Jet-O-mized eggshell membrane powder: A novel hydrolysis strategy to unlock gut-promoting bioactivities

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DEDICATIONS

To my dearest husband, Farouk, whose unwavering support and encouragement have been my greatest strength. Your belief in my passion for research, despite its challenges and stresses, has been a constant source of motivation. I am forever grateful for your sacrifices, especially for those late-night drives to complete my measurements – your love and dedication know no bounds.

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ABSTRACT

In developed countries like Canada, significant amounts of eggshell (ES) and eggshell membrane (ESM) waste are generated by egg-breaking plants serving the food and biopharmaceutical industries. ESM is composed of 90% proteins with diverse bioactivities, including antibacterial, immunomodulatory, anti-inflammatory, and antioxidant properties. As a supplement, ESM shows promise in promoting gut health by modulating the microbiome through its bioactive effects. However, advanced solubilization strategies for creating ESM-based functional platforms remain underexplored. This study aimed to develop novel ESM formulations for gut health. Size-reduced ESM particles (JEM) were prepared via jet-O-mizing and hydrolyzed under optimized alkaline conditions using dilute potassium hydroxide, yielding a 50% solubilization of bioactive proteins and peptides. The hydrolyzed JEM suspension (WJ) was centrifuged to obtain the soluble fraction (SJ). Both WJ and SJ were further processed through simulated gastrointestinal digestion (SGID) to produce hydrolyzed metabolites (SJ-G, WJ-G, SJ-GI, and WJ-GI). JEM formulations demonstrated a 15-fold increase in Trolox-equivalent antioxidant capacity (TEAC) compared to non-hydrolyzed JEM (NJEM), with 10 mg/mL SJ showing 667.3 ± 25 μM Trolox versus 43.7 ± 7.1 for NJEM. SJ also exhibited bacteriostatic effects, inhibiting *E. coli* growth by 50 % over 24 hours compared to untreated culture. Additionally, SJ and WJ-G reduced Lipopolysaccharide-induced nitric oxide production by up to 80% in RAW 264.7 macrophages. The impact on intestinal barrier function was evaluated using Caco-2 cells, showing no significant changes in cell permeability. These findings highlight the development of bioactive ESM formulations with antibacterial, antioxidant, and anti-inflammatory properties, positioning them as promising dietary supplements for gut health.

TABLE OF CONTENTS

ABBREVIATIONS

ABTS	2,2'-Azino-bis (3-ethylbenzothiozoline-6-sulfonic acid)
AvBDs	Avian β-defensins
BATF	Basic Leucine zipper transcription factor
BBF	Burnbrae Farms
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Caco-2	Cancer Coli - Human epithelial colorectal adenocarcinoma
CD44	Cluster of Differentiation 44
CRP	C-reactive protein
CUPRAC	Cupric (Cu2+) reducing antioxidant capacity
CREMPs	Cysteine-rich ESM proteins
DMEM	2,2-diphenyl-1-picrylhydrazyl
DMEM-CCM	DMEM Complete Culture Media
DPPH	Dulbecco's Modified Eagle medium
DSS	Dextran Sodium Sulfate
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ES	Eggshell
ESM	Eggshell membrane
ESM-CH	Chemical hydrolysate of eggshell membrane

ESMF	Eggshell membrane fiber
FITC-CM-Dextran	Fluorescein isothiocyanate–Carboxymethyl–Dextran
GAGs	Glycosaminoglycans
GalNAc	N-acetyl-d-glucosamine
GI	Gastrointestinal
HA	Hyaluronic acid
HCl	Hydrochloric acid
HJEM	Hydrolyzed JEM
HPEM	Hydrolyzed PEM
IBD	Inflammatory bowel disease
IC	Ionomycin control
IL-8	Interleukin 8
JEM	Jet-O-mized eggshell membrane
KCl	Potassium Chloride
KOH	Potassium hydroxide
LOXL2	Lysyl oxidase-like type 2
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinases
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NJEM	Non-hydrolyzed JEM
NO	Nitric oxide

OCX-32	Ovocalyxin-32
OCX-36	Ovocalyxin-36
OD600	Absorbance value measured at 600nm
OP	Protex 26L
PBS	Phosphate buffered saline
PBMCs	Human peripheral blood mononuclear cells
PEM	Particalized eggshell membrane
Pen-Strep	Penicillin + streptomycin
PKA	cAMP-protein kinase A
p-value	Probability value
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron micrograph
SGD	Simulated Gastric Digestion
SGF	Simulated Gastric Fluid
SGID	Simulated gastrointestinal digestion
SIF	Simulated intestinal Fluid
SID	Simulated intestinal Digestion
SJ	Soluble fraction of JEM hydrolysate
SJ-G	SJ after gastric digestion
SJ-GI	SJ after gastrointestinal digestion

STAT	Signal transducer and activator of transcription
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TEER	Transepithelial electrical resistance
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha
WJ	Whole JEM hydrolysate
WJ-G	WJ after gastric digestion
WJ-GI	WJ after gastrointestinal digestion

LIST OF TABLES

LIST OF FIGURES

Chapter 1: GENERAL INTRODUCTION

Worldwide, approximately 100 million tons of unfertilized chicken eggs, equivalent to 1.65 trillion table eggs, are produced each year (Ahmed et al., 2023). Over 10 billion chicken eggs were produced in Canada in 2023, with around 33% of egg production directed to the processing sector (Statistics Canada, 2023), generating significant amounts of eggshell (ES) and associated eggshell membrane (ESM) waste. Large amounts of this waste are currently disposed of in landfills, where it negatively impacts the environment by attracting pests and promoting microbial growth. Due to ES resistance to natural degradation, it accumulates over time, leading to persistent waste issues. As a result, ES waste is classified as hazardous under European Union regulations (Mignardi et al., 2020). These by-products hold potential as valuable raw materials for various applications and further processing (Kulshreshtha et al., 2022). The ESM is approximately 1% of egg weight and consists of a bilayered proteinaceous fibrous meshwork, which is composed mainly of proteins (~90%), lipids (~3%), and carbohydrates (~2%) (Ahmed et al., 2019). ESM fibers contain various structural proteins, including collagens and cysteine-rich eggshell membrane proteins (CREMPs), in addition to a significant content of bioactive proteins. ESM is considered a functionally equivalent extracellular matrix (ECM); however, it is greatly stabilized by extensive desmosine, isodesmosine, and disulfide cross-linkages, which challenge the identification of its protein constituents by standard proteomic approaches and shield their activities (Ahmed et al., 2019b). Despite these difficulties, >500 protein constituents of ESM have been identified, with numerous predicted biological functions such as antioxidant, antimicrobial, antihypertensive, immunomodulatory and anti-inflammatory properties (Ahmed et al., 2017).

Processed ESM powder and ESM-derived carbohydrate fractions have demonstrated

immunomodulatory effects *in vitro* using a lipopolysaccharide (LPS)-induced inflammation model (Vuong et al., 2017). Studies have shown that commercially available ESM powder, NEM®, effectively reduced tumor necrosis factor α (TNF-α) production by peripheral blood mononuclear cells (PBMCs) following stimulation (Benson et al., 2012).Additionally, ESM powder has been found to mitigate LPS-induced inflammatory cytokine production and promote Caco-2 cell proliferation. Furthermore, ESM hydrolysate inhibited the secretion of the pro-inflammatory cytokine Interleukin 8 (IL-8) *in vitro* (Shi et al., 2014). In an *in vivo* mouse model of dextran sodium sulphate (DSS)-induced colitis, ESM supplementation significantly upregulated gene expression related to inflammatory mediators, intestinal epithelial cell proliferation, and antimicrobial peptides. Increased epithelial proliferation, along with reduced inflammatory cytokines and microbiota modulation helps preserve tight junctions and prevent leaky gut - key for conditions like IBD (Jia et al., 2017). Moreover, human clinical studies have reported that ESM consumption helps reduce pro-inflammatory cytokines and alleviates joint pain in patients with osteoarthritis (Ruff et al., 2015).

Several ESM supplements, including Kordel's BiovaFlex® eggshell membrane, Swanson Ultra Natural Eggshell Membrane, and Jamieson Natural Eggshell Membrane (NEM®), are commercially available. However, there is currently a lack of reported advancements in the development of novel technologies to create ESM-based functional platforms for gut health improvement. Given that egg products are inherently classified as "food grade", and that ESM is abundantly available as a by-product of egg product manufacturing, developing a natural ESM supplement presents a promising, non-pharmaceutical nutritional intervention for maintaining overall health.

Enhancing the solubility of ESM is a key research focus aimed at improving its bioavailability and maximizing its potential as a high-value dietary supplement for promoting gut health. This study aimed to optimize the alkaline hydrolysis process of jet-milled (jet-o-mized) ESM (JEM) to develop novel ESM formulations enriched with soluble peptides that exhibit enhanced bioavailability and bioactivities. To achieve this, we evaluated the antioxidant, antibacterial, and anti-inflammatory properties of the resulting peptide formulations. Additionally, their impact on epithelial cell integrity and permeability was assessed using the *in vitro* Caco-2 intestinal model, which are human epithelial colorectal adenocarcinoma cells and can replicate certain aspects of the gut epithelium. The immunomodulatory potential of the ESM formulations was further investigated in an *in vitro* murine model utilizing RAW 264.7 macrophages. Findings from this study establish a solid foundation for future *in vivo* investigations using a mouse feeding model to explore the biological efficacy of these novel ESM-based formulations as a promising dietary supplement for gut health.

Chapter 2: LITERATURE REVIEW

1. Avian egg

The avian egg is structured in layers, starting from the innermost yolk, followed by the egg white (albumen), the ESM, the calcified eggshell (ES), and finally the outer cuticle (Figure I). Its formation is a complex yet highly organized process (Hincke et al., 2012). After ovulation, the yolk passes through specialized sections of the oviduct, where it gathers essential components of the developing egg. In the infundibulum, the yolk is encased in the vitelline membrane, followed by the addition of the albumen in the magnum. Subsequently, precursors of the tightly cross-linked network of ESM fibers are secreted and assembled around the albumen in the white isthmus (Figure II) (Hincke et al., 2012). The double-layered ESM forms on the surface of the rotating egg, consisting of an inner thin membrane and an outer thick membrane (Cordeiro & Hincke, 2011).

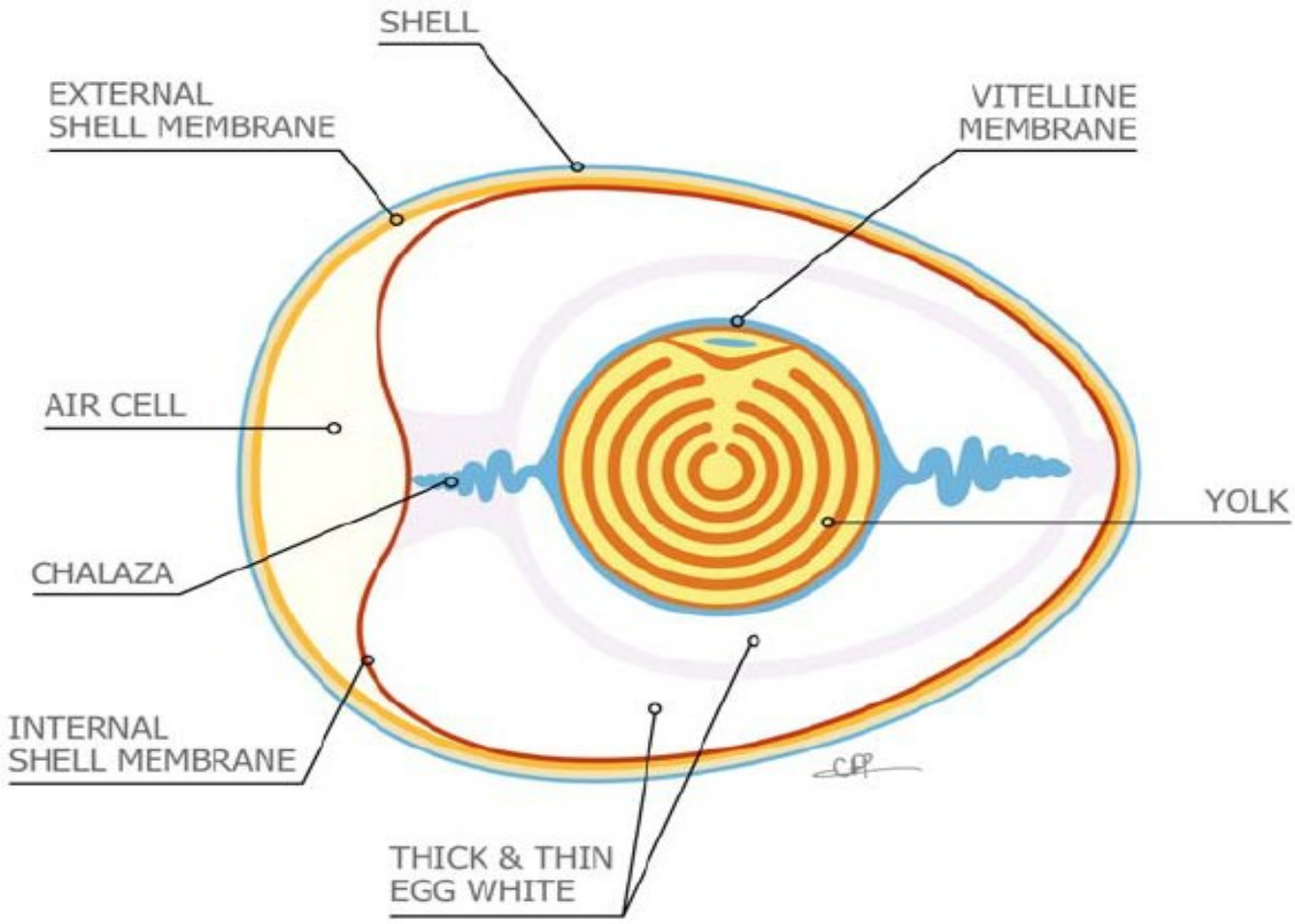

Figure I. Longitudinal section to depict the interior contents of a chicken egg (Hincke et al., 2012). "Copyright (2012) IMR Press"

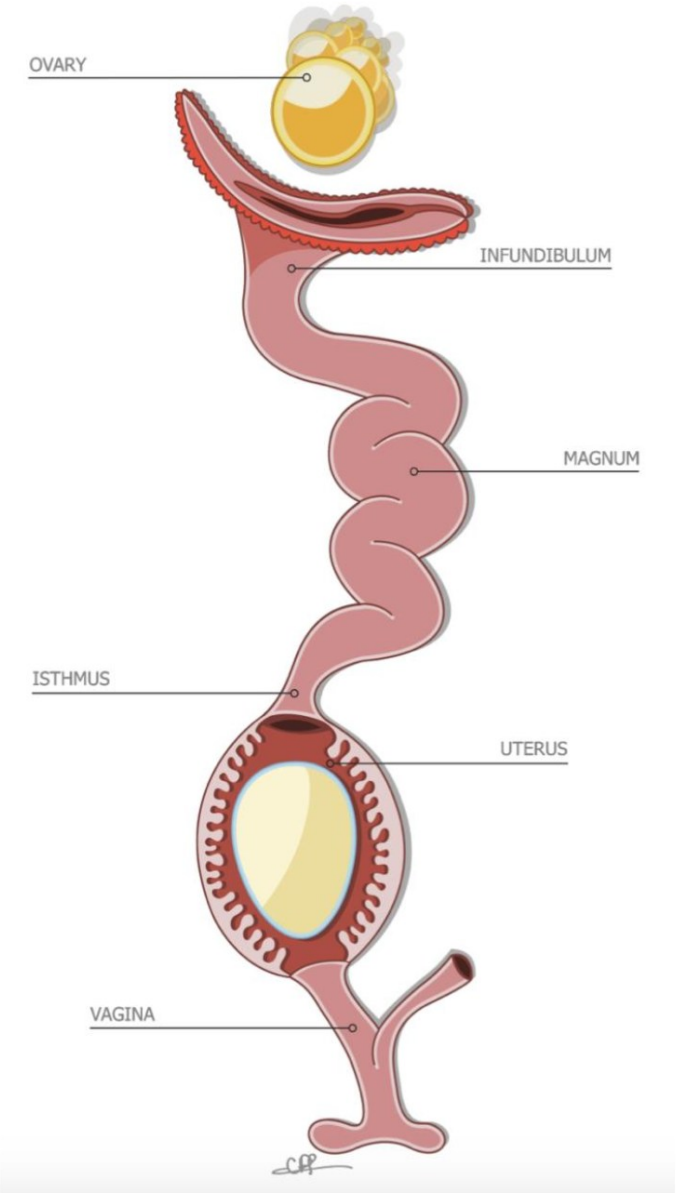

Figure II. Stylized depiction of the reproductive system of the hen, containing an incomplete egg in the uterus (Hincke et al., 2012). “Copyright (2012) IMR Press”

2. *Eggshell Membrane (ESM)*

2.1. Functions of Eggshell membranes

The ESM functions to maintain the albumen and is crucial for the formation of the ES by providing the structural support upon which biomineralization occurs during egg formation (Ahmed et al., 2017), while preventing mineralization towards the inner egg white (Hincke et al., 2000). It also provides a physical and biological homeostatic environment that supports the developing embryo in fertilized eggs. Abnormal ESM fiber formation, organization or cross-linking will inhibit normal ES biomineralization, which results in diminished ES quality and strength (Ahmed et al., 2023b).

2.2. Physical structure and chemical composition of Eggshell membranes

The ESM are a double-layered, mechanically stable, and insoluble cross-linked fibrous meshwork (figure III) (Ahmed et al., 2017). The outer and inner ESM layers differ slightly in terms of morphology, chemical composition, and the degree of protection they provide against pathogen invasion (Ahmed et al., 2023b). The features of the ESM are revealed by scanning electron microscopy (SEM) (figure III), where the thickness of the outer ESM is 50–70 μm and inner membrane is 15–26 μm. The outer ESM is adherent to the ES; its fiber diameters range between 1 and 7 μm. In comparison, the fibers of the inner membrane are smaller in diameter (0.1 to 3 μm) (Zhou et al., 2011). A top-down view of the outer ESM after removal from the ES revealed the tips of the mammillary cones still attached to the outer ESM fibers (figure IV) (Chien et al., 2008). As a result, preparations derived from the outer ESM are more likely to be contaminated with calcium carbonate from the ES, compared to those derived from the inner ESM (Kulshreshtha et al., 2022). Calcium carbonate contamination complicates accurate dosing in health formulations, interfering with processing, affecting solubility and yield, and poses regulatory and labeling challenges in functional food or supplement markets (Baláž et al., 2021a).

