FEASTS
Fostering European Cellular Agriculture for Sustainable Transition Solutions

Deliverable 3.3: Catalogue of media compositions and scaffold materials by target application and opportunities for cheaper and more sustainable formulations

Work Package 3 *Sustainability by design*

Task no: 3.2 and 3.4

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Executive Summary

In the last several years, multiple research papers as well as review articles were published focusing on the optimization of cell culture media for production of cultured meat and cultured seafood (CM/CSF). Due to the vast amount of available data and characters limits in articles, most reviews focus on a small part of media development. This leads to fragmented knowledge and stakeholder ambiguity in this complex area. Thus, to address this problem, FEASTS D3.3 delivers several items, which will facilitate strategic decision-making for stakeholders, identify current bottlenecks and promote future research in the area:

- **Catalogue of serum free media components for terrestrial and aquatic species by target application** (see **Annex 1** below), which lists all the components of the basal media, as well as media add-ons, consisting of the growth factors, stabilizing proteins or non-protein components. For each component, a function, final concentration, CAS number (if available), and components which can alternatively be used, are listed.
- **Catalogue of scaffold materials by target application** (see **Annex 2** below), which lists common scaffold materials used in cellular agriculture. The catalogue presents the most used scaffold materials in cellular agriculture categorized by type. Each entry presents their functional role, nutritional features, CAS nº, digestibility and allergenicity and possible alternatives.
- **Review on published serum-free media compositions** (see the preprint (Leber et al., 2025) or **Annex 3** below, and a summary in chapter **1** below), which revealed that multiple serum-free media formulations are published for terrestrial, as well as for the aquatic cells, but some areas are still lagging behind.
- **Review on texturizing meat/seafood analogues** (see a summary in chapter **2** below, or in **Annex 4** in detail)**,** showing that the use of functional ingredients and advanced structuring techniques has taken texturization to the next level, laying the grounds to produce cultivated animal and seafood foods that closely mimic the structural characteristics of conventional products.
- **A conceptual framework for a more efficient Circular Cell Culture system and a waste valorization concept** (see chapter **3** below) was proposed to further lower media costs, reduce media ecological footprint and allow media production based on the local agricultural side product sources.
- In **Annex 5** below we present our experimental results further investigating the opportunities for cheaper and more sustainable media formulations. We present a **hydrolysate-based alternative** to the chemically fully defined basal media DMEM/F12.

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1. Review on published serum-free media compositions

A full review on published fully disclosed serum-free media compositions is available as a preprint (Leber et al., 2025). This review aims to cover the fully disclosed published compositions of serum-free (SF) media formulations for applications in cultured meat and seafood, as well as pinpoint development bottlenecks in certain areas, and critically evaluate possible solutions. The following summary represents the key points of the review:

- Media for cell isolation and storage:
  - **No SF isolation medium** has been published so far.
  - Chemically defined and SF cryopreservation media are commercially available, but **composition is not publicly available**.
  - Food-grade chemically defined and SF cryopreservation media are required on the market.
- Media for cell proliferation:
  - There is **no universal** chemically defined proliferation medium applicable to all cell types and species.
  - **SF proliferation media** for **muscle** stem cells and **fibro-adipogenic** progenitor cells from terrestrial species have been developed.
  - **SF proliferation media** for Pluripotent Stem Cells (i.e. ESCs/iPSCs) of non-food species (e.g. human, mouse) are available but are mostly lacking for food species.
  - **Proliferation** of **aquatic muscle** and **fat** precursors still depends on media supplemented with serum.
  - **Cell line adaptation** can lead to extremely **cost-effective** media solutions, but risk factors have to be considered (e.g. collection of background mutations).
- Differentiation media:
  - Several SF media formulations are published for **myogenesis** of **terrestrial** species' cell lines.
  - For **myogenesis** of **aquatic** species' cell lines, no SF formulations have been reported.
  - SF formulations for **adipogenic** differentiation of both **terrestrial and aquatic** species are published.
  - Further search for efficient differentiation inducers, ligands and stimulatory factors suitable for the food sector is required.

- Evaluation of the published strategies to lower cell culture media costs was performed:
  - **Substitution of fetal bovine serum** (FBS) with hydrolysates: only **partial** replacement possible for cultured meat/cultured sea food (CM/CSF) cell lines.
  - Recombinant growth factors (GFs) and cell line adaptation are **effective alternatives to FBS**.
  - **Increasing stability** and/or biological activity of GFs in culture media significantly cut costs (for the list see Table 5 in the review (Leber et al., 2025)).
  - A growing number of potentially low-cost **growth factor alternatives** can reduce media costs very significantly, but their regulatory approval status is not clear.
  - Low-cost food-grade **human serum albumin (HSA) alternatives** are available.
  - **Hydrolysates are a valid alternative to basal medium**.
  - Circular cell culture (CCC) can further lower the medium price and reduce spent medium generation.

2. Review on texturizing meat/seafood analogues

A synopsis of an overview on published methodologies to structuring and **texturization culture meat /seafood analogues** is reported in Annex 4. The **following corner points are presented:**

- An overview on scaffolding materials provided (Catalogue, Annex 2) revealed that most texturization techniques have been developed for plant-based meat analogues. More recently, these strategies have been applied for cultivated products;
- A comprehensive overview of the state-of-the-art processing techniques and plant-based functional ingredients used in the texturization of cultivated meat products;
- The challenges and the future perspective on the texturization of cellular meat analogues are presented.

In detail it is highlighted that:

- The most commonly used techniques for texturization of cultivated meat analogues are:
    - Electrospinning.
    - Shear cell technology.
    - High-moisture extrusion.
    - Freeze alignment.
    - 3D manufacturing (moulding and printing).

Additionally, the use of different physical stimulation of cells was proposed, such as electrical and/or mechanical stimulation, to biologically enhance cell differentiation, maturation and tissue organization.

- Binders and structuring agents used for scaffolding and texturization play an important role, they are made of:
    - Vegetable proteins.
    - Polysaccharide and gum-based materials.
    - Lipids and waxes.
    - Decellularized materials (e.g. decellularized plants).
    - Synthetic materials.
- The key challenges are identified:
    - Replicating the various animal tissues.
    - Limited availability of suitable materials for scaffolding.
    - Scalable scaffolding materials.
    - Cost-effective production of texturized culture meat.

3. Conceptual framework for a more efficient Circular Cell Culture (CCC) system and a waste valorization concept

Literature study was conducted to identify existing possibilities of CCC systems for CM/CSF production. Please see the extensive analysis of the topic in our review available as a pre-print (Leber et al., 2025). Shortly, the existing variants are based on the microalgae species (*Synechococcus* sp. PCC 7002), which can propagate in spent cell culture medium, and consume ammonia and - if genetically modified - consume lactate produced by the cultivated mammalian cells (Haraguchi et al., 2023). This system could be further potentially used to convert mammalian cell produced CO_2 into O_2. Microalgae CO_2 fixation rates are 10–50 times

higher than those of plants due to the existence of CO_2 concentration mechanisms (Kim et al., 2024), and this could lead to the feasibility of such application.

Two main disadvantages of the published CCC systems were identified (one must take into consideration that published CCC systems are proof-of-concept studies and can overcome these limitations upon optimization):

(1) GFs, necessary for the growth of mammalian cells, were produced by the RL34 rat liver cell line, which can hardly be used in production of food, but can of course be supplemented extra, or produced by genetically modified microalgae;

(2) Microalgae species used have quite a low doubling time under autotrophic conditions (8 hours to several days) and slow metabolism (Hidayati et al., 2020; Ma'mun et al., 2022), which would require quite equal cell quantities of microalgae as of the cultured mammalian cells, to metabolize ammonia and lactate. Although that can potentially be avoided, if heterotrophy is used instead of autotrophy, which at an industrial scale could lead to costs close to autotrophic growth. On the other hand, such slow growth rate could be of use if the co-culturing is envisioned. Another possibility is the search for other, faster growing *Synechococcus* strains of the same species. For example, *Synechococcus* sp. PCC 11901 is reported to have doubling times of less than 2.5 h and very high cell mass productivity (32.6 g/L in 14 days) when cultured with CO_2 concentrations of ~5% (Kim et al., 2024).

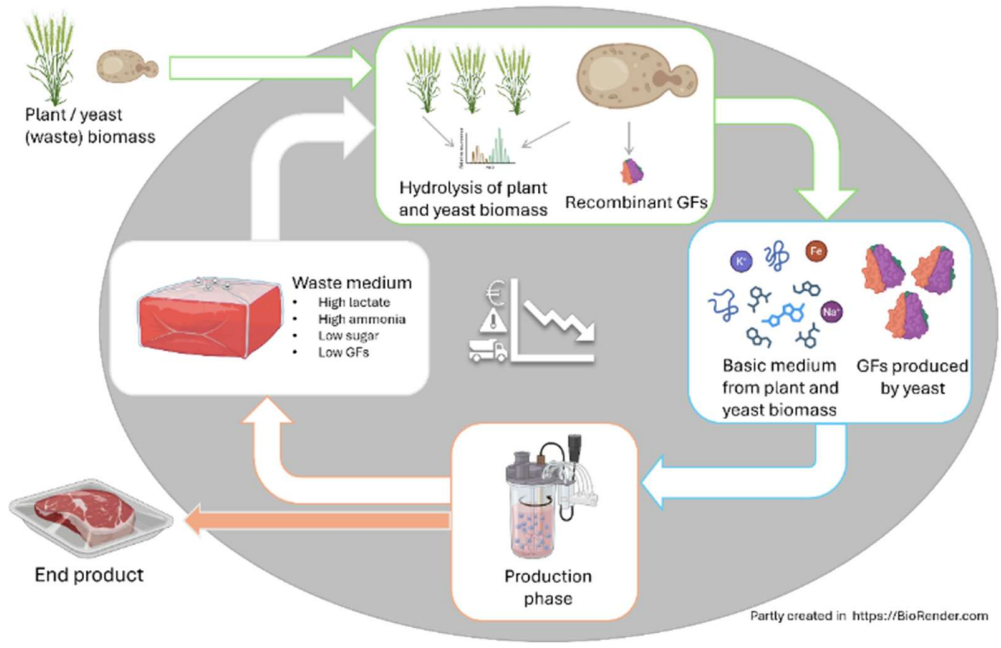

Figure 1: *Potential application scheme of using yeast species as medium recycling organisms could be a more efficient and easier to adapt system, than that is based on microalgae.*

But it might be possible to produce a much more efficient CCC system. We thus propose to utilize yeast as a recycling organism in a CCC system as following advantages can be made use of:

1. Yeast doubling time is only 1.5-2 hours (Bergman, 2001);
2. It can naturally metabolize ammonia, e.g. to produce glutamate and glutamine (Kevvai et al., 2016);
3. Some yeast species – e.g. brewer's yeast – can naturally utilize such non-sugar carbons as lactate (Turcotte et al., 2010);
4. Yeast can be very easily genetically modified to produce required GFs or to metabolize ammonia and lactate at even higher rates;
5. Several yeast species are defined as Generally Recognized As Safe (GRAS) organisms by United States Food and Drug Administration (FDA), with some species used in food industry for several hundred years (*S. cerevisie*), and others are more and more widely used in the recent time (e.g. *P. pastoris* aka *Komagataella phaffii*).

This fulfils the essential requirements for the development of yeast-based CCC systems, similar to those utilizing microalgae and rat liver cells, as discussed above. Additionally, first work laying ground for establishing such CCC system was started within this project and will be presented in full in the Final Project Reports. Please see the Report on Task 3.2 Case Study 2 (see Annex 5 below), where we demonstrate, that hydrolysates from plant and yeast can be used as a full substitution of DMEM/F12 basal medium without affecting the proliferation efficiency of the bovine satellite cells (BSCs). This is the first step in producing a closed yeast- and plant-based CCC system, which should allow much lower spent medium production rates and lower production prices.

6. Current gaps

Based on our extensive literature study, we have identified several knowledge and technology gaps in the area of media formulation and materials for scaffolding, which are summarized in the following table (partly from (Leber et al., 2025)):

Table 1: *Key challenges that remain to be addressed (white spaces).*

Topic	**Needed action / Possible solution**
Food-grade, stable and animal component - free **media**, especially for proliferation of aquatic cell lines, and isolation/cryopreservation of primary cells	Depending on species (e.g. bovine, porcine, avian, fish) and cell types: define **essential medium components** for cells' isolation, proliferation and differentiation.
Food-grade, stable and animal component - free **materials for scaffolding** compatible with animal cell biomass processing, with high nutritional and sensorial value, especially considering hybrid products formulation	Depending on the food manufacturing approach, i.e. the structuring process and whether animal cells should be included on the scaffold alive or after inactivation, different studies should be performed.
Food-safe and low-cost **growth promoting components**	Depending on species (e.g. bovine, porcine, avian, and especially fish) and cell types: identify **appropriate types of GFs** or growth promoting compounds.
	Engineer **minimal active and stable GF** versions.
	Identify low-cost **stabilizers for GFs or immobilization strategies.**
	Define **regulatory and safety constrains** for media components (Species-specific/not? Wild type/mutants? Naturally occurring/artificial? Safe rest concentrations? Reliable methods for quantification?).
Low-cost **carbohydrate sources**	**Analyse** the potential of agro-side products (e.g. molasses).
High amount of **procurement** of materials for media and scaffoldings with low environment footprint	Analyse value supply chain and availability of materials recommended on the catalogue for nutritional, sensorial, safety, but also considering the raw materials availability at scale considering different geographic locations and environmental performance of their use.
Low-cost **protein sources** (peptide or amino acid)	Analyse the potential of agro-side products (e.g. brewer's yeast, plant hydrolysates).
Low-cost and high volume of edible, safe materials (e.g. **free fatty acids, starch**, etc)	Analyse the potential of plant-based materials including agro-side products.
Better optimization and iteration of **basal media components** for scalable and highly productive cell cultures	Basal media formulation can be further optimized beyond the "DMEM/F12" baseline.

Development of **SOPs**	**Standardized** extraction methods for different nutrients (e.g. proteins, vitamins, lipids) and materials for scaffoldings from agricultural sources or industrial side-streams. Standard practices to prepare medium and materials and sterilize them on large scale.
Low level of high throughput of methodologies for **food structuring** proposed and need to have **compatible cell biomass processing and scaffolding materials** (either for viable cells or inactivated cells), promoting the adequate structural properties and without losing nutritional and sensorial properties	Develop novel approaches, processes and devices for CM/CSF structuring, including techniques with high throughput and adequate degree of resolution for food texturization, namely leveraging on knowledge from textile fabrication.
Spent medium recycling / waste management	Optimize **recovery and recycling of non-consumed** media components (glucose/amino acids/vitamins and minerals, albumins, growth factors).
	Develop techniques to **remove/upcycle harmful cellular metabolites** e.g. ammonia and lactate.
CCC-systems using mixed cell cultures	Establish and optimize a fully animal-source independent, highly efficient **multi species cell consortia** for CCC systems applicable for CM/CSF production processes.
AI-based optimization of cell culture medium and food structuring	Collect data and establish stable **models for AI/ML** approaches. Develop digital twins for mastication, electronical nose and tongues.

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8. Abbreviations

SF - serum free
FBS - fetal bovine serum
CM - cultured meat
CSF - cultured sea food
GFs - growth factors
HSA - human serum albumin
BSA - bovine serum albumin
CCC - circular cell culture
BSCs - bovine satellite cells
ESCs – embryonic stem cells
(i)PSCs – (induced) pluripotent stem cells

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Annex 1 of Deliverable 3.3:

Catalogue of media compositions and scaffold materials by target application and opportunities for cheaper and more sustainable formulations

Work Package 3 *Sustainability by design*

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1. General comments regarding media composition

The exact cell culture media compositions vary depending on the step in the CM/CSF production step, but quite a lot of the single components are overlapping. Here we present a list of the published serum free cell culture media components, used for the proliferation and differentiation of the cell lines suitable for cultured meat and seafood production. As most of the components originate from the published R&D formulations, caution must be taken in regard of regulation approval of using some of them for the product manufacturing. For example, the usage of human growth factors (GFs) should be avoided, and **preference should be given to species-specific GFs**. Components of isolation and storage media were not included as it still strongly relies on FBS, which is a serious drawback and must be addressed in the nearest future.

For the full overview of the available serum free media compositions used in the R&D and for manufacturing in cellular agriculture (state June 2025) please see our review:

- Preprint version: (Leber et al., 2025)

For the safety of the single components of the media please see following publications:

- Safety of the basal media components: (Ong et al., 2025)
- Safety of the growth factors: (Huang et al., 2024)
- Example of the additional sources of information on the safety of the media components:
    - SAFETY EVALUATIONOF CULTURED CHICKEN CELLS PRODUCED BY BELIEVER MEATS, Media Component Risk Assessment Tables 7.1.1-1 to 7.1.1-3 (https://www.fda.gov/media/188067/download)
    - RESPONSE TO FDA FOR CLARIFICATION OF QUESTIONS RELATING TO BELIEVER MEATS CULTIVATED CHICKEN SUBMISSION CCC 000039 Safety Assessment of Media Inputs (page 25) (https://www.fda.gov/media/188063/download)
    - PREMARKET NOTICE FOR INTEGRAL TISSUE CULTURED POULTRY MEAT SUBMITTED ON BEHALF OF UPSIDE FOODS (page 12 to 19) (https://www.fda.gov/media/163262/download?attachment).

1.1. Components of basal media (based on DMEM, DMEM-F12, Leibovitz L15, Neurobasal medium)

Basal medium: most commonly used is 1:1 mixture of DMEM and Ham's F12 media named **DMEM/F12;** commercially available from different suppliers. Contains 52 components including amino acids, vitamins, inorganic salts, glucose, lipids (G. Chen et al., 2011). DMEM/F12 is commonly supplemented with growth factors and proteins. Adaptions needed depending on species and cell types. DMEM/F12 with the lowest price of **€125.00 / 50 Liter** in powder form (Gibco™ DMEM/F-12 #32500043; Fischer Scientific #11504466), which leads to approx. € 107.50 / kg meat (using a high-medium scenario with 43 L medium needed for the production of 1 kg meat cells, see Table D.7 in (Sinke et al., 2023)).

Glycine		
Amino acid in basal media		
Function: Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 18.75 mg/L (DMEM/F12) 30 mg/L (DMEM; Neurobasal) 200 mg/L (Leibovitz L15)	**CAS number:** 56-40-6
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Arginine hydrochloride / L-Arginine*		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 84 mg/L (DMEM, Neurobasal medium) 126.4 (MEM AA solution, 1x) 147.5 mg/L (DMEM/F12) 500 mg/L* (Leibovitz L15)	**CAS number:** 1119-34-2 ***CAS number:** 74-79-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Cystine 2HCl / L-Cystine*		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-cysteine also serves as a precursor of essential molecules like glutathione, taurine and coenzyme A (Stipanuk et al., 2006). As cysteine is a thiol-containing molecule, it contributes to the intracellular redox-state (Bin et al., 2017) and acts as a major source of sulfur in cell culture (Chevallier et al., 2021).	**Final concentration:** 24 mg/L* (MEM AA solution, 1x) 31.29 mg/L (DMEM/F12) 31.5 mg/L* (Neurobasal medium) 63 mg/L (DMEM) 120 mg/L* (Leibovitz L15)	**CAS number:** 30925-07-6 ***CAS number:** 56-89-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Glutamine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Glutamine is a major source of energy, carbon, and nitrogen for mammalian cells, but in aqueous solutions, L-glutamine can spontaneously break down into ammonia and pyrrolidone carboxylic acid (Arii et al., 1999; Vriezen et al., 1997).	**Final concentration:** 300 mg/L (Leibovitz L15) 365 mg/L (DMEM/F12) 584 mg/L (DMEM)	**CAS number:** 56-85-9
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025), L-alanine-L-glutamine, GlutaMax™		

L-Histidine hydrochloride-H_2O / L-Histidine*		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 31.48 mg/L (DMEM/F12) 42 mg/L (DMEM; Neurobasal medium; MEM AA solution, 1x) 200 mg/L* (Leibovitz L15)	**CAS number:** 5934-29-2 ***CAS number:** 71-00-1
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Isoleucine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-Isoleucine is a branched-chain amino acid (BCAA). BCAAs are key regulators of protein synthesis, and their optimal ratio is essential to induce nutrient sensors to signal myocyte proliferation and differentiation leading to muscle growth and development (Kim et al., 2023).	**Final concentration:** 52.4 mg/L (MEM AA solution 1x) 54.47 mg/L (DMEM/F12) 105 mg/L (DMEM; Neurobasal medium) 250 mg/L (Leibovitz L15)	**CAS number:** 73-32-5
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Leucine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-Leucine is a branched-chain amino acid (BCAA). It is a key amino acid for the control of translation initiation in muscle cells (Atherton et al., 2010; Deldicque et al., 2008). BCAAs are key regulators of protein synthesis, and their optimal ratio is essential to induce nutrient sensors to signal myocyte proliferation and differentiation leading to muscle growth and development (Kim et al., 2023).	**Final concentration:** 52.4 mg/L (MEM AA solution 1x) 59.05 mg/L (DMEM/F12) 105 mg/L (DMEM; Neurobasal medium) 125 mg/L (Leibovitz L15)	**CAS number:** 61-90-5
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Lysine hydrochloride / L-Lysine*		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Lysine promoted protein synthesis in bovine mammary epithelial cells (Lin et al., 2018). It provides nitrogen for biosynthesis of nonessential amino acids (Lin et al., 2018).	**Final concentration:** 72.5 mg/L (MEM AA solution 1x) 75 mg/L* (Leibovitz L15) 91.25 mg/L (DMEM/F12) 146 mg/L (DMEM; Neurobasal medium)	**CAS number:** 657-27-2 ***CAS number:** 56-87-1
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Methionine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Methionine is an essential amino acid with many key roles in mammalian metabolism such as protein synthesis, methylation of DNA and polyamine synthesis (Cavuoto & Fenech, 2012).	**Final concentration:** 15.1 mg/L (MEM AA solution 1x) 17.24 mg/L (DMEM/F12) 30 mg/L (DMEM; Neurobasal medium) 75 mg/L (Leibovitz L15)	**CAS number:** 63-68-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Phenylalanine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 33 mg/L (MEM AA solution 1x) 35.48 mg/L (DMEM/F12) 66 mg/L (DMEM; Neurobasal medium) 125 mg/L (Leibovitz L15)	**CAS number:** 63-91-2
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Serine		
Amino acid in basal media		
Function: In cell cultures, L-serine is conditionally essential, because of its prominent role in cell proliferation (De Koning et al., 2003). L-serine functions as the predominant source of one-carbon units for the *de novo* synthesis of purine nucleotides and the pyrimidine nucleotide deoxythymidine monophosphate (Snell et al., 1987).	**Final concentration:** 26.25 mg/L (DMEM/F12) 42 mg/L (DMEM; Neurobasal medium) 200 mg/L (Leibovitz L15)	**CAS number:** 56-45-1
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Threonine		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993). L-threonine is the limiting amino acid in swine and poultry diets (Q. Tang et al., 2021).	**Final concentration:** 47.6 mg/L (MEM AA solution 1x) 53.45 mg/L (DMEM/F12) 95 mg/L (DMEM; Neurobasal medium) 300 mg/L (Leibovitz L15)	**CAS number:** 72-19-5
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Tryptophan		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-Tryptophan has a critical role in regulating skeletal muscle mass. Low levels of tryptophan reduced C2C12 myoblast cell proliferation and differentiation (Ninomiya et al., 2020). Dietary supplementation of L-tryptophan increased muscle development, adipose tissue catabolism and fatty acid transportation in the muscles of Hanwoo steers (Priatno et al., 2020).	**Final concentration:** 9.02 mg/L (DMEM/F12) 10.2 mg/L (MEM AA solution 1x) 9.02 mg/L (DMEM/F12) 16 mg/L (DMEM; Neurobasal medium) 9.02 mg/L (DMEM/F12)	**CAS number:** 73-22-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Tyrosine disodium salt dihydrate / L-Tyrosine*		
Amino acid in basal media		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993). L-Tyrosine is among the least soluble amino acids but a critical media component, as tyrosine depletion can result in the substitution of phenylalanine (H. Tang et al., 2019).	**Final concentration:** 36 mg/L* (MEM AA solution 1x) 55.79 mg/L (DMEM/F12) 72 mg/L* (Neurobasal medium) 104 mg/L (DMEM) 300 mg/L* (Leibovitz L15)	**CAS number:** 122666-87-9 ***CAS number:** 60-18-4
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Valine		
Amino acid in basal medium		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-Valine is a branched-chain amino acid (BCAA). BCAAs are key regulators of protein synthesis, and their optimal ratio is essential to induce nutrient sensors to signal myocyte proliferation and differentiation leading to muscle growth and development (Kim et al., 2023).	**Final concentration:** 46.8 mg/L (MEM AA solution 1x) 52.85 mg/L (DMEM/F12) 94 mg/L (DMEM;Neurobasal medium) 100 mg/L (Leibovitz L15)	**CAS number:** 72-18-4
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Alanine		
Amino acid in basal media		
Function: Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 2 mg/L (Neurobasal medium) 4.45 mg/L (DMEM/F12) 225 mg/L (Leibovitz L15)	**CAS number:** 56-41-7
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Asparagine-H_2O /L-Asparagine*		
Amino acid in basal media		
Function: Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 0.83 mg/L (Neurobasal medium) 7.5 mg/L (DMEM/F12) 250 mg/L* (Leibovitz L15)	**CAS number:** 5794-13-8 ***CAS number:** 70-47-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Aspartic acid		
Amino acid in basal medium		
Function: Amino acids are the basic building blocks of proteins. The demand of amino acids is determined by the metabolic requirements of cells and can differ from one cell line to another (Salazar et al., 2016; Yamamoto & Niwa, 1993).	**Final concentration:** 6.65 mg/L (DMEM/F12)	**CAS number:** 56-84-8
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Cysteine hydrochloride-H_2O		
Amino acid in basal medium		
Function: One of the 13 amino acids which were found to be essential for the survival and growth of cultured cells (Eagle, 1959). L-cysteine also serves as a precursor of essential molecules like glutathione, taurine and coenzyme A (Stipanuk et al., 2006). As cysteine is a thiol-containing molecule, it contributes to the intracellular redox-state (Bin et al., 2017) and acts as a major source of sulfur in cell culture (Chevallier et al., 2021).	**Final concentration:** 17.56 mg/L (DMEM/F12)	**CAS number:** 7048-04-6
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Glutamic Acid		
Amino acid in basal medium		
Function: L-Glutamate is the most abundant intracellular amino acid (Newsholme et al., 2003). At physiological pH, glutamic acid exists in anionic form and is referred to as glutamate. Glutamate along with glutamine plays a major role in amino acid metabolism, and in regulating nitrogen balance in the body. Glutamate plays a central role in transamination reactions (Kulkarni et al., 2005). Consumption of L-glutamate or its monosodium salt as a food additive is responsible for the umami taste (Albarracin et al., 2016).	**Final concentration:** 7.35 mg/L (DMEM/F12)	**CAS number:** 56-86-0
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

L-Proline		
Amino acid in basal media		
Function: L-proline is a non-essential amino acid that plays a crucial role in cell culture. It was shown to be necessary for growth of mature rat hepatocytes and that its requirement by the cells for induction of DNA synthesis is correlated closely with synthesis of collagen in the cells (Nakamura et al., 1984). Proline serves as a pro-oxidant in apoptotic signalling (Rivera & Maxwell, 2005) and the intracellular accumulation of proline is an adaptive stress response that affords oxidative stress protection in certain mammalian cells (Krishnan et al., 2008).	**Final concentration:** 7.76 mg/L (Neurobasal medium) 17.25 mg/L (DMEM/F12)	**CAS number:** 147-85-3
Alternatives: Extracts from yeasts, plants, algae (Leber et al., 2025)		

Choline chloride		
Vitamin precursor in basal media		
Function: One of the seven components which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). A synthesized compound choline chloride (formerly also known as vitamin B4) is used as a source of choline, which is necessary for the biosynthesis of two major cell membrane components: phosphatidylcholine and sphingomyelin. It is a standard component of cell culture media as the capacity to synthesize choline *de novo* is inadequate to meet the demand for phospholipid synthesis (Yamamoto & Niwa, 1993).	**Final concentration:** 1 mg/L (Leibovitz L15) 4 mg/L (DMEM; Neurobasal medium) 8.89 mg/L (DMEM/F12)	**CAS number:** 67-48-1

D-Calcium pantothenate		
Vitamin in basal medium		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Pantothenic acid is a vitamin of the B group. It stimulated cell proliferation in a dose-dependently manner (Weimann & Hermann, 1999). It is an essential cofactor in the synthesis of fatty acids and for the co-translational and post-translational acetylation of proteins (Driessen et al., 1985; Persson et al., 1985).	**Final concentration:** 1 mg/L (Leibovitz L15) 2.24 mg/L (DMEM/F12) 4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 137-08-6

Folic acid		
Vitamin in basal media		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Folic acid is the synthetically form of folate, a naturally water-soluble B vitamin. *In vitro* studies enumerate a cell-type and dose-dependent effect of folic acid on cell viability, proliferation, and differentiation (see review from (Bendahan et al., 2020)).	**Final concentration:** 1 mg/L (Leibovitz L15) 2.65 mg/L (DMEM/F12) 4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 59-30-3

Niacinamide		
Vitamin in basal media		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Niacinamide (nicotinamide; amide of vitamin B3), promotes cell survival and differentiation in human pluripotent stem cells. It affects human embryonic stem cell pluripotency and differentiation as a selective kinase inhibitor (Meng et al., 2018).	**Final concentration:** 1 mg/L (Leibovitz L15) 2.02 mg/L (DMEM/F12) 4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 98-92-0

Pyridoxine hydrochloride		
Vitamin in basal media		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Pyridoxine (alcohol of Vitamin B6) is serving as a co-enzymes in the glutathione based antioxidant defence system. It supports cells maintenance in cell culture, protects them from death (Danielyan & Simonyan, 2017).	**Final concentration:** 1 mg/L (Leibovitz L15) 2.013 mg/L (DMEM/F12) 4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 58-56-0

Riboflavin		
Vitamin in basal media		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Riboflavin (vitamin B2) act as cofactor for enzymes involved in oxidation–reduction reactions (Hoppel & Tandler, 1975). Riboflavin depletion caused irreversible loss of proliferative potential of colon cells *in vitro* (Nakano et al., 2011).	**Final concentration:** 0.219 mg/L (DMEM/F12) 0.4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 83-88-5

Riboflavin-5'-phosphate sodium		
Vitamin in basal medium		
Function: Most important biologically active form of Vitamin B2 - beside flavin adenine dinucleotide (Powers, 2003). One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Riboflavin (vitamin B2) act as cofactor for enzymes involved in oxidation–reduction reactions (Hoppel & Tandler, 1975). Riboflavin depletion caused irreversible loss of proliferative potential of colon cells *in vitro* (Nakano et al., 2011).	**Final concentration:** 1 mg/L (Leibovitz L15)	**CAS number:** 130-40-5

Thiamine hydrochloride / Thiamine monophosphate		
Vitamin in basal media		
Function: One of the seven vitamins which have been identified as essential for mammalian cells *in vitro* (Eagle, 1955). Thiamine hydrochloride or the phosphorylated derivative of thiamine (Vitamin B1) is an essential coenzyme in various processes in energy metabolism and the metabolism of glucose to form pentoses (see review by (Schnellbaecher et al., 2019).	**Final concentration:** 2.17 mg/L (DMEM/F12) 4 mg/L (DMEM; Neurobasal medium)	**CAS number:** 67-03-8 ***CAS number:** 10023-48-0

i-Inositol (also called myo-inositol or meso-inositol)		
(Pseudo-) Vitamin in basal media		
Function: Myo-Inositol - considered as a member of the B vitamin complex - has proven to be essential for the survival and growth of cultured human cells (Eagle et al., 1957). It plays an important role in cell morphogenesis and cytogenesis, lipid synthesis, structure of cell membranes and cell growth (Downes, 1989).	**Final concentration:** 2 mg/L (Leibovitz L15) 7.2 mg/L (DMEM, Neurobasal medium) 12.6 mg/L (DMEM/F12)	**CAS number:** 87-89-8

Biotin		
Vitamin in basal medium		
Function: Biotin (vitamin B7) functions as prosthetic group of carboxylases (Dakshinamurti et al., 1985). It plays a key role in metabolic processes, such as the citric acid cycle, fatty acid synthesis, and the catabolism of branched-chain amino acids (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1998). Biotin was identified to be required for the survival of mouse fibroblasts in cell culture (Sanford et al., 1963).	**Final concentration:** 0.0035 mg/L (DMEM/F12)	**CAS number:** 58-85-5

Vitamin B12 (also known as cyanocobalamin)		
Vitamin in basal media		
Function: Vitamin B12 is a coenzyme, playing a key role in propionate, amino acid, and single-carbon metabolism (*Encyclopedia of Human Nutrition*, 2012; *The Vitamins*, 2017). It is known to play an important role in genomic stability (G. M. Li et al., 2003), and it is also required for the synthesis of methionine and S-adenosyl methionine (Zingg & Jones, 1997). Requirements of Vitamin B12 in cell culture media seems to be cell line specific (Yamamoto & Niwa, 1993).	**Final concentration:** 0.0068 mg/L (Neurobasal medium) 0.68 mg/L (DMEM/F12)	**CAS number:** 68-19-9

Calcium Chloride ($CaCl_2$) (anhyd.)		
Inorganic salts in basal media		
Function: Calcium is involved in a wide range of vital cell functions. Therefore, $CaCl_2$ is generally supplied to cell culture media and adjusting the concentration of calcium ions is essential for cell growth because either excess or deficient calcium ions induce cell apoptosis (Hashizume et al., 2023).	**Final concentration:** 116.6 mg/L (DMEM/F12) 140 mg/L (Leibovitz L15) 200 mg/L (DMEM; Neurobasal medium)	**CAS number:** 10043-52-4

Ferric Nitrate ($Fe(NO_3)_3x9H_2O$)		
Inorganic salts in basal media		
Function: Iron is an essential transition metal in cell culture as it plays a critical role in electron transport and cellular respiration, proliferation and differentiation, and the regulation of gene expression (Arigony et al., 2013).	**Final concentration:** 0.05 mg/L (DMEM/F12) 0.1 mg/L (DMEM; Neurobasal medium)	**CAS number:** 7789-02-8

Ferric sulfate ($FeSO_4$-$7H_2O$)		
Inorganic salts in basal medium		
Function: Iron is an essential transition metal in cell culture as it plays a critical role in electron transport and cellular respiration, proliferation and differentiation, and the regulation of gene expression (Arigony et al., 2013).	**Final concentration:** 0.417 mg/L (DMEM/F12)	**CAS number:** 7782-63-0

Magnesium Sulfate ($MgSO_4$) (anhyd.)		
Inorganic salts in basal media		
Function: Magnesium is involved in multiple functions in cellular processes, which also include DNA replication and protein synthesis (Arigony et al., 2013).	**Final concentration:** 48.84 mg/L (DMEM/F12) 97.67 mg/L (DMEM; Leibovitz L15)	**CAS number:** 7487-88-9

Magnesium chloride (anhyd.)		
Inorganic salts in basal media		
Function: Magnesium is involved in multiple functions in cellular processes, which also include DNA replication and protein synthesis (Arigony et al., 2013).	**Final concentration:** 28.64 mg/L (DMEM/F12) 77.3 mg/L (Neurobasal medium) 93.7 mg/L (Leibovitz L15)	**CAS number:** 7786-30-3

Potassium Chloride (KCl)		
Inorganic salts in basal media		
Function: Monovalent ions K^+, Na^+, and Cl^- have been described as a contributing factor to the regulation of cell proliferation (Marakhova et al., 2024). KCl at the concentrations of 7–12 mM activated the expression of IGF-1 and mechano growth factor (MGF) in murine myoblasts as well as in myotubes. It also stimulated myoblast proliferation (Kravchenko et al., 2019).	**Final concentration:** 311.8 mg/L (DMEM/F12) 400 mg/L (DMEM, Leibovitz L15; Neurobasal medium)	**CAS number:** 7447-40-7

Potassium Phosphate monobasic (KH_2PO_4)		
Inorganic salts in basal medium		
Function: KH_2PO_4 is a source of phosphorus and potassium as well as a buffering agent in cell culture media. Monovalent ions e.g. K^+, Na^+, and Cl^- have been described as a contributing factor to the regulation of cell proliferation (Marakhova et al., 2024).	**Final concentration:** 60 mg/L (Leibovitz L15)	**CAS number:** 7778-77-0

Sodium Bicarbonate ($NaHCO_3$)		
Inorganic salts in basal media		
Function: Sodium bicarbonate is a commonly used buffer compound in mammalian cell cultures. Particularly, media exposed to an atmosphere enriched in CO_2 must include an appropriate concentration of HCO_3^- salt to stabilize at the required pH (Michl et al., 2019).	**Final concentration:** 2200 mg/L (Neurobasal medium) 2438 mg/L (DMEM/F12) 3700 mg/L (DMEM)	**CAS number:** 144-55-8

Sodium Chloride (NaCl)		
Inorganic salts in basal media		
Function: NaCl is a ubiquitous osmolyte in the human body. Cell culture medium is designed to have osmolarity in the range of 260 and 320 mOsm (Ozturk & Palsson, 1991). Cell proliferation rate of rat keratinocytes increased as the NaCl concentration increased up to 100 mM and thereafter appeared to plateau (Oku et al., 1999).	**Final concentration:** 3000 mg/L (Neurobasal medium) 6400 mg/L (DMEM) 6999.5 mg/L (DMEM/F12) 8000 mg/L (Leibovitz L15)	**CAS number:** 7647-14-5

Sodium Phosphate monobasic (NaH_2PO_4-H_2O)		
Inorganic salts in basal media		
Function: NaH_2PO_4-H_2O is a reagent with very high buffering capacity. Useful in conjunction with sodium dibasic phosphate in biological buffers (https://www.mpbio.com/).	**Final concentration:** 62.5 mg/L (DMEM/F12) 125 mg/L (DMEM; Neurobasal medium)	**CAS number:** 10049-21-5

Sodium Phosphate dibasic (Na_2HPO_4) anhydrous		
Inorganic salts in basal media		
Function: Sodium phosphate is used in conjunction with sodium phosphate dibasic in the preparation of biological buffers (https://www.mpbio.com/).	**Final concentration:** 71.2 mg/L (DMEM/F12) 190 mg/L (Leibovitz L15)	**CAS number:** 7558-79-4

Cupric sulfate ($CuSO_4$-$5H_2O$)		
Inorganic salts in basal medium		
Function: Copper is an essential trace element, serving as a cofactor for many enzymes in different biological processes (Arigony et al., 2013). It is contained in serum (e.g. 0.7 µg mL^{-1} in bovine serum). Addition of copper sulfate (5 µM) to cell culture medium significantly decreased lactate accumulation while increasing viable CHO cell density (Y. Qian et al., 2011).	**Final concentration:** 0.0013 mg/L (DMEM/F12)	**CAS number:** 7758-99-8

Zinc sulfate ($ZnSO_4$-$7H_2O$)		
Inorganic salts in basal media		
Function: Zinc is one of the most important micronutrients due to the prevalence of zinc-dependent enzymes in metabolic processes. It has a vital role in cell reproduction, growth, and immunity (Arigony et al., 2013).	**Final concentration:** 0.194 mg/L (Neurobasal medium) 0.432 mg/L (DMEM/F12)	**CAS number:** 7446-20-0

D-Glucose (Dextrose)		
Carbon source in basal media		
Function: Glucose is the major carbon source for cellular biosynthesis and energy generation. Cells consume large amounts of glucose, nearly 90% of which is converted to lactate during aerobic glycolysis (Mulukutla et al., 2010).	**Final concentration:** 3151 mg/L (DMEM/F12) 4500 mg/L (DMEM; Neurobasal medium)	**CAS number:** 50-99-7
Alternatives: Maltose (Leong et al., 2017); Raffinose and other polysaccharides (Hossler et al., 2017); molasses (no ref for mammalian cell culture found).		

