Title: Towards cost-effective and sustainable media formulations for 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 but also identified challenges yet to be solved. 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 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.

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1. Introduction

Meat and seafood have both played crucial roles in human evolution and are important components of a healthy and well-balanced diet due to its nutritional richness (Pereira and Vicente 2013). The world's population continues to grow, thus requiring more consistent protein supply for an increased global consumption of animal-derived protein. Despite the availability of plant-based protein alternatives, animal meat remains a popular, high-quality protein source (Shaikh et al. 2021). Meat production needs to nearly double by 2050 to meet growing global demand for animal proteins. But the way the world currently produces meat cannot meet this demand and still achieve global climate, food security, public health, and biodiversity goals (Battle et al. 2024).

Cultured meat (CM) and seafood (CSF) is produced by cultivating animal cells *in vitro* (Elliot Swartz 2021). This might involve the isolation of a few cells from living farm animals through a biopsy, followed by their expansion in bioreactors to produce a substantial biomass (Levi et al. 2022). 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). To create a muscle-like tissue product, cells can be cultured on an edible scaffold that structurally and biologically supports their assembly and maturation. Additional processing steps can be further applied to better mimic the flavour, texture, and nutritional values of traditional meat (Levi et al. 2022).

Despite the increasing number of companies focussing on CM or CSF - at least 156 in 2024, more 107 companies since 2021, there are still major challenges yet to be solved, such as the high costs associated with manufacturing and gaining consumer acceptance (Battle et al. 2024). Furthermore, to

reach the full potential of CM/CSF technologies, significant technological challenges need to be addressed, such as **cell line procurement**, **efficient, sustainable and cost-effective serum free (SF) cell culture media**, CM/CSF-aimed bioprocessing design including bioreactor design, and scaffolding technology (Levi et al. 2022; Battle et al. 2024).

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). Adipocytes make up most of the fat tissue volume and 95% of their entire cell body is composed of one large lipid droplet, containing triglycerides (Puri and Czech 2008; Fujimoto and Parton 2011; Mitić et al. 2023a). Fat has been reported as the most important contributor to meat flavour across species (Wood et al. 2008; Webb and O'Neill 2008; Arshad et al. 2018), and will most probably be part of the first CM hybrid products reaching the market in Europe (Mosa Meat 2025). In this review we will therefore focus on the SF medium requirements for the proliferation and differentiation of muscle, fat and other cells which can be sources for CM/CSF products.

Growing cells *ex vivo* in bioreactors requires cell culture media to provide the same fundamental compounds as required *in vivo:* a mixture of a carbon-based energy source (e.g. glucose), amino acids, salts, vitamins, water, and other components to support cell viability and vitality (Elliot Swartz 2021). Once adequate cell quantities are reached, differentiation into muscle or fat cells can be triggered by changing the medium composition (Martins et al. 2024). The differentiated cells are then harvested, prepared, and packaged into final products.

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 as primary cells or 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 (Laura Pasitka et al. 2024). Thus, **the selection/isolation of the appropriate cell line(s) will impact the choice and composition of cell culture media**, as discussed in more detail below. Cell lines are extensively reviewed by Rosa et al. for aquatic cell lines and by Schiavo et al. for terrestrial cell lines in this volume, also providing an overview of standard methods for cell line isolation and handling as an SOP Database.

This review aims to evaluate **non-animal and cost-effective alternatives to animal serum-based** cell culture media for CM/CSF production as the field still faces challenges around scale-up and cost reduction. Cell culture medium – an essential input for all CM and CSF productions - accounts for 55%–90% of production costs (Gomez Romero and Boyle 2023). Significant cost savings are predicted from using animal SF media, food-grade raw materials and good manufacturing practices (GMP) (Specht 2020). 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 (GFI, state of the industry report, 2022). We further review possible strategies which may also contribute to a cost reduction for cell culture media and discuss regulatory issues and key challenges that remain to be addressed.

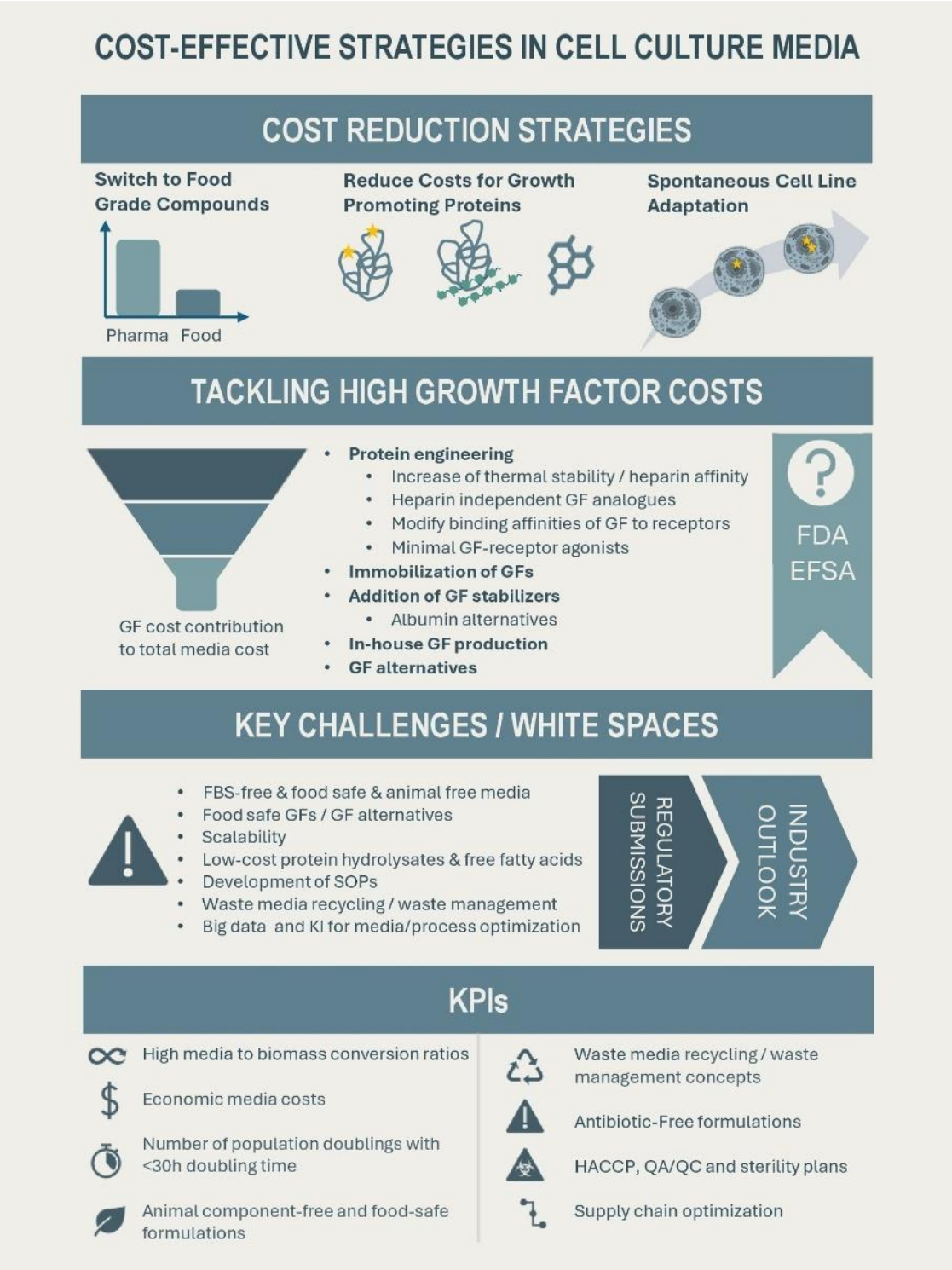

Figure 1: Infographic summarizing topics discussed in the review.

2. History of serum free cell culture media development

Cell cultures have many clinical applications in medicine, such as the production of vaccines, diagnostic tests, and cell therapy (Hawkes 2015). 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 cell attachment, proliferation, and maintenance, such as serum albumin, fetuin, hormones, vitamins, trace elements, 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 (Hawkes 2015; Gagnieur et al. 2014), 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 (Shuai Liu 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, Stewart, and Weaver 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 and 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, transforming growth factor b3, and neuregulin 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, Robertson, and Suzuki 2021), no medium optimization efforts were reported so far for edible species, except for porcine ESCs and iPSCs (Ma et al. 2018; H. Choi et al. 2024, 20).

