ^c	1.790 ± 0.002 ^b	1.20 ± 0.005 ^e	1.130 ± 0.001 ^f
Alanine	0.51 ± 0.005 ^e	0.14 ± 0.023 ^g	0.58 ± 0.006 ^e	0.46 ± 0.024 ^e	0.26 ± 0.006 ^f	0.86 ± 0.002 ^d	1.74 ± 0.081 ^b	2.05 ± 0.004 ^a	0.28 ± 0.008 ^f	1.07 ± 0.074 ^e

^a Values are mean ± SD; they are followed by different alphabets in each row which indicate significantly different values ($p < 0.05$). The letter 'a' represents the highest value.

Table 2 Vitamin quantification in ethanolic extracts of cultivars^a

Vitamins	Concentration in the samples ($\mu\text{g mL}^{-1}$)									
	NEN	Kappa	MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN
Thiamine	0.007 ± 0.013^a	—	—	—	0.005 ± 0.007^a	—	—	—	—	0.002 ± 0.076^a
Pyridoxine	0.060 ± 0.001^c	0.790 ± 0.065^a	0.220 ± 0.002^c	0.0610 ± 0.013^c	0.190 ± 0.009^d	0.180 ± 0.006^d	0.078 ± 0.024^c	0.307 ± 0.006^b	0.240 ± 0.005^c	0.164 ± 0.002^d
Folic acid	0.206 ± 0.011^c	0.860 ± 0.051^a	0.018 ± 0.007^h	0.140 ± 0.004^d	—	0.090 ± 0.081^c	0.480 ± 0.003^b	0.068 ± 0.007^f	—	0.054 ± 0.004^g
Nicotinic acid	0.440 ± 0.090^e	0.640 ± 0.007^c	0.730 ± 0.005^a	0.280 ± 0.009^h	0.340 ± 0.008^g	0.640 ± 0.016^c	0.430 ± 0.025^c	0.700 ± 0.005^b	0.601 ± 0.015^d	0.370 ± 0.017^f
Riboflavin	0.150 ± 0.006^a	—	—	—	—	—	—	—	—	0.130 ± 0.008^b
Ascorbic acid	0.280 ± 0.032^d	0.210 ± 0.009^f	0.770 ± 0.067^a	0.307 ± 0.005^c	0.240 ± 0.045^c	0.240 ± 0.076^c	0.360 ± 0.007^b	0.082 ± 0.009^h	0.360 ± 0.008^b	0.180 ± 0.014^g

^a Values are mean $\pm$ SD; they are followed by different alphabets in each row which indicate significantly different values ($p < 0.05$). The letter 'a' represents the highest value.

glutamine.⁴⁸ Examining wild fruits in Assam revealed a high content of aspartic acid, arginine, glutamic acid, histidine, valine, and leucine, aligning with our findings.⁴⁹ Studies indicate that phenylalanine, tryptophan, and tyrosine are three essential components in plant metabolism that act as precursors of production of aromatic secondary metabolites such as lignin, anthocyanins, flavonoids, and alkaloids. The amount of aromatic amino acids has significant influences on the production of phenolic compounds.^{50,51}

Unripe bananas are reported to be a good source of vitamins B6, C, and provitamin A.⁵² Raw bananas selected under the present study were analysed for water-soluble vitamins (thiamine, pyridoxine, nicotinic acid, folic acid, riboflavin, and ascorbic acid) using LC-MS/MS (Table 2). Folic acid ($0.860 \pm 0.051 \mu\text{g mL}^{-1}$) and pyridoxine ($0.790 \pm 0.065 \mu\text{g mL}^{-1}$) were found to be abandoned in Kappa, while MA had higher ascorbic acid content ($0.770 \pm 0.067 \mu\text{g mL}^{-1}$) and nicotinic acid ($0.730 \pm 0.005 \mu\text{g mL}^{-1}$). All cultivars showed significant amounts of Vit B6 and Vit C. Prior studies noted vitamin C and B complex vitamins (B1, B2, B3, B9, and B6) in raw bananas.^{53–56} It is observed from the available literature that the vitamin C content varies significantly throughout cultivars, with

Cavendish and Dwarf Brazilian levels varying between 4.5 and 12.7 mg per 100 g.⁵² The varieties investigated in the current study displayed lower vitamin C content than that of the reported ones. This difference may be attributed to factors like variety, growing conditions, maturity, and drying conditions. A recent study showed that increased drying temperatures in ripe Nendran bananas resulted in decreased vitamin content.⁵⁷ The present study highlights the nutritional richness of raw bananas, despite variations, offering essential vitamins in a well-balanced diet.⁵⁸ Representative LC-MS/MS chromatograms of the standard and sample are presented in ESI Fig. 3a and b,† and ion chromatograms in ESI Fig. 4A and B.†

3.4 Phytochemical characterisation

3.4.1 Total phenolic and flavonoid content. Phenolic compounds, owing to their free radical-scavenging properties, act as excellent antioxidants.⁵⁹ Hydro-alcoholic extracts of banana flour were analysed for total phenolic content and total flavonoid content. The phenolic content of raw banana flour followed the order MA > PALA > ZAN > Kappa > ME > ROB > PEYA > YAN > NEN > MON, ranging from 50.78 ± 0.15 to 96.14 ± 0.64 mg GAE per 100 g (Fig. 3a). Comparable TPC was observed

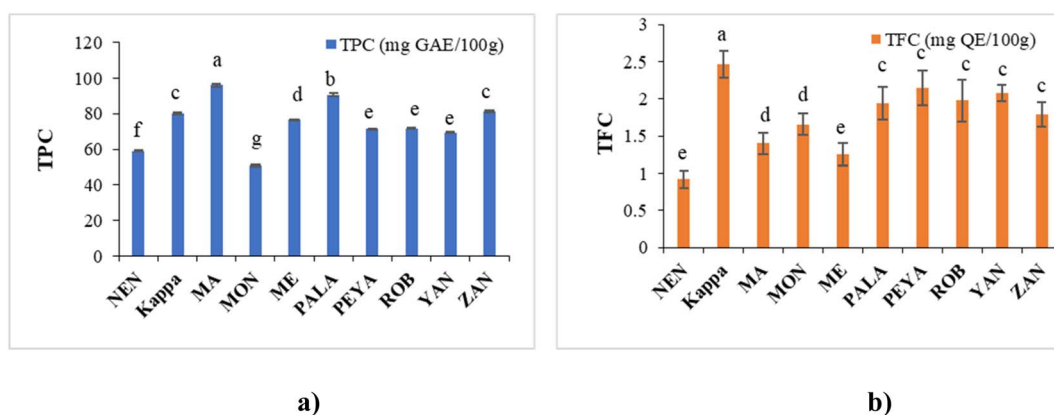


Fig. 3 Phytochemical assays: (a) TPC and (b) TFC of different banana cultivars. Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

in *Musa acuminata* by Menezes *et al.*,⁶⁰ while Kumar *et al.*³⁰ reported 44.50 mg GAE per 100 g TPC in Monthan. Higher TPC in Nendran was recorded earlier; variations in TPC are attributed to genomic content, planting area, or geographical conditions affecting banana varieties.^{10,61}

The TFC in this study ranged from 0.92 ± 1.32 to 2.46 ± 1.88 mg QE per 100 g, in the order Kappa > PEYA > YAN > ROB > PALA > ZAN > MON > MA > ME > NEN (Fig. 3b). Siji and Nandini⁵⁵ reported TFC of 3.58 mg QE per 100 g for Kappa, aligning closely with our study. Suniati and Purnomo⁶² reported comparable TFC for raw Goroho (*Musa acuminata* sp.). A study on Dwarf Cavendish also documented similar findings (1.69 ± 0.34 mg RE per g) in terms of TFC.⁶³ Flavonoid synthesis, stimulated by sunlight, concentrates them in the outer portions of fruits.⁶⁴ The most prevalent polyphenols in our regular diets are flavonoids, which exhibit antioxidant, antiviral, antibacterial, antiallergenic, antithrombotic, anti-inflammatory, and hepatoprotective actions.^{65,66} In banana fruit, soluble flavonoids were shown to have a higher concentration and stronger antioxidant activity than bound flavonoids.⁶⁷ Moreover, flavonoids have a complex structure, which makes them quite poor in bioavailability. Since only small amounts of them can be absorbed by the small intestine, a large amount reaches the large intestine and interacts with intestinal microbes to promote the growth of beneficial microbiota.⁶⁸ Gut microbiota, like *Bifidobacteriaceae*, *Lactobacillaceae*, *Lachnospiraceae*, and *Enterococcaceae*, contribute to flavonoid transformation (O-deglycosylation of flavonoids), whereas other species are involved in the degradation of aglycones or cleaving of flavonoid-C-glucosides.^{69,70} Dietary flavonoid intake can improve gut microbiota regulation, impacting host immunity, inflammation, and diversity of microbiota.⁷¹ The regulation of gut microbes could be associated with regulating short-chain fatty acid metabolism and bile acid synthesis and uptake, potentially promoting bile acid homeostasis.^{72,73} Thus, the phenolic and flavonoid composition established from the present study further unveils the potential application of raw banana flour in functional foods and health food applications.

3.4.2 Antioxidant activity. The antioxidant activity of raw banana flour was studied by DPPH and ABTS free radical scavenging assays and is summarised in Fig. 4a and b. These assays are used to assess the free radical scavenging properties based on spectrophotometric measurements of the DPPH and ABTS concentration changes resulting from the reaction with an antioxidant. The results of the DPPH assay showed that the free radical scavenging potential was higher in ROB and PALA as compared to other varieties. The IC₅₀ values for ROB and PALA were 35.27 ± 0.0062 and 35.39 ± 0.0063 $\mu\text{g mL}^{-1}$, respectively. The minimum DPPH activity shown by Kappa was 48.105 ± 0.0053 $\mu\text{g mL}^{-1}$. A lower IC₅₀ value indicates strong free radical inhibitory activity. The DPPH activity of ethanolic extracts in this study was in agreement with that reported by Siji and Nandini, 2017.⁷⁴ Consistent with the DPPH activity, the ABTS free radical scavenging capacity of ROB (520.74 ± 0.0068 $\mu\text{g mL}^{-1}$) and PALA (832.53 ± 0.0021 $\mu\text{g mL}^{-1}$) was considerably higher than that of others; ME and Kappa had the lowest ABTS activity, 1680.68 ± 0.0024 $\mu\text{g mL}^{-1}$ and 1504.78 ± 0.0084 $\mu\text{g mL}^{-1}$, respectively. The ABTS activity of six Australian banana cultivars (Cavendish, Ladyfinger, Plantain, Ducasse, Monkey, and Red Dacca) was reported in the range of 0.98–2.35 mg AAE per g, and these were close to those of the NEN, MON, YAN, ZAN and PEYA cultivars of our study.⁷⁵ Similar results are reported for crude ethanolic extracts of other plant species wherein the radical scavenging activity increased in a dose dependent manner.^{76–78} It is also reported that TPC and antioxidant activity are positively correlated, and the variation in TPC and TFC is influenced by changes in climate, environmental conditions, and the cultivar.^{79–81}

3.5 Quantification of polyphenols

TPC analysis highlighted raw bananas as excellent sources of phenolic compounds. LC-MS/MS is generally used for the quantitative estimation of phytochemicals in plant-based extracts.^{82–84} In the present study, we have employed the technique to analyse 28 polyphenolic compounds in raw banana flour extracts.

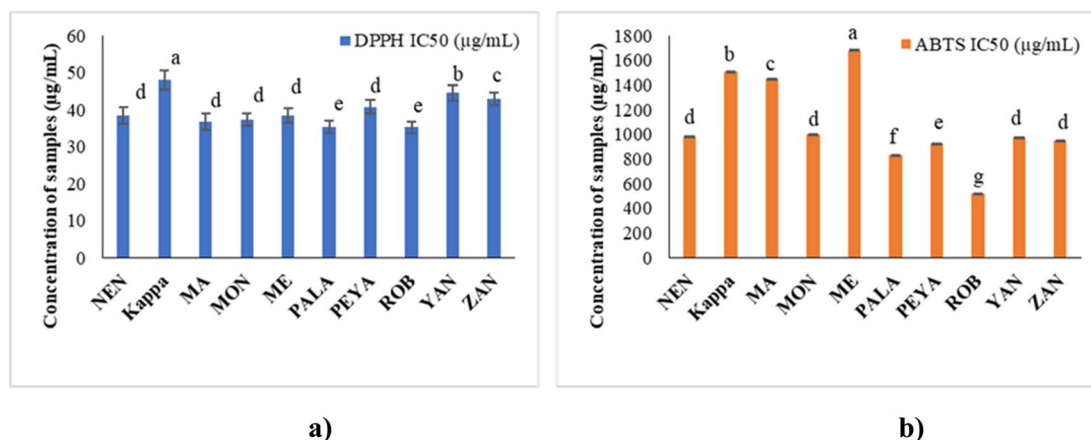


Fig. 4 Antioxidant assays: (a) DPPH and (b) ABTS activity of different banana cultivars. Data presented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).