D-Galactose		
Carbon source in basal media		
Function: Carbon source, which led to a low survival rate of CHO cells when cultured with galactose instead of glucose as the sole carbon source (Neermann & Wagner, 1996). But when cultured in a medium containing both glucose and galactose, cells reduced lactate generation (H.-Y. Zhang et al., 2021). A simultaneous replacement of glucose and glutamine by the combination of galactose and glutamate allows the maintenance of cells with minimal consumption of nutrients and minimal generation of lactate and ammonium, although cell growth is reduced (Altamirano et al., 2000). In a static culture of a lymphoblastoid cell line, lactate levels were significantly lowered when the cells were cultured on galactose as a carbon source (Marquis et al., 1996).	**Final concentration:** 900 mg/L (Neurobasal medium)	**CAS number:** 59-23-4

Hypoxanthine Na		
Component in basal medium		
Function: Hypoxanthine is a nutrient additive for a variety of cell culture applications. It stimulated DNA synthesis in serum-starved mouse L cells treated with platelet protein (Klenow & Flodgaard, 1983). A combination of hypoxanthine and thymidine stimulated the initial cell growth of CHO cells in serum-free medium (F. Chen et al., 2012). Hypoxanthine is also a major metabolite in the degradation of ATP. It accumulates continuously in fish and beef, as well as in organs e.g. skeletal muscle after death (Mao et al., 2001).	**Final concentration:** 2.39 mg/L (DMEM/F12)	**CAS number:** 68-94-0

Linoleic Acid		
Component in basal medium		
Function: Linoleic acid (18:2, n-6) is an essential nutrient for animals and must be provided in serum-free cell culture media to enhance proliferation of mammalian cells (Bandyopadhyay et al., 1987; Butler et al., 1999). In addition, the survival rate of cells grown in media supplemented with linoleic acid improved significantly under high stirring rates (Butler et al., 1999).	**Final concentration:** 0.042 mg/L (DMEM/F12) 10 mg/L (Gibco™ 11905031) 0.042 mg/L (Oku et al., 2006)	**CAS number:** 60-33-3
Alternatives: Plant seed oils (e.g. soy bean oil, 53 % linoleic acid (Dunford, 2018), lipid concentrate (Gibco™ 11905031)		

Lipoic Acid		
Component in basal medium		
Function: Lipoic acid is synthesized by eukaryotic cells; it is involved in several aspects of cell energy and amino acid metabolism, as well as in the defence against oxidative stress and apoptosis (Abd El-Fadile & Hussine, 2016). Lipoic acid increased the *ex vivo* expansion of steady state peripheral blood cells (Debeissat et al., 2022).	**Final concentration:** 0.105 mg/L (DMEM/F12)	**CAS number:** 1077-28-7
Alternatives: Plant seed oils (Lodge & Packer, 1999).		

Putrescine 2HCl		
Component in basal medium		
Function: Polyamines such as putrescine, spermine, and spermidine, are ubiquitous in living organisms. They have various important functions in eukaryotic cell proliferation and differentiation, gene regulation, cell signalling, and apoptosis (Mandal et al., 2013; Sagar et al., 2021). Rapidly dividing cells contain a higher amount of polyamines (Sagar et al., 2021).	**Final concentration:** 0.081 mg/L (DMEM/F12)	**CAS number:** 333-93-7
Alternatives: Plant extracts (González-Hernández et al., 2022).		

Sodium Pyruvate		
Component in basal media		
Function: Pyruvate functions as a key molecule in energy production and as an antioxidant (Yako et al., 2021).	**Final concentration:** 25 mg/L (Neurobasal medium) 55 mg/L (DMEM/F12) 550 mg/L (Leibovitz L15)	**CAS number:** 113-24-6

Thymidine		
Component in basal medium		
Function: Thymidine primarily serves as nucleoside for DNA synthesis. It is also utilized to obtain synchronous cultures of mammalian cells (Bergeron, 1971). A combination of hypoxanthine and thymidine stimulated the initial cell growth of CHO cells in serum-free medium (F. Chen et al., 2012).	**Final concentration:** 0.365 mg/L (DMEM/F12)	**CAS number:** 50-89-5

HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid))		
Buffering component in basal medium		
Function: HEPES is a zwitterionic buffering agent commonly used in cell culture media. HEPES has more buffering capacity than sodium bicarbonate (Itagaki & Kimura, 1974). However, cells in low density cultures were limited in their proliferative capacities because of lack of a critical level of CO_2 in HEPES-buffered medium (Itagaki & Kimura, 1974).	**Final concentration:** 2600 mg/L (Neurobasal medium) 4.9 mM (Mitić et al., 2023a)	**CAS number:** 7365-45-9
Alternative: sodium bicarbonate buffering		

1.2. Components of proliferation media

These components are typically added to basal medium, such as DMEM/F12. See review (Leber et al., 2025) for the full list of compositions.

rInsulin (recombinant Insulin)		
Proliferation media; part of standard adipogenic induction cocktail and differentiation medium for myogensis		
Function: Polypeptide hormone; has pleiotropic effects on the regulation of cellular growth and differentiation (Khil et al., 1997).	**Final concentration:** 3 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2020; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021) 1.8 µM (Messmer et al., 2022)	**CAS number:** 11061-68-0 Recombinant bovine insulin is available from GenScript, # Z03735-1
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Ascorbic acid 2-phosphate		
Proliferation and differentiation media		
Function: At low concentration, ascorbic acid stimulates cell proliferation without reciprocal loss of phenotype and differentiation potency. Stimulates collagen and glycosaminoglycan secretion in MSCs. High concentrations induce osteogenesis and adipogenesis (Sisakhtnezhad et al., 2017).	**Final concentration:** 50 – 200 mg/L (G. Chen et al., 2011; Kolkmann et al., 2020; Kuo et al., 2020; Stout et al., 2021) 40 µM (Messmer et al., 2022)	**CAS number:** 23313-12-4

rTransferrin (recombinant Transferrin)		
Proliferation and differentiation media		
Function: Transferrin, an iron chelator regulates the bioavailability of iron to cells and supports growth in serum-free media (Keenan et al., 2006).	**Final concentration:** 5.5 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Skrivergaard et al., 2023; Stout et al., 2021). 135 nM (Messmer et al., 2022).	**CAS number:** 11096-37-0 Bovine Transferrin is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Sodium selenite		
Proliferation media and differentiation medium (myogenesis)		
Function: Sodium selenite protects cells from oxidative damage by reducing the production of free radicals and inhibits lipid peroxidation (Abedelahi et al., 2008).	**Final concentration:** 6.7 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021). 80 nM (Messmer et al., 2022).	**CAS number:** 10102-18-8

rFGF-2 (recombinant Fibroblast Growth Factor-2, also known as basic FGF)		
Growth factor in proliferation media		
Function: FGF-2 is a pleiotropic cytokine and a member of the heparin-binding FGF family. It regulates a variety of processes including cell proliferation, differentiation, survival, adhesion, etc (Huang et al., 2024).	**Final concentration:** 2 – 100 µg/L growth: (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021).	**CAS number:** 148348-15-6* Available for several species from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

*Most companies do not provide a CAS number for their rFGF-2.

rFGF-2_G3 (recombinant thermostable engineered form of FGF-2 (basic FGF))		
Growth factor in proliferation media		
Function: FGF2-G3 is a thermostable engineered form of FGF-2 (bFGF). It comprises a 145 aa form of FGF-2 with increased functional half-life (from <10 h (wild-type) to >7 days (FGF2-G3) (Dvorak et al., 2018).	**Final concentration:** 40 µg/L growth: (Kuo et al., 2020; Stout et al., 2021).	**CAS number:** NO CAS number registered. Available as bovine and human FGF2-G3 from https://definedbioscience.com/
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rTGFß1 (recombinant Transforming Growth Factor beta 1)		
Growth factor in proliferation media		
Function: TGF-ß1 is one of the three isoforms of growth promoting TGF-ß´s. In long-term growth studies with porcine SMCs, TGF-β promoted the gene and protein expression of collagen (Zheng et al., 2023). TGF-β1 treatment improved myogenesis in 3D mono- and co-cultures containing human muscle cells, but not in 2D (Krieger et al., 2018). TGF- ß 1 is a homodimer of two TGFB1 gene products, making its recombinant expression in an inexpensive *E.coli* impossible.	**Final concentration:** 0.1 – 2 µg/L growth: (G. Chen et al., 2011; Kuo et al., 2020).	**CAS number:** NO CAS number registered. Several companies offer this recombinant growth factor.
Alternatives: In-house production in yeasts, plants, etc.		

rTGFß3 (recombinant Transforming Growth Factor beta 1)		
Growth factor in proliferation media		
Function: TGF-ß3 is one of the three isoforms of growth promoting TGF-ß´s. TGF-β3 is structurally less complex than TGF-β1 and be expressed biologically active in *E.coli*. It was also shown to be more potent than TGF-β1 in a chemoregulated cell migration assay for dermal fibroblasts (Huang et al., 2014), and had a comparable activity to TGF-β1 in a hiPSC line 19c3 using a long-term five-passage, 4-day growth assay (Kuo et al., 2020).	**Final concentration:** 0.1 µg/L growth: (Kuo et al., 2020; Stout et al., 2021).	**CAS number:** NO CAS number registered. Several companies offer this recombinant growth factor.
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rNGR1 (recombinant Neuregulin 1)		
Growth factor in proliferation media		
Function: NRG1 growth factor proteins play essential roles in the nervous system, heart, and breast as well as in the development and function of several other organ systems (Falls, 2003). NRG1 can act as a potent mitogen in cardiomyocytes, induced pluripotent stem cells (iPSCs), and satellite cells (Gemberling et al., 2015; Kuo et al., 2020; Stout et al., 2021).	**Final concentration:** 0.1 µg/L growth: (Kuo et al., 2020; Stout et al., 2021).	**CAS number:** NO CAS number registered. Several companies offer this recombinant growth factor.
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rVEGF (recombinant Vascular Endothelial Growth Factor)		
Growth factor in proliferation media		
Function: VEGF is a potent growth and angiogenic cytokine. It is known to have an antiapoptotic effect and it has been shown to increase muscle-derived stem cell proliferation in a dose-dependent manner (Germani et al., 2003; H. D. Wang et al., 2017).	**Final concentration:** 10 µg/L growth: (Kolkmann et al., 2022)	**CAS number:** 127464-60-2 Bovine available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rIGF-1 (recombinant Insulin-like Growth Factor)		
Growth factor in proliferation and differentiation media		
Function: IGF-1 is one of the most potent natural activators of the AKT signalling pathway, which stimulates cell growth and proliferation, and inhibits cell apoptosis. The IGF-1 analogue LR3 IGF-1 has increased stability and biological activity (https://www.oryzogen.net/view/27.html)	**Final concentration:** 100 µg/L growth: (Kolkmann et al., 2022) 10 ng/mL differentiation: (McAleer et al., 2015; Stout et al., 2023)	**CAS number:** 67763-96-6 Bovine/porcine available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rHGF (recombinant Hepatocyte Growth Factor)		
Growth factor in proliferation media		
Function: HGF is a heparin-binding protein that activates satellite cells and regulates cell proliferation in a dose-dependent manner (White & Smythe, 2016; Yamada et al., 2010). HGF acts synergistically with FGF-2 or PDGF (Syverud et al., 2016).	**Final concentration:** 5 - 20 µg/L growth: (Kolkmann et al., 2022; Skrivergaard et al., 2023)	**CAS number:** NO CAS number registered. Several companies offer this recombinant growth factor. **Accession No:** Human (P14210)
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rPDGF-BB (recombinant Platelet-Derived Growth Factor-BB)		
Growth factor in proliferation media		
Function: PDGF-BB is a powerful promoter of cell proliferation and increases the expression of stem cell markers (Mihaylova et al., 2018). A combination of TGF-ß, PDGF, and FGF promoted human mesenchymal stem cell (MCSs) proliferation in a serum-free medium up to 5 passages (Ng et al., 2008). This combination was also shown to be important for the differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages (Ng et al., 2008).	**Final concentration:** 5 - 10 µg/L growth: (Kolkmann et al., 2022; Skrivergaard et al., 2023)	**CAS number:** NO CAS number registered. Several companies offer this recombinant growth factor. **Accession No:** Human (P01127) Porcine PDGF is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Sodium Bicarbonate ($NaHCO_3$)		
Buffering agent used in cell cultures.		
Function: Media exposed to an atmosphere enriched in CO_2 must include an appropriate concentration of HCO_3^- salt to stabilize the pH in the physiological area (Michl et al., 2019).	**Final concentration:** 543 – 2428 mg/L growth: (G. Chen et al., 2011; Kuo et al., 2020; Stout et al., 2021). 6.5 mM (Messmer et al., 2022)	**CAS number:** 144-55-8

<table>
<tr><td colspan="4">rHSA (recombinant Human Serum Albumin)</td></tr>
<tr><td colspan="4">Proliferation (CM) and differentiation media</td></tr>
<tr><td colspan="2">Function:
As albumin is the major protein in FBS, it is used in serum-free media formulations (Francis, 2010). Albumins are relevant in mammalian cell culture due to their antioxidant properties and as transporter of biologically important ligands (Francis, 2010).
Albumins have also a protective role for cells against physical damage in bioreactors (Chisti, 2001) (Chisti, 2001 in Francis 2010) and for stabilization of GFs (Benington et al., 2021).</td><td>Final concentration:
800 mg/L (Stout et al., 2021)
5 g/L (Kolkmann et al., 2022)
0.5 mg/ml (Messmer et al., 2022)</td><td>CAS number:
70024-90-7</td></tr>
<tr><td>Alternatives:</td><td>Methylcellulose: preservation of cell function in suspension (Stewart et al., 1995), cell aggregation for 3D spheroid formation (Maritan et al., 2017), growth factor stabilizer (Schenzle et al., 2025).</td><td>Final concentration:
0.5 % (w/v) (Benington et al., 2021)
0.05 – 0.1 % (w/v) (Pasitka et al., 2024)
0.1125 g/L (Schenzle et al., 2025)</td><td>CAS number:
9032-42-2</td></tr>
<tr><td></td><td>Plant protein isolates: can support BSCs growth in vitro to the same extent or better than HSA (Egger et al., 2025; Stout et al., 2023).</td><td>Final concentration:
0.4 g/L (Stout et al., 2023)
0.12 g/L (Egger et al., 2025)</td><td></td></tr>
</table>

BSA (Bovine Serum Albumin, isolated from bovine serum)			
Proliferation (CM) and differentiation media			
Function: As albumin is the major protein in FBS, it is used in serum-free media formulations (Francis, 2010). Albumins are relevant in mammalian cell culture due to their antioxidant properties and as transporter of biologically important ligands (Francis, 2010). Albumins have also a protective role for cells against physical damage in bioreactors (Chisti, 2001) and for stabilization of GFs (Benington et al., 2021).		**Final concentration:** 75 mg/L BSA solution from Sigma, A8412 (Skrivergaard et al., 2023)	**CAS number:** 9048-46-8
Alternatives:	**Methylcellulose** preservation of cell function in suspension (Stewart et al., 1995), cell aggregation for 3D spheroid formation (Maritan et al., 2017), growth factor stabilizer (Schenzle et al., 2025).	**Final concentration:** 0.5 % (w/v) (Benington et al., 2021) 0.05 – 0.1 % (w/v) (Pasitka et al., 2024) 0.1125 g/L (Schenzle et al., 2025)	**CAS number:** 9032-42-2

Fetuin (isolated from fetal bovine serum)		
Proliferation (CM) and differentiation media (Adipogenesis)		
Function: Fetuin is a multifunctional, heterodimeric plasma glycoprotein. It is involved e.g. in regulation of insulin signalling, calcium metabolism, and it displays antiapoptotic activities (Chekol Abebe et al., 2022). These diverse effects of fetuin are brought about by its interaction with a plethora of receptors, like for insulin and growth factors (Chekol Abebe et al., 2022). Fetuin has also adipogenic properties that increase the uptake and storage of FFA in adipocytes (Chekol Abebe et al., 2022).	**Final concentration:** 600 mg/L growth: (Skrivergaard et al., 2023)	**CAS number:** 9014-81-7
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Hydrocortisone		
Proliferation media		
Function: The steroid hormone hydrocortisone naturally occurs in FBS; it is commonly used as a supplement for cell culture media as it is known to support adhesion and growth of various mammalian cells (Croisille et al., 1994; Shipunova et al., 2013).	**Final concentration:** 36 µg/L to 2 mg/L (Kolkmann et al., 2022; Pasitka et al., 2024)	**CAS number:** 50-23-7

GlutaMax™		
Proliferation media		
Function: GlutaMAX-I is a stable L-glutamine derivative; a dipeptide which slowly releases L-alanine and L-glutamine into the culture medium (https://www.thermofisher.com/). Glutamine is regarded as the most abundant amino acid in the body, is mainly synthesized in skeletal muscle (Curi et al., 2005). It promotes cell proliferation as a precursor for the synthesis of purine and pyrimidine nucleotides and by serving as an auxiliary energy source, especially when the cells are rapidly dividing (Zhao et al., 2021).	**Final concentration:** 1% (Kolkmann et al., 2022)	**CAS number:** NO CAS number registered.
Alternatives: Glutamine, L-alanine-L-glutamine.		

L-alanine-L-glutamine		
Proliferation media		
Function: Stable Glutamine (also known as L-Alanyl-L-Glutamine Solution) is a substitute for glutamine in mammalian cell culture media (https://www.thermofisher.com/). Glutamine is regarded as the most abundant amino acid in the body, is mainly synthesized in skeletal muscle (Curi et al., 2005). It promotes cell proliferation as a precursor for the synthesis of purine and pyrimidine nucleotides and by serving as an auxiliary energy source, especially when the cells are rapidly dividing (Zhao et al., 2021).	**Final concentration:** 2 mM = 0.434 g/L (Pasitka et al., 2022) 4 mM (Pasitka et al., 2024)	**CAS number:** 39537-23-0
Alternatives: GlutaMax™		

rIL-6 (recombinant Interleukin 6)		
Proliferation media		
Function: Interleukin 6 (IL-6) is a pleiotropic cytokine produced and secreted by skeletal muscle (Pedersen et al., 2001). Recent evidence indicates that IL-6 plays an essential role to SC dynamics during periods of compensatory muscle growth (Brandt et al., 2018). Human IL-6 stimulates bovine satellite cell proliferation; however, bovine satellite cells did not respond to bovine IL-6 *in vitro* (Brandt et al., 2018).	**Final concentration:** 20 µg /L growth: (Kolkmann et al., 2022)	**CAS number:** NO CAS number registered. Accession No: Human (P05231) Bovine IL-6 is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Alpha-Linoleic Acid		
Proliferation media		
Alpha-linolenic acid (18:3, n-3) is a plant-based essential polyunsaturated fatty acid. It exhibits anticancer, anti-inflammatory, and antioxidative effects (Zhou et al., 2022). Supplementation with alpha-linolenic acid and essential amino acids improved the proliferation and differentiation of C2C12 cells. Moreover, it increased the expression levels of myogenic factors 4 (myogenin) and 5 during C2C12 cell differentiation (Zhou et al., 2022). Addition of polyunsaturated fatty acids to cell cultures modifies the total lipid fatty acid composition of cells. Alpha-linolenic acid supplementation resulted in a significant decrease in arachidonic acid in macrophage cell lines (Wiesenfeld et al., 2001).	**Final concentration:** 1 mg/L growth: (Kolkmann et al., 2022)	**CAS number:** 463-40-1
Alternatives: **Flaxseed oil:** Alpha-linolenic acid accounts for approximately 55% of the total fatty acids in flaxseed (Wiesenfeld et al., 2001).		

Canola lipid mixture		
Proliferation media		
Canola was developed from rapeseed using traditional plant-breeding techniques. The name canola refers to those cultivars containing less than 5 percent erucic acid in the oil and 3 mg/g aliphatic glucosinolates in the meal (Dunford, 2018). Edible oils are primarily composed of triacylglycerides. Canola oil is rich in oleic acid (62 %) and linoleic acid (22 %) (for fatty acid analysis see (Dunford, 2018)). It is also a good source of vitamin E (alpha tocopherol) (Dunford, 2018).	**Final concentration:** 10 mg/L growth: (Pasitka et al., 2024)	**CAS number:** 120962-03-0

1.3. Components of differentiation medium

Differentiation medium differ greatly depending on the required final cell type (muscle or fat cell). Full medium compositions can be found in the review (Leber et al., 2025). Components listed here are supplemented additionally to the basal medium (typically DMEM/F12). In some cases 1:1 L15 and Neurobasal media was used as basal medium for differentiation (McAleer et al., 2015; Stout et al., 2023), and their components are listed in the chapter 1.1. Components of basal media.

1.3.1. Myogenesis media

rEGF (recombinant Epidermal Growth Factor)		
Growth factor in differentiation media		
Function: Epidermal growth factor (EGF) is a 53 amino acid polypeptide that stimulates a variety of cellular processes, including proliferation, survival, and differentiation (Wells, 1999). EGF promotes mammalian cell growth by suppressing cellular senescence (Alexander et al., 2015).	**Final concentration:** 100 ng/mL (Diff: (McAleer et al., 2015; Stout et al., 2023)) 10 ng/mL (Messmer et al., 2022)	**CAS number:** 62229-50-9 Sec. no existing: 62253-63-8 Porcine EGF is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Acetylcholine		
Differentiation medium (myogenesis)		
Function: Acetylcholine is the synaptic transmitter between motoneurons and skeletal muscle fibres of vertebrates. Myogenic cells arising by cell division *in vitro* can become sensitive to acetylcholine and construct myofibrils without cell fusion (Fambrough & Rash, 1971).	**Final concentration:** 10 µM (Messmer et al., 2022)	**CAS number:** 51-84-3

Lysophosphatic acid (LPA, also known as 1-oleoyl-*sn*-glycero-3-phosphate sodium salt)		
Differentiation medium (myogenesis)		
Function: The bioactive phospholipid lysophosphatidic acid possesses hormone- and growth-factor-like properties (van der Bend et al., 1992). LPA induced keratinocyte proliferation through transforming growth factor-α induction (Piazza et al., 1995) and induced keratinocyte differentiation (Sumitomo et al., 2019). LPA present in conditioned media increased the growth of a preadipose cell line in culture (Pagès et al., 2000).	**Final concentration:** 1 µM (Messmer et al., 2022)	**CAS number:** *325465-93-8*

rIGF-1 (recombinant Insulin-like Growth Factor)		
Growth factor in proliferation and differentiation media		
Function: IGF-1 is one of the most potent natural activators of the AKT signalling pathway, which stimulates cell growth and proliferation, and inhibits cell apoptosis. The IGF-1 analogue LR3 IGF-1 has increased stability and biological activity (https://www.oryzogen.net/view/27.html)	**Final concentration:** 100 µg/L growth: (Kolkmann et al., 2022) 10 ng/mL differentiation: (McAleer et al., 2015; Stout et al., 2023)	**CAS number:** 67763-96-6 Bovine/porcine available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

1.3.2. Adipogenesis-Media:

L-alpha-Phospathidylcholine (soy lecithin)		
Differentiation medium (adipogenesis)		
Function: Phosphatidylcholine is one of the most abundant phospholipids in mammalian cell membranes (Van der Veen, 2017: https://doi.org/10.1016/j.bbamem.2017.04.006). L-a-phosphatidylcholine at 80 µg/ml supported long-term cell proliferation of gibbon ape lymphoid cells in serum-free medium (Brown et al., 1983).	**Final concentration:** 12 µg/mL (Pasitka et al., 2022)	**CAS number:** 8002-43-5
Alternatives: Plant oils		

Putrescine		
Differentiation medium (adipogenesis)		
Function: Polyamines such as putrescine, spermine, and spermidine, are ubiquitous in living organisms. They have various important functions in eukaryotic cell proliferation and differentiation, gene regulation, cell signalling, and apoptosis (Mandal et al., 2013; Sagar et al., 2021).	**Final concentration:** 57 µM each (Mitić et al., 2023a)	**CAS number:** 110-60-1
Alternatives:	Plant extracts (Ali et al., 2011)	

Progesterone		
Differentiation medium (adipogenesis)		
Function: Steroid hormones, as estrogen and progesterone, regulate a wide range of physiological processes including cell differentiation and development, cellular homeostasis and reproduction (Baker, 2003).	**Final concentration:** 17.8 nM (Mitić et al., 2023a)	**CAS number:** 57-83-0

Hydrocortisone		
Differentiation medium (adipogenesis)		
Function: Hydrocortisone (or cortisol) is the most well-known glucocorticoid in the human body. Cortisol adversely affects animal growth and development (Siddiqui et al., 2021). It altered the morphology of satellite cells and suppressed myogenesis (Pandurangan et al., 2014), whereas the addition of cortisol to a chemically defined serum-free medium resulted in a potent dose-dependent stimulation of the adipose differentiation process (Hauner et al., 1989).	**Final concentration:** 25 nM (Mitić et al., 2023a) 50 ng/mL (Oku et al., 2006)	**CAS number:** 50-23-7

Calcium Chloride ($CaCl_2$) (anhyd.)		
Inorganic salts in differentiation medium (adipogenesis)		
Function: Calcium is involved in a wide range of vital cell functions. Therefore, $CaCl_2$ is generally supplied to cell culture media and adjusting the concentration of calcium ions is essential for cell growth because either excess or deficient calcium ions induce cell apoptosis (refs 40–43 in Hashizumo, 2023).	**Final concentration:** 1 mM (Mitić et al., 2023a)	**CAS number:** 10043-52-4

Ascorbic acid 2-phosphate		
Differentiation medium (adipogenesis)		
Function: At low concentration, ascorbic acid stimulates cell proliferation without reciprocal loss of phenotype and differentiation potency. High concentrations induce osteogenesis and adipogenesis (Choi, 2008).	**Final concentration:** 227 µM (Mitić et al., 2023a)	**CAS number:** 23313-12-4

rFGF-2 (recombinant Fibroblast Growth Factor-2, also known as basic FGF)		
Growth factor in proliferation media		
Function: FGF-2 is a pleiotropic cytokine and a member of the heparin-binding FGF family. It regulates a variety of processes including cell proliferation, differentiation, survival, adhesion, etc (Huang et al., 2024).	**Final concentration:** 2 – 100 µg/L growth: (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021).	**CAS number:** 148348-15-6* Available for several species from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

*Most companies do not provide a CAS number for their rFGF-2.

rEGF (recombinant Epidermal Growth Factor)		
Growth factor in differentiation media		
Function: Epidermal growth factor (EGF) is a 53 amino acid polypeptide that stimulates a variety of cellular processes, including proliferation, survival, and differentiation (Wells, 1999). EGF promotes mammalian cell growth by suppressing cellular senescence (Alexander et al., 2015).	**Final concentration:** 100 ng/mL (Diff: McAleer, 2015; Stout, 2023) 10 ng/mL (Messmer et al., 2022)	**CAS number:** 62229-50-9 Sec. no existing: 62253-63-8 Porcine EGF is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rBMP-4 (recombinant Bone Morphogenetic Protein 4)		
Differentiation medium (adipogenesis)		
Function: TGF-β superfamily (Huang & Hinck, 2016). They play significant roles in adipocyte development. BMP-4 is an important regulator of white adipogenesis (Hoffmann et al., 2017); however, BMP-4 also enhances browning of white adipocytes (S.-W. Qian et al., 2013).	**Final concentration:** 10 ng/mL (Mitić et al., 2023a)	**CAS number:** No CAS number available

rInsulin (recombinant Insulin)		
Proliferation media; part of standard adipogenic induction cocktail and differentiation medium for myogensis		
Function: Polypeptide hormone; has pleiotropic effects on the regulation of cellular growth and differentiation (Khil et al., 1997).	**Final concentration:** 3 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2020; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021) 1.8 µM (Messmer et al., 2022)	**CAS number:** 11061-68-0 Recombinant bovine insulin is available from GenScript, # Z03735-1
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

rTransferrin (recombinant Transferrin)		
Proliferation and differentiation media		
Function: Transferrin, an iron chelator regulates the bioavailability of iron to cells and supports growth in serum-free media (Keenan et al., 2006).	**Final concentration:** 5.5 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Skrivergaard et al., 2023; Stout et al., 2021). 135 nM (Messmer et al., 2022).	**CAS number:** 11096-37-0 Bovine Transferrin is available from ORFgenetics
Alternatives: In-house production in bacteria, yeasts, plants, etc.		

Sodium selenite		
Proliferation media and differentiation medium (myogenesis)		
Function: Sodium selenite protects cells from oxidative damage by reducing the production of free radicals and inhibits lipid peroxidation (Abedelahi et al., 2008).	**Final concentration:** 6.7 – 20 mg/L (G. Chen et al., 2011; Kolkmann et al., 2022; Kuo et al., 2020; Pasitka et al., 2022; Skrivergaard et al., 2023; Stout et al., 2021). 80 nM (Messmer et al., 2022).	**CAS number:** 10102-18-8

<table>
<tr><td colspan="4">Rosiglitazone (5-[[4-[2-(methyl-2-pyridinylamino)ethoxy]phenyl]methyl]-2,4-thiazolidinedione)</td></tr>
<tr><td colspan="4">Differentiation medium (adipogenesis)</td></tr>
<tr><td colspan="2">Function:
Thiazolidinediones (TZDs) like Rosiglitazone are selective activators of peroxisome proliferator-activated receptor γ (PPARγ) (Lehmann et al., 1995) through which they trigger adipocyte differentiation and adipose tissue remodelling (Fürnsinn & Waldhäusl, 2002).</td><td>Final concentration:
5 µM (Mitić et al., 2023a)</td><td>CAS number:
122320-73-4</td></tr>
<tr><td>Alternatives:</td><td>A number of natural compounds e.g. alpha-linolenic acid, linoleic acid, narigenin, quercetin etc are agonists of PPARγ (see review (L. Wang et al., 2014).</td><td></td><td></td></tr>
</table>

<table>
<tr><td colspan="4">Lipid Concentrate (Chemically Defined) – see full composition below</td></tr>
<tr><td colspan="4">Differentiation medium (adipogenesis);</td></tr>
<tr><td colspan="2">Function:
Chemically Defined Lipid Concentrate (Gibco™ 11905031) is a concentrated lipid emulsion that contains a defined combination of saturated and unsaturated fatty acids, cholesterol, vitamin E and emulsifiers
(https://www.thermofisher.com/at/en/home/technical-resources/media-formulation.249.html).
Free fatty acids are promoting lipid accumulation during adipogenesis (Yanting et al., 2018).</td><td>Final concentration:
0.1 % (Mitić et al., 2023a)</td><td>CAS number:
No Cas number registered for whole mixture.</td></tr>
<tr><td>Alternatives:</td><td>Mixture of 6 free fatty acids
Tested with bovine adipose-derived stem cells (Louis et al., 2023).</td><td>Final concentration:
100 µM each (Louis et al., 2023).</td><td></td></tr>
<tr><td></td><td>Oleic acid only (no DEX and IMBX; bovine SCs, (X. Z. Li et al., 2019))</td><td>Final concentration:
100 µM (X. Z. Li et al., 2019);</td><td></td></tr>
<tr><td></td><td>Plant oils (e.g. olive oil contains 55 - 83 % oleic acid; (Casado-Díaz et al., 2019).</td><td>Final concentration:
2 % (w/v) Intralipid for bovine preadipocytes (Wu et al., 2000).
[Intralipid® 20% is a sterile fat emulsion for intravenous administration, contains 20 % soybean oil, egg phospholipids and glycerin; https://www.accessdata.fda.gov/]</td><td></td></tr>
</table>

1.3.2.1. Single components of Chemically Defined Lipid Concentrate (Gibco™ 11905031)

This mixture is used as one of the components in the adipogenic media compositions used by (Mitić et al., 2023b), see (Leber et al., 2025) for an overview.

Arachidonic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Arachidonic acid (AA, a major n-6 PUFA) is an essential fatty acid which has been identified as one of the adipogenic components of serum. It is required for the induction of differentiation of preadipocytes (Gaillard et al., 1989). AA can be converted to prostacyclin which activates PPARg and stimulates adipocyte differentiation. Additionally, AA can activate PPARg activation through an increase cAMP (Naughton et al., 2016). AA can program the differentiation potential of preadipocytes by regulating gene expression at the early stages of adipogenesis. Short-term AA treatment of preadipocytes inhibits their differentiation potential via upregulation of the transcription factor Fra-1 (Nikolopoulou et al., 2014).	**Concentration in the Lipid Concentrate:** 2 mg/L	**CAS number:** 506-32-1

Cholesterol
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)

Function: Cholesterol is a lipophilic molecule with several vital functions for mammalian cells. It is an important component of cell membranes. Beyond this, cholesterol has several other biological functions e.g. it is a precursor molecule for the synthesis of vitamin D and hormones etc. (Huff et al., 2025).	**Concentration in the Lipid Concentrate:** 220 mg/L	**CAS number:** 57-88-5
DL-alpha-Tocopherol Acetate		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: DL-alpha-Tocopherol Acetate is a synthetic form of vitamin E, a fat-soluble vitamin with potent antioxidant properties. Vitamin E supplementation enhances adipocyte differentiation, lipid transport, adipogenesis, and mitochondrial function (Alcalá et al., 2015).	**Concentration in the Lipid Concentrate:** 70 mg/L	**CAS number:** 7695-91-2

Ethyl Alcohol 100 % (Ethanol)		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Ethanol is used a solvent for water-insoluble compounds like lipids. Ethanol has been demonstrated to inhibit adipogenesis in human ASCs at a concentration of 50 mM, corresponding to 0.3 % (v/v) (Ardenkjær-Skinnerup et al., 2024).	**Concentration in the Lipid Concentrate:** confidential	**CAS number:** 64-17-5

Linoleic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031); differentiation medium (adipogenesis)		
Function: Linoleic acid (18:2, n-6) is an essential nutrient for animals and must be provided in serum-free cell culture media to enhance proliferation of mammalian cells (Bandyopadhyay et al., 1987; Butler et al., 1999). In addition, the survival rate of cells grown in media supplemented with linoleic acid improved significantly under high stirring rates (Butler et al., 1999).	0.042 mg/L (Oku et al., 2006) **Concentration in the Lipid Concentrate:** 10 mg/L (Gibco™ 11905031)	**CAS number:** 60-33-3
Alternatives: Plant seed oils (e.g. soy bean oil, 53 % linoleic acid (Dunford, 2018), DMEM/F12.		

Linolenic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Linolenic acid, especially alpha-linolenic acid (18:3, n-3) is a plant-based essential polyunsaturated fatty acid. It exhibits anticancer, anti-inflammatory, and antioxidative effects (Zhou et al., 2022). Linolenic acid is a natural ligand for PPAR-g. Supplementation with alpha-linolenic acid and essential amino acids improved the proliferation and differentiation of C2C12 cells (Zhou et al., 2022). Long-chain fatty acids (FAs) stimulate clonal preadipocyte differentiation with a-linolenic acid being very potent (Ding et al., 2003).	**Concentration in the Lipid Concentrate:** 10 mg/L	**CAS number:** 463-40-1
Alternatives: Flaxseed oil: Alpha-linolenic acid accounts for approximately 55% of the total fatty acids in flaxseed (Wiesenfeld et al., 2001).		

Myristic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Myristic acid significantly promotes the differentiation of intramuscular porcine adipocytes in a dose-dependent manner. It also led to a parallel increase in the expression PPARγ and adipose-related genes (Lu et al., 2014).	**Concentration in the Lipid Concentrate:** 10 mg/L	**CAS number:** 544-63-8

Oleic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031); differentiation medium (adipogenesis)		
Function: Long-chain FA are building blocks for lipid droplets in adipocytes and can be actively involved in the regulation of adipogenesis and lipogenesis (Yanting et al., 2018). Oleic acid (OA; C18:1) and C18:2, stimulated porcine preadipocyte differentiation to a greater extent than C18:3 (Ding et al., 2003). In bovine adipocytes, OA increased lipid content during adipogenesis and during the maturation stage (i.e. lipogenesis) (Yanting et al., 2018). OA effectively increased adipogenic gene expression in bovine SCs in the absence of a synthetic PPARγ agonist (X. Z. Li et al., 2019). OA, alone or in combination with palmitic acid, enhanced lipid accumulation in subcutaneous adipocytes - potentially through PPARα signalling (Abou-Rjeileh et al., 2025).	**Concentration in the Lipid Concentrate:** 10 mg/L (Gibco™ 11905031) 200 µM (Pasitka et al., 2022)	**CAS number:** 112-80-1
Alternatives: Plant oils		

Palmitic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Palmitic acid (16:0) is a saturated long-chain fatty acid (FA); it is one of the most common saturated fatty acids in animals, plants, and microorganisms (https://www.vitas.no/articles/fat-matters-lipids-and-health). However, saturated FAs were less effective regarding adipogenesis and lipogenesis of bovine and porcine adipocytes than unsaturated FAs (Ding et al., 2003; Yanting et al., 2018). Moreover, these effects were dose-dependent with an optimal concentration of 250 µM for palmitic acids (Yanting et al., 2018).	**Concentration in the Lipid Concentrate:** 10 mg/L	**CAS number:** 57-10-3
Alternatives: Plant oils		

Palmitoleic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Palmitoleic acid (C16:1n7) is an omega-7 monounsaturated long chain fatty acid, whose main vegetable sources are macadamia seed oil and sea buckthorn pulp (Bolsoni-Lopes et al., 2013). It increases the metabolic and oxidative capacity of adipocytes (Cruz et al., 2018). Palmitoleic acid increased 3T3-L1 proliferation and differentiation, as well as the expression of genes involved in adipogenesis (Simão et al., 2022).	**Concentration in the Lipid Concentrate:** 10 mg/L	**CAS number:** 373-49-9
Alternatives: Plant oils		

Pluronic F-68		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Pluronic F-68 is a synthetic non-ionic surfactant (Z. Zhang et al., 1992). It is frequently used media additive to ameliorate the effects of shear stress in bioreactors (Murhammer & Goochee, 1990). Supplementing serum-free CHO cultures with Pluronic F-68 enhanced cell yield in agitated cultures and reduced cell adhesion in stationary cultures (Tharmalingam et al., 2008).	**Concentration in the Lipid Concentrate:** 90 g/L	**CAS number:** 9003-11-6

Stearic Acid		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Stearic acid (C18:0) is a long-chain saturated fatty acid. Milk and fats contain 5 %–15 % stearate, while lard and cocoa and shea butters contain a higher concentration ranging from 10 % to 35 % stearate (review from (Shen et al., 2025)). For CHO cultures fed with saturated fatty acids, i.e. C16:0 and C18:0, the degree of saturation did not increase; in fact, it decreased slightly. This suggests that CHO cells predominantly metabolized saturated fatty acids further to unsaturated fatty acids as part of their normal metabolism (Priem et al., 2025).	**Concentration in the Lipid Concentrate:** 10 mg/L	**CAS number:** 57-11-4
Alternatives: Plant oils		

Tween 80® (Polysorbate 80)		
Component of Chemically Defined Lipid Concentrate (Gibco™ 11905031)		
Function: Polysorbate 80 is a surface-active, non-ionic agent which is widely used as solubilizer, stabilizer and emulsifier in pharmaceuticals, foods, and cosmetics (Kerwin, 2008).	**Concentration in the Lipid Concentrate:** 2.2 g/L	**CAS number:** 9005-65-6
Alternatives: Plant oils		

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FEASTS
Fostering European Cellular Agriculture for Sustainable Transition Solutions

Annex 2 of Deliverable 3.3:

Catalogue: Components in scaffolds designed for CM/CSF production

Part of the Deliverable 3.3

Work Package 3 ***Sustainability by design***

Task no: 3.2 and 3.4

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1. General comments regarding scaffold materials composition

The use of scaffolds in cultivated meat and seafood and become essential for the production process, either for supporting cell growth, alignment and differentiation, or for structuring the final food formulation. They can serve different purposes working as beads for expansion of anchorage dependent cells during proliferation stage; as fibrous and porous supports for cell alignment, promoting meat-like properties; and as hydrogels or bioinks for cell encapsulation and posterior assembly of a food product through 3D printing or moulding.