For aquatic species, this journey is only starting, 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) showed a significantly enhanced proliferation and extracellular matrix mineralization compared to the FBS control (Rosa et al. 2010). Also, **several embryonic stem cells** require fish embryo extracts (e.g., (Dash et al. 2010; Buonocore et al. 2006) 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 (S. -L. Chen et al. 2003; 2003; Bejar, Hong, and Alvarez 2002; S.-L. Chen, Sha, and Ye 2003). Similarly, **goldfish muscle cells** (N. Li, Guo, and Guo 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 orthologue GFs share 90 to 98% protein sequence identity, allowing cell culture of human cells in bovine serum, the protein identity between fish and human orthologue GFs, as well as between orthologue GFs of some fish species, is in the range of 77% (Yuan and Hong 2017), which may hamper the efficient proliferation of many types of fish cells in FBS or in fish serum from distant species. This highlights the importance of species-specific signalling molecules – e.g. growth factors, hormones - present in fish serum or extracts, which are better suited to support fish cells within the same taxonomic group, as compared to FBS. It should be noted that fish serum/extracts exhibit some of the key 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 growth factors,** critical for different developmental stages of fish cells, before their dependence on FBS and fish extracts can be eliminated.

Given the high specific demand of 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.

One **potential alternative** to the necessity of medium optimization for every cell line is to apply the **spontaneous adaptation** approach to cell lines for 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 and Fent 1998; Brunner 2010; Radošević et al. 2016; Yue et al. 2016; C.-L. Liu et al. 2017). Same approach of was successfully used to reduce the FBS content from 20% to as low as 2.5% for spontaneously immortalized mackerel muscle cell line Mack1 (Lim et al. 2024). It was recently successfully used to **spontaneously immortalize chicken fibroblasts**, which also possessed the ability

to activate adipogenesis (L. 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 2).

These studies offer **valuable methodologies** that can be used for the development of SF media for other adapted meat- and seafood-relevant cell lines. While attractive from the 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 and Mulvaney 1999). Additionally, relying on good adaptation of cell lines from bigger animals could be too optimistic. Indeed, the **spontaneous immortalization of mammalian cells has been negatively correlated with species body mass** (Perillo et al. 2023), which makes this method more applicable to small animals. This and other methods for cell line development are reviewed in extensive detail by Rosa et al. for aquatic cell lines and by Schiavo et al. for terrestrial cell lines in this volume.

3. Media compositions from cell isolation to maturation

The process of CM/CSF production can be divided into four phases, namely: (i) cell isolation; (ii) cell proliferation; (iii) cell differentiation into skeletal muscle cells and fibres, or adipocytes and (iv) assembly of the final meat or fish product.

The successful cell culture of animal cells *ex vivo* using an artificial environment and controlled conditions requires an appropriate medium to support cell survival and growth. Thus, the **choice of cell type and final product will greatly influence the media requirements**, and, consequently, the **price** of the media, as evidently shown in Table 2 and Table 3. Medium composition also depends on the cell **culture stage**, as cell requirements are very different during the proliferation and differentiation phases (Sophie Hubalek, Post, and Moutsatsou 2022). Cell culture media generally comprise an appropriate source of energy and compounds which regulate the cell cycle. A typical culture medium is composed of 1) basal medium containing amino acids, vitamins, inorganic salts, carbohydrates, buffering system and of 2) cell line specific GFs, hormones, and attachment factors (Chaudhry, Bowen, and Piret 2009). For the extensive list of possible components, including available safety information, see also the catalogue “Components in media designed for CM/CSF production” on FEASTS website.

Essential nutrients as well as their concentrations need to be adapted to the individual demands of cell types and species. A non-adapted medium-composition profile can cause inefficient nutrient uptake, leading to inefficient metabolism of carbon and nitrogen, often accompanied by an overproduction of metabolic waste products such as lactate and ammonium (Sophie Hubalek, Post, and Moutsatsou 2022; S. Hubalek et al. 2023). Moreover, it is important that the final formulation meets the boundary requirements, namely simplicity, cost-efficiency, food-safety and scalability (Kolkmann et al. 2022).

Although it does not meet the last of the named requirements – scalability – the selection of an appropriate commercially available basal medium is a good starting point. Chen et al. performed a comparative analysis of 12 different basal media and identified DMEM/F12 as the best choice for human cells (G. Chen et al. 2011), which has also found wide acceptance in CM area (see Table 2). DMEM/F12 is a 1:1 mixture of DMEM and Ham's F12 media that uses sodium bicarbonate as buffering system and therefore requires a 5-10% CO_2 environment to maintain physiological pH. HEPES is often included in the formulation at a final concentration of 15 mM to compensate for the loss of buffering capacity incurred from the elimination of animal serum (Arora 2023).

For fish cells, Leibovitz's L-15 is widely used for a diverse range of cell types and species. Recent studies revealed that cell densities of fish primary SCs in F10 and DMEM/F12 were markedly higher than those in DMEM, 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 (Lakra, Swaminathan, and Joy 2011; Gardell et al. 2014; Solhaug et al. 2024).

Contaminations by microbes can be hazards to mammalian cell culture systems, especially in research environments. Supplementing growth media with antimicrobial agents such as antibiotics and antifungals generally provide an effective method for eliminating contaminating cells. However, antibiotics are biologically active and exhibit concentration-dependent effects on mammalian cells (Hassan, 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 along with a sterile cell culture environment and equipment, as it was 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 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 1, mammalian cells were suspended in basal medium containing **10 % FBS** and 10% (vol/vol) dimethyl sulfoxide (DMSO) (Rønning 2019; X. Z. Li et al. 2019; Vaughn, Phelps, and Gonzalez 2018) or frozen in FBS with 10% DMSO (S. Ding et al. 2018; Mehta, Theunissen, and Post 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 improves cell survival during the freezing and thawing (Verhoeye et al. 2001).

Cryopreservation of **fish-derived cells** utilizes methods and reagents originally developed for mammalian cells. A standard formulation contains 10% DMSO and 10%–50% FBS, with 20% FBS emerging as the optimal concentration, striking a balance between cryoprotection and cellular recovery (Yashwanth et al. 2023; Goswami et al. 2023; Zhengshan Zhao and 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, more sensitive cells may benefit from lower DMSO levels, such as 5%, paired with up to 25% FBS (Dubey et al. 2015). In addition to DMSO, glycerol also serves as an effective alternative or complement, typically utilized at concentrations of 5%–10%, as reported in older publications (Middlebrooks, Ellender, and Wharton 1979). These adaptations highlight the importance of tailoring cryopreservation protocols to the unique needs of individual fish cell lines, ensuring optimal post-thaw viability and functionality.

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 and 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 FGF-2 (5 ng/mL) 1% penicillin and streptomycin	(S. S. Ahmad et al. 2024)
Porcine SCs	DMEM	10% FBS 2% porcine serum penicillin (100U/ mL) streptomycin (100 µg /mL) gentamicin (20 µg /mL)	(Vaughn, Phelps, and Gonzalez 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, Theunissen, and Post 2019)
Fish SCs	L-15	20% FBS Human FGF (rec; 1ng/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 Glu 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 1: Media used for cell isolation after biopsy.

3.2. Proliferation

3.2.1. Serum free proliferation media for muscle cell progenitors of terrestrial species

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.

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 has been demonstrated to work well for expansion and prolonged maintenance of stem cells without triggering differentiation, and its composition is publicly available (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 2).

Info box 1: There is **NO universal** chemically defined medium applicable to all cell types and species.

- ⇨ **Choice of cell type/species** determines composition of culture media
- ⇨ **Adaption to individual cellular demands** for long-term growth and viability essential
- ⇨ **Identification of species-specific signaling molecules** required for maximal growth promoting effect

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 2). Observations of the authors and communications with other researchers led to the assumption 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. 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.

