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SAFETY AND MICROBIAL DYNAMICS OF HIGH-MOISTURE EXTRUDED PROTEIN SYSTEMS UNDER CHILLED STORAGE

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Abstract. Growing consumer demand for environmentally sustainable protein alternatives has intensified research into the safety and quality of plant-based meat alternatives. This study evaluates the microbiological stability of extruded products with high moisture content obtained from soy and pea protein isolates, as well as composite formulations with the addition of soryz flour, hazelnut meal, and chickpea flour. Ten samples were produced using two industrial extrusion temperature profiles (40–60–80–100 °C and 60–80–100–120 °C) and stored in a refrigerator (+4 °C) for 24 days. Pea protein izolate blends with grain and nut components processed at a milder temperature profile demonstrated excellent microbiological stability, which can be explained by improved water-binding and microstructural properties. These results highlight the practical importance of formulation and extrusion parameters in the development of microbiologically safe plant-based meat alternatives. The study provides industry-relevant information for the development of stable, high-moisture extruded protein products without the use of chemical preservatives.

Keywords: *contamination, extrusion, formulation, meat analogues, refrigeration, stability.*

Rezumat. Cererea crescândă a consumatorilor pentru alternative ecologice la proteine a intensificat cercetările în domeniul siguranței și calității analogilor vegetali ai cărnii. În acest studiu se evaluează stabilitatea microbiologică a produselor extrudate cu conținut ridicat de umiditate, obținute din izolate proteice de soia și mazăre, precum și din rețete compozite cu adaos de făină de soriz, șrot de alune și făină de năut. Zece eșantioane au fost produse utilizând două profiluri industriale de temperatură de extrudare (40-60-80-100 °C și 60-80-100-120 °C) și au fost păstrate la frigider (+4 °C) timp de 24 de zile. Amestecurile de izolat proteic din mazăre cu componente din cereale și nuci, prelucrate la un profil de temperatură mai blând, au demonstrat o stabilitate microbiologică excelentă, ceea ce poate fi explicat prin proprietățile îmbunătățite de legare a apei și microstructurale. Aceste rezultate subliniază importanța practică a parametrilor de formulare și extrudare în dezvoltarea de analogi vegetali ai cărnii, siguri din punct de vedere microbiologic. Studiul oferă informații relevante pentru industrie în vederea dezvoltării de produse proteice extrudate stabile, cu conținut ridicat de umiditate, fără utilizarea de conservanți chimici.

Cuvinte-cheie: contaminare, extrudare, formulare, analogi de carne, refrigerare, stabilitate.

1. Introduction

In recent decades, the global food industry has undergone rapid changes related to environmental, demographic and social challenges. The growth in the world's population, projected by the United Nations to reach 8.5 billion by 2030 and 10 billion by 2050, is accompanied by the need to find sustainable solutions to ensure food security [1]. At the same time, interest in alternative sources of protein is growing, as animal husbandry is characterised by low efficiency in converting plant protein into animal protein, a significant impact on the environment and high consumption of natural resources [2-6].

Among the promising solutions, particular attention is paid to plant-based alternatives to meat products, which reduce the burden on natural ecosystems, provide sufficient protein in the diet, and meet the growing consumer demand for 'green' and ethically acceptable products [7-10]. Vegan and vegetarian diets, as well as the flexitarian approach, are becoming increasingly common, forming a new market for plant-based alternatives to meat, which is projected to exceed $422 million by 2026 [9].

The most promising components for creating meat analogues are protein isolates and legume meal, as well as nut and cereal processing products [10-12]. These ingredients are characterised by high nutritional value, optimal amino acid composition and the ability to form a texture similar to meat through green technology, like extrusion.

Under controlled high-moisture extrusion conditions, meat analogs remain microbiologically safe and retain high nutritional quality, despite partial protein denaturation and aggregation [13,14]. Precise adjustment of thermal and moisture parameters enables modulation of the Maillard reaction, thereby minimizing undesirable nutritional effects [15]. The incorporation of lipid-rich materials, such as peanut flour, can increase susceptibility to lipid oxidation under elevated temperature and shear [16]. However, endogenous antioxidants present in plant-based ingredients may counteract these effects by inhibiting lipid peroxidation [17].

