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# Serum-Free Media Development Strategies for Cultivated Meat: A Machine Learning-Assisted Systematic Scoping Review

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**ABSTRACT**

Growing global population and environmental concerns associated with conventional meat production demand sustainable alternatives. Cultivated meat holds significant promise, yet its scalability remains constrained by reliance on fetal bovine serum (FBS) that raises critical ethical, economic, and environmental concerns. This systematic scoping review conducted following PRISMA-ScR guidelines across Scopus, Web of Science, and PubMed (2000–September 2025), synthesizes sustainable strategies for serum-free media development in cellular agriculture. Two principal approaches were identified: circular cell culture (CCC) systems and agrifood side stream valorisation. CCC systems integrate microalgae or cyanobacteria with mammalian myoblasts, enabling medium recycling. Growth rates were comparable to FBS-supplemented controls, with ammonia reduced by 98% and lactate by 68%. However, gradual nutrient depletion and system complexity limit current applicability. Side stream valorisation showed that protein isolates from agricultural by-products can support myoblast proliferation. Rapeseed protein isolate outperformed recombinant albumin by 15%, and enzymatic extraction increased protein yields up to 930% over conventional methods. Remaining challenges include suboptimal proliferation relative to 10% FBS, premature differentiation, extended nutrient depletion, and a critical lack of life cycle assessments. Integrating standardized extraction protocols with optimized culture conditions will be essential to unlock commercial-scale cultivated meat production.

**KEYWORDS**

Circular economy; cultivated meat; machine learning; media development; serum-free; side streams

## Introduction

Over the past few years, the rate of global population expansion has markedly risen. As of 2024, the Earth harbored 8.2 billion inhabitants, and demographic forecasts predict sustained population growth through the next 50–60 years, with global numbers rising to peak at roughly 10.3 billion by the mid-2080s.[1] This demographic challenge is exacerbated by the intensifying environmental crisis, with the food system contributing to up to one-third of global greenhouse gas (GHGs) emissions, ranking as the second-largest producer.[2] Notably, 31% of GHG emissions within the food sector originate from livestock and fisheries.[3] Conventional intensive livestock practices further compound environmental and health issues, including the depletion of vital resources (e.g., land and water), spillovers, and biodiversity loss. Consequently, the challenge extends beyond the mere production and delivery of protein to the growing population; it necessitates a sustainable approach.

While the source of protein significantly influences the sustainability of the current food system, the food supply chain also appears to be flawed, with approximately one-third of food being lost or wasted

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throughout the supply chain from beginning to end.[4,5] GHGs related to food loss and waste primarily originate from two sources: the emissions generated during production (which include storage, processing, distribution, and consumption) of food that ends up being lost or wasted, and the emissions resulting from the management of food loss and waste (specifically, waste management).[6] Moreover, the United States Environmental Protection Agency estimated that 70% of the food waste produced in households is considered edible, while this value goes up to 90% if we consider the waste from the supply chain.[7] Therefore, reducing food loss and optimizing the use of agricultural byproducts are vital for improving food security, decreasing environmental impact, and enhancing economic returns.[8] To address these challenges, the circular economy model built on reduction, reuse, and recycling principles has emerged as a widely advocated solution for managing food waste and agricultural residues.[9,10] This approach is considered more sustainable than the current linear production system, as it emphasizes resource efficiency and transforms waste materials into valuable products, thus supporting sustainable development objectives.[10,11]

In navigating this complex landscape, identifying emergent biotechnologies offers promising strategies for minimizing the environmental impact of current food production. While emergent biotechnologies are currently being investigated to mitigate the environmental impact of existing food systems, their commercial viability depends on the utilization of cost-effective ingredients, as conventional ingredients remain economically prohibitive for large-scale production. Among these innovative approaches, cellular agriculture aims to produce foods (e.g., meat, fish, dairy) via cell culture, adapting established biomedical tissue engineering methods to food production. Tissue engineering for biomedical applications focuses on supporting in vivo regenerative processes by harnessing endogenous cells and providing scaffolding structures along with biological factors and cues to restore lost tissue mass and function.[12] Differently, cellular agriculture aims to develop tissues *in vitro* through biomimetic strategies for the production of edible products that recapitulate the structural, nutritional, and sensory characteristics of conventional meat and fish tissues. While both fields involve tissue development, several key factors distinguish tissue engineering approaches used in regenerative medicine from those applied in cellular agriculture, particularly in areas such as cell culture techniques, medium optimization, and production cost considerations. These distinctions arise from the divergent end goals: tissue repair versus food production. Consequently, the final products differ in their required attributes, i.e., functional recovery in the former, versus sensory qualities, nutritional value, and affordability in the latter. Additional differences include the scale of production, the mode of interaction with the body (implantation and long-term integration versus digestion), and the availability of alternative solutions. Although traditional tissue engineering principles can support cellular agriculture, novel and tailored strategies are likely necessary to meet the specific demands of cultured meat production.[13] In detail, the tissue engineering approach applied in cellular agriculture involves four major steps for manufacturing[14]:

- Biopsy or appropriate cell selection
- Cell isolation
- Cell proliferation
- Cell differentiation on edible scaffolds

Each step encounters several technological (e.g., cell proliferation, cell banking, media development), ethical (e.g., use of fetal bovine serum (FBS)) and financial challenges (e.g., reduction of growth factors and recombinant protein costs) to be optimized for industrial production, underlining that cellular agriculture is still in its nascent phase. Indeed, while the "cell-based" method in food production is groundbreaking, many of the bioprocesses involved have been thoroughly explored in the context of tissue engineering.

Specifically, five main areas have been identified for bioprocess optimization, necessary to scale up cultivated meat production: (1) cell selection and optimization, (2) media composition, (3) expansion strategies, (4) differentiation protocols, and (5) scaffold design and manufacturing.[14] Additionally, it

is necessary to evaluate technical/economic feasibility and environmental impact via Techno-Economic Analysis (TEA) and Life Cycle Assessment (LCA). Sinke et al.[15] demonstrated that cultivated meat exhibits threefold greater crop-to-meat conversion efficiency than conventional chicken, reducing land requirements and emissions through contained production systems. Despite energy-intensive requirements for bioreactor maintenance and medium production, renewable energy integration achieves carbon footprints lower than beef/pork and comparable to chicken. Strategic optimization could establish cultivated meat as a sustainable alternative to conventional production. Given the food system's capacity to generate high-value by-products and cellular agriculture's requirement for cost-effective ingredients, the integration of these complementary aspects represents a potential climate change mitigation strategy, particularly when embedded within circular supply chain models.

The integration between cellular and traditional agriculture takes place on larger scales and throughout value chains. Byproducts from conventional agri-food systems can be utilized as inputs for operations within or near cellular production facilities.[16] Establishing more circular and closely linked resource loops for feedstock and other inputs reduces embodied carbon emissions throughout the life cycle of cellular agriculture products.[17,18] Discovering opportunities for resource circularity necessitates research into materials that have a lower impact and are locally sourced. For instance, circular agriculture production systems might be encouraged as alternatives to FBS are developed, using alternative growth media that are ideally cost-effective, animal-free, and sourced regionally.[19]

This systematic scoping review addresses the knowledge gap between developing sustainable cellular agriculture supply chains and circular food systems, specifically focusing on serum-free media development challenges. Indeed, current cultivated meat and fish production relies on FBS as a supplement, creating significant scale-up barriers. The scientific community aims to identify alternative supplements that could reduce production costs associated with expensive ingredients like recombinant proteins while ensuring safety and affordability. This work presents a comprehensive overview of serum-free media development.

## Cultivated meat: the FBS challenge

Cultivated meat is an edible product derived from cell engineering technologies, where cells are extracted from animal tissues and multiplied using culture media in laboratory settings, eliminating the need for traditional livestock farming.[20] Like many cell culture protocols, cultivated cells are maintained through the use of basal media (e.g., Dulbecco's Modified Eagle Medium (DMEM)) supplemented with FBS.

FBS is the most widely used growth supplement in cell culture technology. It is obtained from the fetuses of slaughtered pregnant cows, and more than two million bovine fetuses are used worldwide to produce approximately 800,000 L of FBS.[21,22] FBS is prepared after removing clotting factors (cruor), such as clotted fibrin, along with blood corpuscles from clotted blood. Although it is difficult to accurately state the ingredients and contents, many studies have been conducted on growth factors, hormones, proteins, cofactors, and minerals that can affect cell culture.[22,23]

For adherent cells, like bovine muscle-derived satellite cells used in cultured meat production, ensuring cells adhere to culture dishes is crucial for initial cell growth. Factors in FBS that influence cell adhesion include fibronectin, laminin, and albumin. While albumin is generally considered a non-adhesive protein, it can modulate the activation of adhesion and spreading factors, thereby affecting the extent of cell adhesion.[24] Specifically, albumin is the most concentrated protein in FBS.[25] Albumin, along with insulin and transferrin, plays a crucial role in promoting cell growth, survival, and differentiation in cultured meat media. It achieves this by stabilizing growth factors, aiding nutrient absorption, regulating metabolic processes, and ensuring the maintenance of the essential iron balance necessary for tissue development.[26] Therefore, in the context of serum-free media development, albumin needs to be replaced. Currently, sources of non-animally derived albumin are available on the market via recombinant DNA methodologies. Despite that, the production process

makes the recombinant albumin not well-suited for food production, due to financial reasons. Therefore, to ensure scaling up and cost reduction, effective and cheap alternatives to FBS and recombinant albumin need to be found.

On the side of albumin, the FBS contains also growth factors, proteins that interact with receptors on the cell surface, triggering multiple downstream pathways that lead to cell movement, growth, and differentiation. In media development for cultivated meat, key growth factors generally include Fibroblast Growth Factor 2 (FGF2), Epidermal Growth Factor (EGF), Insulin-like Growth Factor 1 (IGF1), Neuregulin 1 (NRG1), Transforming Growth Factor Beta (TGFβ1), and Platelet-Derived Growth Factor Subunit B (PDGFB).[27] Although a wide array of GFs is used to support eukaryotic cell culture, many of which are tissue/cell-type specific, there are a subset that are considered essential for supporting cell proliferation in general.[28] Recombinant production of these GFs is challenging and expensive. They are of eukaryotic origin and typically require some level of post-translational modification, such as oxidation or glycosylation, and proper disulfide bond formation is essential for protein folding and maturation into their biologically active conformation. Consequently, the production of these proteins in bacterial expression systems has been challenging.