The LC-MS/MS analysis unveiled the polyphenol profile in various banana cultivars, with shikimic acid, epicatechin, ferulic acid, and rutin as predominant ones (Table 3). NEN demonstrated significant content of polyphenols, with shikimic acid ($78.820 \pm 0.0021 \mu\text{g mL}^{-1}$) and ferulic acid ($3.490 \pm 0.0076 \mu\text{g mL}^{-1}$) as the major ones. Kappa was rich in shikimic acid ($118.940 \pm 0.0042 \mu\text{g mL}^{-1}$) and ferulic acid ($4.120 \pm 0.0032 \mu\text{g mL}^{-1}$). MA contained shikimic acid ($63.490 \pm 0.0078 \mu\text{g mL}^{-1}$) and gallic acid ($3.630 \pm 0.0031 \mu\text{g mL}^{-1}$). MON had shikimic acid ($49.100 \pm 0.0015 \mu\text{g mL}^{-1}$). Shikimic acid ($81.23 \pm 0.0019 \mu\text{g mL}^{-1}$) and ferulic acid ($1.720 \pm 0.0071 \mu\text{g mL}^{-1}$) were the

major phytochemicals in ME. PALA excelled with abundant shikimic acid ($133.380 \pm 0.0013 \mu\text{g mL}^{-1}$) and notable amounts of ferulic acid ($3.820 \pm 0.008 \mu\text{g mL}^{-1}$). Shikimic acid ($65.130 \pm 0.0042 \mu\text{g mL}^{-1}$) and epicatechin (5.830 ± 0.0016) topped in PEYA. ROB featured the highest ferulic acid ($19.430 \pm 0.0023 \mu\text{g mL}^{-1}$) among the cultivars and the highest shikimic acid ($109.180 \pm 0.0065 \mu\text{g mL}^{-1}$). The phytochemical profile in YAN followed the order shikimic acid > ferulic acid > epicatechin, with a shikimic acid content of $107.730 \pm 0.0012 \mu\text{g mL}^{-1}$. Shikimic acid ($80.50 \pm 0.0017 \mu\text{g mL}^{-1}$) and ferulic acid ($2.8360 \pm 0.0033 \mu\text{g mL}^{-1}$) were the major compounds in ZAN.

Table 3 Polyphenol quantification in ethanolic extracts of cultivars^a

Compounds	Concentration in the samples ($\mu\text{g mL}^{-1}$)									
	NEN	Kappa	MA	MON	ME	PALA	PEYA	ROB	YAN	ZAN
Catechin	1.220 ± 0.0011^e	2.280 ± 0.0022^b	1.040 ± 0.0065^f	1.040 ± 0.0054^f	0.510 ± 0.0033^g	1.520 ± 0.0012^c	3.470 ± 0.0021^a	1.380 ± 0.0064^d	0.260 ± 0.0070^h	1.530 ± 0.0052^c
Quinine	0.022 ± 0.0032^b	0.021 ± 0.0063^b	0.011 ± 0.0032^d	0.007 ± 0.0076^e	0.020 ± 0.0023^b	0.023 ± 0.0054^a	0.013 ± 0.0033^d	0.016 ± 0.0018^c	0.005 ± 0.0042^e	0.006 ± 0.0015^e
Naringenin	1.360 ± 0.0055^d	2.140 ± 0.0085^a	1.260 ± 0.0023^e	1.010 ± 0.0011^g	1.110 ± 0.0051^f	0.890 ± 0.0040^h	1.530 ± 0.0013^c	0.980 ± 0.0063^g	0.840 ± 0.0056^i	1.650 ± 0.0012^b
Tocopherol	0.088 ± 0.0057^c	0.130 ± 0.0044^b	0.160 ± 0.0034^a	0.097 ± 0.0066^c	0.095 ± 0.0076^c	0.120 ± 0.0011^b	0.120 ± 0.0025^b	0.100 ± 0.0098^c	0.110 ± 0.0084^c	0.091 ± 0.0067^c
Gallic acid	0.570 ± 0.0121^g	0.950 ± 0.0052^d	3.630 ± 0.0031^a	0.730 ± 0.0011^e	0.610 ± 0.0060^f	1.610 ± 0.0054^c	1.680 ± 0.0010^b	0.380 ± 0.0023^i	0.490 ± 0.0056^h	0.500 ± 0.0032^h
Chlorogenic acid	0.047 ± 0.0225^b	0.044 ± 0.0011^b	0.005 ± 0.0040^e	0.028 ± 0.0022^c	0.021 ± 0.0036^c	0.013 ± 0.0045^d	0.034 ± 0.0014^c	0.005 ± 0.0017^e	0.026 ± 0.0113^c	0.061 ± 0.0055^a
Epicatechin	1.750 ± 0.0064^g	2.590 ± 0.0017^d	3.200 ± 0.0134^b	2.160 ± 0.0012^f	1.290 ± 0.0046^h	3.100 ± 0.0020^c	5.830 ± 0.0016^a	2.400 ± 0.0018^e	1.120 ± 0.0021^i	1.730 ± 0.0042^g
Syringic acid	0.390 ± 0.0414^d	—	0.120 ± 0.0051^e	1.530 ± 0.0013^b	0.008 ± 0.0011^g	0.130 ± 0.0025^e	1.600 ± 0.0050^a	0.020 ± 0.0022^f	—	0.800 ± 0.0048^c
Vanillic acid	0.680 ± 0.0034^e	0.640 ± 0.0021^f	1.800 ± 0.0019^b	1.430 ± 0.0083^c	0.370 ± 0.0064^h	1.400 ± 0.0056^d	2.690 ± 0.0051^a	0.670 ± 0.0017^e	0.370 ± 0.0014^h	0.610 ± 0.0015^g
Caffeic acid	0.270 ± 0.0061^e	0.530 ± 0.0036^a	0.260 ± 0.0076^f	0.520 ± 0.0017^b	0.240 ± 0.0019^g	0.470 ± 0.0026^c	0.290 ± 0.0021^d	0.190 ± 0.0015^i	0.260 ± 0.0028^f	0.230 ± 0.0022^h
Epigallocatechin	0.012 ± 0.0052^c	0.018 ± 0.0020^b	0.021 ± 0.0114^a	0.013 ± 0.0066^c	0.013 ± 0.0026^c	0.018 ± 0.0020^b	0.018 ± 0.0097^b	0.019 ± 0.0037^b	0.021 ± 0.0017^a	0.014 ± 0.0022^c
Ferulic acid	3.490 ± 0.0076^d	4.120 ± 0.0032^b	1.890 ± 0.0086^h	2.180 ± 0.0014^g	1.720 ± 0.0071^i	3.820 ± 0.0087^c	1.520 ± 0.0021^j	19.430 ± 0.0023^a	2.500 ± 0.0045^f	2.860 ± 0.0033^c
Myricetin	0.370 ± 0.0016^g	0.600 ± 0.0019^e	1.260 ± 0.0010^a	0.240 ± 0.0044^h	0.170 ± 0.0035^j	0.780 ± 0.0012^d	0.790 ± 0.0073^c	0.950 ± 0.0017^b	0.180 ± 0.0034^i	0.530 ± 0.0028^f
<i>p</i> -Coumaric acid	0.460 ± 0.0081^d	0.210 ± 0.0023^h	0.920 ± 0.0011^a	0.490 ± 0.0034^c	0.170 ± 0.0072^i	0.340 ± 0.0081^e	0.750 ± 0.0024^b	0.240 ± 0.0055^g	0.290 ± 0.0012^f	0.350 ± 0.0016^e
Apigenin	0.013 ± 0.0049^b	0.018 ± 0.0032^a	0.017 ± 0.0065^a	0.015 ± 0.0006^b	0.012 ± 0.0016^b	0.018 ± 0.0046^a	0.017 ± 0.0010^a	0.017 ± 0.0023^a	0.019 ± 0.0042^a	0.019 ± 0.0016^a
Kaempferol	0.026 ± 0.0032^c	0.065 ± 0.010^a	0.040 ± 0.0020^c	0.052 ± 0.0050^b	0.025 ± 0.0031^e	0.037 ± 0.0003^c	0.030 ± 0.0038^d	0.050 ± 0.0007^b	0.018 ± 0.0011^f	0.052 ± 0.0018^b
Rutin	2.590 ± 0.0011^c	2.650 ± 0.0040^b	1.720 ± 0.0056^d	1.170 ± 0.0077^g	0.550 ± 0.001^i	1.540 ± 0.0010^e	4.350 ± 0.021^a	1.250 ± 0.0027^f	0.790 ± 0.0047^h	2.590 ± 0.0073^c
Hesperetin	0.016 ± 0.0010^b	0.025 ± 0.0015^a	0.025 ± 0.0044^a	0.016 ± 0.0025^b	—	0.024 ± 0.0036^a	0.026 ± 0.0021^a	0.027 ± 0.0014^a	0.024 ± 0.0087^a	0.018 ± 0.0022^b
Shikimic acid	78.820 ± 0.0021^g	118.940 ± 0.0042^b	63.490 ± 0.0078^i	49.100 ± 0.0015^j	81.230 ± 0.0019^e	133.380 ± 0.0013^a	65.130 ± 0.0042^h	109.180 ± 0.0065^c	107.730 ± 0.0012^d	80.500 ± 0.0017^f
Ellagic acid	—	0.410 ± 0.0112^b	0.220 ± 0.0064^c	0.042 ± 0.0045^e	0.160 ± 0.0014^d	—	—	0.600 ± 0.0031^a	—	—
Genistein	0.011 ± 0.0024^b	0.018 ± 0.0025^a	0.019 ± 0.0076^a	0.012 ± 0.0010^b	0.011 ± 0.0086^b	0.019 ± 0.0043^a	0.017 ± 0.0032^a	0.020 ± 0.0015^a	0.020 ± 0.0054^a	0.011 ± 0.0044^b
Cinnamic acid	0.410 ± 0.0132^d	0.390 ± 0.0045^e	0.850 ± 0.0025^a	0.420 ± 0.0016^d	0.140 ± 0.0063^g	0.140 ± 0.0059^g	0.310 ± 0.0047^f	0.640 ± 0.0043^b	0.480 ± 0.0033^c	0.160 ± 0.0034^f

^a Values are mean $\pm$ SD; they are followed by different alphabets in each row which indicate significantly different values ($p < 0.05$). The letter 'a' represents the highest value.



Representative LC-MS/MS chromatograms of the standard and sample are shown in ESI Fig. 5a and b,[†] and ion chromatograms in ESI Fig. 6A and B.[†]

When assessing the polyphenol component of all banana cultivars, it was noted that shikimic acid was the predominant compound, followed by ferulic acid and flavonoids including rutin, epicatechin, and catechin. Earlier studies reported that unripe fruit pulp contains shikimic acid.⁸⁵ In addition to the above-mentioned polyphenols, lower concentrations of compounds like naringenin, vanillic acid, myricetin, syringic acid, and gallic acid were also found in some banana cultivars. Rutin, myricetin, naringenin, gallic acid, and ellagic acid were reported earlier in bananas.⁸⁶ An earlier study of the phenolic and flavonoid contents in unripe and ripe Australian-grown banana types revealed the presence of *p*-hydroxybenzoic acid, quercetin, gallic acid, syringic acid, diosmin, chlorogenic acid, *p*-coumaric acid, protocatechuic acid, quercetin-3-rhamnoside, and kaempferol.⁷⁵ In another study, the pulp and peel of banana cultivars were found to contain ferulic acid, ferulic acid-hexoside, epicatechin, caffeic acid-hexoside, sinapic acid, hydroxycinnamic acid, myricetin-deoxyhexose, rutin, quercetin-deoxyhexose, kaempferol-3-*O*-rutoside, and other unidentified compounds.⁸⁷ The dried pulp powder of *Musa balbisiana* has been found to contain epicatechin, catechin, rutin, quercetin, chlorogenic acid, kaempferol 3-*O*-sophoroside, apigenin-6-*C*-glucoside-7-*O*-glucoside, and rhamnopyranoside.⁸⁸ Rutin, catechin, quinic acid, galocatechin, propionic acid, caffeic acid-3-glucoside, kaempferol-*O*-rhamnosidehexoside, caffeic acid, *L*-dopa, sennoside A, and dopamine have been identified in the hydro-alcoholic extract of *Musa Cavendish* with anticancerous activity.⁸⁹ These findings aligned with the current study on certain phenolics. Despite numerous studies conducted in this field, there is still a gap in the quantification of phenolics due to their complex structures and the way environmental factors affect the secondary metabolite levels, which differ between cultivars.

3.6 Cytotoxicity assay

The MTT assay investigated the cytotoxic effects of raw banana flour extracts on L6 myoblasts, revealing no impact on cell viability up to 500 $\mu\text{g mL}^{-1}$. The cell lines treated with different concentrations showed above 70% cell viability. Among the flour extracts,

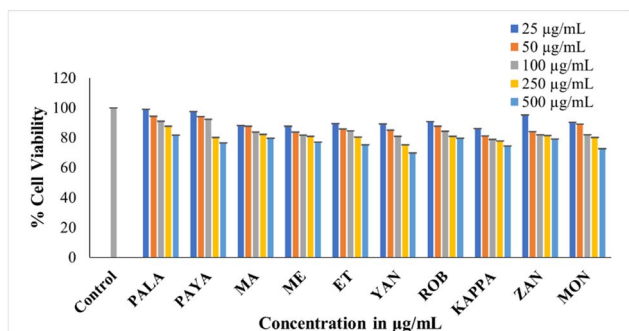


Fig. 5 MTT assay showing the cytotoxicity of the ethanolic extract of ten cultivars against L6 cells. Data presented are mean value $\pm$ SD.