The most suitable scaffolds for cultivated meat production should be edible, food-grade, cost-effective and mechanically suitable for mimicking the various cell tissues. Moreover, they should provide efficient nutrient diffusion, be biocompatible with animal cells, free of toxicity and ultimately be scalable materials. On top of that, more suitable scaffolds would also have valuable nutritional features, be digestible, have no allergenic effects and be of non-animal origin. In most cases, the final scaffold results from the combination of several materials, each contributing for specific aspects pf the final structure.

That said, common scaffold materials used in cellular agriculture include animal- and plant-sourced proteins, polysaccharides and gums from plant or microbial origin, and lipid- and wax-based ingredients. The use of these materials in the field has been reported either for microcarrier formulations, hydrogels, bioinks or fibrous scaffolds.

The following catalogue presents the most used scaffold materials in cellular agriculture categorized by type. Each entry presents their functional role, nutritional features, CAS nº, digestibility and allergenicity and possible alternatives.

1.1. Protein- based materials

Natural proteins, either from plant or animal origin, have shown to have significant use in scaffolds for cultivated meat and seafood production, due to their ECM-like properties. Besides their biocompatibility and mechanical properties, they support cell adhesion, growth and differentiation. This chapter presents the most common and cited proteins used in scaffolding.

<table>
<tr><td colspan="4">Collagen</td></tr>
<tr><td colspan="4">Protein derived from animal ECM</td></tr>
<tr><td colspan="2">Function:
Promotes excellent cell adherence as it mimics animal cells natural ECM. Used in fibrous scaffolds for cell alignment and structuring. Adds gel-like texture to the meat.
(Bomkamp et al. 2022)</td><td>Nutritional features:
Source of glycine, proline, hydroxyproline.</td><td>CAS number:
9007-34-5</td><td>Food safety:
Highly digestible and food-grade safe, but animal origin may raise concerns</td></tr>
<tr><td colspan="2"></td><td></td><td></td><td></td></tr>
<tr><td>Alternatives:</td><td colspan="2">Recombinant collagen/gelatin from plant or microbial source; plant proteins isolates.</td><td></td><td></td></tr>
</table>

Gelatin			
Hydrolysed collagen from animal ECM			
Function: Used in microcarriers and hydrogels formulation, enhances cell adhesion and proliferation. Improves mouthfeel due to its reversible properties according to temperature.	**Nutritional features:** Source of glycine, proline, hydroxyproline.	**CAS number:** 9000-70-8	**Food safety**: Fully digestible, considered to be GRAS. Low allergenicity.
Alternatives:	Recombinant collagen/gelatin from plant or microbial source; plant proteins isolates		

Albumin			
Animal protein			
Function: Used for electrospun fibres and fibrous scaffolds. (Nseir et al. 2013)	**Nutritional features:** High protein content.	**CAS number:** 9048-46-8 (bovine origin) 70024-90-7 (human origin)	**Food safety**: High digestibility. Egg allergen risk.

Alternatives:	Casein; plant proteins: pea, soy.		

Fibronectin			
Animal protein			
Function: Used for electrospun fibres and fibrous scaffolds. (Chantre et al. 2018)	**Nutritional features:** High protein content.	**CAS number:** 86088-83-7	**Food safety**: No reported information.
Alternatives:	Casein; plant proteins: pea, soy.		

Soy protein isolate			
Plant legume protein			
Function: Provides mechanical strength in scaffolds and in bioinks for 3D printing or extrusion. (Ben-Arye et al. 2020)	**Nutritional features:** High-quality protein source containing all essential amino acids.	**CAS number:** 9010-10-0	**Food safety**: Highly digestible. Known allergen risk.
Alternatives:	Other legume plant proteins: pea, chickpea, lentil.		

Pea protein isolate			
Plant legume protein			
Function: Coating for microcarriers (Kong et al. 2023). Provides mechanical strength in scaffolds and in bioinks. Support cell attachment, growth and differentiation. (David et al. 2024)	**Nutritional features:** High-quality protein source rich in all essential amino acids. Source of iron.	**CAS number:** 222400-29-5	**Food safety**: Highly digestible. Low allergenicity.
Alternatives:	Other legume plant proteins: soy, chickpea, lentil.		

Zein			
Corn protein			
Function: Used for electrospun fibres and fibrous scaffolds. Support cell alignment and provide mechanical stability. Functional properties of a viscoelastic material. (Kawecki et al. 2023)	**Nutritional features:** High protein content but deficiency in some essential amino acid (lysine and tryptophan).	**CAS number:** 9010-66-6	**Food safety**: Low digestibility. Low allergen risk.
Alternatives:	Other cereal proteins: gliadin, hordein.		

Wheat gluten			
Wheat proteins			
Function: Used for elastic, porous scaffolds. Mechanical strength. (Xiang et al. 2022)	**Nutritional features:** High protein content.	**CAS number:** 8002-80-0	**Food safety**: High digestibility. Gluten allergen risk.
Alternatives:	Other cereal proteins: zein, hordein.		

Mycoprotein			
Fungi-, mycelium-origin protein			
Function: Microcarrier composition. (Ogawa et al. 2024)	**Nutritional features:** High content in protein and fibre. Low content in fat and cholesterol.	**CAS number:** Not applicable	**Food safety**: High digestibility. Rare allergies.
Alternatives:	Plant protein.		

Yeast extract			
Residues from yeast-derived industries.			
Function: Scaffold for cell proliferation and differentiation. (Wang et al. 2024)	**Nutritional features:** Source of protein, B vitamins and minerals. Low in fat	**CAS number:** 8013-01-2	**Food safety**: High digestibility. No known allergens.
Alternatives:	Plant proteins: pea, soy.		

1.2. Polysaccharide- and gum-based materials

Polysaccharides are essential in edible, biocompatible scaffolds with adequate mechanical properties. Materials such as alginate, carrageenan or gellan, are commonly employed for hydrogel formation, encapsulation and 3D printing. Generally, they contribute to the content of dietary fibre. This chapter presents the most common and cited polysaccharide- and gum-based materials used in scaffolding.

<table>
<tr><td colspan="4">Alginate</td></tr>
<tr><td colspan="4">Brown algae-derived polysaccharide</td></tr>
<tr><td>Function:
Hydrogel porous scaffold, used for encapsulation and 3D bioprinting. (Fe and Dj 2017)</td><td>Nutritional features:
Source of fibres, promotes gut health, Low in other nutrients.</td><td>CAS number:
9005-38-3 (sodium alginate)</td><td>Food safety:
Low digestibility. Reported allergy cases associated to the algae-origin.</td></tr>
<tr><td colspan="2"></td><td></td><td></td></tr>
<tr><td>Alternatives:</td><td>Agar, carrageenan, gellan gum.</td><td></td><td></td></tr>
</table>

Carrageenan			
Red algae-derived polysaccharide			
Function: Thermo-reversible properties for hydrogel and bioink formulation. (Dmc et al. 2022)	**Nutritional features:** High fibre and carbohydrate content.	**CAS number:** 11114-20-8	**Food safety**: Low digestibility. Rare reports of allergy cases.
Alternatives:	Alginate, gellan gum, agar.		

Agar/Agarose			
Red algae-derived polysaccharide			
Function: Thermo-reversible material used in hydrogel formulations. supports cell encapsulation. Provides structure to cell encapsulation matrixes.	**Nutritional features:** High in carbohydrates and fibres.	**CAS number:** 9002-18-0 (agar) 9012-36-6 (agarose)	**Food safety**: Indigestible fibre. No reported allergens.
Alternatives:	Alginate, gellan gum, carrageenan.		

Gellan gum			
Bacterial-origin polysaccharide			
Function: Used for formulation of clear and firm gels. (Chen et al. 2023)	**Nutritional features:** High fibre content.	**CAS number:** 71010-52-1	**Food safety**: Indigestible fibre. No reported allergens.
Alternatives:	Alginate, agar, carrageenan.		

Xanthan gum			
Bacterial-origin polysaccharide			
Function: Used as a thickener and stabilizer in hydrogels and bioinks. (Wollschlaeger et al. 2022)	**Nutritional features:** High fibre content.	**CAS number:** 11138-66-2	**Food safety**: Indigestible fibre. Rare allergies.
Alternatives:	Guar, gellan gum, agar.		

Cellulose			
Plant fibre			
Function: Used in microcarriers, fibrous scaffolds and to strengthen and stabilize hydrogels. (Bodiou et al. 2020)	**Nutritional features:** High fibre content. Improves chewiness.	**CAS number:** 9004-34-6	**Food safety**: Indigestible fibre. No reported allergens.
Alternatives:	Bacterial cellulose. Other plant fibres: pectin.		

Pectin			
Bacterial-origin polysaccharide			
Function: Used in microcarriers. Provides thickening and stabilizes scaffolds. (Martins et al. 2018)	**Nutritional features:** High fibre content.	**CAS number:** 9000-69-5	**Food safety**: Indigestible fibre. Rare allergies.
Alternatives:	Guar, gellan gum, agar.		

Chitosan			
Polysaccharide from crustacean shell origin			
Function: Used in hydrogels and aligned scaffolds. Biodegradable and antibacterial properties. (Bomkamp et al. 2022)	**Nutritional features:** High fibre content.	**CAS number:** 9012-76-4	**Food safety**: Indigestible fibre. Shellfish-related allergies.
Alternatives:	Fungi chitosan. Chitin, konjac glucomannan.		

Starch			
Plant-derived polysaccharide			
Function: Gelatine behaviour that improves hydrogel properties. (Xian et al. 2024)	**Nutritional features:** Rich in glucose, provides energy.	**CAS number:** 9005-25-8	**Food safety**: Digestible carbohydrate. No reported allergens.
Alternatives:	Other plant polysaccharides: cellulose, pectin.		

1.3. Lipid- and wax- based materials

Lipids and waxes are primarily used to replicate the sensory properties of fat animal tissue in cultivated products (MULLER-AUFFERMAN and WALKER 2022). When mixed with other materials, such as proteins, lipids form an oleogel matrix that can help achieve the marbling, juiciness and mouthfeel attributes of conventional meat. Beyond texturization, these materials also contribute to the nutritional enhancement of the final product by introducing functional lipids such as omega-3 and omega-6 fatty acids. This chapter presents the most common and cited lipid-based materials used in scaffolding.

<table>
<tr><td colspan="4">Rapeseed oil</td></tr>
<tr><td colspan="4">Vegetable oil</td></tr>
<tr><td>Function:
Add fat content to the scaffolds, hydrogels or bioinks. (Albergaria et al. 2025)
Used to form oleogels that mimic fat tissue.</td><td>Nutritional features:
Healthy fat.</td><td>CAS number:
8002-13-9</td><td>Food safety: High digestibility. No reported allergens.</td></tr>
<tr><td></td><td></td><td></td><td></td></tr>
<tr><td>Alternatives:</td><td>Other plant oils: sunflower, soybean.</td><td></td><td></td></tr>
</table>

Sunflower oil			
Vegetable oil			
Function: Mimic the fat animal tissue, structure and juiciness. Added to form oleogels. (Lambert et al. 2024)	**Nutritional features:** Healthy fat. High content in vitamin E, omega-6 fatty acids (linoleic acid) and monounsaturated fats (oleic acid).	**CAS number:** 8001-21-6	**Food safety**: High digestibility. No reported allergens.
Alternatives:	Other plant oils: rapeseed, soybean.		

Coconut oil			
Vegetable oil			
Function: Provides a meaty, firm texture to tissues. Solid at room temperature. Added to form oleogels. (Lambert et al. 2024)	**Nutritional features:** Healthy fat. High content in saturated fats (lauric acid).	**CAS number:** 8001-31-8	**Food safety**: High digestibility. Rare reported allergies.
Alternatives:	Palm oils.		

Cocoa butter			
Vegetable oil			
Function: Provides a rich, mouthfeel fat sensory properties. Added to form oleogels. (Lambert et al. 2024)	**Nutritional features:** High calorie fat. High content in vitamin D2, polyphenols, flavonoids and saturated fatty acids.	**CAS number:** 8002-31-1	**Food safety**: High digestibility. No reported allergies.
Alternatives:	Coconut or palm oil.		

Algae oil			
Macro- and microalgae derived oil			
Function: Mimics the fat tissue of meat. Added to form oleogels.	**Nutritional features:** Healthy fat. High content in polyunsaturated fats, mainly omega-3 (DHA, EPA).	**CAS number:** 6217-54-5 (for DHA, not for algae oil)	**Food safety**: High digestibility. Possible allergens related to particular algae proteins.
Alternatives:	Plant oils or fish oil.		

Glycerol Monostearate (GMS)			
Food emulsifier			
Function: Gelation properties, provides thermal stability and acts as an oil structuring. Added to form oleogels. (Yen et al. 2023)	**Nutritional features:** Nutritional value not relevant. Used as an emulsifier and stabilizer in foods.	**CAS number:** 31566-31-1	**Food safety**: Classified as GRAS.
Alternatives:	Other monoglycerides.		

1.4. Decellularized materials

Decellularized tissues provide a biomimicry of the natural occurring network on animal tissue. These materials provide appropriate nutrient delivery, oxygenation, support to cells, promoting growth and adhesion. Common examples include spinach and other green leaves, algae or bamboo, which undergo a treatment process to solely preserve the ECM-like architecture. This chapter presents the most common materials used in decellularized scaffolds.

<table>
<tr><td colspan="4">Spinach</td></tr>
<tr><td colspan="4">Plant origin</td></tr>
<tr><td>Function:
3D natural scaffold that allows nutrient perfusion. Supports cell differentiation and alignment. (Jones et al. 2021)</td><td>Nutritional features:
Dietary fibre. Rich mineral content.</td><td>CAS number:
-</td><td>Food safety:
High digestibility. Low allergenic potential.</td></tr>
<tr><td colspan="2"></td><td></td><td></td></tr>
<tr><td>Alternatives:</td><td>Decellularize leafy scaffolds or synthetic polymers.</td><td></td><td></td></tr>
</table>

Bamboo			
Plant origin			
Function: Rigid, lignocellulosic scaffolds with high porosity supporting cell infiltration.	Nutritional features: Dietary fibre. Rich mineral content.	CAS number: -	Food safety: Low digestibility on scaffold form. Low allergenic potential.
Alternatives:	Decellularize celery or synthetic polymers.		

Algae			
Algae-derived scaffolds			
Function: Hydrated scaffolds used for hydrogel formulation.	**Nutritional features:** Dietary fibre.	**CAS number:** -	**Food safety:** Inedible fibre. No reported allergies.
Alternatives:	Edible hydrocolloids or fibre sources.		

1.5. Synthetic materials

Synthetic scaffolds, consist on polymers and esters designed to be biocompatible and replicate the biochemical properties of natural tissues. These materials allow for a more precise control over scaffold characterization such as biodegradability, porosity, adhesion rates, etc.(Bomkamp et al. 2022). However, since they are not edible, they need to be removed before consumption, which difficult the downstream process. This chapter presents the most common synthetic materials used in scaffolding.

<table>
<tr><td colspan="4">Poly(lactic-co-glycolic Acid) (PLGA)</td></tr>
<tr><td colspan="4">Synthetic copolymer</td></tr>
<tr><td>Function:
Biodegradable and biocompatible material for microcarriers, electrospun fibres, porous scaffolds, hydrogels and films.</td><td>Nutritional features:
-</td><td>CAS number:
26780-50-7</td><td>Food safety:
Inedible.</td></tr>
<tr><td colspan="2"></td><td></td><td></td></tr>
<tr><td>Alternatives:</td><td>Natural protein and decellularized scaffolds.</td><td></td><td></td></tr>
</table>

Poly-ε-caprolactone (PCL)			
Aliphatic polyester			
Function: Biodegradable and biocompatible material for fibrous scaffolds. Supports cell alignment and proliferation.	**Nutritional features:** -	**CAS number:** 24980-41-4	**Food safety**: Inedible.
Alternatives:	Natural protein and decellularized scaffolds.		

Polylactic Acid (PLA)			
Synthetic polymer			
Function: Biodegradable material for spinning-produced fibres. Supports cell adhesion, differentiation and growth.	**Nutritional features:** -	**CAS number:** 26100-51-6	**Food safety**: Inedible.
Alternatives:	Natural protein and decellularized scaffolds.		

Polyethylene Glycol (PEG)			
Synthetic polymer			
Function: Biodegradable material for hydrogels production. Supports cell adhesion, differentiation and growth.	**Nutritional features:** -	**CAS number:** 25322-68-3	**Food safety**: Food additive.
Alternatives:	Natural protein and decellularized scaffolds.		

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FEASTS
Fostering European Cellular Agriculture for Sustainable Transition Solutions

Annex 3 of Deliverable 3.3:

Review on published serum-free media compositions

Work Package 3 *Sustainability by design*

Task no: 3.2 and 3.4

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Title: Towards cost-effective and sustainable media formulations for terrestrial and aquatic cellular agriculture

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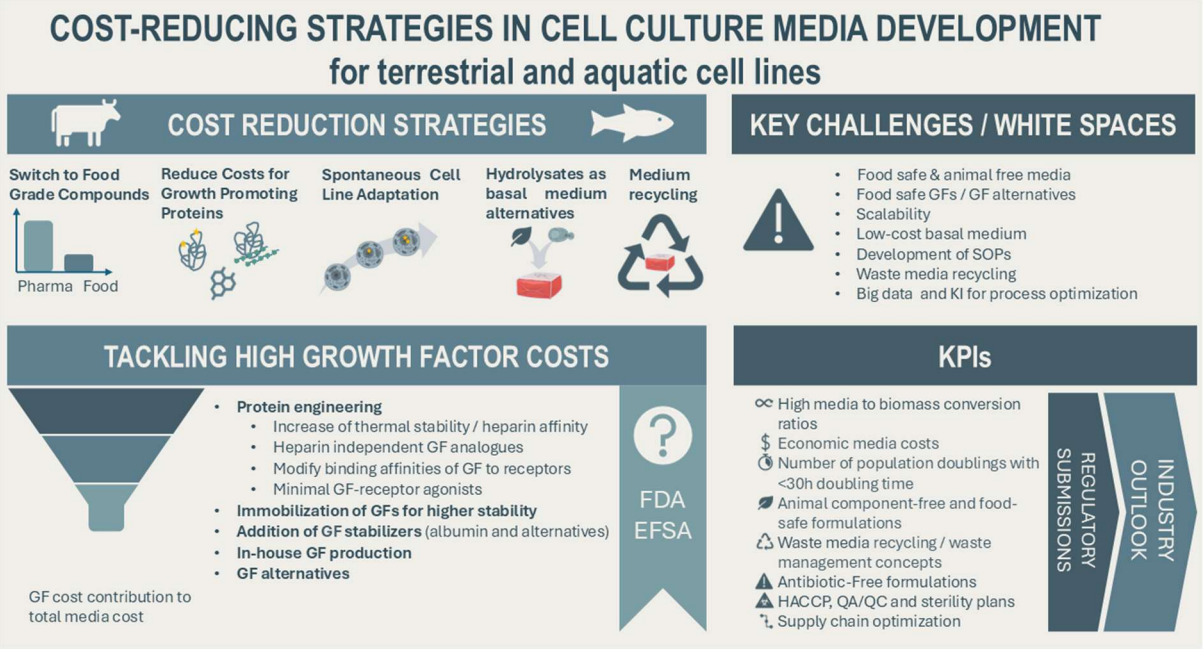
Abstract:

Over more than a decade of research on media for cultured meat and seafood production has resulted in multiple highly efficient serum-free and chemically defined formulations for some species, but also identified challenges yet to be solved – especially for aquatic cell lines. Depending on the product and cell type, the approach to develop highly efficient, sustainable and low-priced media can diverge greatly. In this review, we provide an in-depth overview of this complex research area to facilitate strategic decision-making for stakeholders. We evaluate serum-free media formulations published for cultured meat production and research efforts on developing adequate media for cultured seafood regarding their advantages and limitations. We critically analyze strategies aimed at reducing medium costs and enhancing sustainability of cultured meat/seafood production, as well as summarizing topics that require further exploration. Additionally, we consider possible emerging regulatory issues and current trends in consumer acceptance and their impact on media formulation development. Finally, key performance indicators (KPIs) for media formulations are proposed to guide future strategic and operational improvements regarding an economical and sustainable production process.

Keywords:

Cultured meat, cultured seafood, cellular agriculture, serum free media formulations, cost reduction strategies, key performance indicators.

Graphical abstract:



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1. Introduction

Meat and seafood are playing crucial roles in human nutrition because they are rich sources of high-quality protein (Pereira & Vicente, 2013). As the world's population is growing continuously, an increased global demand of animal-derived protein can be expected. Although there are plant-based "meat" analogue products available, genuine meat will remain the preferred protein source for most people in the near future (Shaikh et al., 2021). Meat production needs to nearly double by 2050 to meet the growing global demand for animal proteins. Also, global seafood consumption has doubled since 1960 and it will continue to increase (Guillen et al., 2019). But the conventional way to produce meat and seafood cannot meet this high demand without having significant environmental impacts (Battle et al., 2024).

Cultured meat (CM) and seafood (CSF) is produced by cultivating animal cells *in vitro* (Swartz, 2021). Efficiency of cellular agriculture is largely determined by the culture conditions, cell type and its respective cell doubling time, cell culture medium and its biomolecular composition, and the type of bioreactor used (Shaikh et al., 2021).

Despite the increasing number of companies focussing on cellular agriculture, there are still major challenges yet to be solved, such as the high costs associated with manufacturing mainly due to expensive media components and gaining consumer acceptance (Battle et al., 2024). Significant technological challenges need to be addressed, such as isolation and maintenance of stable **cell lines**, efficient, sustainable and cost-effective **serum free (SF) cell culture media** – especially for CSF, CM/CSF-aimed **bioprocessing design** including bioreactor design, and **scaffolding** technologies, and upscaling (Battle et al., 2024; Levi et al., 2022).

Traditionally, meat and seafood quality is mainly determined by three components - i.e., muscle fibres, connective tissue, and adipose tissue - which vary depending on species and muscle type (Listrat et al., 2016) – a parameter which is freely variable in CM/CSF, thus allowing for a very precise,

most desirable end-product composition. Although meat is mainly composed of muscle tissue, fat typically accounts for up to 10% of biomass (Fish et al., 2020). Fat is the most important contributor to meat flavour across all species (Arshad et al., 2018; Webb & O'Neill, 2008; Wood et al., 2008), and will most probably be part of the first CM hybrid products reaching the market in Europe (Mosa Meat, 2025).

Growing cells *ex vivo* in bioreactors requires cell culture media that provide all essential compounds to promote cell proliferation and maintain long-term viability: a liquid mixture of a carbon-based energy source (e.g. glucose), amino acids, salts, vitamins, and cell proliferation promoting components to support cell viability and vitality (Swartz, 2021). Once adequate cell biomass is generated, differentiation into muscle or fat cells is generally triggered by changing the medium composition (Martins et al., 2024). The tissue composition of the final product can be varied depending on consumers preferences.

Generally, three main categories of cell types with diverging media preferences can be considered for CM/CSF production: (1) adult progenitors (e.g. here mentioned primary cells, muscle satellite cells, fibro-adipogenic progenitor cells (FAPs) and mesenchymal stem cells (MSCs)), which can produce limited range of cell types; (2) pluripotent stem cells (PSCs, including induced pluripotent stem cells and embryonic stem cells (ESCs)); and (3) immortalized cell lines. Cells isolated from various species, as well as from diverging sex, race and age of the animal may show differences in medium requirements (Burton et al., 2000; Pääsuke et al., 2016; Skrivergaard, Krøyer Rasmussen, et al., 2023). Finally, continuously proliferating cell lines can be adapted to tolerate less rich/expensive medium formulations (Pasitka et al., 2024). Thus, **the selection/isolation of the appropriate cell line(s) will impact the choice and composition of cell culture media**.

This review aims to evaluate published **serum-free, sustainable and cost-effective alternatives to animal serum-based** cell culture media proposed for CM/CSF production as the field still faces challenges around scale-up and cost reduction. Only fully declared media compositions were

reviewed, as it is a prerequisite for further medium optimisation efforts. Cell culture medium – an essential input for all CM and CSF productions - accounts for 55%–90% of production costs (Gomez Romero & Boyle, 2023). To be economically competitive with traditional meat/fish products, CM/CSF need to be produced using low-cost medium formulations that match the metabolic requirements of selected cell lines, in addition to creating a supply chain of more affordable, animal-free, food-grade and sustainable ingredients (Battle et al., 2024). We further review possible strategies which may also contribute to a cost reduction and higher sustainability for cell culture media and discuss regulatory issues and key challenges that remain to be addressed.

2. History of serum free cell culture media development

Traditionally, cell culture media are supplemented with animal serum (particularly fetal bovine serum, **FBS**) to support growth of animal cells. FBS contains essential components required for proliferation and maintenance of mammalian, as well as fish cells. Most important are growth promoting proteins like hormones and growth factors (GFs) (Subbiahanadar Chelladurai et al., 2021). Although FBS is traditionally a universal supplement for cell culture media, it has several **limitations** and there are rising **ethical concerns** as FBS is obtained from the foetuses of pregnant slaughtered cows. FBS is a byproduct of the meat industry, and its **availability** may be subject to fluctuations caused by climate change, limited water supply or extreme weather events (*FBS Availability and Uses*, n.d.). Additionally, as the pharmaceutical industry is currently the largest user, the growing CM/CSF industry would have to compete with it, potentially resulting in **even higher prices and increased availability challenges** due to the rising demand.

As a biological component, FBS can be a source of contamination by viruses or other infectious agents, which can raise **concerns over the safety** of FBS for food production. In Europe, FBS must

be tested for 7 specific viruses according to the Code of Federal Regulations Title 9 (9CFR) guidelines to avoid potential pathogen transmission (*Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products - Scientific Guideline | European Medicines Agency (EMA)*, 2013). However, researchers have detected viruses in commercial FBS batches (Gagnieur et al., 2014; Hawkes, 2015), which may raise **regulatory concerns**.

Further, the exact **composition of FBS is unknown** and have batch-to-batch differences depending on its origin and processing, which may impact on the reproducibility of cell culture processes (S. Liu, Yang, et al., 2023). In addition, FBS does not accurately mimic the physiological environment of interstitial spaces where somatic cells reside, which mostly contains proteoglycans, glycosaminoglycans, fibronectin and collagens, and where albumins are present in very low concentrations, as opposed to serum, where serum albumin is the largest protein component (50–60%) (Frantz et al., 2010).

Despite these limitations and its high cost, FBS is often a major constituent of cell culture media (typically 2-20 % by volume). Hence the development of **SF medium formulations** has gained much attention over the last decades (see the detailed review by Yao and Asayama on the history of SF media (Yao & Asayama, 2017).

The first developments in this regard were achieved by Chen et al. when identifying 7 components including two GFs to be essential for human ESC and iPSC cultures (G. Chen et al., 2011). These 7 compounds were added to the chemically defined basal medium DMEM/F12. The resulting so-called **Essential 8 medium** (E8) is a SF cell culture medium providing chemically defined conditions for human cells used in research and clinical applications (G. Chen et al., 2011). E8 medium was further optimized (and named B8 medium) regarding medium constituents and concentrations required to culture human PSCs by Kuo et al. (Kuo et al., 2020). Costs of **B8 medium** could be substantially reduced by the in-lab generation of three *Escherichia coli*-expressed, codon-optimized

recombinant GFs (fibroblast growth factor 2 – FGF-2, transforming growth factor β3 (TGFβ-3), and neuregulin 1(NRG-1)) (Kuo et al., 2020).

These formulations optimized for human cell lines provided the starting point for developing SF-media for CM production, with most of them optimized for muscle satellite cells (SCs) (Kolkmann et al., 2022; Skrivergaard, Young, et al., 2023; Stout et al., 2022). For other cell types such as ESC, iPSC (Reiss et al., 2021), no medium optimization efforts were reported so far for edible species, except for porcine ESCs and iPSCs (H. Choi et al., 2024, p. 20; Ma et al., 2018).

For aquatic species, research in this area is still in its early stages, with initiatives focusing on **low serum and SF proliferation media for muscle-derived cell lines and fibroblasts** often yielding suboptimal results. The research is further complicated by the fact that bovine serum is not an ideal growth promoting medium component for fish cells. When FBS, routinely used in fish cell culture media, was replaced with gilthead seabream (*Sparus aurata*) serum, **seabream mesenchymal-derived cells** VSa13 and VSa16 performed significantly better regarding proliferation and extracellular matrix mineralization (Rosa et al., 2010). Also, **several embryonic stem cells** require fish embryo extracts (Buonocore et al., 2006; Dash et al., 2010) or fish serum (Sha et al., 2010) for their isolation and proliferation in addition to FBS. Both components may be sourced from the same or different fish species (Bejar et al., 2002; S. -L. Chen, Sha, et al., 2003; S. -L. Chen, Ye, et al., 2003). Similarly, **goldfish muscle cells** (N. Li et al., 2021) rely on **species-specific muscle extracts and fish serum**, with fish serum demonstrated to be essential for their long-term viability (J.-W. Kim et al., 2020).

While human and bovine GF orthologs share 90 to 98% protein sequence identity, allowing an efficient culture of human cells in bovine serum, the protein identity between fish and bovine GF orthologs, as well as between fish orthologs, is in the range of 77% (Yuan & Hong, 2017), which may result in the suboptimal proliferation of many types of fish cells in FBS or in fish serum from distant species. This highlights the importance of using species-specific serum or extracts, rather than

FBS, to efficiently support the proliferation of fish cells. It should be noted that fish serum/extracts also exhibit some of the limitations associated with FBS, such as limited availability, batch-to-batch variability and ethical acceptability, which impede their application in the production of CSF. Collectively, these findings underscore current **critical reliance of fish cell lines on FBS and fish extracts**. **More research** is needed to identify **species-specific GFs,** critical for different developmental stages of fish cells, before their dependence on FBS and fish extracts can be eliminated.

Given the high demand for different cell types and species (especially for fish), a universal chemically defined SF-medium for meat or fish cell cultures appears to be unrealistic (Van Der Valk 2018; O'Neill et al. 2021). Thus, the development of low-cost, customizable and sustainable media allowing optimal growth and differentiation of cells for the large-scale production of high-quality CM/CSF products remains a big challenge.

A **possible alternative** to the need for medium optimization for every cell line is the **spontaneous adaptation** of cells to SF and generally less nutrient-rich media conditions. This approach has been successfully employed in the context of fish cell lines not directly relevant to CSF (Ackermann & Fent, 1998; Brunner, 2010; C.-L. Liu et al., 2017; Radošević et al., 2016; Yue et al., 2016) but also to reduce FBS content from 20% to 2.5% in the medium for the culture of mackerel muscle cell line Mack1 (Lim et al., 2024). This approach was also successfully used to **spontaneously immortalize chicken fibroblasts** capable of adipogenesis (Pasitka et al., 2022). While proliferation of bovine SCs in SF medium requires multiple GF supplementation, chicken fibroblasts derived from several chicken breeds were adapted to not only grow in single cell suspension, but also in an albumin free and SF culture medium containing practically no GFs (see Table 3).

These studies report **methodologies** that may be adapted to the development of SF media for other meat- and seafood-relevant cells. While attractive from a media development point of view, this approach, by relying on random mutagenesis, harbours **serious limitations** - such as

ineffectiveness, collection of large number of background mutations and very limited screening possibilities for the required phenotypes (Riquelme-Guzmán et al., 2024). For instance, attempts to adapt the **silver seabream myofibroblast precursor** Catmus1PFR cell line to lower FBS concentrations resulted in a progressive decline in cell proliferation, ultimately leading to cell death upon complete removal of FBS (Chong et al., 2022). Similar trends were observed in other fish cell lines (Cyrino & Mulvaney, 1999).

223 However, the adaptation to SF conditions of cell lines from bigger animals may not work out that
224 well as **spontaneous immortalization of mammalian cells has been negatively correlated with**
225 **species body mass** (Perillo et al., 2023), which makes this method more applicable to small animals.

226 3. Media compositions from cell isolation to maturation

227 The process of CM/CSF production can
228 be divided into four phases, namely: (i)
229 cell isolation and maintenance; (ii)
230 generation of cell biomass
231 (proliferation); (iii) cell differentiation
232 and maturation into skeletal muscle
233 cells and fibres, or adipocytes and (iv)
234 assembly of the final meat or fish
235 product.

Info box 1: Media for cell isolation and storage

- ⇨ **No SF isolation medium** has been published so far.
- ⇨ **Chemically defined** and **SF cryopreservation media** are commercially available, but composition is not publicly available.
- ⇨ **Food grade** chemically defined and SF cryopreservation media are required on the market.

236 The successful cell culture of animal cells *ex vivo* in an artificial environment under controlled
237 conditions requires an appropriate medium providing a source of energy and essential nutrients like
238 amino acids, vitamins and inorganic salts (Eagle, 1955), whereas a buffer system is necessary to
239 maintain a physiological pH over time (Chaudhry et al., 2009). For most cell lines, additional
240 supplementation with cell line- and phase-specific growth promoting factors like cell line specific
241 GFs and/or hormones is crucial for SF media to be effective (O'Neill et al., 2021). Medium
242 composition further depends on the cell types and species (Hubalek et al., 2022).
243 Thus, the **choice of cell type and final product will greatly influence the media requirements**,
244 and, consequently, the **price** of the media, as evidently shown in Table 3 and Table 4. For an

economically viable CM and CSF production process it is important that the final formulations are cost-efficient, food-safe and scalable (Kolkmann et al., 2022).

Further, contamination by microbes can be hazardous to cell culture systems used in the CM/CSF sector as contaminated products must be discarded. Supplementing growth media with antimicrobial agents such as antibiotics and antifungals generally provides an effective method to prevent contamination. However, antibiotics are biologically active and exhibit concentration-dependent effects on mammalian cells (Hassan & Ahmad, 2020). Therefore, prophylactic antibiotics supplements should be limited to cell isolation processes. During proliferation and differentiation, microbial contamination can be prevented by following standard aseptic techniques which have been established in pharmaceutical industry decades ago. Following this, we do not address the impact of antimicrobial agents on cell proliferation and differentiation, nor do we include their costs in media cost comparisons.

3.1. Media for cell isolation and storage

During cell isolation, a medium composed of basal medium supplemented with 1% antibiotics & antimycotics to prevent contamination, and **10 - 20% FBS** to block activity of proteases (see Table 2 and Info box 1) is usually used. To our knowledge, no SF isolation medium has been published so far. Beside factors necessary for cell maintenance and proliferation, FBS also contains protease inhibitors like α1-antitrypsin and α2-macroglobulin (D. Y. Lee et al., 2022), which are important for ending protease treatment during cell isolation. Studies evaluating the possibility to replace FBS by protease inhibitors for cell isolation processes are lacking.

For long-term storage, isolated animal cells are routinely **cryopreserved** at ultra-low temperatures (< -150°C) in the presence of a range of cryoprotectants. In reports mentioned in Table 2, mammalian cells were suspended in basal medium containing **10 % FBS** and 10% (vol/vol) dimethyl sulfoxide (DMSO) (X. Z. Li et al., 2019; Rønning, 2019; Vaughn et al., 2018) or frozen in FBS with 10%

DMSO (S. Ding et al., 2018; Mehta et al., 2019; Stout et al., 2022). Chemically defined and **SF cryopreservation media** with and without DMSO, which provide a safe and protective environment during the freezing, storage and thawing process for mammalian cells, are available on the market. Their composition, however, is proprietary and it is unclear if the ingredients can be rated as food-grade. Interestingly, plant hydrolysates have been successfully used to cryopreserve CHO cells, which improved cell survival during the freezing and thawing (Verhoeye et al., 2001).

Species	Basal medium	Supplements	References
Bovine SCs	DMEM	20% FBS 1% penicillin-streptomycin	(S. Ding et al., 2018)
Bovine SCs	DMEM	10% FBS	(Rønning, 2019)
Bovine SCs	DMEM	10% FBS 2% L-glutamine 1% antibiotic and antimycotic mix	(Leng & Jiang, 2019)
Bovine SCs	DMEM +Glutamax	20% FBS human FGF-2 (1 ng/mL) 1% primocin	(Simsa et al., 2019; Stout et al., 2022)
Bovine SCs	DMEM	10% FBS 1× antibiotic-antimycotic	(X. Z. Li et al., 2019)
Bovine, porcine, and chicken SCs	Ham's F-10	20% FBS mouse FGF-2 (5 ng/mL) 1% penicillin and streptomycin	(S. S. Ahmad et al., 2024)
Porcine SCs	DMEM	10% FBS 2% porcine serum penicillin (100 U/mL) streptomycin (100 µg /mL) gentamicin (20 µg/mL)	(Vaughn et al., 2018)
Porcine SCs	Alpha MEM Eagle	20% FBS 1% penicillin–streptomycin 1% amphotericin B 0.5% gentamicin	(Y. Zhao et al., 2021)
Bovine adipocytes	DMEM	10% FBS 1% penicillin/streptomycin/amphotericin	(Mehta et al., 2019)
Fish SCs	L-15	20% FBS human FGF (rec; 1 ng/mL) 20 mM HEPES 1% antibiotic–antimycotic 10 µg/mL gentamicin (coating: 0.25 µg/cm^2 iMatrix recombinant laminin-511-E8)	(Saad et al., 2023)
Fish myoblasts	L-15	20% FBS 10 ng/mL IGF 10 ng/mL EGF 10 ng/mL bFGF 0.8 mM CaCl2 2 mM glutamine 100 µg/mL penicillin/streptomycin	(Krishnan et al., 2023)
Fish adipocytes	DMEM/L-15 (1:1)	10% FBS 1% antibiotic/antimycotic solution	(Goffette et al., 2024)

Table 2: Media used for cell isolation after biopsy.