The FBS also contains hormones, fatty acids and lipids, carbohydrates, vitamins, minerals, inorganic compounds, nitrogen sources, enzymes, and transport proteins. The FBS is considered a supplement and added to a basal medium containing the necessary nutrients for the cells to grow. Despite being essential for cell culture, FBS presents multifaceted challenges. Technical limitations stem from its variable composition due to individual genetic factors, making it difficult to standardize. Economically, media constitutes over 99% of current production costs, significantly hampering commercial viability.[29–31] Ethically, FBS harvesting methods face substantial criticism for compromising animal welfare standards, with animal rights organizations and ethicists advocating for its prohibition in cell culture applications, driving industry efforts toward developing animal-free alternatives.[32]

## Methods

### *Protocol, main question, and definition*

This systematic review aimed to answer the question: "What is the state-of-the-art of serum-free media development for cultivated meat and fish production using plant-based and circular approaches?" The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guideline for scoping review was used as a reporting guideline for the methodology. Despite that, the review was not registered into the PROSPERO registry. The inclusion and exclusion criteria were defined focusing on the formulation/ingredients of the cell culture medium described.

### *Eligibility criteria*

The literature included in this study is peer-reviewed journal articles published between the years 2000 and September 2025.

To be considered for inclusion in this review, articles needed to satisfy the following criteria:

- Must be a scholarly (peer-reviewed) publication.
- It should present the formulation/list of ingredients for the medium.
- Focusing on cultivated meat/fish production.
- The study uses at least one animal-derived cell type employed to evaluate serum-free or serum-reduced culture conditions relevant to cellular agriculture, including but not limited to myoblasts, satellite cells, adipocyte-derived stem cells, fibroblasts, or established cell lines used as proliferation models.
- The article tests the substitution of at least one biomolecule present in FBS
- The article is written in English.

Studies with the following characteristics were excluded:

- Review or non-original research articles.
- The article is not focused on serum-free medium testing for cultivated meat/fish production.
- The article does not include the list of ingredients/formulation of the cell culture medium.
- Non-peer review sources.
- The article focuses on vegetarianism, veganism, plant-based meat alternatives, organic meat, and high animal welfare meat.
- The article is not written in English.

### *Information sources and search strategy*

The papers included in this study were retrieved (day of search: 13th October 2025) through keyword research of the following databases: Scopus, Web of Science, and PubMed. The keywords used are cultivated meat, cultured meat, cell cultured meat, lab-grown meat, lab-grown meat, cell-based meat, cell-based meat, cellular agriculture, synthetic meat, artificial meat, clean meat, cell-grown meat, cellular meat, stem cell meat, in-vitro meat, serum-free, plant-based, vegan, vegetal, medium, media. The list of different queries used for keyword research is summarized in Table 1.

### *Record screening*

The total number of papers extracted as described above was screened by two independent researchers using the following parameter:

- Records were identified.
- Duplicate records were removed.
- Identification and elimination of review, book, chapter books, notes, commentary.
- Identification and elimination of paper related to consumer behavior and diet.
- Identification and elimination of records focused on plant-based products.
- Identification and elimination of records focused on social impacts.
- Identification and elimination of records not in English.
- Identification and elimination of human cell-related records.
- Identification and elimination of non-open access records.

The exclusion of non-open access records may represent a source of selection bias. This decision was operationally driven by the requirement for full-text access to verify medium formulation composition. This limitation may have resulted in the exclusion of relevant studies and should be considered when interpreting the scope of this review.

**Table 1.** Queries used for keywords research.

| Database | Query |
|---|---|
| Scopus | ("cultivated meat" OR "cultured meat" OR "cell cultured meat" OR "lab-grown meat" OR "lab grown meat" OR "cell-based meat" OR "cell based meat" OR "cellular agriculture" OR "synthetic meat" OR "artificial meat" OR "clean meat" OR "cell-grown meat" OR "cellular meat" OR "stem cell meat" OR "in-vitro meat") AND ("serum-free" OR "plant-based" OR "vegan" OR "vegetal") AND ("medium" OR "media") |
| Web of Science | ((TS=("cultivated meat" OR "cultured meat" OR "cell cultured meat" OR "lab-grown meat" OR "lab grown meat" OR "cell-based meat" OR "cell based meat" OR "cellular agriculture" OR "synthetic meat" OR "artificial meat" OR "clean meat" OR "cell-grown meat" OR "cellular meat" OR "stem cell meat" OR "in-vitro meat")) AND TS=("serum-free" OR "plant-based" OR "vegan" OR "vegetal")) AND TS=("medium" OR "media") |
| PubMed | ((("cultivated meat" OR "cultured meat" OR "cell cultured meat" OR "lab-grown meat" OR "lab grown meat" OR "cell-based meat" OR "cell based meat" OR "cellular agriculture" OR "synthetic meat" OR "artificial meat" OR "clean meat" OR "cell-grown meat" OR "cellular meat" OR "stem cell meat" OR "in-vitro meat") AND ("serum-free" OR "plant-based" OR "vegan" OR "vegetal")) AND ("medium" OR "media") |

The record screening was performed as shown in Fig. 1.

The record screening was performed via automation software. Specifically, a set of keywords per each step was identified and used to perform a title and abstract screening using RStudio (R version 4.4.2). The list of keywords used is reported in Table S2 , Supplementary Material. The 332 records remaining after automated screening were subjected to k-means (k = 2, assessed using Silhouette and Elbow plots) and hierarchical clustering to stratify papers by technical content. Cluster 1, characterized by technical terminology related to cell biology and media formulation, was retained for full-text eligibility assessment. Cluster 2, predominantly containing papers on socio-economic, consumer, and policy aspects, was excluded as outside the scope of this review. Finally, the records assessed for eligibility were screened to ensure strict adherence to the inclusion criteria and to obtain the final dataset. Specifically, the final screening step consisted of a full-text review of the 113 records assessed for eligibility, performed by two researchers (B.A., S.F.G). Records were evaluated against all inclusion and exclusion criteria, with particular attention to the requirement for medium formulation disclosure. Discrepancies or borderline cases were discussed until consensus was reached.

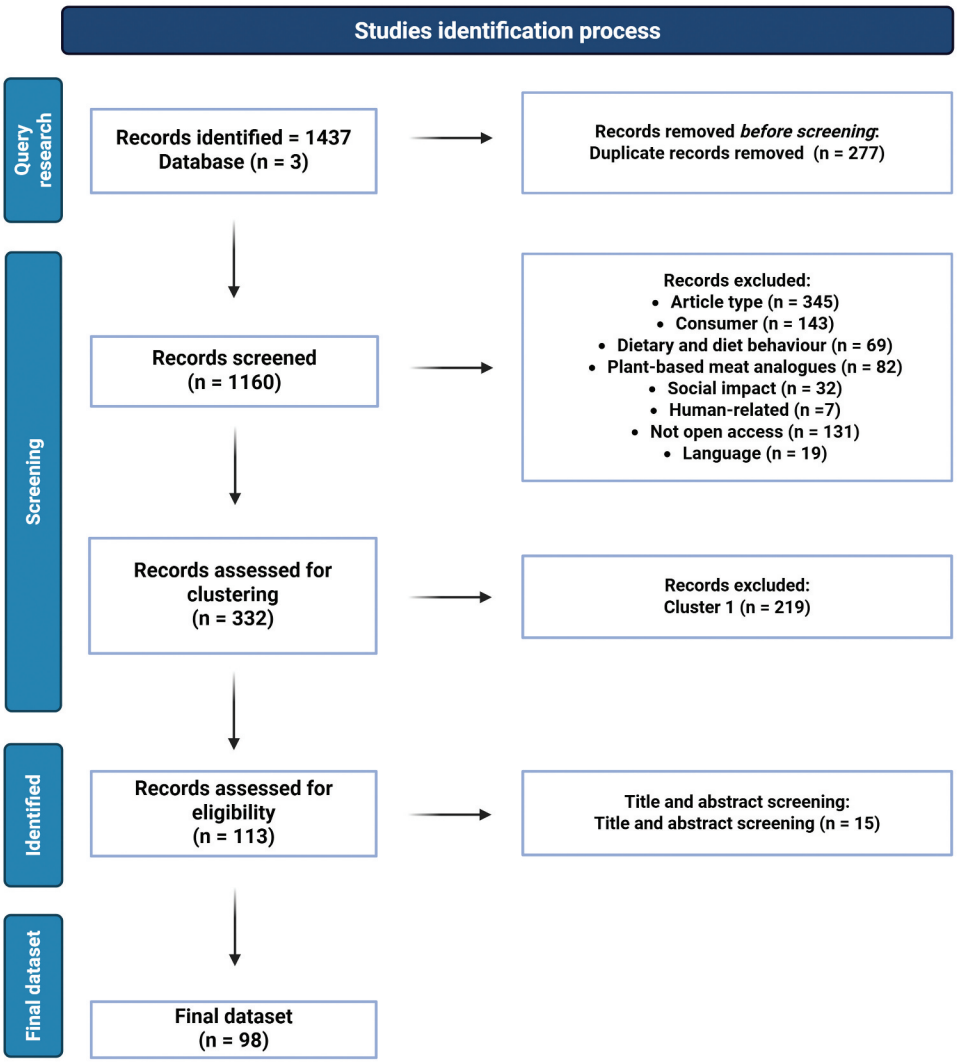



**Figure 1.** Records screening diagram.

### Data extraction

To categorize the retrieved literature ($n = 98$), we developed a hybrid machine learning pipeline combining unsupervised topic modeling with rule-based classification refinement. The approach leveraged BERTopic (https://maartengr.github.io/BERTopic/),[33] a neural topic modeling framework that employs transformer-based embeddings to identify semantic clusters within textual data. Each paper's title and abstract were concatenated and encoded using SciBERT,[34] a variant specifically pre-trained on scientific literature (allenai/scibert_scivocab_uncased). SciBERT embeddings capture domain-specific semantic relationships more effectively than general-purpose language models for scientific text analysis. The resulting 768-dimensional embeddings were reduced to a lower-dimensional space using Uniform Manifold Approximation and Projection (UMAP),[35] a non-linear dimensionality reduction technique. UMAP was configured with default parameters (n_neighbors = 15, n_components = 5, min_dist = 0.0) to balance preservation of fine-grained semantic relationships with computational efficiency.