PALA (81.78%) and PEYA (79.62%) exhibited higher viability, while YAN (69.83%) showed lower viability compared to other cultivars at 500 $\mu\text{g mL}^{-1}$ (Fig. 5). Many previous studies have reported the poor cytotoxicity of banana extracts against normal cell lines. Dahham *et al.*⁹⁰ tested the cytotoxic activity of *Musa sapientum* fruit extract against the normal human umbilical vein endothelial cell line (HUVEC) and the human colorectal carcinoma cell line HCT-116. The result revealed that the banana extracts showed lower cytotoxicity against normal cell lines than cancer cells. Extracts from 20 different fruit extracts were analysed for cytotoxicity against leukemic cell lines and normal human peripheral blood mononuclear cells (PBMCs). Some of these extracts exhibited cytotoxicity against both normal and leukemic cell lines, but some extracts specifically showed toxicity against leukemic cell lines, and others did not show any toxicity. Among these, the *Musa sapientum* L. extracts presented no cytotoxic effect on normal PBMC cell lines.⁹¹ The non-toxic nature of the extracts indicates that they could be used as an ingredient or additive without causing harm to normal cells. The lower cytotoxicity implies that bananas could be considered safe for a wide range of applications, particularly in the food and pharmaceutical industries.

3.7 α -Amylase inhibition assay

Hyperglycemia results from the rapid breakdown of starchy meals by carbohydrate-hydrolyzing enzymes such as α -amylase and α -glucosidase. The therapeutic approach for diabetes involves synthetic inhibitors of α -amylase to impede glucose absorption, which is reported to cause undesirable side effects.⁹² Many dietary bioactive components are reported to inhibit these enzymes. Banana extracts (200 $\mu\text{g mL}^{-1}$) and acarbose (100 $\mu\text{g mL}^{-1}$) were evaluated against PPA. The findings demonstrate that the α -amylase inhibitory activity of flour extracts varied across samples, with PALA and ROB with higher inhibitory potential (66.96% and 50.50%) (Fig. 6). The standard acarbose showed 76.84% inhibition. The results are in agreement with previous reports on *Musa paradisiaca*'s antidiabetic potential.⁹³ The α -amylase inhibitory activity may be correlated with the bioactive phytochemicals, in line with previous reports.^{94,95} The results could have implications for managing

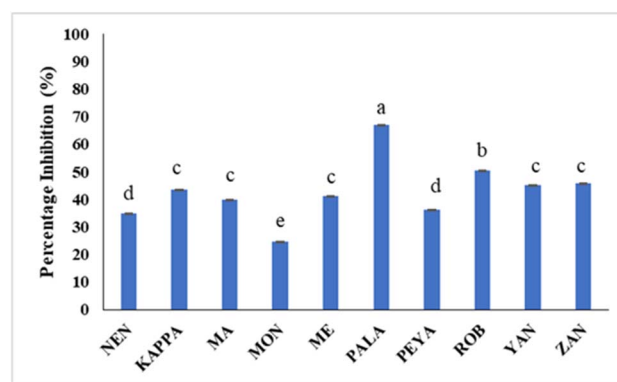
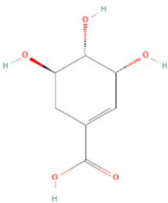
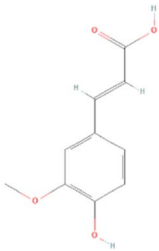
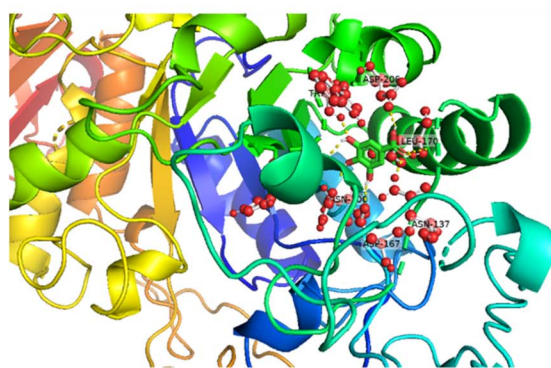


Fig. 6 The percentage inhibition of PPA by different banana cultivars. Data represented are mean value $\pm$ SD, while data bars with different alphabets show significant differences ($p < 0.05$).

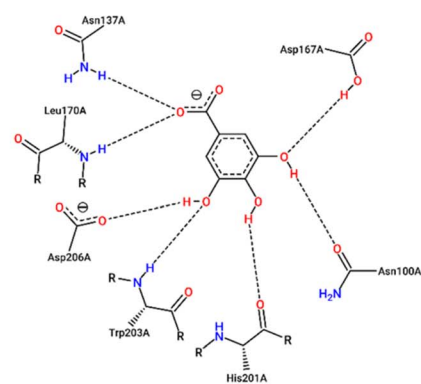


Table 4 Molecular docking results of banana cultivars phenolics towards α -amylase (PDB ID: 4W93)

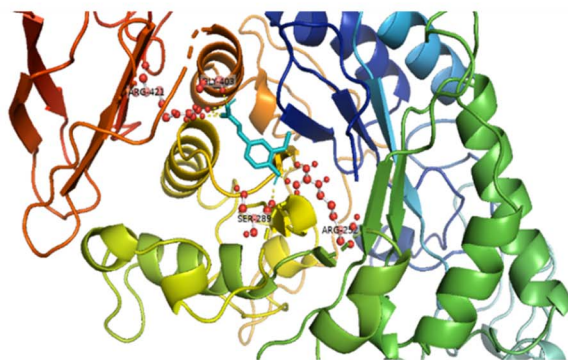
Ligands	PubChem CID	Structure	H-bonded residues	Binding energy (kcal mol ⁻¹)
Shikimic acid	8742		Asn137 Leu170 Asp206 Trp203 His201 Asn167 Asn100	−5.45
Ferulic acid	445 858		Arg421 Gly403 Arg252 Ser289	−5.48



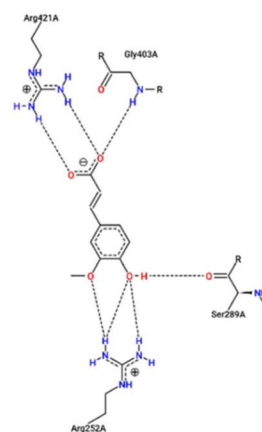
a)



b)



d)



c)

Fig. 7 Molecular docking for shikimic acid and ferulic acid with α -amylase amino acid residues; (a and b) 3D & 2D interaction image of shikimic acid; (c and d) 3D & 2D interaction image of ferulic acid.

postprandial blood sugar levels, making them potentially useful in the dietary management of diabetes.

3.8 Molecular docking studies

According to LCMS/MS analysis, shikimic acid and ferulic acid were identified as the major phytochemicals in the raw banana flour. Both shikimic acid and ferulic acid are reported to have hypoglycemic activity due to their inhibition potential of α -amylase.^{96,97} We adopted molecular docking to correlate the same. The docking analysis identified the optimal poses for both, with the lowest energies at -5.45 and -5.48 kcal mol⁻¹ respectively, for shikimic acid and ferulic acid, suggesting high binding affinity. Table 4 and Fig. 7 illustrate the hydrogen bond residues involved in their interactions with α -amylase. Earlier studies reported a binding energy of -5.3 kcal mol⁻¹ for the ferulic acid- α -amylase complex.⁹⁷ However, the binding energy was lower compared to acarbose (-8.8 kcal mol⁻¹).⁹⁸ PALA and ROB were found to have higher estimated amounts of ferulic acid and shikimic acid in comparison to the other variants. Thus, the α -amylase inhibitory activity of the banana extracts can be correlated with the presence of phytochemicals such as shikimic acid and ferulic acid, which was confirmed by docking studies.

4 Conclusion

This study delved into the nutritional composition, starch, resistant starch, phytochemicals, antioxidants, and cytotoxicity of 10 raw banana cultivars at stage II maturity. While proximate composition remained comparable across varieties, distinctions in phenolic, flavonoid, and antioxidant potential were noted, reflecting variations in genomic and chemical constituents. LC-MS/MS analysis quantified significant polyphenols (shikimic acid, ferulic acid, epicatechin, and rutin), amino acids (phenylalanine, histidine, glutamine, lysine, and arginine), and vitamins (pyridoxine, ascorbic acid, and nicotinic acid) among cultivars. Unripe banana flour emerged as a notable source of resistant starch, which can play an important role in contributing to gut health. With the presence of bioactive phytochemicals and hypoglycemic properties enriched with resistant starch, there is great scope for the value addition of unripe bananas. The study underscores the potential of raw banana flour as a functional food ingredient, enriching food products with enhanced nutritional properties. The findings present raw banana flour as a viable substitute, offering sustainable and nutritious alternatives for food development. Future studies in the direction of identifying and quantifying the bioactive components in green bananas that are responsible for health benefits and their potential uses as functional ingredients in nutraceuticals and functional foods are warranted. Development of gluten-free products and meal replacements looking into their potential in diabetic management, prebiotic potential and effects on gut microbiota can also be investigated and established by *in vivo* studies. Exploring the untapped potential of these cultivars opens avenues for innovative and sustainable food production, aligning with current trends in health-conscious and environmentally friendly consumer choices.

Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AlCl ₃	Aluminum chloride
CH ₃ COOK	Potassium acetate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESI	Electrospray ionisation
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
I ₂	Iodine
KI	Potassium iodide
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MA	Malayannan
ME	Mysore Ethan
MON	Monthan
MRM	Multiple reaction-monitoring
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaOH	Sodium hydroxide
NEN	Nendran
PALA	Palayankodan
PBMCs	Peripheral blood mononuclear cells
PEYA	Peyan
PPA	Porcine pancreatic amylase
RADD	Refrigerated adsorption dehumidified drier
ROB	Robusta
RS	Resistant starch
SD	Standard deviation
TFC	Total flavonoid content
TPC	Total phenolic content
YAN	Yangambi
ZAN	Zanzibar

Data availability

Data will be made available on request.

Author contributions

SVS: conceptualization, data curation, methodology, investigation, formal analysis, and writing – original draft; AB: investigation, writing – review and editing; AS: investigation; PN: conceptualization, supervision, resources, writing – review and editing.

Conflicts of interest

The authors declare no conflict of interest in this work.

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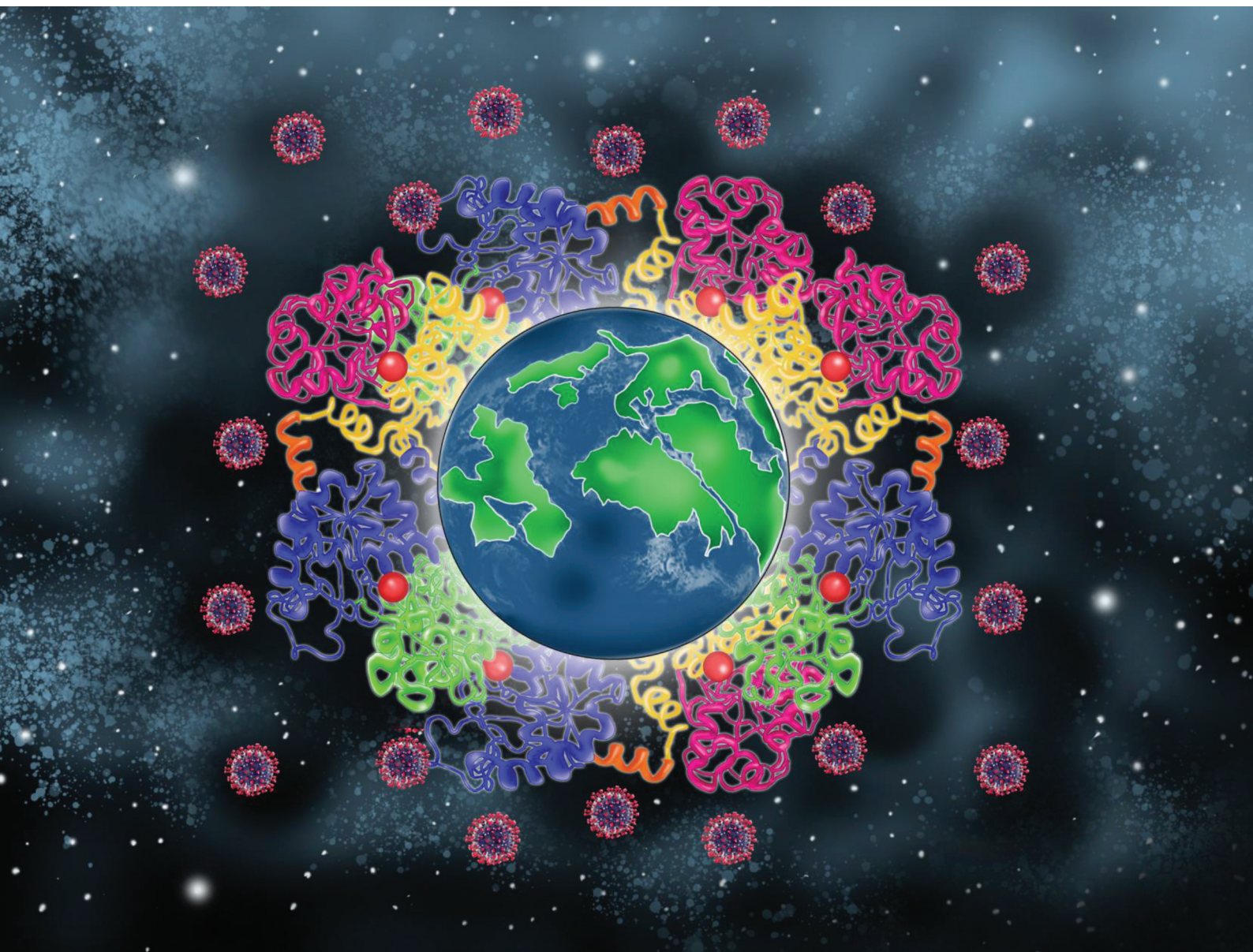


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A comprehensive review on lactoferrin: a natural multifunctional glycoprotein

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Lactoferrin (Lf) is a natural iron-binding globular glycoprotein, present mainly in milk. It maintains human health through its multifunctional activities, including immunomodulation, iron metabolism, and anti-oxidant and prebiotic efficacy. It also shows anti-microbial, anti-fungal, and anti-viral activities against a broad spectrum of viruses, including SARS-CoV-2 that causes COVID-19. In addition, several investigations established that Lf is involved in bone metabolism, neural development, and metabolic disorders. In this review, we summarize the *in vitro* and *in vivo* studies on the health benefits of Lf and its bio-availability. Furthermore, we briefly describe the production, industrial applications and future prospects of Lf.