Bovine satellite cells (BSCs) isolated from a 2-week-old calf proliferated only for 3 days in SF **B8** medium, indicating that a complete substitution of serum by B8 compounds did not meet the requirements of BSCs (Stout et al. 2022). In this study, some of the serum-related functions could be replaced with **recombinant human serum albumin (rHSA),** which restored expansion of BSCs over at least seven passages, 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 2) due to its component number and design towards bovine muscle cell culture. Increasing the concentration of albumin in B9 improved growth over one month of cell expansion, with total doublings exceeding 19

and 20 for B9 containing 3.2 and 6.4 mg/mL albumin, respectively (compared with 18 total doublings for the original B9 containing 0.8 mg/mL albumin) (Stout et al. 2022). This line of research – stabilization of media – is further discussed below in the chapter 4.4.4.

Kolkmann et al. successfully developed another SF and chemically defined medium for **BSCs** which supported exponential cell growth up to 97% of the 20 % FBS containing golden standard growth medium (Kolkmann et al. 2022). The authors performed a 5-step optimization process which started with the substitution of serum with single proteins based on stem cell literature (Kolkmann et al. 2022). A relatively rich mix of components was used, including one basal medium, five of the most abundant components in serum, at least one mitogenic GF and at least two attachment factors. In step 2, the substitution of certain components with their animal-free homologues as well as the possibility to exclude some of them was evaluated in order to tailor it towards a cultured meat production application. In step 3, a two-level full factorial design of experiments was applied, to identify the optimal combination of GFs associated with satellite cell proliferation. In step 4, the concentration of the high-price components was optimized, and the most effective formulation was finally tested in step 5 as for its long-term performance over six passages and ability to maintain the cell differentiation capacity (Kolkmann et al. 2022). Components added to DMEM/F12 are listed in Table 2, with 20% of the total medium price attributed to HSA, approx. 60% to fibronectin (cell adhesion agent), and IL-6, HGF and PDGF as most costly GFs.

Skrivergaard et al. developed a SF-medium containing less components compared to the medium published by Kolkmann et al. (Kolkmann et al. 2022; Skrivergaard, Young, et al. 2023). Their complex screening-type DOE setup showed that only three main components, namely fetuin, BSA and FGF-2, are essential for the proliferation of bovine satellite cells (**BSCs**). Fetuin is a serum carrier protein like albumin, with multiple important biological functions (e.g. regulation of calcium metabolism, insulin signaling), and it is prevalent in the fetal serum instead of serum albumin, whereas albumin is the predominant protein in serum of adult mammalians (Chekol Abebe et al. 2022). Fetuin had much higher proliferative effect on BSCs than BSA, when 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). An additional supplementation with ITS (insulin-transferrin-selenium) resulted in significant positive effects on the proliferation of BSCs over a 5-day period compared to the 10% FBS-containing control. The so-called Tri-basal 2.0+ medium is using only six components - **FGF-2, fetuin, BSA and ITS** - to supplement the DMEM/F12 basal medium, but costs for fetuin accounts for approximately 80 % of this medium formulation (see Table 2) (Skrivergaard, Young, et al. 2023). This medium also enhanced proliferation of porcine and murine muscle cells. The inclusion of **PDGF** (5 ng/mL) showed further positive effect in 5-day proliferation assay with SCs of all three species compared with Tri-basal 2.0+, whereas lipid concentrate, and linoleic acid decreased proliferation (Skrivergaard, Young, et al. 2023). PDGF is an important mitogen that was shown to promote human MSCs proliferation when being supplemented in combination with FGF-2 and TGF-β1 (F. Ng et al. 2008).

The addition of **HGF** (20 ng/mL) to Tri-basal 2.0+ significantly enhanced long-term performance of BSCs in a multi-passage live assay. The hepatocyte growth factor HGF is a key factor that regulates cell proliferation activity of satellite cells in a dose-dependent manner (Yamada et al. 2010). HGF was further shown to act synergistically with FGF-2 or PDGF (Syverud, VanDusen, and Larkin 2016). In **Tri-**

basal 2.0+ medium with HGF, BSCs proliferated for **19 days** through 3 passages without trypsin inactivation or centrifugation to a much higher extent than in 10% FBS (Skrivergaard, Young, et al. 2023).

It is obvious that the costs for fetuin, albumin and fibronectin are at a restricting height. Upscaling will certainly reduce the production price, but other strategies are also required to alleviate the burden on CM/SCF production. Recent evaluations of the costs and volumes of recombinant proteins necessary for cost-competitive CM production suggest that 96.6% of the production volume is anticipated to be derived from albumins, with slightly less than 4% consisting of transferrin, insulin, growth factors, and other proteins. Based on these assumptions, producing CM that accounts for 1% of the global meat market would necessitate millions of kilograms of albumin, far exceeding the current production volumes of many recombinant industrial enzymes as well as serum-derived albumins (E. Swartz 2023). Also, fetuin and fibronectin will be required at very high amounts, which would lead to the same bottleneck.

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

Although research on SF medium formulations for **fish muscle cells** is in its infancy compared to mammalian systems, 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 growth factors**. 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.

In this line, **embryonic fish stem cells** require basic fibroblast growth factor (FGF-2) and leukaemia inhibitory factor (LIF) (Dash et al. 2010; Sha et al. 2010; S.-L. Chen, Sha, and Ye 2003; S. -L. Chen et al. 2003), 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, Sabin, and Paboeuf 2010; Kong et al. 2021; Krishnan et al. 2023). IGF-1 promoted proliferation of Catmus1PFR myofibroblast precursors, but not of brown **bullhead catfish (*Ameiurus nebulosus*) fibroblasts** (Cyrino and Mulvaney 1999) nor of continuous 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, Sabin, and Paboeuf 2010). Some **fibroblast cultures** rely on FGF-2 and epidermal growth factor (EGF), additionally to FBS (Rougée et al. 2007; Zhao1 et al. 2004; L. Zhao et al. 2020). 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.

Beyond the GFs mentioned, additional candidates could be explored for their potential to enhance the proliferation of fish cell lines. A recent review listed some of 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). Authors listed several proliferative factors - such as myostatin inhibitors, IGF

mimics, and the adenylate cyclase activator forskolin - that could be tested to improve these processes *in vitro*. IGF-2 could also be tested in combination with other cytokines, as medaka IGF-2 (*Oryzias latipes*) and its human orthologue were able to support viability and pluripotency of medaka ESCs in culture media containing only basic medium (Yuan and Hong 2017). The morphology of the ESCs cultured in basic 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.

Another interesting study has explored non-animal-derived alternatives to reduce animal serum dependency. For instance, Dong et al. (Dong et al. 2024) evaluated 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 growth factors. 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 (Wirawan et al. 2012; ShiZuo Liu et al. 2023).

To further address the remaining 5% FBS requirement, Dong et al. (Dong et al. 2024) screened a range of additives, including antioxidants, glucocorticoids, and an ITS-based supplementation. Through this screening, they identified L-ascorbic acid, a vitamin with antioxidants properties (Gęgotek and Skrzydlewska 2022), and ITSE (insulin-transferrin-selenium-ethanolamine) as effective components at optimal concentrations of 10 µM and 5×, 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 (Skrivergaard, Young, et al. 2023; Kolkmann et al. 2022). 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, Gilchriest, and Lorenz 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, **knowledge on fish GFs crucial for 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.

3.2.3. Serum free proliferation media for fat cell progenitors

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). Hausman et al., however, showed that porcine adipose tissue stromal-vascular cells (preadipocytes) could be cultured in SF medium (DMEM supplemented with a mixture of insulin, transferrin, and selenium). Short-term proliferation (24-48 h) was facilitated in presence of specific extracellular matrix proteins (Hausman, Wright, and Richardson 1996).

Another SF medium composition was published by Hubalek et al. (S. Hubalek et al. 2023), where bovine fibro-adipogenic progenitor cells (FAPs), suitable for cultured fat production, were propagated in Kolkmann proliferation medium for bovine satellite cells (Kolkmann et al. 2022), as well as in the same medium adapted for low ammonia genicity. 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. Also, final volume of lipid droplets after differentiation was significantly higher in adapted medium (S. Hubalek et al. 2023). Thus, the **study presents four SF medium compositions for proliferation of cultured bovine fat precursors.**

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 2)(L. 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 (Laura 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 2).