The dimensionality-reduced embeddings were then clustered using Hierarchical Density-Based Spatial Clustering of Applications with Noise (HDBSCAN),[36] an algorithm that identifies clusters of varying densities and shapes without requiring pre-specification of cluster numbers. HDBSCAN parameters were set to min_cluster_size = 2 to ensure comprehensive coverage of niche research areas, while allowing the algorithm to determine the optimal number of topics based on data density patterns. This approach resulted in 10 distinct semantic topics. A custom CountVectorizer was implemented with stopword removal (including domain-generic terms such as "study," "method," and "result") and bigram extraction (n-gram range 1–2) to enhance topic interpretability and capture multi-word technical expressions (e.g., "serum-free," "recombinant protein"). Representative terms for each topic were generated using class-based TF-IDF (c-TF-IDF), which identifies words that are important for a specific topic by comparing their frequency within that topic to their frequency across all other topics. Next, BERT-derived topics were mapped to seven predefined research categories relevant to cultivated meat serum-free media development:

(1) Plant-based and Circular Approaches
(2) Microbial and Recombinant Proteins
(3) Animal-based Ingredients
(4) Bioprocess Optimization
(5) Cell Line Development
(6) Computational Modelling
(7) Life Cycle Assessment.
(8) Other

Each category was associated with a comprehensive lexicon of domain-specific keywords derived from expert knowledge and iterative refinement. Topic-to-category assignment was performed using a scoring algorithm that calculated keyword overlaps between each BERT topic's representative terms and the predefined category lexicons, with topics assigned to the category achieving the highest matching score. The full keywords lexicons used are reported in Table S3, Supplementary Material checklist 1. Moreover, we implemented a hierarchical rule-based refinement system with three priority levels. Super-priority rules (Level 1) addressed unambiguous classification cases, (e.g., terms as "egg extract," "milk whey," "plant hydrolysate" being categorized according to ingredient origin). Enhanced rules (Level 2) were derived from systematic error analysis of preliminary classification results and targeted recurrent misclassification patterns, (e.g., distinguishing papers on recombinant protein production from cell culture studies). Standard keyword rules (Level 3) provided catch-all classification for papers not captured by higher-priority rules. Classification accuracy was improved through iterative manual validation. After each round, misclassified papers were flagged and corrected, while correctly classified papers were whitelisted to retain their category in subsequent iterations. Papers

manually verified as correctly classified were whitelisted to retain their assigned category across subsequent iterations, ensuring internal classification consistency. While being internally evaluated, an external validation was not performed. Finally, the category "Plant-based and circular approaches" was selected as focus of this review and an article screening was done, to identify the final dataset constituting the state-of-the-art in "Plant and Circular approaches" in serum-free media development for cultivated meat production.

The following data were extracted and reported in Table 2:

- Title: full reference of the study
- Substrate tested: the plant-based or circular material used as serum substitute or medium component (e.g., microalgae species, protein isolate)
- Class: characterization of the study approach, distinguished as "Extraction followed by cell testing", "Cell testing only", "Circular system"
- Cell line: the animal cell line used to evaluate the medium formulation (e.g., C2C12 mouse myoblast, primary bovine satellite cells, porcine satellite cells)
- Extraction method: the technique used to obtain the bioactive fraction from the substrate (e.g., alkaline extraction, enzymatic hydrolysis, ultrasonication)
- Extraction conditions: key process parameters (e.g., pH, temperature, solid-to-liquid ratio)
- Extract characterization: analytical methods used to characterize the extract prior to cell testing e.g., BCA protein quantification, SDS-PAGE, LC-MS)
- Reference: citation of the original study.

Data extraction was performed by one researcher (B.A.) and verified by a second researcher (S.F.G). Studies reporting circular co-culture systems without an extraction step are indicated with "//" in the extraction-related columns.

Figures 1, 8 and 9 were done using BioRender (https://www.biorender.com/). Figures 2–7 were done using python, version 3.14 (https://www.python.org/downloads/release/python-3140/).

## Results

### *K-MEANS and hierarchical clustering*

K-means and hierarchical clustering successfully separated the 332 candidate papers into two distinct thematic clusters with markedly different content profiles. Cluster 1 ($n = 113$, 34.1%) was characterized by technical terminology related to cell biology and bioprocess engineering. The most frequent title keywords were "serum" (39 occurrences), "cell/cells" (71 combined), "bovine" (30), "culture/cultured" (57 combined), "free" (23), "media" (19), "satellite" (15), and "proliferation" (15). Representative papers focused on serum-free media development, cell line optimization, scaffold design, and bioreactor scale-up core technical challenges in cultivated meat production. This cluster showed concentrated temporal distribution, with 90.3% of papers published in 2022–2025, reflecting the recent intensification of technical research.

Cluster 2 ($n = 219$, 65.9%) included papers with broader socio-technical vocabulary. Top keywords included "food" (41), "agriculture" (12), "sustainable" (10), "alternative" (9), "public" (9), "future" (12), "challenges" (10), and "analysis" (11). Sample abstracts revealed this cluster encompassed consumer perception studies, policy analyses, life cycle assessments, review papers, and general discussions of alternative proteins. The temporal distribution was more dispersed (2010–2025), with earlier papers (pre-2022) more prevalent than in Cluster 1 (32.1% vs. 9.7%), consistent with the longer history of socio-economic and sustainability research preceding intensive technical development. This binary clustering effectively stratified papers by depth of technical content, enabling efficient manual screening. Cluster 1 papers were prioritized for full-text review as they represented original research on cultivation methodologies, media formulation, and bioprocessing—the primary scope of this

**Table 2.** Substrate, classification (e.g., extraction vs circular), method, extraction condition and characterization. "//" indicate that the condition/method is not used or specified in the paper.

| Title | Substrate tested | Class | Cell line | Extraction method | Extraction condition | Extract characterization | Reference |
|---|---|---|---|---|---|---|---|
| A Beefy-R culture medium: Replacing albumin with rapeseed protein isolates. | Inca Peanut, cottonseed, rapeseed, soybean | Extraction followed by cell testing | Primary bovine satellite cells | Alkali-isoelectric precipitation with modification | pH = 12.5 via NaOH 5N; pH = 4.5 via HCl 6N | Bicinchoninic Acid protein (BCA) quantification, Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) | Stout et al.[37] |
| A circular cell culture system using microalgae and mammalian myoblasts for the production of sustainable cultured meat. | *Chlorococcum littorale* | Extraction followed by cell testing | C2C12 mouse myoblasts | Acid hydrolysis | HCl 0.5 N | Hexokinase method, liquid chromatography-mass spectrometry (LC-MS), and colorimetric method. | Haraguchi et al.[38] |
| A serum-free culture medium production system by co-culture combining growth factor-secreting cells and L-lactate-assimilating cyanobacteria for sustainable cultured meat production | *Synechococcus sp. KC0110* | Circular system | C2C12 mouse myoblasts | // | // | // | Chu et al.[39] |
| Auxenochlorella pyrenoidosa extract supplementation replacing fetal bovine serum for Carassius auratus muscle cell culture under low-serum conditions. | *Auxenochlorella pyrenoidosa* | Extraction followed by cell testing | Carassius auratus muscle cells | Hydrothermal extraction | 0, 0.1, 0.2, 0.5, 1 mg/ml | Basic composition analysis, Amino acid composition determination, Monosaccharide composition analysis, Proteomic analysis | Dong et al.[40] |
| Circular cell culture for sustainable food production using recombinant lactate-assimilating cyanobacteria that supplies pyruvate and amino acids. | *Synechococcus* sp. PCC 7002 | Circular system | C2C12 mouse myoblasts | // | // | // | Haraguchi et al.[41] |
| Development of serum-free and grain-derived nutrient-free medium using microalga-derived nutrients and mammalian cell-secreted growth factors for sustainable cultured meat production. | *Chlorella vulgaris* | Circular system | Bovine myoblasts | Acid hydrolysis and ultrasonic fractionation | 0.25, 0.5, 1 N HCl and (50 g/L and 100 g/L microalgae/water) | Glucose and aminoacid quantification | Yamanaka et al.[42] |

(*Continued*)

**Table 2.** (Continued).

| Title | Substrate tested | Class | Cell line | Extraction method | Extraction condition | Extract characterization | Reference |
|---|---|---|---|---|---|---|---|
| Effects of enzymes on protein extraction and post-extraction hydrolysis of non-animal agro-industrial wastes to obtain inputs for cultured meat. | Peanut meal, soybean meal, and residual brewery yeast | Extraction followed by cell testing | // | Conventional, alkaline and enzyme-assisted | 1) S/L 1:10, *t* = 30°C, pH = 7.0, mixing rate of 200 rpm, and extraction time of 120 min. 2) Same as 1) but pH = 10 3) S/L = 1:10, *t* = 50°C, Agitation = 200 rpm, pH = 5, pH = 7 4a) S/L = 1:10, *t* = 50°C, Agitation = 200 rpm, time = 120 min, enzyme = Viscozyme L® at 5 FBG per gram dry mass, pH = 5,0 - 6.5, 7.0 4b) *t* = 30°C, pH = 10, Agitation = 200 rpm, time = 120 min, | Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE), Degree of hydrolysis (DH) | Flaibam and Goldbeck[43] |
| Evaluation of fermented soybean meal and edible insect hydrolysates as potential serum replacement in pig muscle stem cell culture. | Fermented soybean meals | Cell testing | Pig muscle stem cells | Enzymatic hydrolysis | t = 55°C, time = 8 h, enzyme = Alcalase, substrate-to-enzyme ratio = 100:1 | Degree of hydrolysis (DH) | Kim et al.[44] |
| *In vitro* culture of bovine fibroblasts using select serum-free media supplemented with *Chlorella vulgaris* extract. | *Chlorella vulgaris* | Extraction followed by cell testing | Embryonic bovine tracheal (EBTr) fibroblasts | Hot water extraction | // | Liquid Chromatography - Mass Spectrometry (LC-MS) | Defendi-Cho and Gould[45] |
| Muscle cell proliferation using water-soluble extract from nitrogen-fixing cyanobacteria Anabaena sp. PCC 7120 for sustainable cultured meat production | *Anabaena sp. PCC 7120* | Extraction followed by cell testing | C2C12 muscle cells and quail muscle clone 7 (QM7) | Ultrasonication extraction | Ice-cooled water bath 60 Hz, 200 W, S/L: 0.1 g/ml | MW separation via centrifugation, UV spectral analysis | Ghosh et al.[46] |

(*Continued*)

**Table 2.** (Continued).

| Title | Substrate tested | Class | Cell line | Extraction method | Extraction condition | Extract characterization | Reference |
|---|---|---|---|---|---|---|---|
| Proliferation of mammalian cells with Chlorococcum littorale algal compounds without serum support | *Chlorococcum littorale* | Extraction followed by cell testing | C2C12 mouse myoblasts, 3T3 cells, Chinese hamster ovarian cells | Ultrasonication extraction | Ice-cooled water bath 60 Hz, 200 W, S/L: 0.1 g/ml | Liquid Chromatography - Mass Spectrometry (LC-MS), chemiluminescent enzyme immunoassay, liquid chromatography-tandem mass spectrometry, high performance liquid chromatography, gas chromatography, Bicinchoninic Acid protein (BCA) quantification | Ghosh et al.[47] |
| Sustainable food-grade serum-reducing biomaterial: Plant protein hydrolysates for scalable cultivated meat manufacturing | Soy, wheat, peanut protein isolates | Extraction followed by cell testing | Porcine satellite cells, C2C12 | Enzymatic extraction | Enzyme (2% W/W), protein (5% W/V) in deionized water | Proximate Composition Analysis (PCA), degree of hydrolysis by formaldehyde titration method, molecular weight via size exclusion chromatography, amino acid via amino acid analyzer | Mi et al.[48] |
| The potential function of soy protein hydrolysate to induce myogenic differentiation of C2C12 cells | Soy protein isolate | Cell testing | C2C12 | // | // | // | Chen et al.[49] |
| Ultrafiltrated Extracts of Fermented Okara as a Possible Serum Alternative for Cell Culturing: Potential in Cultivated Meat Production. | Fermented okara | Cell testing | HEK293, HepG2, and C2C12, Immortalized Porcine Myoblasts (IPM) | Heat extraction | // | Gas Chromatography - Mass Spectrometry (GC-MS) | Teng et al.[50] |