1. Introduction

Glycoproteins are molecules containing glycans (oligosaccharide chains) covalently linked to side chains of amino acids through a process called glycosylation. Glycosylation mainly occurs on proteins during co-translational or post-translational modification. Mainly, there are two types of glycosylation: *N*-glycosylation, which occurs when the glycans are attached to the nitrogen present in the side chain of arginine or aspara-

gine, and *O*-glycosylation, which occurs at the hydroxyl group of threonine, serine or tyrosine group of proteins. These glycoproteins have multiple physiological functions, such as acting as transport molecules, hormones, enzymes, receptors, structural molecules, lubricating agents, immunological molecules, blood group determinants, and also cell attachment sites. In mammals, glycosylation mainly occurs in the cell membrane and in most secreted proteins. Several glycoproteins are predominantly present in milk, and they play a vital role in the bio-modulatory effects of milk and eventually determine its nutritional quality.¹ Milk is of high nutritious value for infants and adult humans. Milk has a variety of biomolecules – proteins, oligosaccharides, and lipids that perform non-nutritional roles. Milk proteins are mainly divided into caseins, whey pro-

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teins, and mucins; the latter surrounds the milk fat globule membrane. Whey protein contains many proteins, such as immunoglobulins, Lf, α -lactalbumin, lactoperoxidase enzymes, β -lactoglobulin and glycomacropeptides. In human milk, the major part is constituted by whey proteins while in bovine milk casein is predominant.

In human milk, the major glycoproteins present are Lf, secretory immunoglobulins, lactoperoxidase, mucin, lactadherin, leptin, adiponectin, α -lactalbumin and lysozyme. Among these, Lf is a globular glycoprotein, which is the second most abundant whey protein in human milk. In 1939, Lf was identified from bovine milk in its pure form.² The Lf amount in milk differs among mammalian species during the lactation period. In human milk, Lf concentration is usually 2–4 mg mL⁻¹, but it is high in colostrum (6–8 mg mL⁻¹). Lf concentration in bovine milk ranges between 0.02 and 0.2 mg mL⁻¹, and in pig, mouse, and horse milk, it is between 0.2 and 2 mg mL⁻¹. It is also present in exocrine secretions such as tears, saliva, bile, semen vaginal fluids, and pancreas, gastrointestinal tract, nasal and bronchial secretions. In response to inflammatory reactions, secondary neutrophil granules, macrophages, and glandular epithelial cells produce significant amounts of Lf. Lf has multifunctional activities, as shown in Fig. 1, namely immunomodulatory, antimicrobial, antifungal, antiviral, anti-inflammatory, antitumoral, and antioxidant activities. Besides, it modulates gut microbiota growth and iron metabolism, and stimulates osteoblast proliferation and differentiation.³

In recent years, there has been renewed interest in natural compounds. Hence, as an innate substance, Lf is capable of resisting viral infections, and simultaneously, it can maintain inflammatory homeostasis and rebalance iron. Lf has a broad spectrum of antiviral activities against many viruses, such as, severe acute respiratory syndrome coronavirus (SARS-CoV), which is similar to the severe acute respiratory syndrome coro-

navirus 2 (SARS-CoV-2) that caused the recent pandemic coronavirus disease 2019 (COVID-19) outbreak. The clinical outcomes of individuals affected by COVID-19 are severe and may even result in mortality. At present, as a preventative measure, vaccinations are available, but COVID-19 treatments mainly include symptom alleviation methods and use of synthetic non-specific wide-spectrum antiviral drugs. Lf can act as a potent nutraceutical in the treatment of SARS-CoV-2 infection by preventing binding to the surface of the target cell, which could be specifically effective during the phase of viral replication in the salivary glands, throat, and upper respiratory tract.⁵

Thus, due to the current value of Lf in preventing and managing COVID-19, the objective of this article is to review the production, multifunctional properties, and potential clinical and nutritional applications of the natural glycoprotein Lf. This literature review summarizes the recent scientific information regarding this biological molecule and its activities and aims to elucidate the future prospects of Lf in other applications.

2. Structure of lactoferrin

Lf is an iron-binding globular glycoprotein belonging to the transferrin family, also known as lactotransferrin. Its molecular weight is 80 kDa and is composed of about 796 amino acids (aa). Lf shows a great extent of homology between species. Human Lf and bovine Lf have 711aa and 689aa, respectively; they share approximately 70% sequence identity. Lf's tertiary structure has a single chain of polypeptide which is folded into two equal domains – N and C lobes. It consists of α -helical and β -sheet structures, and the domains are covalently linked by a short α -helix. The two domains are further divided into two sub-domains: N1 and N2 and C1 and C2, which form two clefts for each lobe, where the ferric ion is bound. Aspartic acid, histidine, and tyrosine are the essential iron-binding amino acids present in Lf. Each ferric ion is bound tightly to Lf through a carbonate ion. When a ferric ion reversibly binds to Lf, it can exist in the free iron form called apo-Lf, monoferric Lf, and in the bound iron form called holo-Lf. Also, Lf can bind with metal ions like Cu²⁺, Zn²⁺, Mn²⁺ and Al³⁺. Lf is a cationic protein; hence it can bind with anionic biomolecules like DNA, lipopolysaccharide and heparin. Lf is prominently an N-linked glycosylated protein; mannose and N-acetyl glucosamine are the main sugars coupled with it. The gene expression in individuals determines the types of sugars and the number of glycosylation sites in Lf. The difference in glycosylation sites involved in Lf not only affects its susceptibility towards thermal denaturation and proteolysis but also alters its immunogenicity.⁶

3. Synthesis and digestion of lactoferrin

The biosynthesis of Lf is regulated by the type of cells producing it. Lf produced in the mammary gland is determined via



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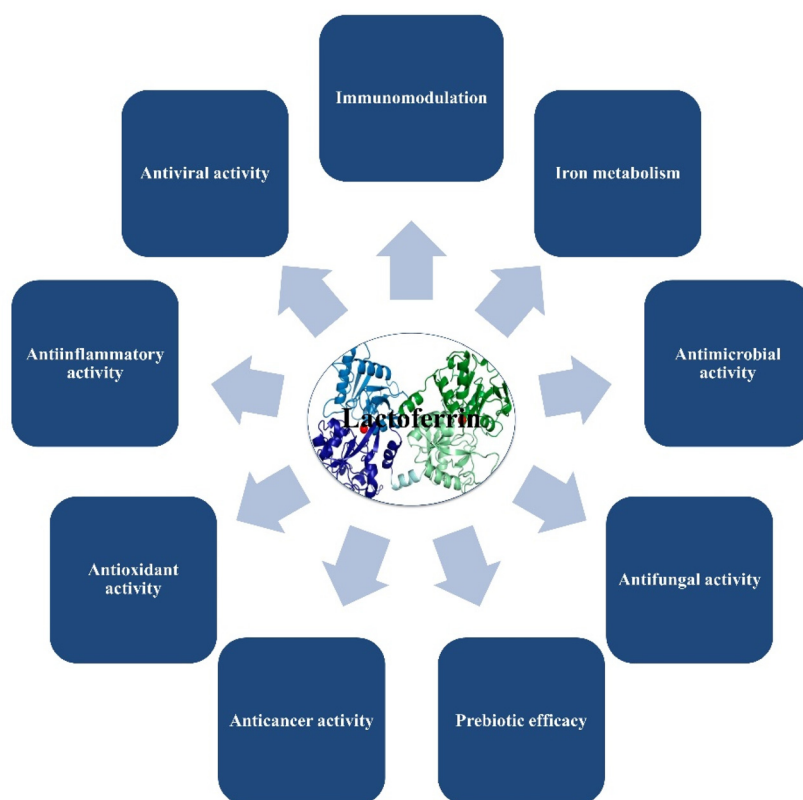


Fig. 1 Physiological functions of lactoferrin (Lf).⁴

prolactin, while the estrogens influence protein production in reproductive tissues. In the endometrium, Lf synthesis is controlled by the estrogens and epidermal growth factor. Lf is synthesized and secreted by exocrine glands in a continuous manner.

Lf production occurs in neutrophils during their differentiation from promyelocytes into myelocytes, and it is stored in particular granules, perhaps in the tertiary granules. Lf is not produced as a large precursor and appears as an unphosphorylated form. Depending on the acidification mechanisms, Lf moves towards its storage granules, and this takes place in the trans and medial cisternae of the Golgi body; hence it is treated like proteins designed for secretion. The Lf present in neutrophil granules has two targets: it can be either released into the blood or nearby tissues, or it can fuse with phagosomes.⁷ The polymorphonuclear cell's secretion into blood circulation is determined by the degranulation factors, that in turn seem to be dependent on the cGMP, protein kinase C and guanylate cyclase activation.

Digestion of food is essential because the body requires nutrients from drinks and food to stay healthy and work well. The digestive system breaks down nutrients like carbohydrates, fats, proteins, minerals, vitamins, and water into smaller subunits adequate for the body to take up and use for energy, growth, and cell repair. The human digestive system consists of three digestive segments: salivary, gastric, and intestinal. In the salivary phase, the breakdown of carbohydrates begins,

while the digestion of protein occurs in the gastric and intestinal phases. Lf is a glycoprotein, digested into peptides and essential amino acids, which are absorbed by the intestine and later transported into the blood. The *in vivo* digestion of Lf in the gastrointestinal (GI) tract is not clearly understood. The degree of iron saturation determines the breakdown of Lf. holoLf is more resistant to digestion than apoLf. Due to the digestion of orally supplemented Lf, its biological activity diminishes in the intestine. However, in a recent study, the systemic activities of intravenous (IV) and orally administered recombinant human Lf (rhLf) were examined. It confirmed that the orally administered rhLf showed systemic biological effects *via* stimulating the signal transduction mechanisms similar to the IV administered Lf. In both cases, the upregulation genes (transforming growth factor beta 1 (TGFβ1), interleukin-1β (IL-1), colony-stimulating factor 1 (CSF-1), xanthine dehydrogenase (XDH), cytochrome B alpha chain (CYBA) *etc.* were specifically involved in the inflammatory response and oxidative stress.⁸ Additional genome analysis is required to understand signaling pathway crosstalk. But some studies revealed that the bioactive peptide derived from pepsin digestion of Lf showed more antibacterial activity than an intact one.⁹ Orally administered Lf improves gut health; to exert its effects in the human colon, intact Lf is required.¹⁰ So the studies suggested that both intact Lf as well as peptides derived from Lf retain their biological activity after oral administration.

4. Production of lactoferrin

4.1 Purification from milk

Industrial development in processing technologies and equipment has led to the separation of vital nutritional components from the milk of goats, cows, buffalo, sheep, and other species. In 1939, Lf was discovered in its pure form and was easily identified by its pink color.² Heat treatment leads to the denaturation of Lf, so for the purification of bLf, pasteurized milk is unsuitable. Hence, Lf is isolated and purified from cheese whey and skim milk on an industrial scale. According to the amino acid composition, Lf exists as a cationic form; hence it can be readily adsorbed using a cation-exchange resin such as carboxymethyl (CM)-Sephadex and later eluted using salt solutions; so, this method is mainly used for the purification of bLf. The purified freeze-dried Lf powder can be obtained with 95% or higher purity; otherwise, after sterile filtration bLf is spray-dried. Pasteurization of Lf is very important to inactivate microbes and viruses; it was observed that during heat treatment at acidic pH Lf is stable, but protein denaturation occurs under heat treatment at neutral pH. Gel formation was observed in apo-Lf at alkaline and neutral pH.¹¹ The suitable methods for pasteurization of Lf are treating to 90–100 °C, at pH $\approx$ 4 for 5–10 min; besides, UHT treatment is used, and it can be applied in the manufacturing of broad-spectrum products containing Lf such as infant formula. Furthermore, from the hydrolysate of Lf, lactoferricin is purified through dual-step hydrophobic chromatography.¹² In safety tests, the toxicity of purified Lf was very low; studies showed that Lf has no adverse effects in rats at 2000 mg per kg per day and did not show any mutagenic activity.¹³ The results suggested that the purified Lf remains safe as a food additive.

