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ULTRASONICATION-ENHANCED MICROBIAL TRANSGLUTAMINASE CROSSLINKING OF BOTANICAL AND FOREIGN PROTEIN FOR SUSTAINABLE HIGH-MOISTURE EXTRUDED MEAT ANALOGUE: A COMPREHENSIVE MULTIDIMENSIONAL CHARACTERIZATION

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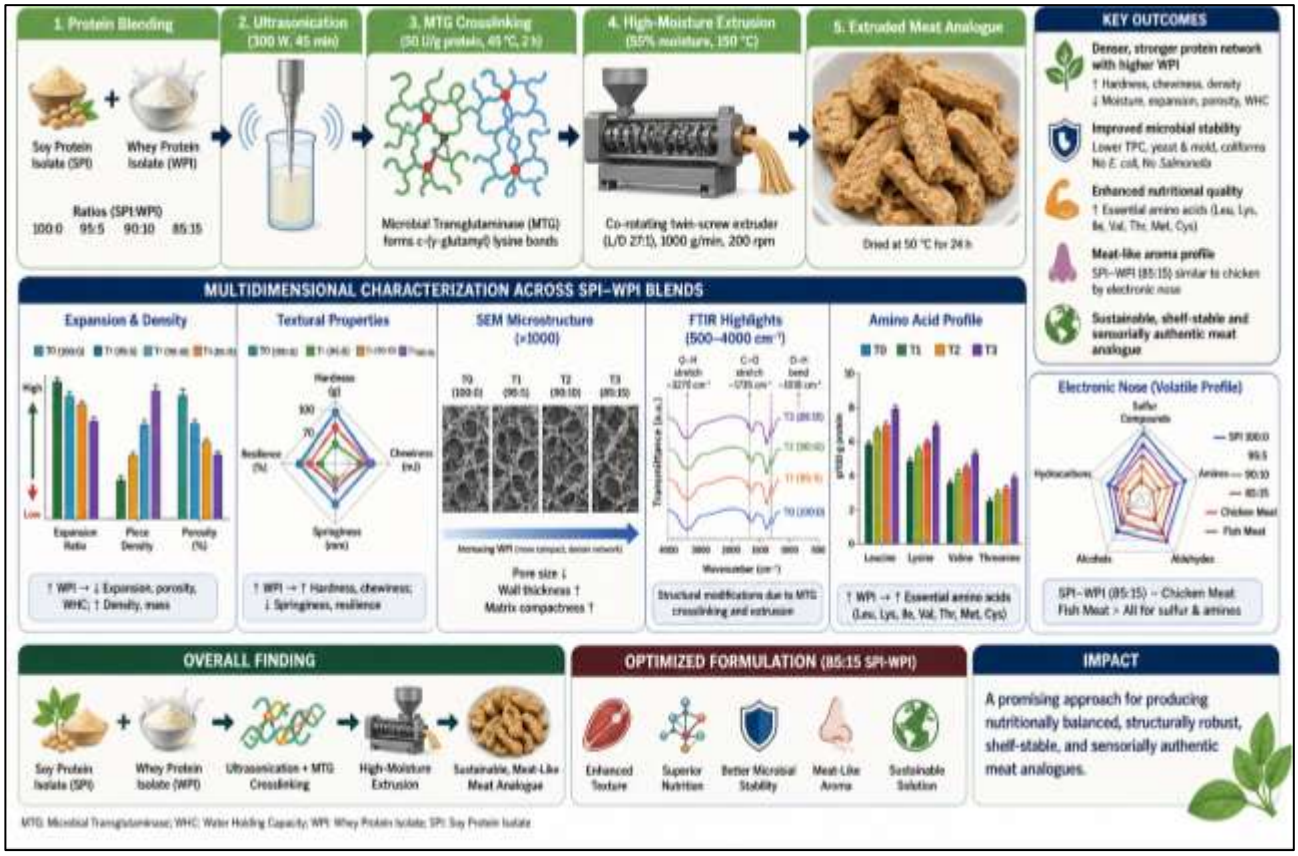
Graphical Abstract



ABSTRACT

The growing global population and environmental concerns around conventional meat production are driving demand for sustainable, nutritionally balanced protein alternatives. Plant proteins, while environmentally favorable, often have incomplete amino acid profiles, necessitating targeted fortification and structural modification. This study developed high-moisture extruded meat analogues via ultrasonication-assisted microbial transglutaminase (MTG) crosslinking of soy protein isolate (SPI) and whey protein isolate (WPI) at 100:0, 95:5, 90:10, and 85:15 ratios. The extrudates were characterized for physicochemical, textural, structural, microbial, nutritional, and volatile (electronic nose) properties. Increasing WPI content reduced moisture, expansion, porosity, and water-holding capacity, while increasing density, hardness, and chewiness, indicating denser protein networks. Lightness and pH shifted slightly with whey addition, and microbial counts decreased, suggesting improved shelf stability. FTIR confirmed structural

modifications, and amino acid profiling revealed enhanced essential amino acids, including leucine, lysine, isoleucine, valine, and threonine. E-nose analysis demonstrated that controlled whey fortification progressively increased sulfur- and amine-responsive volatile signals, with the SPI–WPI (85:15) formulation exhibiting aroma profiles statistically similar to chicken meat. These findings indicate that strategic protein blending can enhance both the nutritional quality and the meat-like sensory profile of soy-based high-moisture extrudates. Ultrasonication-assisted MTG crosslinking combined with high-moisture extrusion offers a promising approach for producing nutritionally balanced, structurally robust, shelf-stable, and sensorially authentic meat analogues.