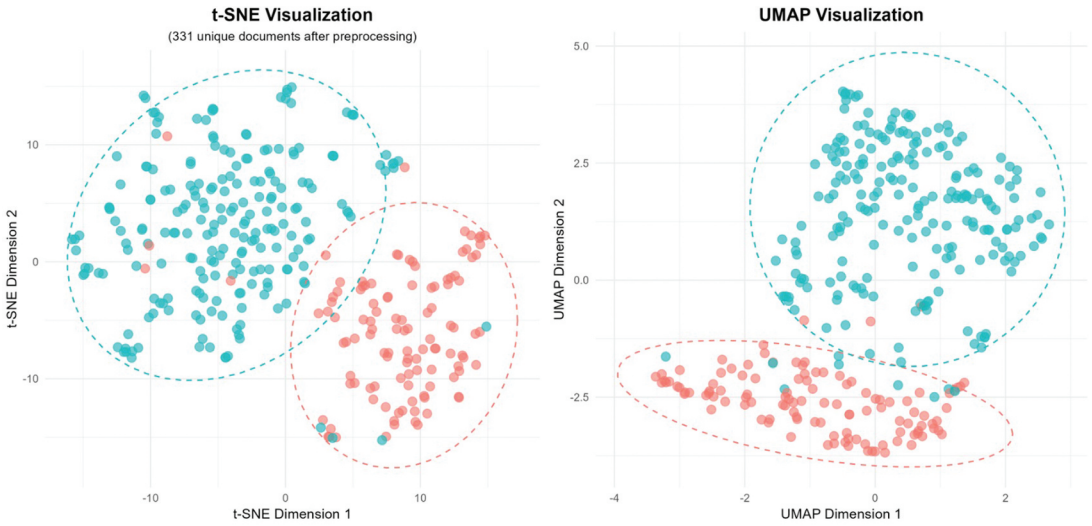



**Figure 2.** Two-dimensional visualization of binary screening results using t-SNE (left) and UMAP (right) dimensionality reduction techniques. Each point represents a scientific article, with colors indicating the assigned thematic cluster. Red: Cluster 1 Blue: Cluster 2.

systematic review. Cluster 2 papers, while topically relevant to cultivated meat, largely addressed adjacent issues (consumer acceptance, environmental impact, economic feasibility, regulatory frameworks) rather than experimental technical development. The clear separation (t-SNE and UMAP visualizations showed minimal overlap between clusters) showed in the Fig. 2 validated the unsupervised approach and reduced manual screening burden by immediately identifying the 113 high-priority technical papers for detailed evaluation.

The two clusters were identified by the following terms:

- Cluster 1 (113): muscl, differenti, serum, medium, bovin, growth, prolifer, media, serumfre, tissu, satellit, extract, fbs, myogen, cultiv
- Cluster 2 (219 papers): anim, agricultur, consum, consumpt, chang, environment, vegan, industri, altern, social, market, research, increas, technolog, system

The tree structure of the dendrogram in Fig. 3 validates the optimal number of clusters identified through k-means clustering, with distinct branches corresponding to thematic groupings in the literature.

## *Dataset overview*

The data were categorized into 10 categories by BERT and were later switched to knowledge-related categories. Figure 4 shows the Sankey diagram that explains the flow from unsupervised BERTopic clustering to knowledge related categories.

The workflow demonstrates how ten initial BERT-generated topics were consolidated into eight final categories through domain-specific keyword rules. Most topics exhibited clear thematic alignment, with Topic 2 predominantly mapping to Cell Line Development, Topic 1 to Plant-based & Circular Approaches, and Topic 0 to Microbial & Recombinant Proteins. Specifically, 23.5% of papers were multidisciplinary and being in the Topic 10. These papers were successfully reclassified using keyword-based rules targeting specific technical terms and methodologies, with the majority distributed across Cell Line Development and Plant-based & Circular Approaches. The flow width represents

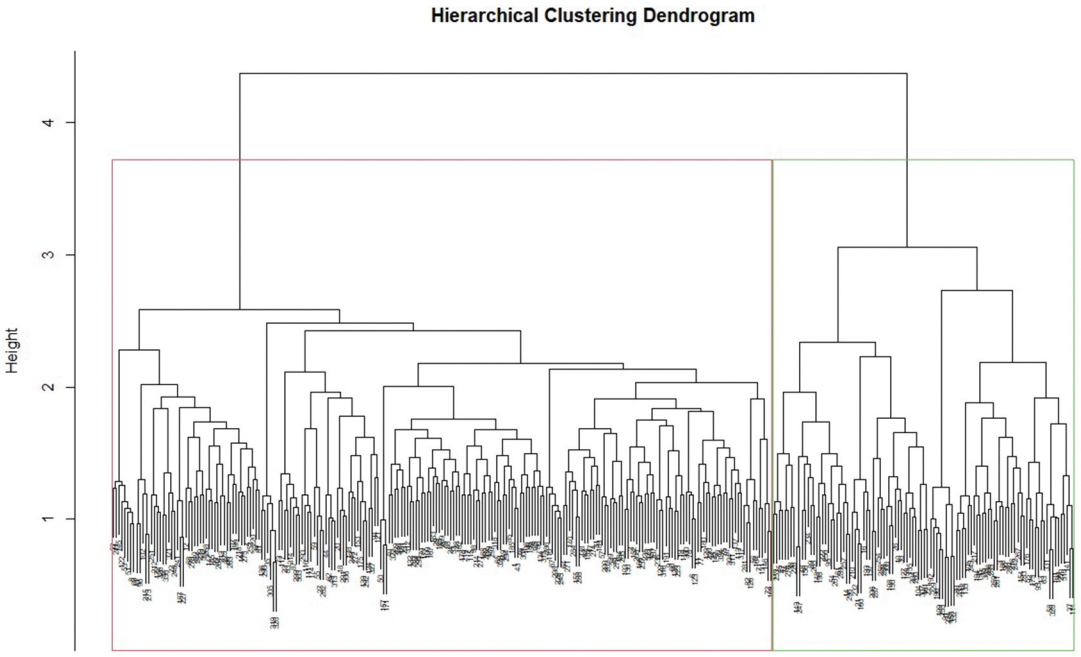



**Figure 3.** Hierarchical clustering dendrogram.

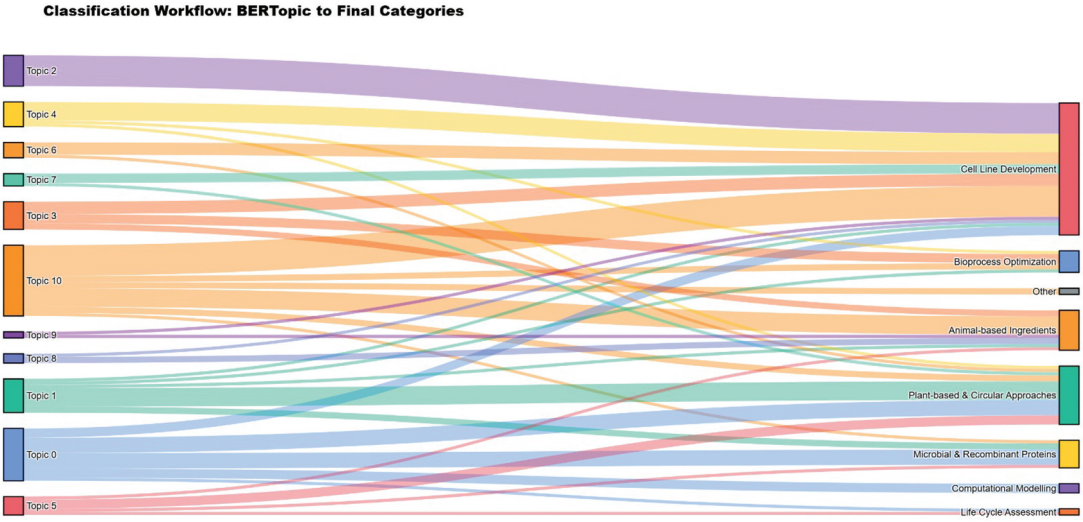



**Figure 4.** Flow diagram showing the hybrid classification methodology from unsupervised BERTopic clustering (left nodes) to expert-curated research categories (right nodes).

the number of papers transitioning between topics and categories, with broader flows indicating stronger topic-category associations. This hybrid approach effectively addressed the limitations of purely unsupervised methods while maintaining reproducibility and reducing manual annotation burden.

The data retrieved showed that there has been a growing number of publications from 2019 to September 2025, as shown by the growing number of papers from 2019 to today. Figure 5 shows the temporal evolution of research categories.

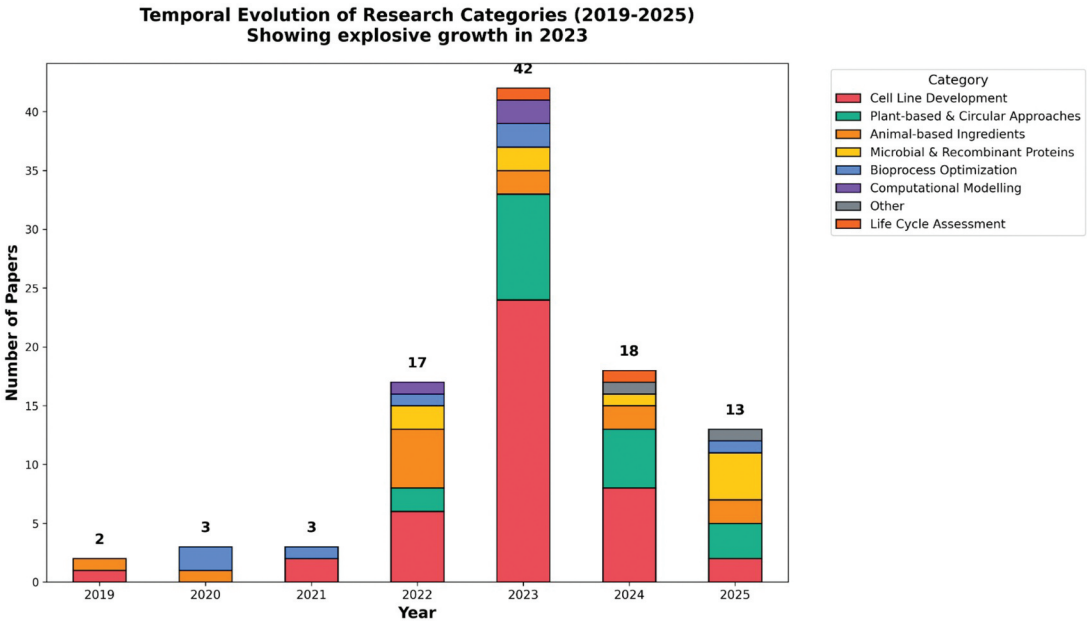



**Figure 5.** Temporal evolution of research categories from 2019 to 2025.

The field experienced minimal activity in its nascent phase (2019–2021, $n = 8$ total), followed by rapid expansion in 2022 ($n = 17$) and peak productivity in 2023 ($n = 42$). The reduction in 2024 ($n = 18$) and 2025 ($n = 13$, data current to September) may reflect publication lag times rather than reduced research activity. Cell Line Development dominated the 2023 surge, contributing 24 of 42 publications and maintaining substantial representation across all years post-2021. Plant-based & Circular Approaches showed sustained growth from 2022 onwards, while Animal-based Ingredients exhibited a more scattered temporal pattern. Emerging categories such as Computational Modelling and Life Cycle Assessment appeared exclusively in recent years (2022–2024), suggesting maturation of the field toward system-level analyses and sustainability assessments. A complementary distribution of paper per research category is shown in Fig. 6.