On a laboratory scale, many chromatographic means have been used for Lf purification, like heparin-sepharose affinity chromatography, heparin-agarose affinity chromatography and cationic exchange expanded bed chromatography (EBC).^{14–16} Moreover, several new methods have been evaluated to increase the purity of Lf. An aptamer affinity column combined with high-performance liquid chromatography and novel multimodal chitosan mini-spheres were used for the purification of Lf from cheese whey without any pretreatment.^{17,18} These developed processes and matrices offer potential application in industry.

The commercially available method for bLf manufacturing is shown in Fig. 2. Skim milk is used as feedstock for the chromatographic separation of bLf. The unpasteurized skim milk is cooled below 8 °C to prevent the growth of microbes, passed through a clarifier, followed by 1 μ m filter to remove fat and any insoluble substances and to decrease the microbial load. The filtrate is subjected to an ion exchange column; the column bound with the filtrate is washed first with demineralized water and then with sodium chloride solution to remove the weakly bound contaminants. The column is washed with concentrated sodium chloride to elute out the bound bLf, and desalted using ultrafiltration; pH is adjusted to 6.5, followed by pasteurization at 73.5 °C for 18 s, followed by evaporation

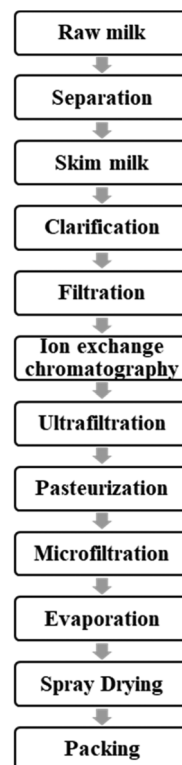


Fig. 2 Process of production of bovine milk-derived lactoferrin.

and spray drying. The manufacture of bLf *via* freeze drying followed by pulverization in the final step instead of spray drying has also been reported.¹⁹

4.2 Recombinant production

Human Lf production (hLf) from human milk has safety problems including human pathogen contamination and limited supply. Thus, for the clinical and animal studies, recombinant hLf (rhLf) has been developed mainly in transgenic cows, plants and microbial species. In transgenic cows, the hLf gene is under the control of the bovine casein promoter, which produces rhLf; therefore, using mono S chromatography, the transgenic cow milk which contains both bLf and rhLf were separated. A new cloning method was developed for the large-scale production of rhLf using a bovine mammary bioreactor. It has a large capacity for protein synthesis and an efficient production system with a 4.5–13.6 g L⁻¹ yield (R).²⁰ However, the chances of potential contamination with infectious pathogens create public concerns about its biological safety. In rice, rhLf produced has similar biochemical and physicochemical properties as that of native hLf, and is expressed as 25% of total soluble protein.²¹ Furthermore, transgenic tomatoes with hLf genes showed antibacterial and antifungal activities.²² Using plants as a transgenic vector provides an efficient, safe, and cost-effective alternative, but the lack of research and change in yield depending on various factors like genomic content, plant species, and growing conditions reduce its com-

mercial application. It has been reported that rhLf produced from *Aspergillus oryzae* and *Aspergillus niger* by fermentation possess similar characteristics as hLf. Another yeast *Pichia pastoris* is used for expressing the Lf gene from Tibetan sheep and the purified rLf demonstrated antibacterial, iron-binding and inhibitory activities against cancerous cells. It can rapidly reproduce up to 2 g L^{-1} yield; however, it has low secretion efficiency and is unsuitable for high-density protein production.²³ Mammalian cells are also used to produce rhLf. Researchers examined the efficacy of Chinese hamster ovary (CHO) expressed rhLf to boost the BCG vaccine. The results showed that rhLf not only modulates its activity but also reduces hypersensitive response by the host immune system.²⁴ The CHO-derived rhLf is fully compatible with hLf; thus, it is most promising for therapeutic applications in humans in the future. In a recent news, a US and Singapore based technology company, Turtle Tree has announced that it is ready to launch a cell-based Lf for infant formula and also as a nutrient supplement.²⁵ This will be a stepping stone for both food and pharmaceutical industries in the area of hLf commercialization.

5. Health benefits of lactoferrin

5.1 Immunomodulation

Lf has the capability of altering the innate and adaptive immune systems by either inducing or suppressing the components of immune response. It is the first-line defence that influences the development of adaptive immunity. Cytokine secretion is altered by iron sequestration of Lf, which in turn decreases the oxidative stress. Human colostrum has a higher concentration (7 g L^{-1}) of Lf, but comparatively, there is a low concentration of Lf in breast milk (1 g L^{-1}). The modulation of the immune response of Lf depends on the degree of glycosylation on a molecule. One of the main functions of colostrum is to supply infants with the vital components for immune system development and provide protective functions. It provides systemic as well as localized protection in the intestine of infants by aiding in the development of immature intestines and sustaining postprandial pH. Lf controls the immune reaction of lymphoid tissue related to the intestine and stimulates the small epithelial cell proliferation and differentiation, thus influencing the length, mass, and expression of digestive enzymes in the small intestine.²⁶

Many immune-modulatory activities of Lf are associated with its potential to bind with numerous proinflammatory pathogen associated microbial patterns (PAMPs), which are mainly identified by specific innate immune cell receptors, the toll-like receptors (TLRs). This interaction has been reported between unmethylated CpG-containing DNA and Gram-negative bacterial lipopolysaccharide (LPS). Lf inhibits the TLR-4 related pathway, thereby interfering with the pulmonary inflammatory processes induced by LPS.²⁷ The Lf subunit Lfc binds with LPS, which is a prototypical PAMP through the lipid-A moiety. In an *in vitro* study, such an interaction was

reported to block the signaling of LPS by receptors, like L-selectin and TLR-4, not only in immune cells such as neutrophils, lymphocytes, and monocytes-macrophages but also in endothelial cells.²⁸ Moreover, Lf has a high affinity towards the co-receptor of TLR-4, namely soluble CD14 (sCD14), alone or combined with LPS. In the inflammatory regions, all this binding was shown to suppress the LPS-induced stimulation of immune cells, causing a lower secretion of proinflammatory cytokines, such as IL-6, IL-1, and tumor necrosis factor- α (TNF- α). This is similar to IL-8 expression (an essential constituent in inflamed tissues) and adhesion molecules (E-selectin and intercellular adhesion molecule-1). As a result, polymorphonuclear leukocyte (PMNL)-secreted Lf could reduce responses, and after that at the site of inflammation, immune cells are recruited, thereby decreasing further tissue damage. Lf immune response in rats intoxicated with diazinon was evaluated and it was found that the Lf treated group had a significantly decreased number of WBCs and increased IL-10, total protein, globulin, and albumin levels compared to other groups and this study has proved that Lf can be used as a prevention aid rather than as a treatment drug.²⁹

LF interacts with antigen-presenting cells (APCs), such as macrophages, dendritic cells, Langerhans cells, and B cells in the immune system. Macrophages are phagocytic cells; their primary role is to prevent microbial infection through the microorganism's destruction intracellularly or by inhibiting their replication by cytokine secretion or some mediators like nitric oxide. Also, they play a role in inflammation by the secretion of inflammatory mediators and proinflammatory cytokines. When Lf binds with its receptor, macrophages are activated, thereby increasing their phagocytic ability and IL-12 synthesis; this recruits more macrophages to the site of inflammation and, thus, activates T-lymphocytes (CD4^+). Dendritic cells are a heterogeneous cell population; they are specific in antigen detection and have an essential role in the immune system *via* regulating tolerance and immunity. When the dendritic cells interact with Lf through specialized surface receptors, this binding promotes their maturation, differentiation and its functional activities. The rhLf talactoferrin induces dendritic cell maturation, and this stimulates the secretion of IL-8 and CXCL10, with a significant decrease in the production of IL-10, IL-6, and CCL20.³⁰ Many review articles have discussed the immunomodulation and anti-inflammatory activities of Lf. Recently the bioavailability and the role of different forms of Lf in maintaining immune homeostasis and inflammatory processes and its recent clinical studies investigating Lf application have been discussed by researchers. Similarly, the vital role of Lf in immune responses against infectious attacks and injury and also the importance of Lf in immunomodulation and the anti-inflammatory mechanism to retain physiologic homeostasis were reviewed. Some researchers specifically reviewed the immunomodulatory action of Lf on both innate and adaptive immune responses. All these articles support the role of Lf in immunomodulation activity.^{31–34}

5.2 Iron metabolism

Iron acts as a cofactor for key enzymes involved in several critical metabolic pathways and cell functions; its deficiency leads to fatigue and reduced immunity. LF has excellent iron affinity with an equilibrium dissociation (K_D) constant of approximately 10–20 and is required to maintain a regular iron balance and eliminate iron deficiency and iron overload. LF, being an iron-binding glycoprotein, has an excellent iron binding capacity and is essential to sustain an even iron balance. One molecule of Lf can bind with two ferric ions in the presence of two molecules of carbonate ions; thus, it can act as an iron transporter. Lf inhibits microbial growth in an iron-binding dependent manner. In the absence of an iron-bound form of Lf, microbial adhesion and entry occur in host cells. In infected cells, anti-inflammatory activity exerted by Lf against interleukin-6 leads to the upregulation of transferrin receptor-1 and ferroportin and downregulation of ferritin which are the vital players in maintaining inflammation and iron homeostasis. Many studies have been done to discover the role of bLf in iron absorption; in a study, when pregnant women were supplemented with oral bLf, they have shown an increased level of hemoglobin, ferritin, and total serum levels and a decreased occurrence of iron deficiency anemia.³⁵ Due to the occurrence of particular Lf receptors, Lf is assumed to have a vital role in the intestinal absorption of iron across the enterocyte's brush border membrane. In a current meta-analysis study, oral administration of bLf improved the iron supplies similar to that of ferrous sulfate with reduced gastrointestinal side effects in iron-deficient pregnant women.³⁶ In an *in vivo* study with Kenyan infants, the effects of holo-bLf, apo-bLf, and iron sulfate were compared, showing that both forms promote iron absorption.³⁷ Moreover, the complexity of Lf and iron interactions remains unknown, so iron-dependent pathologies in humans should be precisely evaluated.

5.3 Antimicrobial activity

Lf structural features impart further functionality other than iron homeostasis which is similar to that of the transferrin family. Mainly, Lf shows a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts, fungus, and parasites; in addition, Lf stimulates the selective growth of gut beneficial bacteria such as *Lactobacillus*, *Bifidobacterium* etc. Being an iron-binding protein, Lf has the iron sequestration ability; thus, it creates a nutrient shortage, which is essential for the growth and division of siderophores. Also, Lf binds to the surface of bacteria and generates LPS release from the outer membrane, leading to cell damage and death. One of the significant factors involved in bacteriostatic Lf properties is the disruption of the osmotic function of membranes. Lf has a highly cationic N-terminal region that interacts with a negatively charged component of bacteria: lipoteichoic acid in Gram-positive bacterial membrane and LPS in Gram-negative bacteria, and thereby exerts a strong bactericidal activity. This interaction destroys the lipid bilayer of the microbial cell membranes, causing greater permeability, loss

of cellular contents, and finally leads to death. However, the growth of some bacteria that have a lower iron requirement is promoted by Lf, which is beneficial for human health. Several *in vitro* investigations have revealed that Lf suppresses pathogen adhesion to the epithelial cells and intestinal mucus. Furthermore, an *in vivo* study conducted by researchers discovered that rectal administration of Lf has a dual effect of stopping *Escherichia coli* O157:H7 colonization and improving the local immune response to the infection of 6-month-old Holstein-Friesian calves with this organism.³⁸ The antimicrobial activity of Lf against *Streptococcus dysgalactiae* and *Streptococcus mutans* is by reducing its invasion into cells and the bacteriostatic effect. It inhibits *Cronobacter sakazakii* adhesion and internalization and reduces the oxidative stress caused by these microbes. Lf has been explored to treat *Helicobacter pylori* infection by inhibiting growth and reducing expression of the virulence factors. It has therapeutic activity against *Mycobacterium tuberculosis* and reduces the immune-mediated tissue damage in tuberculosis disease. Lf also reduces the bacterial load of *Salmonella enterica* sv. *Typhimurium* and decreases its infectious inflammation.^{39–44}

After the Lf proteolytic digestion with pepsin, lactoferricin (Lfc), a bioactive peptide, is released from the N-terminal region of Lf that shows higher antimicrobial action than Lf.¹² The Lf and Lfc antimicrobial activity has not been restricted to human pathogenic microbes. A study evaluated the lactoferricin activity in transgenic plants too. When tobacco plants are exposed to *Pseudomonas syringae* pv. tabaci and *Botrytis cinerea* infection, they showed that after 30 days of infection, transgenic plants survived, but due to pathogen damage, wild-type plants perished.⁴⁵ A combination of milk proteins, like Lf and lactoperoxidase, had an inhibitory effect against multidrug-resistant *Acinetobacter baumannii* and showed a synergistic effect on their immunomodulatory activity against this organism.⁴⁶ The Lf nanoparticle produced through the electrostatic complexation to gellan gum showed intensified antimicrobial activity against *Staphylococcus aureus* and *E. coli* than Lf in its pure state.⁴⁷