KEYWORDS: Soy protein isolate, whey protein isolate, essential amino acids, aroma profile, electronic nose

INTRODUCTION

The global population is projected to reach 9.7 billion by 2050 and approximately 11.1 billion by 2100 [1], a demographic shift that will inevitably intensify the demand for food [2], including protein. While animal-derived proteins are traditionally recognized for their comprehensive essential amino acid profiles and superior digestibility [3], their production is associated with significant environmental and potential health concerns. Conversely, plant-based protein sources offer sustainability benefits but may present challenges in terms of bioavailability and complete nutritional profiles. This paradigm necessitates a balanced approach, integrating nutritional excellence with sustainable practices in protein provision. Meat analogues, designed to mimic the sensory and nutritional attributes of animal meat [4], represent a promising avenue. This study develops sustainable meat analogues using the complementary strengths of soy protein isolate (SPI) and whey protein isolate (WPI). Their selection was deliberate: both are high-quality sources; SPI offers a Protein Digestibility Corrected Amino Acid Score (PDCAAS) comparable to whey, indicating similar bioavailability [5]. Nutritionally, their amino acid profiles are complementary, particularly in leucine (crucial for muscle protein synthesis [6–7]). Although excellent proteins in their respective classes, neither is nutritionally perfect alone: WPI contains higher amounts of Threonine, Methionine, Lysine, Valine, Serine, Glutamic acid, Proline, Cysteine, Alanine, Tyrosine, and Isoleucine than SPI, while SPI possesses more Phenylalanine, Histidine, Arginine, and Glycine [6]. Hence, their strategic blending covers these nutritional gaps, yielding a more comprehensive and balanced amino acid profile. Soy protein, the major constituent, with whey protein as a supplementary component or fortifier, was chosen for SPI's functional advantages—excellent gelling ability, thermal stability, and fibrous texture formation (ideal for texturization)—plus its cost-effectiveness and abundant availability [8]. To enhance these combined proteins' structural and functional properties beyond simple blending, enzymatic crosslinking using microbial transglutaminase (MTG) was employed [9]. MTG catalyzes covalent ϵ−(γ−glutamyl) lysine bonds, creating more stable, uniform protein networks. This enzymatic modification significantly improves texture, structural integrity, water retention [10], emulsifying stability [11], viscosity, and rheological strength [11-12]. MTG also enhances individual soy protein [13] and whey protein [14] properties, improves thermal stability [15], contributes to a meatier mouthfeel [16], and can reduce heat-induced coagulation in whey proteins [17] (beneficial during thermal processing). The resultant enzymatically crosslinked soy-whey hybrid proteins were subjected to high-moisture extrusion (HME), a thermo-mechanical process pivotal for creating a fibrous, meat-like texture characteristic of textured vegetable proteins (TVP) and contemporary plant-based meat analogues (PBMA) [4]. The objective was to develop novel, sustainable meat analogues via HME of MTG-crosslinked soy-whey protein blends, followed by comprehensive structural, functional, physicochemical, nutritional and microbial properties characterization to assess commercialization potential.

2. MATERIALS AND METHODS

2.1. Preparation of Enzymatically Crosslinked Hybrid Proteins and High-Moisture Extrusion of Meat Analogues

This study was conducted in the Department of Food Science, Faculty of Life Sciences, Government College University, Faisalabad. Soy protein isolate (SPI; Nutrena), whey protein isolate (WPI; 90%, Protein Factory), and microbial transglutaminase (MTG; Sunson Industry, China) were used. Four formulations (100% SPI, 95:5, 90:10, 85:15 SPI:WPI) were dissolved in 0.2 M PBS (pH 7, 100 mg protein/100 mL), stirred 2 h at room temperature, and stabilized at 4 °C for 12 h. Samples (30 mL) were ultrasonicated at 300 W for 45 min (25–35 °C ice bath), MTG added (50 U/g protein), and incubated at 45 °C for 2 h. Enzyme deactivation was performed at 80 °C for 10 min. Crosslinked proteins were separated, drained (4 °C, 12 h), and lyophilized (–50 °C, 150 µmHg, 8 h). High-moisture extrusion was conducted using a co-rotating twin-screw extruder (L/D 27:1) at 55% feed moisture, 1 cm die, 150 °C die temperature, 1000 g/min feed rate, 200 rpm screw speed, with thermal zones: feeding 100 °C, compression 130 °C, die 150 °C. Extrudates were dried at 50 °C for 24 h. After that certain analysises of different types were performed that are discussed in the later sections. All measurements were performed in triplicate (n = 3), and mean values were reported.

Figure.1 Pictorial depiction of extruded crosslinked proteins

2.2. Expansion and Density Characteristics

The expansion and density properties of extruded proteins were evaluated to characterize their textural and structural behavior, following methodologies adapted from Zhang et al. [21] and Mohamad Mazlan et al. [22]. Moisture content was first determined by drying samples at 105 °C, and moisture loss (%) was calculated as:

Moisture loss (%) = (Weight before drying (g) - Weight after drying (g)) / Weight before drying (g) × 100

For expansion characteristics, the diameter of extrudates (n = 5) was measured using a Vernier caliper, and the expansion ratio was calculated as:

Expansion ratio (%) = (Sample diameter (cm) / Die diameter (1 cm)) × 100

Apparent density (ρapp, kg/m³) was determined from extrudate dimensions (width, height, length) measured with a digital Vernier caliper and weight measured using an analytical balance, using the formula:

$\rho app = 4\ W / \pi\ D^2\ L$

where W is the extrudate weight (kg), D^2 = width × height (m^2), and L is the length (m). Piece density was calculated by dividing the mass of 2 cm extrudate segments by their volume, obtained from Volume = $\pi\ r^2\ L$, where r is the radius (cm) and L is length (cm). True density was measured by pulverizing extrudates, sieving (100-mesh), filling 5 mL graduated cylinders, and compacting with 50 strikes. True density (g/cm³) was calculated as Mass of Extrudate Powder (g) / Volume of Extrudate Powder (cm³). Porosity (%) was then calculated as:

Porosity (%) = (True density (g/cm³) – Piece density (g/cm³)) / True density (g/cm³) × 100

2.3. Physicochemical and Rehydration Characteristics

Physicochemical and rehydration properties of extruded proteins were evaluated to assess their quality, functionality, and water-related behavior, following established methodologies. Color parameters (L*, a*, b*) were measured using an ST-C P60 colorimeter [21], and total color difference (ΔE) was calculated as:

$\Delta E = \sqrt{[(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2]}$,

where L_0, a_0, b_0 correspond to measurements of a white ceramic plate. Color was recorded at three positions on each sample. The pH of extrudates was measured using a CpH-103 pH meter following Wu et al. [23]. Water absorption percentage (Wp) and water holding capacity (WHC) were determined to assess rehydration properties. For Wp, 2-cm extrudates were immersed in 100 mL distilled water at 25 °C for 2 hours, blotted, and weighed, with Wp (%) calculated as:

$Wp\ (\%) = (Wt - W_0) / W_0 \times 100$

where Wt = sample mass at time t, and W_0 = initial mass. WHC was measured using 0.2 g extrudate powder dissolved in 5 mL deionized water, vortexed, incubated at 4 °C for 12 hours, and centrifuged at 4000 rpm for 15 minutes. The supernatant was discarded, and WHC was calculated as:

WHC (ml/g) = (W2 - W1) / W0

where W0 = powder mass, W1 = tube + powder mass, and W2 = tube + pellet mass [21, 24].