Regarding **fish preadipocytes**, the selection of an appropriate basal culture medium can also impact on cell proliferation as shown for primary preadipocytes from gilthead seabream. Their growth rate was higher in DMEM than in L-15 (C. 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 growth factors 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 (Tian et al. 2016; P. Liu et al. 2018). Cytokines such as IGF-1 and insulin, and growth hormone (somatotropin) were used to improve **gilthead seabream preadipocyte proliferation** (C. Salmerón et al. 2013). Similarly, insulin enhanced the proliferation of preadipocytes from the large yellow croaker (X. Wang, Huang, and Wang 2012), while tumor necrosis

factor-alpha (TNFα) and docosahexaenoic acid (DHA) inhibited it. **In rainbow trout**, supplementation with IGF-I stimulated preadipocyte proliferation, particularly during early phases of culture (Bouraoui, Gutiérrez, and Navarro 2008). Interestingly, low doses of TNFα slightly enhanced proliferation, though higher doses were less effective (Bouraoui, Gutiérrez, and Navarro 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, 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, Dick, and Sargent 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.

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 (L. Pasitka et al. 2022)	Chicken fibroblasts (Laura 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	Upon request from Oryzogen
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	Sigma # S5261
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 2: 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 (Laura 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.

3.3. Differentiation and maturation

3.3.1. Myogenesis

Skeletal muscles consist of bundles of **highly differentiated** and multinucleated cells which are oriented to facilitate contraction in a single plane. Therefore, important criteria for *in vitro* muscle engineering are high cell densities, a high degree of precursor cell fusion to form multinucleated cells (myotubes) and unidirectional alignment of cells to facilitate orientated myotube formation (Mudera et al. 2010).

Info box 2: For robust and efficient differentiation processes required for the CM/CSF production further efforts are needed:

- ⇨ Search for **species-specific transcription factors** and genes upregulated during early and late phases of myogenesis and adipogenesis **to identify targets for activation of differentiation**.
- ⇨ Search for differentiation inducers/ligands/stimulatory factors **suitable for the food sector**.

After the proliferation phase, differentiation and maturation of myoblasts into multinucleated myofibers occurs typically within 7 – 14 days *in vitro*, compared to months or years *in vivo* (Martins et al. 2024). Differentiation initiates through an irreversible cell cycle arrest of the precursor cells (myoblasts) and proceeds with a gradual increase in expression of muscle function genes, and the fusion of myoblasts into multinucleated myofibers (Blais, 2005, Martins 2024). *In vitro*, cell cycle arrest is often induced through **serum starvation** in various mammalian species (Ostrovidov et al. 2014), a concept also successfully applied to fish myosatellite cells (Gabillard, Sabin, and Paboeuf 2010; Krishnan et al. 2023; Ulagesan et al. 2024) and myoblasts (Saad et al. 2023; Kong et al. 2021). Despite its effectiveness, this method still depends on the use of animal-derived serum, albeit at reduced 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 (Krishnan et al. 2023; Kong et al. 2021; Saad et al. 2023; Lautaoja et al. 2020) in media such as DMEM or L-15.

Generating **SF formulations** that drive robust differentiation across a range of donor animals (and even 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 the transcriptomic remodelling of bovine satellite cells 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 is still have 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** 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). In another study, supplementing culture medium with differentiation-stimulatory factors such as **nitric oxide (NO) and arachidonic acid** promoted the differentiation of muscle stem cells (K. Choi et al. 2021). NO stimulated the production of follistatin through cGMP signaling, causing myoblast fusion both *in vitro* and *in vivo* (Pisconti et al. 2006). Also, both **mechanical and electrical stimulations** are well documented to improve the myogenic differentiation of muscle stem cells and the contractile force thereof (A. Ito et al. 2014; Langelaan et al. 2011; K. Choi et al. 2021). The ways to realize these stimuli in a bioprocess for CM production are discussed in the bioreactor review in this issue by Ngwa et al.

For muscle cells of aquatic animals, development of a fully SF medium for their differentiation has yet to be reported in literature. Till now, several components were reported to induce differentiation. **Retinoic acid** at varying concentrations (from µM to mM) has been shown to promote differentiation in fish embryonic stem cells (ESCs) (Dash et al. 2010; V. Parameswaran et al. 2007; Vijayakumar Parameswaran et al. 2012; S.-L. Chen, Sha, and Ye 2003; S. -L. Chen et al. 2003; Bejar, Hong, and Alvarez 2002). Several other reagents known to enhance myogenesis in fish stem cells—including FGF-2, GSK3β inhibitors, calpain inhibitors, and adenylate cyclase activators—may offer promising avenues for improving myogenic differentiation protocols (Bomkamp et al. 2023), see Table 2).

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

Alternative approaches to induce differentiation in fish primary SCs have also been tested. For instance, a combination of DMEM/F12 medium supplemented 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) (adapted from (E. Xu et al. 2023)) effectively induced differentiation in *Larimichthys crocea* satellite cells (S. Zhang et al. 2023). But suitability of these components for the food sector has yet to be investigated. For fibroblast differentiation into muscle-like states, non-starvation-based methods have also been explored. In

thread-sail filefish *Stephanolepis cirrhifer*, a transition from L-15 basic medium containing 10% FBS to AIM V basic medium (Thermo Fisher Scientific) containing 10% FBS successfully stimulated myogenesis of fibroblast-like cells (Tsuruwaka and Shimada 2022). Interestingly, the same thread-sail filefish fibroblast cell line was able to 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). Myotubes adhere extremely well to a fibroblast monolayer, which functions as an elastic support for contracting myotubes. Further, fibroblasts also secrete extracellular matrix components and growth factors that may also contribute to the improved survival and 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. In contrast, **more efforts are needed** towards the development of SF differentiation media for muscle cells from aquatic animals.

3.3.2. Adipogenesis

The differentiation of precursor cells into mature adipocytes requires the sequential activation of a battery of transcription factors. In white adipocytes, this sequence initiates with the activation of members of the activating protein-1 (AP-1) family of transcription factors and continues with the induction and expression of PPARγ, a critical pro-adipogenic transcription factor (Sarjeant and Stephens 2012). During adipogenesis cells undergo a significant shift in metabolism and accumulate large amounts of lipids (predominantly triglycerides) (Sarjeant and Stephens 2012).

Standard protocols for mammalian cells use an adipogenic cocktail containing **insulin, glucocorticoids**, and 1-methyl-3-isobutylxanthine (**IBMX**) (Hauner et al. 1989; Wabitsch et al. 1995; Hube et al. 1999). A 30- to 70-fold increase in the number of developing fat cells was achieved by the addition of cortisol or related corticosteroids in the presence of insulin. Either of the two hormones alone was ineffective (Hauner et al. 1989). Progenitor or stem cell types such as preadipocytes and MSCs differentiate well into mature adipocytes in presence of the standard adipogenic cocktail containing DEX, IBMX and insulin (Sugii et al. 2023). This cocktail together with one of PPARγ activators was used in most of the *in vitro* differentiation studies with cells of edible species until recently (Sugii et al. 2023; Mitić et al. 2023b). PPARγ is a key transcriptional regulator of adipogenesis, and agonists such as thiazolidinediones have been reported to be necessary for successful maturation of adipocytes that exhibit full adipogenic characteristics (Cheng et al. 2016), and can also trans-differentiate myoblasts into adipocyte-like cells (Teboul et al. 1995).