5.4 Antifungal activity

LF and LF-derived peptides have a wide spectrum of antifungal activity due to their iron sequestration ability and binding with the fungal cell surface, which leads to cell membrane damage and leakage. Studies have shown that Lf changes the cell membrane permeability in *Candida krusei*, *Candida albicans* and *Cryptococcus neoformans*, leading to cell death. In *Candida* isolates, cell surface alterations are seen through scanning electron microscopy, such as protein leakage, surface bleb formation, swelling and cell collapse.⁴⁸ A recent study revealed that Lf binding protein Pma1p, a proton pump which is inhibited by Lf through interruption of the membrane ergosterol and sphingolipid rich lipid rafts connected with Pma1p, promotes the intracellular accumulation of ergosterol, and it also reduces the V-ATPase activity to stimulate antifungal activity.⁴⁹ Furthermore, hLf prevents oral candidiasis caused by *C. albicans*, via upregulation of anti-inflammatory cytokines

and downregulation of virulent candida genes.⁵⁰ The antifungal drug like fluconazole combined with Lf from various species significantly enhanced the inhibitory effect and decreased the minimum inhibitory concentration (MIC) against *Candida* species, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*.⁵¹

5.5 Antiviral activity

The Lf antiviral activity against enveloped or non-enveloped RNA and DNA viruses has always been well-known (Table 1). Lf can prevent virus infections by blocking the viral attachment to host cells and thus inhibit viral replication within the cell, and it also enhances systemic immune responses. Several mechanisms of action of Lf have been proposed to prove its antiviral activity; the most commonly accepted one is based on its capacity to bind with viral receptors like glycosaminoglycans, chiefly heparan sulfate (HS). This interaction prevents the viral attack by blocking the primary contact between target cells and virus. Another most widely accepted mechanism is by modulating the immune response; the phagocytic action of macrophages is increased by binding with Lf. It also improves the response of TH1 lymphocytes and natural killer (NK) cell activity, which produce cytokines that defend against viral infection.⁵

Direct Lf administration can prevent human respiratory syncytial virus (RSV) attacks at ten times smaller amounts than those present in human milk. Lf also blocks the infection from non-enveloped viruses like enteroviruses and adenoviruses. The antiviral activity of Lf is not only limited to viruses that affect humans, but its activity is extended against animal viruses, like the Friend virus complex, which causes erythroleukemia rodents, feline herpesvirus, feline immunodeficiency virus and feline calicivirus.⁶⁹ It seems that the Lf activity

against viruses is exhibited in the early phase of infection. During the infection process, several pathogenic viruses, such as herpes simplex virus, have been shown to utilize the surface of host cells as a binding receptor. Results imply that a functional GAG-binding site of Lf is formed by synergistic binding of GRRRRS sequence at the amino terminus of human Lf with an RKVR sequence.⁵

A study determined that when the target cell is infected, instead of hindering the replication of the virus, bLf blocks the host cell infection. When they monitored the bLf activity on the infection by Mayaro virus (MAYV), they found that it has potent antiviral activity, involving prevention of viral entry into the cell; this finding may provide a method to develop a potent strategy for resistance to MAYV infection.⁷⁰ Moreover, it is demonstrated that bLf prevents influenza A virus infection by interacting with fusion peptide hemagglutinin.⁷¹ However, *in vitro* studies depict that the efficacy of Lf against HIV may be through its prevention of viral replication within the host cells. Thus, three Lf antiviral mechanisms of action were identified. Firstly, it can inhibit the cellular virus internalization (including poliovirus type 1); secondly, it can suppress the replication of virus within the cell (including HCV); and finally, it can block the glycosaminoglycan viral receptor heparan sulfate, thereby preventing the interaction between virus and host cell.⁵⁵

SARS-CoV-2 is a highly contagious and easily replicating virus that causes the acute disease COVID-19, which is characterised by acute respiratory distress syndrome and pneumonia. SARS-CoV-2 is a spherical, enveloped virus containing positive-sense single-stranded RNA genome. This genome encodes 29 proteins including sixteen nonstructural, four structural and nine accessory proteins. The four structural proteins are nucleocapsid, envelope protein, matrix, and spike protein. The

Table 1 Antiviral activity of lactoferrin (Lf)

References	Virus	Mode of action of lactoferrin
I. Enveloped viruses		
Hara <i>et al.</i> (2002) ⁵²	Hepatitis viruses	Inhibition of viral entry, inhibits intracellular replication
Andersen <i>et al.</i> (2003) ⁵³	Herpes simplex viruses (HSV)	Inhibition of viral adsorption, inhibits viral replication, inhibition of cytopathic effect
Beljaars <i>et al.</i> (2004) ⁵⁴	Cytomegalovirus (CMV)	Inhibits viral replication
Berkhout <i>et al.</i> (2002) ⁵⁵	Human immunodeficiency virus (HIV)	Blocks viral entry, inhibits viral replication
Mistry <i>et al.</i> (2007) ⁵⁶	Human papillomavirus	Blocks viral entry, inhibition of cytopathic effect
Taha <i>et al.</i> (2010) ⁵⁷	Influenza A (H1N1)	Blocks viral entry, inhibition of cytopathic effect
Portelli <i>et al.</i> (1998) ⁵⁸	Respiratory syncytial virus (RSV)	Inhibition of viral adsorption and growth
Yamamoto <i>et al.</i> (2010) ⁵⁹	Parainfluenza virus (PIV)	Inhibition of viral adsorption
Waarts <i>et al.</i> (2005) ⁶⁰	Alphavirus	Blocks viral entry
Chien <i>et al.</i> (2008) ⁶¹	Japanese encephalitis virus (JEV)	Inhibition of viral attachment
Campione <i>et al.</i> (2021), ⁶² Rosa <i>et al.</i> (2022) ⁶³	SARS-CoV-2	Inhibition of viral attachment and replication
II. Non-enveloped viruses		
Superti <i>et al.</i> (2001) ⁶⁴	Rotavirus	Inhibition of viral adsorption, inhibits assembly of new viral particles, inhibition of cytopathic effect
Marchetti <i>et al.</i> (1999) ⁶⁵	Poliovirus	Blocks viral entry, inhibition of cytopathic effect
Arnold <i>et al.</i> (2002) ⁶⁶	Adenovirus	Inhibition of viral attachment, inhibits viral replication
Oda <i>et al.</i> (2021) ⁶⁷	Norovirus	Inhibition of cytotoxic damage
McCann <i>et al.</i> (2003) ⁶⁸	Feline calicivirus (FCV)	Inhibition of viral attachment

spike protein contains two subunits S1 and S2, which are located on the envelope. Through the S1 subunit, the virus attaches to the cell receptor ACE2 and S2 is involved in membrane fusion. SARS-CoV-2 also uses the HSPG proteins present on the cell membrane, as an attaching site, which have a vital role in the early phase binding of the virus. SARS-CoV-2 is an emerging virus that presently lacks curative treatments and the recent SARS-CoV-2 outbreak had made life come to a standstill all over the world. Lf is a natural, non-toxic protein that has established its antiviral efficacy against a wide-spectrum of viruses that causes human infection, including SARS-CoV-2 (Fig. 3). Lf has many mechanisms of action against SARS-CoV-2. Lf directly binds with SARS-CoV-2 and thereby prevents viral entry and replication. Also, Lf competes with the SARS-CoV-2 for the cell membrane receptors ACE2 and HSPGs which block the entry and replication of the virus. Moreover, Lf is an immunomodulator that stimulates the immune cells like natural killer cells to fight against the virus. It promotes the antigen presenting capacity of B cells and regulates T cell function thereby reducing inflammation. Lf reduces the iron containing ferritin protein level by iron chelation, thus limiting SARS-CoV-2 viral replication. Lf's ability to bind with iron leads to a reduction in the formation of reactive oxygen species, and this antioxidant activity impairs SARS-CoV-2 replication. The invasion of SARS-CoV-2 stimulates the cytokine storm in a COVID-19 patient, and this may lead to acute respiratory distress and later death. Lf reduces the level of cytokines like TNF- α , IL-6, and acute phase reactants like ferritin. The SARS-CoV-2 virus decreases the production of interferons, that

usually fight off viral infections. Lf restores interferon action by increasing its production which leads to the reduction in the replication of the SARS-CoV-2 virus. Lf also has the ability to reduce platelet aggregation by regulating the protein plasminogen, which is released during a SARS-CoV-2 viral invasion. SARS-CoV-2 viral replication starts at the respiratory system and continues its replication after reaching the gastrointestinal tract. So, Lf protects the gastrointestinal tract by exerting anti-inflammatory activity through maintaining a balance between good and bad gut microbes and reducing the intestinal damage caused by the SARS-CoV-2 virus.⁶³

In a meta-analysis it was revealed that Lf reduces the risk of respiratory tract infections, so it can be used as an adjunct for the prevention and management of COVID-19.⁷² *In vitro* studies reported that due to the availability, safety, and *in vitro* efficacy, Lf has protective effects against SARS-CoV-2 by modulating the antiviral immune response with a modest effect on infection.^{73,74} It also revealed that bLf binds to the heparan sulfate proteoglycans, thereby blocking the attachment of SARS-CoV-2 to the host cell.⁷⁵ Furthermore, *in vitro* administration of Lf with hypothiocyanite enhances the antiviral activity of this compound against SARS-CoV-2.⁷⁶ Recently, a novel Lf-derived peptide GSRY has been identified as a SARS-CoV-2 inhibitor; it has potential to inhibit main protease (M^{pro}), which is involved in the corona virus life cycle.⁷⁷

5.6 Anti-inflammatory activity

Lf acts as a natural host defense regulator with major anti-inflammatory activities by catalyzing the tissue-toxic hydroxyl

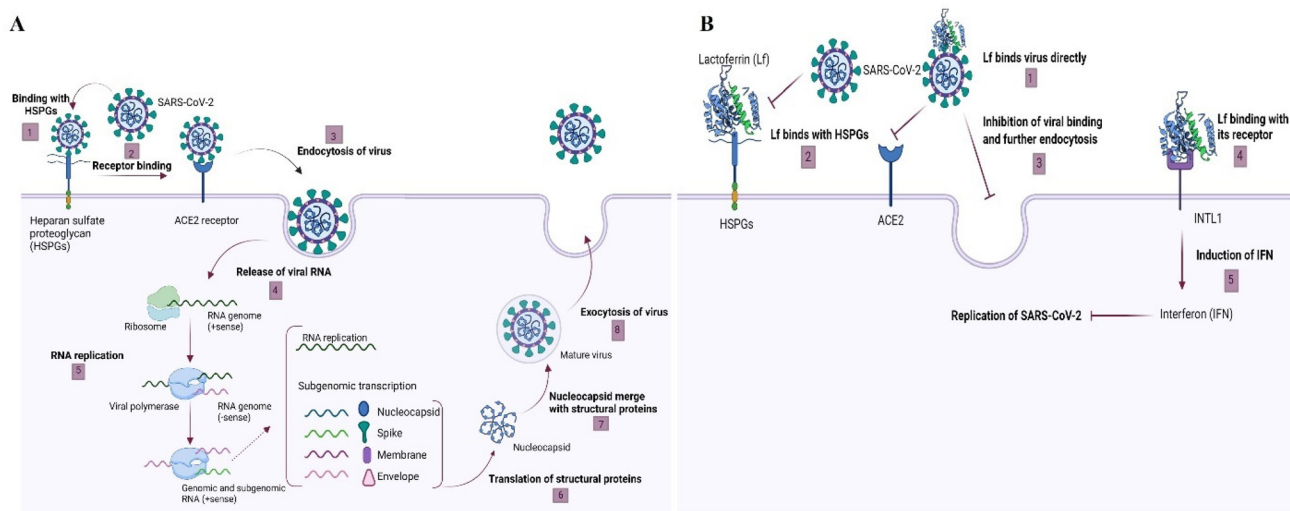


Fig. 3 (A) SARS-CoV-2 attachment and replication in host cells. SARS-CoV-2 first binds to heparan sulfate proteoglycans (HSPGs) (1) and then attaches with angiotensin-converting enzyme 2 (ACE2) receptor (2) that facilitates the fusion between host cell and SARS-CoV-2 and its endocytosis (3). After endocytosis, the viral genomic RNA is released into the cytoplasm (4). Replication occurs in the cytoplasm, where using the genomic RNA as a template, subgenomic RNA is formed (5). Using viral RNA genes, nucleocapsids and viral structural proteins are transcribed and translated (6). The nucleocapsids combine with structural proteins and allow the release of mature new viruses (7) via exocytosis (8). Created with <https://www.biorender.com>. (B) Anti-viral mechanism of lactoferrin (Lf). Direct binding of Lf with SARS-CoV-2 (1); Lf binds with heparan sulfate proteoglycans (HSPGs) and thus blocks the SARS-CoV-2 attachment with angiotensin-converting enzyme 2 (ACE2); hence prevents endocytosis and viral replication (2 and 3). Lf might also inhibit virus replication through the stimulation of intracellular cell signals like interferon (IFN) activation by binding with its receptor intelectin-1 receptor (INTL1) (4). Created with <https://www.biorender.com>.