2.4. Microbial Analysis

Total plate count, yeast and mold, coliforms, E. coli, Salmonella evaluated following Butt et al. [25]. 10 g samples homogenized in 90 mL sterile saline; serial dilutions (10^{-1} to 10^{-5}) plated on selective media: Nutrient agar (TPC), Potato Dextrose agar (yeast/mold), Violet Red Bile agar (coliforms), Eosin Methylene Blue agar (E. coli), Xylose Lysine Deoxycholate agar (Salmonella).

2.5. Scanning Electron Microscopy (SEM):

Microstructural characterization of the extruded protein samples was performed using scanning electron microscopy (SEM) to evaluate the internal fibrous network, porosity, and crosslinked structural morphology of the SPI–WPI meat analogues. Dried extrudate samples from each treatment (T0: 100:0, T1: 95:5, T2: 90:10, T3: 85:15 SPI:WPI) were sectioned into small pieces and mounted on aluminum stubs using conductive carbon adhesive tape. Samples were

sputter-coated with a thin layer of gold under vacuum to enhance conductivity. Micrographs were obtained using a scanning electron microscope operated at an accelerating voltage of 5.0 kV under low (×300) and high (×1,000) magnifications. Images were captured to observe variations in pore size, fibrous alignment, network compactness, and structural integrity as influenced by ultrasonication-assisted microbial transglutaminase crosslinking and whey protein incorporation [49].

2.6. Structural and textural analysis

The structural and textural properties of extruded proteins were evaluated to assess molecular interactions, functional groups, and mechanical behavior. Textural properties were measured using a TX-700 texture analyzer [21]. Rehydrated 2-cm extrudates (200 min at 25 °C, blotted, rested 30 min at 25 °C) were subjected to a double compression to 50% of their original height in TPA mode, with settings of 3 mm/s pre-test, 2 mm/s test, 5 mm/s post-test, and 3 s interval. Parameters obtained included Hardness (g), Resilience (%), Springiness (mm), and Chewiness (mJ). Fourier transform infrared (FTIR) spectroscopy was performed using a Cary 630 spectrometer to identify molecular interactions and functional groups in the extruded proteins [14]. Spectra were acquired over 500–4000 cm^{-1} following a 4 cm^{-1} resolution blank scan.

2.7. Amino acid analysis

Amino acid profiling was performed using High-Performance Liquid Chromatography (HPLC) fitted with a UV detector set at 338 nm. Separation was achieved on a C18 column (2.5 × 200 mm, 5 µm). The mobile phase consisted of a 1:2:2 (v/v/v) mixture of 100 mM sodium sulphate (pH 7.2), acetonitrile, and methanol, delivered at a flow rate of 0.45 mL min^{-1}, with the column temperature maintained at 40 °C [45].

2.8. Volatile Characterization

Fresh chicken (thigh) and fish (cod fillet) samples were purchased locally, and 0.5 g portions were placed in 20 mL vials to allow accumulation of volatiles prior to measurement. Samples were stored at either room temperature (24 ± 1 °C) or under refrigeration (2 ± 1 °C) for up to 96 hours. Disposable colorimetric sensor arrays (CSAs) consisting of twenty chemically responsive dyes printed onto polypropylene membranes were used for e-nose measurements. The arrays were dried under vacuum, stored in N_2-filled bags, and exposed to sample headspace for 2 minutes using a handheld optoelectronic nose, with images recorded before and after exposure. Each sample was measured in triplicate with three independent arrays. Color changes were quantified from RGB differences for each sensor element, and overall responses were expressed as Euclidean distances. Data were analyzed using principal component analysis (PCA), hierarchical cluster analysis (HCA), and support vector machine (SVM) to evaluate discrimination among sample types and storage durations, while reproducibility was confirmed through independent array batches and repeated trials. This methodology enabled sensitive detection of volatile compounds, including sulfides and biogenic amines, providing a rapid assessment of meat freshness and spoilage progression [46].

2.8. Statistical analysis

All analyses were performed in triplicate, and results are presented as means. Data were statistically analyzed using IBM SPSS Statistics 25. Analysis of variance (ANOVA) in a completely randomized design (CRD), following Montgomery [26], was performed, and means were interpreted using Tukey's HSD test.

3. RESULTS AND DISCUSSIONS

3.1. Expansion and Density Statistics

Mean moisture and expansion characteristics of extrudates are presented in Table 1. Extruded crosslinked soy protein exhibited higher moisture content compared to soy–whey protein hybrids, with sample R3 (highest whey protein proportion) showing the lowest moisture. This trend aligns with Zhang et al. [17], who reported that whey protein isolate (WPI) enhances hardness, cohesiveness, and fibrous structure in pea protein extrudates through increased disulfide bonds and hydrogen bonding. The resulting denser, more cross-linked network reduces internal porosity, limiting water retention and explaining the lower moisture in high-whey formulations. Regarding expansion, pure soy protein isolate (SPI) demonstrated superior properties, with higher expansion ratio, radius, and porosity, while mass, piece density, extrudate powder mass, true density, and apparent density were lower. Conversely, R3 showed the least favorable expansion characteristics. These observations are consistent with Zhang et al. [21], who found whey protein addition decreases expansion due to enhanced cross-linking, and Devi et al. [27], who noted similar effects in sorghum-based extrudates. Kreger et al. [28] further reported that soy protein promotes lighter, crispier textures, whereas whey protein produces denser, crunchier products. The increased density in high-whey extrudates contributes to higher mass and reduced porosity, confirming the structural influence of whey protein on the extrudate matrix.

3.2. Physicochemical and Rehydration Characteristics

Mean physicochemical and rehydration characteristics of the extrudates are presented in Tables 2 and 4. Color values were inconsistent, with T0 (pure soy) showing the highest lightness (L*), T2 the highest redness (a*), and T3 the highest yellowness (b*), while ΔE varied, peaking in T1. These trends reflect the complex effects of protein

composition on pigment stability and surface color, influenced by protein structure, pigment type, and processing conditions [29–32]. pH increased with higher whey protein content, shifting from acidic toward less acidic conditions, which can alter protein hydrophobicity, secondary and tertiary structures, and emulsification, particularly in SPI's glycinin (11S) fraction [33,34]. Rehydration properties declined with increasing whey content, as both water holding capacity (WHC) and water absorbance (WP) decreased. This is attributed to the denser, more cross-linked protein network in whey-enriched samples, which reduces porosity and limits water retention [21,28,36]. Overall, higher whey concentrations produced extrudates that were denser, less porous, less hydrophilic, and displayed variable surface color and pH, highlighting the interconnected effects of protein composition on physicochemical and functional properties.