The induction of cell differentiation with a standard adipogenic cocktail was shown to function **independently of animal serum** being present or not, however the **food compatibility** of molecules present in this cocktail **remains very questionable** (Martins et al. 2024). While DEX, IBMX and PPAR-γ antagonists are synthetic molecules (Mitić et al. 2023b), IBMX and PPAR-γ antagonist rosiglitazone are

toxic and therefore not food-compatible (Nissen and Wolski 2007; Fort et al. 1998). Although the use of such compounds may only be required transiently during the initial induction of adipogenesis, searching for non-toxic options may be relevant for large scale food production scenarios (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 **replace the function of IBMX** in the adipogenic cocktail (Katafuchi, Garbers, and Albanesi 2010). In 3T3-L1 preadipocytes, CNP potently elevated cGMP production through activation of guanylyl cyclase-B (GC-B), a membrane-bound guanylyl cyclase, in the presence of insulin and DEX. Replacing IBMX (0.5 mM) in the adipogenic cocktail with CPN (10 nM) enhanced levels of adipogenesis marker genes to almost the same range compared to the IBMX-containing cocktail (Katafuchi, Garbers, and Albanesi 2010). However, costs for synthetic CPN peptides elevate costs for large-scale CM production (1 mg of CPN-22 peptide costs appr. €150 (Bachem); appr. €1/L medium).

In another attempt to simplify and deliver a food-grade formulation of SF differentiation medium, Mitić et al. demonstrated that out of the four inducers, **insulin** and PPAR-γ antagonist **rosiglitazone were sufficient** to trigger adipocyte differentiation (Mitić et al. 2023b). Also, the addition of low amounts of two glucocorticoid receptor activators, namely **hydrocortisone and progesterone**, was found to be important for a homogeneous cell distribution during differentiation, as well as for cell viability and spreading. The inclusion of DEX and IBMX was not necessary for adipocyte differentiation of bovine stromal vascular cells. The **simplified one-step adipogenic protocol** with a reduced number of adipogenic inducers and phases was not species specific and outperformed serum-containing medium. The percentage of positive cells and total lipid area were significantly higher in the SF differentiation conditions used by Mitić et al. This highly efficient SF and animal component free medium, designated DMAD (Defined Medium for Adipogenic Differentiation), was based on DMEM/F-12 and contained 10 µg/ml insulin and 5 µM rosiglitazone as adipogenic inducers, 17.8 nM progesterone and 25 nM hydrocortisone as endogenous alternatives to DEX, and several other components, e.g. 2 ng/ml FGF-2, 2 ng/ml EGF1, 10 ng/ml BMP4 (see Table S3 for full composition in (Mitić et al. 2023b)). This study efficiently **narrowed down toxic adipogenic inducers** to only rosiglitazone.

Beside some of them being adipogenic inducers, **adipogenesis requires a constant supply of free fatty acids (FFA)** for triglyceride production and lipid droplet accumulation (Cristina Salmerón 2018). In experiments with bovine stromal vascular cells and conventional differentiation media supplemented with FFAs**, oleic and linoleic acid** were found to be the most efficacious for promoting lipid accumulation during the maturation stage of adipocytes (Yanting et al. 2018). Mitić et al. included 0.1 % of a chemically defined and commercially available **lipid concentrate** in their differentiation medium. Beside a mixture of seven free fatty acids (C14 to C20, saturated and unsaturated) and cholesterol, it contained Pluronic F-68 (used to reduce foaming in stirred cultures and to protects cells from hydrodynamic damage) and Tween 80 as emulsifying and dispersing substance (Mitić et al. 2023b).

For a **more food-compatible strategy**, Mehta et al. proposed the use of FFAs for induction of adipogenesis in bovine precursors, as unsaturated and branched-chain fatty acids are agonists of PPAR-family transcription factors (Mehta, Theunissen, and Post 2019). However, it is important to keep in mind that FFA administration can have both beneficial and deleterious effects on adipogenic differentiation, depending on the type of fatty acids and treated cells or species (Yuen Jr et al. 2022). Indeed, individual FFAs differentially bind to or activate members of the PPAR family of transcription factors (Schoonjans et al. 1997; Kliewer et al. 1997). The n-6 PUFAs stimulate, whereas n-3 PUFAs

inhibit adipocyte differentiation and PPARγ *in vivo* (Thoennes et al. 2000). Porcine adipocyte differentiation was increased in a dose-related fashion by addition of oleic acid (C18:1) or linoleic acid (C18:2), but not by addition of α-linolenic acid (C18:3) to SF differentiation medium (DMEM/F12 containing 100 nM bovine insulin, 50 ng/mL hydrocortisone and 10 µg/mL transferrin) (S.-T. Ding, Wang, and Mersmann 2003). Oleic acid also induced a strong increase in the accumulation of lipid droplets in **chicken preadipocytes** isolated with the stromal-vascular fraction (Shang et al. 2014) and was found to promote adipogenic gene expression and lipid accumulation in **bovine muscle satellite cells** (X. Z. Li et al. 2019), however, all these studies included serum in their differentiation media.

The effectiveness of **oleic acid and linoleic acid** to induce *in vitro* cell adipogenesis presents a sustainable and probably more cost-efficient opportunity for large scale cultured fat production, as these FFAs are present at high concentrations in plant-based oils such as olive and soybean oil (Casado-Díaz, Dorado, and Quesada-Gómez 2019; Matthaus and Özcan 2014). The direct use and efficacy of **soybean oil** in promoting adipogenesis has been explored in clinical applications for adipose tissue engineering (X. Li et al. 2018; Hadri et al. 2004; Yuen Jr et al. 2022).

Also in case of spontaneously **immortalized chicken fibroblasts** from the study of Pasitka et al., discussed in chapter 3.2.3, the most efficient transdifferenciation inducers in SF medium – reaching over 80% of the cell population – were 200 µM **oleic acid** combined with 12 µg/ml L-α-**phosphatidylcholine**, which is a major component of plant lecithin. This combination showed a 2.5-fold induction in PPAR activation assay, as compared to rosiglitazone with oleic acid, and resulted in the formation of adipocyte-like cells in the absence of IBMX or rosiglitazone, delivering the **first SF food-grade differentiation medium** for CM applications (L. Pasitka et al. 2022).

The composition of fatty acids in adipose tissue plays a critical role in determining both the firmness and oiliness, as well as the oxidative stability of the resulting muscle tissues, all of which significantly impact the flavour and colour of the meat product (Louis et al. 2023). For high quality beef meat, such as Wagyu meat, the presence of high levels of **oleic acid** is vital for **tenderness and juiciness**. In contrast, a high content of **poly-unsaturated fatty acids** is associated with **undesirable flavours** due to their fast oxidation (Calkins and Hodgen 2007; Louis et al. 2023). In addition, the **nutritional aspect** of alternative 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 samples from bovine white adipose tissue (García-Rojas et al. 2010). Other natural components occasionally added to adipogenic induction medium that may enhance adipogenesis include **biotin** and **pantothenate** (Scott et al. 2011; Schiller et al. 2013), as well as diverse vitamins and acetate (see Table S1 in (Mitić et al. 2023b)). 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 used for mammalian cells, a **differentiation cocktail for fish cells** generally consists of three main components: insulin, IBMX, and DEX (reviewed in Dang, 2024 and Bomkamp

2023, (Dang 2024; Bomkamp et al. 2023)). Additionally, a lipid mixture, such as oleic and linoleic acids or an adipogenic cocktail containing fatty acids from Sigma-Aldrich (Product L5146) was added to 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 **aquaculture-reared fish species** - impacting production levels, meat quality, consumer perception, and animal welfare (Regost et al. 2001; J.-T. Wang et al. 2005) - have led to increased research in fish adipogenesis (reviewed in (Cristina Salmerón 2018). Hence, additional molecules that enhance adipocyte differentiation, such as triiodothyronine (T3), biotin, pantothenic acid, ciglitazone, transferrin, and hydrocortisone, have also been identified (reviewed in (Dang 2024; Bomkamp et al. 2023)).

However, 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 effects of insulin, T3, and fat-soluble vitamins (*all-trans* retinoic acid, retinylacetate and 1,25 dihydroxyvitamin D3) on adipocyte differentiation were also investigated by the authors. They found 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, the study revealed that fat-soluble vitamins had no effects on adipocyte differentiation. Previous research has also confirmed insulin role in stimulating adipocyte differentiation in both gilthead seabream (C. Salmerón et al. 2013) and large yellow croaker (X. Wang, Huang, and Wang 2012). Interestingly, in gilthead seabream, IGF-1 was identified as a more effective inducer of differentiation compared to insulin (C. Salmerón et al. 2013).

While current progress in serum- and IBMX-free media for fish adipogensis 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 (Cristina 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. But more research is necessary to cover further cell types and species.