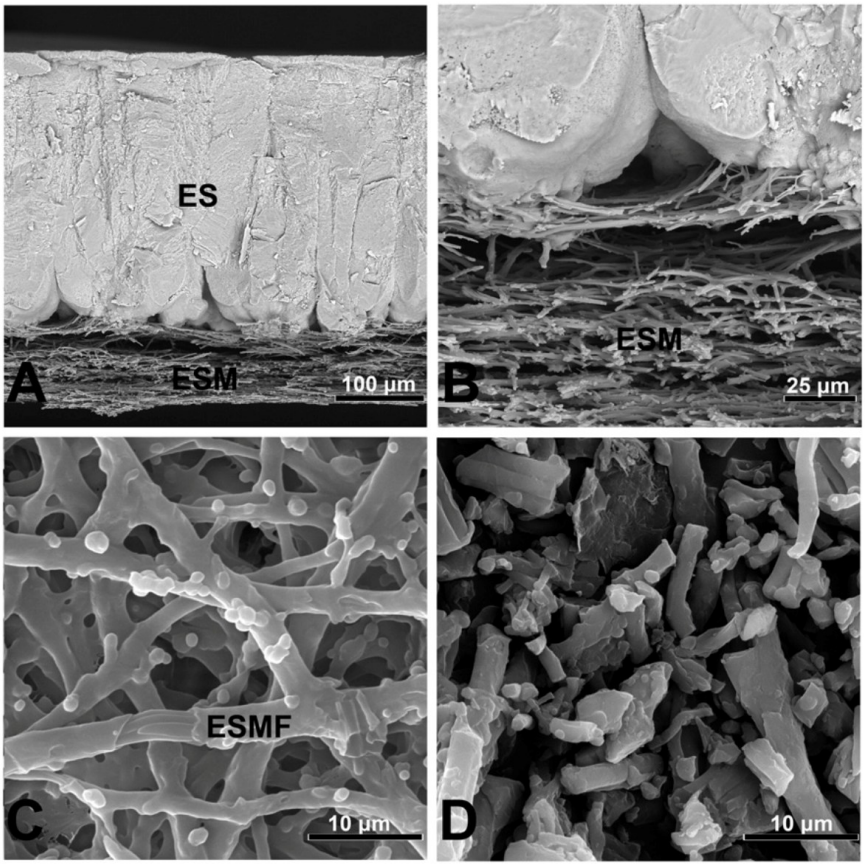

Figure III. Scanning electron micrographs illustrating the morphology of the eggshell and eggshell membranes. A. Eggshell cross-fractured to reveal the eggshell membranes (ESM) and calcified eggshell (ES); B. higher magnification of the ESM/mammillary cone interface; C. hand-peeled ESM, showing an eggshell membrane fiber (ESMF); D. processed ESM, showing fragmented eggshell membrane fibers (Ahmed et al., 2017).

Source: Ahmed et al., 2017. In-depth comparative analysis of the chicken eggshell membrane proteome. *Journal of Proteomics*, *155*, 49–62.

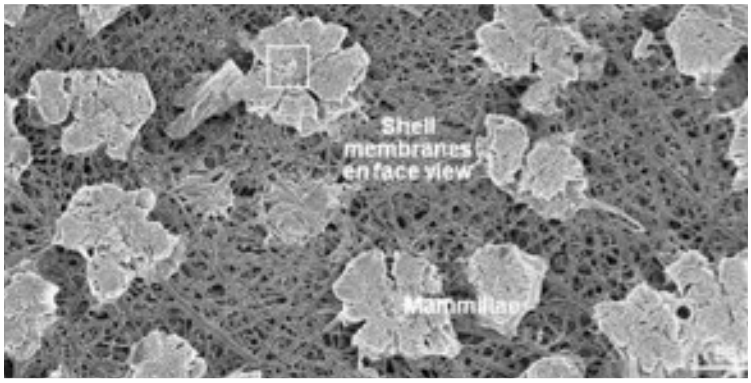

Figure IV. SEM showing mammillae patterned on the ESM in a pseudo-periodic manner (Chien et al., 2008)**.**

Source: Chien et al., 2008. Ultrastructural matrix-mineral relationships in avian eggshell, and effects of osteopontin on calcite growth in vitro. *Journal of Structural Biology*, *163*(1), 84–99.

The composition of ESM is traditionally characterized as approximately 90% proteins, 3% lipids, and 2% carbohydrates (Chen et al., 2014). The protein constituents of the ESM consist of approximately 10% types I, V, and X collagens, along with lysozyme, CREMPs, and a variety of other proteins and glycoproteins, such as ovotransferrin, lysyl oxidase-like type 2 (LOXL2), and others (Ahmed et al., 2017; Makkar et al., 2015).

The carbohydrate component of the ESM primarily consists of uncharged glycosaminoglycans (GAGs), predominantly hyaluronic acid (HA), which constitutes 81% of the carbohydrate fraction (Liu et al., 2014). HA is a polysaccharide made up of repeating disaccharide units of N-acetyl-d-glucosamine (GalNAc) and d-glucuronic acid (GlcA). It is the only GAG that is neither sulfated nor bound to proteins (Lindahl et al., 2015).

The mechanical properties of the ESM are attributed to the extensive cross-linking of desmosine, isodesmosine, and disulfide bonds (Kodali et al., 2011). The extensive lysine-mediated crosslinking of collagens, involving desmosine and isodesmosine and catalyzed by lysyl oxidase, imparts the ESM with its exceptional insolubility and structural flexibility. This enables the ESM to enclose the egg white effectively while providing a robust framework to support ES calcification. This crosslinking renders ESM intractable to dissolving in reducing agents and detergents, and subsequently, the ESM fibers possess poor solubility and low bioavailability, which hinders the identification of their proteins and their applications (Ahmed et al., 2017; Ahmed et al., 2023b). Hincke et al. (2000) identified and localized lysozyme as a component of ESM using colloidal gold immunocytochemical staining (Hincke et al., 2000). Kodali et al. (2011) discovered a major disulfide-rich structural protein, termed CREMP, by digesting reductively alkylated ESM proteins (Kodali et al., 2011). Ahmed et al. (2017) identified 472 proteins in the ESM through a combination of different extraction and solubilization methods using various

proteomic approaches. Among these, 163 and 124 proteins were found to be relatively or highly specific to the ESM compared to other egg compartments (Ahmed et al., 2017). Key protein families enriched in the ESM (Table I) include collagens, CREMPs, histones, avian β-defensins (AvBDs), LOXL2, and ovocalyxin-36 (OCX-36) (Ahmed et al., 2017).

Table I. ESM protein families.

	Protein family name	Family members identified in ESM
1.	Avian β-defensins	Avian B-defensins 9 and 10
2.	Collagens	Collagens Ill (a1 chain), IV (a1, a3, and a6 chains), V (a2 chain), VII (a1 chain), VIII (a1 chain), X (a1 chain), XII (a1 chain), XXII (a1 chain).
3.	Cysteine-rich eggshell membrane proteins (CREMPS)	CREMP – CREMP6
4.	Enolases	Enolases 2 and 3
5.	Heterogenous nuclear ribonucleoproteins	Heterogenous nuclear ribonucleoproteins A2/B1, A3, and D-like
6.	Histones	Histones H1.11L, H1.11R, H2A, H2B, H3 family 3C, and H5
7.	Motor proteins	Dyneins (axonemal heavy chain 1, 9, and 12), dynein (cytoplasmic heavy chain 1), and kinesin (21B and 26A).
8.	Myeloid/lymphoid or mixed-lineage leukemia	Myeloid/lymphoid or mixed-lineage leukemia 2 and 3
9.	Neuron navigator	Neuron navigators 2 and 3
10.	Protein-coupled receptor kinase interactors	Protein-coupled receptor kinase interactors 1 and 2
11.	Serpin peptidase inhibitors	Serpin peptidase inhibitors clade B (ovalbumin) members 1 and 5, and clade E (nexin, plasminogen activator inhibitor type 1) member 2
12.	Transient receptor potential cation channel	Transient receptor potential cation channel subfamily M member 1 and Subfamily V member 2
13.	Zinc finger proteins	Zinc finger protein 185-like and 335

Various protein families identified in ESM, modified from (Ahmed et al., 2017).

A variety of multifunctional proteins with antimicrobial, anti-inflammatory, and antiviral properties have been directly identified in the ESM. These include lysyl oxidase (Akagawa et al., 1999), lysozyme (Hincke et al., 2000), ovotransferrin (Gautron et al., 2001), OCX-36 (Cordeiro et al., 2013), histones (Ahmed et al., 2017), and AvβD 11 (Guyot et al., 2020), among others. Additionally, other bioactive proteins, identified in hen reproductive tissues or the ES matrix, are suggested to potentially exist in the ESM. These include OCX-32 (Gautron et al., 2001), and OCX-36, (Gautron et al., 2007), as well as AvβD types 1 - 5 and 7 - 12 (Abdel Mageed et al., 2008). These proteins not only provide innate immune protection to the embryo but also offer significant potential for biomedical research and the application of ESM as a valuable biomaterial (Baláž, 2014; Kulshreshtha et al., 2022).

3. Eggshell membrane Separation Methods

Since the fibers of the outer ESM are embedded within the ES mamillary cones, it must be separated from the mineralized ES layer before purifying the ESM (Romero et al., 2024). Common techniques for ESM separation are summarized in Table II. The primary methods for separating the ESM include physical, chemical, and enzymatic approaches. The physical methods for separating the ESM rely mainly on differences in mechanical strength and specific gravity between the ES and the membrane. In this process, the shell and membrane are mechanically crushed together, then mixed with water and allowed to settle. This stratification enables the separation of the shell from the membrane (Han et al., 2023). The outer fibers of ESM embedded in the tips of the mammillary cones of the ES can only be separated through chemical treatments, such as using dilute acids (e.g., acetic acid or hydrochloric acid (HCl) or calcium chelators like

ethylenediaminetetraacetic acid (EDTA) (Patra et al., 2020). Enzymatic methods achieve the highest recovery rate of ESM after separation. These methods utilize various enzymes to cleave peptide bonds within the fibrous connections of the ESM and between the outer ESM and the mineralized ES layer (Han et al., 2023). Major caveats of the current separation technologies include: modification and/or denaturation of ESM components (Ahmed et al., 2017; Zhang et al., 2016), high levels of calcium contamination (Pasarin & Rovinaru, 2019), contamination with egg white with and egg yolk (Snyder, 2014), accumulation of chemical and aqueous waste that negatively impacts the environment and microbial contamination (Kulshreshtha et al., 2022).

Table II. ESM Separation methods.

Type	Characteristics	Membrane recovery rate
Physical	Crushing, ultrasonic radiation or sieve separation. Recovery depends on specific gravity difference between ES and ESM	85 – 95 %
	Pressurization and heating (above 100°C and 1.2 Mpa)	High efficiency and film purity
	Cyclone airflow sorting devices	94 %
	Method and apparatus for separating ESM	High efficiency and film purity
Chemical	20 mL of 0.5 mol/L HCl for 20 minutes	89.21 %
	3.68 mol/L of HCl for 36.25 minutes at 48.96°C	96.52 %
	3 times excess CH_3COOH solution	93 %
Enzymatic	Alkaline Protease and Papain	98.9 %
	50 μg/mL Proteinase K for 48 hours	Unclean removal of outer ESM
	0.2 g/100 mL alkaline protease for 2 hours	Optimizing egg film properties

Typical ESM separation technology statistics, modified from (Han et al., 2023).

4. Methods to enhance Eggshell membranes Solubility & Bioavailability

ESM is composed of antimicrobial and antioxidant proteins as well as collagens, and GAGs such as HA, chondroitin sulfate, and glucosamine. However, its limited solubility and bioavailability, caused by the extensive cross-linking of ESM fibers (Ahmed et al., 2017; Du et al., 2015) poses a significant challenge to its effective use as a bio-functional material. To address these limitations, various processing and extraction methods have been developed and investigated.

4.1. Top-down size reduction

Studies have shown that size reduction of natural products significantly increases their solubility and bioavailability both *in vitro* and *in vivo*. Reducing the particle size of ESM flakes through grinding, pulverizing, or milling techniques such as ball milling, bead milling, jet milling, or vortex milling, followed by sieving and screening for size selection, can enhance the bioavailability of active components for targeted applications (Kulshreshtha et al., 2022). Ball milling is an effective technique for expanding the applications of egg-based biomaterials; however, it has notable drawbacks, including potential contamination of the processed material with fragments from the milling balls and the generation of elevated temperatures within the milling chamber (Baláž et al., 2021b). Specific size reduction methods can be applied to either dry ESM or ESM suspended in a liquid medium. Micronized ESM with an average particle size of less than 100 μm has been shown to support the healing of chronic wounds when used in wound dressings and implantable medical devices (Schmidt et al., 2016). We have recently introduced a novel, eco-friendly method for producing particalized eggshell membrane (PEM) intended for skin health applications (Kulshreshtha et al., 2020). Using cryo-grinding and homogenization techniques, the particle size of ESM was reduced to include submicron dimensions, approaching 200 nm. PEM demonstrated enhanced anti-inflammatory properties and antimicrobial activity against skin-associated

pathogens, including Gram-positive *S. aureus* and Gram-negative *P. aeruginosa*. These novel particles hold potential as topical ingredients in skincare products to promote skin health by reducing bacterial infections and inflammation (Kulshreshtha et al., 2020).

4.2. Chemical Hydrolysis

Chemical hydrolysis to cleave peptide bonds of ESM includes oxidation, reduction and acid/ alkali methods. The oxidation enhances ESM solubility by oxidizing the disulfide bonds between fibers into sulfonic acid groups, which exposes more soluble fibrin to the solvent. Reduction enhances the solubility of ESM by breaking its disulfide bonds using reducing agents like sulfhydryl compounds, which open disulfide bonds through exchange reactions without disrupting the peptide chains of the proteins (Han et al., 2023). ESM solubilization using acid-based methods can utilize inorganic acids, such as HCl and acetic acid, as disulfide bonds remain stable under acidic conditions. These methods can also incorporate reducing agents, such as 3-mercaptopropionic acid, to extract proteins from the ES membrane. In contrast, the alkali methods typically employ strong alkali solutions like sodium hydroxide (NaOH) to break disulfide bonds or even peptide bonds, resulting in a protein solution with lower molecular weights (Chi et al., 2019). Marcet et al. (2017) investigated the impact of ultrasonication on alkaline extraction of proteins from ESM, by treating ESM with 4% NaOH solution at 70°C for 60 minutes, combined with ultrasonication, led to the detachment of large protein clumps from the membrane, enhancing solubilization of its components. However, the antioxidant properties of unmodified proteins (alkaline treatment alone) were comparable to those of modified proteins (alkaline treatment with ultrasonication), indicating that ultrasonication did not enhance biological activity (Marcet et al., 2017). The chemical hydrolysate of eggshell membrane (ESM-CH), prepared by suspending powdered ESM in 5% (w/v) NaOH at 50°C for 4 hours, demonstrated significant NF-κB activation in human

peripheral blood mononuclear cells (PBMCs) (Ruff et al., 2015). In another study, solubilizing ESM powder in 5% (w/v) acetic acid and 1.25 M 3-mercaptopropionic acid at 90°C for 12 hours achieved a 63% recovery of ESM proteins (Santana et al., 2016). While chemical hydrolysis is a cost-effective method for extracting bioactive components from ESM, certain processing methods may produce toxic byproducts, leading to environmental concerns (Kulshreshtha et al., 2022).

4.3. Enzymatic Hydrolysis

Enzymes are used to solubilize ESM, which is a controlled process that can be easily managed through temperature regulation. However, it is often associated with incomplete reactions. This method is typically employed for solubilizing specific proteins (Han et al., 2023). Enzymes like alcalase (Pasarin et al., 2023), papain, bromelain (García & González, 2018), pepsin (Lee & Huang, 2019), trypsin (Ahmed et al., 2017) and actinase E (Liu et al., 2014) have been used to solubilize ESM. The solubilization of ESM can be enhanced by using a single enzyme or a combination of enzymes in optimized buffer conditions, supplemented with denaturing and reducing agents. This approach can reduce costs and/or improve efficiency during downstream processing. The addition of sodium dodecyl sulfate (SDS) or sodium taurocholate facilitates protein denaturation, while reducing agents such as sodium hydroxymethanesulfinate (CH_3NaO_3S) and sodium metabisulfite ($Na_2S_2O_5$) break disulfide bonds. This enhances the activity of enzymes like proteases, enabling the production of hydrolysates suitable for nutraceutical and cosmetic applications (García & González, 2022). Ultrasonic treatment of enzymatic hydrolysates of ESM demonstrated superior functional properties compared to untreated hydrolysates. Digestion of ESM with a particle size of less than 180 μm using alcalase (2:100 g/g) for 8 hours achieved maximum protein solubility (84%), along with enhanced water-holding capacity, foaming ability,

and emulsifying properties (Jain & Anal, 2016). Another study showed that enzymatic hydrolysis of ESM using alkaline protease in a sodium bicarbonate/sodium carbonate buffer at 60°C over five days produced a hydrolysate that significantly activated NF-κB in PBMCs (Ruff et al., 2015). Proteolysis of ESM powder with actinase E (0.25 mg actinase/dry membrane) at 55°C for 24 hours enabled the purification of HA with a yield of 0.07%. A higher yield was achieved when ESM powder ground in dry ice was used as the starting material, compared to intact ESM, which yielded 0.04% HA. A study employed a combination of chemical extraction (Tris-HCl/SDS/urea) and enzymatic hydrolysis using papain at 37°C for 48 hours to extract carbohydrates from ESM. The resulting carbohydrate fraction, containing both sulfated and non-sulfated GAGs, exhibited NF-κB-mediated immunomodulatory and anti-inflammatory activities in lipopolysaccharide (LPS)-stimulated human immune cells (Vuong et al., 2017).

4.4. Use of Nanotechnology

Nanotechnology is a rapidly advancing field increasingly used to develop ESM-based biomaterials for biomedical applications like tissue engineering and regenerative medicine (Kulshreshtha et al., 2022). ESM powder was recently used to crosslink a gelatin-chitosan cryogel, creating bioengineered skin substitutes for large, deep wounds (Saha et al., 2021). An eco-friendly approach developed a biocompatible composite membrane of polydopamine-modified ESM nano/microfibers coated with KR-12 antimicrobial peptide and HA for wound healing (Liu et al., 2019). The micro-fibrous network of ESM fibers serves as an extracellular matrix for tissue-engineered scaffolds. For instance, bioactive nanopatterned ESM (N-ESM) scaffolds were developed for bone tissue regeneration. These scaffolds were non-toxic to osteoblast MG63 cells, promoting osteogenic differentiation without affecting cell viability (Park et al., 2021). ESM has

been used as a template for composites with bioactive molecules or embedded nanoparticles for wound healing. Incorporating silver nanoparticles into ESM microfibers enhanced re-epithelialization, granulation tissue formation, cell proliferation, and inflammation control (Liu et al., 2017).

5. Inflammatory process & gut health

The role of the immune system is to protect the body from invasive pathogens and harmful stimuli (Lee & Park, 2016). However, in the last few decades, increasing evidence indicates that the immune system and inflammatory processes are involved in a wide variety of health problems that are leading causes of morbidity and mortality worldwide.

More than 50% of all deaths are attributable to inflammation-related diseases including diabetes mellitus, ischemic heart disease, cancer, kidney diseases, non-alcoholic fatty liver disease (NAFLD), and auto-immune and neurodegenerative conditions (Furman et al., 2019). Inflammation is a process characterized by immune cell activation to protect the host from bacteria, viruses, and toxins by eliminating pathogens and promoting tissue repair and recovery. The protective effects of inflammatory processes and potential for tissue destruction are balanced in the healthy state. However, if uncontrolled, inflammation is implicated in numerous diseases like rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease (IBD), psoriasis, immune-inflammatory ailments, and neoplastic transformations. Furthermore, chronic inflammation is also associated with tumorigenesis and is recognized as a risk factor for various cancers (Patil et al., 2019).