Extrusion provides several technological and nutritional advantages in the formulation of plant-based meat analogs. It effectively reduces or eliminates antinutritional factors such as trypsin inhibitors, lectins, and phytic acid, which are inherent to legumes and cereals [18,19]. Their inactivation enhances protein digestibility and improves the overall nutritional value of the final product. Furthermore, extrusion-induced protein denaturation increases substrate accessibility to proteolytic enzymes, facilitating amino acid release and absorption [20]. The high processing temperatures also achieve efficient microbial inactivation, thereby improving product safety and extending shelf life [21].

However, despite numerous technological and market studies, issues of safety and microbiological stability of meat analogues during storage remain insufficiently studied.

Microbiological stability is a key factor determining the quality and safety of finished products. Plant-based protein matrices provide a favourable environment for the growth of microorganisms, and storage and transportation pose additional risks of contamination. In this regard, research into the stability of meat analogues under various heat treatment and storage conditions is particularly relevant. The aim of this study was to evaluate the microbiological stability and safety of meat analogues obtained from soy protein isolate (SPI) and pea protein isolate (PPI), as well as the combination of PPI with sorzy flour (S), huzelnut meal (HM), and chickpea flour (C), produced by extrusion with a high moisture content (HME) at two different heating temperature profiles: **40-60-80-100 °C and 60-80-100-120 °C and stored refrigerated at +4 °C for 24 days.**

2. Materials and Methods

2.1. Chemical materials

Buffered peptone water, potato dextrose agar with chloramphenicol, plate count agar (casein-peptone glucose yeast extract agar) was obtained from Altmann Analytik GmbH & Co. KG - Analytics-Shop (Munich, Germany).

2.2. Materials

For the production of high-moisture meat analog samples, **chickpea flour** (Vegrano, Kimbiotek Kimyevi Maddeler San. Tic. A.Ş., Istanbul, Türkiye), **hazelnut meal (**Altaş Yağ Sanayi Ticaret A.Ş., Giresun, Türkiye), **pea protein isolate** (Vegrano, Kimbiotek Kimyevi Maddeler San. Tic. A.Ş., Istanbul, Türkiye), and **soryz flour** (variety *Alimentar 1*, Chisinau, Republic of Moldova) were used.

Based on previous researches of Bulgaru et al., the analyzed raw materials presented valuable chemical compounds that can contribute to obtaining quality meat analogues that are safe for consumption [22,23].

SPI exhibited the highest protein content (80.5%) among all samples, with negligible lipid and carbohydrate fractions, confirming its high purity and suitability as a concentrated protein source. Its amino acid profile was dominated by glutamic acid, aspartic acid, leucine, and lysine, reflecting both high nutritional quality and functional versatility. Potassium and magnesium were identified as the predominant minerals, accompanied by lower levels of calcium and iron, which together indicate a balanced mineral composition beneficial for metabolic and physiological processes. Pea protein isolate (PPI) displayed a similarly high protein concentration (76.0%), with minimal fat and ash contents. Its amino acid profile was characterized by elevated levels of glutamic acid, aspartic acid, leucine, and arginine, but comparatively lower amounts of methionine and cysteine, a feature typical of legume-derived proteins. The mineral composition was dominated by potassium and magnesium, with trace quantities of calcium, iron, and zinc, confirming PPI as a nutritionally valuable and functionally effective plant-based protein source.

Chickpea flour contained a moderate protein content (22.5%). Its amino acid composition included significant quantities of leucine, valine, phenylalanine, and lysine, though limited in methionine, reflecting a balanced yet legume-typical amino acid profile. The mineral fraction was rich in phosphorus, potassium, and calcium, with moderate levels of iron and magnesium, designating chickpea flour as a nutrient-dense raw material that combines protein, minerals, and complex carbohydrates suitable for incorporation into plant-based formulations. Hazelnut meal was characterized by a high protein content (34.98%). Its amino acid composition was dominated by glutamic acid, arginine, and leucine, consistent with the profile of nut-derived proteins. The mineral composition revealed relatively high levels of magnesium and potassium, together with measurable amounts of calcium and iron, contributing to its nutritional and functional value in blended formulations [22]. The chemical composition of *Sorghum oryzoidum* (soryz) indicates its potential as a valuable raw material for plant-based product development. The grains contain 70.8% starch, 10.1% protein, 3.9% fat, 1.9% crude fiber, and 1.6% ash. The protein fraction, although limited in lysine and sulfur-containing amino acids, is rich in glutamic acid, leucine, and proline. Soryz also provides significant levels of potassium and magnesium, with moderate calcium and trace amounts of iron and manganese. This balanced composition highlights soryz as a nutrient-dense cereal suitable for incorporation into functional foods and meat analog formulations [23].