Adipogenesis								
Porcine and ovine FAPs (Mitić et al. 2023b)			Chicken fibroblasts (L. 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	Sigma # S5261						
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**	

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

4. Strategies to reduce costs of cell culture media

A critical component to produce CM/CSF is the 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 growth medium (Fernandes et al. 2022; Elliot Swartz et al. 2021; Specht 2020). Assuming that novel technologies will be developed to reduce the cost of the medium, including growth factor substitutes and the purchase of ingredients in bulk, 1 kg of cell-cultured meat is estimated to cost $63/kg (around €56/kg) in a large-scale facility setting (Garrison, Biermacher, and Brorsen 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 (Elliot 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 industry requires game-changing innovations to facilitate an economically high-volume production capacity, which are discussed in this chapter.

Info box 3: The **focus** will be on strategies leading to **increased stability of growth promoting proteins** in culture media, as this is crucial for their long-term functionality (see also Table 5):

- ⇨ Raising GF stability or activity to avoid or at least lower the need in GF stabilizing albumins;
- ⇨ Use of low-cost stabilizing agents instead or in combination with HSA;
- ⇨ Use of alternatives to GFs to avoid stability issues;
- ⇨ Explore the potential of medium recycling for media cost reduction;
- ⇨ Adaption of continuously proliferating cell lines to less protein rich media.

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).	(Sophie Hubalek, Post, and Moutsatsou 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 and 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).	(L. Pasitka et al. 2022; Lim et al. 2024)
Hydrolysates	Glucose, vitamins, amino acids, minerals	Plant extracts can replace nutrients in media, allowing lower FBS concentration.	(S. H. Kim and Lee 2009; C. H. Kim et al. 2023; Charlesworth, Jenner, and Le Coutre 2024)
		Microalgae can replace nutrients in media, including glucose, allowing lower FBS concentration.	(Okamoto et al. 2020, 20; 2022; Yamanaka et al. 2023; Dong et al. 2023)
		Microbial cells can replace nutrients in media but also substitute HSA after adaptation period.	(Andreassen et al. 2020; Sung et al. 2004; Dolgin et al. 2024)
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.	(Shimizu et al. 2021; Haraguchi et al. 2023; 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.	(Zakrzewska et al. 2005; 2009; G. Chen et al. 2012)
	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)

Table 5: Strategies for the reduction of the costs associated with cell production and differentiation for cell-based food, discussed in this review.

4.1. Switch from pharma grade to food grade compounds

Cost reductions may be achieved by altering the medium formulation or by using cheaper food grade alternatives, e.g. by-products of agar or food industry like plant or yeast extracts (Flaibam, Meira, et al. 2024; Ho et al. 2021), which will be discussed in detail below. An obvious step to take is the switch from pharma grade to food or feed grade nutrient components in cell culture media. As an example, the list price of 1 kg of pharma-grade glucose is €90 (Thermo Fisher), whereas food-grade table sugar can be purchased for €1 (any supermarket). This is also the case for amino acids, as 1 kg of high-purity glutamine is at €280 (Thermo Fisher) but food-grade glutamine can be purchased at €40 (Sophie Hubalek, Post, and Moutsatsou 2022). Both might be equally effective as a medium supplement for *in vitro* meat production. 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: Over 80x Cost Reduction in Our Animal-Free Medium," 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.

4.2. Reduce the concentration of growth promoting proteins

Around 2020 Specht et al. stated that GFs are the most dominant cost drivers (Specht 2020). A **reduction of the concentration of all 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 applies to reducing the prevalence of 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, 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 their stabilization. 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). Further studies will be required to identify appropriate concentrations of medium supplements for specific cell lines used for *in vitro* meat production.

4.3. Appropriate choice of growth factors or orthologs

GFs are proteins secreted by cells to communicate with each other. They are commonly added to cell culture media to mimic the endogenous signalling pathways that 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 and decreased with higher concentrations. The most pronounced effect was observed when bovine SCs were treated with PDGF-BB from Eagle – however at a concentration of 100 ng/mL. In contrast, human TGF-β1, showed no improvement at all concentrations tested compared to FBS controls (Venkatesan et al. 2022). Also, replacing bovine recombinant IL-6 with human recombinant IL-6 not only led to an overall 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 History of serum free cell culture media development2, 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 3.2.2, 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 appropriate GF ortholog and determining the most suitable GF concentration and GF combination 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 6 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 cells. They are of eukaryotic origin and typically require some level of post-translational modification, such as oxidation or glycosylation, and proper disulfide bond formation is essential for protein folding and maturation into their biologically active conformation. GF recombinant production is therefore challenging and is a major contributor to their high cost (Venkatesan et al. 2022). Moreover, GFs tend to be inherently unstable molecules because they are biologically intended to act as short-term signals within the body (L. Benington et al. 2020). Hence, multiple administrations and/or supraphysiological doses are often necessary to sustain an effective concentration of GFs, resulting in higher costs and also 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 cultured food production. This is also reflected in the recent surveys, showing high interest of manufacturers and CM producers in modified GF (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, truncations, immobilizations, addition of stabilizers or chemical modifications, 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' thermal 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, Rinas, and Scheper 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 charges 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 during incubation 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 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 and 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. However, despite the increased affinity of the mutant D82R for heparin, the cell proliferation activity of the D82R variant 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 (Q40P, S47I, and H93G), 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). Additionally, triple (Jeong 2015) and quintuple (Nölle 2015) **FGF-2** mutants with improved stability and up to 10-fold prolonged activity in cell culture were recently prepared by aligning protein sequences of wild-type FGF-2 with previously reported stabilized FGF-1 mutants. Finally, 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.

Most ECM proteins and components found in vertebrates had evolved in early chordates prior to the emergence of vertebrates (Huxley - Jones, Robertson, and Boot-Handford 2007), which is supported

by the similarities in their function in fish and mammals. 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 induction of FGF-FGFR complex formation and FGFR signalling (Zakrzewska et al. 2009), demonstrating the effectiveness of this strategy. Similar observations were made regarding FGF2-STAB variants with improved thermal stability. FGF2-STABs showed higher efficiency in induction of FGFR-mediated proliferation, lower affinity to heparin and were less dependent on heparin than wild-type FGF-2 for induction of FGFR-mediated mitogenic response (Koledova et al. 2019). Thus, protein engineering resulting in strong and powerful stabilizing effects may abolish heparin dependence of FGF proteins.

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 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 the 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). However, high affinity ligands may also trigger a fast receptor internalization and degradation whereas low affinity ligands may preserve the receptor leading to a longer lasting signalling (Zaiss et al. 2015). Although this strategy has the potential to produce highly effective GFs, it may require longer development as the effects of modified signalling may be less predictable than those of improved stability (Ren et al. 2020).

As **FGF-2 dimerization** is essential for the formation of active ligand–receptor complex, a covalent linkage of two FGF-2 molecules provides another strategy to improve the biological properties of GFs (Nawrocka et al. 2020). To engineer FGF-2 dimers, mutations in the wild-type FGF-2 were designed to obtain variants with a single surface-exposed reactive cysteine for the chemical conjugation with bis-functionalized linear PEG linkers. All eight FGF-2 dimers of defined topology stimulated the downstream signalling at the same level as FGF-2 WT, which indicates that all dimers are fully competent in the activation of receptor-dependent signalling cascades (Nawrocka et al. 2020). For its mitogenic activity, FGFs not only need to activate FGFRs, but long-term stability in the cell culture medium is essential to induce cell division. All dimeric proteins showed enhanced mitogenic potential, as compared to FGF-2 monomer; the most statistically significant differences were noticed at 10 ng/mL protein concentration (Nawrocka et al. 2020). The advantage of dimers over FGF-2 WT in the context of mitogenic potential was equalized by the addition of heparin to the cell culture. Although heparin

supplementation increased the mitogenic response of all investigated proteins, the effect was most pronounced for cells grown in the presence of FGF-2 WT (Nawrocka et al. 2020). Dimeric FGF-2 variants did not show any tendency to aggregate and preserved the native conformation. Thus, FGF-2 dimerization provides the possibility to reduce external heparin administration as dimeric proteins possess increased stability in cell culture medium. The amount of functional dimers was not significantly altered after 48 h in the presence of cells, whereas the concentration of active FGF-2 WT was strongly reduced already after 24 h (Nawrocka et al. 2020).