radical formation and scavenging free iron deposition in tissues, and this makes Lf a potential therapeutic means in the treatment of inflammatory disorders. After exposure to pathogens, Lf is one of the molecules released by neutrophils and promotes innate activation of adaptive responses through neutrophil release into the inflammation site. Studies have proved that the released Lf has a response not only against pathogens, but also against neutrophils released at the site of inflammation. Depending upon the iron saturation status it has an anti-apoptotic activity against neutrophils that may lead to chronic inflammatory diseases.⁷⁸ Thus, the iron saturation status of Lf is important to exerts its action against both pathogens and neutrophils. When the host encounters skin allergens, Lf has the capacity to trigger the immune system against it through dendritic cell accumulation and prevention of Langerhans cell migration in lymph nodes. Furthermore, Lf modulates the cytokine secretion from leukocytes; it promotes the anti-inflammatory cytokine production of IL-10; in addition, Lf inhibits the synthesis of proinflammatory cytokines like IL-1 β , IL-6, TNF- α , and IL-8. Depending upon the signal type recognized by the immune system, the synthesis of these components occurs. Lf amplifies the number of adaptive T cells (CD4+ and CD8+) and natural killer (NK) cells, and increases PMN recruitment in the blood, which causes phagocytosis and regulates myelopoiesis. IL-12 is well known for its vital role in helper T-cell development. Hence, the Lf role in controlling IL-12 and proinflammatory cytokines reveals the interaction between innate and adaptive immunity.⁷⁹

The wound healing ability of Lf is well reported: a recombinant human Lf, talactoferrin (TLf), aids in the secretion of proinflammatory cytokines TNF- α , IL-8, IL-6, and MIP-1 α . Also, it may upregulate the platelet-derived growth factor, stimulate the proliferation of keratinocytes and enhance hyaluronan expression needed for producing tissue granulation and migration necessary for wound re-epithelization, thereby protecting the cell from apoptosis.⁸⁰

5.7 Antioxidant activity

During cellular respiration, along with energy, an excess amount of reactive oxygen species (ROS) production and accumulation lead to metabolic disorders in humans. When the ROS production exceeds the capacity of the antioxidant system, oxidative stress occurs. Oxidative stress has a vital role in the disablement of endothelium-dependent vasodilation and the stimulation of hypertrophy, inflammation, and apoptosis by various signaling pathway activation. The excess formation of ROS causes endothelial cell damage *via* protein oxidation, lipid peroxidation, and DNA fragmentation. The Lf antioxidant activity is associated with its iron binding ability, thereby reducing ROS production. Lf has a lethal effect on pathogens by stimulating the ROS production by granulocytes; in contrast, at extracellular pH, the ROS production is inhibited by Lf through the activation of iron binding by monocytes; this prevents the lipid peroxidative activity of neutrophils, thus preventing oxidative tissue damage by ROS. However, Lf has a direct effect on hydroxyl and oxygen scavenging capacity,

which is independent of iron binding ability. It is shown that Lf protects DNA from direct oxidative damage by acting as an ROS scavenger. Lf has a cytoprotective effect against oxidative stress induced by hydrogen peroxide (H₂O₂). It reduced the hydrogen peroxide concentration and increased FRAP value in the extra- and intra-cellular fluids. The pretreatment of human umbilical vein endothelial cells (HUVECs) with Lf significantly reduced the intra- and extracellular hydrogen peroxide levels assessed using the FOX-1 assay. The results also showed a significant increase in total antioxidant capacity in intra- and extracellular fluids after pretreatment of HUVECs with Lf.⁸¹

In healthy individuals, LF supplementation has been related to an increase in hydrophilic antioxidant activity. LF has also shown antioxidant effect on erythrocytes through the inhibition of lipid peroxidation and hemolysis. It has been observed that subsequent treatment with Lf reduces the intracellular ROS levels induced by glucose oxidation, implying its capacity to decrease oxidative stress-induced apoptosis. The study showed that Lf pretreatment could guard human mesenchymal stem cells (hMSCs) against oxidative stress-induced senescence and apoptosis. It is revealed that Lf inhibited peroxide-induced senescence by preventing Akt and caspase-3 activation.⁸²

In pregnant women, some pregnancy complications have also been associated with oxidative stress. Lf administration to the vagina reduces the oxidative stress in pregnant women's amniotic fluid with mid-trimester genetic amniocentesis by reducing lipoperoxide formation through directly mediating Fe³⁺ elimination by Fenton reaction or ROS scavenging.⁸³

5.8 Anticancer activity

LF has an antineoplastic effect against various types of cancer cells by different mechanisms of action, such as, immunomodulation, alteration of the cell membrane, apoptosis, cell cycle arrest, inhibition of metastasis, anti-angiogenic action and necrosis of cells. LF has been recommended as a potent chemo preventive agent against many cancerous cells. When Lf is orally administered, it cannot be absorbed or degraded by the small intestine, so it remains in the digestive system and thereby stimulates gastrointestinal immune response against carcinogenesis.

The chelating activity of Lf against tumor formation is the most commonly studied mechanism of action. The free iron in the tissue causes oxidative stress on genomic material, which may lead to carcinogenesis, and Lf captures the free iron ions at the tissue level. Moreover, Lf reduced the cytotoxicity induced by aflatoxin B1 and M1, oxidative stress, DNA damage and levels of ERK1/2 and JNK in four cancerous cell lines – Caco-2, Hep-G2, HEK and SK-N-SH.⁸⁴ The findings suggested that Lf has a chemopreventive effect against diethylnitrosamine-induced hepatocarcinogenesis in rats by decreasing the expression and activation of the PKB/Akt-pathway.⁸⁵ In addition, it has been proved that a nano combination of Lf and lactoperoxidase is more effective in apoptosis mediating anticancer activity than free forms in various human cancer cell lines.⁸⁶ Lf can produce cytokines in the cancer cell, arrest

tumor growth and induce apoptosis; it can also prevent the G1 to S phase transition in malignant tumor. Furthermore, the treatment with hLf inhibits tumor growth by increasing the anticarcinogenic cytokine levels by IL-18, and NK cell and T cell (CD8+) activation. In mice, oral administration of bLf inhibits tumor-induced angiogenesis, and hLf has a particular pro-angiogenic effect.⁸⁷ Studies in animal models reported that bLf inhibits colon cancer, and clinical trials have proved that hLf reduces the risk of colorectal cancer, and it is also revealed that goat Lf (gLf) shows anticancer activity.^{88,89} Furthermore, the antiproliferative function of colon cancer cells (SW480) was inhibited by the synergistic effect of the chlorogenic acid–Lf stable complex; this complex promotes the cell cycle arrest at the G₀–G₁ transition phase.⁹⁰ The evidence suggested that for the treatment of cancers, the most promising approach may be the Akt signaling pathway inhibition. Lf has shown to reduce the growth of breast cancer cells, and it also inhibits the G1–S phase transition by extrinsic Lf addition into culture media of MDA-MB-231 (breast cancer cell lines). Moreover, in HeLa cells, Lf promotes cell cycle arrest, apoptosis, and Smad-2 nuclear accumulation.^{91,92}

In addition, it is commonly known that the Lf receptors are overexpressed in cancerous cells to provide the increasing nutritional demands of these fast multiplying cells. Therefore, for the specific targeting of these cells, one of the excellent approaches is to develop Lf nanocarriers. A Lf-conjugated lipid polymer-based nanoparticles (LLPBNPs) is used for the targeted delivery of methotrexate into the cancerous cells.⁹³ A high drug loading and stable Lf dual drug (docetaxel and celastrol) nanoconjugate delivery system was developed for synergistic breast cancer therapy.⁹⁴ Also, an Lf targeted-*N*-isopropylacrylamide (NIPAM)-*co*-acrylic acid (AA) nanohydrogel was developed for the delivery of a bi-phenolic lignin, honokiol (HK), as a promising breast cancer therapy because it has a high affinity towards LDL and transferrin receptors present in various cancerous cells; also Lf receptors (LRP1, LRP2) are overexpressed in breast cancer cells.⁹⁵ Zein–Lf micelles showed tumor-specific targeted co-delivery of rapamycin/wogonin with a low drug dose, maximized antitumor efficacy, and minimized side effects. Besides, a pH-dependent release of drug is observed in Lf nanocarriers. A faster release of drug is found at acidic pH; it might increase the release of drug at acidic sites like the tumor tissue microenvironment; so this improves the therapeutic efficiency of the hydrophobic bioactive encapsulated within the nanocarriers.⁹⁶ Additionally, in the brain endothelial cells, surface Lf receptors are present; thus, when Lf binds to its cell surface receptor, it can easily cross the blood–brain barrier by receptor-mediated transcytosis. Because of this ability, Lf can be used to fabricate nanocarriers that specifically target brain tumor cells.⁹⁷

5.9 Prebiotic efficacy

Lf is a glycoprotein that stimulates the growth of specific probiotics. Milk is a rich source of Lf, and its bioactive peptide lactoferricin (Lfc), is obtained by pepsin digestion of Lf, which has an antimicrobial effect against pathogenic organisms.¹²

Many *in vitro* and *in vivo* studies are reported about Lf activity that promotes the growth of the probiotics. Both peptide Lf and Lfc enhance the growth of selected probiotics, including *Lactobacilli* sp. and *Bifidobacteria* sp.⁹⁸ It may be due to the physicochemical properties of surface components and enzyme secretion from probiotics that stimulate the growth-promoting activity of Lf than antimicrobial activity. In an *in vivo* study, piglets were taken as a model to study Lf's role in the growth and on the varieties of colon microbes and their influence on the wellness of the neonatal intestine.⁹⁹ Through intestinal microbiota, Lf can be involved in many processes, like providing energy for the growth of the intestine, intestinal cell maturation and differentiation, signaling of gut–brain–microbiota axis for nerve fiber maturation, and regulating inflammatory and immune responses for a beneficial effect on intestinal wellness.

Many studies have demonstrated the effects of human and bovine milk samples on probiotic growth. The growth of the *Bifidobacterium* species like *B. bifidum*, *B. breve* and *B. infantis* were similarly enhanced by human and bovine milk.¹⁰⁰ It is found that the collected one to six month postpartum human milk showed varying growth stimulating activity on *B. breve*, *B. pennsylvanicus*, and *B. infantis*; however, in bovine milk, a stable bifidogenic activity was noted.¹⁰¹ These results may signify differences in bovine and human milk composition due to genetic variation, changes in bioactive components like Lf present in milk in different postpartum periods, and the diverse fermentability of bifidobacterial strains. In a study, it was documented that unpasteurized human breast milk had inhibitory effects on neonatal pathogens like *Staphylococcus* and *E. coli*, as compared to the pasteurized one. This phenomenon was attributed to the higher amount of Lf in unpasteurized breast milk. Supplementation of hLf to the pasteurized human breast milk resulted in the inhibition of pathogenic as well as probiotic cultures. And at higher dosages, bLf also showed antimicrobial activity against all tested species, including *B. breve*.¹⁰² The findings implied that the simultaneous administration of Lf and probiotics might affect the efficacy of probiotic colonization. This result is contradictory to those found in other studies.⁹⁸

Many studies have revealed that Lf iron saturation has varying impacts on the growth of probiotic strains and inhibitory action against pathogens. The antimicrobial effect of Lf and Lfc B against *Bacteroides* species found on the human gastrointestinal tract was investigated in an *in vitro* study; the results showed that the binding to laminin and biofilm formation were intensely inhibited by Lf at low concentrations.¹⁰³ In a probiotic culture, assays have shown that apobLf exhibited inhibitory activity against the growth of foodborne pathogenic organisms like *Enterococcus fecalis* and *Salmonella typhimurium*; in contrast, it inhibited or was not involved in the growth of bifidobacterial and lactobacilli species.¹⁰⁴ A study with different forms of metal-bound Lf like apobLf, Fe-bLf and Cu-bLf showed an enhanced growth of bifidobacterial species. Besides, Zn-bLf and Cu-bLf have greater inhibitory activity on pathogenic growth, such as *Staphylococcus aureus* and

Escherichia coli, than apobLf. These outcomes support that an iron-depleted form of bLf reduces the growth of pathogens without altering certain probiotic proliferation and provides innate means for retaining a balance between gut homeostasis and microbiota.¹⁰⁵