The cryopreservation of **fish cells** relies on methods and reagents originally developed for mammalian cells. A standard formulation of the cryopreservation medium contains 10% DMSO and

10%–50% FBS, with 20% FBS emerging as an optimal concentration to boost cell recovery (Goswami et al., 2023; Yashwanth et al., 2023; Z. Zhao & Lu, 2006). For cells requiring an enhanced protection, DMSO concentration may be increased to 20% in combination with 20-40% FBS (Rougée et al., 2007; G. Wang et al., 2003). Conversely, less sensitive cells may be cryopreserved in 5% DMSO and 25% FBS (Dubey et al., 2015). Glycerol has been reported in the past as an effective cryoprotectant (as an alternative or a complement) and is typically utilized at concentrations ranging 5%–10% (Middlebrooks et al., 1979). These different protocols highlight the importance of tailoring cryopreservation to the unique needs of each fish cell line, ensuring optimal post-thaw viability and functionality.

3.2. Media for cell proliferation

Several serum free media formulations have been published for growing cells deriving from **terrestrial animals** *in vitro* and have been very shortly reviewed by Quek et al., 2024 (Quek et al., 2024). These media are based on DMEM/F12 basal media which was originally identified to be the best choice for human cells by Chen et al. (G. Chen et al., 2011). DMEM/F12 is a 1:1 mixture of DMEM and Ham's F12 medium and uses sodium bicarbonate as buffering system to maintain a physiological pH. Alternatively, HEPES is included to support the buffering capacity of DMEM/F12 (Arora, 2023). This basal medium typically requires

Info box 2: There is **NO universal** chemically defined medium applicable to all cell types and species.

- ⇨ SF proliferation media for **muscle stem cells and fibro-adipogenic progenitor cells from terrestrial species** have been developed
- ⇨ SF proliferation media for **not edible species ESCs/(i)PSCs** are available, but lack for edible species
- ⇨ Proliferation of **aquatic muscle and fat precursors still depends on media supplemented with serum**
- ⇨ Cell line adaptation leads to extremely cost-effective media solutions, but risk factors have to be considered

supplements to support and maintain cell proliferation. The components as well as their concentrations vary depending on the cell type and species (see overview in Table 3 and Info box 2).

Leibovitz's L-15 is used as basal medium for the cultivation of a wide range of **fish cells**, but recent studies revealed that F10 and DMEM/F12 could be more suitable for fish primary SCs, with F10 demonstrating a more pronounced effect on proliferation compared to DMEM/F12 (S. Zhang et al., 2023). Notably, **marine fish cells** typically require a **higher osmolarity** compared to both freshwater fish and mammals (Gardell et al., 2014; Lakra et al., 2011; Solhaug et al., 2024).

It is clear from world-wide efforts that there is no single alternate or chemically defined medium or even a formulation of a group of components that can replace FBS in culture medium for all possible cell types (Subbiahanadar Chelladurai et al., 2021). Still, several published compositions proved to be efficient for multiple CM-relevant cell lines and can be taken as a basis for specific cell line adaptations.

In the following section we summarize the efficacy of SF-media for different cell types.

Medium	E8	B8 and B8 + MC + starch from corn	B9	Kolkmann	Skrivergaard	Pasitka SF 2022 medium	ACF medium[&]	Component (bulk) cost	Product information
Cell type (Ref.)	Human stem cells (G. Chen et al., 2011)	Human stem cells (Kuo et al., 2020), BSCs, PSCs (Schenzle et al., 2025)	BSCs (Stout et al., 2022)	Bovine SCs (Kolkmann et al., 2022)	Bovine SCs (Skrivergaard, Young, et al., 2023)	Chicken fibroblasts (Pasitka et al., 2022)	Chicken fibroblasts (Pasitka et al., 2024)		
DMEM/F12 *	1	1	1	1	1	1	1	€24/L	Thermo Fisher #11320033
ITSE Animal-Free**	-	-	-	1%	-	-	-	€488.64/100 mL	InVitria 777ITS032
Not animal-origin free ITS**	-	-	-	-	1%	-	-	€55.5/10 mL	Thermo Fisher #41400045
rInsulin	19.4 mg/L	20 mg/L	20 mg/L	**10 mg/L**	**10 mg/L**	3 mg/L	-	€41,920/100 g	Upon request from InVitria
Ascorbic acid 2-phosphate	64 mg/L	200 mg/L	200 mg/L	50 mg/L	-	-	-	€513/100 g	Sigma #49752
rTransferrin	10.7 mg/L	20 mg/L	20 mg/L	**5.5 mg/L**	**5.5 mg/L**	-	-	€70/g	Upon request from Oryzogen
Sodium selenite	14 µg/L	20 µg/L	20 µg/L	**6.7 µg/L**	**6.7 µg/L**	7 µg/L	7 µg/L	€240/100 g	Sigma # S5261
rFGF-2 (FGF-2-G3***)	100 µg/L	40 µg/L***	40 µg/L	10 µg/L	2 µg/L	10 µg/L	-	€22/mg	Upon request from Oryzogen
rTGFß1	2 µg/L	0.1 µg/L	0.1 µg/L	-	-	-	-	€1,235/100 µg	Upon request from Oryzogen
rNGR1	-	0.1 µg/L	0.1 µg/L	-	-	-	-	€1,484.00/mg	Upon request from Peprotech
rVEGF	-	-	-	10 µg/L	-	-	-	€150/mg	Upon request from Oryzogen
rIGF-1	-	-	-	100 µg/L	-	-	-	€30/mg	Upon request from Oryzogen
rHGF	-	-	-	5 µg/L	20 µg/L	-	-	€4,735.00/mg	Thermo Fisher #100-39-01M
rPDGF-BB	-	-	-	10 µg/L	5 µg/L	-	-	€3,845.00/mg	Thermo Fisher #100-14B-1MG
rhIL-6	-	-	-	20 µg/L	-	-	-	€2,965/mg	Thermo Fisher #200-06-1MG
Sodium bicarbonate	543 mg/L	2428 mg/L	2428 mg/L	-	-	-	-	€59.30 per 500 g	Sigma #S5761
HSA	-	-	800 mg/L	5 g/L	-	-	-	€25/g	Upon request from Oryzogen
BSA	-	-	-	-	75 mg/L	-	-	€154/100 mL 7.5% in DPBS (x100)	Sigma #A8412
rFibronectin	-	-	-	10 mg/L	-	-	-	€35/mg	Upon request from Oryzogen
Hydrocortisone	-	-	-	36 µg/L	-	2 mg/L	-	€305/10g	Sigma # H0888
L-alanine-L-glutamine	-	-	-	-	-	2 mM	4 mM	€18.700,71/50 L of 200 mM	Sartorius SKU: 03-022-1B, upon request
Glucose	-	-	-	-	-	-	5 g/L	€170,10/25 kg	Laboratoriumdiscounter #GLUC1.3
Methylcellulose (MC)	-	0.01%[§]	-	-	-	-	0.1%	€339/kg	Sigma #M0512
Others		• 0.4 g/L starch from corn[§]		• 1 mg/L α-linoleic acid • **0.2 g/L ethanolamine** • 1 % GlutaMAX	• 600 mg/L fetuin	• 10 mg/L in-house lipid mixture[§]	• 10 mg/L in-house lipid mixture[&] • 7 U/ml antioxidant solution[&&] • 0.3 mg/ml HPBCD[§§§]	• Starch from corn €221.00/5 kg • Alpha-linoleic acid €1,680/10 g • GlutaMAX €77/100 mL • Fetuin €3,240/25 g • HPBCD €1,470/100 g • § Price?	• Starch from corn Sigma #S4126 • Alpha-linoleic acid Sigma #L2376 • Fetuin Sigma #F2379-25G • GlutaMAX Thermo Fisher #35050061 • HPBCD[§§§§] Sigma #H107-100G
Price per Liter (no basal medium)	**€36.17**	**€12.70** **€12.75[§]**	**€32.70**	**€658,18** **€308,18[§§]**	**€262.67** **€148.74[§§§]**	**€5.30**	**€12.29**		

Table 3: SF proliferation media formulations, components and their concentrations. Basal medium costs – DMEM-F12 – were not considered. Bulk prices – when available – are included.

* DMEM/F12 (Thermo Fisher #11320) contains L-glutamic acid (7.35 mg/L) and L-glutamine (365 mg/L).

** 100x ITS formulations contain 1 g/L insulin, 0.55 g/L transferrin and 0.67 mg/L sodium selenite, and ITSE additionally contains 0.2 g/L ethanolamine. If ITS/ITSE is included into formulation, components concentrations contained therein are marked **bold underlined**, and only ITS/ITSE costs are considered.

*** In commercially available B8, provided by Defined Biosciences, a stabilized version of FGF-2 (Dvorak et al., 2018) is used.

[§] B8 additionally stabilized by methylcellulose (MC) and starch from corn. [§§] Without fibronectin. [§§§] Without HGF and PDGF-BB. [§§§§] HPBCD - 2-hydroxypropyl-β-cyclodextrin.

[&] ACF medium seem to contain undefined GF(s), listed in Fig 2b (Pasitka et al., 2024), which we include into this calculation with the listed price of $0.008/L = €0,0071/L.

[&&] Undefined composition, undefined price.

3.2.1. (Induced) pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs)

As shortly mentioned in chapter 2, **Essential 8 (E8)** is a widely used and commercially available animal component-free medium, originally designed for **human** PSC and ESC culture (G. Chen et al., 2011). It works well for expansion and prolonged maintenance of stem cells without triggering differentiation (G. Chen et al., 2011; Specht, 2020). E8 is based on the SF basal medium DMEM/F12 supplemented with L-ascorbic acid-2-phosphate, magnesium, sodium selenium, FGF-2, insulin, sodium bicarbonate, transferrin, transforming growth factor (TGF)β1 or Nodal (G. Chen et al., 2011; J. Xiao et al., 2018) (for their concentrations see Table 3).

The concentrations of the eight components in E8 were further optimized to support a higher growth rate of human iPSCs under low seeding density conditions (Kuo et al., 2020). The resulting medium formula termed B8 could support both hiPSC generation and long-term culture for more than 100 passages (see composition in Table 3). Observations of the authors and communications with other researchers led to the conclusion that neither E8 nor B8 media were universally suited for all human iPSC lines. However, there is a lack of medium alternatives suitable for iPSC/ESCs from edible species. A medium published for porcine iPSCs, which includes three inhibitors known for enhancing human embryonic stem cells self-renewal (CHIR99021, SB431542, and PD0325901), and three cytokines (BMP4, SCF, and IL-6), also contained human platelet lysates, which makes it not applicable for the CM/CSF area (Ma et al., 2018). Significantly more data is available for human iPSCs, but such media compositions would require verification and possibly adaptation for iPSCs from edible species. For example, a simplified SF culture medium containing FGF-2 and the WNT and SRC pathway inhibitors IWR-1 and WH-4-023 (H. Choi et al., 2024) has been reported, but it is formulated using the commercial medium mTeSR™ with a proprietary composition, thus not ideal for CM/CSF applications.

3.2.2. Bovine satellite cells (BSCs) and porcine satellite cells (PSCs)

Stout et al. adapted the SF-medium B8 from Kuo et al. after they observed that BSCs isolated from a 2-week-old calf proliferated only for 3 days in this medium, indicating that a complete substitution of serum by B8 compounds did not meet the requirements of BSCs (Stout et al. 2022; Kuo, 2020). The addition of **recombinant human serum albumin** (rHSA, 800 mg/L) enabled BSCs to grow for at least seven passages over 28 days (Stout et al., 2022), underlining the importance of molecules with stabilizing properties, as also present in serum. This adapted B8 medium was termed **Beefy-9 or B9,** (see Table 3) due to its component number and it specific design for bovine muscle cells. Increasing the concentration of albumin in B9 further improved growth of BSCs over one month of cell expansion. Total cell doublings reached 19 and 20 for B9 containing 3.2 and 6.4 mg/mL albumin, respectively, as compared to 18 cell doublings for 0.8 mg/mL albumin (Stout et al., 2022). But it also substantially increased costs for this medium. Further, B8 stabilized with methylcellulose and B9 was also demonstrated to perform well for both BSCs, PSCs and chicken satellite cells (CSCs), with the more expensive B9 performing slightly worse in case of PSCs and CSCs (Schenzle et al., 2025). This line of research – stabilization of media – is further discussed below in the chapter 4.4.5.

Kolkmann et al. performed a 5-step medium optimization process which led to reduced concentrations of all components present in B9 except for rHSA (Kolkmann et al., 2022). HSA was increased to 5 g/L and also the number of GFs was elevated (Table 3). Consequently, costs for this medium are appr. 20x higher than for B9, with 20% of the total medium price attributed to HSA and approx. 60% to fibronectin (cell adhesion agent), and IL-6, HGF and PDGF as most costly GFs. This medium supported growth of BSCs over 6 passages almost as good as the control growth medium containing 20 % FBS (Kolkmann et al., 2022).

Skrivergaard et al. applied a Design of Experiment (DOE) approach in combination with a Response Surface Methodology to develop and optimize a SF-medium for BSCs (Skrivergaard, Young, et al. 2023).

This approach reduced the number of constituents, especially of GFs. Instead of rHSA they used a combination of 75 mg/L serum derived bovine serum albumin (BSA) and 600 mg/L recombinant fetuin. The fetal serum component fetuin is a serum carrier protein like albumin with multiple important biological functions including insulin signaling (Chekol Abebe et al., 2022). Fetuin had much higher proliferative effect on BSCs than BSA, when the latter was added at low concentration of 75 µg/mL because higher amounts of BSA seemed to hinder cell attachment (as also noted for HSA by Stout (Stout et al., 2022)). The addition of 600 µg/mL fetuin and 75 µg/mL BSA to DMEM/F12 allowed a reduction of the amount of FGF-2 from 10 ng/mL (Kolkmann et al., 2022) to 2 ng/mL (Skrivergaard, Young, et al., 2023). The final minimal formulation of Skrivergaard et al. resulted in significant positive effects on the proliferation of BSCs over a 5-day period compared to the 10% FBS-containing control, and this medium also sustained proliferation of PSCs at a significantly higher level than 10% FBS in a short term experiment. Costs for this medium are 2.5 times lower as compared to the medium from Kolkmann et al., but fetuin accounts for approximately 80 % of the costs (see Table 3) (Skrivergaard, Young, et al., 2023).

Similarly to Kolkmann et al., the inclusion of PDGF (5 ng/mL) and HGF (20ng/ml) showed further positive effects in short term proliferation assays with BSCs and PSCs, as well as in long term cultivation of BSCs (Skrivergaard, Young, et al., 2023). The same effect was reported by Schenzle et al. in methylcellulose stabilized B8 medium for both BSCs and PSCs during long term proliferation (Schenzle et al., 2025).

3.2.3. Towards serum free proliferation media for fish muscle cell progenitors

Although research on SF medium formulations for **fish muscle cells** is clearly behind the research on mammalian cells, initial studies highlight the cell type-specific effects of different GFs and demonstrate the strong responsiveness of fish muscle-derived cells to these stimuli.

In addition to FBS and fish embryo extract, most fish cell lines require supplementation with **additional GFs**. But The knowledge in this area is still extremely sparse and patchy, with only a handful of suppliers offering FGF-2 and IGF-1 of fish origin. Thus, GFs and hormones typically used in fish cell culture are derived from mammalian sources (human, mouse, or rat). Identification of such crucial GFs is still a very important step towards SF media for fish cell lines.

Embryonic fish stem cells require basic fibroblast growth factor (FGF-2) and leukaemia inhibitory factor (LIF) (S. -L. Chen, Sha, et al., 2003; S. -L. Chen, Ye, et al., 2003; Dash et al., 2010; Sha et al., 2010), while muscle-derived cell lines primarily depend on FGF-2, insulin-like growth factor 1 (IGF-1), and insulin , additionally to FBS (Chong et al., 2022; Gabillard et al., 2010; Kong et al., 2021; Krishnan et al., 2023). IGF-1 promoted the proliferation of Catmus1PFR myofibroblast precursors, but not brown bullhead catfish (*Ameiurus nebulosus*) **fibroblasts** (Cyrino & Mulvaney, 1999) nor Atlantic mackerel (*Scomber scombrus*) **skeletal muscle** Mack1 cell line (Saad et al., 2023). IGF-1 at a concentration of 50 nM was also shown to stimulate the proliferation of trout satellite cells under low serum conditions (2%) in both DMEM and F10 media, with a fivefold increase observed when cells were cultured in F10 medium (Gabillard et al., 2010). Some **fibroblast cultures** rely on FGF-2 and epidermal growth factor (EGF), additionally to FBS (Rougée et al., 2007; L. Zhao et al., 2020; Z. Zhao et al., 2004). Additionally, the olive flounder **satellite muscle cell line** (OFMC) showed diminished proliferation at 10% FBS (Krishnan et al., 2023), while FGF-2 supplementation was found to enhance cell growth.

A recent article reviewing the mechanisms and signalling pathways involved in the formation of muscle, fat, and other relevant tissues within the context of cultured fish (Bomkamp et al., 2023) highlighted several factors – e.g. myostatin inhibitors, IGF mimics, and the adenylate cyclase activator forskolin - as possible enhancers of cell proliferation *in vitro*. IGF-2 could also be tested in combination with other cytokines, as medaka IGF-2 (*Oryzias latipes*), and its human ortholog, were able to support viability and pluripotency of medaka ESCs in culture media containing only basal medium (Yuan & Hong, 2017). The morphology of the ESCs cultured in basal medium with 200 nM medaka IGF-2 was approaching the stem cells in rich medium containing 15% FBS, medaka embryo extract, 2% of seabass serum and 10 ng/ml of human FGF-2.

In another recent study, Dong et al. (Dong et al., 2024) explored non-animal-derived alternatives to reduce animal serum dependency by evaluating the potential of freshwater green algae *Auxenochlorella pyrenoidosa* protein extract (APE) as a partial replacement for FBS in **goldfish muscle cells** (CAM). These cells are traditionally cultured in L-15 medium supplemented with 10% FBS and 10% goldfish muscle extract. While the study did not explicitly state whether goldfish muscle extract was replaced, suggesting continued reliance on animal-derived components, the results were promising. Short- and long-term assays showed that APE supplementation significantly increased proliferation at reduced FBS levels (e.g., 5% and 2%), compared to 10% FBS controls. Notably, Myf5 expression remained consistent across all conditions suggesting that differentiation potential was maintained (Dong et al., 2024). However, longer-term studies are necessary to confirm the sustained viability of APE as a serum replacement. Under nutrient-deficient conditions, cells often activate autophagy, a survival mechanism that recycles intracellular components to compensate for the lack of serum, amino acids, or GFs. While autophagy initially provides protective benefits, prolonged or excessive activation can induce transition into autophagic cell death. This dual role has been well-documented, with autophagy

serving as a critical adaptive response under short-term stress but becoming detrimental under sustained nutrient deprivation (S. Liu, Yao, et al., 2023; Wirawan et al., 2012).

To further address the remaining 5% FBS requirement, Dong et al. screened a range of additives, including antioxidants, glucocorticoids, and an ITS-based supplementation (Dong et al., 2024). Through this screening, they identified L-ascorbic acid, a vitamin with antioxidants properties (Gęgotek & Skrzydlewska, 2022), and ITSE (insulin-transferrin-selenium-ethanolamine) as effective components at optimal concentrations of 10 µM and 5× (exact concentrations are not disclosed, producer: Beyotime Biotechnology), respectively. L-ascorbic acid has been previously shown to stimulate the proliferation of **pacu (*Piaractus mesopotamicus*) myoblasts** (Duran et al., 2019), while ITSE has demonstrated similar effects on mammalian muscle cells (Kolkmann et al., 2022; Skrivergaard, Young, et al., 2023). APE+ITSE+L-ascorbic acid combination is shown to effectively promote cell proliferation in the absence of FBS, with total cell numbers in the scale-up culture on day 3 almost reaching the level of 10% FBS. Another study describes a medium containing **dried Maitake mushroom extracts** used to culture **goldfish** (*Carassius auratus*) cells at comparable efficiency as the FBS containing medium (Benjaminson et al., 2002), but it was **not successful** to support proliferation of **Mack1 cell line** (Saad et al., 2023).

These studies provided important insights into the development of SF medium formulations for fish cells. However, the **knowledge on fish GFs that are central to muscle development is lacking**, and our understanding of the mechanisms governing their functionality at different stages of development is rudimentary. The field remains at an early stage of development, **requiring further research** to establish reliable, scalable, and cost-effective alternatives to FBS and species-specific derived extracts for SF proliferation media of fish muscle cell precursors.

3.2.4. Fibro-adipogenic progenitor cells from terrestrial species

Only in 2022 a comprehensive review on **growth media for adipogenic progenitor cells** and cell culture to generate alternative fat was conducted by Sugii et al. showing that almost all the studies supplemented basal media with FBS in addition to diverse GFs (Sugii et al., 2022).

But recently, Hubalek et al. showed that bovine fibro-adipogenic progenitor cells (FAPs) which are suitable for cultured fat production, were able to grown in a SF-proliferation medium originally developed for bovine satellite cells (Kolkmann et al., 2022), as well as in the same medium adapted for low ammonia genicity (Hubalek et al., 2023). Accumulation of ammonia is a result of the metabolization of glutamine by the cells or of its spontaneous degradation in the medium. Substitution of glutamine with a-ketoglutarate, glutamate and pyruvate in multi-passaging experiments led to a slightly higher doubling time during proliferation, with a-ketoglutarate performing best. But more importantly, both during proliferation and differentiation, the ammonia concentration in adapted medium was significantly lower than in glutamine containing control and barely increasing (Hubalek et al., 2023). Thus, the **study presents four SF medium compositions for proliferation of cultured bovine fat precursors.**

3.2.5. Spontaneously immortalized chicken fibroblasts

A further SF medium composition for proliferation of fat cell progenitors was mentioned in chapter 2. Pasitka et al. were able to adapt a **spontaneously immortalized chicken fibroblast** cell line to an albumin and SF culture medium containing only one GF and insulin (see Table 3; (Pasitka et al., 2022). And eventually, this cell line was shown to perform even better in an animal-component-free (ACF) medium, based on DMEM/F12 medium supplemented with three components to substitute albumin: 0.1% methylcellulose, 7 U/ml antioxidant solution and 0.3 mg/ml 2-hydroxypropyl-β-cyclodextrin; with 7 ng/ml sodium selenite, 4 mM L-alanine-L-Glutamine, 5 g/L glucose and 10 µg/ml of in-house prepared lipid mixture, and with a very low concentration of undefined GF (Pasitka et al.,

2024). This made a **medium price of under €1/L** possible (calculated by Pasitka et al.), though several components were produced in-house, and their industrial scale costs could result in a higher final price (see Table 3).

3.2.6. Fish preadipocytes

The selection of an appropriate basal culture medium is critical for the efficient proliferation of **fish preadipocytes**, and DMEM was found to promote higher growth rates than L-15 (Salmerón et al., 2013). **No SF formulations** have yet been published, nor have strategies for a FBS reduction or elimination in fish preadipocyte cultures been explored. While studies have investigated **specific molecules to enhance preadipocyte proliferation**, these approaches have exclusively used FBS-supplemented media.

Preadipocytes are routinely cultured without the addition of specific GFs in proliferation media. Some – e.g. preadipocytes from grass carp – required additional supplementation with fish serum, indicating that fish serum may contain crucial fish-specific GFs (P. Liu et al., 2018; Tian et al., 2016). Cytokines such as IGF-1 and insulin, and growth hormone (somatotropin) were used to improve **gilthead seabream preadipocyte proliferation** (Salmerón et al., 2013). Similarly, insulin enhanced the proliferation of preadipocytes from the large yellow croaker (X. Wang et al., 2012), while tumor necrosis factor-alpha (TNFα) and docosahexaenoic acid (DHA) inhibited it. **In rainbow trout**, medium supplementation with IGF-I stimulated preadipocyte proliferation, particularly during early phases of culture (Bouraoui et al., 2008).

Interestingly, low doses of TNFα slightly enhanced proliferation, though higher doses were less effective (Bouraoui et al., 2008). Furthermore, DHA was shown to induce the Wnt/β-catenin signaling pathway, suppressing late-stage adipogenic differentiation and maintaining preadipocytes in an undifferentiated, potentially proliferative state (P. Liu et al., 2018).

Previous studies involving polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), DHA, and arachidonic acid (AA), have also demonstrated their capacity to enhance proliferation in skeletal VSa16 cell lines of **gilthead seabream** without exhibiting toxic effects (Viegas et al., 2012). Similarly, the proliferation of EPC-EFAD, a cell line derived from carp epithelial papilloma cells that can survive and proliferate in essential fatty acid-deficient (EFAD) medium, was stimulated by AA, EPA, and DHA in short-term cultures, while (alpha-)linolenic acids (LA and ALA) inhibited it. However, a long-term exposure to these fatty acids increased cytotoxicity, an effect that was partially mitigated by the inclusion of vitamin E in the culture medium (Tocher et al., 1996). These reports emphasize the potential **role of several components** in optimizing media composition to enhance preadipocyte proliferation, while also highlighting the **variations** in their proliferative effects **across different fish cell species**. Investigating these components may present opportunities for developing SF media specifically tailored for preadipocyte proliferation. Furthermore, sourcing PUFAs from non-animal organisms, such as microalgae (W. Chen et al., 2023), could make them more suitable for cultured fish applications.

3.3. Media for cell differentiation and maturation

3.3.1. Myogenesis

Info box 3: Differentiation media:

- ⇨ Several SF media formulations are published for **myogenesis of terrestrial** species' cell lines
- ⇨ **For myogenesis of aquatic** species' cell lines, no SF formulations are reported
- ⇨ SF formulations for adipogenic differentiation of both terrestrial and aquatic species are published
- ⇨ Further search for efficient differentiation inducers / ligands / stimulatory factors **suitable for the food sector** is required

After the proliferation phase of SCs, differentiation and maturation of myoblasts can occur within 7 – 14 days *in vitro*, compared to months or years *in vivo* (Martins et al., 2024). An irreversible cell cycle arrest of the precursor cells (myoblasts) is a prerequisite for initiating differentiation. Myogenesis proceeds with an increasing expression of muscle function genes and ends with the formation of multinucleated myofibers by fusion of myoblasts (Blais et al., 2005; Martins et al., 2024). *In vitro*, cell cycle arrest is generally induced through **serum starvation** (Ostrovidov et al., 2014), a concept also successfully applied to fish myosatellite cells (Gabillard et al., 2010; Krishnan et al., 2023; Ulagesan et al., 2024) and myoblasts (Kong et al., 2021; Saad et al., 2023). Despite its effectiveness, this method still depends on animal-derived serum, although at low concentrations (e.g., 2% FBS) (Kirkpatrick et al., 2022; Kolkmann et al., 2022). In both, terrestrial animals and fish culture, alternative protocols often substitute FBS with horse serum due to less ethical concerns although it is of animal origin (Kong et al., 2021; Krishnan et al., 2023; Lautaoja et al., 2020; Saad et al., 2023).

The design of **SF formulations** that drive robust differentiation of SCs independent of species has proven to be challenging (Martins et al., 2024), still, several efficient formulations have been published. Functional myotube formation using SCs extracted from adult rats was observed with a SF differentiation medium containing 10 ng/mL **IGF-1** and 100 ng/mL **EGF** in a 1:1 mixture of

Neurobasal and L-15 medium (McAleer et al., 2015). SCs isolated from bovine muscle and grown in B9 medium also differentiated efficiently into myotubes in SF-differentiation medium developed by McAleer, reaching higher fusion indices as compared to FBS containing medium (Stout et al., 2022). Messmer et al. investigated transcriptomic changes in BSCs during myogenic differentiation induced by serum starvation and identified several surface receptors upregulated during the early phase of differentiation (IGF1R, TFRC and LPAR1) (Messmer et al., 2022). Supplementing SF DMEM basal medium with **ligands** for these receptors resulted in differentiation efficiencies comparable to serum starvation. It still has to be kept in mind, that these SF formulations often use animal-derived coating proteins, such as Matrigel (Messmer et al., 2022) or collagen (Sugii et al., 2023), which could bind and bring in undefined cytokines of animal origin into SF medium.

Several other supplements are known to induce myogenesis. Research on the signaling pathways that regulate myogenic differentiation unveiled that ERK1/2 inhibition induces robust myoblast differentiation and fusion of primary mouse myoblasts (Eigler et al., 2021). Although, **kinase inhibitors** could provide a powerful tool to enhance efficiency of myoblast differentiation and fusion for the CM industry, cytotoxicity of kinase inhibitors must be considered as studies showed that some kinase inhibitors are highly toxic to primary cultures of hepatocytes (J. Zhang et al., 2018). Multiple studies report **nitric oxide (NO)** as a differentiation-stimulatory factor, promoting activation, renewal and differentiation of satellite cells and ESCs (Buono et al., 2012; Caballano-Infantes et al., 2022). Also, both **mechanical and electrical stimulations** are well documented to improve the myogenic differentiation of muscle stem cells and the contractile force thereof (K. Choi et al., 2021; A. Ito et al., 2014; Langelaan et al., 2011).

The development of a SF medium for the myogenic differentiation of fish muscle cells has not been reported in the literature yet, but several ingredients that could induce differentiation have been identified. **Retinoic acid** at varying concentrations (from µM to mM) has been shown to promote differentiation in fish embryonic stem cells (ESCs) (Bejar et al., 2002; S. -L. Chen, Sha, et al., 2003;

S. -L. Chen, Ye, et al., 2003; Dash et al., 2010; Parameswaran et al., 2007, 2012). Compounds known to enhance myogenesis in fish stem cells—including FGF-2, GSK3β inhibitors, calpain inhibitors, and adenylate cyclase activators—may also offer promising avenues for improving myogenic differentiation protocols ((Bomkamp et al., 2023), see Table 3).

Alternative approaches to induce myogenic differentiation in fish primary SCs have been tested, and the supplementation of DMEM/F12 medium with 8% FBS, 2.5 µM RepSox (a TGFβR-1/ALK5 inhibitor (Ichida et al., 2009)), 5 nM LY411575 (a γ-secretase inhibitor (Curry et al., 2005)), and 0.125 µM dexamethasone (DEX, a synthetic glucocorticoid (E. Xu et al., 2023)) triggered the differentiation of *Larimichthys crocea* satellite cells (S. Zhang et al., 2023). The suitability of these components for the food sector remains to be established. For the differentiation of fibroblasts into muscle-like cells, non-starvation-based methods have shown promising results. In thread-sail filefish *Stephanolepis cirrhifer*, a transition from L-15 basal medium containing 10% FBS to AIM V basal medium (Thermo Fisher Scientific) containing 10% FBS successfully stimulated myogenesis of fibroblast-like cells (Tsuruwaka & Shimada, 2022). Interestingly, the same thread-sail filefish fibroblast cells could differentiate into adipocytes when cultured in L-15 with 10% heat-inactivated SeaGrow serum (EastCoast Biologics, North Berwick, ME).

Although the **maturation of myofibers** has not been extensively studied, there are several documented components and conditions that promote this process, e.g. anabolic GFs including IGFs and androgen (K. Choi et al., 2021). **Co-culturing** of C2C12 myotubes with fibroblasts resulted in **higher maturity** and contractility of differentiated muscle fibres (Cooper et al., 2004). Fibroblast monolayers can function as an elastic support for contracting myotubes. Further, fibroblasts secrete extracellular matrix components and GFs that may be important for the differentiation of myotubes (Cooper et al., 2004). Myogenic fusion events and the organization of muscle fibres can also be influenced by the structural properties of **scaffolds** (for details see review from (Ostrovidov et al., 2014).

To summarize, substantial **knowledge exists** regarding the SF differentiation of mammalian muscle cells, and several effective SF differentiation media have been published (see Table 4 and Infobox 3). In contrast, **more efforts are needed** towards the development of SF differentiation media for muscle cells from aquatic animals.

	Myogenesis			
Component	**McAleer**	**Messmer**	**Component (bulk) cost**	**Product information**
Cell type (Ref.)	Rat myoblasts (McAleer et al., 2015)	BSCs (Messmer et al., 2022)		
Neurobasal	500 ml/L	-		Invitrogen #21103049
L15	500 ml/L	-		Invitrogen #11415064
DMEM/F-12	-	1 L		Invitrogen #53003018
rEGF	100 µg/L	10 ng/ml	€371.41/mg	Fisher #50-197-6254
rIGF-1	10 µg/L	-	€225.00/mg	Qkine #Qk047
rHuman serum albumin	-	0,5 mg/ml	€25/g	Upon request from Oryzogen
L-ascorbic acid 2-phosphate	-	40 µM	€513/100 g	Sigma #49752
MEM AA solution	-	1x	€546.00/2000 ml of 50x	Fisher #11580386
$NaHCO_3$		6.5 mM	€59.30 per 500 g	Sigma #S5761
Sodium selenite	-	80 nM	€240/100 g	Upon request from Oryzogen
rInsulin	-	1,8 µM	€41,920/100 g	Upon request from InVitria
Lysophosphatidic acid (LPA)	-	1 µM	€212.57/25 mg	Focus #10-1449
rTransferrin	-	135 nM	€70/g	Upon request from Oryzogen
Acetylcholine	-	10 µM	€313.00/500 g	TCI #A0084
Price per Liter (no basal medium)	**€39,39**	**€26,95**		

Table 4: SF myogenic media formulations, components and their concentrations. Basal medium costs were not considered. Bulk prices – when available – are included.

3.3.2. Adipogenesis

Differentiation of precursor cells into mature adipocytes that accumulate large amounts of lipids is a complex process with multiple sequentially activated transcription factors driving the process,

with PPARg being identified as the most critical transcription factor among them (Sarjeant & Stephens, 2012).

Standard protocols for mammalian cells use an adipogenic cocktail containing **insulin, glucocorticoids** (e.g. cortisol or dexamethasone (DEX), a synthetic glucocorticoid) and 1-methyl-3-isobutylxanthine (**IBMX** – a non-specific inhibitor of cyclic nucleotide phosphodiesterases, which prevents the break down of cyclic AMP (cAMP) and cyclic GMP (cGMP) within cells) (Hauner et al., 1989; Hube et al., 1999; Wabitsch et al., 1995). Progenitor or stem cell types such as preadipocytes and MSCs differentiate well into mature adipocytes in presence of this standard adipogenic cocktail (Sugii et al., 2023). This cocktail together with one **PPARγ activator** was used in most of the *in vitro* differentiation studies with cells of edible species until recently (Mitić et al., 2023; Sugii et al., 2023). PPARγ agonists, such as thiazolidinediones, have been reported to be also necessary for successful maturation of adipocytes that exhibit full adipogenic characteristics (Cheng et al., 2016), and can even trans-differentiate myoblasts into adipocyte-like cells (Teboul et al., 1995).

However, some ingredients of this classical adipogenic cocktail are not food-compatible, like the synthetic molecules IBMX and the PPAR-g antagonist rosiglitazone which are classified as toxic (Fort et al., 1998; Nissen & Wolski, 2007). Although these compounds are required only transiently during the initial induction of adipogenesis, searching for non-toxic options will be necessary for food production processes (Yuen Jr et al., 2022). In this regard, Katafuchi et al. provided evidence that the C-type natriuretic peptide (CNP), an endogenous adipogenesis regulator, can **substitute IBMX** in the adipogenic cocktail (Katafuchi et al., 2010). Replacing IBMX (0.5 mM) with CPN (10 nM) enhanced levels of adipogenesis marker genes to almost the same range compared to the IBMX-containing cocktail (Katafuchi et al., 2010). However, the costs for synthetic CPN peptides will elevate costs for large-scale CM production (1 mg of CPN-22 peptide costs appr. €150 (Bachem); appr. €1/L medium).

631 In another attempt to design and simplify a food-grade formulation for adipogenesis, Mitić et al.
632 demonstrated that out of the four inducers, **insulin** and PPAR-g antagonist **rosiglitazone were**
633 **sufficient** to trigger adipocyte differentiation (Mitić et al., 2023). In addition, low amounts of two
634 glucocorticoid receptor activators, namely **hydrocortisone and progesterone**, were found to be
635 important for a homogeneous cell distribution during differentiation, as well as for cell viability and
636 spreading. Thus, Mitić et al. demonstrated that DEX and IBMX were not necessary for adipocyte
637 differentiation of bovine stromal vascular cells. The **simplified one-step adipogenic protocol** with
638 a reduced number of adipogenic inducers and phases was not species specific and outperformed
639 serum-containing medium. The percentage of positive cells and total lipid area were significantly
640 higher in the SF differentiation conditions used by Mitić et al. This highly efficient SF and animal
641 component free medium, designated DMAD (Defined Medium for Adipogenic Differentiation), is
642 based on DMEM/F-12 and efficiently **narrowed down toxic adipogenic inducers** to only
643 rosiglitazone (see Table S3 for full composition in (Mitić et al., 2023)).
644 Beside adipogenic inducers, **adipogenesis requires a constant supply of free fatty acids (FFA)**
645 for triglyceride production and lipid droplet accumulation (Salmerón, 2018). In experiments with
646 bovine stromal vascular cells and conventional differentiation media supplemented with FFAs**, oleic**
647 **and linoleic acid** were found to be the most efficacious for promoting lipid accumulation during
648 maturation of adipocytes (Yanting et al., 2018). Mitić et al. included 0.1 % of a chemically defined
649 and commercially available **lipid concentrate** in their differentiation medium. Beside a mixture of
650 seven free fatty acids (C14 to C20, saturated and unsaturated) and cholesterol, it contains Pluronic
651 F-68 (used to reduce foaming in stirred cultures and to protects cells from hydrodynamic damage)
652 and Tween 80 as emulsifying and dispersing substance (Mitić et al., 2023).
653 For a **more food-compatible strategy**, Mehta et al. proposed the use of FFAs for induction of
654 adipogenesis in bovine precursors, as unsaturated and branched-chain fatty acids are agonists of
655 PPAR-family transcription factors (Mehta et al., 2019). However, FFAs can have both beneficial and

656 unfavorable effects on adipogenic differentiation, depending on the type of fatty acids and cell
657 type or species (Yuen Jr et al., 2022). E.g. n-6 PUFAs stimulate, but n-3 PUFAs inhibit adipocyte
658 differentiation and PPARg *in vivo* (Thoennes et al., 2000). Porcine adipocyte differentiation was
659 increased in a dose-related fashion by addition of oleic acid (C18:1) or linoleic acid (C18:2), but not
660 by addition of α-linolenic acid (C18:3) to SF differentiation medium (DMEM/F12 containing 100 nM
661 bovine insulin, 50 ng/mL hydrocortisone and 10 µg/mL transferrin) (S.-T. Ding et al., 2003). Oleic acid
662 also induced a strong increase in the accumulation of lipid droplets in **chicken preadipocytes**
663 isolated with the stromal-vascular fraction (Shang et al., 2014) and was found to promote
664 adipogenic gene expression and lipid accumulation in **bovine muscle satellite cells** (X. Z. Li et al.,
665 2019), however, all these studies included serum in their differentiation media.
666 The effectiveness of **oleic acid and linoleic acid** to induce *in vitro* cell adipogenesis presents a
667 sustainable and probably more cost-efficient opportunity for large scale cultured fat production, as
668 these FFAs are present at high concentrations in plant-based oils such as olive and soybean oil
669 (Casado-Díaz et al., 2019; Matthaus & Özcan, 2014). The efficacy of **soybean oil** in promoting
670 adipogenesis has been explored in clinical applications for adipose tissue engineering (Hadri et al.,
671 2004; X. Li et al., 2018; Yuen Jr et al., 2022).
672 Also in case of spontaneously **immortalized chicken fibroblasts** from the study of Pasitka et al.,
673 discussed in chapter **3.2.5**, the most efficient transdiffereniation inducers in SF medium – reaching
674 over 80% of the cell population – were 200 µM **oleic acid** combined with 12 µg/ml L-α-
675 **phosphatidylcholine**, which is a major component of plant lecithin. This combination showed a 2.5-
676 fold induction of PPAR-g, as compared to rosiglitazone with oleic acid, and resulted in the formation
677 of adipocyte-like cells in the absence of IBMX or rosiglitazone, delivering the **first SF food-grade**
678 **differentiation medium** for CM applications (Pasitka et al., 2022).
679 The nutritional value as well as flavour and oxidative stability of CM/CSF products depend on the
680 composition of fatty acids in adipose tissue (Louis et al., 2023). For high quality beef meat, like

Wagyu meat, **tenderness and juiciness** are related to high levels of **oleic acid**. A high content of **poly-unsaturated fatty acids**, however, makes meat prone to fast oxidation which leads to **undesirable flavours** (Calkins & Hodgen, 2007; Louis et al., 2023). In addition, the **nutritional aspect** of cultivated fat in CM/CSF products **may be adjusted** by varying concentrations of certain fatty acids in cell culture media during the differentiation process. For example, when FFA-mixtures or oleic acid only were used in adipogenesis studies with isolated **primary bovine adipose-derived stem cells**, differentiation **solely with oleic acid** in serum containing medium resulted in cultured bovine fat which exhibited a fatty acid composition that closely resembled that of the bovine cheek fat and suet controls (Louis et al., 2023).