A similar strategy was applied to improve the limited stability of native hepatocyte growth factor/scatter factor (HGF). The mature, biologically active species of HGF is a two-chain (α/β), disulphide-linked protein. The α-chain comprises an N-terminal and four kringle (K) domains (64 kD) and contains 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 it was demonstrated in both *in vitro* and *in vivo* models that K1K1 is stable and displays biological activity, equivalent or superior compared with native HGF (De Nola et al. 2022). With this approach a cost-effective expression of the HGF minimal variant K1K1 in *E. coli* was possible, whereas recombinant production of the full-length heterodimeric HGF with posttranslational modifications in mammalian expression hosts is more expensive.

For GF engineering approaches, the design of short GF variants mimicking full length GF activities would provide an **additional possibility to reduce costs** for recombinant expression of growth promoting proteins required for media supplementation for CM 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 and chemical modifications of growth factors

GF immobilization has emerged as a possible strategy to optimize GF use in cell culture and to avoid costly and frequent labour-intensive changes of growth media. To date, several immobilization techniques have been reported for attaching GFs onto a variety of biomaterials, but must of them have been applied to tissue engineering for regenerative medicine (Enriquez-Ochoa et al. 2020). Studies performed on suspended-cells culture systems showed a significant reduction of the total amount of GFs required for cell growth (Enriquez-Ochoa et al. 2020). Thus, the use of **immobilized GFs (iGFs)** may be a promising approach for cultured meat production as it gives the possibility to achieve a sustained GF release, which may considerably reduce the need for multiple doses and effectively decrease 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. Advantages of the physical encapsulation involve technical accessibility, low cost of reagents and preservation of iGF bioactivity (Enriquez-Ochoa et al. 2020). The interaction between GFs and the selected material for entrapment relies on hydrophobic, hydrophilic-hydrophilic, and electrostatic interactions (K. Lee, Silva, and Mooney 2011). This technique was successfully applied to the culture of non-adherent cells since iGFs can be encapsulated and added to suspension culture systems, such as flasks and bioreactors. Furthermore,

hydrogels used for physical immobilization techniques are suitable for cell scaffolding (Enriquez-Ochoa et al. 2020).

The use of microencapsulation matrices for food applications is limited to edible, biodegradable, and preferably inexpensive materials, with biopolymers based on proteins or polysaccharides being the most suitable candidates (Gómez-Mascaraque et al. 2018). **Polysaccharides** are abundant and relatively inexpensive biopolymers and most of those used for encapsulation have a plant origin, such as starch, pectin, cellulose, **alginate** or guar gum, although animal-derived polysaccharides, such as **chitosan,** or those from bacterial origin, such as **dextran,** are also widespread for this application (Gómez-Mascaraque et al. 2018).

A biomaterial-based system allowing simultaneously the encapsulation and the sustained delivery of GFs would offer an excellent possibility to **reduce the amount of GFs required for cell culture**, hence lowering costs for media. For example, in their study using alginate microencapsulation of viable islets as a potential treatment for type 1 diabetes, Khanna et al. used **multilayered alginate** microcapsules coated with a permselective poly-L-ornithine (PLO) membrane for the encapsulation and sustained delivery of FGF-1 (Khanna et al. 2010). Release kinetics of FGF-1 in the outer alginate layer of the microbeads 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 it was co-encapsulated with heparin in the outer layer (Khanna et al. 2010).

Alginate microspheres, however, **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 to study the release of TGF-b1, and to evaluate the effect of released TGF-b1 on chondrogenesis in the scaffold. **Chitosan** has been reported to stimulate and protect the activity of GFs (Ueno et al. 2001; Ishihara et al. 2019) and it possesses a cationic nature resulting from primary amine groups. Chitosan microspheres were loaded with TGF-b1 prepared by the emulsion-ionic crosslinking method, in the presence of tripolyphosphate, which carries five negative charges and allows electrostatic interaction with protonated chitosan in an aqueous acidic solution (J. E. Lee et al. 2004). Chitosan microspheres showed sustained release of TGF-b1 over time. The shape of the release curve was parabolic. After 3 days the release rate of TGF-b1 stabilized, and the accumulated release of TGF-b1 by day 7 was 44.8% of the initial loading (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 bound and stabilized in the chitosan/fucoidan micro complex-hydrogel, and they were gradually released upon biodegradation of the hydrogel *in vivo* (Nakamura et al. 2008). Incorporation of FGF-2 in this hydrogel can thus provide an **excellent controlled release system** and it was shown that it substantially prolonged the biological half-life time of FGF-2 (Nakamura et al. 2008).

Microspheres mixing readily degradable **collagen** with more slowly degradable, mesh-forming **alginate polymers** were also used for the sustained release of FGF-2 (Ali et al. 2018). The degradation of these alginate/collagen mixtures, which controls the release kinetics of embedded FGF-2, can be adjusted depending on the amount of collagen used. Importantly, the amount of pro-angiogenic FGF-2 released from such microspheres led to robust induction of angiogenesis in **zebrafish embryos** (Ali et al. 2018). Collagen, a key structural component of the extracellular matrix and originally extracted from animal

tissues, is now available from recombinant biosynthesis (Zilong Zhao, Deng, and Fan 2023), though at a high price.

Beside natural polymers, synthetic polymers such as **polylactic glycolic acid (PLGA)** have also been described for sustained release formulations of GFs. The encapsulation of **FGF-2 into PLGA** microspheres improved cell culture conditions for human embryonic stem cells (hESCs). The controlled release of FGF-2 from PLGA beads significantly reduced the frequency of media changes needed to maintain stem cell cultures. After 72 h in cell culture, the level of FGF-2 released from the microspheres remained almost unchanged, whereas the level of FGF-2 administrated in soluble form decayed by 90% (Lotz et al. 2013). PLGA is a biocompatible and biodegradable linear co-polymer that can be prepared at different ratios between its constituent monomers 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, biopolymers used for medical applications may not be approved for food industry.

Although microencapsulation is an effective approach to control the release of GFs from polymeric delivery vehicles while maintaining their bioactivity in cell culture media, questions that should be investigated in more detail are for example the estimation of release kinetics that would be the most desirable for *in vitro* meat production or the relationship between microsphere diameter and their degradation-rate (Ali et al. 2018). Further the choice of polymer material will have an impact not only on the encapsulation efficiency and protection of the bioactive ingredient of interest, but also on its release behaviour (Dias, Ferreira, and Barreiro 2015), the selection of the most adequate encapsulation matrix is of outmost importance in the development of microencapsulation structures (Gómez-Mascaraque et al. 2018).

Physical immobilization methods are generally the most cost-effective but entail **disadvantages** such as **lack of control-over-release**. Covalent binding of GFs to surfaces is required when these cannot be adsorbed onto a substrate surface, or when a gradual release from the substrate is necessary (Enriquez-Ochoa et al. 2020). After a covalent conjugation of GFs to biomaterials, GF release depends on biomaterial degradation and/or cleavage (hydrolytic and enzymatic) of the bond between GFs and biomaterials (Ren et al. 2020). Functional groups (e.g. amino-, carboxyl- or thiol-reactive groups) in GFs are usually used for their covalent binding to surfaces. The chemical approach often requires complex methodologies. Carbodiimide coupling represents one of the most common methods for covalently attaching GFs onto substrate surfaces (Enriquez-Ochoa et al. 2020). For further methods used and examples of covalently as well as non-covalently immobilized GFs we refer to the reviews of Enriques-Ochoa and Teixeira (Enriquez-Ochoa et al. 2020; Teixeira et al. 2020).