The inflammatory responses are regulated mainly with an array of immune regulatory cytokines. Pro-inflammatory cytokines trigger both acute and chronic inflammatory reactions, while anti-

inflammatory cytokines regulate the magnitude and the extent of inflammation (Pradervand et al., 2006). . Various signaling pathways trigger the secretion of cytokines, including signal transducer and activator of transcription (STAT), the nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPK) , cAMP-protein kinase A (PKA), and interferon regulatory factor (IRF) (Barnes & Karin, 1997; Ozato et al., 2002) (Figure V).

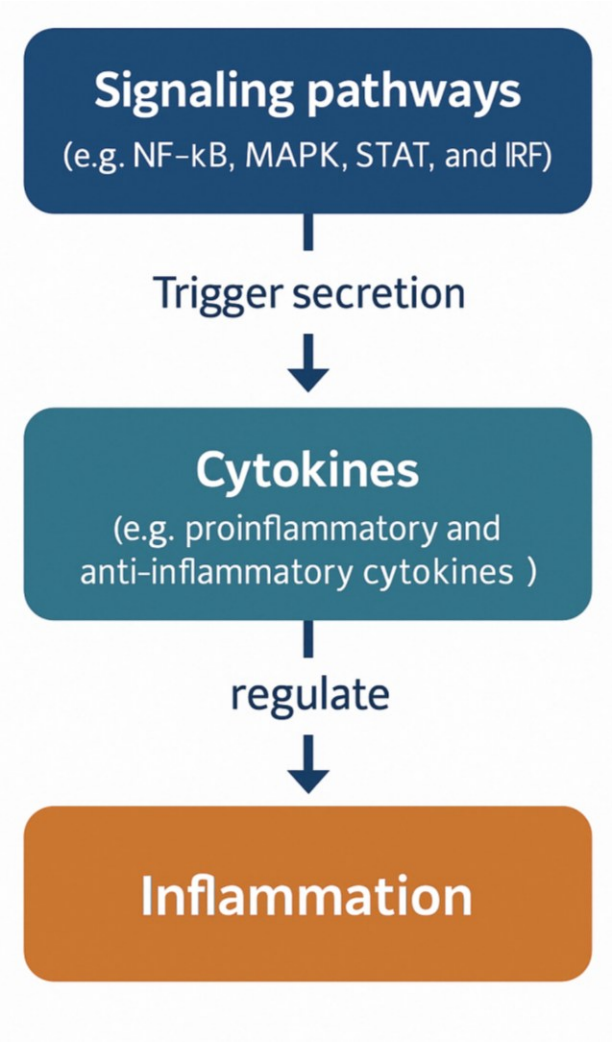

Figure V. A simplistic diagram illustrating an overview of the regulation of inflammatory response.

The gut is continuously exposed to diverse environmental stimuli that can lead to various inflammatory conditions (Cardoso Dal Pont et al., 2020). The human gut plays a vital role in digestion, metabolism, immune function, and inflammation regulation - key factors in chronic diseases such as IBD, obesity, diabetes, and cardiovascular conditions (Potrykus et al., 2021; Vemuri et al., 2020). Gut health is critical for developing and regulating the immune system and excluding pathogens (Yang et al., 2014). In addition, its microbiota regulates gut permeability to maintain health (Biesalski, 2016). Genetic, environmental, and dietary factors affect the gut microbiome profile (Valdes et al., 2018). Maintenance of gut health supports the production of anti-inflammatory mediators like short-chain fatty acids (SCFAs), which preserve gut integrity. Disruptions in gut function can lead to inflammation and increased gut permeability (Bisanz et al., 2019).

Diet significantly influences intestinal health and inflammatory markers such as IL-6, C-reactive protein (CRP), and TNF-α, which are linked to metabolic diseases (Aleksandrova et al., 2021). Protein digestion begins with chewing and saliva in the mouth, followed by acid and pepsin action in the stomach, where proteolysis is initiated (Loveday, 2023). In the distal colon, proteolytic fermentation produces branched-chain fatty acids, meaning that protein quality and quantity impact microbiota composition and metabolite production (Randeni et al., 2024; Rinninella et al., 2023). The digestibility of ESM proteins in the rats GIT is 87%.

6. Eggshell Membrane & Gut Health

The human gut microbiota is a complex ecosystem essential for digestion, metabolism, immune regulation, and even brain function (Vemuri et al., 2020). Diet greatly influences microbiome balance, healthy diets promote beneficial bacteria, while Western diets can lead to dysbiosis (García-Montero et al., 2021). This imbalance weakens the gut barrier, increases inflammation, and reduces protective metabolites like SCFAs, and has been linked to chronic diseases such as IBD, diabetes and cancer (Randeni et al., 2024). Recent studies suggest that modified ESM, rich in bioactive proteins and peptides, can beneficially modulate gut microbiota composition and function, improving markers of gut health such as microbial diversity and SCFA production (Yang et al., 2022).

6.1 Anti-inflammatory activity

Inflammation plays a crucial role in the advancement of various gastrointestinal disorders (Randeni et al., 2024). Jia et al., (2017) found that ESM modulates microbiota, offering new prevention and treatment strategies. In a dextran sodium sulfate - induced murine model of colitis, mice were fed 8% ESM fine powder-supplemented diet and results showed that ESM improved disease activity, colon shortening, and inflammatory gene expression, while enhancing epithelial proliferation and antimicrobial peptide production. In the same study, omics analysis demonstrated improved energy metabolism, increased microbial diversity, and a reduction in pathogenic bacteria like *Enterobacteriaceae* and *E. coli*. ESM also regulated Th17 cell expansion by limiting segmented filamentous bacteria, aiding epithelial repair, energy balance, and reducing mucosal inflammation (Jia et al., 2017).

In a study conducted by Vuong et al., (2107), ESM powder and an ESM- derived carbohydrate fraction reduced the activity of the NF-κB in the LPS-induced human monocytic cell line (U937–3xκB-LUC). In LPS-induced human monocytic leukemia cell line THP-1(in macrophage-like state), ESM down-regulated the expression of toll-like receptor 4 (TLR4) and cell surface glycoproteins Intercellular Adhesion Molecule 1 (ICAM-1) and Cluster of Differentiation 44

(CD44), and increased the secretion of the anti-inflammatory cytokine IL-10, while the ESM carbohydrate fraction reduced secretion of the pro-inflammatory cytokines IL-1β and IL- 6 (Vuong et al., 2017). Another study highlighted ESM's potential for pre-cachexia prevention. In an IL10-knockout mouse model, transcriptomic analysis showed that ESM supplementation suppressed LPS/IL1-mediated RXR pathway inhibition in the liver and reduced colonic chemokine and Th cell marker expression by targeting the upstream Basic Leucine zipper transcription factor (BATF) pathway. ESM also enhanced microbial alpha diversity, reduced inflammation-associated microbiota, and increased organic acid levels, particularly short SCFAs like butyrate (2.3-fold), which inhibit Th1 and Th17 production (Jia et al., 2022). Rønning and coworkers proposed ESM as a promising nutraceutical for addressing skeletal muscle aging through immunomodulation and gut microbiota effects. Mice fed an 8% ESM diet exhibited increased gut microbiota diversity, altered microbial composition, and reduced TNFA expression in both mice and THP-1 macrophages (Rønning et al., 2023).

6.2. Antimicrobial Activity

ESM is naturally rich in antimicrobial proteins and peptides, including lysozyme, histones, and AvBDs (Ahmed et al., 2017; Du et al., 2015; Rose-Martel & Hincke, 2017). ESM glycoproteins like mucin and ovomucin also possess antibacterial and antiviral activities (Lieleg et al., 2012;

Omana et al., 2010). Additionally, minor components such as protease inhibitors (ovalbumin Y, ovomacroglobulin, ovomucoid) enhance ESM antimicrobial effectiveness (Andersen, 2015; Makkar et al., 2015). ESM hydrolysates have demonstrated broad-spectrum antimicrobial activity against pathogens including *S. aureus*, *B. subtilis*, *K. pneumoniae*, *S. marcescens*, and *E. coli* (Yoo et al., 2014).

These observations underscore the potential of ESM for gut health applications, particularly in managing dysbiosis-related disorders to support a balanced gut microbiome.

6.3. Antioxidant Activity

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses, is a key factor in the onset of various GI disorders (Semenova et al., 2024). Excessive ROS levels can harm cellular structures, promote inflammation, and contribute to diseases like IBD and colorectal cancer. For example, oxidative stress can compromise the mucosal barrier of the GI tract, making it more susceptible to bacterial invasion and triggering immune responses that lead to IBD (Semenova et al., 2024). Antioxidants are able to counteract oxidative stress and restore the redox equilibrium. Due to their safety and accessibility, the search for natural antioxidants as alternatives has attracted wide interest among researchers. Protein hydrolysates exhibit better antioxidant activity than their intact form due to structural changes in the secondary and tertiary structure including reductive cleavage of disulfide bonds, that increase the free radical scavenging and prooxidative metal chelating capacity (Elias et al., 2008). A majority of peptides derived from natural sources and industrial by-products possess potential radical scavenging activity, inhibition of liposome oxidation or inhibition of thiobarbituric acid reactive substances (TBARS, degradation products of fats) (Shahidi & Zhong,

2008). Ultrafiltered ESM enzymatic hydrolysate fractions demonstrated a range of antioxidant activity including iron (Fe^{3+}) reducing, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging, and Fe^{2+} chelating activity which were further verified by a cell-based study where ESM hydrolysate reduced proinflammatory cytokine IL-8 secretion in oxidative stress-induced human intestinal epithelial cells (Cancer Coli - Human epithelial colorectal adenocarcinoma Caco-2 cells) (Shi et al., 2014). Similarly, ESM enzymatic hydrolysates and corresponding identified synthetic peptides showed a strong ability to quench 2,2'-Azino-bis (3-ethylbenzothiozoline-6-sulfonic acid) ABTS, inhibit TBARS and had a high total antioxidant activity in a study by Zhao and coworkers (X. Zhao et al., 2019).

6.4. Effect on Intestinal Barrier Function

The integrity of the intestinal barrier is crucial for maintaining gut function and overall health. Disruptions, such as increased intestinal permeability, are associated with various diseases, including IBD (McGuckin et al., 2009), irritable bowel syndrome (IBS) (Piche et al., 2009), metabolic syndrome (Teixeira et al., 2012), and central nervous system disorders (Julio-Pieper et al., 2014). These observations underscore the vital role of gut integrity in sustaining host health.

There is growing interest in food-derived bioactive peptides for improving intestinal barrier function and their potential in disease management (Bao & Wu, 2021; Wang et al., 2021). Studies have shown that proteins and bioactive peptides from food regulate the physical, chemical, biological, and immunological aspects of the intestinal barrier (Bao & Wu, 2021). Examples include casein hydrolysate and peptides (Visser et al., 2010; Yasumatsu & Tanabe, 2010), fish protein hydrolysate (Marchbank et al., 2009), soybean β-conglycinin hydrolysate (Ren et al., 2014), and wheat gluten-derived peptides A5 and C5 (Maggioni et al., 2016).

Transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)-dextran transport assays are commonly employed to assess epithelial barrier integrity in Caco-2 cell monolayers. TEER measurements provide real-time, non-invasive monitoring of barrier function by evaluating the electrical resistance across the epithelial layer. Elevated TEER values indicate well-formed tight junctions and robust barrier properties, while decreased values suggest compromised integrity. Complementarily, FITC-dextran transport assays measure the permeability of the monolayer to fluorescently labeled dextran of specific molecular sizes. The extent of FITC-dextran passage through the monolayer reflects the tightness of the paracellular pathway, with increased permeability signifying disrupted barrier function. Together, these assays offer comprehensive insights into the structural and functional status of epithelial barriers *in vitro* (Felix et al., 2021)

For example, the egg white protein ovomucin hydrolysate, Protex 26L (OP), effectively restored transepithelial electrical resistance (TEER) and reduced FITC-dextran (4 kDa and 40 kDa) permeability in LPS-treated Caco-2 cells at concentrations of 0.1, 0.5, and 1.0 mg/mL. OP also preserved the expression and structural integrity of tight junction proteins, including occludin and ZO-1, ensuring their proper localization on the cell surface (Bao et al., 2024).

Further research is essential to explore the direct effects of ESM on intestinal barrier function. Current evidence highlights the bioactive properties of ESM, including its antimicrobial, anti-inflammatory, and antioxidant activities, which are promising for gut health applications. However, ESM specific role in maintaining or enhancing the structural and functional integrity of the intestinal barrier remains largely unexplored.

Studies should investigate how ESM impacts key components of the intestinal barrier, such as tight junction proteins (e.g., occludin, claudins, and ZO-1), mucosal integrity, and the regulation

of intestinal permeability. Additionally, it is important to determine whether ESM-derived bioactive peptides or hydrolysates can restore barrier function under conditions of stress or inflammation, such as those induced by LPS, cytokines, or other gut-disrupting factors.

Improving the solubility of eggshell membrane (ESM), a structurally resilient biomaterial, remains a critical challenge due to its extensive disulfide and lysine-mediated cross-linking, which limits its digestibility and functional applications. Despite its rich composition of bioactive proteins and peptides with antioxidant, antimicrobial, and anti-inflammatory properties, ESM remains underutilized, particularly in gut health interventions. This study addresses this gap by introducing a novel, enzyme-free, scalable alkaline hydrolysis method using diluted KOH to solubilize jet-milled ESM (JEM). The resulting formulations were evaluated for their bioactivity and safety using *in vitro* models (Caco-2 and RAW 264.7), revealing significant improvements in solubility, functional bioactivity, and epithelial barrier compatibility. These findings support the development of ESM-based supplements with gut-targeted benefits and form the basis for future *in vivo* investigations.

Chapter 3: HYPOTHESIS AND OBJECTIVES

1. Hypothesis

Improved extraction and processing strategies for ESM will lead to enhanced anti-inflammatory and immunomodulatory activities, solubility, bioavailability and bioactivities of ESM formulations. These formulations will have potential to promote gut health.

2. Objectives

1. Optimization of alkaline hydrolysis conditions for JEM to produce an ESM hydrolysate with enhanced solubility.
2. Digestion of the produced ESM hydrolysate by simulated gastrointestinal digestion (SGID) to produce ESM metabolites *in vitro.*
3. Characterization of bioactivities of the developed ESM formulations, including antimicrobial and antioxidant activities.
4. Assessment of anti-inflammatory activity of the developed ESM formulations *in vitro*.
5. *In vitro* evaluation of the effect of new formulations on intestinal barrier integrity and permeability.

Chapter 4: MANUSCRIPT SUBMITTED TO FOOD RESEARCH INTERNATIONAL

Jet-O-mized eggshell membrane powder: A novel hydrolysis strategy to unlock gut-promoting bioactivities

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Abstract

This study aimed to create novel Eggshell membrane (ESM) formulations suitable for gut health benefits. Size-reduced ESM particles (JEM) were prepared by Jet-O-mizing (high-efficiency vertical jet milling) and subjected to alkaline hydrolysis using diluted potassium hydroxide, with heating and stirring. This resulted in a 50% solubilization yield of bioactive proteins and peptides, outperforming other chemical hydrolysis methods. The soluble protein fraction (SJ) was separated from the whole hydrolysate (WJ), and both underwent simulated gastrointestinal digestion to generate hydrolyzed JEM metabolites. The antioxidant capacities of the JEM derivative formulations showed a significant 15-fold increase compared to the soluble portion of non-hydrolyzed JEM (NJEM), where SJ showed antioxidant power equivalent to 667.3 ± 25 μM Trolox versus 43.7 ± 7.1 in case of NJEM. In addition, the SJ exhibited bacteriostatic effects against *E. coli*, where the bacterial growth was inhibited by 50% in 24 hours, compared to the untreated bacterial culture. The formulations demonstrated superior anti-inflammatory properties when tested on lipopolysaccharide (LPS) - induced RAW 264.7 macrophages, where they showed up to 80% reduction in LPS-stimulated nitric oxide (NO) production following 24 hours of treatment, compared to untreated cells. The permeability of the intestinal barrier model human epithelial colorectal adenocarcinoma (Caco-2) was not significantly affected upon treatment with the developed JEM formulations. This study provides novel formulations of ESM with high bioavailability and bioactivities including antibacterial, antioxidant, and anti-inflammatory effects, positioning them as promising candidates for dietary supplements aimed at promoting gut health.

Keywords: eggshell membrane, alkaline hydrolysis, proteins, antibacterial, antioxidant, anti-inflammatory, gut permeability.

1. Introduction

Globally, about 100 million tons of unfertilized chicken eggs (1.65 trillion table eggs) are produced annually (Ahmed et al., 2023). ES and ESM in the egg structure are approximately 10-12% and 1.02% (wt. %), respectively (Han et al., 2023). In Canada, approximately 33 % of the egg production goes to the processing sector (Statistics Canada, 2023), producing considerable ES and ESM waste, which are potentially valuable raw materials for various applications and secondary processing (Ahmed et al., 2021). Structurally, ESM is a fibrous semi-permeable membrane wrapped around the egg white and embedded in the ES mammillary cones as the innermost shell component (Ahmed et al., 2023). ESM prevents egg white exudation, protects the egg from external pathogens, provides the initial support for ES mineralization and improves the mechanical properties of the egg (Ahmed et al., 2017; Hincke et al., 2000; Mine et al., 2023) Chemically, the fibrils of the ESM consist mainly of proteins (~90%), with low amounts of lipids (~3%) and carbohydrates (~2%) (Ahmed et al., 2019b). Over 500 proteins have been identified in the ESM (Ahmed et al., 2017), with collagens being the most plentiful, along with other proteins and glycoproteins (Torres-Mansilla et al., 2023), including Cysteine-rich ESM proteins (CREMPs) (Ahmed, Suso, Maqbool, et al., 2019), egg white proteins (i.e., ovotransferrin and lysozyme) and ES matrix proteins (i.e., ovocalyxin-36) (Ahmed et al., 2017, 2019b; Shi et al., 2021). The ESM's distinctive physicochemical, biological properties, compositional, and mechanical characteristics, as well as its availability, make it a promising biomaterial for diverse biotechnological applications. ESM has been studied as a natural supplement for diverse application including the treatment of joint and connective tissue disorders (Ruff et al., 2018), wound healing (Ahmed, Suso, & Hincke, 2019), tissue regeneration (Farjah et al., 2020) and food packaging (Li et al., 2020). ESM has various applications for its anti-inflammatory (Kulshreshtha et al., 2020), antimicrobial

(Preda et al., 2020), antioxidant (Nimalaratne et al., 2015), and biosorbent (Parvin & Biswas, 2019) activities.

A number of pre-clinical studies have suggested that isolated ESM could be repurposed as a beneficial dietary intervention because of its immunomodulatory and anti-inflammatory effects, along with its capacity to modulate gut microbiota (Jia et al., 2022; Ramli et al., 2020; Yang et al., 2022). Yang et al. (2022) showed that supplementation of an 8% (w/w) KOH treated fraction of ESM improved survival and body composition in IL-10 knockout mice, a model for IBD. ESM increased gut microbial diversity restored the Firmicutes/Bacteroidetes ratio, elevated beneficial bacteria (e.g., *Ruminococcus*), and reduced pro-inflammatory *Enterobacteriaceae*. It also boosted butyrate levels and downregulated inflammatory genes (*Il-1β*, *TNF-α*), highlighting its anti-inflammatory potential. They demonstrated that ESM supplementation improved survival, enhanced gut microbial balance, increased butyrate production, and reduced inflammatory markers, supporting its anti-inflammatory and gut health benefits (Yang et al., 2022). Jia et al. (2022) showed that ESM supplementation in IL10-knockout mice improved body composition, muscle strength, and reduced inflammation, while enhancing microbiota diversity and short chain fatty acids (SCFA) production, suggesting its potential for cachexia prevention (Jia et al., 2022). ESM may be effective in obesity management, since ESM supplementation in mice fed a high-fat diet lowered plasma triglycerides and liver cholesterol, while increasing expression of lipid metabolism genes such as carnitine palmitoyltransferase 1A. Microbiota analysis revealed increased relative abundance of the anti-obesity bacterium *Lactobacillus reuteri* and decreased abundance of inflammation-related bacteria such as *Blautia hydrogenotrophica* (Ramli et al., 2020). Ronning and coworkers reported that a diet with 8% ESM increased gut microbiota diversity and lowered TNFA expression in mice and *in vitro* in THP-1 macrophages. In their

human study, dietary ESM reduced the inflammatory marker C-reactive protein (CRP) (Rønning et al., 2023).