Besides unsaturated fatty acids, the naturally occurring **carotenoids** (β-carotene and lutein) and **retinoids** were also shown to induce preadipocyte differentiation in cells from bovine white adipose tissue (García-Rojas et al., 2010). Other natural components that may enhance adipogenesis include **biotin** and **pantothenate** (Schiller et al., 2013; Scott et al., 2011), as well as diverse vitamins and acetate (see Table S1 in (Mitić et al., 2023)). These compounds derived from natural sources may be more suitable for the food-compatible process of producing cultured adipocytes as compared to the standard adipogenic induction cocktail.

In line with protocols established for mammalian cells, the **differentiation cocktail used for fish cells** is composed of insulin, IBMX, and DEX (reviewed in (Bomkamp et al., 2023; Dang, 2024)). Additionally, a lipid mixture containing oleic and linoleic acids or an adipogenic cocktail containing fatty acids (Sigma-Aldrich reagent L5146) could enhance the adipogenic differentiation of fish preadipocytes. The adipogenic potential of fatty acids regarding fish adipocytes was assessed in bone-derived MSCs from gilthead seabream, where FFA supplementation resulted in cells adopting a rounded morphology and increased lipid accumulation, particularly with **linoleic acid**. Alpha-linolenic acid, linoleic acid and their combinations significantly upregulated the expression of fatty

acid transporters FATP1 and FABP11, correlating with the enhanced lipid content of adipocytes (Riera-Heredia et al., 2019).

Concerns regarding excessive fat accumulation in **farmed fish,** which could impact production levels, meat quality, consumer perception, and animal welfare (Regost et al., 2001; J.-T. Wang et al., 2005), have stimulated the research on fish adipogenesis (reviewed in (Salmerón, 2018)) and identified molecules that enhance adipocyte differentiation, such as triiodothyronine (T3), biotin, pantothenic acid, ciglitazone, transferrin, and hydrocortisone (reviewed in (Bomkamp et al., 2023; Dang, 2024))

Adipogenesis								
Porcine and ovine FAPs (Mitić et al., 2023)			Chicken fibroblasts (Pasitka et al., 2022)			Stromal-vascular cells of red sea bream (Oku et al., 2006)		
Components	**Component (bulk) cost**	**Product information**	**Components**	**Component (bulk) cost**	**Product information**	**Components**	**Component (bulk) cost**	**Product information**
DMEM/F-12	1 L		DMEM10	1 L		DME/F12 (1:1)	1 L	
HEPES 4.9 mM	€3,290/5 kg	Sigma #H3375-5KG	200 µM oleic acid	€1,082.90/100 g	Thermo #270291000	0.042 mg/L linoleic acid	€639/kg	Sigma W338020-1KG
Lipid concentrate 0.1%	€100/100 ml	Thermo #11905031	12 µg/ml L-α-phosphatidylcholine (soy lecithin)	€820,00/g	Sigma #P7443-1G	65 mM NaCl	€162.75/25 kg	Roth #3957.5
Putrescine 57 µM	€120.95/50 g	Roth #4141.3				50 µg/mL rTransferrin	€70/g	Upon request from Oryzogen
Progesterone 17.8 nM	€105.70/25 g	Glentham #GP3663-25g				5 ng/mL sodium selenite	€240/100 g	Upon request from Oryzogen
Hydrocortisone 25 nM	€305/10 g	Sigma #H0888				50 ng/mL hydrocortisone	€305/10 g	Sigma # H0888
Calcium Chloride 1 mM	€162.50/25 kg	Roth #A119.5				50 µg/ml bovine insulin	€3,162/g	Cell Appl.#128-1000
L-Ascorbic acid 2-phosphate 227 µM	€513/100 g	Sigma #49752						
Glucose 17 mM	€170.10/25 kg	Laboratoriumdiscounter #GLUC1.3						
FGF-2 2 ng/ml	€22/mg	Upon request from Oryzogen						
EGF-1 2 ng/ml	€371.41/mg	Fisher #50-197-6254						
BMP-4 10 ng/ml	€4,140/mg	Thermo #120-05ET-01M						
rInsulin 10 µg/ml	€41,920/100 g	Upon request from InVitria						
Rosiglitazone 5 µM	€284.85/200 mg	Fisher # 16481246						
Price per Liter (no basal medium)	**€60,53**		**Price per Liter (no basal medium)**	**€10.45**		**Price per Liter (no basal medium)**	**€161.63**	

713 **Table 5:** SF adipogenic media formulations, components and their concentrations. Basal medium costs were not considered. Bulk prices – when available – are included.

To date, only **one study** (Oku et al., 2006) has investigated **SF formulations for fish adipocyte differentiation**, notably also excluding IBMX. For **stromal-vascular cells** of **red sea bream** adipose tissue, a differentiation medium consisting of DMEM/F12 (1:1) containing 0.042 mg/L linoleic acid, supplemented with 65 mM NaCl, 50 µg/mL transferrin, 5 ng/mL sodium selenite, and 50 ng/mL hydrocortisone, has been used to induce terminal adipocyte differentiation (Oku et al., 2006). In the absence of FBS, hydrocortisone was found to be essential. The authors also reported that insulin significantly enhanced adipocyte differentiation and facilitated lipid accumulation in a concentration-dependent manner. While T3 alone did not impact differentiation, it did increase differentiation-linked lipoprotein lipase (LPL) gene expression when combined with insulin. In contrast, fat-soluble vitamins had no effects on adipocyte differentiation. Other studies confirmed the role of insulin in stimulating adipocyte differentiation in both gilthead seabream (Salmerón et al., 2013) and large yellow croaker (X. Wang et al., 2012). Interestingly, IGF-1 was identified as a more effective inducer of differentiation compared to insulin in gilthead seabream (Salmerón et al., 2013). While current progress in serum- and IBMX-free media for fish adipogenesis is promising, further research is needed to **assess gene expression profiles** and **signaling pathways** involved in adipocyte precursor proliferation and differentiation. These studies are critical for identifying species-specific needs and developing targeted supplements to optimize (pre)adipocyte culture. For instance, gilthead seabream MSCs from adipose tissue and bone were shown to differentiate into adipocyte-like cells, with distinct lineage-specific regulation of adipogenic gene expression (Salmerón et al. 2016). This highlights the influence of cell origin on adipogenic pathways and the need for tailored culture conditions. The study also identified key genes regulating adipogenesis in gilthead seabream, providing valuable insights into lipid storage regulation in fish (Salmerón et al., 2016). These findings underscore the importance of further investigations into species- and cell-specific mechanisms to improve media formulations and cell culture techniques.

To summarize, in both **CM** and **CSF** areas, efficient **SF adipogenesis promoting media formulations are published**, which also include food grade components only (see **Table 5**). But more research is necessary to cover further cell types and species

4. Strategies to reduce costs of cell culture media

A critical prerequisite to produce CM/CSF is an adequate cell culture medium which provides all essential components required for cells to grow, replicate, and differentiate. Approximately 50% to 90% of the material costs for cell-based food production refer to the cell culture medium (Fernandes et al., 2022; Specht, 2020; Swartz et al., 2021). Garrison et al: estimated that 1 kg CM produced in a large-scale setting will cost $63/kg (around €56/kg) assuming that future technologies will reduce costs for media (Garrison et al., 2022). For reference, the wholesale price of lean pork was €8.12/kg and lean beef was €14.12/kg in 2024 (*Fresh Meat - Europe | Statista Market Forecast*, n.d.). CM manufacturers (Swartz et al., 2021) as well as researchers (Sinke et al., 2023) anticipate that the medium price has to fall to **€1/Liter** to reach price parity with conventional meat. Therefore, the cell-cultured meat and seafood industry requires game-changing innovations to facilitate an economically high-volume production capacity, which are discussed in this chapter (see **Table 6** for an overview).

Info box 4: Strategies to lower cell culture media costs

- ⇨ **Substitution of FBS with hydrolysates: only partial replacement** possible for CM/CSF cell lines
- ⇨ **Recombinant GFs** and **cell line adaptation** are effective alternatives to FBS
- ⇨ **Increasing stability and biological activity of GFs** in culture media significantly cut costs (for the list see **Table 6**)
- ⇨ Low-cost food-grade **HSA alternatives** are available
- ⇨ Hydrolysates are a valid **alternative to basal medium**
- ⇨ Circular cell culture can further **lower the medium price** and **reduce waste generation**

762 Cost reductions can be achieved by altering the medium formulation or by using cheaper food grade
763 alternatives, e.g. by-products of agar or food industry like plant or yeast extracts, strategies which
764 have

Approach	Components	Costs and Comments	References
General approaches			
Switch from pharma grade to food grade compounds	Glucose, amino acids	Glucose (€1/kg instead of €90/kg); Glutamine (€40/kg versus €280/kg).	(Hubalek et al., 2022)
Reduce concentration of growth promoting proteins	FGF-2, TGF-ß, insulin, transferrin	Reduction by app. 90% of the costs for in-house prepared E8; Possible negative effects – determination of appropriate concentration for each factor and cell line/species required	(Specht, 2020)
	Growth promoting proteins in B8	A 3:1 dilution of B8 in DMEM-F12 in presence of stabilizers like methylcellulose or alanine sustained cells viability for four days.	(Schenzle et al., 2025)
Appropriate choice of GFs or orthologs	Human recombinant IL-6	Cost reduction by 80%, Increased proliferation of bovine myoblasts	(Kolkmann et al., 2022)
	Atlantic salmon FGF-2	Best FGF-2 ortholog to promote bovine SC proliferation. Best results with 25 ng/mL. B8 contains 40 ng/mL recombinant FGF-2.	(Venkatesan et al., 2022)
GF alternatives	Peptides, peptoids, small molecules	Lower production costs, higher stability, but unclear safety and regulatory status.	(Atkinson & Dickman, 2023)
Spontaneous cell line adaptation	Adhesion→suspension, lower GF requirements	Allows adaptation of continuously proliferating cell lines to lower GF concentrations (up to 10-20 times lower medium price) and/or to growth as single-cell suspensions (40-fold higher cell densities).	(Lim et al., 2024; Pasitka et al., 2022)
Hydrolysates	Glucose, vitamins, amino acids, minerals	Plant extracts can replace nutrients in media, allowing lower FBS concentration.	(Charlesworth et al., 2024; C. H. Kim et al., 2023; S. H. Kim & Lee, 2009)
		Microalgae can replace nutrients in media, including glucose, allowing lower FBS concentration.	(Dong et al., 2023; Okamoto et al., 2020, p. 20, 2022; Yamanaka et al., 2023)
		Microbial cells can replace nutrients in media but also substitute HSA after adaptation period.	(Andreassen et al., 2020; Dolgin et al., 2024; Sung et al., 2004)
Recycling of waste media	Glucose, vitamins, amino acids, minerals, lactate	Microalgae *Chlorococcum littorale* and RL34 hepatocytes allowed at least two full media cycles, after replenishing GFs and media components using microorganisms. Genetically modified cyanobacteria reduced lactate assimilation.	(Haraguchi et al., 2023; Shimizu et al., 2021; Yang et al., 2023)
Protein engineering			
Increase of GF thermal stability by mutations	FGF-1	Two single mutations led to increased proteolytic stability, but did not increase thermostability.	(Kobielak et al., 2014)
	hFGF-1	Mutating three sites increased stability.	(G. Chen et al., 2012; Zakrzewska et al., 2005, 2009)
	FGF-2	Triple and quintuple mutations improved stability and caused 10-fold prolonged biological activity.	(Jeong, 2015; Nölle, 2015)

Increase affinity of GF for heparin	hFGF-1	Engineering of hFGF-1 heparin binding pocket increased affinity for heparin, but cell proliferation activity of mutated variant was reduced. Heparin is of animal origin and this may be problematic.	(Davis et al., 2018)
Heparin independent GF analogues	FGF-1	FGF-1 variants with increased stability were less dependent on heparin.	(Zakrzewska et al., 2009)
	FGF-2	Similar observations for FGF-2 variants as for FGF-1	(Koledova et al., 2019)
	FGF-2	GF-dimerization by chemical conjugation of two mutated FGF-2 preserved its native conformation and reduced heparin dependence.	(Nawrocka et al., 2020)
Modify binding affinities of GF to receptors	EGF	Site-directed mutagenesis resulted in 30-fold higher affinity of EGF for its receptor. This strategy may lead to rapid receptor internalization or unknown modified signalling effects.	(Cochran et al., 2006; Ren et al., 2020)
Minimal receptor agonists	HGF	Two copies of the kringle 1 (K1) domain in tandem orientation displayed biological activity like native HGF. K1K1 is one fifth of the full-length protein, thus decreasing costs for recombinant GF production.	(De Nola et al., 2022)
Immobilization of GFs			
Physical immobilization	bFGF	Multilayered biodegradable microspheres (heparin-conjugated alginate covered by chitosan and dextran-sulfate). Sustained release for two weeks.	(Zuo et al., 2015)
	FGF-1	Multilayered alginate microcapsules coated with poly-L ornithine membrane. Sustained release for three days. But only co-encapsulation with heparin retained FGF-1 active.	(Khanna et al., 2010)
	TGF-b1	Chitosan microspheres; encapsulation in presence of tripolyphosphates; sustained release for 7 days	(J. E. Lee et al., 2004)
	FGF-2	Hydrogel carrier made of chitosan and fucoidan; gradual release of FGF-2 upon biodegradation of hydrogel.	(Nakamura et al., 2008)
	FGF-2	Microspheres made of collagen and alginate polymers; sustained release.	(Ali et al., 2018)
	FGF-2	PLGA microspheres; sustained release for 72 h. Synthetic polymer! Food grade?	(Lotz et al., 2013)
Covalent binding of GFs to surfaces		Achieve gradual release, but requires complex methodologies, thus result in increased costs.	See reviews from (Enriquez-Ochoa et al., 2020; Teixeira et al., 2020)
Addition of stabilizers	FGF-2	High concentrations of methylcellulose, alanine and HSA, etc. massively increased GF stability.	(L. Benington et al., 2020; L. R. Benington et al., 2021)
	GFs in B8	Rapeseed protein isolates successfully substitute HSA in B8 medium.	(Stout, Rittenberg, et al., 2023)
	GFs in B8 and B9	Methylcellulose extended greatly the stability of GFs in DPBS.	(Schenzle et al., 2025)

765 **Table 6:** Strategies for the reduction of the costs associated with cell production and differentiation for cell-based food, discussed in this review.

een described in recent reviews (Sophie Hubalek, Post, and Moutsatsou 2022; Flaibam, Meira, et al. 2024; Quek, 2024). Some companies are already producing and selling food-grade media at industrial scale – e.g. Nutreco (Netherlands), Multus (UK). Using this strategy, Mosa Meat reported to have reduced its media costs by the factor of 88 (*Milestone*, n.d.). Still, caution must be taken, as food/feed grade products may have performance issues compared to pharma-grade alternatives. This includes increased potential for contaminants, as well as having different (lower) efficiencies due to differences in processing/storage/etc., which must be monitored.

The constituents of FBS, such as GFs and albumins, have been identified as the most dominant cost drivers for the media (Specht, 2020; Stout, Rittenberg, et al., 2023; Swartz et al., 2021). However, they are required for most of the CM/CSF relevant cell lines. Basal media presents further possibilities to reduce media production costs, but also to simplify logistics of its sourcing. In the following sections, we will therefore focus on strategies to reduce the associated costs.

4.1 Efforts to substitute FBS with hydrolysates

4.1.1 Partial substitution of FBS by plant hydrolysates

For cell lines not applicable in the CM/CSF area, research data indicated that protein-rich plant hydrolysates, such as those derived from soy, wheat, and rice, are non-toxic and can fully substitute FBS. Protein hydrolysates improved cell viability, proliferation rate, and resistance against shear stress for CHO, HEK, VERO, BHK as well as insect cell lines (Ballez et al., 2004; Siemensma et al., 2008; Sung et al., 2004). The effect of hydrolysates seemed to be not only nutritional, which gave rise to a theory that higher-MW oligopeptides present in plant hydrolysates could act as growth or survival factors.

The same effect was also anticipated for CM relevant cell lines, but as for now, only partial substitution of FBS was demonstrated, and is discussed in detail by Flaibam et al. (Flaibam, Da Silva, et al., 2024).

Thus, plant-derived hydrolysates can enhance cell growth by contributing nutritional value, but they can only **partially replace FBS** in CM/CSF cell culture media. The limited possibility of plant extracts to substitute FBS may be attributed to the absence of essential GFs, which are necessary for the proliferation of primary or generally not adapted cell lines. It appears that GFs could not be replaced by the protein/peptide mixture derived from the hydrolysis of the plant material. However, plant extracts provide a diverse source of nutrients like amino acids and peptides, carbohydrates, lipids and minerals (Ho et al., 2021) which could contribute to the basic ingredients of cell culture media formulations.

4.1.2. Partial substitution of FBS by microalgae hydrolysates

The potential of microalgae extracts **to lower FBS** concentrations in cell culture medium has been proved using goldfish *Carassius auratus* muscle cells (Dong et al., 2024). Supplementation of Leibovitz's L-15 medium with ***A. pyrenoidosa* extract (1 mg/mL)** and 5% FBS significantly promoted the proliferation of fish muscle cells which could be continually cultured for 4 passages (Dong et al., 2024). **Low serum conditions** in combination with the use of extracts from the microalgae ***C. vulgaris*** as a cell culture additive also successfully promoted the growth of CHO cells and MSCs for up to 21 days (Ng et al., 2020; Nickolay Rojas-Tavara & Jesus Donayre-Torres, 2023). Cell proliferation with a maximum increase in cell viabilities was observed at algae extract concentration of 0.001 g/L. These results highlight that microalgae extracts can act as a functional supplement enhancing the nutritional properties of mammalian culture media.

An **SF medium** supplemented with algae extract was studied by Yamanaka et al. (Yamanaka et al., 2023). First the authors used ***C. vulgaris*** extracts in addition to an inorganic salt solution as nutrient source for maintaining rat liver epithelial RL34 cells which secreted **IGF-1 and IGF-2**. The culture supernatant containing these GFs supplemented with *C. vulgaris* extracts was then provided to bovine

myoblasts as cell culture medium. Compared to FBS-containing media, cell numbers of bovine myoblasts cultured in such medium were reduced indicating that this SF medium with GFs IGF-1 and IGF-2 and *C. vulgaris* extract could replace only partly FBS (Yamanaka et al., 2023). Also, the combination of *C. vulgaris* extract with GFs **FGF and TGF plus insulin** as supplement for SF DMEM was not sufficient to reach proliferation rates comparable to 10 % controls as shown for bovine fibroblasts (Defendi-Cho & Gould, 2023). In June 2021, the South Korean company SeaWith published an announcement stating that they are using microalgae as an FBS replacement for manufacturing CM (https://seawith.net/news/media). Media details, however, are not available.

4.1.3. Partial substitution of FBS by yeast hydrolysates

Yeast-based hydrolysates have been widely used to improve CHO cell proliferation and as supplements for CHO cells cultured in a SF medium (see review by Ho et al. (Ho et al., 2021)). **Yeast extracts** have the potential to partly **replace serum** in mammalian cell culture media used for CM production. The growth of bovine primary skeletal muscle cells in SF conditions, in short term (48h), could be partly restored by the addition of a commercially available yeast extract (Andreassen et al., 2020). Yeast extract enhanced cell metabolic activity more than 100% and compared to the control without FBS, cell proliferation raised by almost 50%. However, this effect was dose-dependent with an upper limit of 1 g/L yeast extract. Higher concentrations showed cytotoxic effects to the cells. Furthermore, the authors observed that especially hydrolysates rich in **peptides** with approximately **2–15 amino acids** in length enhanced cell growth (Andreassen et al., 2020). Also, as discussed in the chapter 4.4.6, 100 mg/L yeast hydrolysate has significantly improved proliferation of Mack1 fish cell line in 2.5% FBS containing medium (Dolgin et al., 2024).

In the light of the findings that yeast hydrolysates are non-toxic to animal cells, and able to support short term cell proliferation, they can potentially be used as an alternative to basal medium (discussed in 4.5.3). Further, a complementing strategy could work for long-term proliferation, as required in CM/CSF production. A paper of Lei et al. (Lei et al., 2023) has demonstrated, that the addition of sterile filtrated wet yeast lysate of a *S. cerevisiae* strain, simultaneously expressing four cytokines (human bFGF, EGF, PDGF-BB, and LR3-IGF-1), was able to promote cell proliferation of porcine SCs for 3 days in presence of 5% FBS to the same level as 10% FBS. Although the yeast lysate with the above-mentioned recombinant GF combination was also not able to completely substitute FBS, the application of GFs as part of yeast lysates avoids the need of GF purification which can make up to 50% of the GF production cost.

4.2. Reducing the concentration of growth promoting proteins

A reduction of the concentration of four recombinant growth promoting proteins (insulin, transferrin, FGF-2, and TGF-β) by one tenth of their current level resulted in $41 (around €36) per L of medium, a price which accounts for only 11 % of the costs estimated for an in-house prepared E8 (Specht, 2020). Low-serum adaptation is routine, and the same principle could be applied for individual GFs. However, a reduced concentration of these supplements may have negative effects on cell proliferation and maintenance. In this regard, Stout et al. have demonstrated that in the long-term culture of primary non-adapted BSCs, the attempt to lower FGF-2 concentration in B8-based medium led to a significantly lower cell number (Stout et al., 2022). On the other hand, they have shown that concentration reduction of growth promoting proteins could still be a reasonable strategy if combined with stabilization approaches. This was confirmed in the paper by Schenzle et al., where the dilution to 70% of the original concentrations of B8 and B9 media could be rescued by addition of stabilizers,

namely HSA or, alternatively, methylcellulose (MC) with starch from corn (Schenzle et al., 2025). Another strategy to lower the GF concentration is the cell line adaptation, discussed on an example of immortalized chicken fibroblasts in chapter 3.2.5. and on fish cell lines in chapter 2.

4.3. Appropriate choice of growth factors or orthologs

GFs are proteins secreted by cells to communicate with each other. They are essential supplements for SF cell culture media as they mimic endogenous signalling pathways to promote growth, proliferation, and differentiation of cells. The choice of GFs used in CM/CSF production is dependent on the animal species, cell type, and culture process (Huang et al., 2024) and is discussed in chapter 3.2.

The available supply of recombinant GFs from a variety of species provides a substantial pool of GFs that can be analysed for its effect on the growth of cells *in vitro*. When testing various GF orthologs for their ability to promote bovine SCs proliferation, Venkatesan et al. showed that Atlantic salmon FGF-2 was the most potent among all the FGF-2 orthologs tested (Venkatesan et al., 2022). Highest effect was observed at 25 ng/mL but this decreased with higher concentrations. The most effective GF for bovine SCs was PDGF-BB from Eagle – however at a concentration of 100 ng/mL. In contrast, human TGF-b1, showed no improvement at all concentrations tested compared to FBS controls (Venkatesan et al., 2022). In another study, replacing bovine recombinant IL-6 with human recombinant IL-6 not only led to a staggering cost reduction of 80% but also significantly increased the proliferation of bovine myoblasts (Kolkmann et al., 2022).

Most of the GFs used for fish cell cultures are recombinant and based on mammalian GF sequences. But, as mentioned in the chapter 2, protein identity of GFs between fish and mammals, as well as between different fish species is sometimes quite low (Yuan and Hong 2017). Thus, effective species-

cross-reactive GFs in case of fish cell lines are less probable. As discussed in more detail in 3.2.3, only a handful of providers produce fish GFs, although the situation is gradually improving, including more research on species-specific GFs being done.

Thus, identifying the most effective GF ortholog and determining its minimal concentration or finding appropriate GF combinations for individual species and cell types will be a prerequisite to enable long-term cell proliferation and to maintain cells in an FBS-free environment for an economic *in vitro* propagation and differentiation of non-adapted cells. However, it should be kept in mind that the industry is currently leaning towards **using species-specific GFs** due to regulatory requirements, although it is **not always the most efficient choice** (https://gfi.org/resource/cultivated-meat-media-growth-factor-survey/#species-specific-growth-factors) (see also chapter 5 about Regulatory Submissions).

4.4. Addressing the issue of growth factors' high costs

GFs are key components of SF medium used for cellular agriculture applications of non-adapted mammalian cells. Many of them require post-translational modifications for their functionality which makes the production of recombinant GFs challenging and expensive (Venkatesan et al., 2022). Moreover, GFs are inherently unstable molecules because they are biologically intended to act transiently as short-term signals (L. Benington et al., 2020). Hence, sequential multiple administrations and/or supraphysiological doses are required to sustain an effective concentration of GFs in media, resulting in higher costs and possibly adverse effects (Ren et al., 2020). Thus, technological approaches with the aim to significantly improve GF stability and bioactivity are crucial factors for reducing GF doses and costs for CM/SCF production. This is also reflected in the recent surveys,

showing high interest of manufacturers and CM producers in modified GFs (https://gfi.org/resource/cultivated-meat-media-growth-factor-survey/#modifications). In this chapter we will discuss approaches aiming at GFs with (1) **higher stability –** e.g. through specific point mutations, immobilizations or addition of stabilizers, and (2) **higher potency** through enhanced binding affinity to appropriate cell receptors(s). Other approaches (3) to lower the medium costs by substituting GFs with **peptides** from plant hydrolysates or synthetic peptides, or with **small molecules** will be also discussed.

4.4.1. Increase growth factors' stability by mutations

A characteristic feature of the structurally related and highly conserved members of the FGF family proteins is their low thermodynamic stability and relatively short half-life *in vivo*. At physiological pH and temperature almost 50% of the **FGF-1** protein is unfolded within less than an hour and therefore prone to proteolytic degradation (Zakrzewska et al., 2005). Prolonged biological activity of FGFs can be achieved by increasing their proteolytic resistance. Proteolytic cleavages occurred mainly within the C-terminal region of the wild-type FGF-1, pointing on its significant role in GF degradation (Kobielak et al., 2014). Based on bioinformatic analysis, two single mutations (C117P, K118V) were introduced within the C-terminal region and combined in a double mutant. This led to a rigidification of the FGF-1 segment highly sensitive to proteases and susceptibility to trypsin and chymotrypsin degradation was significantly reduced *in vitro* compared to the wild-type FGF-1. Thermostability, however, was not improved by introducing these mutations (Kobielak et al., 2014).

Significant efforts have been made to clarify the function and mechanism of action of heparin binding to GFs, which appears to strongly affect GFs stability and affinity for receptors. Heparin-binding sites

are prevalent in the GFs utilized in the CM industry, and are present e.g. in FGF-2, HGF, IL-6, PDGF, VEGF (Bolten et al., 2018). Heparin, which is a stabilizing glycosaminoglycan that is commonly found in the extracellular matrix (ECM) of mammalian cells, binds to a cluster of positively charged residues of FGFs, which are located at the C-terminal end of the molecules (Canales et al., 2006). Sulfate-rich polymers like heparin and other heparinoids can help to preserve GF activity by extending their half-life at 37°C (Ishihara et al., 2019). **However, the use of heparin** derived from animal sources as an additive in cell culture media may be problematic for CM/CSF production. It is also susceptible to batch-to-batch variability and contamination, as it is isolated from animal tissues. Fractionated heparin is associated with fewer side effects, but the process of fractionation is costly (Merli & Groce, 2010). Alternatively, **other heparinoids** could be investigated for applications in cellular agriculture (Ishihara et al., 2019).

The stability of **hFGF-1** could be increased by selective engineering of the heparin binding pocket via charge reversal mutations, but the cell proliferation activity of the resulting hFGF-1 mutant was reduced compared to the wild type hFGF-1 (Davis et al., 2018). The weak thermostability of wild type hFGF-1, which loses its activity after 6 hours at 37°C even in presence of heparin, could be significantly improved by point mutations at amino acids Q40, S47, and H93 (G. Chen et al., 2012; Zakrzewska et al., 2009). By mutating these three sites, FGF-1 became significantly stabilized, while its activity was maintained. This increased stability could compensate for reduced affinity to heparin when in addition the heparin binding site K112 was mutated to asparagine (Zakrzewska et al., 2005).

Based on sequence alignments of stabilized FGF1 mutant sequences and wild-type **FGF-2**, triple (Jeong, 2015) and quintuple (Nölle, 2015) FGF-2 mutants with significantly improved stability were generated. Further, a hyperstable nine-point mutant named FGF2-G3 (or FGF2-STAB2) was constructed by employing focused directed evolution and combining individual stabilizing mutations published

elsewhere (Dvorak et al., 2018; Koledova et al., 2019). In this mutant, functionally important positions for the heparin binding were left unchanged. The melting temperature of this mutated FGF-2 variant increased by 19°C and its functional half-life at 37°C improved from 10 h to more than 20 days in vitro (Dvorak et al., 2018). This variant (FGF2-G3 or FGF2-STAB2) is commercially available from several distributors.

In their stability studies using **FGF-2** from human and zebrafish, Chen et al. found that both GFs were not degraded after 24-hour incubation at 37°C but aggregation caused a loss of FGF-2 biological activity (G. Chen et al., 2012). Such aggregation events were prevented by **heparin-binding** and point mutations in the conserved heparin binding sites of FGF-family proteins. Interaction with heparin restored the optimal FGF protein folding and increased its thermostability (G. Chen et al., 2012; Zakrzewska et al., 2009).

Generally, FGF-1 variants with increased stability were shown to be less dependent on heparin binding for receptor binding and signalling (Zakrzewska et al., 2009), demonstrating the effectiveness of this strategy. Similar observations were made regarding FGF2-STAB variants with improved thermal stability. Compared to wild-type FGF-2, FGF2-STABs showed higher efficiency in promoting proliferation and lower affinity to heparin (Koledova et al., 2019). Thus, protein engineering resulting in strong and powerful stabilizing effects may abolish heparin dependence of FGF proteins. Therefore, the development **of soluble, heparin independent GF analogues** with improved stability by protein engineering is the preferred strategy for obtaining stable and bioactive variants of most crucial GFs used in CM/CSF area. This approach was successfully applied for HGF (De Nola et al., 2022), leading to a more potent minimal variant, as well as for VEGF (S. S. Ahmad et al., 2023). Although, regulatory status of such stabilized GF mutants is still to be determined.

4.4.2. Strengthen signaling response by modifying binding affinities of growth factors to receptors

Modifying **binding affinities** between GFs and their receptors may influence the magnitude and duration of the signalling response. A prolonged cellular response to GFs at lower doses would also contribute to cost reduction for culture media. The affinity of EGF for its receptor was up to 30-fold higher after amino acid residues Ile 38, Glu 51 and Leu 52 in EGF were changed by site-directed mutagenesis (Cochran et al., 2006).

As **FGF-2 dimerization** is essential for the formation of an active ligand–receptor complex, a covalent linkage of two FGF-2 molecules provides another strategy to improve the biological activity and stability of GFs (Nawrocka et al., 2020). To engineer FGF-2 dimers, mutants with single surface-exposed reactive cysteine for the chemical conjugation with PEG were generated. All dimeric proteins showed enhanced mitogenic potential, as compared to FGF-2 monomer; the most significant differences were detected at 10 ng/mL protein concentration (Nawrocka et al., 2020). Dimeric FGF-2 variants did not show any tendency to aggregate and – in contrast to wild-type FGF-2 - the amount of functional dimers was not significantly altered after 48 h in the presence of cells (Nawrocka et al., 2020). Thus, FGF-2 dimerization provides the possibility to reduce the need for heparin as well as costs as dimeric proteins possess increased stability in cell culture medium.

A similar strategy was applied to improve the limited stability of native hepatocyte growth factor/scatter factor (**HGF**). Wild-type HGF is comprised of two-chains (α/β). The 64 kDa α-chain contains four kringle (K) domains and the high affinity binding site for the MET receptor. The β-chain (34 kD) contains a secondary binding site for MET (Donate et al., 1994). A **minimal receptor agonist** termed K1K1 consisting of two copies of the kringle 1 domain of HGF in tandem orientation was designed and tested

both *in vitro* and with *in vivo* models. K1K1 was stable and displayed biological activity which was equivalent or superior compared with native HGF (De Nola et al., 2022).

For GF engineering approaches, the design of short GF variants mimicking full length GF activities would provide a powerful tool **to reduce costs** for recombinant expression of growth promoting proteins required for media supplementation for CM/CSF production. This strategy, however, requires extensive information about binding mechanisms of GFs to their receptors as well as detailed structural data regarding receptor-activating configurations. The creation of realistic models to simulate distinct receptor-ligand interactions is a prerequisite for predicting minimal GF variants or mimetics with increased bioactivities and stabilities.

4.4.3. Immobilization of growth factors

The use of **immobilized GFs (iGFs)** may be a promising approach for CM/CSF production as it gives the possibility to achieve a higher GF stability and a sustained release, which may considerably reduce the need for multiple doses, facilitate recycling of GFs and consequently decrease associated costs for cell cultures.

Physical immobilization is technically the simplest method to immobilize GFs, commonly achieved by adding GFs into a polymer matrix before its gelatinization (Enriquez-Ochoa et al., 2020). This and other GF immobilization strategies are extensively reviewed elsewhere (Enriquez-Ochoa et al., 2020). Several natural and inexpensive biopolymers can be used for microencapsulation and regarding food-safety, polymers of plant origin like alignate or starch are of special interest (Gómez-Mascaraque et al., 2018). Below we present examples of GF immobilization in food-relevant matrixes, showing sustained release.

Multilayered alginate microcapsules coated with a permeable poly-L-ornithine (PLO) membrane were used for the encapsulation of FGF-1 (Khanna et al., 2010). Release kinetics showed a burst release of FGF-1 within the initial 5 hours, followed by a low-dose continuous release for up to thirty days. However, FGF-1 released from microbeads was active only when heparin was co-encapsulated in the outer layer (Khanna et al., 2010).

In contrast, alginate microspheres **failed to release TGF-b1** (J. E. Lee et al., 2004), possibly because of the interaction between the carboxyl groups of alginate and the amine groups of the GF. Therefore, TGF-b1 was loaded in **chitosan microspheres** which were incorporated in porous chitosan scaffolds. **Chitosan** has been reported to stimulate and protect the activity of GFs (Ishihara et al., 2019; Ueno et al., 2001) and it possesses a cationic nature resulting from primary amine groups. Chitosan microspheres were loaded with TGF-b1 by the emulsion-ionic crosslinking method (J. E. Lee et al., 2004). The release curve showed a strong release within the first 3 days, followed by a slow sustained release of TGF-b1 over several days (J. E. Lee et al., 2004).

A 1:2 (wt) mixture of **chitosan and fucoidan** - a sulfated polysaccharide derived from brown seaweeds - was used to encapsulate FGF-2 for controlled GF release (Nakamura et al., 2008). FGF-2 molecules were incorporated in the chitosan/fucoidan micro complex-hydrogel, which substantially prolonged the biological half-life time of FGF-2. Moreover, a gradual release of FGF-2 from the hydrogel was determined *in vivo* (Nakamura et al., 2008).

Microspheres composed of readily degradable **collagen** with more slowly degradable, mesh-forming **alginate polymers** were used to study sustained release of FGF-2 in relation to varying amounts of collagen (Ali et al., 2018). *In vivo* experiments using zebrafish embryos showed that FGF-2 released from such microspheres induced angiogenesis (Ali et al., 2018). Collagen, which is usually extracted from

animal tissues, is now available from recombinant biosynthesis (Z. Zhao et al., 2023), though at a high price.

Beside natural polymers, synthetic biocompatible and biodegradable polymers such as **polylactic glycolic acid (PLGA)** have also been described for sustained release formulations of GFs as shown for FGF-2 (Lotz et al., 2013). PLGA is a biodegradable linear co-polymer composed of lactic and glycolic acid, which has been approved for clinical use in humans by the American Food and Drug Administration (Lanao et al., 2013). However, such biopolymers used for medical applications may not become approved for food industry.

The **impact of GF immobilization** on reducing the overall manufacturing cost for CM/CSF will depend on a number of parameters e.g. the cost for (recombinant) GFs, the selected immobilization method including the costs for biomaterials used or immobilization surfaces. Loading efficiency, release kinetics, stability and biological activity of each GF will have to be tested and evaluated for every immobilization technique together with cell lines/species used for CM/CSF production processes. These are main challenges that lies ahead in the development of engineered and immobilized GFs and will require further research in this field.