3.3. Microbial analysis

Table 1 indicates that total plate count decreased significantly with increasing whey protein incorporation, with the highest microbial load observed in the pure soy treatment (T0). A similar declining trend was observed for yeast and mold as well as coliform counts, suggesting improved microbial stability in formulations containing higher whey protein levels. This behavior is likely attributable to reduced moisture content and water activity, which are critical factors governing microbial growth and shelf stability of extruded protein matrices [25,44]. Furthermore, *E. coli* was not detected, and *Salmonella* was absent in all treatments, confirming satisfactory hygienic quality and processing safety.

Table-1. Moisture Content, Expansion, Rehydration Behavior, and Microbial Quality of Textured Protein Treatments

Analysis	T_0	T_1	T_2	T_3
Moisture (%)	7.11 ± 0.12^{a}	6.5 ± 0.16^{b}	6.17 ± 0.21^{c}	5.16 ± 0.17^{d}
Expansion ratio (%)	251 ± 5.4^{a}	245 ± 4.1^{ab}	233 ± 6.1^{bc}	224 ± 5.2^{c}
Extrudate mass (g)	0.48 ± 0.01^{d}	0.54 ± 0.01^{c}	0.60 ± 0.02^{b}	0.64 ± 0.01^{a}
Extrudate radius (cm)	1.3 ± 0.10^{a}	1.2 ± 0.10^{ab}	1.1 ± 0.10^{bc}	1 ± 0.10^{c}
Piece density (g/cm^3)	0.045 ± 0.002^{d}	0.060 ± 0.003^{c}	0.079 ± 0.004^{b}	0.102 ± 0.005^{a}
Extrudate powder mass (g)	0.50 ± 0.02^{d}	0.52 ± 0.02^{c}	0.58 ± 0.03^{b}	0.66 ± 0.02^{a}
True density (g/cm^3)	0.100 ± 0.004^{d}	0.104 ± 0.004^{c}	0.116 ± 0.006^{b}	0.132 ± 0.004^{a}
ρapp (kg/m³)	1230.21 ± 7.96^{d}	1285.52 ± 8.25^{c}	1342.67 ± 8.55^{b}	1398.80 ± 8.88^{a}
Porosity (%)	55 ± 1.50^{a}	42.31 ± 1.80^{b}	31.90 ± 2.20^{c}	22.73 ± 2.00^{d}
WHC (ml/g)	5.15 ± 0.08^{a}	4.92 ± 0.07^{ab}	4.68 ± 0.06^{b}	4.45 ± 0.05^{b}
WP (%)	232.0 ± 2.9^{a}	223.3 ± 2.8^{b}	211.9 ± 2.6^{c}	203.4 ± 2.5^{d}
TPC (log CFU/g)	2.73 ± 0.15^{a}	2.61 ± 0.13^{ab}	2.48 ± 0.14^{bc}	2.35 ± 0.12^{c}
Yeast & Mold (log CFU/g)	2.10 ± 0.12^{a}	1.92 ± 0.10^{ab}	1.74 ± 0.09^{bc}	1.56 ± 0.08^{c}
Coliforms (log CFU/g)	1.42 ± 0.08^{a}	1.28 ± 0.07^{ab}	1.11 ± 0.06^{bc}	0.95 ± 0.05^{c}
E. coli	ND	ND	ND	ND
Salmonella	Absent	Absent	Absent	Absent

3.4. Microstructural Characterization by Scanning Electron Microscopy (SEM)

Scanning electron microscopy revealed substantial differences in the internal morphology and fibrous architecture of ultrasonication-assisted microbial transglutaminase (MTG) crosslinked SPI–WPI extrudates as influenced by whey protein incorporation (Figure X). All treatments exhibited porous, interconnected protein matrices typical of high-moisture extruded products; however, progressive structural modifications were observed with increasing WPI concentration.

The control treatment (T0; 100% SPI) displayed a comparatively larger pore structure, thinner cell walls, and a more open, expanded network, indicative of greater expansion and higher porosity. These observations align with the measured higher expansion ratio, lower density, and superior water holding capacity of pure soy extrudates. The relatively loose matrix suggests that SPI alone formed a less compact crosslinked network during extrusion.

With incremental WPI incorporation (T1–T3), SEM images demonstrated a gradual reduction in pore size, increased wall thickness, and enhanced compactness of the fibrous matrix. Treatments containing higher whey protein levels, particularly T3 (85:15 SPI–WPI), exhibited a denser and more organized microstructure with reduced void spaces and stronger interlinked protein strands. This denser architecture is likely attributable to enhanced protein–protein interactions, including disulfide bonding, hydrogen bonding, and ε-(γ-glutamyl) lysine covalent crosslinks catalyzed by MTG, further intensified by ultrasonication-induced unfolding of protein structures.