The distribution of research papers across the thematic categories identified showed that Cell Line Development emerged as the predominant research focus, accounting for nearly half of all publications (43.9%, $n = 43$), reflecting the field's emphasis on fundamental cell culture technologies. Plant-based & Circular Approaches constituted the second-largest category (19.4%, $n = 19$), followed by Animal-based Ingredients (13.3%, $n = 13$) and Microbial & Recombinant Proteins (9.2%, $n = 9$). Bioprocess Optimization represented 7.1% ($n = 7$) of publications, while Computational Modelling (3.1%, $n = 3$), Life Cycle Assessment (2.0%, $n = 2$), and Other (2.0%, $n = 2$) included minor but emerging research directions. This distribution reveals the field's current priorities and identifies underexplored areas requiring further investigation. Indeed, the results of the GAP analysis performed on BERT categories followed by knowledge-related categories are shown in Fig. 7.

Gap analysis revealed substantial differences in research priorities. While Cell Line Development dominated with 3.51× expected coverage ($n = 43$), critical translational areas remained severely under-researched: Bioprocess Optimization (0.57×, $n = 7$), Computational Modelling (0.24×, $n = 3$), and Life Cycle Assessment (0.16×, $n = 2$). This 22-fold disparity between most and least represented categories suggests the field remains technology-push rather than market-pull oriented. At the topic level, 50% of BERT topics fell below the median threshold (8.0 papers), with Topics 7–9 representing nascent research directions requiring further investigation. The higher focus on cell culture technologies is on accordance

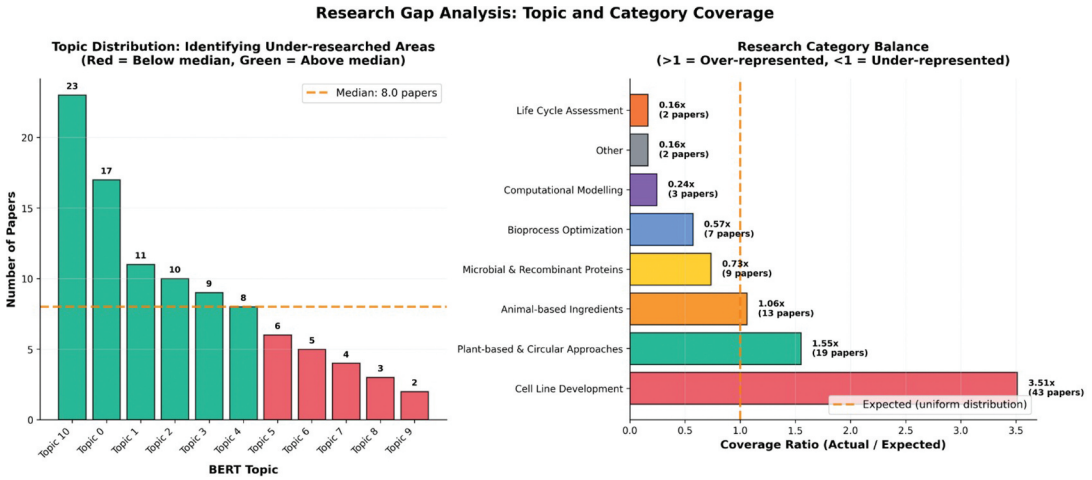



**Figure 6.** Distribution of paper per research category.

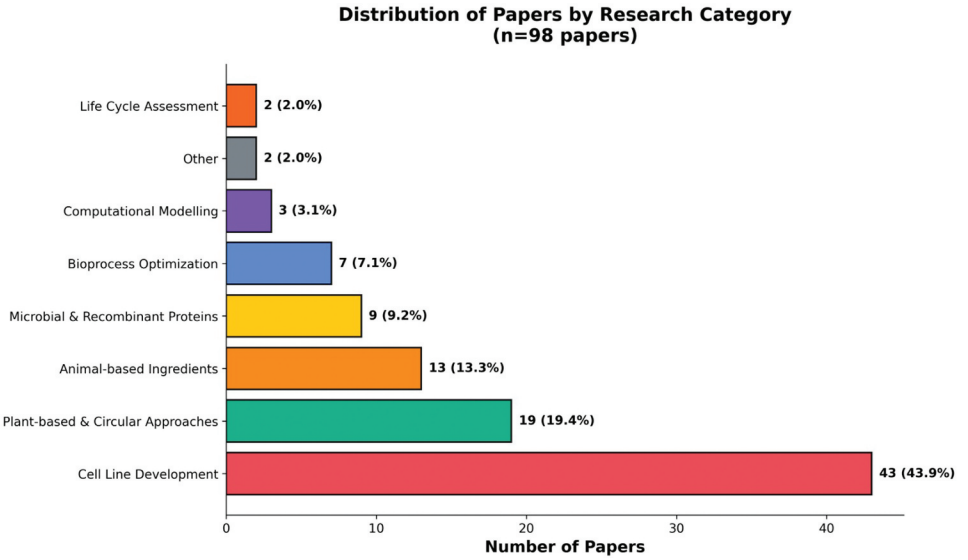



**Figure 7.** GAP analysis results. Left panel shows per-topic paper count with median threshold (orange dashed line), highlighting under-researched topics (red bars, $n <$ median) versus well-established topics (green bars, $n >$ median). Right panel displays category coverage ratios relative to expected uniform distribution, where values > 1.0 indicate over-representation and <1.0 indicate under-representation.

with the technology stage. Despite that, the gaps identified presents strategic opportunities for interdisciplinary research addressing the translational barriers to cultivated meat commercialization. Despite the BERTopic pipeline identified eight research categories, this review focuses on Plant-based and Circular Approaches as the primary category of interest, given its direct relevance to sustainable and circular serum-free media development.

## *Plant based and circular approaches*

Analysis of the "Plant-based and Circular Approaches" category identified two distinct methodological approaches to developing serum-free culture media from plant-based resources. Both agro-

industrial side streams (waste valorization) and primary plant raw materials served as sources for bioactive macromolecule extraction. Table 2 summarizes the extraction methods (e.g., isoelectric precipitation, ultrasound-assisted extraction) and the characterization analysis.

## Discussion

The data reported in Table 2 shows that two main categories of research have been conducted to substitute the FBS:

- Circular cell culture (CCC) systems
- Biomolecule extraction and testing

### *CCC SYSTEMS*

CCC systems represent an innovative approach to sustainable medium recycling for mammalian cell cultivation. These systems efficiently repurpose spent medium containing substantial residual biomolecules (e.g., amino acids) as nutrient sources for subsequent cell populations, thereby enhancing resource utilization while maintaining cellular productivity. The general workflow for CCC systems and preferred analytical methods is shown in Fig. 8.

#### *First generation cell culture systems*

Haraguchi et al.[38] introduced circular cell culture (CCC) as a strategy to decouple cultured meat production from two structural dependencies of conventional protocols: competition with food-grade grain for nutrient inputs, and the disposal of nutrient-rich but cytotoxic spent medium, which is estimated to leave up to 74% of supplied amino acids unused and contributes to aquatic eutrophication when discarded.[51] Their system closed the loop by alternating C2C12 myoblast culture with *Chlorococcum littorale* cultivation: myoblast waste medium fed the microalgae, which cleared ammonia (91.8 ± 8.6%) while consuming minimal glucose and amino acids, and the resulting algal biomass was acid-hydrolyzed to replenish these depleted nutrients for a second myoblast cycle, adding it to the spent medium from initial culture process. Proliferation in this recycled medium (4.5 ± 1.5-fold) approached that of serum-supplemented DMEM (5.0 ± 1.2-fold), demonstrating that a fully serum-free, grain-independent cycle can sustain proliferation comparable to conventional culture over at least two cycles.

For viability assessment, the researchers used the trypan blue exclusion method and counted the cells with a hemocytometer. On the other hand, the microalgae growth was analyzed using

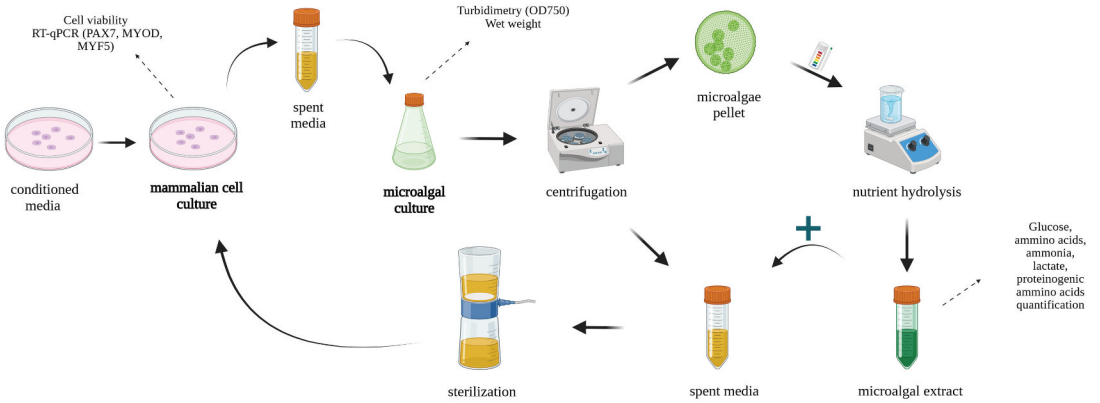



**Figure 8.** Workflow and preferred parameters for CCC systems.

Turbidimetry (OD750). Microalgal extract was added at 5% concentration (V/V) in C2C12 cell culture. Glucose, amino acids, and ammonia levels of the microalgal extract and waste medium were analyzed by the hexokinase method, LC-MS, and a colorimetric method.[52–54]

The CCC systems investigated by Haraguchi et al.[38] present a promising approach for sustainable cellular agriculture, while also revealing several areas that would benefit from further research and development. While the systems elegantly recycle carbon dioxide through microalgal photosynthesis, it still requires supplementation of phosphorus and nitrogen compounds—though this might be addressed through nitrogen-fixing microalgal species. On one hand, in this study serum-free mediaum still depends on RL34 rat hepatocyte-conditioned medium as the growth factor source: the system removes FBS but substitutes it with a second animal-cell-derived input, so the circularity applies to nutrients and waste, not to growth factor supply. On the other hand, equivalence to FBS was demonstrated only across two culture cycles; whether growth factor activity, ammonia clearance efficiency, and amino acid balance remain stable over the longer cycle counts required for industrial-scale production is untested, and, as the authors themselves acknowledge, growth factor retention across repeated media transfers remains an open question.