Overall, the chemical and nutritional profiles of these ingredients demonstrate strong complementarity – protein isolates providing structural and functional proteins, while chickpea flour and hazelnut meal contribute carbohydrates, lipids, and minerals—making their combination highly suitable for the formulation of nutritionally balanced, high-protein plant-based foods such as meat analogs.

2.3. Production of High-Moisture Meat Analogs

C, S, and HM were mixed with PPI at a 1:1 ratio on a dry weight basis. Control samples consisted solely of SPI, PPI without any added flour. All formulations were processed at a fixed moisture content of 60% and subjected to two different barrel temperature profiles during extrusion. The first temperature profile was **40–60–80–100 °C**, with the corresponding samples designated as „**I”,** while the second profile was **60–80–100–120 °C**, with samples designated as „**II”**. The **cooling die temperature profiles,** including the adapter, were set according to the final barrel temperature:

- for a final barrel temperature of **100 °C** → **100–60–40–30 °C,**
- for a final barrel temperature of **120 °C** → **120–60–40–30 °C.**

The feed moisture content was adjusted to 60% using the extruder's integrated water pump. After extrusion, all products were **vacuum-packed** using a **Packtech (Turkey)** packaging machine and **stored at −18 °C** until further analysis.

Table 1

Sample codification

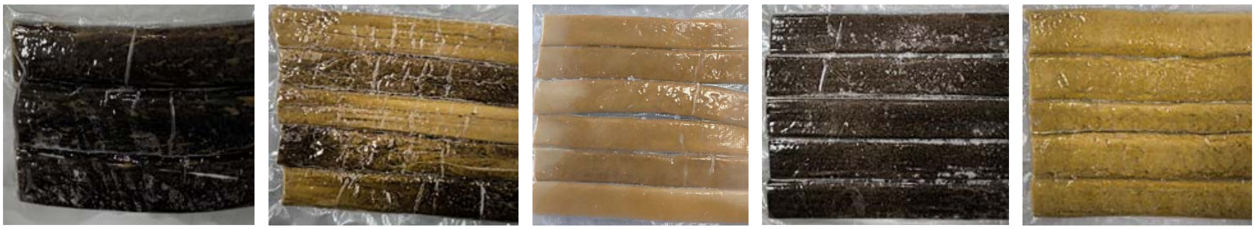
Heating temperature profile, °C	Codification
40-60-80-100	SPI-I PPI-I S-I HM-I C-I
60-80-100-120	SPI-II PPI-II S-II HM-II C-II

Figures 1 and 2 show meat analogues produced by HME at two different heating temperature profiles: 40-60-80-100 °C and 60-80-100-120 °C.

a. b. c. d. e.

Figure 1. Samples of meat analogues obtained at extrusion temperatures of 40-60-80-100 °C

a) SPI-I, b) PPI-I, c) S I, d) HM-I, e) C-I.



a. b. c. d. e.

Figure 2. Samples of meat analogues obtained at extrusion temperatures of 60-80-100-120 °C
a) SPI-II, b) PPI-II, c) S-II, d) HM-II, e) C-II.

Samples of high-moisture meat analogues were stored at -18 °C, then thawed and stored at +4 °C for 24 days. Microbiological parameters were determined on days 1, 6, 12, 18, and 24 of storage.

The analyzed high-moisture meat analogues (HMMA), produced by extrusion of PPI and its blends with soriz flour, chickpea flour, and hazelnut meal, exhibit physicochemical and functional characteristics that collectively confer favorable resistance to microbiological spoilage and stable behavior during storage [11]. The formulations exhibited near-neutral pH values, ranging from 5.96 to 7.95, depending on the blend composition. Although such pH values may not inherently inhibit microbial growth, the structural and biochemical transformations induced by extrusion – including protein denaturation, starch gelatinization, and formation of compact fibrous matrices – contribute to reduced water mobility and limited nutrient accessibility for spoilage microorganisms. The high water-holding and oil-holding capacities (up to 4.09 mL/g and 2.89 mL/g, respectively) indicate stable protein–lipid–water interactions that bind free moisture and decrease water activity, thereby indirectly supporting microbiological stability during storage. The relatively high protein content (up to 37.8 %) and low-fat content (<1.5 % except for HM-containing samples) create a nutrient composition less prone to oxidative rancidity and lipid-mediated microbial growth. The ash and crude fiber fractions, particularly in HM-based analogues, further enhance product robustness by contributing minerals with mild antimicrobial potential (e.g., phenolic-bound iron and zinc) and indigestible matrix components that encapsulate nutrients. The incorporation of HM notably increased antioxidant activity, with ABTS values up to 1.27 mg TE/g DW, surpassing PPI controls by 70.7 %. Elevated antioxidant capacity limits oxidative stress and the formation of reactive substrates that can stimulate microbial metabolism, supporting both biochemical and microbiological stability. Taken together, the parameters obtained indicate favourable conditions for microbiological stability during cold storage and standard cold chain logistics.