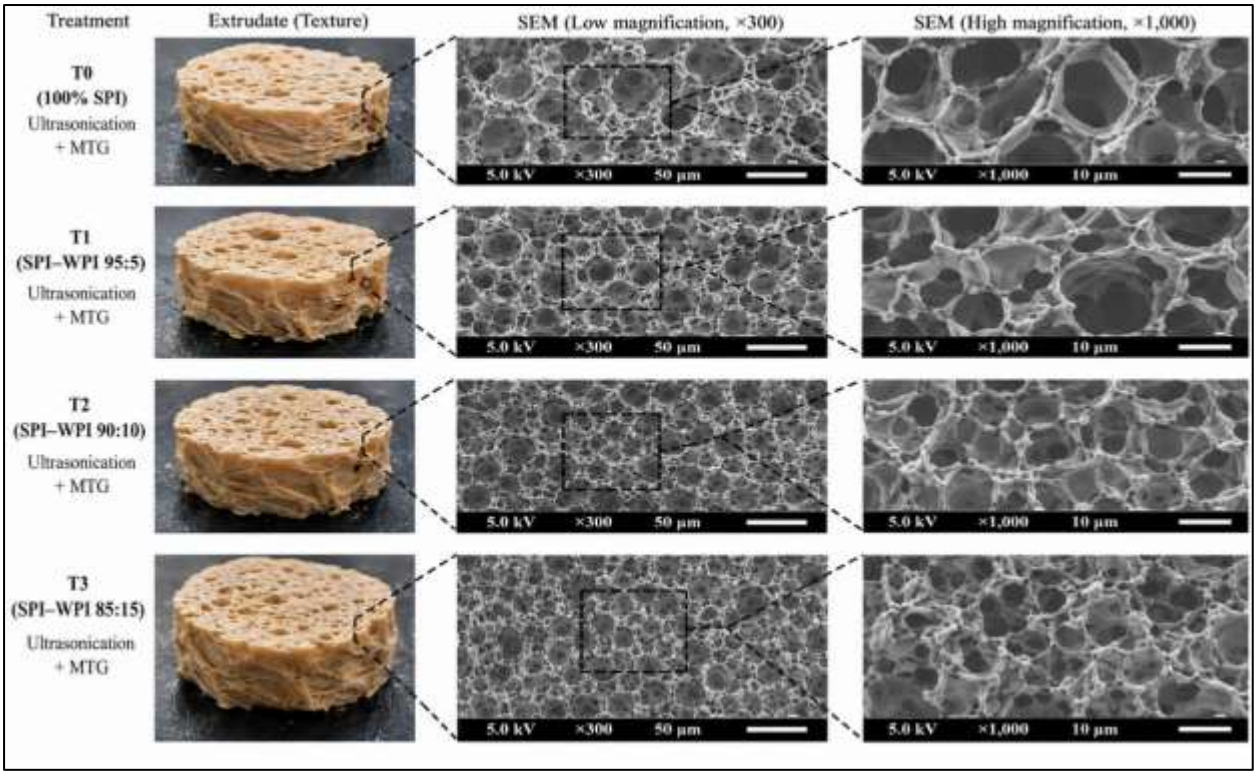
The observed microstructural densification corroborates physicochemical and textural findings, where increased whey protein content significantly elevated hardness, chewiness, and density while reducing moisture retention, expansion,

and rehydration properties. The compact matrix likely restricted water penetration and expansion during extrusion, resulting in products with more meat-like textural properties.

At higher magnification, T3 samples demonstrated more continuous fibrous layers and improved structural integrity, suggesting that controlled whey fortification may enhance anisotropic fibrous formation, a critical quality attribute for realistic meat analogue development. These findings are consistent with previous studies reporting that whey protein contributes to stronger gelation and denser extrudate networks due to its superior sulfur-containing amino acid profile and crosslinking potential.

Overall, SEM analysis confirmed that ultrasonication-assisted MTG crosslinking combined with strategic SPI–WPI blending systematically modified the microstructure of high-moisture extrudates, producing progressively denser, structurally robust, and commercially desirable meat analogue matrices. The T3 formulation exhibited the most compact and meat-like microstructure, supporting its suitability as an optimized formulation for sustainable protein-based meat alternatives.

Figure 2. SEM micrographs of ultrasonication-enhanced MTG crosslinked SPI–WPI extrudates showing microstructural variations across different whey protein incorporation levels.



3.5. Structural and Textural properties

Table 2 demonstrates a consistent textural trend in the extruded proteins: increasing whey protein content led to higher hardness and chewiness, while springiness and resilience decreased. In contrast, pure soy protein exhibited the lowest hardness and chewiness and the highest springiness and resilience. These observations are well supported by literature. Zhou et al. [35] and Zhang et al. [21] reported increased hardness with whey protein addition, while Onwulata et al. [36] showed that higher whey levels reduce expansion and water absorption, thereby increasing hardness. Springiness declined as hardness increased, consistent with reports that compression elevates hardness, gumminess, and chewiness but reduces springiness [37,38]. Chewiness followed the same pattern as hardness, reflecting their direct relationship [37,38]. Resilience also decreased with increasing whey protein content, with pure soy protein showing the highest value, aligning with findings of simultaneous reductions in springiness and resilience [39]. Moreover, sensory evaluations of cohesiveness, hardness, and springiness are known to correlate closely with instrumental texture measurements [40].

Table-2. Physicochemical and textural properties of the textured protein treatments

Analysis	T_0	T_1	T_2	T_3
L*	21.81 ± 1.10^{a}	13.77 ± 1.30^{d}	19.07 ± 1.90^{b}	20.27 ± 1.30^{c}
a*	1.83 ± 0.20^{a}	2.15 ± 0.30^{b}	2.37 ± 0.12^{c}	2.35 ± 0.14^{c}
b*	4.59 ± 0.20^{a}	6.17 ± 0.11^{b}	6.18 ± 0.29^{b}	7.06 ± 0.12^{c}
ΔE	16.42 ± 1.12^{a}	24.62 ± 1.24^{c}	19.49 ± 1.23^{b}	18.60 ± 0.12^{b}

pH	6.30±0.10[a]	6.50±0.20[ab]	6.70±0.20[b]	6.80±0.10[b]
Hardness (g)	86.67±5.36[a]	88.35±5.50[a]	90.81±5.65[a]	93.37±5.70[a]
Springiness (mm)	103.11±1.89[a]	101.94±1.72[a]	100.54±1.75[a]	99.24±1.80[a]
Chewiness (mj)	56.40±3.74[a]	57.29±3.60[a]	58.56±3.68[a]	60.02±3.80[a]
Resilience (%)	42.25±1.33[a]	41.64±1.13[a]	40.94±1.15[a]	40.14±1.20a

FTIR analysis (Figure 3, Table 3) revealed consistent spectral features across all samples (T0–T3), with the longest peak around 1017–1019 cm^{-1} (O-H bending, carboxylic acid), the broadest peak around 3270–3274 cm^{-1} (O-H stretching, carboxylic acid), and the shortest peak around 1733–1744 cm^{-1} (C=O stretching, ester). Distinct peaks were observed at 1101.4 cm^{-1} (C-O stretching, aliphatic ether) in T0 and 2374.3 cm^{-1} (S-H stretching, thiol) in T1. These variations align with Sadat and Joye [41], who reported FTIR differences based on protein origin and treatment, and reflect changes in amide structures induced by crosslinking, high-temperature, and high-moisture extrusion, consistent with Moharram et al. [42]. Chemical modifications of proteins, as Zhao et al. [43] demonstrated, create new C=O stretching vibrations, and in this study, transglutaminase treatment produced similar effects, confirming the structural impact of enzymatic crosslinking during extrusion.