ESM proteins are highly insoluble due to their inter- and intra-molecular disulfide bonds, as well as cross-linking of lysine-derived desmosine and isodesmosine. Previous studies used several solubilization approaches to cleave the highly cross-linked structure of ESM using mechanical and chemical methods or a combination of these techniques. Physical ESM solubilization strategies included heating, pressurization, extrusion, and ultrasound energy (Han et al., 2023). Mechanical size reduction and sieving of ESM do not alter its amino acid composition. The amino acid profile of particalized ESM (PEM), regardless of particle size, remains consistent with that of unprocessed ESM (Kulshreshtha et al., 2020). Enzymes have also been used for ESM proteolysis (Lien et al., 2022). Lee and coworkers estimated that the solubility of ESM proteins could reach 91.4% through an orthogonal test using acetic acid and pepsin, combined with a segmental extraction technique (Lee & Huang, 2019).

Increasing ESM solubility is an important research direction to improve its bioavailability and potential as a high-value dietary supplement to promote positive gut health.

This study introduces a novel, simplified, and scalable approach for solubilizing jet-o-mized eggshell membrane (JEM) using mild alkaline hydrolysis, marking a first-of-its-kind strategy for generating gut-targeted bioactive peptides from ESM. Unlike traditional enzyme- or acid-based protocols, this process combines jet milled ESM with low-concentration KOH treatment under moderate conditions to achieve high solubilization efficiency while preserving bioactivity. The optimized method offers a cost-effective and safe alternative for producing functional ESM formulations with demonstrated antioxidant, anti-inflammatory, and gut barrier-supporting properties laying the foundation for their use as high-value dietary supplements for gut health

2. Materials and Methods

2.1. *Materials*

The bacterial strain used in this study was *E. coli* (0157:H7 derived from ATCC® 43888™)**.** Bacterial cultures were maintained and grown using Luria-Bertani (LB) broth (BioShop, Canada). The cell lines used in the study are: RAW 264.7 macrophages (ATCC® TIB-71™, Lot#70012232) and Caco-2 cells (ATCC, HTB-37). Unless stated otherwise, all additional chemicals and supplies were procured from Fisher Scientific, On, Canada or Sigma Aldrich, ON, Canada.

2.2.Jet-o-Mized (JEM) biomaterial

In this study, the material under investigation consisted of ESM powder provided by our industrial partner Burnbrae Farms (BBF, Lyn, Ontario), which was subjected to further processing via a high-efficiency vertical jet mill (Fluid Energy Processing and Equipment Company, USA) designed for grinding dried powders to 1–50-micron average particle size, to produce JEM. Microbiological assessments and chemical analyses of JEM were conducted by Eurofins Environex (Quebec, Canada) to quantify microbial contamination and characterize chemical constituents, providing essential data to evaluate quality. Particle size distribution of a suspension of JEM in 2-propanol was determined using a Beckman Coulter LS Particle Size Analyzer.

2.3.Alkaline Hydrolysis of JEM

In preliminary trials (data not shown), the alkaline hydrolysis of PEM using potassium hydroxide (KOH) under various conditions was extensively investigated to optimize the production of PEM hydrolysate (HPEM, including temperature, time, PEM and KOH concentrations and stirring intensities). Building on that protocol, the current study focused on hydrolyzing JEM with further

investigation of the hydrolysis time to achieve a suitable degree of hydrolysis. The optimal conditions for producing hydrolyzed-JEM (HJEM) were determined (subject to invention disclosure). The aim was to achieve partial hydrolysis of JEM to produce HJEM. Thereafter, samples were collected at fixed intervals and analyzed by SDS-PAGE. The reaction was terminated through neutralization with 11.56 M hydrochloric acid, followed by centrifugation to separate the soluble fraction (SJ, containing soluble proteins and peptides) and remaining insolubilized JEM pellet from the whole hydrolysate (WJ). WJ and SJ were stored at -20°C for subsequent analysis and processing, which is summarized in Figure 1.

2.4.Simulated Gastrointestinal Digestion of JEM Hydrolysate

WJ and SJ were separately subjected to SGID, following a standardized method detailed by Minekus and coworkers (Minekus et al., 2014). This method simulates the physiological conditions of the human gastrointestinal tract and consists of two separate phases: Simulated gastric digestion (SGD) and simulated intestinal digestion (SID). In the gastric phase, the JEM formulations were mixed with the simulated gastric fluid (SGF) and pepsin, followed by pH adjustment to 3.0 using concentrated HCl and incubation at 37°C for 2 hours to simulate stomach digestion. At the end of the gastric digestion, samples were collected and stored at -20°C for subsequent analysis and processing. The resultant simulated gastric metabolites were abbreviated WJ-G, and SJ-G. In the intestinal phase, the gastric metabolites WJ-G, and SJ-G were mixed with simulated intestinal fluid (SIF), bile salts, and pancreatin, and the pH was adjusted to 7.0 using 10 N NaOH. The mixture was incubated at 37°C to simulate digestion in the small intestine. The resultant simulated intestinal metabolites WJ-GI and SJ-GI were collected after 4 hours of simulated digestion and stored at -20°C for subsequent analysis and processing.

2.5.Desalting/Concentration of JEM Formulations

Before proceeding with the evaluation of bioactivities of the developed various JEM formulations (SJ, SJ-G, SJ-GI, WJ-G and WJ-GI), desalting was conducted to remove residual KCl formed upon neutralization of the HJEM with HCl to stop the hydrolysis reaction. Centrifugal filter units (Millipore™ Amicon™ Ultra-15 Centrifugal Filter Units, MWCO: 3 KDa) were used to remove the salt and concentrate the JEM formulations. The manufacturer's procedures were followed, which involved the loading of 15 mL of the clear supernatant into the desalting column, followed by centrifugation at 5,500 RPM for 40 minutes at 4°C (16 Lynx 4000 Centrifuge). To enable sample desalting and washing, the sample volume inside the column was completed to 15 mL with either phosphate buffered saline (PBS) or Dulbecco's Modified Eagle medium (DMEM; Gibco, NY, USA) and then re-centrifuged. This washing step was repeated three times to ensure the thorough removal of KCl. At the end of the desalting process, proteins retained at the upper compartment of the column were collected, analyzed for their protein content, and stored at -20°C for subsequent processing.

2.6.Protein Quantification

The outcome of the hydrolysis and digestion reactions was assessed using the Bicinchoninic acid (BCA) assay and SDS-PAGE before and after desalting/concentration.

BCA Assay: The soluble protein content of the hydrolysate SJ and the metabolites SJ-G SJ-GI, WJ-G, and WJ-GI were quantified. Before analysis, thawed samples were centrifuged for 5 minutes, 13000 rpm at 4°C (accuSpin Micro 17R, Fisher Scientific) to obtain a soluble supernatant for analysis. The sample (25 µL) of the soluble, neutralized protein samples were aliquoted into microplate wells, followed by the addition of 200 µL of the Pierce BCA Protein Assay Kit reagent

(Thermo Fisher Scientific, Rockford, USA) and incubation at 37°C for 30 minutes in the dark. The absorbance of the developed color was measured at 562 nm and corrected for absorbance at 600 nm using a microplate reader (BioTek Eon, BioTek, USA) operating in the dual wavelength mode. Bovine serum albumin (BSA) standard curves were prepared in appropriate solvents (neutralized KOH, SGF or SIF), in order to calculate the protein concentration of samples.

SDS-PAGE: this method was used to visualize the soluble protein apparent molecular weight distribution of the JEM formulations, in order to determine the endpoint for reaction termination. Briefly, 19.5 µL soluble protein fractions were mixed with 7.5 µL sample application buffer and 3 µL reducing agent and heated at 95°C for 5 minutes. Samples (20 µL) were loaded into lanes of a precast polyacrylamide gel (Blot™ 4-12% Bis-Tris Plus, Thermo Fisher Scientific, USA). Prestained protein molecular weight markers (Blue Standards 161-0373) were used. The gel was run (Blot™ MES SDS Running Buffer, Invitrogen, USA) for 20 minutes at 220 volts, then stained overnight using a 0.25 % Coomassie Brilliant Blue R-250 solution, and destained (50 % v/v Methanol, 10 % v/v Acetic acid) to visualize the protein bands (Brunelle & Green, 2014).

2.7.Assessment of Bioactivities of the Developed JEM formulations

Based on the concentration of soluble proteins and peptides obtained from each formulation after desalting, serial dilutions were prepared, and their activities were measured.

2.7.1. Antibacterial Activity

Antibacterial activity of serially diluted SJ against *E. coli* strain was measured using the broth dilution assay. Dilutions to 1.25, 2.5, 5 and 10 mg/mL were prepared in Luria-Bertani (LB,

BioShop, Canada) broth and then mixed 1:1 with bacteria ($2x10^5$ CFU/ml) in 96 well microplate and incubated at 37°C in a microplate reader, where OD600 was measured at time zero, then hourly for 24 hours. Each treatment was replicated in 5 wells to verify reproducibility. Wells with only bacteria were included as a negative control, while wells with 0.62 µg/mL of the broad-spectrum antibiotic gentamicin (Sigma Aldrich) served as a positive control. Blank wells contained only LB broth. Growth curves were obtained by plotting OD600 against time for each treatment. The antibacterial effect was determined by comparing the growth curves of the treated samples with those controls.

2.7.2. Total Antioxidant Activity

The Trolox equivalent antioxidant capacity (TEAC) of non-hydrolyzed JEM (NJEM) and the JEM formulations SJ, SJ-G, SJ-GI WJ-G and WJ-GI at the concentrations 1.25, 2.5, 5 and 10 mg/mL was measured using the Total Antioxidant Power Kit (Oxford Biomedical Research, Product Number: TA02). The assay is based on cupric (Cu^{2+}) reducing antioxidant capacity (CUPRAC). Trolox, a water-soluble vitamin E analog, served as an antioxidant standard. Briefly, the Trolox standard curve was prepared from the 2 mM Trolox standard stock by serial dilution using sterile deionized water. NJEM, JEM formulations and Trolox standards were diluted 1:40 in the provided dilution buffer. An aliquot of 100 µL JEM formulations or Trolox standards was added to a 96-well plate prior to a reference dual-wavelength absorbance measurements at 450 and 600 nm ($Abs_{450} - Abs_{600}$ to correct for sample background and turbidity). Copper solution (25 µL) was added to each well and incubated for 10 min at room temperature. Next, 25 µL stop solution was added to each well, and the absorbance at 450 and 600 nm was measured a second time. The net

absorbance was calculated by subtracting the reference absorbance readings. The dilution buffer served as a reagent blank.

2.7.3. In vitro Anti-inflammatory activity

The anti-inflammatory activity of JEM formulations was assessed by evaluating their impact on the accumulation of the pro-inflammatory mediator nitric oxide (NO) in Lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

2.7.3.1. *RAW 264.7 macrophage cell line and growth conditions*

RAW 264.7 macrophages were cultured in DMEM complete culture medium (DMEM-CCM): DMEM enriched with 10% heat-inactivated fetal bovine serum (FBS; Corning, collected in Canada, processed in USA), 100 U/mL Penicillin, and 100 mg/mL streptomycin (Pen-Strep; Gibco, NY, USA), and 2 mM glutamine (Glutamax, Gibco, NY, USA) in a humidified atmosphere containing 5% carbon dioxide (CO_2), at 37°C. The cells were incubated in a 25 cm^2 flask (Corning) and sub-cultured to 75 cm^2 flask (Corning) at approximately 70% confluency using an initial seeding density of 2×10^4 cells/ml. Cells were renewed by replacing the medium 3 times per week, and cells were sub-cultured at approximately 70% confluency using 0.25 % trypsin-EDTA (Ethylenediaminetetraacetic acid) (Gibco, NY, USA). Inflammation was induced in the macrophage cultures using LPS (LPS, *Escherichia coli* O55:B5; Sigma-Aldrich).

2.7.3.2.Cell viability assay

To determine the optimal LPS concentration for inducing inflammation in RAW 264.7 macrophages without compromising cell viability, a range of LPS concentrations was evaluated, using the alamarBlue® assay to assess cell health. Briefly, RAW 264.7 cells were seeded at a

seeding density of 25×10^3 cells/well into 48-well culture plates (Falcon, n = 3 per treatment) and incubated for 24 hours to facilitate cell adhesion and proliferation. The conditioning media was then replaced with fresh media containing various concentrations of LPS (1, 5, 10, and 20 µg/mL) and incubated for an additional 24 hours. The media was replaced with fresh DMEM-CMM containing 10% alamarBlue® reagent (Thermo Fisher Scientific, USA) and incubated for 1 hour at 37°C. After incubation, 150 µL from each well was transferred to a microcentrifuge tube and centrifuged at 4˚C (13,000 rpm, 5 minutes). The supernatant was then transferred to a 96-well microplate (Corning™ Clear Polystyrene 96-Well Microplates), and fluorescence intensity was measured using Tecan Microplate Reader Spark® (Grödig, Austria) at excitation and emission wavelengths of 560 nm and 590 nm, respectively. The negative control was culture media containing LPS without cells, while the positive control was cells cultured in the absence of LPS. Normalized cell viability for each LPS concentration was determined using the following equation:

$$Normalized\ cell\ viability = \frac{(Fluorescence_{LPS\ treatment} - Fluorescence_{Negative\ control})}{(Fluorescence_{Positive\ control} - Fluorescence_{Negative\ control})} \times 100\%$$

The same protocol was applied to evaluate the effect of JEM formulations at concentrations of 1.25, 2.5, 5, and 10 µg/mL on the viability of RAW 264.7 macrophages induced with 1 µg/mL LPS after 24 hours of treatment. The negative control was culture media containing LPS and JEM formulations without cells, and the positive control was cells cultured with LPS and without exposure to JEM formulations. Normalized cell viability for each JEM treatment was determined using the following equation:

$$Normalized\ cell\ viability = \frac{(Fluorescence_{JEM\ treatment} - Fluorescence_{Negative\ control})}{(Fluorescence_{Positive\ control} - Fluorescence_{Negative\ control})} \times 100\%$$

2.7.3.3. *Nitric Oxide production Assay*

RAW 264.7 cells were seeded at a seeding density of 25×10^3 cells/well into 48-well culture plates (Falcon, n = 3 per treatment) and incubated for 24 hours to facilitate cell adhesion and proliferation. The conditioning media was then replaced with fresh media containing 1 µg/mL LPS along with JEM formulations SJ, SJ-G, SJ-GI WJ-G and WJ-GI at the concentrations 1.25, 2.5, 5 and 10 mg/mL, and incubated for an additional 24 hours. An aliquot (150 µL/well) of culture media was collected, and centrifuged (4˚C, 13,000 rpm) for 5 min and added to a 96-well plate. The accumulation of NO in the culture supernatant was detected using the Griess Reagent System (Promega, Madison, WI) following the manufacturer's instructions. Cells treated with JEM formulations and without LPS were used as a negative control, while cells with LPS without JEM formulations served as a positive control. Accumulated NO levels were determined using a standard curve of known concentrations of nitrite. NO (%) by cells compared to the positive control was determined from the standard curve using the following equation:

$$Production\ of\ NO\ (\%) = \frac{(NO_{JEM\ treatment} - NO_{negative\ control})}{(NO_{Positive\ control} - NO_{Negative\ control})} \times 100\%$$

2.7.4. Effect of JEM Formulations on Intestinal Barrier Function

The effect of the newly developed JEM formulation on modulating intestinal barrier function was assessed in terms of change in transepithelial electrical resistance (TEER) and paracellular permeability of the differentiated Caco-2 monolayer.

2.7.3.4.Caco-2 Cell line and growth conditions

Caco-2 cells were cultured in DMEM-CCM at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every other day, and cells were sub-cultured at approximately 70% confluency using trypsin-EDTA.

2.7.3.5. Preparation of Cell Monolayers

Caco-2 cells were seeded at a density of 25×10^3 cells/insert (n = 3 per treatment) onto Thincert cell culture insert (0.4 μm pore size, Greiner bio-one, Germany) and placed in 12-well plates. Each insert was maintained in DMEM-CCM, 0.5 mL onto the apical compartment and 1 mL in the basolateral compartment. The medium was changed every other day, and the formation of a confluent monolayer was monitored by measuring the TEER of the Caco-2 monolayer (Epithelial Vol-Ohm meter, Milli cell® ERS-2, Millipore). When the TEER values reached a stable reading of approximately 300 $\Omega \cdot cm^2$ (about 3 weeks), indicating the formation of tight junctions, the cells were considered ready for treatment with the test material.

2.7.3.6. Assessment of the effect of JEM formulations on TEER of Caco-2 and Transport of FITC through the monolayer

The JEM formulations were tested at four different concentrations: 1.25, 2.5, 5 and 10 mg/mL. The material was diluted with sterile PBS containing 100 U/mL penicillin and 100 mg/mL

streptomycin to achieve the desired concentrations. Once the TEER reached 300 Ω·cm², the conditioning medium in the apical compartment was carefully removed and replaced with 250 µL of the tested JEM formulation diluted in PBS, and the volume was completed to 0.5 ml with 250 µL of DMEM-CCM containing 1mg/mL Fluorescein isothiocyanate–Carboxymethyl–Dextran (FITC-CM-Dextran, FD-4) (Sigma Aldrich, USA). The basolateral medium was replaced with 1 mL of fresh DMEM-CCM. Each concentration of the JEM formulations was tested in triplicate to ensure reproducibility. TEER measurements were taken at zero time (pre-treatment) and 24 hours (post-treatment) to evaluate the effect of JEM formulations on the integrity of the Caco-2 cell monolayer. The measurements were performed in triplicate, and the values were recorded. TEER values were normalized to the initial reading (pre-treatment TEER value) to account for any variations in baseline resistance between wells. The negative control consisted of Caco-2 cells with DMEM-CCM alone, without any test material, to account for any changes in TEER due to the medium replacement. The positive control was treatment with the known tight junction disruptor ionomycin (IC, 1 µM). To assess the effect of the treatments on the paracellular permeability of Caco-2, the FITC-CM-Dextran permeability assay was performed where the polysaccharide dextran serves as a carrier for the FITC fluorescent dye and allows for the tracking of its movement across the Caco-2 monolayer. At zero-time, 100 µL of basolateral medium was collected from each well and transferred to a black 96-well microplate. Fluorescence intensity was measured using a Tecan Microplate Reader Spark® (Grödig, Austria) at 490 nm excitation and 520 nm emission wavelengths. This was repeated after 24 hours of incubating the cells with the JEM formulations. The fluorescence intensity of FITC that crossed the Caco-2 monolayer in treatment and control wells was measured and evaluated.