2.1. Methods

The microbiological characteristics of meat analogues were assessed according to quantity of mesophilic aerobic and facultative anaerobic microorganisms (QMAFAnM) and the amount of mould and yeast. The microbial analysis of the meat analogues was carried out according to ISO standard methods [24-27].

The stock solution was prepared by aseptically taking 10 g of each sample and placing it in a 100 mL flask containing 90 mL of sterile 0.1% buffered peptone water, then stirring and homogenizing for two minutes [26]. The suspensions were then diluted and serial dilutions of 10^1 to 10^3 were obtained in triplicates and used for the specific medium. Aliquots of 1 mL of each dilution were used for pour plating and spread plating, respectively, into the various media. Spread plating using potato dextrose agar with chloramphenicol (2%) was used to determine yeast and molds; the plates were incubated for 7 days at 25 °C. Spread plating using plate count agar (casein-peptone glucose yeast extract agar) was used to determine total number of live, aerobic bacteria; the plates were incubated at 37 °C for 48 h [27].

After incubation, the results were analyzed and different types of microorganisms were identified. Colonies grown after preliminary microscopy were isolated in cultures and subjected to diagnostic testing for cultural and morphological characteristics, according to which yeast identification was performed. The bacterial culture was further identified by Gram staining. Nivelurile acceptabile de microorganisme s-au bazat pe norme și standarde sanitare și epidemiologice Uniform Sanitary and Epidemiological and Hygienic Requirements for Products Subject to Sanitary and Epidemiological Supervision (Control). Approved by Decision of the Customs Union Commission No. 299. 28 May 2010. Acceptable levels of microorganisms were based on sanitary and epidemiological rules and standards [28].

The **water activity (a_w)** of the samples was determined using a **LabSwift-aw analyzer** (Novasina AG, Lachen, Switzerland).

4. Results

As part of the study, 10 samples of plant-based meat analogues were obtained by HME using two temperature profiles (I: 40-60-80-100 °C; II: 60-80-100-120 °C) from SPI, PPI, and compositions of PPI with S, HM and C. Microbiological safety and stability in refrigerated storage (+4 °C, 24 days) were assessed for colony-forming unit (CFU), yeast and mould, the presence of coliforms and pathogens, as well as water activity (aw). The final threshold levels and results of the initial screening are presented in Table 2.

Table 2

Microbiological indicators for meat analogues

Microbiological indicators	Admitted level	Meat analogues/extrusion process									
		I – 40-60-80-100 °C					II – 60-80-100-120 °C				
		SPI-I	PPI-I	S-I	HM-I	C-I	SPI-II	PPI-II	S-II	HM-II	C-II
QMAFAnM, CFU, max.	1×10^4	1×10^1	1×10^2	1×10^1	1×10^1	1×10^1	1×10^2	2×10^2	4×10^2	3×10^2	6×10^2
Yeasts, CFU, max.	50	Were not found									
Molds, CFU, max.											
Coliform bacteria	Not allowed in 0.1 g	Were not found									
Pathogenic microorganisms, including *Salmonella*	Not allowed in 25 g	Were not found									

Note: QMAFAnM – quantity of mesophilic aerobic and facultative anaerobic microorganisms; CFU – colony-forming unit; SPI-I, SPI-II – meat analogues using 100% soy protein isolate; PPI-I, PPI-II – meat analogues using 100% pea protein isolate; S-I, S-II – meat analogues using soryz flour and pea protein isolate in a 1:1 ratio; HM-I and HM-II – meat analogues using huzelnut meal and pea protein isolate in a 1:1 ratio; C-I, C-II – meat analogues made from chickpea flour and pea protein isolate in a 1:1 ratio.