Figure 3. FTIR results (frequency range: 500-4000 cm-1) of textured protein treatments

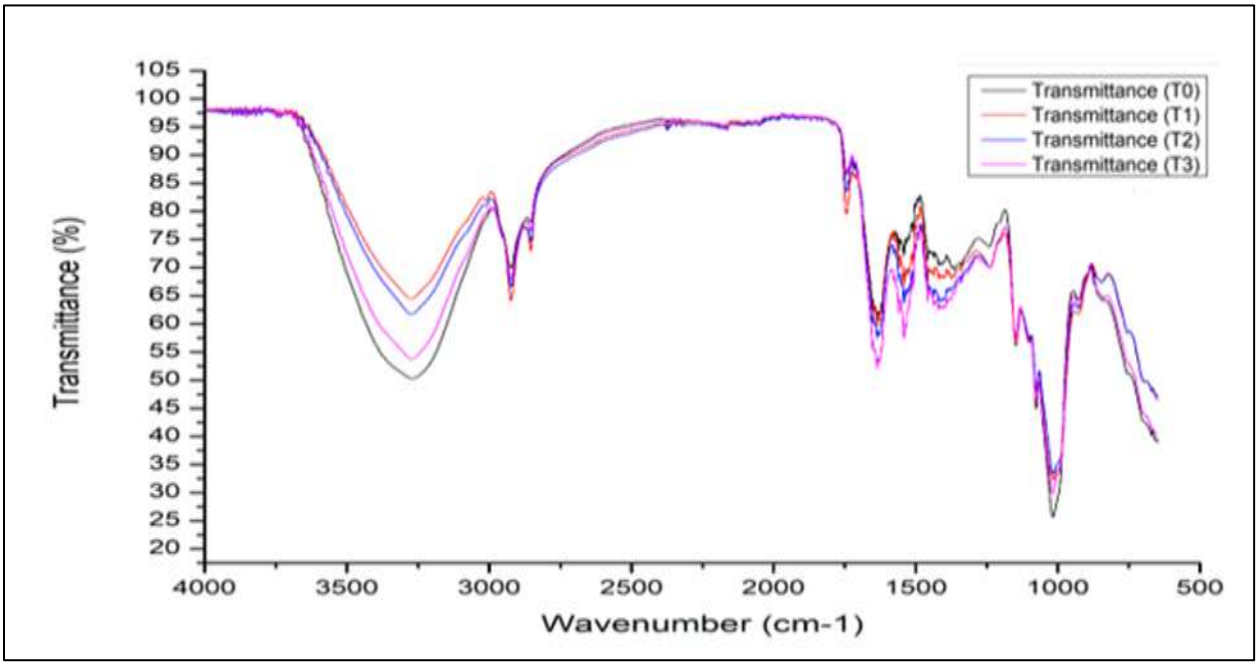


Table 3. Tabulated FTIR (frequency range: 500-4000 cm-1) of textured proteins

bsorption (cm^{-1})	**Type of bond**	**Class of compound**
T_0 textured protein (100% soy protein isolate)		
3274.5	O–H stretching	carboxylic acid
2924.1	N–H stretching	amine salt
2855.1	N–H stretching	amine salt
1742.5	C=O stretching	Ester
1636.3	N-H bending	Amine
1541.3	N-O stretching	nitro compound
1457.4	N-O stretching	nitro compound
1418.3	O-H bending	carboxylic acid
1364.2	S=O stretching	sulfonamide
1148.0	S=O stretching	sulfone
1101.4	C-O stretching	aliphatic ether
1075.3	C-N stretching	amine
1017.6	O-H bending	carboxylic acid
928.1	C=C bending	alkene
T_1 textured protein (95% soy protein isolate crosslinked with 5 % whey protein isolate)		

3274.5	O–H stretching	carboxylic acid
2924.1	N–H stretching	amine salt
2853.3	N–H stretching	amine salt
2374.3	S-H stretching	thiol
1744.4	C=O stretching	ester
1636.3	N-H bending	amine
1541.3	N-O stretching	nitro compound
1457.4	N-O stretching	nitro compound
1418.3	O-H bending	carboxylic acid
1375.4	O-H bending	phenol
1148.0	S=O stretching	sulfone
1075.3	C-N stretching	amine
1017.6	O-H bending	carboxylic acid
846.1	C=C bending	alkene
T_2 textured protein (90% soy protein isolate crosslinked with 10 % whey protein isolate)		
3274.5	O–H stretching	carboxylic acid
2924.1	N–H stretching	amine salt
2853.3	N–H stretching	amine salt
1744.4	C=O stretching	ester
1636.3	N-H bending	amine
1541.3	N-O stretching	nitro compound
1457.4	N-O stretching	nitro compound
1418.3	O-H bending	carboxylic acid
1239.3	C-N stretching	amine
1148.0	S=O stretching	sulfone
1075.3	C-N stretching	amine
1017.6	O-H bending	carboxylic acid
928.1	C=C bending	alkene
848.0	C=C bending	alkene
T_3 textured protein (85% soy protein isolate crosslinked with 15 % whey protein isolate)		
3270.7	O–H stretching	carboxylic acid
2924.1	N–H stretching	amine salt
2853.3	N–H stretching	amine salt
1744.4	C=O stretching	ester
1636.3	N-H bending	amine
1541.3	N-O stretching	nitro compound
1457.4	N-O stretching	nitro compound
1436.9	O-H bending	carboxylic acid
1148.0	S=O stretching	sulfone
1075.3	C-N stretching	amine
1019.4	O-H bending	carboxylic acid
926.2	C=C bending	alkene
840.6	C=C bending	alkene

3.8. Amino Acid content

The amino acid composition of the four hybrid cross-linked extruded proteins (T0–T3) showed a clear trend of increasing essential amino acids with higher whey protein content. T0, containing 100% soy protein, had the lowest levels of branched-chain amino acids (leucine, isoleucine, valine), lysine, threonine, and sulfur-containing amino acids (methionine, cystine). Incorporation of whey protein in T1–T3 progressively enhanced these amino acids, with T3 (15% whey) exhibiting the highest levels, indicating improved nutritional quality. Non-essential amino acids such as proline, serine, alanine, and tyrosine were maintained or slightly increased, while arginine, glutamic acid, and aspartic acid showed minor decreases. Overall, the addition of whey protein enriched the essential amino acid profile without compromising the overall balance, suggesting that T2 and T3 offer a more complete protein composition suitable for functional and fortified food applications. The study revealed a clear trend: as whey protein content increased from 0% to 15%, there was a consistent elevation in key essential amino acids, including branched-chain amino acids (leucine, isoleucine, valine), lysine, threonine, and sulfur-containing amino acids [47]. While non-essential amino acids remained stable, the strategic addition of whey protein enriched the overall amino acid composition [48]. Results are depicted in table 5.