2.8.Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 10). Differences between the treatment groups and the control group were analyzed using One-way ANOVA. Data are presented as the mean ± standard error derived from three independent experiments conducted in triplicate for each assay. For all analyses, a p-value < 0.05 was considered statistically significant. Graphs were generated in GraphPad Prism, ensuring a clear representation of data trends and statistical results.

3. Results

3.1.Production and Quality Assessment of JEM

JEM represents size-reduced particles of ESM. The ESM coarse powder provided by BBF was analyzed for its particle size distribution using Keck sieve shaker kit, which revealed: <53 μm (4.8%), 53 – 106 μm (30.7%), 106 – 381 μm (63.5%), 381 – 508 μm (1.1%) and > 508 μm (0%). The ESM powder was processed via a high-efficiency vertical jet mill to produce JEM, which was thoroughly evaluated for chemical composition, microbiological profile (table 1) and particle size distribution (figure 2). Chemical composition analysis showed that JEM powder is predominantly composed of proteins, accounting for 82.9% by weight, in addition to other constituents such as glucosamine, chondroitin, and hyaluronic acid (HA). A comprehensive microbiological assessment was conducted to assess the safety and quality of the JEM powder, indicating that JEM is free from harmful bacterial contamination such as *E. coli* and Salmonella, as follows: Aerobic Colony Count (<5 colony-forming units per gram (CFU/g)), total Coliforms (<10 CFU/g), *E. coli* (<10 CFU/g) and *Salmonella* spp. (Not detected, as verified by the MFLP-29 method). Particle size analysis of JEM revealed that 99 % is <20.49 μm and 100 % is <47.94 μm with an average particle size of 5.7 μm.

3.2.Hydrolysis of PEM and JEM

In preliminary experiments using PEM, we investigated various acid- and base-mediated hydrolysis conditions (Concentration: 0.125 to 5 N; temperature: 22-55^{o}C; different stirring intensities and durations: 30 minutes to 7 days) of PEM material (reduced size particles <100 μm) (unpublished data subjected to invention disclosure agreement). Following a comprehensive

evaluation of these parameters, we identified the optimal conditions for producing HJEM (Younes, 2024). In the current study, an optimized protocol was used to hydrolyze JEM with KOH (exact conditions subject to invention disclosure). SDS-PAGE analysis of the separated supernatant at the start of the hydrolysis process and the clear supernatant separated from the produced hydrolysate after 24 and 48 hours of hydrolysis revealed significant changes in protein content. Prior to hydrolysis, the soluble JEM protein pattern showed a faint smear; however, after 24 hours of treatment, the HJEM displayed an intense smear, especially at higher molecular weights, indicating the increasing solubility of proteins and peptides, and their release into the supernatant. After 48 hours of hydrolysis, the smear intensity was observed to shift to a lower molecular weight pattern (figure 3).

3.3.Quantification of liberated soluble proteins and peptides

The concentration of soluble proteins in both SJ and WJ was monitored during successive stages of simulated GI digestion (SGID: SGD and SID) using the BCA protein quantification assay and visualization by SDS-PAGE analysis. The initial concentration of soluble proteins in the clear desalted portion of both SJ and WJ was 22.9 ± 4.4 mg/mL. Throughout the SGID, the samples were diluted 1:1 twice, initially with the components of the SGD reaction and then with the components of the SID reaction. As shown in table 2, SGD of the WJ showed a significant increase in the concentration of soluble proteins, where the concentration increased from 11.5 mg/mL at the start of the digestion process to 21.6 ± 0.4 mg/mL after 2 hours of SGD. The SID of WJ showed a lower increase in liberated protein concentration, where it increased from 10.5 mg/mL to 14 ± 1.3 mg/mL. SGID of SJ did not change the concentration of soluble proteins (figure 4).

3.4.Assessment of Bioactivities of the Developed JEM formulations

The bioactivities of the developed JEM formulations are summarized in table 3.

3.4.1. Antibacterial activity

The growth inhibition of *E. coli* by SJ was evaluated by measuring OD600 over 24 hours at five different concentrations (1.25, 2.5, 5, 10 mg/mL) and compared to a negative control (no treatment) and a positive control of *E. coli* culture treated with gentamicin. At SJ concentrations of 1.25 mg/ml, 2.5 mg/ml and 5 mg/ml, *E. coli* growth was significantly inhibited compared to the control. After 24 hours of incubation, the bacteria reached a maximum OD600 of 0.69 ± 0.01, 0.64 ± 0.02 and 0.59 ± 0.13, respectively, which is significantly lower than the control (maximum OD600 was 0.88 ± 0.01). At the highest concentration of SJ (10 mg/mL), the inhibition of bacterial growth was more significantly pronounced (0.44 ± 0.07 at 24 hours), which is 50 % inhibition compared to the untreated bacterial culture (Figure 5).

3.4.2. Antioxidant Activity

In this study, Trolox Equivalent Antioxidant Capacity (TEAC) of JEM formulations was evaluated by the Total Antioxidant Power Kit; the total antioxidant activity is expressed as μmol of Trolox equivalents (figure 6). TEAC was evaluated across various concentrations (1.25, 2.5, 5 and 10 mg/ml) for five JEM formulations: SJ, SJ-G, SJ-GI WJ-G and WJ-GI, and compared to non-hydrolyzed JEM (NJEM). At all concentrations, the newly developed JEM formulations showed a significant increase in TEAC compared to the NJEM. At the lowest concentration of 1.25 mg/ml, all formulations displayed a minimal but still significant increase in TEAC compared to NJEM, where SJ exhibited the highest TEAC value 235 ± 50 μM, which is five-fold the observed value for the NJEM. However, as the concentration increased, clear patterns of activity emerged, where

TEAC values increased with increasing concentration for all treatments, demonstrating a dose-dependent response. At the highest concentration of 10 mg/mL, the antioxidant capacity of SJ and WJ-G significantly increased reaching 667 ± 25 μM and 697.7 ± 15.8 μM, which is approximately 15-fold higher than the TEAC of the NJEM (43 ± 7.1 μM).

3.4.3. Anti-inflammatory Activity

Cytotoxicity of LPS: The effect of various concentrations of LPS (0. 1, 5, 10 and 20 μg/mL) on the viability of RAW 264.7 macrophages was assessed (figure 7). Only the lowest concentration of LPS (1 μg/mL) did not show a significant deleterious effect on cell viability after 24 hours of treatment.

LPS induction of nitric oxide production: The effect of the various LPS concentrations (1, 5, 10 and 20 μg/mL) on NO production in RAW 264.7 macrophages is illustrated in figure 8. No significant change in the nitrite concentration was recorded upon increase of LPS concentration from 1 μg/mL (NO: 1.8 ± 0.05 μM) to 20 μg/mL (NO: 1.5 ± 0.2 μM). Based on these results, the concentration of 1 μg/mL LPS was selected for subsequent experiments to induce NO production in RAW 264.7 macrophages, as this concentration showed the highest level of NO production without compromising cell viability.

Impact of JEM Formulations on RAW 264.7 macrophages: The effect of various concentrations of SJ, SJ-G, SJ-GI WJ-G and WJ-GI (1.25, 2.5, 5 and 10 mg/ml) on the viability and NO production in LPS-induced RAW 264.7 macrophages were assessed and compared to a negative control of untreated macrophages. No significant cytotoxicity was observed for different concentrations of various JEM formulations, in comparison to the negative control, as the cell viability remained above 80% at all concentrations.

Inhibition of nitric oxide production by JEM Formulations: The effect of the various concentrations of SJ, SJ-G, SJ-GI, WJ-G and WJ-GI (1.25, 2.5, 5 and 10 mg/ml) on the relative production of NO in LPS-induced RAW 264.7 macrophages was assessed and compared to the untreated positive control of LPS-induced RAW macrophages. Results showed that inhibition of LPS-stimulated NO production was dose-dependent, with greater concentrations yielding a stronger reduction in NO levels across the various tested formulations in comparison to the positive control, as shown in figure 9. The SJ, at the lower concentrations of 1.25 and 2.5 mg/mL, did not significantly affect LPS-stimulated NO production in comparison to the positive control. However, at the higher concentrations of 5 and 10 mg/mL, SJ demonstrated a highly significant NO suppression by 60 and 80%, respectively, and therefore can be considered anti-inflammatory. After SGID, the gastric metabolite SJ-G exhibited anti-inflammatory activity only at a concentration of 5 mg/mL (60% NO reduction). SJ-GI did not show any significant effect at either tested concentration (1.25 or 2.5 mg/mL). For the whole metabolites, WJ-G and WJ-GI, a significant anti-inflammatory effect was observed at a concentration of 2.5 mg/mL, where LPS-stimulated NO production was reduced by 38% and 57%, respectively. The anti-inflammatory activity further increased at higher concentrations. At all tested concentrations, WJ-GI demonstrated higher anti-inflammatory potency compared to WJ-G. This effect peaked at a concentration of 5 mg/mL, where WJ-GI reduced NO production by 80%. In contrast, WJ-G required a higher concentration of 10 mg/mL to achieve the same level of NO reduction.

3.4.4. Effect on Caco-2 Integrity and permeability

Effect on TEER: The impact of the JEM formulations on the TEER of Caco-2 cell monolayers was evaluated after 24 hours of treatment at concentrations of 1.25, 2.5, 5, and 10 mg/mL. In the negative control wells (Treatment concentration: 0 mg/mL), TEER increased by 28 ± 10 Ω/cm^2 over 24 hours as the cells differentiated under culture conditions. The newly developed JEM formulations showed a concentration-dependent effect, with low concentrations generally enhancing barrier integrity (increase in TEER). At the lower SJ concentrations, TEER was increased by 79.6 Ω/cm^2 at 1.25 mg/mL, 41.6 Ω/cm^2 at 2.5 mg/mL and 31.6 at 5mg/mL. The first negative effect of SJ on TEER appeared at the highest concentration of 10 mg/mL, where the TEER increased only by 20.6 mg/mL in comparison to 28 Ω/cm^2 in the negative control. However, post-gastric digestion (SJ-G) reduced TEER, peaking at a decrease of 13 Ω/cm^2 at 5 mg/mL. Whole metabolites (WJ-G and WG-GI) enhanced TEER at low concentrations (51 Ω/cm^2 and 57.6 Ω/cm^2, respectively, at 1.25 mg/mL) and showed non-significant differences at higher concentrations compared to controls. Across all formulations, the extent of TEER enhancement decreased as the concentration increased, suggesting a concentration-dependent effect on Caco-2 monolayer integrity (figure 10).

Effect on paracellular permeability: The effect of JEM formulations on the transport of FITC-Dextran across the Caco-2 monolayer was evaluated by measuring fluorescence in the basolateral compartment after 24 hours of exposure to the formulations at various concentrations (1.25, 2.5, 5 and 10 mg/mL). The data is represented as a change in fluorescence between 0 and 24 hours post-treatment and compared to the negative control (0 mg/mL). The fluorescence changes at different

concentrations did not significantly differ (ns), although a non-significant upward trend was observed at higher concentrations.

4. DISCUSSION

The human gut is essential for digestion, metabolism, immune function, and inflammation regulation, and influences chronic diseases like IBD, obesity, and diabetes (Potrykus et al., 2021; Vemuri et al., 2020). Gut health supports anti-inflammatory mediators such as short-chain fatty acids (SCFAs), while imbalances disrupt these processes, increasing inflammation and permeability (Bisanz et al., 2019). Diet affects markers like CRP and IL-6, linked to metabolic diseases (Aleksandrova et al., 2021). Protein digestion starts with chewing in the mouth, continues with proteolysis in the stomach through the action of acid and pepsin, and concludes with fermentation in the distal colon, resulting in the production of metabolites such as SCFAs. Thus, the quality and quantity of protein in the diet influence microbiota composition and generation of metabolites (Randeni et al., 2024; Rinninella et al., 2023). In this study, ESM powder was processed into JEM using a high-efficiency jet mill, achieving an average size of 5.7 μm. Quality control assessment demonstrated that JEM was free from harmful bacterial contamination and consisted mainly of proteins (82.9%), making JEM a safe, high-protein material for dietary applications.

These results align with our previous demonstration that reducing the particle size of ESM enhances its anti-inflammatory and antimicrobial activity (Kulshreshtha et al., 2020).

Bioactive peptides, composed of 2–20 amino acids, exhibit diverse bioactivities once released from parent proteins (Majumder & Wu, 2010). Increasing ESM solubility is crucial for improving its bioavailability and dietary supplement potential. Previous methods, including acid or alkali

treatments and fermentation with lactic acid bacteria, have shown promise in enhancing ESM solubility and bioactive properties (Chi et al., 2019; Han et al., 2023). For example, Pasarin et al. (2023) achieved 14.23% hydrolysis using NaOH and Alcalase protease (Pasarin et al., 2023), while ultrasonic pre-treatment has been shown to enhance enzymatic hydrolysis efficiency (Wang et al., 2024).

The primary objective of the current study was to hydrolyze ESM to enhance protein solubility and bioavailability by employing a combination of mechanical size reduction (Jet-O-mizing) and chemical hydrolysis using KOH. KOH is widely used in the food and pharmaceutical industries as a pH regulator, emulsifier, and processing aid, and is approved by the FDA (E525), making it a safe and scalable reagent. Its established use in drug synthesis and diagnostic preparations further supports its suitability for health-focused product development (OMRI, 2016; Food Standards Agency, 2025).

In a preliminary study (data not shown), chemical hydrolysis of PEM was assessed using five different acids and two alkalis, and evaluated at different temperatures, concentrations, reaction durations and stirring intensities. Building on this study, an optimized protocol was followed to hydrolyze JEM in the current study, as it showed the highest degree of generation of soluble peptides and subsequent increase in antioxidant activity (protocol subject to invention disclosure). The process successfully produced soluble proteins and peptide fractions, yielding approximately 50% protein liberation at lower molecular weights as verified by SDS-PAGE. The subsequent SGID of the two forms of the JEM hydrolysates (the whole WJ and the soluble/supernatant SJ) provided insights into their biological stability and further peptide release. WJ formulations released additional soluble peptides during both the gastric and intestinal phases, while SJ formulations showed less change, likely due to pre-digestion solubilization. This highlights the

potential of WJ as a protein-rich dietary supplement capable of withstanding gastrointestinal conditions while releasing bioactive peptides. The desalting step ensured the removal of residual salt formed upon neutralization of the hydrolysate after alkaline hydrolysis, which stabilized the hydrolysate and metabolites and made them suitable for downstream bioactivity assays, establishing a reproducible protocol that can be scaled up for industrial applications.

Our study evaluated the antibacterial activity of SJ against the gut-associated pathogen *E. coli*, building on our previous findings of PEM antimicrobial activities. SJ exhibited a bacteriostatic effect, inhibiting *E. coli* growth by 50 % at 10 mg/mL. The antibacterial potential of developed JEM formulations may be attributed to the liberated peptides and enhanced bioavailability upon alkaline hydrolysis. ESM proteins, such as OCX-36, exhibit a high affinity for bacterial pyrogens such as LPS and Lipoteichoic acid (LTA) (Cordeiro et al., 2013). ESM is naturally enriched with antimicrobial proteins and peptides, including lysozyme, histones, Avian β-defensins (AvBDs), and ovalbumin(Ahmed et al., 2017; Du et al., 2015; Rose-Martel & Hincke, 2017). Additionally, glycoproteins such as ovomucin and mucin have antibacterial and antiviral properties (Lieleg et al., 2012; Omana et al., 2010). Similarly, ESM hydrolysates demonstrated antimicrobial activity against a broad spectrum of pathogens, including *S. aureus*, *B. subtilis*, *K. pneumoniae*, *S. marcescens*, and *E. coli* (Yoo et al., 2014). It is also found that peptide derivatives of ESM histones retain anti-microbial activity (Jodoin & Hincke, 2018) These findings highlight the potential of JEM formulations in gut health applications, particularly for managing dysbiosis-associated disorders.

Oxidative stress is a key factor in the onset of various GI disorders as it can harm cellular structures, promote inflammation, and contribute to diseases like IBD and colorectal cancer (Semenova et al., 2024). Safe antioxidants from natural and accessible sources are alternatives

that have attracted wide interest among researchers. Protein hydrolysates exhibit better antioxidant activity than their intact forms (Elias et al., 2008). Ultrafiltered ESM enzymatic hydrolysate fractions demonstrated a range of antioxidant activity including iron (Fe^{3+}) reducing, 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical scavenging, and Fe^{2+} chelating activity which were further verified by a cell-based study that ESM hydrolysate reduced proinflammatory cytokine IL-8 secretion in oxidative stress-induced human intestinal epithelial Caco-2 cells (Shi et al., 2014). Similarly, ESM enzymatic hydrolysates and corresponding identified synthetic peptides showed a strong ability to quench ABTS, inhibit TBARS and high total antioxidant activity in a study by Zhao and coworkers (Q.-C. Zhao et al., 2019) Therefore, based on the above evidence and our previous proteomic results (Ahmed et al., 2017), we propose that the proteinaceous JEM has the potential to be an alternative antioxidant for biomedical applications.

In this study, we evaluated the antioxidant power of the soluble proteins liberated upon JEM alkaline hydrolysis followed by SGID, using the Trolox equivalent antioxidant capacity assay. SJ showed antioxidant activities up to 15 times greater than NJEM, peaking at 667 μM Trolox equivalents, at 10 mg/mL concentration. This antioxidant capacity was retained after digestion, demonstrating potential to mitigate oxidative stress in biological systems. These results underline the potential of JEM formulations as dietary antioxidants capable of neutralizing oxidative stress, which plays a pivotal role in gut health and chronic inflammation.

ESM comprises proteins such as CREMP, ovalbumin ovotransferrin, and cystatin that contain amino acids, such as cysteine, histidine, tryptophan, and β-hydroxyl tryptophan, that exhibit antioxidant activities (Huang et al., 2010; Kodali et al., 2011; Nimalaratne et al., 2015; Yi et al., 2004). Therefore, we propose that the hydrolysis of cysteine-rich CREMPs and other ESM proteins contributed to the enhanced antioxidant activity of JEM formulations.

Inflammation plays a pivotal role in the development of numerous gut disorders (Randeni et al., 2024). *In vitro*, ESM reduced LPS-induced inflammatory cytokines and enhanced Caco-2 proliferation. In a murine dextran sodium sulfate-induced colitis model, dietary ESM improved inflammatory mediator gene expression and regulated Th17 cell expansion, aiding mucosal inflammation reduction (Jia et al., 2017). ESM improved microbial alpha diversity and reduced inflammation-associated microbiota, while increasing total organic acid levels, particularly SCFAs such as butyrate (2.3-fold), which are known to inhibit Th1 and Th17 production (Jia et al., 2022). A form of particalized ESM (PEM<53 μm) exhibited a 56 ± 7% reduction in LPS-stimulated NO levels after 24 hours, compared to the 80 ± 0.6 % reduction achieved with KOH-hydrolyzed JEM in the current study (Wu, 2020).