In all samples, regardless of the protein matrix and extrusion temperature profile, coliform bacteria and pathogenic microorganisms, including *Salmonella*, were not detected, confirming the sufficient lethality of heat treatment and the absence of post-process faecal contamination. The maximum QMAFAnM values were 10–600 CFU/g (1×10^1–6×10^2), significantly below the permissible limit of 1×10^4 CFU/g, with slightly higher values in extrusion samples with profile II (PPI-II, S-II, HM-II, C-II in the range of 2×10^2–6×10^2 CFU/g) compared to the corresponding profile I variants (typically $1×10^1–1×10^2$ CFU/g). This may reflect differences in the residual microflora associated with the formulation (available carbohydrates from cereal/nut components) and microstructure after a higher temperature gradient, affecting surface adhesion and moisture retention in micropores [4,29]. Yeasts and moulds were not detected in the screening at the sensitivity level of the method, which is consistent with the subsequent observation of stable low aw values. A review of the structure and barrier properties of HME confirms the relationship between fibre structure/porosity and moisture retention and substrate availability [4,29].

When assessing the microbial stability of chilled meat analogue samples, analyses were performed immediately after thawing, after 6, 12, 18 and 24 days of storage in a refrigerator at 4 ˚C. The developed colonies were counted, and the results were expressed as log_{10}CFU/g. (Table 3).

Table 3.

Change in the number of microorganisms and water activity in chilled meat analogues during 24 days of storage

Microbiological indicators/ Storage time	**Meat analogues/extrusion regime**									
	I – 40-60-80-100 °C					**II – 60-80-100-120 °C**				
	SPI-I	**PPI-I**	**S-I**	**HM-I**	**C-I**	**SPI-II**	**PPI-II**	**S-II**	**HM-II**	**C-II**
QMAFAnM, log_{10}CFU/g										
- 1st day	1.00	2.00	2.00	1.00	1.00	2.00	2.30	2.60	2.48	2.78
- 6th day	2.30	2.70	2.30	2.18	2.00	2.48	2.00	2.70	2.70	2.90
- 12th day	2.00	2.60	2.48	2.30	2.18	2.70	2.18	2.70	2.70	2.90
- 18th day	2.48	3.00	2.70	2.41	2.30	2.70	2.30	2.85	2.48	3.00
- 24th day	2.70	3.08	2.70	2.48	2.30	3.00	2.30	3.00	2.41	3.00
Yeasts and molds, log_{10}CFU/g										
- 1st day	-	-	-	-	-	-		-	-	-
- 6th day	2.30	<1	<1	<1	<1	<1	<1	1.48	<1	1.30
- 12th day	2.00	1.30	<1	<1	<1	1.30	<1	1.60	<1	1.48
- 18th day	2.48	1.30	<1	<1	<1	<1	1.30	1.48	<1	1.60
- 24th day	2.70	<1	<1	1.48	<1	<1	1.30	1.48	<1	1.60
a_w, c.u.										
- 1st day	0.781	0.780	0.789	0.780	0.783	0.778	0.784	0.781	0.788	0.781
- 6th day	0.781	0.780	0.789	0.782	0.783	0.778	0.784	0.781	0.788	0.781
- 12th day	0.781	0.780	0.788	0.785	0.783	0.778	0.784	0.781	0.788	0.781
- 18th day	0.781	0.780	0.785	0.784	0.783	0.778	0.784	0.781	0.788	0.781
- 24th day	0.781	0.780	0.785	0.785	0.783	0.778	0.784	0.781	0.788	0.781

Note. QMAFAnM – quantity of mesophilic aerobic and facultative anaerobic microorganisms; CFU – colony-forming unit; SPI-I, SPI-II – meat analogues using 100% soy protein isolate; PPI-I, PPI-II – meat analogues using 100% pea protein isolate; S-I, S-II – meat analogues using soryz flour and pea protein isolate in a 1:1 ratio; HM-I and HM-II – meat analogues using huzelnut meal and pea protein isolate in a 1:1 ratio; C-I, C-II – meat analogues made from chickpea flour and pea protein isolate in a 1:1 ratio; a_w - water activity.