4.4.4. Growth factor alternatives

As GFs account for a significant portion of the total media costs in most media compositions, there has been an ongoing effort to substitute them with alternative molecules that are more cost-effective. The substitution of the GFs with defined peptides, peptoids or small molecules, which mimic GFs and can specifically activate respective receptors might be a usable strategy. They are generally more stable, less susceptible to proteolysis and cheaper in production (Atkinson & Dickman, 2023). For example,

Kenichiro Ito et al. presents an HGF mimicking peptide, which at about 2 orders higher concentrations than wild type HGF was able to achieve the same level of Met-selective activation of Erk signaling pathway (K. Ito et al., 2022). Another mode of action was described for commercially available oligopeptide Dihexa (N-hexanoic-Try-Ile-(6)-amino hexanoic amide) and its parent compound Norleucine 1-AngIV, which induce c-Met phosphorylation in the presence of subthreshold concentrations of HGF and augment HGF-dependent cell scattering (Benoist et al., 2014). Peptide and peptiod mimetics for other crucial GFs used in cell cultures have also been developed, for example for FGF-2 and PDGF-B, which, together with multiple HGF mimetics are extensively reviewed elsewhere (Atkinson & Dickman, 2023). Such peptides offer notable advantages, including reduced immunogenicity and lower production price compared to proteins, along with the potential for enhanced interaction with larger receptor surfaces in comparison to small molecules (L. Wang et al., 2022).

Finally, GFs can be substituted with small molecules, which efficiently activate respective receptors. Researchers, connected to the German CM start-up The Cultivated B have recently identified a guanylhydrazone-based molecule (TCB-32) via structure-based virtual screening on the orthosteric binding site of FGFR1. They have then further optimized it to yield three highly potent agonists of FGF receptor (Feofanov et al., 2025). Also, Believer Meats identified small molecules – agonists of FGF-2 (Nahmias et al., 2023). This demonstrates interest of CM industry for such compounds. Other small molecules mimicking FGF-2 and HGF were also reported, though for pharmaceutical applications (Atkinson & Dickman, 2023).

However, a **significant challenge** in utilizing such GF mimetics is the **lack of their safety assessments for food applications**, as most of them were originally developed for medical purposes. Additional

challenge could be the focus of food industry on delivering a "natural product" that should differ as little as possible from conventional meat.

4.4.5. Extending the activity of growth factors by addition of HSA and other known stabilizers

From a cost perspective, the addition of excipients for stabilizing GFs in media would be the preferred approach which is also relatively simple to implement in bioprocesses. Substances like salts, sugars, polymers, proteins, and amino acids may be used for this purpose (L. Benington et al., 2020). In their attempt to preserve functional FGF-2 molecules in **aqueous solutions**, Benington et al. tested a number of approved pharmaceutical excipients - alone or in combinations - in order to identify potential stabilizers for soluble FGF-2 (L. R. Benington et al., 2021). With this systematic approach, addition of 0.5% w/v methylcellulose (MC) - an emulsifier used in food industry also known as E461 – strongly improved protective effect of the alanine (20 mM) and human serum albumin (HSA) (1 mg/mL) on FGF-2 against rapid thermal degradation at 37°C. Insignificant losses of FGF-2 contents after 2 h at 37°C were determined under these conditions. The FGF-2 formulation with 0.5% w/v MC and 20 mM alanine was also stable when incubation time at 37°C was prolonged to 8 h, retaining 97% of FGF-2) (L. R. Benington et al., 2021).

Polymers like **methylcellulose** form hydrogels which increase molecular density and viscosity of aqueous solutions and prevent aggregation of proteins like GFs. This effect was more pronounced at low MC concentrations (L. R. Benington et al., 2021). **Amino acids** such as alanine and glycine, also used as cryoprotectants for protein formulations, contribute to protein stability through weak electrostatic interactions (Kruuv & Glofcheski, 1992). Benington et al. reported that alanine, but not glycine, met the

criteria of an effective stabilizer for FGF-2. Like for MC, lower concentrations of alanine were significantly more effective at stabilising FGF-2 at higher temperatures (L. R. Benington et al., 2021).

Albumins, which are major constituents of mammalian serum, are known to stabilize the structure of other proteins through ionic, electrostatic, and hydrophobic interactions and could be preferred over amino acids due to their metabolic inertness (L. Benington et al., 2020). This stabilizing effect on GFs was most probably the reason for the clearly improved growth of bovine satellite cells (BSCs) in B8-medium supplemented with **recombinant HSA** (800 µg/mL) (Stout et al., 2022). The use of stabilizers, like **recombinant albumin**, however, would result in a substantial increase in medium costs.

In search of solutions to this problem, several groups tested known but also previously unreported stabilizers for their ability to substitute this function of HSA in the cell culture. In the interest of minimizing costs for cell culture media, Schenzle et al. tested the addition of different concentrations of low-price stabilizers for extending the activity of GFs in SF and fully defined B8 and B9 medium, concerning proliferation of bovine, porcine and chicken satellite cells (Schenzle et al., 2025). B8 supplemented with MC resulted in a significant GF stabilizing effects, extending the activity of GFs up to 10 days as determined by ELISA. In the short- and long-term proliferation experiments, B8 with MC, additionally supplemented with starch from corn supported the proliferation of BSCs almost as good as B9, and for PSCs, a combination of B8 with MC was shown to be more effective than B9. With the price for the two excipients only about 0.1% of recombinant HSA, they allow for a substantial cost reduction for some cell lines (Schenzle et al., 2025). Interestingly, these stabilizers at the same concentrations were detrimental to the proliferation of fish cell lines DLEC and MACK1, underlining the differences between terrestrial and fish cell lines (Schenzle et al., 2025).

Further, the addition of stabilizers to culture media allows a reduction of GF concentrations required for prolonged cell proliferation. A 3:1 dilution of B8 with the basal medium DMEM-F12 and addition of either 0.1125 g/L MC or by 0.8 g/L HSA was shown to sustain cell viability for four days (Schenzle et al., 2025). Thus, further studies on optimizing the type and concentration of GFs as well as **effective combinations of stabilizers** could result in economically viable costs for cell culture media.

Although GFs are considered as the most dominant costs drivers (Specht, 2020), **rHSA or BSA** in SF-media formulations designed for bovine cells also substantially contribute to costs for media (Table 3 and (Stout et al., 2022)). Upscaling will certainly reduce the production price of CM/CSF, but other strategies are also required to alleviate the burden of costs for media. Moreover, based on assumptions by Swartz et al, (2023), millions of kilograms of albumin would be needed to produce CM that accounts for 1% of the global meat market. This quantity is far exceeding the current production volumes of many recombinant industrial enzymes as well as serum-derived albumins (Swartz, 2023).

4.4.6. Substitution of HSA with plant and microbial isolates

Substituting albumin by plant hydrolysates was already suggested by Siemensma et al. to lower shear stress for cells and to increase their robustness (Siemensma et al., 2008). Results of some recent studies suggest that a replacement of albumins by plant or microbial protein extracts has the potential to be transferable to large-scale production of CM/CSF.

Non-hydrolysed oilseed protein isolates (particularly rapeseed protein isolate, RPI) were shown to be effective albumin alternatives in SF medium Beefy-9 (Stout, Arnett, et al., 2023). The isolation of RPI from rapeseed protein meal is simple and gives a sustainable stabilizer for GFs. It was shown to even exceed recombinant albumin in promoting bovine SC growth (Stout, Arnett, et al., 2023). Thus, it could

be a viable alternative to recombinant albumins, provided that large-scale production and storage costs remain low. But it is hard to assess at the moment, as the product is currently not available on the market, and according to the publication the isolates must be stored at -80°C to retain biological activity (Stout, Arnett, et al., 2023). Stout et al. estimated the costs of RPI required for one Liter of medium to $0.002/€0.0018 (without considering the isolation costs), whereas the price for recombinant albumin required for one liter of Beefy-9 is approximately $24.56/€21 when purchasing from available suppliers (Stout, Arnett, et al., 2023; Stout et al., 2022).

A recent report shows a simple solution allowing to avoid the necessity of -80°C storage of RPI. Egger et al. demonstrated that lyophilized or even spray-dried protein isolates retain their biological activity if the concentration step is omitted prior to drying (Egger et al., 2025). They further show that Styrian pumpkin protein isolate can slightly outperform RPI in short term proliferation experiments, which demonstrates the possibility for using various local agricultural waste streams. Finally, a four times higher yield of soluble protein was achieved only by raising the grinding efficiency (Egger et al., 2025). These findings underscore that cake protein isolates can become a highly economical alternative to HSA.

Non-protein-based alternatives to HSA (discussed in 4.4.5) might be even cheaper. The cost of MC and starch from corn proposed by Schenzle et al. to substitute the same amount of albumin for achieving comparable effects, is assessed at $0,054/€0,048 per Liter, including production costs, and these excipients are available on the market in big quantities, are highly stable and are routinely stored at room temperature.

Very recently, Dolgin et al. reported the effective **substitution of HSA** in B9 medium with different **microbial lysates** (Dolgin et al., 2024). In this study, 0.1 g/L lysates produced from the yeast *Saccharomyces cerevisiae* were able to substantially improve proliferation of mackerel cell line Mack1 (Saad et al., 2023) in 2.5% FBS medium without any adaptation period. Interestingly, 40 mg/L lysates of Gram-negative marine bacterium *Vibrio natriegens* surpassed HSA stabilizing effect in long term culture of immortalized BSCs (Stout, Arnett, et al., 2023) after an adaptation period of 4 weeks (Dolgin et al., 2024). However, the authors did not indicate the levels of endotoxin produced by *Vibrio natriegens,* which, if present in culture medium, may represent a safety issue, although it is possible to dramatically reduce endotoxin production in *V. natriegens* using genetic engineering (Weinstock et al., 2021). But the fact that microbial lysates can substitute HSA as stabilizing agent is very remarkable.

Additionally, successful proliferation of chicken fibroblasts in SF media was achieved with water-soluble **plant proteins** extracted from soybeans, peas, and chickpeas, with only 0.1 mg/mL of plant proteins replacing 2 mg/mL of costly bovine serum albumin (BSA) (patent WO 2021/148955A1 (Nahmias et al., 2021)). Hemp and wheat proteins were not able to deliver comparable results. Higher amount of plant protein extracts lead to cytotoxicity, which was eliminated after depletion of the <10 kDa fraction from the plant proteins (Nahmias et al., 2021).

4.5. Hydrolysates as basal medium alternatives

Hydrolysates obtained from **plants and microorganisms** by enzymatic or acidic digestion were discussed as a cell culture component for several decades. As discussed in chapter 4.1.1a, hydrolysates from plant materials can be used as a substitution of FBS for pharmaceutically relevant cell lines, such as CHO, HEK, VERO, BHK as well as insect cell lines. For these cell lines they can further act as albumin

substitutes in media to ensure high cell resistance to shear stress and high viability in bioreactors (Siemensma et al., 2008). This application did not show the same efficiency for CM/CSF cell lines, allowing only partial substitution of FBS, but, due to their rich nutritional composition, they may substitute essential basal cell culture media components in CM/CSF production (Flaibam, Da Silva, et al., 2024; Flaibam, Meira, et al., 2024).

4.5.1. Plant cell extracts as a basal media substitution

Media components derived from plants can be a sustainable alternative as plants grow photoautotrophically which reduces the environmental footprint and resource consumption (Blankenship et al., 2011; McConnell et al., 2010; Santos Correa et al., 2023). However, so far most of the research focused on substituting FBS, which so far showed only limited success in CM field (Charlesworth et al., 2024; C. H. Kim et al., 2023).

Plant hydrolysates may be used as nutrient source to replace components of chemically defined basal media used for the CM/CSF production, but some points should be considered. Ideally, a plant used to produce organic media components has GRAS (Generally Recognized As Safe) status but is not a food or feed crop to avoid direct competition with human and animal nutrition. At the same time, the plant should have a high biomass productivity and nutrient content, few antinutrients (e.g. phytates and lectins; (López-Moreno et al., 2022)) and minimal demands regarding fertilization and irrigation as well as a high adaptability to different climate zones.

Importantly, the **composition of plant biomass and corresponding extracts** can vary substantially between different plant species, tissues (e.g. leaves vs roots), over time and depending on the cultivation and extraction conditions, contributing to batch-to-batch variations. For example, more

than 20 small molecule compounds were identified by mass spectrometric analysis in *Moringa oleifera* leaf extracts (Y. Xu et al., 2021) as well as extracts of *Sanguisorba minor* (salad burnet) prepared from either leaves/stems or roots (Karkanis et al., 2019). Among these compounds, kaempferol-O-glucuronide was found at >20 mg/g in extracts of leaves/stems but was not detected in roots. In contrast, (+)-catechin was detected at constant levels of 25 mg/g in all tissues.

Also, light sources show effects on plant growth and metabolite accumulation. The effects of light conditions on anthocyanins, carotenoids and flavonol concentrations in various plant species have recently been reviewed (Thoma et al., 2020). Similarly, tobacco (*Nicotiana tabacum*) leaves contained high concentrations of rutin if cultivated in a greenhouse, but the compound was undetectable if plants were cultivated in a phytotron using LED light (Buyel et al., 2015). The metabolite rutin was shown to stimulate proliferation of SCs isolated from Hanwoo steers (Yu et al., 2023). The geographic location of plants also affects the molecular composition of extracts as reported for *Moringa oleifera* leaves for which antioxidant capacities varied between 0.5 and 1.1 mmol Fe^{2+}/g (Y. Xu et al., 2021). Cell culture media are frequently deficient in antioxidants, although they appear to have positive effects when added to cell cultures (Halliwell, 2014). Thus, correcting this deficiency by supplementing media with plant-derived extracts containing antioxidants can have additional beneficial effects on cells.

The composition of the plant biomass may be tailored to the requirements of cultured meat applications through selected cultivation conditions of specific plants. The best **control of cultivation conditions** to obtain a desired reproducible composition of plant extracts can be achieved by in-door farming. One such option is vertical farming, which can reduce the footprint by a factor of >5, increase biomass output two-fold and can be installed regardless of the climate at a given geographic location (Huebbers & Buyel, 2021). The most reproducible plant (cell) biomass can be obtained from plant cell

suspension cultures in bioreactors. However, the number of large-scale plant cell culture processes may be too small to yield meaningful amounts of biomass from which basal media components can be extracted. The by-far largest operation is at 75 m^3 (Tabata, 2004), probably with an average weekly biomass output of 15,000 kg wet mass (~1000 kg dry mass).

Further, **preparation methods** have a substantial impact on the nutrient composition of hydrolysates (Buyel & Fischer, 2014, 2015). The buffer type and concentration, pH, conductivity, temperature and presence of detergents will affect the resulting extraction efficacy for individual plant components. Hydrolysates can be produced by enzymatic digestion and/or acid hydrolysis of protein-rich plants. Plant raw materials are usually pretreated with heat before digestion or hydrolysis by enzymes or chemicals. Plant biomass can be boiled at 100°C or more for 1 h at low pH (~1.0) to break down proteins to peptide or even amino acid level (Yamanaka et al., 2023). Whereas this can increase the accessibility of nutrients and inactivate pathogenic contaminations, the harsh conditions can also destroy heat sensitive molecules like vitamins (Berry Ottaway, 2010). Further, many plant proteins precipitate at a pH below 5.0 and temperatures above 60°C (Opdensteinen et al., 2021). Enzymatic treatment at pH 5.0 and 55°C is milder and can achieve about 90% of hydrolysis (Min et al., 2024), but it can take up to 48 h (Tao et al., 2023), and the degree of hydrolysis may be <40% for various proteases even at high enzyme concentrations (Chabanon et al., 2008). The resulting plant hydrolysates **are complex mixtures** of oligopeptides, free amino acids, vitamins and carbohydrates, minerals, and other bioactive components (Ho et al., 2021). Regarding the composition of plant hydrolysates, further safety issues, such as bioactivity of the resulting peptides and risks of contamination have to be considered and are discussed together with an overview of preparation methods in a detailed review by Flaibam et al. (Flaibam, Da Silva, et al., 2024).

Additionally, plants contain **various metabolites** that can be difficult to identify and quantify but they may have an impact on cell growth of mammalian cells. These metabolites are usually pooled based on the available detection assays. For example, the concentration of antioxidants can be determined via ABTS (named after the detection reagent 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH (named after 2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing ability of plasma) assays. So instead of quantifying each compound individually, the respective IC_{50} values are reported for ABTS and DPPH (Y. Xu et al., 2021).

Some plant-derived small molecules have also shown anti-cancer activity. In biomanufacturing, this activity can be a drawback because these molecules may also reduce the growth of non-cancer cells or immortalized cells. This has been demonstrated for HeLa and HEK 293 cells (Russell et al., 1977; Scherer et al., 1953) but it could be relevant for immortalized CM/CSF cell lines (Pasitka et al., 2022; Stout, Arnett, et al., 2023).

Specific plant-derived compounds were identified as toxic to animals at high concentrations, e.g. sanguinarine (Selvi B et al., 2009), genistein (an isoflavone angiogenesis inhibitor), lycopene (a carotenoid found in many fruits) and resveratrol (a stilbene found in grape berries and therefore also in wines) (Buyel, 2018). The median lethal dose (LD_{50}) of these compounds can range from ~30 to >5000 mg kg^{-1} body mass (Table 7). They probably still pose only a small risk in bioprocesses because most of these toxic compounds are present at low levels in the plant biomass and are extracted predominantly in non-aqueous buffers which are incompatible with cell culture applications. Interestingly, all the compounds listed here have been extensively researched as nutraceuticals and at low concentrations are widely used in anti-aging and anti-cancer interventions (McCubrey et al., 2017). This might open a new exciting possibility to produce CM/CSF with anti-aging / anti-cancer properties.

Moreover, some plant extracted components were shown to have specific positive effects on muscle cells: e.g. quercetin - a plant flavonoid widely distributed in nature - boosted myogenesis, and led to increased myotube thickness and length for several species (S. S. Ahmad et al., 2024); naringenin - flavanone present in citrus fruits - promoted myogenic differentiation of porcine satellite cells (PSCs) *in vitro* and increased the content and maturity of generated myotubes (Yan et al., 2022) (for the full list see Table 8).

Plant species	Compound	Concentration in biomass [mg/kg dry mass]	Reference	LD50 [mg/kg]	Organism	Route of administration	Reference
Sanguinaria canadensis	Sanguinarine	1400	(Campbell et al., 2007)	1658	Rat	Oral	(Becci et al., 1987)
Sanguinaria canadensis	Sanguinarine	1400	(Campbell et al., 2007)	29	Rat	Intravenous	(Becci et al., 1987)
Berberies vulgaris	Berberine sulfate	20000	(S. Ahmad et al., 2019)	205	Rat	Intraperitoneal	(Kulkarni et al., 1972)
Berberies vulgaris	*Berberies vulgaris* root extract	20000	(S. Ahmad et al., 2019)	1280	Rat	Oral	(Kulkarni et al., 1972)
Glycine max	Genistein	4.6	(Fukutake et al., 1996)	>2000	Rat	Oral	(Michael McClain et al., 2006)
Glycine max	Genistein	4.6	(Fukutake et al., 1996)	300	Mice fibroblasts Balb/c 3T3 cell line	In vitro	(Popiołkiewicz et al., 2005)
Solanum lycopersicum	Lycopene	300	(Matulka et al., 2004)	>5000	Rat	Oral	(Matulka et al., 2004)
Vitis vinifera	Resveratrol	50 - 700	(Langcake & Pryce, 1976)	5	Human LX-2 hepatic stellate cells	In vitro	(Bechmann et al., 2009)
Vitis vinifera	Resveratrol	50 - 700	(Langcake & Pryce, 1976)	>3000	Rat	Gavage	(Crowell, 2004)

Table 7: Examples of toxic compounds identified in plant extracts. Plant species potentially useful for cell culture media component extraction based on their food status are highlighted in **bold font**.

Some authors have raised concerns that pesticides or herbicides could be present in plant extracts used in cell culture and thus pose a potential safety risk (Yao & Asayama, 2017). However, it seems

unlikely that plant material destined for fermentation or cell culture applications would be deliberately exposed to such substances. In contrast, it seems more likely that closed cultivation facilities would be used for plant manufacturing as discussed above (Huebbers & Buyel, 2021). Still, other sources of environmental contamination – e.g. air, water, soil, as well as those from processing – can pose an issue, making good traceability of materials essential.

Plant extracts may result in reduced nutrient uptake (e.g. of iron) compared to animal-derived counterparts due to reduced levels of vitamin B12 (Ghosh & Arcot, 2022). Accordingly, it will be important to test extracts from different plant types and tissues for their performance in cultured meat applications. Therefore, to obtain plant extracts with desired components for culture media used for CM/CSF production, it is also necessary to determine adequate types of starting material and to standardize hydrolysis processes.

Compounds (natural source)	Mechanism of action	Effect on cells	Cell type tested	References
Quercentin (vegetables, fruits, herbs)	Myostatin inhibitor	Best results with 10 and 100 nM quercetin; promotion of **differentiation**; increased myotube thickness for bovine SCs and C2C12; increased the length of myotubes (chicken); no significant morphological change of SCs (porcine).	Bovine, pig, chicken and C2C12	(S. S. Ahmad et al., 2024)
Licochalcone A and B (Glycyrrhiza uralensis)	Myostatin inhibitor	Enhance muscle **proliferation** and **differentiation**		(S. S. Ahmad et al., 2024)
Gingerol, Curcumin (Ginger, Curcuma)	Curcumin and gingerol attenuate the effect of advanced glycation end products on myoblasts	Both promote **differentiation** at concentrations of 1 µM and 5 µM.	C2C12	(Baig et al., 2017)
Rosmarinic acid (Aromatic plants)	Activation of the unfolded protein response and the mTOR pathway	Enhances CHO cell **proliferation** and viability (Final conc. 0.5 mM).	CHO	(Mao et al., 2024)
Rutin (various fruits, plants)	Novel Stimulators of Pax7 and/or MyoD	Stimulation of cell **proliferation** (100 and 200 µM); No effect on differentiation.	Bovine SCs from Hanwoo steers	(Yu et al., 2023)
Parthenolide (plant feverfew)		Stimulation of cell **proliferation** (0.5 and 1µM); No effect on differentiation.		
Naringenin (tomato, grapefruit, and citrus fruits)	Naringenin upregulates the IGF-1/AKT/mTOR anabolic pathway during myogenesis of SCs by activating the estrogen receptor β.	Naringenin (20 µM) promotes myotube formation (**differentiation**) and maturation.	Porcine SCs	(Yan et al., 2022)
Vitamin K2 (vegetables, fish, meat, cheese and egg)	Vitamin K2 is speeding up the transfer of electrons in mitochondria, which results in more efficient ATP production. It further slightly reduced the LDH release into the media.	Vitamin K2 (10 µM) improves **proliferation** and migration of bovine skeletal muscle cells.	Bovine SCs	(Rønning et al., 2018)
Vitamin D (Fish and mammals)	1,25-D3 promotes the expression of myogenic markers, myotube formation and modulates myogenic GFs; 1,25-D3 upregulates the expression of follistatin (a myostatin inhibitor) and it downregulates expression of myostatin (a negative regulator of muscle mass).	Vitamin D (100nM 1,25-D3) exerts a pro-myogenic effect on satellite cells (promotion of **differentiation**).	SCs from mice	(Braga et al., 2017)
β-carotene (fruits and vegetables)	At a dose of 30 µ*M* increased PPARγ mRNA expression.	Enhancement of adipogenesis (**differentiation**).	Bovine white adipocytes	(García-Rojas et al., 2010)
Lutein (fruits and vegetables)				
Spermidine (present in all plant and animal derived food types)	Linked to biological processes which modulate cellular proliferation, differentiation and apoptosis	Enhancement of growth rate	Bovine SCs	Patent WO 2021/158103 (Moutsatsou et al., 2021); (Xuan et al., 2023)
Sericin (silk protein; waste in silk industry)	Mitogenic factor involved in cell proliferation and attachment.	Accelerates the proliferation, Replacement of FBS in SF-media	Bovine SCs	Patent US 2022/0098546A1 (Akcali et al., 2022); (L. Liu et al., 2016; Terada et al., n.d.)

Table 8: Substances / compounds promoting cell proliferation / differentiation.

Also, for aquatic species the same picture can be observed. For example, wheat gluten hydrolysate resulted in a slight increase in fish cell proliferation when cells were cultured in Ultra-CULTURE medium, a SF medium based on DMEM/F12 supplemented with insulin, transferrin and a purified mix of bovine serum proteins, including albumin (Radošević et al., 2016). However, higher concentrations of wheat gluten (10 g/L) led to a reduction in cell proliferation by 40–65% and soy hydrolysates were found to inhibit growth. These findings are consistent with previous reports by (Sung et al., 2004), which documented reduced maximal cell densities for CHO cells when high concentrations of wheat gluten hydrolysate were incorporated into UC medium. Elevated levels of nutrients, particularly oligopeptides, can disrupt the nutritional equilibrium of the culture medium (Chun et al., 2007), potentially leading to significant alterations in its pH.

In addition to hydrolysates obtained from cultivated plants, the utilization of **hydrolysates from plant-based agro-industrial waste**, such as soybean meal, peanut meal, sunflower bran, etc., are of **special interest** regarding sustainability and costs of media compounds (Flaibam, Da Silva, et al., 2024). Other protein-rich plant-based by-products, such as press cakes, meals, rice bran, and waste potato juice (Hadidi et al., 2024) should be also considered as viable sources of ingredients for culture media in future to support the principles of a circular economy.

For further research in this area, **CM/CSF relevant cell lines** should be used to ensure usability of the results – e.g. bovine primary and non-spontaneously immortalized cell lines are now commercially available, and primary cells can be easily isolated using well established protocols – e.g. in the SOP Database of the FEASTS project on FEASTS website or (Stout et al., 2022).

4.5.2. Microalgae extracts as a sustainable basal medium alternative

Microalgae may provide a sustainable supplement source for cell culture media because they store large quantities of digestible proteins and lipids. The most abundant classes of micro-algae are the diatoms in phytoblankton, the green algae e.g. *Chlorella vulgaris*, and the golden algae (Vigani et al., 2014). The main advantage of microalgae is their content of essential amino acids, which is much higher than those from terrestrial plant proteins (see Table 1 in (Naik et al., 2024) and (Torres-Tiji et al., 2020). Microalgae are also a source of carbohydrates, poly-unsaturated fatty acids, sterols, minerals, antioxidant vitamins and carotenoids (Wells et al. 2017, Naik et al. 2024)).

Due to their high protein content and nutritional values, microalgae extracts were studied as an alternative to replace nutrients in basal chemically defined media or to substitute FBS in animal cell culture. The capacity of algae extracts as **replacement for DMEM** was tested by Okamoto et al. with C2C12 mouse myoblasts (Okamoto et al., 2020) and primary bovine myoblasts (Okamoto et al., 2022). Nutrients extracted from microalgae rescued cell growth of C2C12 mouse myoblasts cultured in a glucose- and amino acid-free medium (Okamoto et al., 2020).

In the presence of serum, replacement of DMEM by green microalgae *Chlorella vulgaris* extracts (5% of the total culture volume) resulted in the same proliferation rates for primary bovine myoblasts as in DMEM after 6 days in cell culture. Moreover, after induction of differentiation the myotube area was comparable to that of bovine myoblasts cultured in DMEM (Okamoto et al., 2022). However, excess addition of algal extracts to cell culture medium decreased cell viability of myoblasts, indicating that algae extract includes also factor(s) that counter its positive effects on cell growth.

Algal extracts obtained by acid hydrolysis contained glutamic acid (Okamoto et al., 2020), whereas the basal medium DMEM/F12 contains glutamine and glutamic acid

(https://www.thermofisher.com/at/en/home/life-science/cell-culture/mammalian-cell-culture/cell-culture-media/dmem-f12.html). Cell viability was lower in presence of glutamic acid compared to a glutamine containing medium. Further, different salt concentrations in the microalgal culture medium, as compared to mammalian cell culture media, may also have an impact on cell viability (Okamoto et al., 2020). On the other hand, the concentrations of some nutrients in algae extracts, such as tryptophan and vitamins B6 and B9, were lower than those in DMEM, which may lead to rapid depletion of these basic nutrients (Okamoto et al., 2022).

Cell-cultured food also requires glucose in culture medium as a source of energy for cells. Most of the glucose is produced from starch obtained from grains such as corn and wheat, which causes negative environmental impacts. Microalgae could supply glucose that has a much smaller environmental footprint than glucose from grains (Shimizu et al., 2021).

One of the main **drawbacks of using microalgae** is that its protein contents can vary greatly, from few percentages up to almost 50% or higher – depending on algal species, harvesting season, and different natural environments (Espinosa-Ramírez et al., 2023). This high variability complicates standardization processes which are essential for incorporating microalgae extracts in media formulations used in CM / CSF production. Another major disadvantages are their **low doubling rates under autotrophic conditions**, ranging from 8 hours to several days (Hidayati et al., 2020; Ma'mun et al., 2022). Although that can potentially be avoided, if heterotrophy is used instead of autotrophy, which at an industrial scale could lead to costs close to autotrophic growth.

4.5.3. Brewer's Spent Yeast potential for basal medium alternative

Hydrolysates obtained from agro-industrial wastes like Brewer's spent yeast could provide sustainable sources of nutrients for the cultured food sector. Moreover, this would offer opportunities to valorise

waste and reduce its negative impacts on environment (Smith et al. 2024). Further, the yeast *Saccharomyces cerevisiae*, also commonly known as brewer's yeast, is regarded as Generally Recognized as Safe (GRAS) (Jaeger et al. 2020). Although most residual biomass from big breweries is already subjected to a cascading use in food and feed industry, smaller-scale breweries frequently dispose their spent yeast which is significantly contributing to the organic load of wastewater (Łukaszewicz et al., 2024). Integration of such local waste into the production of highly valuable cell culture components for culture medium could solve this problem.

Brewer's spent yeast is potentially well suited as a substitute for basal medium. It contains **45–60% protein** and 40% of the total amino acids are comprised of essential amino acids, is rich on vitamins and minerals (Jaeger et al., 2020; Vieira et al., 2016). Nutrient composition, however, can vary depending on extraction processes and conditions used, and consequently have different impacts on cell growth *in vitro*. E.g. when autolysis was carried out at 47°C, autolysates with varying free amino acid content and peptides of different molecular weights were obtained depending on the autolysis time (Podpora et al., 2016). For a comprehensive review on this topic we refer to a review from Tao et al. (Tao et al., 2023).

In regard to the additional potential of yeast for circular bioeconomy, it is exciting to see that the search for identifying low-cost carbon sources among agro-industrial wastes (e.g. molasses, sugarcane bagasse, high fructose syrup by-products, etc.) for recombinant protein production in yeast is progressing (Álvarez-Cao et al., 2018; Ergün et al., 2022).

The **usability of yeast-based hydrolysates** for CM/CSF production will depend on **additional studies** regarding optimization and standardization of extraction methods for the preparation of extracts from spent brewer's yeast. The formulation of a tailor-made brewer's yeast-based supplement for SF basal media could provide an economical and sustainable option for cell culture media, thereby enhancing the circular economy within the cellular and traditional agricultural sectors.

4.6. Circular cell culture as a waste reduction strategy

Solutions to recycle or treat the enormous volume of liquid waste, i.e. spent culture medium, which will be generated during big scale CM/CSF production must be considered as it cannot be simply discarded. This would be inherently wasteful and will lead to a tremendous environmental burden and loss of valuable resources (Shimizu et al., 2021): after three days in cell culture, mouse myoblasts consume 99% of glucose but only 69% of glutamine initially present in DMEM. However, the amount of 14 other amino acids decreased by only 26% (Haraguchi & Shimizu, 2021). In addition, more than 80 % of the phosphorus in the medium was not consumed by the myoblasts after three days. The amount other medium ingredients like pyruvate or vitamins as well as minerals barely changed (Haraguchi & Shimizu, 2021) which also indicates that the concentration of these components could be optimized to reduce costs. Recycling these non-consumed nutrients from spent media would allow a resource-saving cultured meat production.

Bioprocess technology approaches can be also used to recycle spent media, and applicable strategies in this direction were reviewed by Quek et al. (Quek et al., 2024). In the following sections, the recycling approaches developed by Shimizu et al., Haraguchi et al. and Yang et al. (Haraguchi et al., 2023; Shimizu et al., 2021; Yang et al., 2023) will be discussed. Also, the potential of yeast species in medium recycling will be discussed. This strategy would significantly reduce the requirements for regular media exchanges and the production of waste media, drastically reducing the overall medium costs.

4.6.1. Microalgae and circular cell culture for sustainable meat production

Haraguchi et al. proposed concepts for the recycling of substances in **spent culture media** of animal cells by a **co-culture system of algae and animal cells** (Haraguchi & Shimizu, 2021). In this system,

algae supply oxygen to animal cells, while animal cells produce carbon dioxide and ammonia, components which are nutrients for algae (Haraguchi et al., 2017; Haraguchi & Shimizu, 2021). However, in this setting animal muscle cells excreted much more lactate than ammonia, which is not a metabolite for microalgae. Therefore, a different approach was required for removing lactate from spent media. The L-lactate dehydrogenase gene from *E. coli* was introduced into **cyanobacterium** *Synechococcus sp.* PCC 7002. This genetically modified **microalgae were able to consume lactate** and convert it into pyruvate, which can be converted into glucose by mammalian cells (Kato et al., 2023). Thus, an advanced circular cell culture (CCC) system was developed in which genetically modified *Synechococcus* microalgae and animal cells mutually exchanged substances of their metabolic waste products: (i) microalgae utilized lactate and ammonia excreted by animal muscle cells, (ii) the animal cells metabolized pyruvate and some amino acids excreted by microalgae, and (iii) rat liver cells produced GFs required to promote animal cell proliferation.

Applying this CCC system allowed mouse muscle C2C12 cells to grow efficiently for several cycles **without the need of animal serum, and without producing waste medium to be disposed** (Haraguchi et al., 2023). The use of microalgae is a key feature of this CCC system, which makes it possible to use repeatedly cell culture medium, opening possibilities for a low-cost and low-environmental-load cultured food production (Haraguchi et al., 2023). CCC systems would allow to reduce the environmental impact of large-scale CM/CSF production and support the idea that cultured food provides a cleaner alternative to conventional meat.

4.6.2. Brewer's Spent Yeast potential for circular cell culture

As discussed in 4.5.3, **yeast cell hydrolysates** could contribute constituents to **basal cell culture media**, thereby reducing the overall medium costs, and can be easily genetically modified to allow the

production of GFs independent of human-consumable carbon sources. Moreover, some yeast species – e.g. brewer's yeast – can naturally utilize such non-sugar carbons as **lactate** (Turcotte et al., 2010) and use **ammonia** as a nitrogen source, e.g. to produce glutamate and glutamine (Kevvai et al., 2016). This fulfils the essential requirements for the development of **yeast-based CCC systems**, similar to those utilizing microalgae and rat liver cells, as discussed in the previous chapter. Such systems would offer the **significant advantage** of a markedly higher proliferation rate of yeasts (doubling time 1,5 - 2 hours (Bergman, 2001)) as compared to microalgae (doubling time 8 hours to several days (Hidayati et al., 2020; Ma'mun et al., 2022)), easy handling and a wide range of methods and manipulation tools.

5. Regulatory submissions and industry outlook

The CM/CSF industry has seen an increase in regulatory approvals worldwide, with Australian start-up Vow announcing its entry in Hong Kong's and Singapore's markets with a cultured duck foie gras (Vow, 2024), which followed earlier regulatory approvals in Singapore for GOOD Meat's chicken products, and in the United States where Upside Foods and GOOD Meat have received Food and Drugs Administration (FDA) no-questions memoranda for their cultured avian meat products (Fasano et al., 2022; Folmer et al., 2023).

From a regulatory standpoint, culture medium formulations must meet various legal requirements regarding their chemical composition. These primarily focus on identifying hazardous compounds, compounds that pose risks of allergenicity, or compounds that are present in the final products due to their presence in media and are in concentrations not traditionally found in conventional meat products (Fasano et al., 2022; Folmer et al., 2023; FSANZ, 2023).

The recent regulatory engagement report from Australian start-up Vow indicates that the company has conducted extensive safety testing on specific media components, evaluating their presence in the final cell-based quail products. While exact tests were not highlighted in the public version of the report, it is indicated there were no toxicological concerns associated with cell culture medium components (FSANZ, 2023).

In the United States, toxicological assessments for both Upside Foods and GOOD Meat included testing for heavy metals (e.g., lead, arsenic, cadmium, and mercury) and comprehensive microbiological analysis covering aerobic plate counts, coliforms, *E. coli*, *Enterobacteriaceae*, molds, *Salmonella*, *Enterobacter cloacae*, and influenza types A and B, with additional screening for a broad panel of human and bovine viruses in GOOD Meat's submission. These results were compared to conventional ground chicken products (GOOD Meat, 2022; UPSIDE Foods, 2021). Interestingly, one analysis found that cultured chicken contained higher levels of arsenic, cadmium, and lead compared to conventional chicken. Notably, the lead content was found to be 20 times higher in avian cells cultured in serum-free media than in conventional chicken tissue (UPSIDE Foods, 2021).

In most regulatory submissions, companies are addressing allergenicity by assessing the presence of allergens in cultured meat products (FSANZ, 2023; GOOD Meat, 2022). For instance, Vow's culture medium, which contains barley proteins, prompted the company to test the gluten content in their cell-based quail products. Notably, the results indicated that gluten levels in the final product were below detectable limits, posing no safety risk for consumers with gluten sensitivities (FSANZ, 2023).

Another important consideration for culture media is its nutritional composition and the need for comprehensive safety and toxicological assessments of nutrients and compounds present in CM products, particularly when their levels differ from those found in conventional meat products. These

concerns are emphasized in the FSANZ assessment of Vow's quail products, as well as in regulatory submissions by start-ups in the U.S. (Fasano et al., 2022; Folmer et al., 2023; FSANZ, 2023).

Regulatory dossiers often contain full nutritional analysis of CM products that includes protein, fat, ash, moisture content, as well as content of minerals, vitamins and cholesterol, and their potential differences to conventional chicken products (GOOD Meat, 2022; UPSIDE Foods, 2021). In instances where a deviation in concentration of any given nutrient is noted, a comprehensive description of its historical consumption in food by humans and potential toxicology is followed. For example, folic acid is not traditionally found in chicken products in relevant concentrations as a nutrient, and therefore safety concerns related to its content in Upside Foods' and GOOD Meat's cultured chicken products were addressed thoroughly in both companies' regulatory submissions (Fasano et al., 2022; Folmer et al., 2023). This was attributed to folic acid use in culture medium to support metabolic processes related with cell proliferation, while consequently accumulating in the food but nonetheless being within the concentration range of other commonly consumed foods.