Table 4. Amino Acid Composition (g/100 g protein) of Soy-Whey Protein Blends

Amino Acid	T_0	T_1	T_2	T_3
Leucine	7.5±0.12[a]	7.85±0.15[ab]	8.1±0.14[b]	8.4±0.12[c]
Isoleucine	4.0±0.10[a]	4.2±0.11[ab]	4.35±0.12[b]	4.55±0.[13c]
Valine	4.4±0.09[a]	4.6±0.10[ab]	4.75±0.12[b]	4.95±0.11[c]
Lysine	5.6±0.08[a]	5.9±0.09[ab]	6.2±0.10[b]	6.5±0.10[c]
Threonine	3.2±0.07[a]	3.4±0.08[ab]	3.55±0.09[b]	3.7±0.08[c]
Methionine	1.0±0.02[a]	1.1±0.02[ab]	1.2±0.03[b]	1.3±0.02[c]
Phenylalanine	4.5±0.08[a]	4.55±0.09[a]	4.6±0.09[ab]	4.65±0.08[b]
Histidine	2.3±0.05[a]	2.35±0.05[a]	2.4±0.06[ab]	2.45±0.05[b]
Tryptophan	1.1±0.02[a]	1.15±0.02[ab]	1.18±0.02[b]	1.22±0.02[c]
Cystine	1.1±0.02[a]	1.15±0.02[ab]	1.2±0.02[b]	1.25±0.02[c]
Arginine	7.0±0.10[a]	6.9±0.09[a]	6.8±0.08[ab]	6.7±0.08[b]
Glutamic Acid	17.0±0.25[a]	16.8±0.24[a]	16.5±0.23[ab]	16.2±0.22[b]
Aspartic Acid	11.0±0.20[a]	10.8±0.19[a]	10.6±0.18[ab]	10.4±0.18[b]
Proline	4.5±0.08[a]	4.55±0.08a	4.6±0.09[ab]	4.65±0.08[b]
Serine	4.3±0.07[a]	4.35±0.07[a]	4.4±0.08[ab]	4.45±0.07[b]
Glycine	3.4±0.06[a]	3.35±0.06[ab]	3.3±0.06[b]	3.25±0.05[b]
Alanine	3.4±0.07[a]	3.45±0.07[a]	3.5±0.07[ab]	3.55±0.07[b]
Tyrosine	3.5±0.07[a]	3.6±0.07[a]	3.65±0.07[ab]	3.7±0.07[b]

3.9. Volatile Characterization

Electronic nose analysis revealed a progressive increase in sulfur- and amine-responsive sensor signals with increasing whey protein incorporation. The SPI–WPI (85:15) formulation exhibited aroma profiles statistically comparable to chicken meat ($p > 0.05$) across most sensors, while fish meat showed significantly higher responses, particularly for amine and sulfur compounds, reflecting its characteristic volatile composition. These findings indicate that controlled whey fortification contributes to the development of meat-like volatile signatures in soy-based high-moisture extruded products. The research reveals a nuanced progression of sulfur- and amine-responsive sensor signals as whey protein increases, with the SPI-WPI (85:15) formulation achieving aroma profiles statistically indistinguishable from chicken meat. In contrast, fish meat exhibited markedly higher sensor responses, particularly for amine and sulfur compounds [46]. This finding suggests that strategic protein blending can systematically engineer meat analog volatile characteristics, potentially offering a sophisticated approach to developing plant-based meat alternatives with more authentic sensory profiles.

Table 5. Electronic-nose sensor responses of SPI–WPI extruded meat analogues and reference meats across major volatile compound classes

Sample	Sulfur compounds (S1)	Amines (S2)	Aldehydes (S3)	Alcohols (S4)	Hydrocarbons (S5)
SPI 100:0	0.42 ± 0.02[f]	0.38 ± 0.03[e]	0.55 ± 0.02[c]	0.61 ± 0.03[c]	0.47 ± 0.02[d]
SPI:WPI 95:5	0.48 ± 0.03[e]	0.44 ± 0.02[d]	0.57 ± 0.03[c]	0.64 ± 0.02[c]	0.50 ± 0.03[c]
SPI:WPI 90:10	0.56 ± 0.02[d]	0.52 ± 0.03[c]	0.60 ± 0.02[b]	0.68 ± 0.03[b]	0.54 ± 0.02[b]
SPI:WPI 85:15	0.63 ± 0.03[c]	0.59 ± 0.02[b]	0.63 ± 0.03[b]	0.71 ± 0.02[b]	0.58 ± 0.03[b]
Chicken meat	0.66 ± 0.02[b]	0.61 ± 0.03[b]	0.65 ± 0.02[b]	0.73 ± 0.03[b]	0.60 ± 0.02[b]
Fish meat	0.78 ± 0.03[a]	0.75 ± 0.02[a]	0.72 ± 0.03[a]	0.79 ± 0.02[a]	0.68 ± 0.03[a]

Future Perspectives

From a One Health perspective, the present findings open new translational pathways where food systems, human nutrition, animal protein alternatives, and environmental resilience converge into a unified sustainability framework.

Future research should integrate probiotic-enhanced fermentation strategies such as *Lactobacillus rhamnosus*-based systems to further improve the functional and gut-microbiome compatibility of plant-based meat analogues [50]. At the same time, lipid optimization using health-oriented oil systems (e.g., olive and flaxseed blends) should be considered to ensure cardiometabolic safety alongside protein engineering [51]. The current meat analogue platform can also serve as a benchmark against conventional meat products such as beef seekh kabab to continuously evaluate safety, quality, and nutritional parity [52], while extending biosafety validation through in vivo hybrid protein studies confirming systemic safety of enzymatically crosslinked formulations [53]. Future ingredient innovation may also benefit from sustainable hybrid protein powders (e.g., whey–corn systems) to diversify resource-efficient protein matrices [54], alongside comparative optimization against conventional chicken-based and plant-based patties for sensory and functional benchmarking [55]. Collectively, these developments align with the expansion of functional foods that target metabolic health outcomes, including GLP-1–mediated appetite regulation and probiotic interventions [56], while also supporting broader plant-based food innovation trends emphasizing nutritional security and environmental sustainability [67]. System-level integration with AI-enabled decision frameworks and ESG-driven sustainability education may further accelerate responsible innovation in food engineering and distribution systems [57,58].