This study assessed the anti-inflammatory effects of JEM formulations using the model of LPS-induced inflammation in RAW 264.7 macrophages. The focus was on modulating NO production, a key inflammation marker, via TLR4-mediated signaling pathways (Hommes et al., 2003). Optimal LPS concentration (1, 5, 10, 20 μg/mL) was evaluated in RAW 264.7 macrophages, where 1 μg/mL was observed to induce NO production similar to 20 μg/mL, but without affecting viability. According to ISO 10993-5, cell viability above 80% is defined as non-cytotoxic (*ISO 10993-5*, 2009). Thus, JEM formulations (1.25–10 mg/mL) were non-toxic, maintaining >80% cell viability. JEM showed dose-dependent anti-inflammatory activity, achieving up to 80% suppression of NO production at 10 mg/mL. Post-gastrointestinal digestion products retained potent anti-inflammatory effects, highlighting the stability and bioactivity of hydrolyzed peptides under physiological conditions. Metabolites of the whole JEM hydrolysate (WJ: WJ-G and WJ-GI) showed the most significant anti-inflammatory effects, possibly due to the presence of diverse bioactive peptides generated during digestion of WJ. The findings from this model provide

valuable insights into the potential of JEM formulations as anti-inflammatory agents against gut-related disorders.

The integrity of the intestinal barrier is essential for maintaining its function and its disruptions has been linked to various diseases (Chelakkot et al., 2018), including IBD (McGuckin et al., 2009), irritable bowel syndrome (IBS) (Piche et al., 2009) and metabolic syndrome (Teixeira et al., 2012). Previous *in vitro* and *in vivo* studies showed the impact of proteins and derived bioactive peptides on regulating intestinal physical, chemical, biological and immunological barrier function (Bao & Wu, 2021), such as fish protein hydrolysate (Marchbank et al., 2009), soybean β-conglycinin hydrolysate (Ren et al., 2014), and wheat gluten exploring A5 and C5 (Maggioni et al., 2016). Alaska pollock skin-derived collagen and its tryptic hydrolytic fractions significantly mitigated TNF-α-induced barrier dysfunction in Caco-2 cells monolayer, by alleviating disruption of tight junction proteins ZO-1 and occludin, while also inhibiting MLC phosphorylation and MLCK expression, and suppressing the activation of NFκB and Elk-1 (Q. Chen et al., 2017). Casein hydrolysate and derived peptides improved intestinal barrier function in diabetes-prone (DP)-BioBreeding (BB) rats, where improvement was expressed as decrease in lactulose: mannitol ratio, decrease in serum zonulin levels and increase in ileal TEER (Visser et al., 2010),

The Caco-2 cell line is widely used to investigate barrier function after achieving differentiation. When used in LPS-treated Caco-2 cells, a hydrolysate from egg white protein ovomucin, Protex 26L (OP), demonstrated effective restoration of TEER and a reduction in paracellular permeability of FITC-dextran (4 kDa and 40 kDa), and significantly preserved the expression and structural integrity of tight junction proteins, including occludin and ZO-1, (Bao et al., 2024). Here, we used differentiated Caco-2 cells to study the effect of our developed JEM formulations on a model of

intestinal barrier function. We evaluated TEER and paracellular tracer (FITC-Dextran) transport to identify potential impact on intestinal barrier function.

Our results revealed that a low concentration (1.25 mg/mL) of the soluble potion of JEM hydrolysate (SJ), and the metabolites of the whole hydrolysate (WJ-G and WJ-GI) significantly increased TEER, indicating enhanced intestinal barrier integrity, whereas higher concentrations did not show significant effects on TEER.

Fluorescence analysis of the basolateral compartment showed no significant differences in FITC-Dextran transport across Caco-2 monolayers treated with JEM formulations after 24 hours. These *in vitro* findings indicate that JEM formulations do not disrupt paracellular intestinal permeability and integrity even at the highest concentration of 10 mg/mL, at which the highest anti-inflammatory effect was observed. This highlights their potential to protect intestinal barrier function, a key aspect of gut health.

The current study focused on the potential of the largely underutilized by-product ESM biomaterial as a high-value dietary supplement, with applications in promoting gut health by modulating inflammation while maintaining epithelial barrier integrity. Using a novel hydrolysis strategy, the newly developed ESM formulations demonstrated enhanced antioxidant, antibacterial, and anti-inflammatory, while maintaining epithelial integrity. These properties align with the growing interest in functional food components that support gut and systemic health.

The results of this study have broader implications for the utilization of ESM as a sustainable, functional ingredient in health-focused applications. With millions of tons of ESM generated as an industrial by-product annually, its transformation into value-added products aligns with global efforts to reduce waste and promote circular economies. The compositional richness of ESM, including its collagen content and bioactive peptides, positions it as a versatile material for

nutraceuticals, pharmaceuticals, and functional foods. The demonstrated antioxidant, antimicrobial, and anti-inflammatory activities of JEM formulations highlight their potential as dietary supplements to boost gut health. Furthermore, the ability to enhance gut barrier integrity at appropriate concentrations could make ESM formulations valuable for preventing systemic inflammation and associated diseases.

5. Conclusions

Hydrolyzed ESM dominates global revenue as compared to its unhydrolyzed form due to improved bioavailability of components like collagens, HA, antioxidants, and antimicrobials. ESM hydrolysates are increasingly marketed as premium health supplements (Kulshreshtha et al., 2022). This study explores conversion of ESM waste, into a modified biomaterial, addressing sustainability and health innovation. The results demonstrate the potential of hydrolyzed JEM formulations for use in functional foods, nutraceuticals, and therapies targeting gut health. With bioactivities such as antioxidant, antimicrobial, anti-inflammatory effects, and improved intestinal barrier integrity, these formulations show promise as dietary supplements for gut health.

Future *in vivo* studies must validate these findings and assess the safety and efficacy of dietary JEM formulations. Further exploration of the scalability and environmental impact of the hydrolysis process is essential, offering a model for sustainable and resource-efficient innovation. This research establishes a strong foundational basis to exploit ESM for advancements in health, wellness, and the circular economy.

Declaration of Competing Interest

The authors affirm that they have no known financial or personal conflicts of interest that could have influenced the work presented in this paper.

CRediT: authorship contribution statement

Manar Younes: Investigation, Methodology, Resources, Validation, Formal Analysis, Data Curation, Writing – original draft, Writing – review and editing. ***Tamer Ahmed:*** Project administration, Conceptualization, Methodology, Validation, Resources, Writing – review and editing, Funding acquisition. ***Maxwell Hincke:*** Supervision, Conceptualization, Methodology, Validation, Writing – review and editing, Funding acquisition. **Riadh Hammami:** Funding acquisition, Supervision.

Acknowledgements

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Table 1

The microbiological profile of JEM.

ESM POWDER AFTER JET-O-MIZER (5 sub-samples@ 150 grams each)		
Aerobic colony count	<5	CFU/g
Total coliforms	<10	CFU/g
Escherichia coli	<10	CFU/g
Salmonella spp - Detection - MFLP-29	Negative	-

Microbiological assessment of JEM powder (performed by Eurofins Environex, Quebec, Canada).

Table 2

The change in soluble protein content of SJ and WJ upon SGID.

Concentration of Soluble Proteins & Peptides (mg/mL)

	WJ	**SJ**
	22.9 ± 4.4	22.9 ± 4.4
SGD		
Initial	11.5	11.5
Final	21.6 ± 0.4	11.2 ± 0.6
SID		
Initial	10.5	5.5
Final	14 ± 1.3	5 ± 1.5

Summary of the concentrations of liberated soluble proteins and peptides measured by BCA assay of SJ and WJ throughout SGID.

Table 3

The bioactivities of JEM formulation

JEM Formulation	Conc.	1.TEAC	2. Anti-inflammatory Activity	3. Change in TERR	4. FITC transport	5. Antibacterial Activity
SJ	1.25	236*** ± 50	126 ± 17	80*** ± 8	2.3 ± 0.6	22%***
	2.5	361**** ± 3	111 ± 5	42 ± 3	2.7 ± 0.6	27%****
	5	363**** ± 51	42** ± 13	32 ± 16	2.7 ± 0.6	33% ****
	10	667**** ± 25	20**** ± 1	21 ± 4	3 ± 1	50%****
SJ-G	1.25	160**** ± 4	96 ± 10	20 ± 8	2.3 ± 0.6	
	2.5	272**** ± 8	78 ± 7	-4* ± 12	2.3 ± 0.6	
	5	377**** ± 10	39*** ± 8	-13** ± 11	4.7 ± 1.2	
SJ-GI	1.25	186*** ± 34	122 ± 27	34 ± 6	4.7 ± 0.6	
	2.5	274**** ± 21	107 ± 10	4 ± 8	5.3 ± 0.6	
WJ-G	1.25	144**** ± 13	89 ± 2	51* ± 6	1.7 ± 0.6	
	2.5	237**** ± 17	62 **± 10	14 ± 5	2.3± 0.6	
	5	381**** ± 6	35 ***± 5	14 ± 3	2.7 ± 0.6	
	10	698**** ± 15	19**** ± 2	8 ± 11	3.3 ± 0.6	
WJ-GI	1.25	220**** ± 41	80 ± 2	58* ± 9	1.7 ± 0.6	
	2.5	325**** ± 18	43*** ± 8	32 ± 5	2.3 ± 0.6	
	5	370**** ± 14	19 ****± 2	20 ± 11	2.7± 0.6	

1.Antioxidant Activity (Trolox Equivalent Antioxidant Capacity, TEAC, uM). 2.Anti-inflammatory Activity (% inhibition of NO production in LPS-induced RAW 264.7 RAW macrophages). 3. Effect on intestinal barrier integrity (change in TEER of Caco-2 cell monolayer

after 24 hours of treatment; positive values indicate an increase, and negative values indicate a decrease in TEER). 4. FITC Transport (Paracellular Permeability Assessment, change in fluorescence (AU) detected in the basolateral compartment of a Caco-2 monolayer model after 24 hours of treatment). 5. Antibacterial activity (percentage inhibition of growth of *E. coli*, at 24 hrs.). Significant differences between treatments and control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

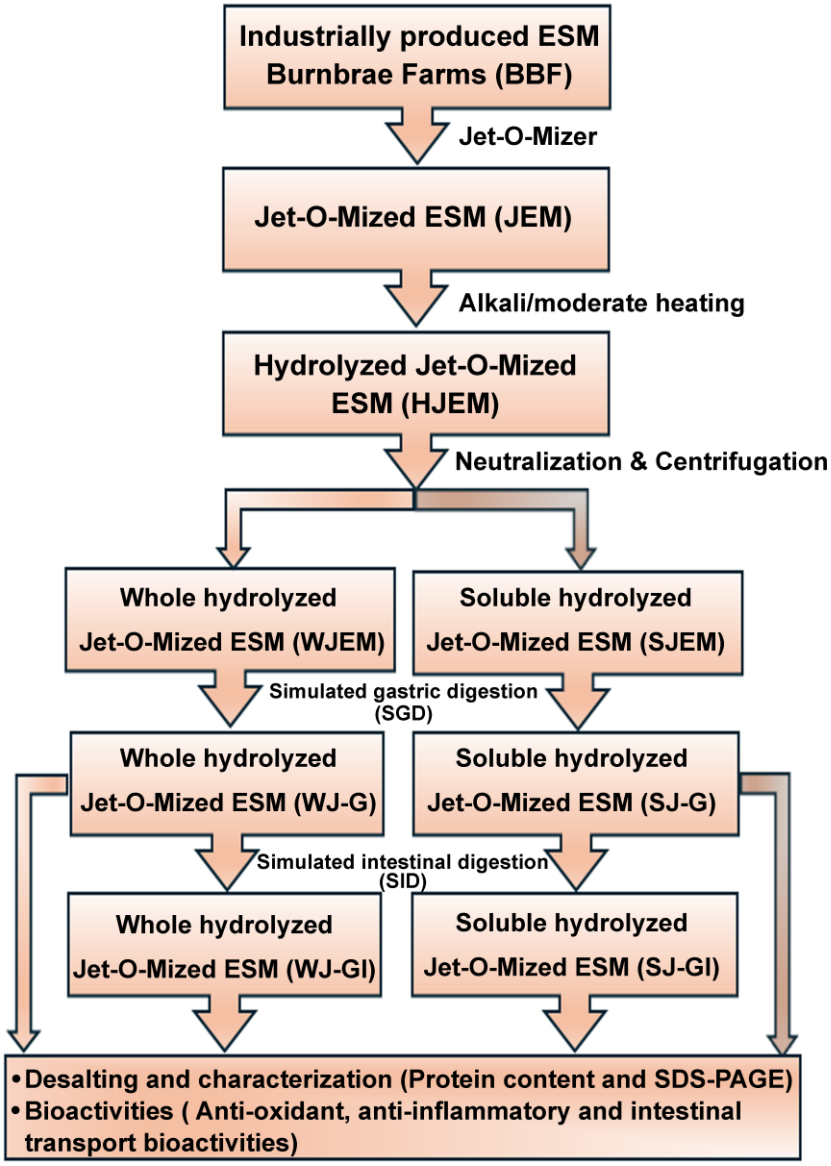
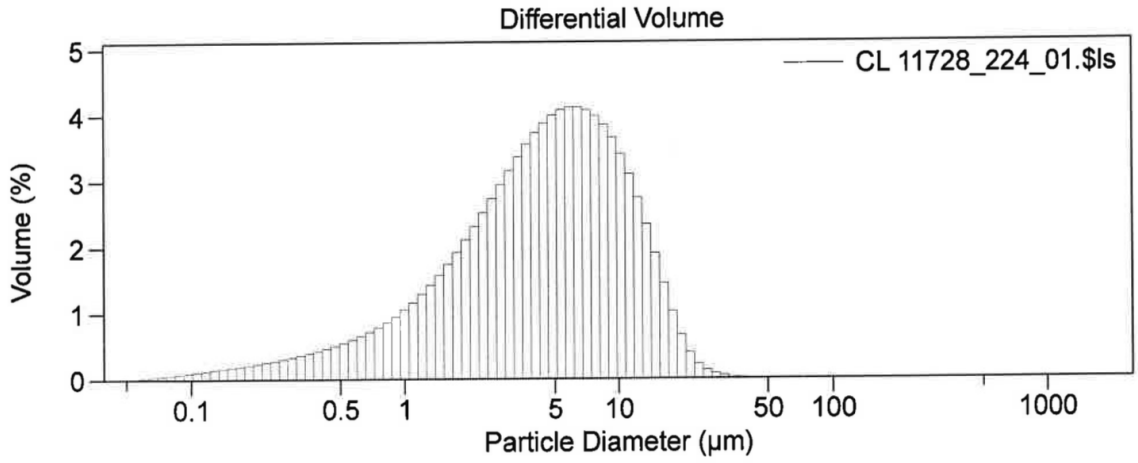


Figure 1. Schematic representation of the JEM processing, purification, characterization, and subsequent *in vitro* assessments.



Volume Statistics (Arithmetic) CL 11728_224_01.$ls

Calculations from 0.040 µm to 2000 µm

Volume:	100%		
Mean:	5.767 µm	S.D.:	4.670 µm
Median:	4.590 µm	Variance:	21.81 µm^2
Mode:	5.878 µm	Skewness:	1.368 Right skewed
		Kurtosis:	2.579 Leptokurtic

<10%	<25%	<50%	<90%	<99%	<100%
0.942 µm	2.231 µm	4.590 µm	12.27 µm	20.49 µm	47.94 µm

Figure 2. Particle Size Distribution of ESM powder after jet-o-mizing (JEM), in 2-propanol, analyzed by Beckman Coulter LS Particle Size Analyzer.

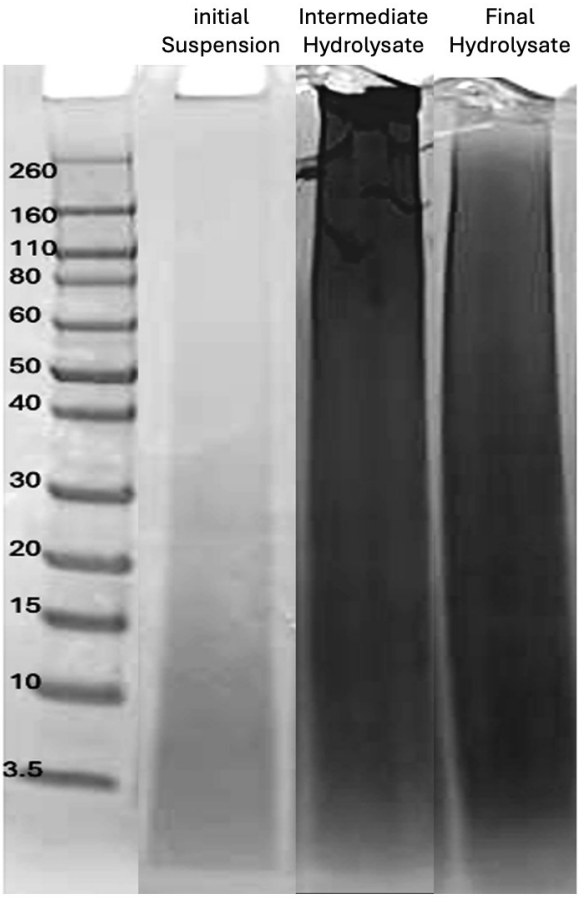


Figure 3. SDS-PAGE analysis showing the release of soluble proteins from JEM during alkaline hydrolysis. The gel displays samples collected at different time points indicating the progression of protein solubilization.

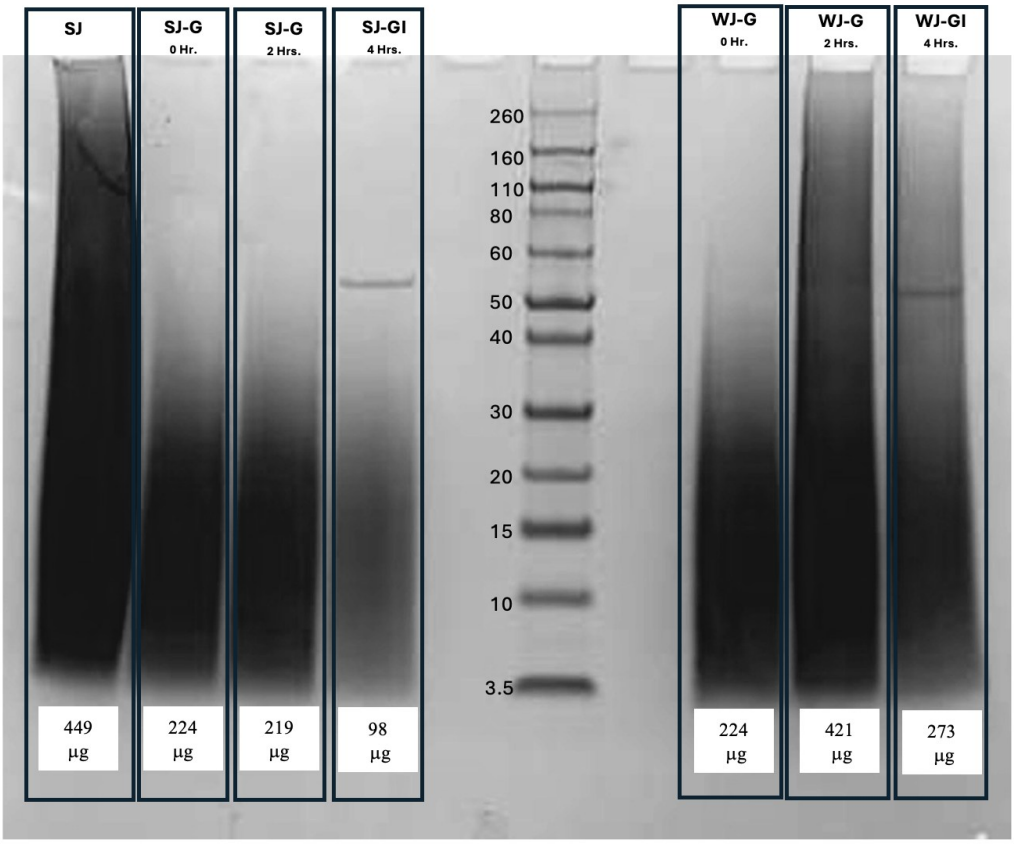


Figure 4. SDS-PAGE gel analysis showing the release of soluble smear of proteins upon SGID of SJ and WJ formulations. Each lane represents a different time point and amount of sample loaded (measured by BCA assay after desalting). Protein marker (kDa) in the center lane provides MW standards from 3.5 kDa to 260 kDa.