A recent overview of CM relevant GFs present in food products, such as meat and milk, was published by Huang et al. This study indicates that historically significant amounts of GFs are being consumed by humans (Huang et al., 2024). Reported concentrations in pasteurized cow milk reached as high as 155 ng/ml EGF, which is 15 times higher than the 10 ng/L utilized in differentiation medium (Messmer et al., 2022). Concentrations ranging from 5-100 ng/ml found in these products are comparable to quantities in most reviewed media formulations. However, a limitation of such comparisons is the lack of published concentrations of GFs in final raw and cooked CM products, which will need to be assessed by the manufacturer during dossier preparation. For example, in FDA's analysis of Upside Foods' dossier, GF safety and their sources were given attention and a request for clarification regarding their use as

process aids, history of safe use and dietary intakes. In addition, Upside Foods has analysed GF concentration in cooked food products and noted that their concentration was below the limit of detection, highlighting that cooking process has likely denatured/destroyed the GFs used for cell culture (Fasano et al., 2022; UPSIDE Foods, 2021), which is in line with the effect of heat treatment in human and cow milk on GFs (Huang et al., 2024). Still, care must be taken, as some GFs – e.g. TGF-β, IGF-1, EGF - when stabilized by e.g. milk albumins, are able to withstand thermal treatments at 85°C to a substantial degree (Huang et al., 2024).

In addition to folic acid, GOOD Meat's FDA dossier highlighted media components that could be present in their products though not explicitly covered by regulation for use as process additives. The list included ferric nitrate, hypoxanthine, thymidine, lipoic acid, putrescine, sodium pyruvate, polyoxamer 188 and FBS (GOOD Meat, 2022). The company has shown the prevalence of most of these components in common foods at similar or lower concentrations. In the case of the non-ionic surfactant polyoxamer 188, it is stated that its content in final products (due to carryover from media) was within the dosage reported in literature where no adverse effects in humans are witnessed (Folmer et al., 2023; GOOD Meat, 2022).

Findings from regulatory submissions underscore region-agnostic visions around food safety regarding culture media components in CM. Recently, a comprehensive report has identified trends in regulatory assessments in different regions, providing a summary of public guidelines available from multiple regulatory agencies globally (JACA, 2024). So far, Gourmey and Mosa Meat have submitted applications to European Union regulators for authorisation of their respective products, but no regulatory dossiers are available so far, thereby hindering a more in-depth analysis of the regulatory process in the region (Monaco, 2025; Mosa Meat, 2025).

6. Key performance indicators

Key performance indicators (KPIs) are specific metrics that highlight the most essential areas of an organization's performance—those that directly influence its present effectiveness and long-term success (Parmenter, 2015). When applied to nascent industries, KPIs provide insights into technological maturity, such as product development or regulatory milestones, and guidance for establishing benchmarks to track a company's progress toward market readiness.

Based on current knowledge from literature cited and discussed throughout this manuscript, expert discussions and KPI validations as well as recent analysis and reports (JACA, 2024; Pasitka et al., 2022, 2024; SuperMeat, 2024; UPSIDE Foods, 2021; Vow, 2024), the following culture media-related KPIs for current CM/CSF processes have been defined:

1. High media to biomass conversion ratios.
2. Medium cost: one of CM/CSF highest costs is culture medium and its contribution to CM/CSF final cost should be in cents of € per L to reach cost parity with conventional meat. However, it is dependent on the process and the conventional counterpart it attempts to emulate. A more descriptive unit of measurement for this KPI at scale is media cost per mass of CM/CSF (€/kg).
3. Number of population doublings supported by proliferation media in relatively low doubling times (<30h): adequate medium performance is needed (upwards of 60 population doublings) to sustain a process from cell bank to large bioreactor expansion.
4. Animal component-free and food-safe formulation: free of animal components like FBS, bovine serum albumin (BSA), as well as from GFs and other recombinant proteins of non-species-specific origin. Formulation should take regulatory constraints in entry market(s) into account.

5. Waste medium recycling / waste management concept.
6. Antibiotic-free formulation: isolation, proliferation and differentiation media formulations free of antibiotics and antimycotics.
7. Hazard Analysis and Critical Control Points (HACCP) in place, Quality control and Quality Assurance (QA/QC) and sterility plans for bulk media production and HACCP.
8. Supply chain plans: supply-chain redundancy and resiliency for medium component sourcing.

7. Conclusion

Enormous quantities of cell culture media will be required for large scale production of CM and CSF. Therefore, a central economic question concerns the costs of medium as it significantly contributes to the price of CM and CSF products which should be competitive with conventional meat and seafood at the end.

Cell culture media need to be high-performing in terms of cell proliferation and differentiation and should be free of any animal-derived material due to ethical and safety concerns (see Table 9). In a recent survey conducted for this review, six out of seven companies offering media for CM and CSF production confirmed that animal-derived ingredients are not part of media. These media are generally made up of a basal medium and several supplements including GFs. However, final medium composition depends very much on the type of cell lines and species. According to the survey (see Supplementary Document 1), companies prefer to customize media for their specific cell lines over available standard media.

But medium optimization still faces challenges as there is lack of data regarding essential nutrients and GFs for single cell lines from various animal species (see Table 9). Once essential components are identified for specific cell lines, their concentration must be optimized. The amount of GFs – one of the main cost drivers for media – could be reduced by prolonging their short half-life by increasing proteolytic stability through mutations, microencapsulation or with stabilizing compounds. In this respect, costly albumins could be substituted with low-cost methylcellulose or protein isolates as stabilizing agents (Schenzle et al., 2025). Based on our survey, the majority (60 %) of companies supplement media with wild-type GFs, which may be linked to regulatory issues. An "in-house" production of stabilized and highly bioactive cell-type specific GFs may facilitate cost savings as well (Venkatesan et al., 2022). The identification of effective minimal GF variants as shown for HGF by deNola (De Nola et al., 2022) could further reduce manufacturing costs for CM and CSF, though regulatory aspect of using mutant variants is not clear (Table 9).

A critical element for an economically viable CM and CSF production is the substitution of costly cell culture medium reagents with low-cost but food grade alternatives. In this regard, hydrolysates from plants and microorganisms represent nutrient rich sources of cell culture medium ingredients (see Table 9). According to the survey, two out of seven companies offer CM/CSF media containing lysates or hydrolysates derived from plants or microalgae. Even waste or by-products of agro- and food-industry could provide proteins, peptides, vitamins or carbohydrates required for basal media. To avoid high charge-variations of compounds extracted from alternative sources, extraction/preparation methods need to be standardized through the establishment of Standard Operating Procedures.

Topic	To do
Food grade and animal component free media, especially for proliferation of aquatic cell lines, and isolation/cryopreservation of primary cells	Depending on species (e.g. bovine, porcine, avian, fish) and cell types: Define **essential medium components** for isolation, proliferation and differentiation
Food safe growth promoting components	Depending on species (e.g. bovine, porcine, avian, and especially fish) and cell types: Identify **appropriate type of GF** or growth promoting compound.
	Engineer **minimal active and stable GF** versions
	Identify low-cost **stabilizers for GFs or immobilization strategies**
	Define **regulatory and safety constrains** for media components (Species-specific/not? Wild type/mutants? Naturally occurring/artificial? Safe rest concentrations? Reliable methods for quantification?)
Low-cost carbohydrate sources	**Analyse** the potential of agro-waste products (e.g. molasses)
Low-cost protein sources (peptide or amino acid)	Analyse the potential of agro-waste products (e.g. brewer´s yeast, plant hydrolysates)
Low-cost free fatty acids	Analyse the potential of plant-based oils including agro-waste
Development of SOPs	**Standard** extraction methods for different nutrients (e.g. proteins, vitamins, lipids) from natural sources or waste
Waste medium recycling / waste management	Optimize **recycling of non-consumed** media components (glucose/amino acids/vitamins and minerals, albumins, growth factors).
	Develop techniques to **remove/upcycle harmful cellular metabolites** e.g. ammonia and lactate.
CCC-systems using mixed cell cultures	Establish and optimize a fully animal-source independent, highly efficient **multi species cell consortia** for CCC systems applicable for CM/CSF production processes.
AI-based optimization of cell culture medium	Collect data and establish stable **models for AI/ML** approaches.

Table 9: Key challenges that remain to be addressed (white spaces).

Further, new technologies for medium recovery or recycling will be essential to prevent that waste medium generated during CM/CSF production processes will have a negative environmental impact (see Table 9). In this regard, circulating cell culture (CCC) systems are promising. In a novel CCC system developed by Haraguchi et al., microalgae supply nutrients for mammalian cells and at the same

time serve as a waste-medium recycler (Haraguchi et al., 2022). Mixed cell cultures have long been used for industrial applications (Goers et al., 2014). Improving culturing success by multi-species cell consortia hold enormous potential but this approach is complex and require extensive optimization.

Current approaches regarding SF media often depend on trial-and-error methods and utilize formulations originally designed for non-food areas or model cell lines used in research, which may not be well-suited for the specific requirements of cell lines proposed for the CM/CSF production. Optimizing one factor at a time or performing random experiments is often used to design new media formulations, but this strategy is very inefficient considering the high number of media components, e.g. the basal medium DMEM/F12 consists of 52 components (G. Chen et al., 2011). Further, possible interactions among media components remain unexplored with this approach (Cosenza et al., 2021). The throughput capacity of medium optimization studies can be enhanced by using statistical experimental designs and the application of high-throughput systems (Z. Xiao et al., 2014). Design-of-Experiments (DoE) methods can manage large numbers of components in fewer experiments and allow the evaluation of a wider design space to identify optimal conditions (Cosenza et al., 2021; Z. Xiao et al., 2014). In general, DoE methods can optimize < 10 variables (Singh et al., 2017), and when including screening designs > 25 variables could be considered (Weuster-Botz, 2000). The DoE approach together with a Response Surface Methodology approach was successfully applied by Skrivergaard et al. when developing a SF medium for the proliferation of bovine muscle cells (Skrivergaard, Young, et al., 2023), and by Schenzle et al. for optimization stabilizer combinations for GFs (Schenzle et al., 2025). To deal with increasing complexity, the use of artificial intelligence (AI)/machine learning (ML) for media optimization could be promising for developing single cell line specific culture conditions for CM/CSF production (see Table 9). ML approaches are generally used to develop predictive models based on the relationships among features of a given dataset. ML commonly involves processing input

data, training of an appropriate model, and predicting new data to be evaluated in lab experiments (Camacho et al., 2018). ML-based approaches have been already successfully applied for medium development for mammalian cell cultures (Grzesik & Warth, 2021; Hashizume et al., 2023; Hashizume & Ying, 2024). An AI approach was also applied to optimize medium formulations for fish cells (Nikkhah et al., 2023). The resulting formulation with optimized quantities of GFs, selenium, and ascorbic acid, and a reduced amount of FBS could enhance zebrafish cell growth rate by >50%, suggesting that AI has the potential to accelerate the development of animal-free medium formulations. However, applying AI technologies to develop highly efficient cell culture media requires a deeper understanding of the physiological and nutritional needs of mammalian and fish cell lines under *ex vivo* conditions and the collection of high throughput data from transcriptomic, proteomic, and metabolic analyses (Hashizume & Ying, 2024).

As media composition depends on multiple factors like cell type, species, number and types of essential supplements, and as companies producing CM/CSF customize and hide their medium formulations, a quantification of expected cost reductions is difficult. Companies offering media for the CM/CSF area stated that prices range from ≤ 1 up to €100/L. According to our survey, five out of seven companies can offer media for CM production with ≤ 1 up to €5/L, whereas only two out of seven companies offer media for CSF in this range. Taking strategies proposed for cell culture media cost reductions mentioned in this review into account, an economically viable and sustainable production of CM/CSF should be possible in the near future.

8. CRediT authorship contribution statement

Regina Leber: Funding acquisition, Conceptualization, Writing – original draft, Writing – review and editing, Joana Rosa: Writing – original draft, Writing – review and editing, Vincent Laizé: Writing – original draft, Writing – review and editing, Gonçalo Filipe Marques Fernando: Writing – original draft, Writing – review and editing, Johannes Buyel: Writing – original draft, Writing – review and editing, Aleksandra Fuchs: Funding acquisition, Conceptualization, Project administration, Writing – original draft, Writing – review and editing, Visualization.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FEASTS
Fostering European Cellular Agriculture for Sustainable Transition Solutions

Annex 4 of Deliverable 3.3: Review on texturizing meat / seafood analogues

Work Package 3 *Sustainability by design*

Task no: 3.2 and 3.4

Funded by
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Duration: 36 months

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Contents

Review on texturizing meat/seafood analogues

1. Introduction

The global population is growing rapidly, with perspectives to reach 9.7 billion people by 2050. There are countless implications for current feeding systems resulting from this demographic trend, including an increased pressure on the food sector, to satisfy the population needs in a sustainable and healthy way. Current dietary patterns remain highly dependent on animal protein, with FAO predicting an increase of 70% of meat consumption, which would cause a significant disparity between the supply and demand (Alexandratos, 2012). Such growth is associated to negative environmental impacts such as an increase in greenhouse gas emissions (GHG), overexploitation of land and water, intensive livestock slaughter and public health issues, which highlights the urgent need for a change of scenario (Kirsch et al., 2023). In this context, alternative proteins are emerging as potential solutions to address the food crises. It includes plant-base products (e.g. tofu, textured plant protein products), precision fermentation-derived proteins (e.g. mycoproteins) and cell-based meat. Despite the growing market trends, the industry of meat substitutes still faces many challenges, being consumer acceptance one of the most critical factors. To address this, research has focused on developing meat analogues that mimic the sensory, structural and nutritional characteristics of conventional meat (Kirsch et al., 2023).

This review focuses on the texturization of cultivated meat analogues, *i.e.* animal meat-like products derived from cellular agriculture. The field of cellular agriculture consists on manufacturing animal-protein products, without the need of living organisms, through cell expansion and posterior food product formulation integrating the cultivated biomass. Whether the ultimate product is structured into whole cut meat food (e.g., steak, fish fillet), or unstructured minced products (e.g. hamburgers, patties, meatballs), it is essential to replicate the conventional texture of such products (Malav et al., 2015).

Besides texturization, consumer acceptance has also been a challenge of cellular agriculture. Presenting meat substitutes that closely replicate the animal derived foods, providing a familiar texture, elasticity, sensory and cooking experience to the consumer, may help mitigate scepticism. Animal meat results from a combination of muscle, fat and connective tissue that are responsible for the fibrous, mouthfeel, chewiness and juicy attributes of meat. The ultimate goal of texturizing meat analogues is to reproduce these natural occurring structural and functional characteristics (Kumar et al., 2017).

In order to achieve it there must be a thorough method of formulation and processing, relying on the combination of functional ingredients (plant proteins, carbohydrates, vegetable oils) with the appropriate texturization techniques (extrusion, shear cell technology, 3D printing, electrospinning).

The aim of this review is to provide a comprehensive overview of the state-of-the-art processing techniques and plant-based functional ingredients used in the texturization of cultivated meat products. This review will also present the challenges and the future perspective on the texturization of cellular meat analogues.

2. Processing techniques

Over the years, the technology used for meat analogues texturization has advanced significantly across plant-based, microbial-based and cellular-based meat. Multiple approaches have been investigated to recreate the fibrous anisotropic meat-like structures, including extrusion, 3D (bio)printing, electrospinning, shear-cell technology, electric or mechanical stimulation, directional freezing and scaffolds-based strategies (Dekkers et al., 2018). In general, all these processing techniques can be categorized into i) bottom-up strategies, in which texturization is primarily done on individual structural elements (cells, proteins, biopolymers or bioinks) that are then assembled into higher-order, organized structures in a stepwise approach (e.g. spinning technology, 3D printing); ii) top-down strategies if the texturization processing starts with the large bulk material that is restructured using mechanical or physicochemical forces (e.g. extrusion, shear-cell, freeze

alignment). This approach, is effective in recreating fibrous matrices but fails in replicating the hierarchical organization of animal muscle from nano- to macroscale (Dekkers et al., 2018).

The most commonly used techniques for texturization of cultivated meat analogues are (*Deep Dive*, 2022; Dekkers et al., 2018):

- Electrospinning: enables the production of fibres at nanoscale (50-500 nm) by applying a high-voltage electric field to a polymer solution. The resulting fibres closely resemble the fibrillar architecture of the ECM, promoting cell adhesion, proliferation and alignment.
- Shear cell technology: controlled shear forces and heat are applied to proteins in a conic device, aligning the materials into fibrous structures. This way, layered, anisotropic scaffolds mimicking fibrous muscle tissue are created.
- High-moisture extrusion: combines high temperatures with precise pressure and shear in a twin-screw extruder in order to unfold vegetable proteins, with posterior cooling, phase separation and realignment of proteins into fibrous textures. Produces dense, meat-like structures with aligned protein fibres and is mostly used in plant-based and hybrid cultivated foods.
- Electrical/mechanical stimulation: cells are exposed to electric pulses or mechanical strains that potentiate muscle differentiation, maturation and alignment. Enables the formation of native-like muscle tissue, leading to functional, contractile and structurally aligned scaffolds of meat analogues.
- Freeze alignment: directional freezing of hydrogels or proteins, causing ice crystals to align in a fibrous-like network. Results in porous, aligned structures that guide cell growth towards a meat-like structure.
- 3D manufacturing (moulding and printing): additive manufacturing technique that allows precise control of the shape and internal structures through the use of moulds (allows a precise control of the external structure) or 3D bioprinting relying to edible

bioinks (customized scaffolds internally and externally). 3D manufacture enables the production of various cultivated products according to the design used, from whole-cut meat to ground meat.

3. Additive manufacturing

The technology of additive manufacturing (AM) has been widely applied in manufacturing processes across various industries, including construction, automotive, food processing and other. In broad lines, AM refers to the production of 3D structures through a layer-by-layer deposition of material, in opposition to subtractive manufacturing, which reduces the size of a larger bulk piece. In food industry, AM enables the creation of customized food products, priorly designed by digital approaches like computer-aided design (CAD), through the consecutive addition of layers of edible materials (Pulatsu & Udenigwe, 2023).

In the food industry, AM has been used with printable materials such as chocolate, dough, purees, etc, to make personalized, original 3D products. More recently, its application in cellular agriculture has drawn attention, aiming to include cultivated animal biomass in the materials to be printed and therefore produce meat-like foods with integrated animal protein (Pulatsu & Udenigwe, 2023). The incorporation of animal cells can be accomplished in two ways: using live cells (bioprinting) or processed, non-viable biomass.

In the first one, edible bioinks combine live animal cells with functional, biocompatible food-grade ingredients. After being harvested, cells are re-suspended in a viscous, nutrient-rich matrices that provides mechanical support, printability and ensures cell viability (Pennarossa et al., 2021). In scaffold-based bioinks cells are loaded into biocompatible hydrogels that mimic the extracellular matrix (ECM). In scaffold-free bioinks larger animal tissues, resultant from cell aggregation into spheroids, pellets or tissue strands, are integrated in the bioink. This approach is motivated by the ability of cells to self-assembly into tissue-like structures where cell-cell interactions are promoted.

On the other hand, 3D printing, utilizes processed biomass combined with other functional ingredients to generate a food product. The harvested cells are incorporated in an edible

matrix leveraging on the nutritional value and rheological properties of other ingredients and creating a 3D product without the complexity of keeping cells alive within the ink (Ogilvie et al., 2023).

4. Binders and structuring agents

Most formulation techniques resort to scaffolds that can provide the appropriate matrices for structuring a 3D product. Nonetheless, it is also possible to avoid the use of scaffolds, leveraging on the secretion of ECM proteins from cells. Non-adherent cells can form spheroids or aggregates, coming together in a compact cellular biomass, or sheets of cells can be stacked into dense tissue structures (Klatt et al., 2024).

Despite these scaffold-free approaches, most reports cases on food structuring rely on functional materials to support cell growth, proliferation, differentiation and/or alignment. Depending on their properties, scaffold materials can be chosen for certain stages of the production process, either as microcarriers for cell expansion of anchorage-dependent cells during proliferation stages, as fibrous and porous matrices promoting cell alignment and providing meat-like properties to the final formulation, or as hydrogels or bioinks for cell encapsulation and subsequent assembly of the product through 3D printing or moulding (Bomkamp et al., 2022).

The ideal scaffolds for cultivated meat should be edible, food-grade, cost-efficient and mechanically suitable to replicate the various tissues of animal muscle. Additionally, they should provide nutrient delivery, be biocompatible, non-toxic, scalable and have nutritional value. In fact, most scaffolds result for the combination of various ingredients, each contributing with specific advantageous traits (Bomkamp et al., 2022).

Commonly used materials include proteins, polysaccharides, gums, and lipids from animal or plant origin. Protein-based matrices support cell adhesion, growth and differentiation due to their ECM-like properties, besides being biocompatible and with favourable mechanical features. Collagen and gelatin are animal proteins usually used for scaffolding, while plant-base proteins include soy, pea or zein proteins and wheat gluten, and also fungi-derive proteins such as mycoproteins and yeast extract.

Polysaccharide and gum-based materials are used for hydrogel formation and 3D printing due to their favourable viscoelastic and gel-like properties, besides contributing to the dietary fibre content of the final product. Polysaccharides extracted from algae (Fe & Dj, 2017) are valuable scaffolding materials that include, alginate, carrageenan or agar, but also bacterial-sourced gums such as gellan and xantham gum (Chen et al., 2023).

Lipids and waxes are primarily used to replicate the sensory properties of fat animal tissue in cultivated products (MULLER-AUFFERMAN & WALKER, 2022). When mixed with other materials, such as proteins, lipids form an oleogel matrix that can help achieve the marbling, juiciness and mouthfeel attributes of conventional meat. Beyond texturization, these materials also contribute to the nutritional enhancement of the final product by introducing functional lipids such as omega-3 and omega-6 fatty acids(Lambert et al., 2024). Frequent used lipids include vegetable and algal oils.

Although edible materials have become more common in cultivated food formulation, since they do not require cell detachment and can be incorporated in the final product, there are synthetic polymers that have been explored as scaffolds. Some polymers and esters, such as PLGA, PCL or PEG, designed to be biocompatible and replicate the biochemical properties of natural tissues, allow for a more precise control over scaffold characterization such as biodegradability, porosity, adhesion rates, etc. However, since they are not edible, they need to be removed before consumption, which difficult the downstream process (Bomkamp et al., 2022).

5. Challenges

Texturizing cultivated meat presents several critical challenges. One major hurdle is the integration of multiple types of tissues into a single, cohesive product that replicates the complexity of the natural muscle animal network of muscle, fat and connective tissue. Moreover, the limited availability of suitable materials for food-grade application, that also need to be biocompatible, cost-effective and non-toxic, presents an impasse to food formulation. Besides, scalability and reproducibility of texturization remain a difficulty due to the variability in cell behaviour and structural outcomes (Kirsch et al., 2023).

As common challenge in cultivated meat is the regulatory constraints associated to novel food technologies, which delays product development and approval. Ultimately, achieving cost-effective production of lifelike, texturized cultivated meat, is essential for commercial viability, requiring optimization of both upstream cell culture and downstream structuring technologies (Dekkers et al., 2018). Altogether the still existing challenges in cultivated products texturization, highlights for the need of advances in research and development.

6. Conclusion

In summary, the texturization of cultivated meat and seafood constitutes an essential aspect tin the overall production process towards realistic, consumer-accepted meat analogues. While significant progress has been made in replicating the structural and sensory attributes of conventional meat, further innovation is required to integrate multiple tissue types, ensure scalability and reduce production costs.

The use of functional ingredients and advanced structuring techniques, has taken texturization to the net level, laying the grounds to produce realist cultivated animal and seafood foods. As the field evolves, addressing regulatory, material, and consumer-related barriers will be essential to unlock the full potential of cellular agriculture and deliver, truthful, sustainable, high-quality meat alternatives to the market.

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FEASTS
Fostering European Cellular Agriculture for Sustainable Transition Solutions

Annex 5 of Deliverable 3.3:

Final report on Task 3.2,

Case Study 2

Substitutes for animal serum

Work Package 3 *Sustainability by design*

Task no: 3.2 and 3.4

Funded by
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Project title: Fostering European Cellular Agriculture for Sustainable Transition Solutions

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Start date of project: 01.12.2023

Duration: 36 months

Deliverable title: Catalogue of media compositions and scaffold materials by target application and opportunities for cheaper and more sustainable formulations

Due date of deliverable: 30/11/2025

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Executive Summary

Task 3.2 Objective: Map the existing and prospective technologies, criteria and methodologies to develop culture-specific medium and scaffold materials, and their ingredients, for CM/CSF production, as well as strategies to obtain culture media and scaffolding materials in a cheaper, sustainable, food safe and ethically responsible way.

In this report we present our experimental results further investigating the opportunities for cheaper and more sustainable media formulations. We present data from **Task 3.2, Case Study 2: Substitutes for animal serum**, with the following **action Plan:** "Direct extracts from plants (soybean; NaT), algae, and yeast (ACIB), as well as partial hydrolysates thereof, and side streams from animal-free agroindustries (ACIB) will be processed to provide amino acids, glucose, albumins or small molecules to media and assessed regarding scalability, costs, batch-to-batch variability and impurities."

In this case study, our literature search has identified the low probability of the plant/yeast/algae-based substitutes for animal serum to perform adequately for cultured meat/seafood cell lines. Thus, the focus was set to apply hydrolysates of plant and yeast extracts to substitute the basal media (e.g. DMEM/F12) and to assess such substitution regarding scalability, costs, batch-to-batch variability and impurities. One hydrolysate-based alternative to the chemically fully defined basal media DMEM/F12, which performed comparably to DMEM/F12, was identified and characterized in short- and long-term experiments.

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1. Task 3.2, Case Study 2: Substitutes for animal serum, literature study

For Case Study 2, we reviewed published media compositions and supplementary components related to isolation, cryopreservation, proliferation, and differentiation media for cultured meat and seafood cell lines, especially focusing on the possibilities to substitute animal serum and other animal-sourced components with animal-free alternatives. Published media components were catalogued based on cell line and end product application (see Annex 1). The analysis, as a part of Deliverable 3.3, is available as a preprint (Leber et al., 2025) (see **Figure 2** for the summary). In the review, we provided a comparison of individual media formulations regarding cost and possible or already available alternatives for some of the high-cost or high-risk components. We critically analysed strategies aimed at reducing the costs and enhancing the sustainability of CM and CSF production, and summarise topics that require further exploration. We also considered possible emerging regulatory issues, current trends in consumer acceptance, and their impact on media formulation development. Finally, we proposed key performance indicators (KPIs) for media formulations to guide future strategic and operational improvements to the production process, making it more economical and sustainable.

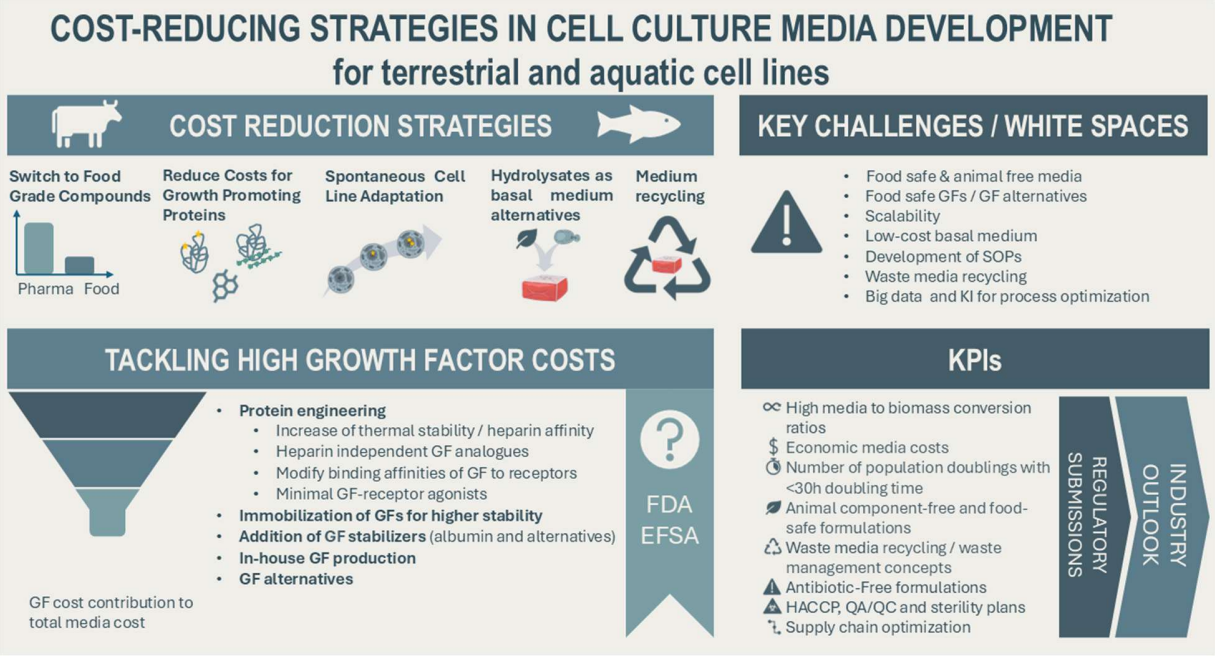

Figure 2: *Infographic summarizing topics discussed in the review (Leber et al., 2025).*

Our initial literature search has shown that for cell lines that are not applicable in the CM/CSF area (immortalized cell lines like CHO, HEK, VERO, BHK, and insect cell lines), research data indicate that protein-rich plant hydrolysates, such as those derived from soy, wheat, and rice, are non-toxic and can fully substitute FBS (Leber et al., 2025). Protein hydrolysates have been shown to improve cell viability, proliferation rates, and resistance to shear stress. The effects of hydrolysates appear to be not only nutritional, which has led to the hypothesis that higher-MW oligopeptides present in plant hydrolysates may act as growth or survival factors. Similar effects were also anticipated for CM/CSF-relevant cell lines; however, to date, only partial substitution of FBS has been demonstrated (Leber et al., 2025).

The restricted ability of plant extracts to substitute FBS may be due to the absence of essential growth factors (GFs), which are necessary for the proliferation of primary or generally less-adapted cell lines. It appears that GFs cannot be replaced by the protein/peptide mixtures derived from plant material hydrolysis. Nevertheless, plant extracts provide a diverse array of nutrients, including amino acids, peptides, carbohydrates, lipids, and minerals, which could serve as foundational components in cell culture media formulations.

Therefore, for this part of the project we evaluated a possibility to substitute a fully chemically defined basal medium (e.g. DMEM/F12) with plant-derived and yeast-derived hydrolysates, and to assess such substitution regarding scalability, costs, batch-to-batch variability and impurities.

2. Substitutes for basal medium:

2.1. Production of the cell lysate and hydrolysates from plant and yeast biomass

ACIB worked in collaboration with NaTurtle to produce several cell extracts and hydrolysates. Conventional food plants (wheat, rice, soy) (see **Figure 3**) as well as biotechnologically relevant *Nicotiana benthamiana* (leaf and stem tissue), *Moringa oleifera* (leaf tissue) and *Nicotiana tabacum* (BY2 cells growing in suspension culture) (see **Figure 4**) were used to produce hydrolysates. Additionally, yeast extracts and autolysates were produced from two yeast species where one could reasonably expect a left-over cell biomass (*Saccharomyces*

cerevisiae from a brewery Sudhaus Graz, as well as CBS7435 Δ Ku70 Ku- His4- HAC strain of *Pichia pastoris*).

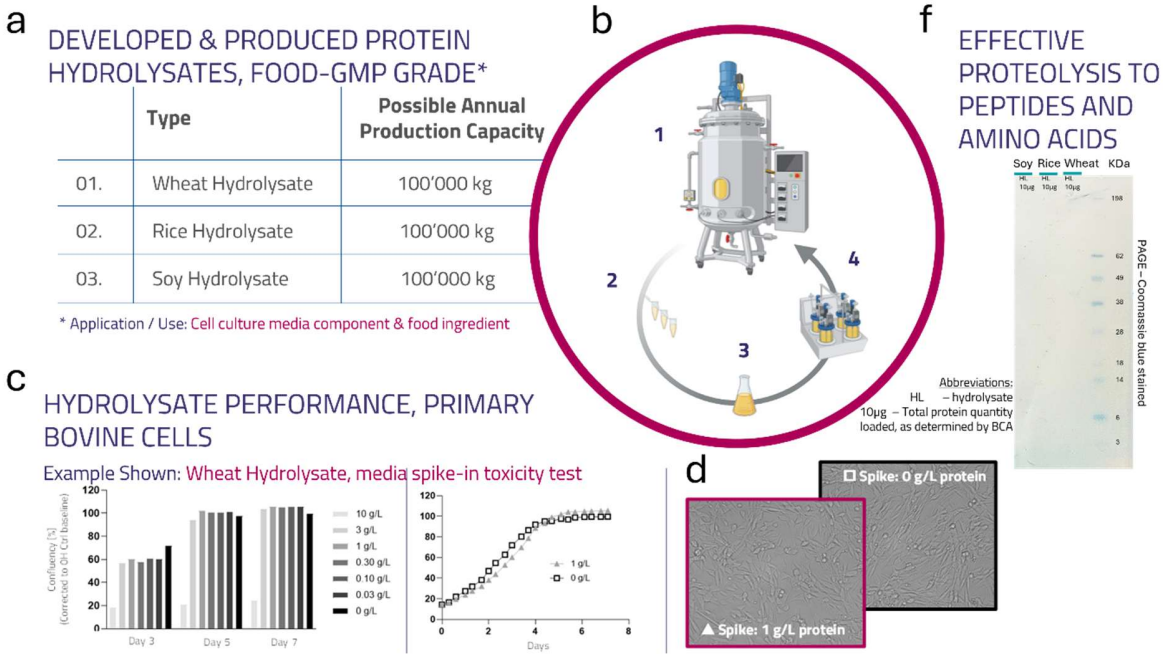

Figure 3: *NaTurtle developed and produced protein hydrolysates from conventional food plants – wheat, rice and soy.* ***(a)*** *List of the plants used and possible annual production capacity using a proprietary process, schematically depicted in* ***(b)****.* ***(c)*** *An example of a toxicity test of the produced hydrolysates, where proliferation medium was spiked with designated concentrations of wheat hydrolysate, and confluency was observed during 7 days of cultivation.* ***(d)*** *Morphology of the BSCs in spiked and control media were compared.* ***(f)*** *Effective proteolysis to peptides and amino acids was demonstrated.*

For production of rice, wheat and soy hydrolysates, a proprietary process was developed by NaTurtle that can be transferred to commercial quantity production without further requirements for scale-up process changes, based on the protocol by (Meinlschmidt et al., 2015). NaTurtle applied following strategy for a proprietary process development:

1. Identify suitable GMP-certified commercial production-plant;
2. Down-scale process flow;
3. Adapt process to crop hydrolyzation assuring tight compatibility with commercial plant work-flow;
4. Upscale to commercial scale without any need for process adjustment or capex.

NaTurtle was able to develop a commercial-scale-ready process for the production of three food plant hydrolysates in powder form (**Figure 3 a** and **b**). A high hydrolysis efficiency was demonstrated (**Figure 3 f**). NaTurtle has further performed toxicity tests showing concentration dependent growth and growth-advantages for hydrolysates in standard cell culture media for BSCs (**Figure 3 c**). Initial morphological observation has further failed to reveal any differences between BSCs cultured in medium with or without hydrolysates (**Figure 3 d**).

For *Moringa oleifera*, *Nicotiana tabacum* and *Nicotiana benthamiana* another adapted version of the (MeinIschmidt et al., 2015) protocol was used by acib, which is planned to be published after additional improvements regarding extraction volume and clarification method in a peer-reviewed scientific journal in an open access format. The latter protocol is new in that it uses plant biomass from technical processes (i.e. *Nicotiana tabacum* and cells thereof) as well as non-standard crops (i.e. *Moringa*) for the extraction of proteins and subsequent hydrolysis (**Figure 4 a** and **b**).

acib used following strategy for scalable process development:

1. Plant material was homogenized;
2. Extract clarification was performed;
3. Proteolytic digestion carried out (pepsin);
4. Sterile filtration applied.

In this way, a rapid, cost-efficient and scalable process for nutrient extraction from different under-utilized plant materials was established, which does not require special and costly biomass pre-treatment (e.g. dehulling, defatting). The process also does not use organic solvents, which is a great advantage for the product applied in food industry. Additionally, a hydrolysis degree of >90% achieved compared to the less than 13% previously reported (MeinIschmidt et al., 2015).

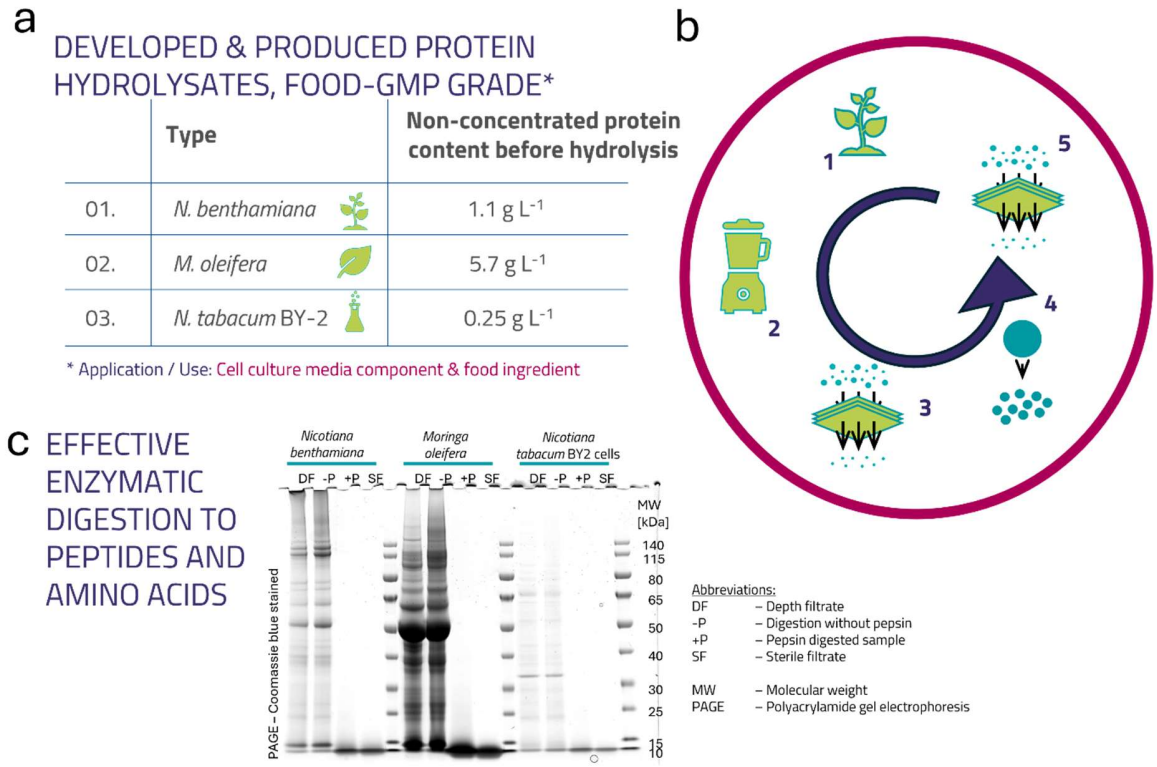

Figure 4: *Developing and producing protein hydrolysates from biotechnology-relevant plants.* ***(a)*** *List of the plants used and non-concentrated protein content prior to hydrolysis.* ***(b)*** *Schematical depiction of the production process.* ***(c)*** *A hydrolysis degree of >90% achieved, as demonstrated via comparison of the digested and non-digested samples on the SDS-PAGE.*

For the production of yeast cell extract and autolysate, a standard in-house protocol was used (see **Yeast cell extracts and autolysate preparation protocol** below). Protein content of *S. cerevisiae* – based autolysate was reproducibly higher than that of *P. pastoris* – based autolysate (**Figure 5 a**). This highlights the need for the method optimization for different yeast species, as the method was originally developed for *S. cerevisiae.* The effectiveness of the autolysis for both species was demonstrated by the SDS-PAGE analysis, showing high autolysis degree (**Figure 5 b**).

a

YEAST CELL EXTRACTS AND AUTOLYSATES

	Species	Protein content after hydrolysis
01.	*S. cerevisiae* Batch 1	11.42 g L^{-1}
02.	*S. cerevisiae* Batch 2	7.2 g L^{-1}
03.	*P. pastoris* Batch 1	0.8 g L^{-1}

b

EFFECTIVE AUTOLYSIS TO PEPTIDES AND AMINO ACIDS

Abbreviations:
S.c. – *Saccharomyces cerevisiae*
P.p. – *Pichia pastoris*
YCE – yeast cell extract
AL – yeast autolysate
B1/B2 – Batch 1 or batch 2
10µg/20µg – Total protein quantity loaded, as determined by BCA

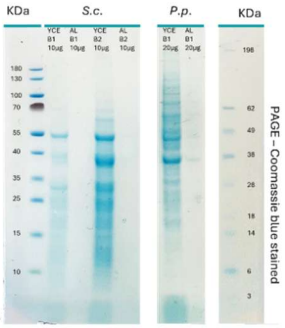

Figure 5: *Production of the yeast autolysates from two yeast species.* ***(a)*** *List of the species and batches produced to compare the inter-species yields and batch-to-batch variations.* ***(c)*** *A high degree of autolysis was achieved, as demonstrated via comparison of the digested and non-digested samples on the SDS-PAGE.*

2.2. Short-term toxicity tests of produced hydrolysates

Short-term toxicity tests were performed, to narrow down the number of lysates to those, which were not only consistently non-toxic in consecutive experiments but also improved cell proliferation rates and cell viability above the rates of the control (DMEM/F12). By screening through a range of concentrations, we identified working concentrations of the non-toxic well performing lysates. Cultivations, assays and media used are described in detail in (Schenzle et al., 2025).