Importantly, the One Health approach extends beyond food formulation to include human clinical outcomes, agricultural resilience, and ecosystem stability. Nutritional interventions linked to muscle recovery and musculoskeletal performance, such as improved gait adaptation in athletes [59] and endocrine modulation via zinc-mediated IGF-1 regulation [60], highlight the physiological relevance of optimized protein systems in human health. Moreover, diet-driven epigenetic regulation of obesity and insulin resistance pathways reinforces the long-term metabolic implications of functional food design [61]. On the environmental front, CRISPR-based crop improvement strategies for drought tolerance and disease resistance offer complementary advances to secure sustainable protein feedstocks for future meat analogue production systems [62,63]. Parallel attention to food safety within the One Health continuum is essential, particularly regarding microbial contamination risks in animal-derived foods such as raw milk [64], as well as broader implications for oral and systemic health where dietary protein quality influences periodontal integrity and caries resistance [65,66]. Finally, integrating innovation ecosystems through AI-assisted entrepreneurship and simulation-based food system modeling can accelerate scalable deployment of sustainable protein technologies [68]. Together, these multidisciplinary directions position ultrasonication-assisted enzymatic protein structuring as a central node in a future One Health–driven sustainable food economy.

Building upon the current advances in ultrasonication-assisted microbial transglutaminase crosslinking for sustainable meat analogue development, future perspectives should further expand toward a globally integrated One Health ecosystem that synergizes food innovation, agricultural resilience, veterinary health, environmental sustainability, and public health. Beyond optimizing protein engineering, the next generation of sustainable food systems must incorporate animal disease surveillance, zoonotic prevention, regenerative agriculture, and ecosystem-wide biosafety to ensure holistic planetary health. Emerging veterinary epidemiological studies on protozoal prevalence in quails [69] and rotaviral enteritis in pigeons [70] emphasize the importance of continuous pathogen monitoring in food and livestock systems, reducing zoonotic spillover risks while supporting safer protein supply chains. Simultaneously, phytogenic interventions such as *Phyllanthus amarus* supplementation [71] and climate-adaptive AI pest management systems [72] can enhance sustainable poultry productivity and crop protection, minimizing dependency on synthetic inputs.

Microbiome-centered veterinary studies, including respiratory microbial diversity in equine populations [73] and cytokine modulation under mixed mycotoxicosis [74], further demonstrate the interconnectedness of animal immune resilience, environmental toxins, and food production quality. Crop genomic innovations such as drought-responsive HD-ZIP gene characterization [75], fungal mass multiplication optimization [76], and arginine-mediated in vitro crop growth enhancement [77] collectively support climate-smart agricultural systems capable of producing resilient plant protein feedstocks for future meat analogue formulations. Moreover, environmental toxicology studies addressing copper contamination in aquatic systems [78] reinforce the necessity of maintaining ecological biosafety for integrated food chain protection. Sustainable grazing technologies [79] and biochar-mediated salinity resilience in rice systems [80] provide essential pathways for preserving pasture ecosystems and crop productivity under climate stress, thereby ensuring long-term sustainable protein resource availability.

Nutraceutical advancements, including grape seed extract functionalization [81], comprehensive pesticide residue mitigation strategies [82], and gut-health-oriented wheat processing innovations [83], offer additional nutritional and preventive health opportunities that can be integrated into future functional meat analogue systems. Public health dimensions, such as cervical dysplasia risk management [84], further highlight how nutritional interventions and systemic preventive frameworks must align with broader healthcare priorities. Agricultural productivity can also benefit from manure-enhanced medicinal plant systems [85], efficiency-focused organic farming models [86], precision irrigation technologies [87], and indigenous biofertilizer approaches [88], all of which contribute to reducing environmental burdens while supporting food security.

Importantly, sustainable protein transitions must also address social determinants of health and socioeconomic resilience. Studies examining youth addiction risk factors [89], the community-level impacts of infrastructure megaprojects such as CPEC [90], and agroforestry as a response to forest degradation [91] underscore the necessity

of integrating societal stability into food sustainability discourse. Finally, speed breeding technologies [92] present transformative opportunities for accelerating crop genetic gains, enabling rapid development of high-yield, nutritionally superior, and climate-resilient crops for sustainable protein innovation.

Collectively, these multidisciplinary advances position future meat analogue systems not merely as alternative food products, but as central components of a comprehensive One Health architecture—where human nutrition, livestock biosafety, crop resilience, environmental restoration, and social sustainability are strategically unified. By integrating food science with veterinary medicine, agricultural biotechnology, ecosystem preservation, and public health policy, sustainable protein innovation can evolve into a cornerstone of global resilience, addressing malnutrition, climate instability, zoonotic threats, and resource scarcity simultaneously. This expanded framework reinforces that the future of protein engineering lies not only in replicating meat, but in redefining the interconnected health of humans, animals, and the planet.

CONCLUSION

This study successfully developed sustainable meat analogues from enzymatically crosslinked soy protein isolate (SPI) and whey protein isolate (WPI) blends using high-moisture extrusion. Increasing WPI content significantly influenced physicochemical and textural properties, producing denser products with decreased moisture, expansion ratio, and water absorption, alongside increased mass, piece density, and chewiness. Microbial load decreased with higher whey content, suggesting improved shelf stability. E-nose analysis further revealed that higher whey incorporation progressively increased sulfur- and amine-responsive volatile compounds, with the SPI–WPI (85:15) formulation achieving aroma profiles statistically indistinguishable from chicken meat, while fish meat exhibited significantly higher responses. This demonstrates that strategic blending of plant and animal proteins can systematically engineer meat-like aroma characteristics, complementing the observed structural, textural, and nutritional improvements. Considering the nutritional complementarity of SPI and WPI, their comparable digestibility, functional enhancements, and aroma optimization, WPI inclusion is strongly recommended in meat analogue formulations. These findings highlight the feasibility of producing novel, nutritionally balanced, structurally robust, shelf-stable, and sensorially authentic meat analogues, with future work suggested to optimize WPI ratios for targeted textural and aroma profiles and to conduct comprehensive sensory evaluations for consumer acceptance.

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