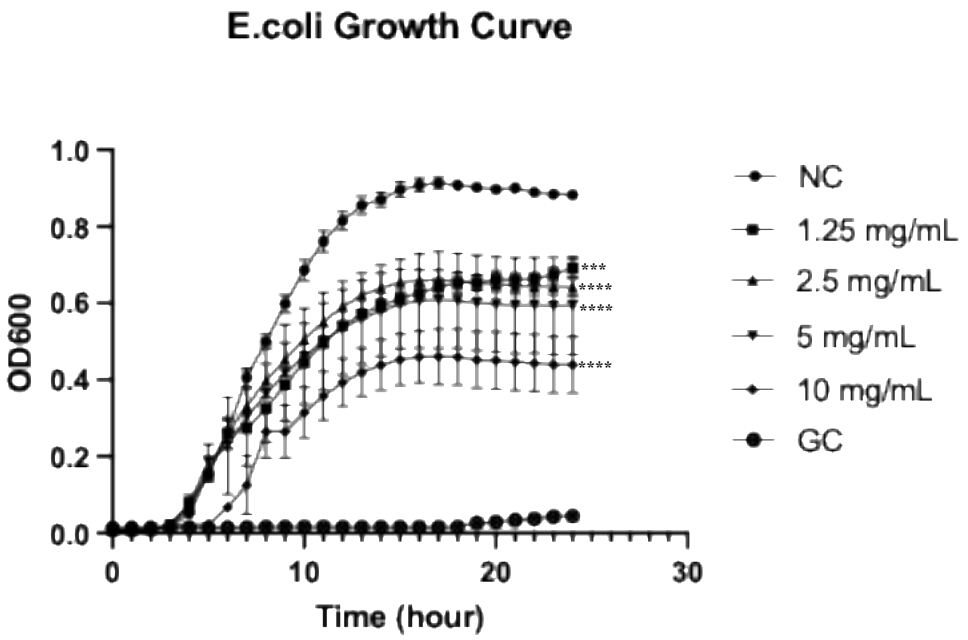


Figure 5. The significant antibacterial effect of various concentrations of SJ on *E. coli* growth over 24 hours. The negative control (NC) is untreated *E. coli,* and the positive control (GC) is *E. coli* culture treated with gentamicin (0.62 µg/mL). The y-axis shows the turbidity due to *E. coli* growth measured as OD600. Significant differences between treatments and negative control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars represent the standard deviation from the mean of five measurements.

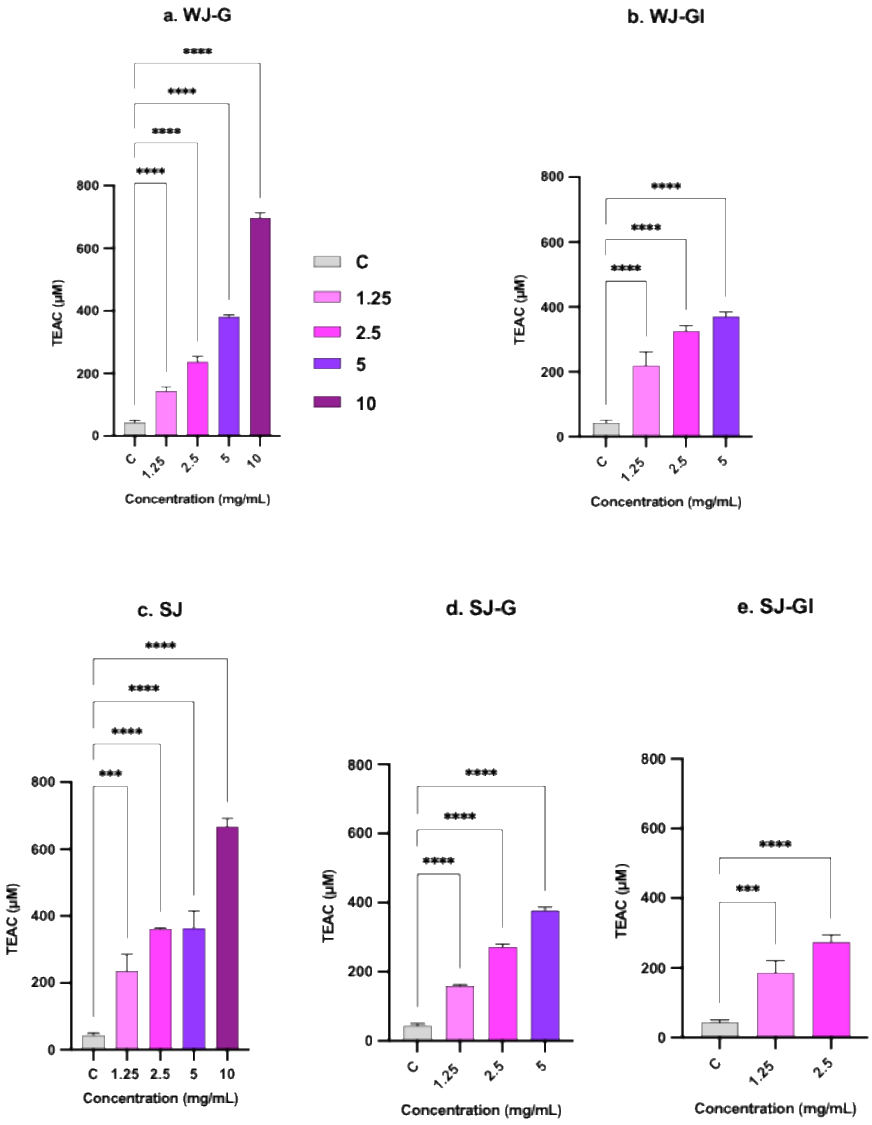


Figure 6. The bar chart illustrates the dose-dependent increase in antioxidant activity of JEM formulations, expressed in TEAC in comparison to the control NJEM. Significant differences between treatments and control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars represent the standard deviation from the mean of three independent experiments, with each experiment performed in triplicate.

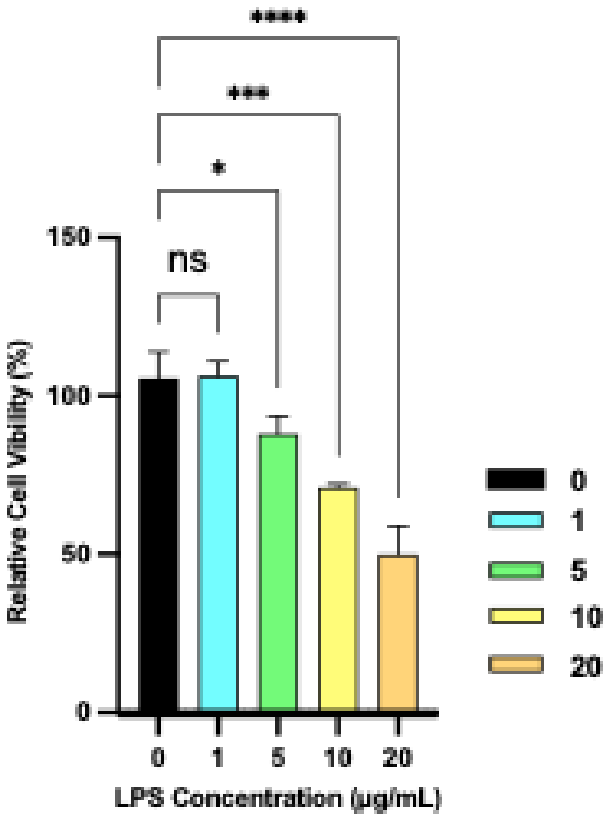


Figure 7. Effect of the concentrations 1, 5, 10 and 20 µg/mL of LPS on the relative viability of RAW 264.7 macrophages after 24 hours of treatment, compared to the negative control (NC: concentration zero). Significant differences between treatments and control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars represent the standard deviation from the mean of three independent experiments.

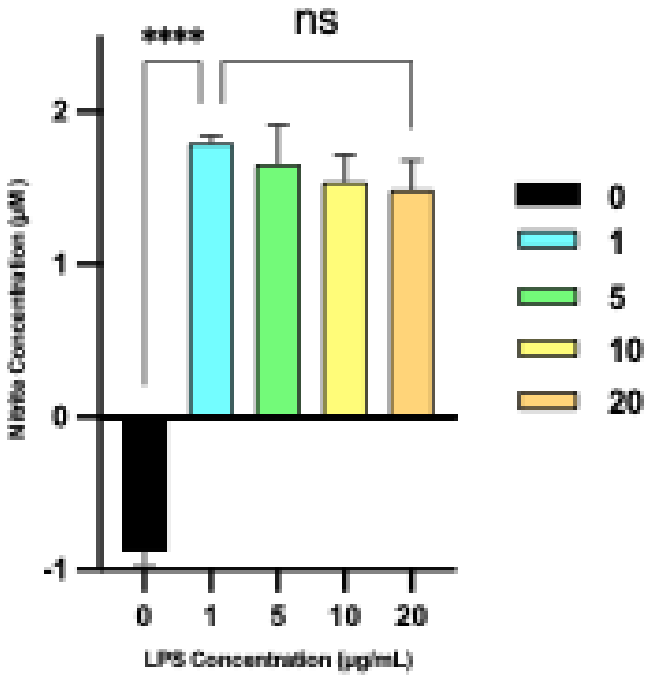


Figure 8. Effect of varying concentrations of LPS on NO production in RAW 264.7 macrophages after 24 hours of treatment. Nitrite levels were measured as an indicator of NO production and expressed in μM. Error bars represent the standard deviation from the mean of three independent experiments, with each experiment performed in triplicate.

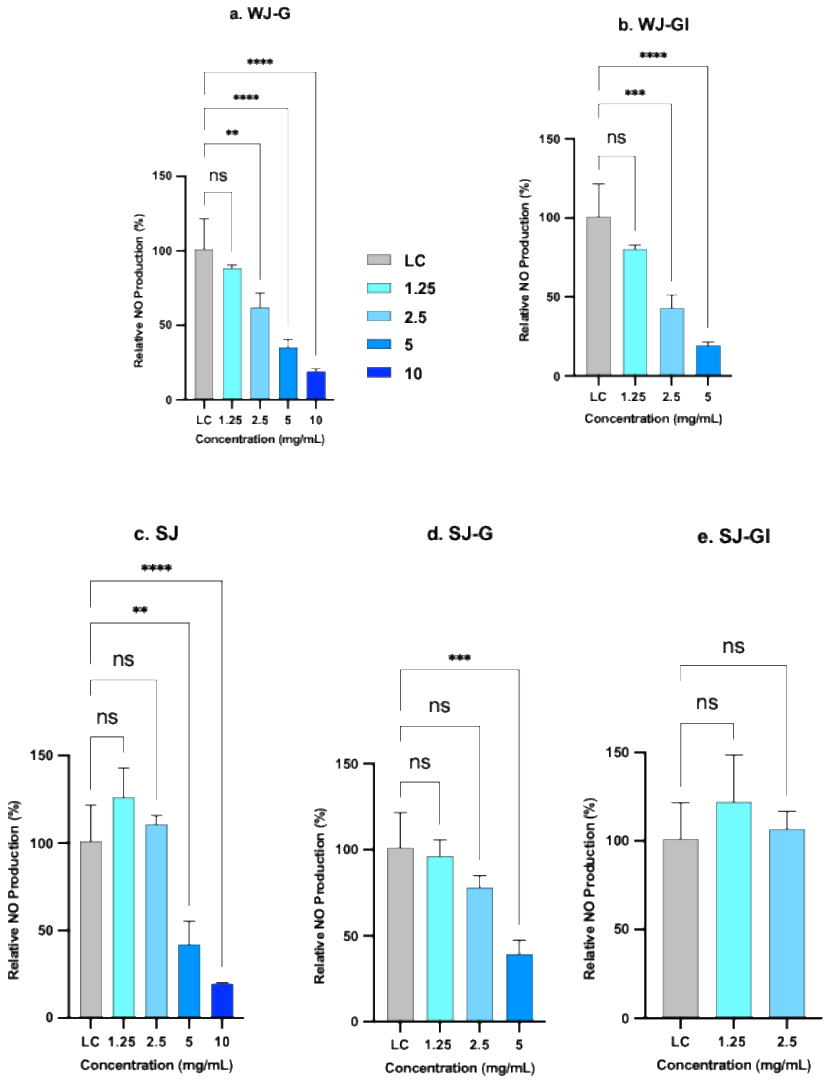


Figure 9. Effect of JEM formulations on the relative production of NO in LPS-stimulated RAW 264.7 macrophages after 24 hours of treatment with different concentrations in comparison to the positive control (LC) (0 mg/mL, untreated LPS-induced RAW 264.7 macrophages). Significant differences between treatments and control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars represent the standard deviation from the mean of three independent experiments, with each experiment performed in triplicate.

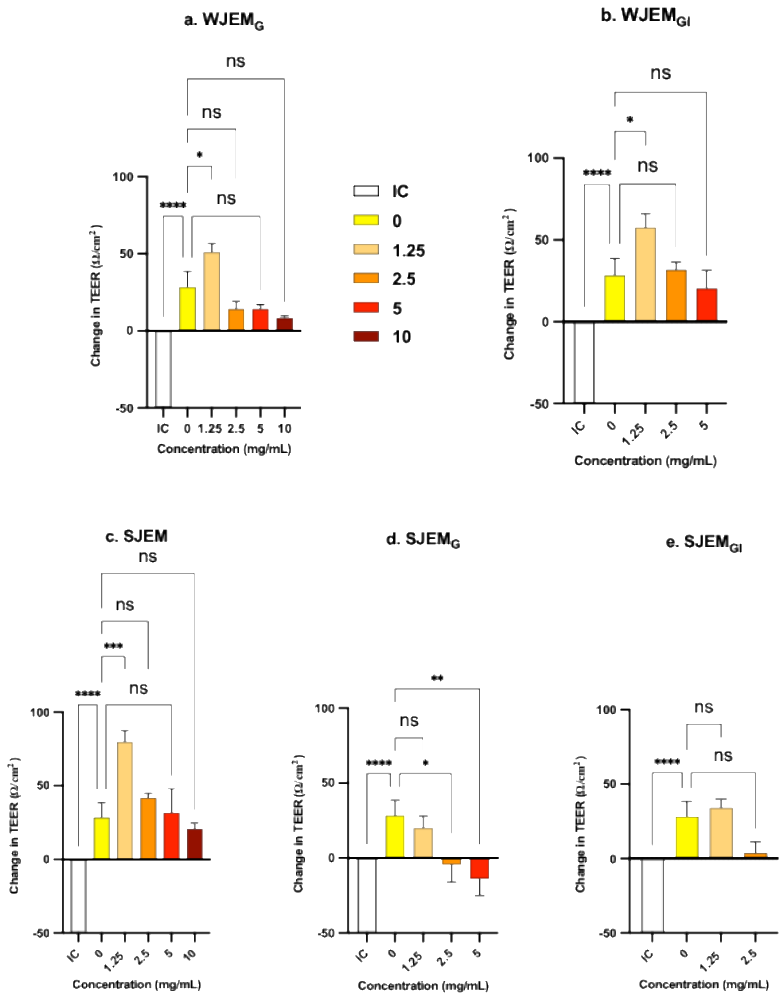


Figure 10. Effect of JEM formulations on the TEER of the Caco-2 cell monolayer. The values are compared to negative control (0 mg/mL, untreated Caco-2 cells), and positive control (IC, Caco-2 cells treated with 1 μM ionomycin). Changes in TEER (Ω·cm²) reflect positive or negative alterations in epithelial barrier integrity. Significant differences between treatments and control are marked (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Error bars denote the standard deviation from the mean of three independent experiments, with each experiment performed in triplicate.

Chapter 5: DISCUSSION AND CONCLUSIONS

1. Background

The human gut plays a crucial role in digestion, metabolism, and immune function, as well as in regulating inflammation - a key factor in chronic diseases, including IBD, obesity, diabetes, and cardiovascular conditions (Potrykus et al., 2021; Vemuri et al., 2020). Maintenance of intestinal health supports the production of anti-inflammatory mediators like SCFAs, which helps to preserve gut integrity. An imbalance in gut function can disrupt these processes, contributing to inflammation and increased gut permeability (Bisanz et al., 2019). Diet is a significant factor influencing intestinal health and inflammatory markers such as CRP, IL-6, and TNF-α (Aleksandrova et al., 2021). Protein digestion begins in the mouth, where food is mechanically broken down by chewing and mixed with saliva before swallowing. In the stomach, acid and pepsin, along with peristaltic mixing, further digest food and initiate proteolysis (Loveday, 2023). Proteins are then subjected to proteolytic fermentation in the large intestine particularly in the distal colon, producing branched-chain fatty acids; thus, the quality and quantity of protein in the diet influence microbiota composition and generation of metabolites (Randeni et al., 2024; Rinninella et al., 2023).

The current study explores the potential of the ESM biomaterial, a largely underutilized by-product of the egg-producing industry, as a high-value dietary supplement for promoting gut health by modulating inflammation and enhancing epithelial barrier integrity. Using a novel hydrolysis strategy, the newly developed ESM formulations demonstrated promising biological activities, including antioxidant, antibacterial, and anti-inflammatory, while maintaining epithelial integrity. These properties align with the growing interest in functional food components that support gut

and systemic health. This discussion examines the methodologies, outcomes, and their implications for future research and applications.

We previously demonstrated that size reduction of ESM particles resulted in enhanced anti-inflammatory activity and increased their antimicrobial activity against skin-associated pathogens (Kulshreshtha et al., 2020). The current study utilized ESM powder sourced from Burnbrae Farms, processed using a high-efficiency vertical jet mill to reduce median particle size to 4.5 microns, which we term JEM. Microbiological assessments demonstrated that JEM was free from harmful microbial contamination, including Aerobic Colony Count, Coliforms, E. coli, and Salmonella, confirming its safety for further applications. Chemical analysis revealed that JEM powder primarily consists of proteins, contributing 82.9% of its weight, along with other constituents like glucosamine and chondroitin. Particle size analysis showed <99% of particles measuring 20.49 μm or smaller, and an average particle size of 5.7 μm. These findings establish JEM as a safe, high-protein material with a narrow particle size distribution, suitable for applications that prioritize both microbiological safety and biochemical functionality.