Insignificant toxicity was demonstrated for *S.cerevisiae* autolysates at concentrations above 0.025g/L, with a slight positive effect for the concentrations below 0.025g/L. *P.pastoris* autolysate had a significant positive effect on proliferation of BSCs in the range of 0.01 to 0.05 g/L (**Figure 6 a-d**). Wheat, rice and soy hydrolysates did not seem to have any negative effect on the proliferation of BSCs, and at high concentrations – 5 g/L in case of wheat and 0.2 g/L and 5 g/L in case of soy – exerted a significant proliferation inducing effect (**Figure 6 e-j**). Biotechnologically relevant plants *Moringa oleifera*, *Nicotiana tabacum* and *Nicotiana benthamiana* did not exhibit any negative effects on BSCs proliferation, and *Nicotiana benthamiana* was even significantly advantageous at low concentrations (0.025-0.5 g/L) (**Figure 6 k-m**).

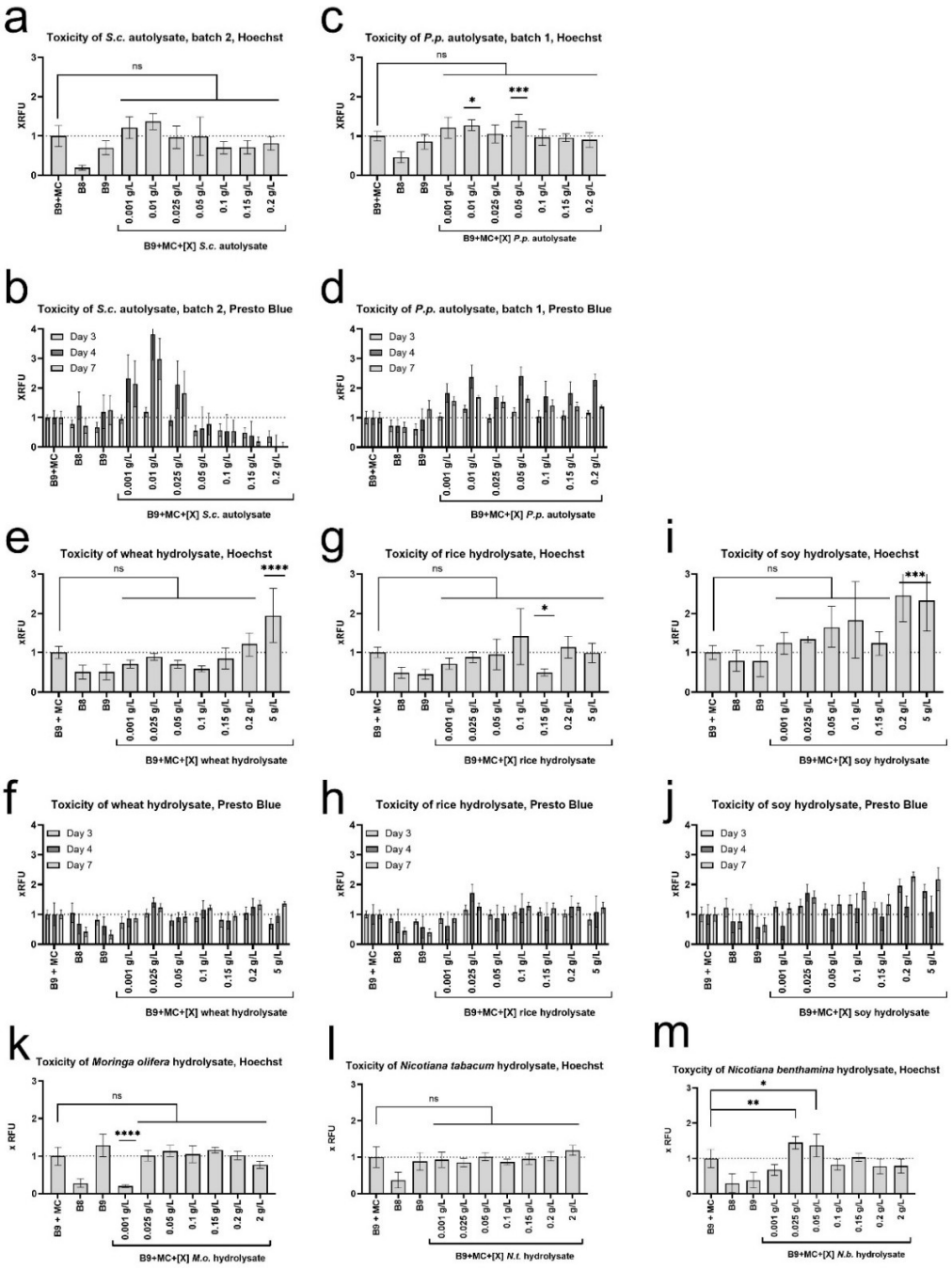

Figure 6: *Short-term toxicity tests of the produced lysates in the serum-free medium B9+MC, using BSCs as reporter cells. 2000 BSCs/cm2 were seeded on day 0 in BSC-GM, and changed on day 1 to the designated medium.* ***(a-d)*** *Yeast autolysates and* ***(e-m)*** *plant hydrolysates in a range of concentrations were supplemented to the B9+MC medium and, as indicated, Presto Blue assay (days 3,4,7) or Hoechst assay (day 7) were performed to assess the viability and proliferation rate of the BSCs. Obtained values were normalized to BSCs in B9+MC. n=6 and repeated twice; statistical significance was calculated by one-way ANOVA combined with Dunnett test for day 7, comparing all samples to B9+MC, and is indicated by asterisks, which are p < 0.05 (*), p <0.01 (**), p < 0.001 (***), p < 0.0001 (****).*

2.3. Short-term tests of hydrolysates-based full substitution of DMEM/F12

In the next step, best performing hydrolysate concentrations of the sourced yeast and plant lysates were combined to identify a combination which could be used as a full substitution of DMEM/F12 with B9 medium supplements (**Figure 7**). Glucose measurement in all yeast and plant hydrolysates has shown a negative result. Thus, a concentration typically used in DMEM was supplemented (17.5 mM). In all yeast and plant hydrolysates the pH was also adjusted to 7.2. B9 and B9+MC, both containing DMEM/F12, were used as controls. Cultivations, assays and media used are described in detail in (Schenzle et al., 2025).

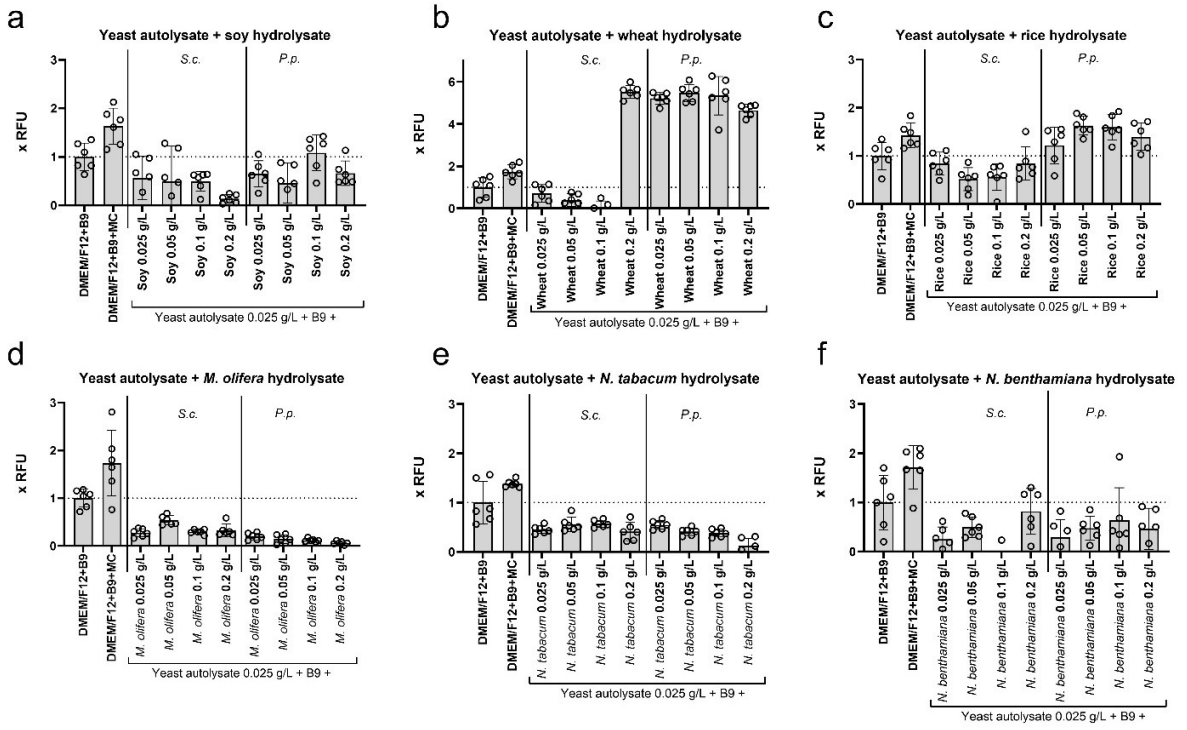

Figure 7: *Full substitution of DMEM/F12 medium with plant and yeast hydrolysates. 2000 BSCs/cm2 were seeded on day 0 in BSC-GM, and changed on day 1 to the designated medium. 0.025 g/L yeast autolysate was chosen as the best working concentration, and plant hydrolysates in a range of concentrations were supplemented to the B9+MC add-on medium without DMEM/F12 and, as indicated, Presto Blue assay (days 3,4,7) or Hoechst assay (day 7) were performed to assess the viability and proliferation rate of the BSCs. Obtained values were normalized to BSCs in DMEM/F12+B9. n=6 and repeated twice.*

0.025 g/L yeast autolysate was chosen as the best working concentration, based on data from **Figure 6**. We show in **Figure 7**, that in a short-term proliferation experiment with BSCs as reporter cells, a combination of 0.025 g/L of *P. pastoris* yeast autolysate and soy, wheat or rice

hydrolysates performed well as a full DMEM/F12 substitute. *Moringa oleifera*, *Nicotiana tabacum* and *Nicotiana benthamiana,* however, were not suitable as a substitute in these combinations. Additionally (data not shown), both yeast autolysates were combined and tested as a full substitute of DMEM/F12, but BSCs had shown slow growth and attachment deficits, which rendered this combination unusable.

2.4. Long-term tests of hydrolysates-based full substitution of DMEM/F12

Best results from the short-term experiments were further validated in long-term proliferation experiments over 6 passages (**Figure 8**).

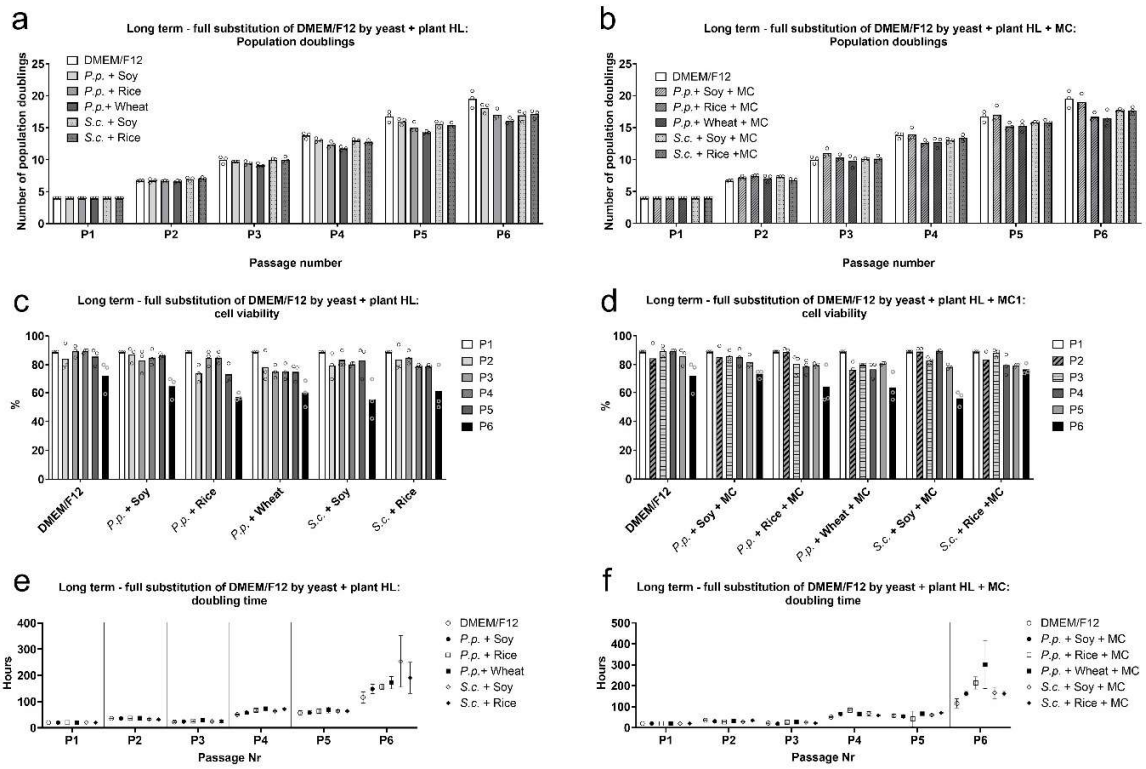

Figure 8: *Long term proliferation experiments using yeast and plant hydrolysates as a full substitution of DMEM/F12. BSCs cells were cultured in 6-well plates in B9 add-on media, using as basal medium either DMEM/F12 or a combination of 0.1 g/L plant hydrolysates combined with 0.025 g/L yeast autolysates. Both hydrolysates were added immediately upon splitting, whereas HSA of the B9 add-on medium was added 24h after the splitting. Average population doublings and doubling time for passage 1 were assessed based on cells count upon isolation of BSCs. Cells were stained with trypan blue and counted upon passaging using Invitrogen Cell Countess, and the number of viable cells* ***(c-d)*** *was used to calculate population doublings* ***(a-b)*** *and doubling time* ***(e-f)****.*

For that, hydrolysates were tested in combinations, which, by our assumption, could compensate their possible bottlenecks. Additionally, a growth factor and cell stabilizer methyl cellulose (MC) addition was tested to assess its effect in combination with alternative basal medium. Cultivations, assays and media used are described in detail in (Schenzle et al., 2025). One of the hydrolysate combinations was performing comparable, though not quite reaching the efficiency of DMEM/F12 in the long run (*P.pastoris* + soy, see **Figure 8 a,c,e**). Its performance could be further slightly improved by addition of stabilizer MC1 (**Figure 8 b,d,f**), still not quite reaching the performance of DMEM/F12.

2.5. Delayed addition of plant hydrolysates enhance viability of BSCs in hydrolysates-based full substitute of DMEM/F12

As the short-term performance of some combined lysates were even exceeding the DMEM/12 level (**Figure 7**), we concluded that the protocol for long-term experiment with such lysates could still be further optimized. For example, plant seeds contain albumin-like molecules (Bérot et al., 2005; Bueno-Díaz et al., 2021; Ntone et al., 2022; Souza, 2020). Such plant albumins were previously shown to constitute a big soluble fraction of the seeds' whole protein isolations (Egger et al., 2025; Stout et al., 2023). And due to similar functionality, they might affect the adhesion capacity of the adherent BSCs when supplemented immediately during plating, as it was demonstrated for Human Serum Albumins (HSA) (Stout et al., 2022). We thus hypothesized that our protein hydrolysates could still contain peptides possessing such a blocking capacity. To test this hypothesis, a long-term proliferation experiment was performed with the supplementation of plant hydrolysates 24 h post splitting.

Addition of plant hydrolysates 24h post splitting had a significant positive effect on cell viability – for soy and especially for rice hydrolysates (**Figure 9 b**). In case of rice hydrolysate this effect led to higher population doublings, but not in case of soy (**Figure 9 a**). Cell morphology upon differentiation (**Figure 9 e**), as well as differentiation capacity, as assessed via fusion index (**Figure 9 d**), were unchanged in the samples grown in fully substituted basal medium, as compared to DMEM/F12.

We thus conclude that plant albumin peptides, present in plant hydrolysates, might have indeed affected cell adhesion. Still, a 24 h delay in addition of plant hydrolysates might be too long, and further effort should be made to optimize the addition of plant hydrolysates as a part of basal medium.

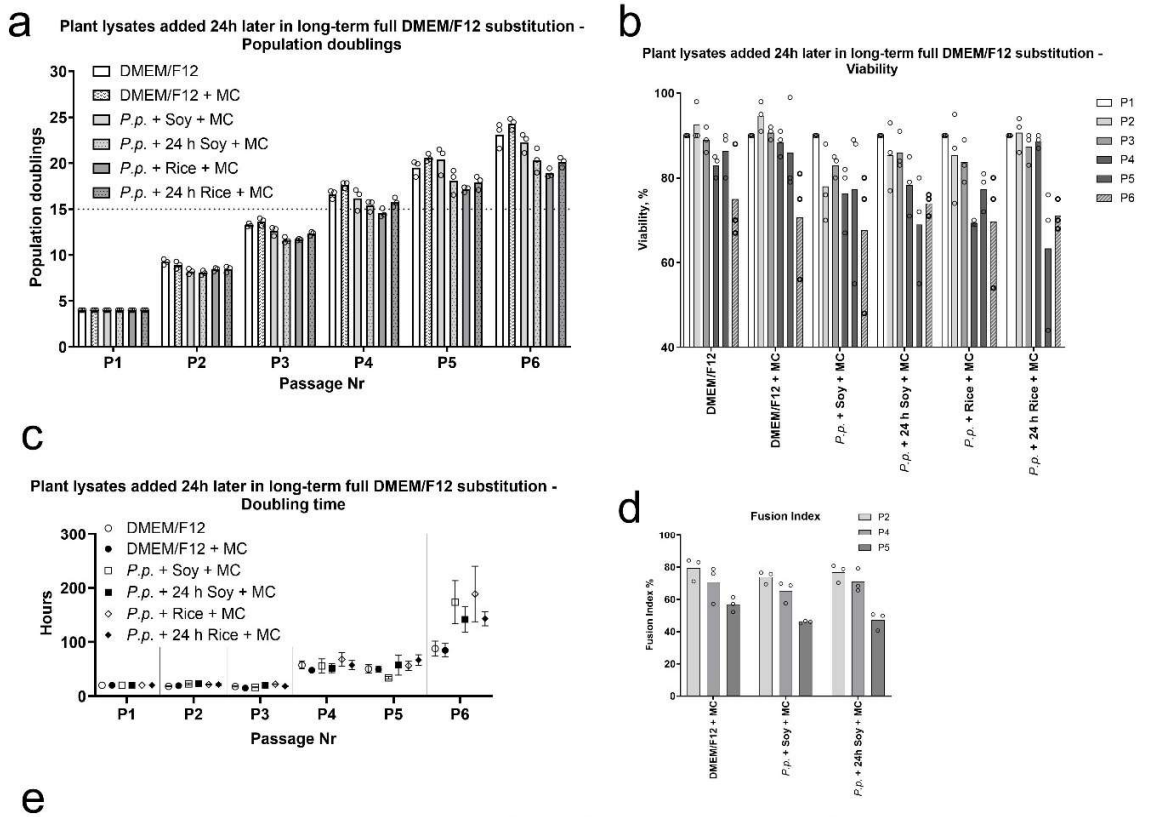
a
Plant lysates added 24h later in long-term full DMEM/F12 substitution - Population doublings
DMEM/F12
DMEM/F12 + MC
P.p. + Soy + MC
P.p. + 24 h Soy + MC
P.p. + Rice + MC
P.p. + 24 h Rice + MC
Population doublings
Passage Nr
P1
P2
P3
P4
P5
P6
b
Plant lysates added 24h later in long-term full DMEM/F12 substitution - Viability
Viability, %
DMEM/F12
DMEM/F12 + MC
P.p. + Soy + MC
P.p. + 24 h Soy + MC
P.p. + Rice + MC
P.p. + 24 h Rice + MC
c
Plant lysates added 24h later in long-term full DMEM/F12 substitution - Doubling time
Hours
Passage Nr
d
Fusion Index
Fusion Index %
e
DMEM/F12 + MC
P.pastoris + Soy + MC
P.pastoris + 24h Soy + MC
P2
P4
P5

Figure 9: *24h delay in addition of plant hydrolysates during long-term proliferation in yeast- and plant-based basal medium. BSCs cells were cultured in 6-well plates in B9 add-on media, using as basal medium either DMEM/F12 or a combination of 0.1 g/L soy hydrolysate combined with 0.025 g/L P.p. autolysate. Plant hydrolysates were added either immediately upon splitting or, where stated, 24h later. HSA (as a part of B9 add-on medium) was added 24h after the splitting. Average population doublings and doubling time for passage 1 were assessed based on cells count upon isolation of BSCs. Cells were stained with trypan blue and counted upon passaging using Invitrogen Cell Countess, and the number of viable cells* ***(b)*** *was used to calculate population doublings* ***(a)*** *and doubling time* ***(c)****. Upon each passage, part of the cells was seeded for differentiation and IF staining* ***(e)*** *and fusion indices were calculated based on the ratio of cells with at least two nuclei to all nuclei. A range of 150-250 nuclei were counted for each image, with three images processed per sample. The DAPI count was performed by the Zeiss Zen tool for cell counting. Immunofluorescence staining was performed for nuclei (DAPI, blue) and desmin (green). Scale bar = 100 µm. Microscope Zeis Axio Imager. Lens: EC Plan-Neofluar 10x; Exposure DAPI: 20 ms.*

2.6. Content analysis of the hydrolysates-based full substitute of DMEM/F12

Further, *P. pastoris* and *S. cerevisiae* autolysate and soy hydrolysate compositions of amino acids, vitamins, sugars and minerals were analysed and compared to DMEM/F12. Possible bottlenecks in amino acid compositions were marked, based on the list of bovine essential amino acids (marked with red E) (Kim & Lee, 2021).

We show that batch-to-batch variations are quite substantial (approx. 30-40%) after the laboratory scale autohydrolysis of the *S. cerevisiae* yeast samples (**Figure 10 a**), demonstrating the need for method optimization and standardisation. Amino acid concentrations of the optimal working combination (0.1 g/L soy hydrolysate combined with 0.025 g/L *P.p.* autolysate) appeared to be in general much lower than used in DMEM/F12 composition (**Figure 10 b**). Still, all essential bovine amino acids were present in both *P. pastoris* and *S. cerevisiae* autolysates, as well as in the combination of 0.1 g/L soy hydrolysate with 0.025 g/L *P.p.* autolysate (**Figure 11**).

Amino acid concentrations in *S. cerevisiae* autolysates, which performed poorly in the full basal medium substitution, were substantially higher than in *P. pastoris* autolysate, which performed better (**Figure 7**). Additionally, the concentrations of the amino acids in the waste medium were not diminished, but higher than in the fresh medium (**Figure 11**). Part of this effect will be due to the quite substantial evaporation during the cultivation in 6-well plates. Still, we can conclude that lower amino acid concentrations in the alternative basal medium, as well as depletion during cultivation are not among the critical bottlenecks of the alternative basal medium. This conclusion supports previous findings, where the concentration of the amino acids is only very slowly depleted, especially at the low cell density (O'Neill et al., 2022).

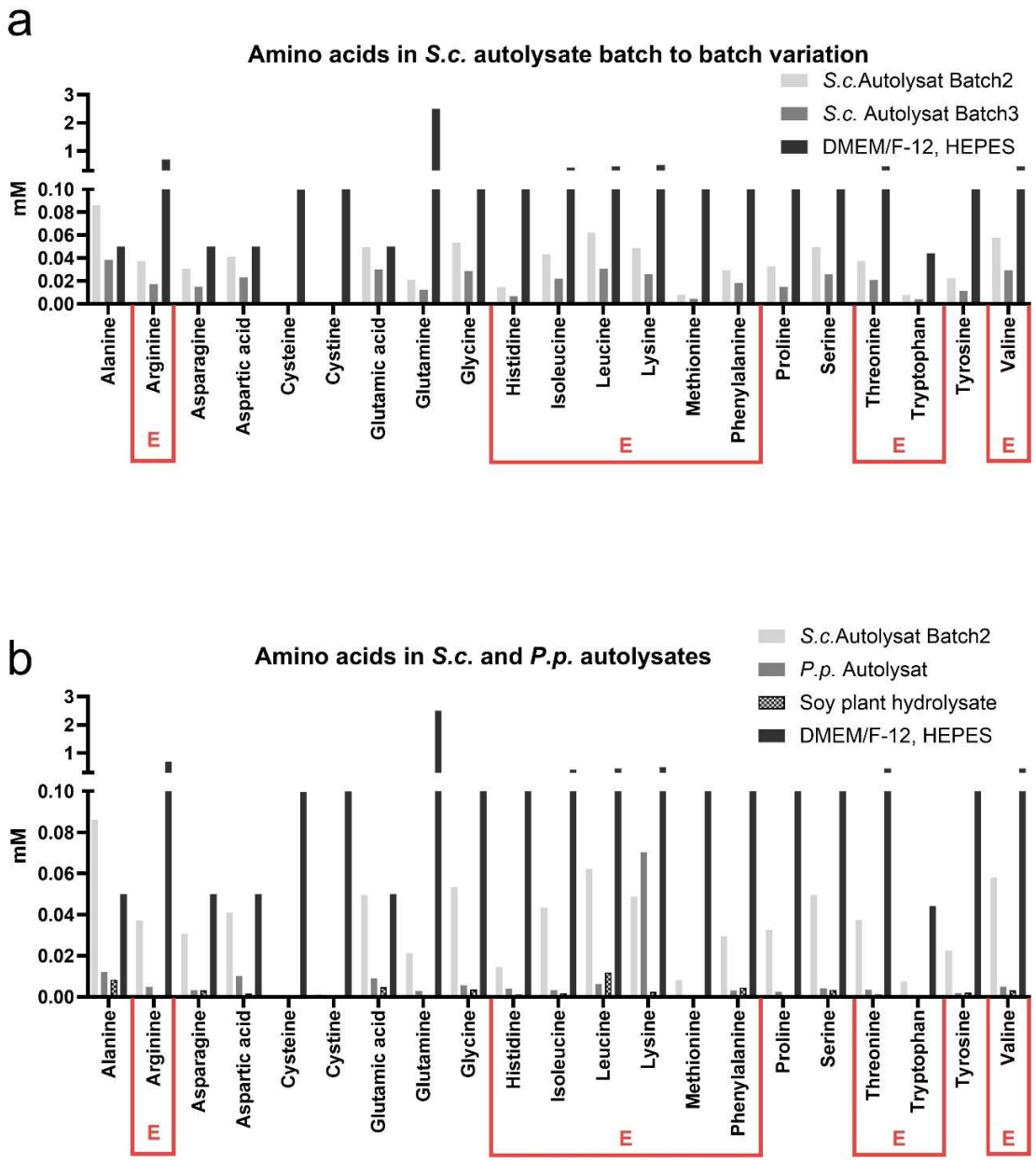

Figure 10: *Comparison of several yeast autolysate preparations of Saccharomyces cerevisiae from Sudhaus Graz and in-house strain of Pichia pastoris. Essential bovine amino acids are marked with red "E".* ***(a)*** *Comparison of two S.c. autolysates to assess batch-to-batch variations.* ***(b)*** *Comparison of S.c. and P.p. autolysates with soy hydrolysate. DMEM/F12 contents are plotted as a control. Analysed concentrations were adapted to reflect the best working concentrations in the cell culture media (0.1 g/L soy hydrolysate combined with 0.025 g/L P.p. autolysate).*

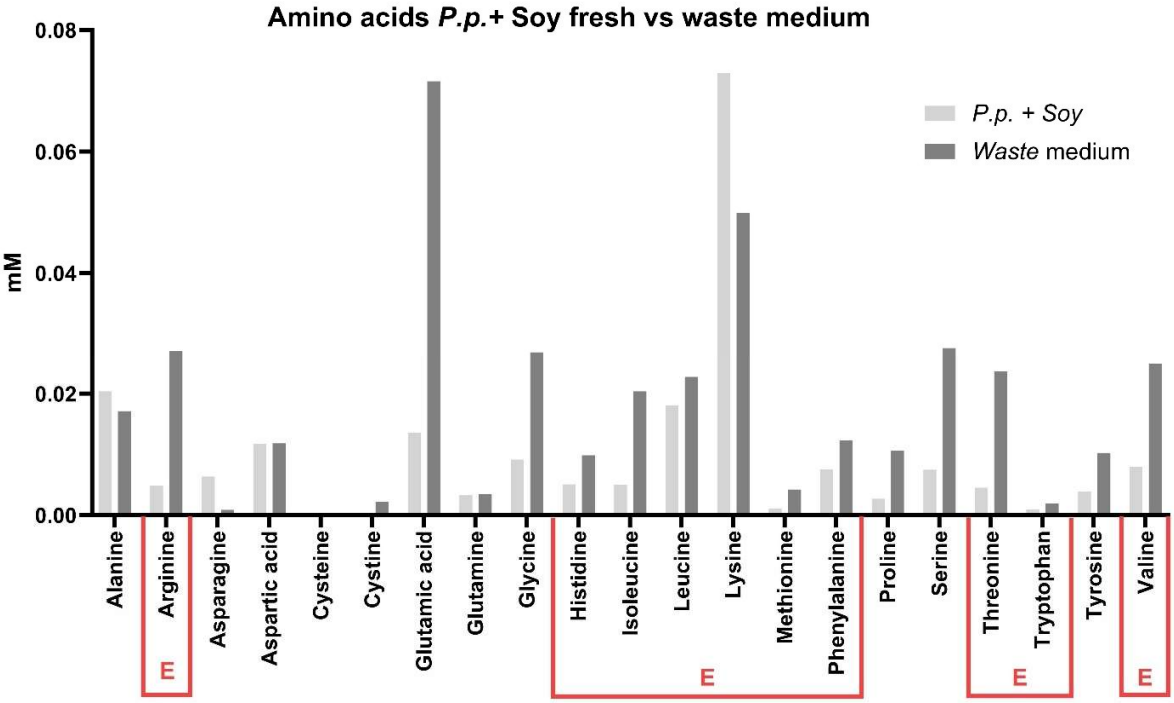

Figure 11: *Comparison of the amino acid content in fresh alternative basal medium and waste alternative basal medium. Essential bovine amino acids are marked with red "E". Analysed concentrations were adapted to reflect the best working concentrations in the cell culture media (0.1 g/L soy hydrolysate combined with 0.025 g/L P.p. autolysate).*

Based on the performed analysis (**Figure 10 b**), following amino acids were identified as possible bottlenecks for *S.c.* autolysate, due to much lower concentrations as compared to DMEM/F12:

- Arg, Met, Trp (Essential)
- Cys, Gln (Non-essential)

Following amino acids were identified as possible bottlenecks for *P.p.* autolysate (same criteria):

- Arg, His, Met, Trp (Essential)
- Cys, Gln (Non-essential)

In attempt to simplify the alternative basal medium even more, these amino acids were supplemented to the corresponding media, containing only either *S.c.* or *P.p.* autolysate as a basal media alternative. Upon supplementation yeast autolysates performed only slightly better as compared to the DMEM/F12 in short-term experiments (data not shown), once again supporting the conclusion that amino acid composition is not the bottleneck of the composition.

Vitamin content in the yeast cell extracts, in yeast autolysates and in soy hydrolysate were analysed and compared to DMEM/F12 concentrations (**Table 10**). Biotin was not detected in any of the lysates, and most yeast strains do not produce biotin at all or only in marginal quantities (Bracher et al., 2017). Plants on the other hand, can produce biotin (Pinon et al., 2005), and although it was not detected, that could be due to its' detection threshold being extremely low. Luckily, the required biotin concentrations are also extremely. Due to that, biotin might be the reason why yeast autolysates alone were not able to sustain the proliferation of BSCs. Low concentrations of pyridoxal (Vitamin B6) could be another bottleneck in the alternative basal medium, as it is needed in quite high concentrations (**Table 10**).

On the other hand, some vitamins (nicotinamide, panthotenic acid, thiamine) were present in very high concentrations, especially in *S.c.* autolysate, which could potentially be detrimental to BSCs growth and viability (**Table 10**).

Table 10: *Vitamin contents of the yeast cell extract, yeast autolysate and soy hydrolysate compared to DMEM/F12. Analysed concentrations were adapted to reflect the best working concentrations in the cell culture media (0.1 g/L soy hydrolysate combined with 0.025 g/L P.p. autolysate). Blue marked are the missing vitamins, red marked are vitamins with very high concentrations.*

Vitamins, µM	*S.c.* cell extract Batch2	*S.c.* Autolysate Batch2	*S.c.* cell extract Batch3	*S.c.* Autolysate Batch3	*P.p.* cell extract Batch1	*P.p.* Autolysate Batch1	Soy plant hydrolysate	DMEM/F-12, HEPES
4-Aminobenzoic acid	0,0000	0,0006	0,0000	0,0000	0,0008	0,0000	0,0018	0
Biotin	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	1.4344263E-5
Choline	0,0529	0,6716	0,3449	0,3756	0,0086	0,0480	0,3549	0,0641
Cyanocobalamin	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0047
Folic acid	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0060
Niacin	0,2334	0,2420	0,2780	0,2938	0,0038	0,2343	0,0084	0,0166
Nicotinamide	0,0000	0,4115	0,0000	0,0000	0,0470	0,0318	0,0110	0,0097
Panthotenic acid	0,0032	0,0453	0,0174	0,0198	0,0038	0,0215	0,0010	5.824468E-4
Pyridoxal	0,0000	0,0004	0,0012	0,0009	0,0002	0,0000	0,0000	0,0064

Pyridoxamine	0,0008	0,0205	0,0112	0,0119	0,0007	0,0033	0,0000	5.0184503E-4
Pyridoxine	0,0029	0,0009	0,0027	0,0031	0,0000	0,0000	0,0000	0,07
Riboflavin	0,0000	0,0132	0,0000	0,0000	0,0016	0,0000	0,0000	0
Thiamine	0,0024	0,0676	0,0207	0,0234	0,0032	0,0149	0,0020	0

Contamination with heavy metals (arsenic, cadmium, mercury, lead, etc.) in these samples was not detected, but caution should be taken when new biomass source or extraction method is being used.

3.Conclusions and future perspectives

We conclude that DMEM/F12 and an alternative basal medium (consisting of 0.1 g/L soy hydrolysate combined with 0.025 g/L *P.pastoris* autolysate, supplemented with glucose and additionally stabilized by 0.1 g/L methyl cellulose) perform equally good in supporting the proliferation and differentiation of BSCs in short- and long-term experiments. Such basal medium substitute can be produced in industrial quantities, as the technology scale-up was already performed: NaTurtle soy hydrolysate can be readily produced in annual quantities of 100.000 kg, and assessed price of 40€/kg. For the production of *P. pastoris* autolysate, the same method and facilities can be used as are existing for the production of baker's yeast extract, with an estimated price equal to the *S.c.* yeast extract of 40€/kg. With the given concentrations (0.1 g/L soy hydrolysate combined with 0.025 g/L *P.pastoris* autolysate and 0.1 g/L methyl cellulose) the price contribution for the alternative basal medium is estimated to around 7 cent/L, whereas standard DMEM/F12 price is around 25€/L bulk.

Additionally, local production and usage of such alternative basal medium instead of DMEM/F12 would minimize the logistic and environmental footprint of the basal media. The input of this case study will feed to Tasks 6.1 (**Prospective Life Cycle Assessment**) and 6.2 (**Techno-economic assessment & Life Cycle Cost Analysis**) in order to calculate actual environmental advantage of such DMEM/F12 substitution. Further analysis and initial draft of the original research paper based on these data is in preparation.

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Yeast cell extracts and autolysate preparation protocol

Preparation/sourcing of the cell mass samples

- Cells to be used
    - Either fresh, cooled at 4°C, preparation right upon sample receipt
    - Or an overnight culture in 50 mL YPD, 130 rpm, 28°C, 250 mL buffled shake flask
- Fresh sample was washed 3x with Fresenius water (centrifuged 3x 150000 rpm)

Yeast cell extract preparation using Merckenschlager:

- Precool Merckenschlager vessels
- Harvest cells with centrifugation at 5000 rpm for 5 min
- Wash once with ddH2O to remove media residues
- Resuspend between 2g and 10g cells (CWW) in 25 mL ddH2O
- Fill Merckenschlager vessels with 1/3 glass beads, 1/3 cell suspension (ca. 25-30 mL) and 1/3 air
- Disrupt for 3 min, cool for 3-5s in the beginning and every 30s afterwards
- Separate cell debris and non-lysed cells via centrifugation (3x 150000 rpm), then collect the supernatant and store at -80°C,
- The glass beads can be washed and reused

Yeast autolysate preparation:

- 5 g cell wet weight in 250 mL buffled shake flasks in 50 mL Fresenius water
- Transfer to the 50°C incubator, 130 rpm shake plate for 50h
- Centrifuge 3x (150000 rpm), store at -80°C

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