A conceptually distinct strategy was pursued by Defendi-Cho and Gould,[45] who tested Chlorella vulgaris extract (CVE) as a direct FBS substitute for embryonic bovine tracheal fibroblasts rather than as part of a circular system. CVE alone yielded only modest viability gains; the best formulation, combining CVE with insulin and growth factors, reached approximately 75% of FBS-supplemented viability. This gap relative to the near-complete equivalence achieved by the CCC approach is instructive: simply supplementing a serum-free base with an algal extract appears insufficient to replicate FBS bioactivity, whereas actively recycling and re-concentrating nutrients through a living intermediate (the microalgal culture itself) came closer to matching serum performance.

These findings indicate that an optimal serum-free medium for these cells would include a combination of algae extract (specifically CVE), insulin, and growth factors. More investigations are needed to elucidate the precise mechanisms by which CVE facilitates cellular growth and to explore potential modifications to supplementary components that could reduce associated costs while maintaining efficacy.

### *Optimized CCC systems*

Building on the first-generation CCC system, several groups targeted its specific limitations through independent engineering strategies. Haraguchi et al.[41] addressed lactate accumulation by metabolically engineering *Synechococcus* sp. PCC 7002 to express lactate dehydrogenase and a lactate permease, converting lactate to pyruvate; over 12 days this reduced lactate by 68% and cleared ammonia by 98%, while pyruvate accumulated to 8 mM and supported $4 \times 10^6$ viable myoblasts/dish after 72 h – but glucose and glutamine, not supplied by the cyanobacterium, declined further with each cycle, and no FBS-supplemented control was included. Their findings indicate that due to reduced levels of glucose and glutamine, animal muscle cells rely on pyruvate and amino acids other than glutamine for energy, leading to metabolic alterations.

Chu et al.[39] instead used a transwell co-culture of RL34 cells with lactate-assimilating *Synechococcus* KC0110, achieving over 3-fold C2C12 proliferation without serum or medium replacement by keeping lactate below cytotoxic thresholds while increasing glucose, pyruvate, and pyruvate-derived amino acids – though the protocol still relied on an initial FBS-containing adhesion step before serum-free culture began. Therefore, the authors mention that complete animal serum elimination will require incorporating extracellular matrix proteins (collagen, laminin) for attachment or implementing suspension culture methodologies.

Yamanaka et al.[42] expanded upon the CCC system by investigating the incorporation of CVE into an inorganic salt solution (ISS) for RL34 cell culture to develop an optimized conditioned medium (CVNM). Their comparative analysis of acidic hydrolysis extraction (AHE) versus ultrasonic fractionation extraction (UFE) revealed critical differences in amino acid profiles. AHE lacked glutamine (likely oxidized to glutamate) and contained minimal asparagine (presumably converted to aspartic

acid), while UFE preserved both amino acids. Optimization experiments demonstrated that supplementation with 6 mg/L glutamine and 5–20 mg/L asparagine to ISS containing 5% 0.5 N AHE yielded maximum RL34 cell viability, with higher concentrations proving cytotoxic. These findings confirmed that UFE effectively compensates for amino acid limitations in AHE, supporting the critical role of balanced amino acid composition in optimizing cell viability. The researchers formulated CVNM by incorporating CVE (an equivolume mixture of 0.5 N AHE and UFE) into ISS at 15% concentration. Osmolality optimization via sodium chloride adjustment revealed peak cell survival at 260–300 mOsm/kg. The resulting conditioned medium matched DMEM-conditioned medium in IGF-1 but showed markedly higher IGF-2, yet myogenic differentiation 1 (MYOD) and myogenic factor 5 (MYF5) expression indicated this growth factor profile was still insufficient to fully replicate FBS-driven myogenic proliferation and differentiation. These findings demonstrate that while RL34-derived cultured medium partially substitutes for FBS, it lacks the complete bioactive profile necessary for optimal myogenic proliferation and differentiation.

Dong, Nannan et al.[40] tested a comparable strategy on Carassius auratus (CAM) muscle cells, where *Auxenochlorella pyrenoidosa* extract (APE) supplementation at 5% FBS outperformed 10% FBS in proliferation and MyoD expression, with no morphological change over four passages. Cell viability was assessed via Calcein staining assay and the MyoD expression via Immunofluorescent staining assay. According to total amino acid composition analysis, glutamate was the most abundant amino acid in the APE, followed by alanine, aspartate, and glycine, with values of 3.96 ± 0.14, 2.93 ± 0.07, 2.89 ± 0.06, and 2.15 ± 0.05 g/100 g, respectively. Free amino acids in the APE were mainly alanine, glutamate, tryptophan, and leucine, with the contents of 0.77 ± 0.01, 0.64 ± 0.01, 0.44 ± 0.20, and 0.29 ± 0.03 g/100 g respectively, indicating that most amino acids in the APE were present in the form of proteins or peptides. The carbohydrate in the APE was composed of several monosaccharides, including galactose, rhamnose, glucose, and mannose suggesting that the monosaccharide composition of APE was like that of serum. Proteomic analysis identified 239 proteins in the *Auxenochlorella pyrenoidosa* extract (APE), all successfully mapped to gene ontology (GO) annotations across three main categories: biological processes, cellular components, and molecular functions. Of the identified proteins, 112 participate in biological processes including cellular metabolism (34.7%), biosynthesis (14.6%), cell communication (1.7%), signal transduction (1.7%), cell cycle (0.8%), cell division (0.4%), and reproduction (0.4%).

Read together, these four studies pursue distinct engineering targets – cyanobacterial metabolism, co-culture density, medium reformulation, and extract supplementation – but none resolves the underlying trade-off common to CCC systems: interventions that clear cytotoxic waste (lactate, ammonia) consistently come at the cost of glucose or growth-factor sufficiency or require a residual animal-derived input to close the gap. Notably, Chu's KC0110 strain is the only system among those reviewed here that increases rather than depletes glucose during waste clearance, suggesting that the specific metabolic pathway engineered into the photosynthetic partner – not just its presence – determines whether a CCC cycle is net nutrient-positive or nutrient-negative. The stronger result obtained by Dong et al. on fish myoblasts, where an algal extract alone outperformed full-serum medium, also raises the question of whether the substitution gap observed in mammalian systems reflects a biological difference between cell types or simply a less optimized extract; this comparison across species is not one the individual studies were designed to make; but it is a testable hypothesis for future comparative work.

### *Current limitations and future research directions in CCC systems*

Across the studies reviewed, CCC systems demonstrate proof-of-concept for medium recycling through microalgae- and cyanobacteria-mammalian cell co-culture, achieving promising metabolite removal efficiencies (e.g., up to 98% ammonia, 68% lactate reduction) and proliferation rates comparable to FBS-supplemented controls in optimized conditions. However, several unresolved technical barriers limit their scaling up opportunities. First, gradual depletion of glucose and glutamine across culture cycles remains a critical unresolved issue – not primarily because these nutrients are costly to replace (glucose in particular is an inexpensive, commodity-grade input for cell culture media), but because their depletion undermines the core rationale of the CCC concept itself. A system that must be

periodically supplemented with exogenous glucose to sustain mammalian cell proliferation is, to that extent, no longer operating as a closed loop: it forfeits much of the "no additional grain-derived nutrients" advantage that motivates CCC over conventional medium replacement in the first place, and the environmental and cost benefits attributed to circularity would need to be re-evaluated against a partially open system. Neither microalgae nor cyanobacteria currently supply glucose or glutamine in sufficient quantities to prevent this drift, leading to progressive metabolic alterations in mammalian cells that rely increasingly on pyruvate and non-glutamine amino acids as energy substrates. Second, growth factor addition remains dependent on feeder cell lines (e.g., RL34 hepatocytes), as shown across multiple studies. Conditioned medium from feeder cells introduces biological variability, regulatory complexity, and scalability constraints that are incompatible with food-grade industrial production. Future work should investigate whether recombinant growth factors produced microbially or plant-derived bioactive fractions with growth factor-like activity could replace feeder cell-derived conditioned medium while maintaining myoblast proliferation rates. Also, validation across diverse and food-relevant cell lines remains limited. Indeed, while some systems tested CCC system on primary myoblasts the majority of studies relied on C2C12 mouse myoblasts. Moreover, no study has yet validated a CCC system on primary satellite cells from commercially relevant species for cultivated meat production, such as porcine or poultry cells. Systematic validation across primary, species-relevant myogenic cell lines and immortalized cell lines is therefore essential before translational claims regarding CCC systems can be substantiated. Finally, no life cycle assessment or techno-economic analysis of any CCC system has been performed to date, leaving their claimed sustainability and cost advantages entirely unquantified. Given that CCC systems add biological and operational complexity relative to conventional batch culture, it cannot be assumed a priori that their environmental footprint is lower.

Future research priorities should therefore include:

- Engineering nitrogen-fixing or glucose-secreting photosynthetic strains to close the nutrient loop;
- Identifying chemically defined, food-grade adhesion factors to eliminate residual FBS;
- Replacing feeder cell-derived conditioned medium with recombinant or plant-derived growth factor alternatives;
- Validating CCC systems on primary, species-relevant satellite cells;
- Demonstrating system scalability in bioreactor configurations;
- Coupling CCC system design with LCA and TEA to quantitatively validate environmental and economic benefits relative to conventional serum-supplemented culture.

### *Biomolecule extraction and testing*

Ghosh et al.[46] performed an ultra-sonication extraction of intercellular components from *Anabaena* sp. PCC 7120. Later, the water-soluble components were separated from the alcohol-soluble components. This water-soluble component (containing phycocyanin showed positive effects on cell proliferation of C2C12 muscle cells and quail muscle clone 7 (QM7) cells. The authors underline that the phycocyanin may have a contrasting effect on cell proliferation depending on the concentration used (e.g., 200 µg/mL phycocyanin concentration showed cell toxicity while 100 µg/mL improved muscle cell proliferation compared to 0%FBS). Moreover, the authors fabricate a three-dimensional cell-dense structure using avian myoblasts.