5.10 Other physiological functions of lactoferrin

5.10.1 Lactoferrin in bone metabolism. The primary bone-forming cell within the bone tissue is the osteoblast and is essential for remodeling of the bone and homeostasis. Bone metabolism is regulated by a dynamic balance between osteoblasts and osteoclasts. Both bLf and native hLf increased the length of the S and G₂/M phases and promoted the proliferation and nourishment of osteoblasts when compared with untreated osteoblasts.¹⁰⁶ A study shows that Lf stimulated MC3T3-E1 osteoblast cell proliferation *via* c-Fos and c-Jun by activating the JNK, ERK, and p38 signaling pathways.¹⁰⁷ LF has been found to promote osteoblast proliferation while inhibiting apoptosis by mediating IGF-1R and in an IGF-1R-independent mechanism, and it also activates the PI3K/RAS signaling pathway.¹⁰⁸ It was demonstrated that bLf and its peptide could enhance osteoblast proliferation and differentiation and prevents osteoclast differentiation. Lf has been shown to induce the secretion of some cytokines or growth factors in osteoblasts and consequently affect the activity and function of osteoblasts along with other skeletal component cells.¹⁰⁹ Studies have shown that bLf is a potent osteogenic factor, which enhances osteoblast formation by the activation of the p38 MAPK and Smad 2/3 signaling pathways.¹¹⁰ It also stimulates the production of angiogenic components, vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2) in osteoblasts, implying that Lf can initiate the cross-talk between osteoblasts and endothelial cells *via* the paracrine activity of angiogenic factors. It has been discovered that Lf increased the level of IL-6 and IL-11 mRNA in osteoblasts. LF treatment significantly upregulated the IL-6, THBS-2, Cyr61, FGF-basic, MCP-1, Serpin E1, and PDGF-AA levels, whereas the Serpin F1 level was downregulated in osteoblasts. LF exerts a beneficial effect on osteoblasts by orchestrating growth factors and cytokines. Furthermore, the peptide LFP-C derived from bLf enhanced the MC3T3-E1 mouse cell line proliferation by increasing the osteoblast proportion in the G₂/M phases and it also increased the calcium deposition and alkaline phosphatase activity.¹¹¹ Osteogenic effects of P1 peptide derived by pepsin hydrolysis of bLf were studied; it also promoted osteoblast proliferation and alkaline phosphatase activity.¹¹² Moreover, a combination of Lf and substance P incorporated within a chitin-PLGA-calcium sulfate hydrogel showed more potent bone regeneration due to the chemotactic agent (SP) and pleiotropic growth protein (Lf).¹¹³ In a study, it was shown that bLf inhibits bone destruction during LPS-induced periodontitis, therefore it can be used as an adequate and safe preventive treatment for periodontitis.¹¹⁴

5.10.2 Lactoferrin in neural development and neuronal diseases. Lf is a glycoprotein abundant in sialic acid, and because of this, it can contribute to neurodevelopment.

Studies in piglets as an animal model have shown that Lf administration can promote cognitive functions, neural development, and memory. It has also been implied that in piglets a Lf rich diet contributes to a reduction in anxiety and impulsivity.¹¹⁵ Evidence showed that cerebral ischemic reperfusion is accompanied by inflammatory reactions, which are stimulated by the triggering of proinflammatory factors, chemokines, and adhesion molecules. TLR-4 widely mediates the inflammatory response during ischemic reperfusion. Many reports have proved the Lf protective effect in cerebral ischemia injury. In a study, they compared the protective effects of Lf, β -lactoglobulin, and α -lactalbumin in cerebral stroke; results indicated that TLR-4 targets these bioactive proteins to block cerebral ischemic reperfusion. In mouse models, Lf is found to suppress the TLR-4 associated pathway in cerebral ischemic reperfusion, anoxia and reoxygenation of cells; however, in a temperature-dependent manner, there is a decrease in Lf activity.^{116,117} Neurological diseases like Alzheimer's (AD) and Parkinson's diseases are related to iron metabolism. In Alzheimer's patients, iron and other metals accumulate in amyloid- β plaques. Lf is a chelator molecule that can pass across the blood-brain barrier; hence, it is used as a potential therapy for this condition. Lf has shown a protective effect in AD by its ability to reduce the cognitive decline and pathological cascade through the p-Akt/PTEN pathway modulation; this affects the vital players in oxidative stress and inflammation involved in the pathology of AD. In naturally aging C57/BL6J mice, Lf showed to improve cognitive ability; moreover, by the effects on inflammatory regulation and iron chelation, Lf reduces the formation of ROS in the hippocampus of mice.¹¹⁸ A high incidence of brain injury due to hypoxia-ischemia occurs in premature infants accompanied by subsequent neural development disabilities, so Lf may have promising applications in protecting infants from neural damage. In addition to this, the specific delivery of drugs like doxorubicin hydrochloride towards glioma is mediated by nanogels coated with Lf to penetrate the blood-brain barrier.¹¹⁹ Being an innate substance that can cross the blood-brain barrier, Lf can be directly used to treat neurological disorders and also used as a delivery system for many neuro drugs without any side effects.

5.10.3 Lactoferrin in metabolic diseases. Recently, a new application of Lf in clinical research has been revealed, which is its use as a potent aid in the treatment of metabolic disorders including diabetes, hypertension, and obesity. The Lf mechanism of action against these metabolic diseases is yet unknown. Studies in high-fat diet streptozotocin-induced diabetic mice showed that Lf supplementation improved pancreatic dysfunction and hepatic insulin resistance by regulating the PI3K/AKT cell signaling pathway.¹²⁰ The interaction of mammalian receptors like omentin-1 with Lf is involved in type-2 diabetes, obesity, and LDL receptor-associated proteins which are implicated in the metabolism of lipids. In high-fat induced obese mice, oral administration of bLf has displayed a positive modulation in gut microbiota, decreased inflammation and regulated glucose and lipid metabolism.¹²¹ Also,

bLf administration in micro mini pigs also showed reduced LDL levels and fat accumulation. These findings indicated that the possible way for the prevention and treatment of metabolic disorders is bLf administration in humans.¹²²

6. Applications

6.1 Infant formula

Lf is a milk protein most extensively used as a supplement due to its wide range of functional activities. WHO recommends breast-feeding throughout six months of age, and human milk is established as the gold standard for the nutrition of infants. However, a significant proportion of infants get complete or partial formula feeding. Many studies are going on to change the infant formula to match the dynamic characteristics of breast milk. Lf is a well-studied bioactive constituent. After the development of freeze-drying and ultrafiltration in the dairy industry, pure and bioactive Lf can be formed, which allows the Lf inclusion in infant formula. Human Lf shares approximately 70% sequence analogy and similar bioactivity with bLf, so it has been extensively used for its health benefits. The ingestion of bLf-fortified milk showed high total iron in the body and effective intestinal absorption of iron in breastfed infants. Dietary bLf was also demonstrated to reduce parasite colonization, invasive fungal infections in infants, and diarrhea and vomiting in older children.¹²³ In infants, Lf in the unsaturated-iron form prevents the growth of bacteria and lowers the incidence of diarrhea by iron scavenging from bacteria. A recent study on the thermal stability of bLf with potassium phosphate (PP) and sodium citrate (SC) additives for application in infant formulas showed that PP and SC do not prevent bLf denaturation, but they inhibit the aggregation of Lf and maintain a relatively large amount of soluble Lf after the heating treatment.¹²⁴ The recommended level of Lf supplementation in powdered infant formula by FDA is 100 mg per 100 g.

6.2 Lactoferrin as a delivery system

There has been an increasing interest in incorporating hydrophobic and hydrophilic bioactives into functional food products, like peptides, proteins, conjugated linoleic acids (CLA), carotenoids, polyphenols, and probiotics. But, the poor chemical stability, oral bioavailability, and water-dispersibility of these bioactives restricted their application. Because of these reasons, there has been a growing interest in developing delivery systems to protect, encapsulate and release the bioactive compounds to improve their bioavailability and increase their food matrix compatibility. Several types of delivery systems have been developed, which include emulsions, solid lipid nanoparticle, liposomes, and biopolymer microgels.

Lf has the capacity to bind with molecules and form covalent conjugates or physical complexes so that it can be used as a nutraceutical carrier in the field of medicine and food. Carboxymethyl tara gum-Lf complex acts as a carrier for vitamin D₃; this encapsulation increases the stability and

improves the controlled release of the vitamin.¹²⁵ Many studies showed that the chemical stability and bioavailability of bioactive molecules like resveratrol and β -carotene are improved when it is encapsulated within Lf-based emulsions and carboxymethylcellulose-Lf complex coacervates.¹²⁶ In a recent study, a novel delivery system was designed using Lf functionalized hollow mesoporous manganese doped silica nanoparticles loaded with resveratrol for ischemic stroke treatment.¹²⁷ Moreover, when polyphenols and polysaccharides form a covalent complex with Lf, it can prevent the bioactive degradation in emulsions and increase their stability. Lf conjugation onto the surface of inorganic nanoparticles modifies their functional properties and stability.

Curcumin encapsulated within Lf nanoparticles improved its cellular uptake, increased bioactivity, and enhanced its retention. The self-assembled nano micelles of Lf peptides to deliver curcumin revealed more dilution, storage, and thermal stability with low cytotoxicity.¹²⁸ Simultaneously, the electrostatic complexation formed of Lf and pectin and an oleogel-based Pickering emulsion stabilized by the transglutaminase-Lf particle used to encapsulate curcumin improved its release profile, chemical stability, and antioxidant activity.¹²⁹ The surface modification of Lf nanoparticles with sodium 2-mercaptoethanesulfonate (MES) enhances its efficacy by specifically targeting the cells infected with HIV-1 through the controlled delivery and effective concentration of the drug at the infected area.¹³⁰ A study reported that Lf nanoparticles (LfNPs) had been used as a delivery system for DNA. It is found to be stable, non-toxic, and resisting digestion against nuclease. Additionally, in Lf receptor-expressing cell lines, LfNPs can allow DNA uptake by receptor-mediated internalization.¹³¹

6.3 Lactoferrin as a dietary supplement

As an immunomodulator, Lf can boost immune response. bLf is produced as a nutraceutical that does not carry any health risk. In *Caenorhabditis elegans*, Lf incorporated nutritional supplement extends lifespan and decreases amyloid β -peptide toxicity by stimulating the immune system.¹³² Studies conducted on healthy volunteers have shown that the consumption of bovine milk-derived Lf has immunomodulatory effects. This suggested that clinically bLf can be used to improve the immunity of patients *via* activating their immune response.¹³³ Lf supplements are presently sold at 96% purity level. To improve the purity of supplements, more research needs to be done to use hLf instead of bLf in nutrient supplements.

6.4 Lactoferrin in the cosmetics sector

Lf has many applications in the cosmetics sector. Lf promotes metabolic processes in the skin and has an anti-aging effect because of its antioxidant capacity, which scavenges free radicals. Due to its anti-bacterial activity, it is an essential ingredient in cleansers. Lf is also used in hairsprays for smoothing and hydrating hair.¹³⁴ Further research is needed to extend the use of Lf in beauty products, including hair, face, make-up, and body care items.

7. Future prospects

Lf is a multifunctional iron-binding or transferring protein. It is a versatile biomolecule that has multiple regulatory activities. This has directed studies to extend the applications of Lf in the pharmaceutical, industrial and nutritional applications, including infant formulas, diet supplements, functional beverages, and cosmetics. Because of the functional activities and safety profile of Lf, it has drawn increased attention towards research in developing novel vaccines and drugs for the treatment of many diseases. An intact Lf is required for its effective functioning in the human body, so to protect the Lf from the gastrointestinal conditions, micro or nano encapsulated formulations are being developed for the oral delivery of Lf. It also has been used as a delivery system for many bioactives in combination with other biopolymers for controlled and site-specific release.

Lf has shown broad-spectrum of antiviral activity, so this indicates its ability for the prevention and treatment of SARS-CoV-2, by inhibiting the binding of the virus to the host cell receptors (ACE2 and HSPGs). In addition, due to the free iron sequestration ability of Lf, it can reduce oxidative stress and the subsequent cytokine storm induced during the SAR-COV-2 infection. Therefore, Lf may be used as an excellent supplement for prophylaxis as well as a therapeutic agent in the COVID-19 treatment. But further research is required for the clinical use of Lf, such as in which state Lf is more active, dosage amount, and effective route of administration, which are still to be determined. More studies need to be conducted to clarify the Lf effects on probiotics. One of the main challenges in the food industry is that bLf is not fully compatible with humans as compared to hLf. Hence, the use of hLf is recommended for the formulation of food products. Further studies are warranted to develop technologies, e.g., recombinant technology, to improve the availability of hLf. Also, more research is needed in the field of plants as transgenic expression vectors to produce rhLf of uniform nature and high quality. Also there need to be more attempts at developing cell-based highly compatible hLf for commercial purposes, especially for use in infant formulas.

8. Conclusions

Lactoferrin is a multifunctional natural glycoprotein, which has been studied for its various physiological processes. The major beneficial activities include immunomodulation, iron metabolism, antibacterial, antiviral, anti-inflammatory, antioxidant, anticancer properties and prebiotic efficacy of Lf protein and its peptides; besides, it is also involved in bone metabolism, neural development, and metabolic disorders. Being a member of the transferrin family, the iron saturation of Lf is reported as one of the key factors in its multifunctional properties. Its reported role in the antiviral activity especially in recent reports of SARS-CoV-2 is very promising in the present scenario. Due to its functional versatility, this health-

enhancing nutraceutical protein has directed scientists in the food and pharmaceutical industries to develop products such as nutrition supplements and infant milk formulas that have immune boosting function. Also, due to its antioxidant property it can be used for the development of skin care products like moisturizing creams. Further research should be conducted in humans to acquire insights into its activity and to further develop the significant therapeutic potential of Lf.

Author contributions

SVS: conceptualization, data curation, investigation, methodology, visualization, writing – original draft, CTU: writing – review and editing, PN: conceptualization, supervision, writing – review and editing.

Conflicts of interest

The authors declare no conflict of interest.

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