Bioactive peptides, typically consisting of 2−20 amino acids, exhibit diverse bioactivities once released from their parent proteins (Majumder & Wu, 2010). Improving ESM solubility by cleaving peptide bonds is a key strategy to enhance its bioavailability and potential as a high-value dietary supplement for intestinal health. Various chemical methods, including oxidation, reduction, and acid-alkali treatments, have been used to solubilize ESM proteins. Acid-alkali methods involve acids such as hydrochloric or acetic acid, which stabilize disulfide bonds and can be combined with reducing agents for protein extraction (Chi et al., 2019). Alkali-based methods, using NaOH, break disulfide bonds and hydrolyze peptide bonds, producing low molecular weight peptides with enhanced solubility and bioactivity (Han et al., 2023). There are, however, other

approaches. Fermentation with lactic acid bacteria has also been employed to produce ESM hydrolysates with excellent solubility and bioactive properties (Jain & Anal, 2017). Pasarin et al. (2023) achieved 14.23% ESM hydrolysis using 1.25 N NaOH combined with Alcalase 2.4 L protease. Jain and coworkers explored ultrasonication before enzymatic hydrolysis with alcalase and papain, resulting in hydrolysates with improved protein concentration, hydrolysis degree, and functional properties compared to untreated samples (Jain & Anal, 2016). Additionally, combining enzymatic hydrolysis with reduction methods has produced ESM hydrolysates suitable for wound healing applications (Wang et al., 2024).

In the current study, we propose that KOH is a suitable solvent to directly hydrolyze ESM with low cost and less impairment in its components. The removal of sulfur odor (sulfur group) using oxidizing agent H_2O_2 might compromise the cysteine disulfide bonds component (Strohbehn et al., 2012). It has been pointed out that high digestion temperatures, especially approaching 90˚C, can lead to irreversible denaturation and loss of ESM protein (Andrew Mackay & Chilkoti, 2008; Schön et al., 2017). Enzymatic hydrolysis limitations include the regulation of enzyme denaturation, access of enzyme to substrate, functional parameter of temperature and pH, which can be hard to overcome on an industrial scale (Khueychai et al., 2019). On the other hand, acid hydrolysis may destroy tryptophan, and partially degrade cystine, serine, and threonine, and can convert asparagine and glutamine residues to their acid versions (Wu, 2020).

The primary objective of the current study was to hydrolyze ESM to enhance protein solubility and bioavailability. In a preliminary study (data not shown), chemical hydrolysis of PEM was assessed using five different acids and two alkalis, and evaluated at different temperatures, concentrations, reaction durations and stirring intensities. Building on this study, an optimized protocol was developed to hydrolyze JEM, based on degree of generation of soluble peptides and

subsequent increase in antioxidant activity (Exact conditions are subject to an invention disclosure). The process successfully produced soluble proteins and peptide fractions, yielding approximately 50% protein liberation. This was verified through SDS-PAGE and BCA assays, revealing a marked increase in soluble protein concentration and the presence of lower molecular weight proteins / peptides after prolonged hydrolysis. The neutralization and desalting steps ensured the removal of residual salts and enhanced sample purity of the produced hydrolysate HEJM in its two forms WJ and SJ. This process not only stabilized the hydrolysate but also made it suitable for downstream bioactivity assays, establishing a reproducible protocol that can be scaled up for industrial applications. The subsequent SGID of the two forms of the JEM hydrolysates (the whole WJ and the soluble/supernatant SJ) provided insights into their biological stability and further peptide release. The results showed that WJ formulations released additional soluble peptides during both the gastric and intestinal phases, while SJ formulations showed less change, likely due to pre-digestion solubilization. This highlights the robustness of WJ as a protein-rich dietary supplement capable of withstanding gastrointestinal conditions while releasing bioactive peptides.

2. Antibacterial Activity of JEM Hydrolysate

Our study evaluated the antibacterial activity of SJ against the gut-associated pathogen *E. coli*, building on our previous findings of PEM antimicrobial activities (Kulshreshtha et al., 2020). SJ exhibited bacteriostatic effect, inhibiting *E. coli* growth by 50 % at 10 mg/mL. The antibacterial potential of developed JEM formulations may be attributed to the liberated peptides and enhanced bioavailability that allowed protein activities to be more exposed and expressed upon alkaline hydrolysis. ESM proteins, such as OCX-36, exhibit high affinity for bacterial pyrogens like LPS

and LTA (Cordeiro et al., 2013), and exposure to other antimicrobial proteins is enhanced during hydrolysis. ESM is naturally enriched with antimicrobial proteins and peptides, including lysozyme, histones, AvBDs, ovalbumin, and gallin (ovotransferrin), all of which exhibit immune-modulating activities (Ahmed et al., 2017; Du et al., 2015; Rose-Martel & Hincke, 2017). Additionally, glycoproteins such as ovomucin and mucin have antibacterial and antiviral properties (Lieleg et al., 2012; Omana et al., 2010). Minor constituents, such as protease inhibitors (ovalbumin Y, ovomacroglobulin, ovomucoid), also contribute to ESM's antimicrobial capabilities (Andersen, 2015; Makkar et al., 2015). However, ESM hydrolysates demonstrated antimicrobial activity against a broad spectrum of bacterial pathogens, including *S. aureus*, *B. subtilis*, *K. pneumoniae*, *S. marcescens*, and *E. coli,* corresponding to increased activity of antimicrobial peptides liberated upon ESM hydrolysis with 2 N NaOH and 40% EtOH at 70ºC for 2 hours (Yoo et al., 2014).

These findings highlight the potential of JEM formulations in gut health applications, particularly for managing dysbiosis-associated disorders. Furthermore, the concentration-dependent inhibition of pathogenic bacteria suggests that JEM may contribute to a balanced gut microbiome, though additional research is needed to assess efficacy across a broader range of gut pathogens.

3. Antioxidant Activity of JEM formulations

Oxidative stress, defined as an imbalance between the production of ROS and the body's antioxidant defenses, is a key factor in the onset of various GI disorders. Excessive ROS levels can harm cellular structures, promote inflammation, and contribute to diseases like IBD and colorectal cancer. For example, oxidative stress can compromise the mucosal barrier of the GI tract, making it more susceptible to bacterial invasion and triggering immune responses that lead

to IBD (Semenova et al., 2024). Antioxidants are able to counteract oxidative stress and restore the redox equilibrium. Regarding the safety of its natural source and accessibility, the search for natural antioxidants as alternatives has attracted wide interest among researchers. Protein hydrolysates exhibit better antioxidant activity than their intact form due to the structural changes of the secondary and tertiary structure, including reductive cleavage of disulfide bonds that increase the free radical scavenging and prooxidative metal chelating capacity (Elias et al., 2008). A majority of peptides derived from natural sources and industrial by-products possess potential radical scavenging activity, inhibition of liposome oxidation or inhibition of TBARS (degradation products of fats) abilities (Shahidi & Zhong, 2008). Ultrafiltered ESM enzymatic hydrolysate fractions demonstrated a range of antioxidant activity, including iron (Fe^{3+}) reducing, DPPH, hydroxyl radical scavenging, and Fe^{2+} chelating activity, which were further verified by a cell-based study which demonstrated that ESM hydrolysate reduced proinflammatory cytokine IL-8 secretion in oxidative stress-induced human intestinal epithelial Caco-2 cells (Shi et al., 2014). Similarly, ESM enzymatic hydrolysates and corresponding identified synthetic peptides showed a strong ability to quench ABTS, inhibit TBARS and high total antioxidant activity in a study by Zhao and coworkers (Q.-C. Zhao et al., 2019).

Therefore, based on the above evidence and our previous proteomic results (Ahmed et al., 2017), we propose that the proteinaceous JEM has the potential to be a suitable antioxidant for biomedical applications. In my study, we evaluated the antioxidant power of the soluble proteins liberated upon JEM alkaline hydrolysis followed by SGID. In our study, the antioxidant potential of JEM formulations was a critical outcome. Using the TEAC assay, SJ showed antioxidant activities up to 15 times greater than NJEM, peaking at 667 μM Trolox equivalents at 10 mg/mL concentration. The antioxidant capacity of the JEM formulations was significantly enhanced after hydrolysis and

was retained after digestion, demonstrating their potential to mitigate oxidative stress in biological systems. These results underline the potential of JEM formulations as dietary antioxidants capable of neutralizing oxidative stress, which plays a pivotal role in gut health and chronic inflammation. In addition to free radical scavenging, the antioxidant mechanism of proteins / peptides can also derive from metal ion chelation, inactivation of ROS, and hydroperoxide reduction, as well as aldehyde adduction. ESM comprises proteins which contain cysteine, histidine, tryptophan, β-hydroxyl tryptophan amino acids (Huang et al., 2010; Yi et al., 2004), in addition to ovalbumin, ovotransferrin, and cystatin that exhibit antioxidant activities (Nimalaratne et al., 2015), as well as CREMPs which contain disulfide bonds and provide cysteine residues (Kodali et al., 2011b). Therefore, we propose the hydrolysis of CREMPs and other ESM proteins contributed to the enhanced antioxidant activity of JEM formulations.

4. Anti-inflammatory Activity of JEM Formulations

Inflammation plays a pivotal role in the development of numerous gut disorders (Randeni et al., 2024). A previous study demonstrated that ESM modulates microbiota and suppresses IBD, offering novel prevention/treatment strategies. Jia et al., (2017) found that ESM reduced LPS-induced inflammatory cytokines and enhanced Caco-2 proliferation by upregulating growth factors. In a murine dextran sodium sulfate-induced colitis model, ESM improved disease activity, colon shortening, and inflammatory mediator gene expression while promoting epithelial proliferation and antimicrobial peptides. Omics analyses highlighted enhanced energy metabolism and reduced pathogenic bacteria, including Enterobacteriaceae and *E. coli*, alongside improved microbial diversity. ESM also regulated Th17 cell expansion by limiting segmented filamentous bacteria, aiding epithelial repair, energy balance, and mucosal inflammation reduction (Jia et al.,

2017). Another study explored the potential of ESM as a complementary intervention for pre-cachexia prevention, where an IL10-knockout mouse model was used to simulate cachexia, transcriptomic analysis showed that ESM supplementation suppressed the LPS/IL1-mediated inhibition of the RXR function pathway in the liver and reduced colonic mucosal expression of chemokines and Th cell differentiation markers by targeting the upstream BATF pathway. ESM also improved microbial alpha diversity and reduced inflammation-associated microbiota, while increasing total organic acid levels, particularly SCFAs like butyrate (2.3-fold), which are known to inhibit Th1 and Th17 production (Jia et al., 2022). Rønning and colleagues proposed that ESM could be a promising nutraceutical for addressing skeletal muscle aging, potentially through its immunomodulatory properties or effects on gut microbiota. Their experiments revealed that mice fed a diet containing 8% ESM exhibited increased gut microbiota diversity and altered microbial composition compared to other groups. Additionally, dietary ESM reduced the expression of the inflammatory marker TNF-α in mice and *in vitro* in THP-1 macrophages. In a human study, ESM capsule supplementation significantly lowered levels of the inflammatory marker CRP (Rønning et al., 2023).

In the current study, the anti-inflammatory properties of JEM formulations were assessed using the well-established pro-inflammatory model of LPS-induced inflammation in RAW 264.7 macrophages. The evaluation focused on the ability of JEM formulations to modulate NO production, a key marker of inflammation, in macrophages stimulated with LPS. LPS induces inflammatory responses through complex signaling pathways mediated by TLR4, as documented in previous studies (Hommes et al., 2003).

Optimal LPS concentration for inducing inflammation without affecting viability was determined using 1, 5, 10, and 20 µg/mL LPS in RAW 264.7 macrophages. Results showed that 1 µg/mL

induced NO production similar to 20 μg/mL, without compromising viability. Cytotoxicity assays of JEM formulations (1.25 - 10 mg/mL) showed non-toxic effect on the cells, even at the highest tested concentration of 10 mg/mL, since, according to ISO 10993-5, cell viability above 80% is defined as non-cytotoxic (*ISO 10993-5*, 2009). Cytotoxicity data confirmed the safety of JEM formulations and ensured observed anti-inflammatory effects were due to formulation mechanisms rather than cytotoxicity. The study demonstrated the potent, dose-dependent anti-inflammatory activity of JEM formulations, evidenced by the suppression of NO production in LPS-stimulated RAW 264.7 macrophages. Macrophages produce NO in response to inflammatory stimuli such as IFN-γ, TNF-α, IL-1β, or LPS (Coleman, 2001).

The developed JEM hydrolysates and their metabolites reduced NO production significantly, achieving up to 80% suppression at the concentration 10 mg/mL. Post-gastrointestinal digestion products retained potent anti-inflammatory effects, highlighting the stability and bioactivity of hydrolyzed peptides under physiological conditions. Interestingly, metabolites of the whole JEM hydrolysate (WJ: WJ-G and WJ-GI) showed the most significant anti-inflammatory effects, possibly due to the presence of diverse bioactive peptides generated during digestion of WJ. The concentration-dependent effects observed in the formulations suggest a nuanced interaction with inflammatory pathways, lower concentrations of certain formulations displayed negligible activity or mild pro-inflammatory effects.

Peptides derived from OCX-36, which is one ESM constituent, by digestion with pepsin and thermolysin showed no impact on NO secretion in LPS-stimulated cells (Kovacs-Nolan et al., 2014). A form of particalized ESM (PEM<53 μm) exhibited a 56.89 ± 7.39% reduction in NO levels after 24 hours, compared to the 80% reduction achieved with KOH-hydrolyzed JEM in the current study (Wu, 2020).

The findings from this model provide valuable insights into the potential of JEM formulations to attenuate inflammatory responses, highlighting their potential as anti-inflammatory agents in protecting against gut-related inflammatory disorders.

5. The Effect of JEM formulations on Intestinal Barrier Function

The integrity of the intestinal barrier is essential for maintaining intestinal function and overall health. Disruptions to this barrier, such as increased intestinal permeability, are linked to various diseases (Chelakkot et al., 2018), including IBD (McGuckin et al., 2009), irritable bowel syndrome (IBS) (Piche et al., 2009), metabolic syndrome (Teixeira et al., 2012), and central nervous system disorder (Julio-Pieper et al., 2014), underscoring its critical role in maintaining host health. Various factors, including intraluminal antigens, toxins, pathogens, and enteric microorganisms, can disrupt the normal biological functions of enterocytes and compromise intestinal barrier function, leading to immune activation and inflammatory damage. Importantly, inflammation can weaken the epithelial barrier, increasing antigen exposure to the epithelium and submucosa, which in turn exacerbates and prolongs inflammatory responses (Bao et al., 2024). There is a growing interest in investigating the effects of food-derived bioactive peptides on intestinal barrier function and their potential applications in disease management (Bao & Wu, 2021; Wang et al., 2021). Previous studies showed the impact of food components, such as proteins and derived bioactive peptides on regulating intestinal physical, chemical, biological and immunological barrier function (Bao & Wu, 2021). Examples of dietary peptides regulating physical barrier function include casein hydrolysate (Visser et al., 2010) and derived peptides from fish protein hydrolysate (Marchbank et al., 2009), soybean β-conglycinin hydrolysate (Ren et al., 2014), wheat gluten A5 and C5 (Maggioni et al., 2016).The casein-derived peptide NPWDQ has been shown to enhance the

expression of occludin and reinforce the tight junction barrier (Yasumatsu & Tanabe, 2010; (ISOBE et al., 2008). Similarly, bovine lactoferrin upregulates tight junction protein expression and improves intestinal barrier function in both Caco-2 cells and suckling piglets. Additionally, the porcine lactoferrin-derived peptide LFP-20, has been found to increase transepithelial resistance, elevate tight junction protein levels, regulate intestinal inflammatory responses, and support functional intestinal structures in LPS-stimulated porcine intestinal epithelial cells and in mice (Zong et al., 2016).

A previous study examined the impact of Alaska pollock skin-derived collagen and its three tryptic hydrolytic fractions - HCP (6 kDa retentate), MCP (3 kDa retentate), and LCP (3 kDa permeate) - on TNF-α-induced barrier dysfunction in Caco-2 cell monolayers. The results showed that collagen and its peptide fractions, particularly LCP, significantly mitigated TNF-α-induced barrier dysfunction compared to TNF-α-treated controls ($P < 0.05$). Pre-incubation with 2 mg/mL LCP for 24 hours notably alleviated TNF-α-induced disruption of tight junction proteins ZO-1 and occludin, while also inhibiting MLC phosphorylation and MLCK expression. Furthermore, LCP suppressed the activation of NFκB and Elk-1(Q. Chen et al., 2017). When used in LPS-treated Caco-2 cells, the hydrolysate of the egg white protein ovomucin, Protex 26L (OP), demonstrated effective restoration of TEER and a reduction in paracellular permeability of FITC-dextran (4 kDa and 40 kDa) at concentrations of 0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL. Furthermore, OP significantly preserved the expression and structural integrity of tight junction proteins, including occludin and ZO-1, ensuring their proper localization on the cell surface (Bao et al., 2024).

The Caco-2 cell line is widely used to investigate *in vitro* epithelial barrier function. Here, we used differentiated Caco-2 cells to study the effect of our developed JEM formulations on intestinal barrier function, aiming to eliminate any negative effect they might have on the barrier integrity.

My experiments measuring effect on TEER and paracellular tracer (FITC-Dextran) transport allowed assessment of intestinal barrier function. The results revealed that a low concentration (1.25 mg/mL) of SJ, WJ-G, and WJ-GI significantly increased TEER, indicating enhanced intestinal barrier integrity, whereas higher concentrations did not show significant effect on TEER. This concentration-dependent response underscores the potential of JEM formulations to protect and maintain intestinal barrier function without disrupting permeability, a crucial factor for gut health supplements. The variability in these effects highlights the importance of formulation and concentration in determining the impact of JEM on epithelial barriers. Future studies should aim to optimize JEM formulations to enhance barrier integrity while minimizing potential adverse effects.

Fluorescence analysis of the basolateral compartment showed no significant differences in FITC-Dextran transport across Caco-2 monolayers treated with JEM formulations after 24 hours. This *in vitro* finding suggests that the developed formulations are unlikely to affect paracellular intestinal permeability following oral administration and gastrointestinal digestion.

The results of this study have broader implications for the utilization of ESM as a sustainable, functional ingredient in health-focused applications. With millions of tons of ESM generated as an industrial by-product annually, its transformation into value-added products aligns with global efforts to reduce waste and promote circular economies. The compositional richness of ESM, including its collagen content and bioactive peptides, positions it as a versatile material for nutraceutical and pharmaceutical applications, and for development of functional foods. The demonstrated antioxidant, antimicrobial, and anti-inflammatory activities of JEM formulations highlight their potential as dietary supplements to boost gut health. Furthermore, the ability to

enhance gut barrier integrity at appropriate concentrations could make ESM formulations valuable for ameliorating systemic inflammation and associated diseases.

Conclusions

Hydrolyzed ESM dominates global revenue due to improved bioavailability of components like collagens, HA, antioxidants, and antimicrobials. ESM hydrolysates are increasingly marketed as premium health supplements. SWOT analysis highlights opportunities and areas for improvement. Advancing technologies to develop functional ESM formats is key to upscaling for commercial applications (Kulshreshtha et al., 2022a).

This study explored the transformation of a waste by-product, ESM, into a high-value biomaterial, addressing both sustainability and health innovation issues. The findings highlight the potential applications of JEM formulations in functional foods, nutraceuticals, and therapeutic interventions targeting gut health and related disorders. The demonstrated bioactivities of hydrolyzed JEM formulations - antioxidant, antimicrobial, anti-inflammatory effects, and the enhancement of intestinal barrier integrity - position them as promising candidates for dietary supplements aimed at promoting gut health.

The future *in vivo* studies are necessary to validate these *in vitro* findings and assess the long-term safety and efficacy of JEM formulations. Additionally, the scalability of the hydrolysis protocol and its environmental implications deserve further exploration, providing a model for resource-efficient, sustainable innovation. This research lays a robust foundation for biological and industrial advancements, paving the way for ESM-based applications in the health and wellness sectors while contributing to circular economy goals.

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