Similarly, Ghosh et al.[47] performed the same extraction process to test the proliferative activity of the ultrasonicated extract of *C. littorale* on C2C12, 3T3 and CHO cells. The authors identified two of the total tested different conditions (named 40CW and 20C) that obtained high proliferation rate and statistical significance between the negative control and the culture conditions in both myoblasts and fibroblasts. Based on promising results, the authors move to a bioreactor set-up for CHO cells that were subject to a gradual decline from passage 1 to 4.

### *Side streams as source of biomolecules*

Side stream valorization or upcycling constitutes the systematic utilization of complete side streams or the extraction and application of specific high-value components from food processing waste streams, thereby transforming these previously underutilized materials into value-added products[55] This methodological approach aligns with circular economic principles, wherein waste materials are reconceptualized as valuable resources amenable to recovery, repurposing, and reintegration into production cycles.[56] Through the implementation of side stream valorization strategies, the food industry can simultaneously minimize waste generation, optimize resource utilization efficiency, and contribute substantively to environmental sustainability objectives.

The valorization of side streams offers multiple advantages, including reduced dependency on virgin raw materials, diminished production costs, and the creation of novel business opportunities within the food sector. To successfully implement circular economy solutions in the agrifood domain, it is imperative to establish viable markets for secondary raw materials and to engage consumers effectively to ensure high levels of acceptance and purchase intention.[57] The significant potential of side stream valorization derives from the fact that food processing waste streams frequently contain valuable biochemical constituents including proteins, dietary fibers, lipids, antioxidants, vitamins, minerals, and bioactive compounds. These components possess functional and nutritional properties that can be strategically harnessed for the development of innovative food ingredients, nutraceuticals, and functional food products with enhanced physiological benefits.[58] The benefits of incorporating these materials, together with additional inputs, for cultivated meat production extend beyond nutritional advantages, as this approach can decrease production expenses and reduce environmental impacts caused by traditional animal agriculture and improper waste disposal methods.[59,60] The general workflow for protein extraction, cell testing, and preferred analytical methods is shown in Fig. 9.

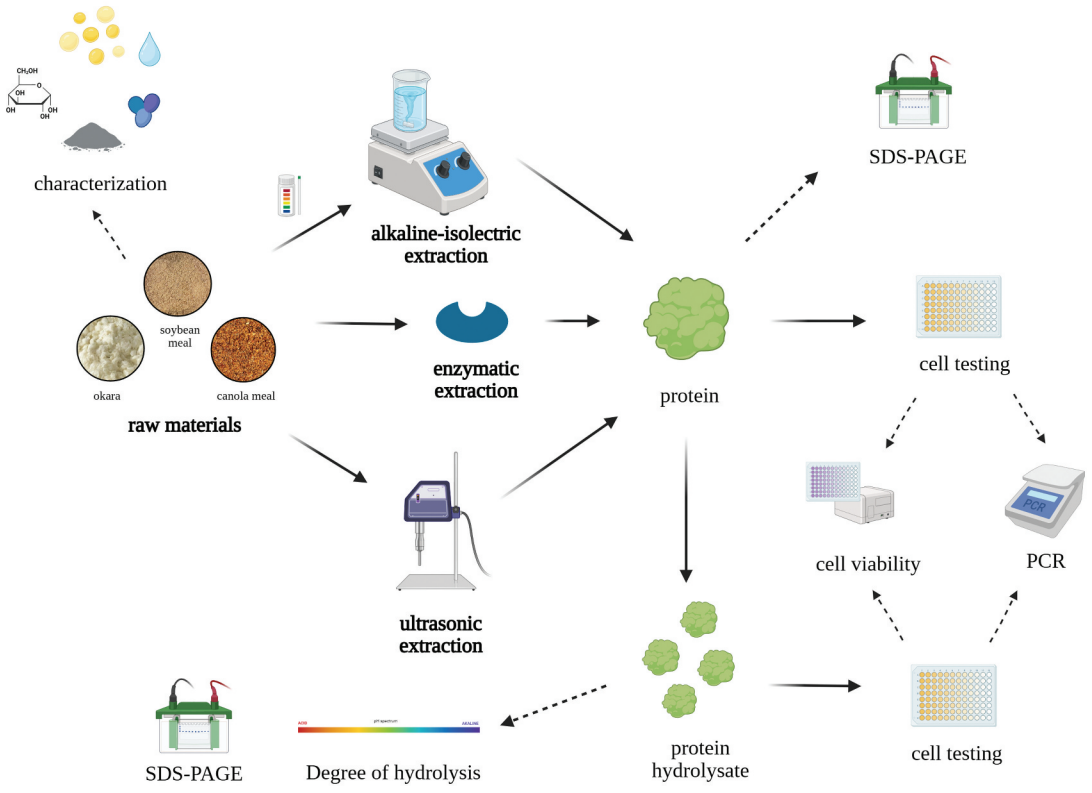



**Figure 9.** Workflow and preferred parameters for protein extraction from sidestreams.

Stout et al.[37] evaluated oilseed protein isolates (OPIs) – from Inca peanut, soybean, rapeseed, and cottonseed, all but the first being sidestreams of food oil production – as albumin replacements in Beefy-9, a serum-free medium for bovine satellite cells (BSCs) built on the iPSC medium B8. Among the four isolates, soybean, cottonseed, and rapeseed protein isolates (RPI) significantly enhanced BSC proliferation over unsupplemented B8, while Inca peanut isolate showed no benefit; RPI at 0.4 mg/mL was the most effective, outperforming recombinant albumin by 15% and yielding the formulation Beefy-R. Across four passages, Beefy-R achieved 11.7 population doublings over 13 days versus 10.6 for albumin-based Beefy-9 — nearly doubling cumulative yield – though both remained below FBS-supplemented BSC growth media (GM) (14.8 doublings), with Beefy-R's doubling time also deteriorating less across passages than Beefy-9's. However, myogenic marker analysis complicated this proliferation advantage: BSC-GM retained a ~ 10-fold higher MyoD and ~6-fold lower myogenin than either serum-free medium, while Beefy-R alone showed a ~ 3-fold increase in myosin heavy chain (MHC) relative to BSC-GM – indicating that both serum-free formulations, and Beefy-R in particular, drove cells toward a more differentiated state during what was intended as expansion culture.

This divergence between proliferation and differentiation marker data arguably deserves as much attention as the raw doubling numbers. A medium that supports faster proliferation while also nudging cells toward earlier differentiation may not be unambiguously advantageous for cultivated meat production, where maintaining an expandable, undifferentiated satellite cell pool across many passages is often a key bottleneck to scale-up; this premature differentiation phenomenon is discussed in more detail in Addressing premature differentiation in serum-reduced cultures. The observation that rapeseed protein isolate outperformed a purified recombinant protein (albumin) by 15% is also worth highlighting: it may suggest that the functional benefit is not attributable to albumin-like activity alone but could instead reflect additional bioactive components co-extracted with the RPI fraction.

### *Fermented side streams*

Fermentation was explored as a complementary processing step to raw extraction, tested on both soybean-derived byproducts and edible insect sources. Kim et al.[44] evaluated two fermented soybean meal hydrolysates – one from *Aspergillus oryzae*/*Bacillus subtilis* (FAB) and one from *Bacillus licheniformis* (FB) – on pig muscle stem cells, finding that FB-H at just 0.1% concentration enabled a 75% FBS reduction while maintaining Paired box protein 7 (PAX7) expression and upregulating MYF5/MYOD, with FAB-H and lower FB-H concentrations supporting a more modest 50% reduction; however, growth diverged from FBS controls by the fourth passage, leaving long-term performance unresolved.

Teng et al.[50] took a size-fractionation approach to fermented okara (a soybean processing byproduct), finding that a 50 kDa ultrafiltration fraction outperformed the unfractionated extract across HEK293, HepG2, C2C12, and porcine myoblast lines, reaching up to 70% relative growth efficiency versus FBS; shorter fermentation (24 h) generally outperformed longer fermentation (72 h) in most cell lines, though this reversed in porcine myoblasts, and antinutritional compounds retained above 50 kDa were proposed as the likely cause of the unfractionated extract's weaker performance.

Together, these two studies point to a common and, in our view, underappreciated principle: the raw fermented or hydrolyzed extract is rarely the optimal formulation, and a secondary purification or fractionation step – however modest, such as simple ultrafiltration by molecular weight – can meaningfully change performance without any change to the source material itself. This suggests that reported "failures" of a given byproduct as an FBS substitute elsewhere in the literature may sometimes reflect an unrefined extraction protocol rather than a genuine biological limitation of the substrate. At the same time, both studies also show that fermentation parameters (microbial strain combination, duration) shift the resulting bioactivity profile in ways that are not yet predictable from first principles.

### *Enzymatic protein extraction*

Enzymatic hydrolysis was also investigated as a route to optimize protein extraction yield and downstream functional performance from non-animal agro-industrial byproducts. Flaibam and Goldbeck[43] focused specifically on extraction optimization, screening soybean meal, peanut meal,

and brewery yeast: a two-step protocol (neutral pH followed by alkaline solubilization at pH 10) combined with Viscozyme L enzymatic treatment increased protein yield by 930% for soybean meal and 698% for peanut meal relative to conventional extraction, consistent with the reduced protein solubility expected near the isoelectric point.[61,62] SDS-PAGE profiles matched the expected native protein families – glycinin and β-conglycinin in soybean,[63] and arachin/conarachin in peanut, whose low-molecular-weight albumin fraction is consistent with previous characterizations of oilseed albumins.[64,65] Notably, this study optimized and characterized the extraction process itself but did not test the resulting extracts in cell culture, leaving functional validation to later work.

Mi et al.[48] addressed that gap directly, comparing multiple proteolytic enzymes (alcalase, protease, neutrase, flavourzyme) on soybean and wheat hydrolysates in porcine satellite cells and C2C12 myoblasts: optimized formulations supported up to 70% FBS reduction, and two conditions (S4, W4) showed PAX7 expression rising then declining in step with proliferation, while fusion indices remained comparable across conditions, indicating that differentiation capacity was preserved despite this shift in timing.

Chen et al.[49] tested soy protein hydrolysate (PU041) on C2C12 cells and found that, at 0.5 g/L, it enhanced not only proliferation but also myogenic differentiation markers (MHC, MyoD, MyoG).