A_w is one of the key factors determining microflora growth, as it regulates the availability of free water for microbial processes. The data in Table 3 show that in all plant-based meat analogues, aw remained within a narrow and low range of 0.778–0.789 during

24 days of storage at +4 °C, with no statistically significant trend. Since the active growth of most bacteria, yeasts, and moulds is usually observed at aw values above 0.88–0.90, values below this threshold indicate limited water availability and, as a result, inhibit the development of microflora [30,31]. Against this background, the dynamics of QMAFAnM were moderate: the starting levels were 1.00–2.78 $\log_{10}$CFU/g, and by day 24 they were within the range of 2.30–3.08 $\log_{10}$CFU/g, which is significantly below the sanitary limit. The most pronounced growth was observed in PPI (PPI-I up to 3.08 $\log_{10}$CFU/g; PPI-II up to 3.00 $\log_{10}$CFU/g) and in some compositions obtained according to profile II (S-II, C-II, HM-II up to 3.00 $\log_{10}$CFU/g), while the corresponding variants of profile I remained lower (S-I up to 2.70 $\log_{10}$CFU/g; HM-I up to 2.48 $\log_{10}$CFU/g; C-I up to 2.30 $\log_{10}$CFU/g). This reflects the recipe-dependent microstructure and sorption properties: mixtures with sorghum, chickpeas and peanut meal obtained according to profile I form a higher water-binding capacity and lower substrate availability for psychrotrophic microflora. Yeast was detected sporadically at low levels (mainly <1.0–1.60 $\log_{10}$CFU/g; occasionally up to 2.70 $\log_{10}$CFU/g in SPI-I), without a consistent trend towards accumulation, which is consistent with a stable low aw [31].

Complementing this, specific samples demonstrate characteristic aw trajectories relevant to microbiological indicators. Variants with minimal changes in water activity – PPI-I and PPI-II, as well as S-I and C-I – maintain stable aw values (around 0.780–0.785), which confirms unfavourable conditions for the development of microflora and correlates with the restrained growth of QMAFAnM. In contrast, samples S-II and C-II show a tendency towards a slight increase in aw at certain times (especially after the 12th day), which potentially increases the risk of microbial activity; this is consistent with slightly higher final QMAFAnM values (up to 3.00 $\log_{10}$CFU/g). Sample HM-II shows a rather stable or slightly decreasing aw, which makes conditions less favourable for yeast and bacterial growth and is reflected in moderate QMAFAnM levels at the end of storage. Thus, formulations with minimal aw fluctuations (e.g., PPI-II and PPI-I, as well as S-I and C-I) are potentially better suited for long-term refrigerated storage, as changes in conditions conducive to microflora growth are minimal. Variants with a tendency to increase aw (S-II and C-II) require stricter control (cooling/packaging hygiene, airtightness, possible use of MAP), as even a small increase in aw can increase the risk of yeast and some bacteria growth during long-term storage [30,31]. A comparison of extrusion temperature profiles confirms that profile II in a number of recipes (SPI-II, S-II, C-II) leads to final QMAFAnM levels 0.2–0.3 $\log_{10}$CFU/g higher, probably due to differences in the formation of the fibrous structure and porosity, which affect surface moisture availability and microbial adhesion niches; at the same time, the dominant aw barrier keeps the indicators within the normal range [29]. These observations are consistent with published data on the effect of maintaining aw <0.90 on slowing the growth of total microflora and yeast in chilled plant products, as well as with mechanistic ideas about the role of structuring in high-moisture extrusion of plant proteins [30].

4. Conclusions

The study demonstrates that high-moisture plant-based meat analogues based on soy and pea isolates and their compositions with soryz, huzelnut meal and chickpeas, obtained by extrusion using two temperature profiles and stored at +4 °C for 24 days, retain microbiological safety: no pathogens or coliforms were detected, and QMAFAnM and yeast-mould microflora remained significantly below the standards. The key barrier is the consistently low water activity (aw≈0.78–0.79), which limits the growth of microflora and smooths out the effects of moderate differences between extrusion profiles. The most favourable options in terms of stability are S-I, HM-I and C-I, are characterised by minimal growth of QMAFAnM and rare detection of yeast, which indicates the advantages of combinations of pea protein with cereal and nut components at a ‘softer’ temperature gradient I in the context of moisture binding and microstructure. The results obtained are consistent with current data on the influence of HME structural factors and aw thresholds on the storage of chilled plant-based analogues, emphasising the practical importance of managing water binding and cooling/packaging hygiene. The novelty of the work lies in the comparison of two industrial HME temperature profiles on a number of real recipes with a quantitative assessment of the dynamics of QMAFAnM /yeast and aw in 24-day chilled storage, which provides applied guidelines for the design of recipes and regimes that ensure regulatory safety without additional barriers

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