Read together, these three studies span the full pipeline from extraction optimization to functional testing, though not within a single study. This separation raises a question none of the three studies addresses directly: Flaibam and Goldbeck[43] optimize extraction specifically to maximize protein yield, through enzymatic hydrolysis and alkaline solubilization conditions that are comparatively aggressive; yet more extensive hydrolysis, which breaks proteins into smaller peptides to increase extractable yield, could plausibly also disrupt the native structures or epitopes. Since no study in this set applies a yield-optimized protocol and a function-optimized protocol to the same substrate and directly compares the resulting bioactivity, it remains unclear whether extraction yield and functional performance are aligned or in tension – a gap worth flagging for future comparative work. Relatedly, degree of hydrolysis (DH), arguably the parameter most predictive of the resulting peptide size distribution and therefore of bioactivity, is not quantitatively reported in any of the three studies; this compounds the challenge that different DH value under the same nominal enzyme and conditions would likely yield a different peptide population and, potentially, a different biological outcome.

### *Current limitations and future research directions in biomolecule extraction and testing*

The studies reviewed showed that plant-derived protein hydrolysates, microalgae extracts, and agro-industrial sidestreams can partially or fully replace FBS in cultivated meat cell culture, with rapeseed protein isolate, enzymatically hydrolyzed soy and wheat proteins, and microalgae-derived extracts emerging as the most promising candidates. However, several critical gaps remain that prevent translation to scalable, standardized production.

First, proliferation rates in serum-free or serum-reduced conditions consistently underperform relative to 10% FBS controls. This performance gap suggests that plant-derived extracts currently lack either the complete growth factor profile, the lipid composition, or the hormonal components necessary to fully replicate FBS bioactivity, and that supplementation with specific recombinant growth factors or bioactive compounds may be necessary to bridge this gap.

Second, premature differentiation in multi-passage cultures (discussed in more detail in the Addressing premature differentiation in serum-reduced cultures) represents a recurrent and under addressed challenge. Systematic investigation of the molecular mechanisms underlying premature differentiation in serum-reduced conditions and identification of the specific extract fractions responsible is therefore essential. Moreover, extraction protocols vary widely across studies, preventing direct cross-study comparison and hindering protocol standardization. Studies employed alkaline isoelectric precipitation, enzymatic hydrolysis with varying enzymes, temperatures, solid-to-liquid ratios and pH values, hydrothermal extraction, ultrasonication, and heat extraction are often under incomparable conditions. The absence of standardized extraction and characterization protocols makes it impossible to establish which method yields the most bioactive fraction for a given substrate and constitutes one of the most significant barriers to field progress. Furthermore, the translation from optimized

extraction processes to validated cell culture applications remains incomplete across the reviewed studies. While several studies systematically optimized extraction parameters and others evaluated the effect of different proteolytic enzymes and peptide size distributions on cell proliferation, these two steps were rarely integrated within a single study in a coherent pipeline. Also, where bioreactor-scale validation was attempted, performance observed at small scale did not consistently translate to larger systems, with gradual declines in cell density across passages suggesting that scale-dependent factors remain unresolved. No study to date has demonstrated a complete, integrated pipeline from substrate selection through extraction optimization, biochemical characterization, cell testing, and scale-up validation. Developing such end-to-end pipelines with consistent biochemical output across batches is a prerequisite for industrial implementation of side stream-based serum-free media.

A further, largely unaddressed limitation concerns the consistency and safety of the raw side stream material itself. Unlike defined recombinant or synthetic inputs, agro-industrial byproducts such as rapeseed meal, okara, or brewery yeast are subject to batch-to-batch variability driven by crop season, geographic origin, processing conditions at the source facility, and storage time before extraction – none of which is characterized or controlled for in any of the reviewed studies, despite its direct bearing on the reproducibility of the extraction yields and bioactivity reported. This variability compounds the protocol standardization issue discussed above: even a perfectly standardized extraction method applied to a variable substrate would be expected to yield batch-dependent biochemical output. Safety considerations are similarly absent from the reviewed literature. Agro-industrial sidestreams can carry microbial contamination, mycotoxins, pesticide residues, or allergenic proteins depending on the source crop and storage conditions and fermented or non-heat-treated extracts introduce an additional risk of microbial contamination if sterilization is not explicitly verified. None of the studies reviewed here report screening for these hazards, which represents a meaningful gap given that any food-grade application would require such characterization as a matter of regulatory compliance.

On the cell sides, the range of cell types tested remains limited and biased towards transformed cell lines. C2C12 mouse myoblasts dominate the dataset with few studies using primary satellite cells (bovine and porcine respectively), and just one study using aquaculture-relevant cells. This bias is not incidental: C2C12 is an immortalized, commercially available, low-cost murine myoblast line with decades of established culture protocols and highly reproducible, donor-independent behavior, making it well suited for the rapid, iterative screening of new extraction methods and serum-free formulations that characterizes most of the studies reviewed here. Primary satellite cells from commercially relevant species, by contrast, require animal sourcing and isolation, are available in limited quantities, undergo replicative senescence after a finite number of passages, and exhibit substantial donor-to-donor variability that complicates cross-study comparison – all of which raise the cost and lower the throughput of screening relative to C2C12. As a result, C2C12 has effectively become the default first-pass model for evaluating candidate serum-free formulations, with validation on species- and application-relevant primary cells reserved for a later, more resource-intensive stage that few of the reviewed studies have reached. Validation of plant-derived extracts on a broader panel of primary, species-relevant myogenic cell lines (e.g., bovine, porcine, and avian satellite cells) is crucial to establish a general protocol for cultivated meat/fish production. Validation of plant-derived extracts on a broader panel of primary, species-relevant myogenic cell lines (e.g., bovine, porcine, and avian satellite cells) is crucial to establish a general protocol for cultivated meat/fish production. When it comes to the environmental impact of the process/product, no study has performed a life cycle assessment or techno-economic analysis of sidestream-based media formulations, leaving the claimed sustainability and cost advantages entirely unquantified. Given that extraction, fractionation, and characterization processes involve energy-intensive steps and generate secondary waste streams, it cannot be assumed a priori that sidestream valorization reduces the environmental footprint of cultivated meat production relative to conventional FBS-supplemented culture.

Future research priorities should therefore include:

- Standardization of extraction and characterization protocols to enable cross-study benchmarking and reproducibility;
- Investigation of premature differentiation in serum-reduced conditions and identification of mitigation strategies;
- Multi-passage long-term culture validation on primary, species-relevant satellite cells;
- Scale-up validation of extraction processes in pilot-scale systems;
- Assessing batch-to-batch variability in sidestreams raw materials;
- Integration of LCA and TEA to quantitatively assess the sustainability and economic viability of sidestream-based media at production scale;
- Exploration of synergistic combinations between plant-derived extracts and minimal recombinant growth factor supplementation to close the proliferation gap relative to FBS controls.

### *Addressing premature differentiation in serum-reduced cultures*

The premature differentiation phenomenon documented in available studies[37,44] was investigated by Katayama et al.[66] who demonstrated that Methyl-β-cyclodextrin (MβCD) maintains myoblast proliferative capacity under serum-reduced culture conditions. The investigators established that C2C12 cell apoptosis during differentiation occurs through a cholesterol-dependent mechanism, with MβCD-induced cholesterol depletion significantly suppressing cell death. Principal component analysis revealed distinct temporal differentiation profiles, with no observable differences between control and MβCD-treated populations at day 1 of differentiation, while significant divergence emerged by day 3. Control cultures exhibited advanced myogenic differentiation characteristics, whereas MβCD-treated cells retained phenotypic features comparable to day 1 differentiation status, indicating that MβCD supplementation inhibits early-stage differentiation progression. The reduction in apoptotic cell death mediated by MβCD treatment represents a potential molecular mechanism underlying its inhibitory effects on myoblast differentiation into myotubes. These findings suggest that cholesterol depletion strategies may provide a viable approach for maintaining myoblast populations in a proliferative state while preventing unwanted differentiation in serum-limited culture systems.

## Methodological strength and limitations

Manually screening and thematically categorizing a large and heterogeneous literature corpus is inherently labour-intensive and susceptible to reviewer-dependent bias. To address this, the present review adopted a machine learning-assisted classification pipeline, which offers several advantages over a fully manual systematic review approach. Transformer-based embeddings and unsupervised clustering allowed a much larger corpus to be processed consistently and at scale, and reduced the subjectivity inherent in manually grouping papers into thematic categories, since topic assignment was based on quantifiable semantic similarity rather than reviewer judgement alone. BERT-based classification and BERTopic-based screening pipelines have similarly been applied directly to systematic review workflows, reporting substantial reductions in manual screening effort and reproducible topic identification when appropriately validated.[67,68]

The findings of this review should be interpreted in light of the following limitations. Specifically, the literature search was restricted to English-language and open-access records, which may have resulted in the exclusion of relevant studies and introduced a selection bias toward well-funded research groups with open-access publishing capacity. Also, the considerable heterogeneity of the included studies (e.g., in terms of cell types, extraction methods, culture conditions, and outcome measures) prevented quantitative meta-analysis and limited cross-study comparability, reflecting the nascent and rapidly evolving nature of the field. Finally, the focus on a single research category (Plant-based and Circular Approaches) means that findings from other categories identified by the BERTopic pipeline were not analyzed.

## Conclusion

CCC systems demonstrate significant potential for reducing resource consumption and environmental impact through efficient medium recycling, with studies showing successful integration of microalgae and cyanobacteria for metabolite remediation and nutrient recovery. The incorporation of microalgal extracts has proven effective in partially replacing FBS while maintaining cellular viability, though optimization of growth factor concentrations and long-term culture stability remains necessary for commercial viability. Despite that, more research is needed to understand the environmental/financial costs of these systems. Additionally, these systems exhibit gradual depletion of essential nutrients such as glucose and glutamine that are not replenished by microbial components, potentially limiting long-term culture viability. However, this could be, again, a gap covered by the overlapping of circular cell culture systems and side streams extraction.

Side stream valorization presents a promising avenue for sustainable biomolecule sourcing, with protein isolates from agricultural waste streams, particularly rapeseed and soybean derivatives, demonstrating comparable performance to conventional supplements in supporting myoblast proliferation. However, challenges persist regarding premature cellular differentiation in serum-reduced conditions, necessitating novel approaches to maintain proliferative capacity. While these innovative approaches collectively offer pathways toward more sustainable cellular agriculture, comprehensive life cycle assessments and scale-up studies are essential to validate their commercial feasibility and environmental benefits. Future research should prioritize the integration of these technologies with emphasis on standardizing extraction methodologies, optimizing culture conditions for extended passages, and developing cost-effective production systems that can compete with traditional animal-derived components.

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## Author contributions

CRediT: **Bruna Anzá:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft; **Silvia Fraterrigo Garofalo:** Project administration, Supervision, Validation, Writing – review & editing; **Edoardo Massarelli:** Writing – review & editing; **Diana Massai:** Validation, Writing – review & editing; **Debora Fino:** Funding acquisition, Project administration.

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