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 each 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. Extending the activity of growth factors by addition of stabilizers / excipients

From a cost perspective, the addition of excipients for stabilizing soluble GFs would be the preferred approach which is also relatively simple to implement in bioprocesses. Excipients for stabilising protein solutions include salts, sugars, polymers, proteins, and amino acids (L. Benington et al. 2020). In their attempt to preserve FGF-2 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 extended to 8 h (97% residual FGF-2 content) (L. Benington et al. 2020). Polymers like **methylcellulose** form hydrogels potentially capable of increasing the molecular density and viscosity of aqueous GF solutions to prevent protein aggregation. This effect appears to be favourable at low polymer concentrations for MC (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 and Glofcheski 1992). Benington et al. reported that alanine, but not glycine, met the criteria of an effective stabiliser for FGF-2. Alanine at 20 mM was significantly more effective at stabilising FGF-2 at higher temperatures than at 100 mM (L. R. Benington et al. 2021). **Proteins like HSA** and BSA are known to stabilise 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).

Supplementing B8-medium with **recombinant HSA** (800 µg/mL) also improved growth of bovine satellite cells (BSCs) approximately 4-fold compared with plain B8, probably due to a substantial GF-stabilizing effect of albumin (Stout et al. 2022).

The use of stabilizers like **recombinant albumin**, however, would result in a substantial increase in medium costs. 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).

Non-hydrolysed oilseed protein isolates (particularly rapeseed protein isolate, RPI) were also shown to be effective albumin alternatives in SF medium Beefy-9 (Stout, Arnett, et al. 2023). RPI is easy to produce through simple and low-cost steps from rapeseed protein meal and was shown to exceed recombinant albumin in promoting bovine SC growth while maintaining myogenicity (Stout, Arnett, et al. 2023). This could be a viable alternative to recombinant albumin, provided that large-scale production costs remain low. But it is hard to assess at the moment, as the proposed isolation method could be costly on an industrial scale, and the isolates must be stored at -80°C to retain biological activity (Stout, Arnett, et al. 2023). The product is currently not available on the market.

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, Gibson, and Strimling 2021). But the fact that microbial lysates can substitute HSA as stabilizing agent is very remarkable.

Also, water soluble **plant proteins extracted** from soy, pea and chickpea were able to replace costly BSA in SF-media for proliferatiton of chicken fibroblasts (patent WO 2021/148955A1 (Nahmias, Cohen, and Caspi 2021)). Only 0.1 mg/mL of plant proteins were required to replace 2 mg/mL BSA. Hemp and wheat proteins were not able to substitute BSA at this concentration. Increasing the amount of plant protein extracts resulted in toxic effects for chicken fibroblasts. However, depletion of the <10 kDa fraction from the plant proteins eliminated toxic effects (Nahmias, Cohen, and Caspi 2021).

Stabilization of GFs in cell cultures by addition of low-cost excipients represents the possibility to dramatically reduce costs for media required for cultured meat production. 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 et al. 2022; Stout, Arnett, et al. 2023). The cost of MC and starch from corn proposed by Schenzle et al. to substitute the same amount of albumin with the very similar effect, 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.

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.

4.4.5. 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. For example, plant protein hydrolysates were successfully used to enhance cell proliferation as well as product yields for industrially applied CHO, HEK, VERO, BHK as well as insect cell lines (Siemensma et al. 2008). However, attempts to apply hydrolysates from plants, yeast or algae for culturing cell lines used for CM production were not successful. But a reduction of FBS concentration in media could be achieved with these approaches – as we discuss in more detail in the chapter 4.5 below.

Another strategy was based on the substitution of the GFs with defined peptides, peptoids or small molecules, which mimic GFs and can specifically activate respective receptors. They are generally more stable, less susceptible to proteolysis and cheaper in production (Atkinson and 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 and 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, growth factors 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, Ayyash, and Pasitka 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 and 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.

4.5. Hydrolysates and Circular Cell Culture

Hydrolysates obtained from **plants and microbes** by enzymatic or acidic digestion have attracted attention as complementary sources of proteins, peptides, amino acids, lipids, carbohydrates and vitamins (Flaibam, Meira, et al. 2024). Due to their rich nutritional composition, they may substitute essential cell culture media components in CM/CSF production. Hydrolysates from plant materials are widely used in pharmaceutical industry as a substitution of FBS for 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).

Info box 4: Hydrolysates are rich in nutrients and may substitute basic media components, however they **cannot fully substitute FBS**.

- ⇨ **Sources**: Plants, fungi, microalgae, bacteria, agro-industrial waste (e.g. brewer`s spent yeasts).
- ⇨ **Standardizations** regarding culture conditions and preparation methods **are needed**.

Protein hydrolysates produced from black soldier fly, cricket, oyster, mussel or lugworm may also be considered as nutrient source – although they are animal sourced. Of particular interest, hydrolysates from the black soldier fly appeared to have superior properties regarding the preservation of cell membrane integrity and to be the most cost-effective alternative of animal serum in culture media

(Batish et al. 2022). Their larvae are 42% crude protein and 29% fat, but their biggest advantage over other insects is their ability to convert waste into food (J. Wang et al. 2017).

Novel routes for animal component-free medium additives may be established by hydrolysing **agro-industrial wastes** which in addition offers opportunities to valorise waste and reduce its negative impacts on environment (Dhara et al. 2023; Smith et al. 2024). Hydrolysates obtained from agro-industrial wastes, e.g. Brewer's spent yeast and molasses, could provide sustainable sources of nutrients for the cultured food sector.

On a different aspect, large volumes of **waste medium** will be generated once large-scale production of cells for CM/CSF production is established. To reduce its environmental impact and to support the idea that cultured food provides a cleaner alternative to slaughtered meat, the development of new technologies for medium recovery or medium upcycling will be essential. Promising strategies to decrease the volume of cell culture waste medium were reported by Haraguchi et al. (Haraguchi and Shimizu 2021; Haraguchi, Okamoto, and Shimizu 2022). The authors have demonstrated successful microalgal culture in mammalian cell culture waste medium. Microalgae are rich in nutrients and biologically active substances; compounds that are in demand in e.g. the pharmaceutical or chemical industry or in the production of feed and functional foods (Bhattacharjee 2016). Furthermore, a **circular cell culture (CCC)** system combining microalgae, RL34 hepatocytes, and C2C12 cells was developed recently (Haraguchi, Okamoto, and Shimizu 2022; Haraguchi et al. 2023). In this novel CCC system, microalgae are used as nutrient supply for mammalian cells and as a waste-medium recycler. In the following section, the potential as well as putative problematic points of hydrolysates from plants, microalgae and yeasts as supplements for cell culture media are reviewed.

4.5.1. Plant cell extracts for cell culture media

Plant-derived nutrients have been extensively studied in a food, feed and cosmetics context where they were found to be safe and able to replace animal-derived components (Abdel-Latif et al. 2022; Saget et al. 2021). Media components derived from plants can be a sustainable alternative as plants grow photoautotrophically which reduces the environmental footprint and resource consumption (McConnell, Li, and Brudvig 2010; Blankenship et al. 2011; Santos Correa et al. 2023).

High amounts of protein were reported for corn, potato, brown rice and pea – up to 80% of the total weight (Gorissen et al. 2018). 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, Garcés-Rimón, and Miguel 2022)) and minimal demands regarding fertilization and irrigation as well as a high adaptability to different climate zones. Testing different plant species, and even more importantly, agricultural or bioprocesses waste products is therefore advisable, as for many of them very high protein contents are reported, which can be used as a source of amino acids: e.g. soybean meal – protein content of 81.4–87.4%; rapeseed meal protein content: 58.39–61.89% (Hadidi et al. 2024). Toxicity does not have to be a problem, as shown in Stout et al., where total protein isolate from rapeseed press cake was successfully used to substitute HSA (Stout, Arnett, et al. 2023).

Further, the **molecular composition** of the plant-based extracts, e.g. the amino acid composition, should be balanced to meet the requirements of the animal cells (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, Chen, and Guo 2021).

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, Chen, and Guo 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 (J. F. 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, Chen, and Guo 2021). Cell culture media are frequently deficient in antioxidants that are normally delivered to cells by the bloodstream. Correcting this deficiency by supplementing media with plant-derived extracts containing antioxidants can have beneficial effects on cells (Halliwell 2014).

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 and 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 basic 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).

Protein digests from diverse plants, termed hydrolysates, have been proposed as alternatives to components in media (Ho et al. 2021). But **preparation methods** have a substantial impact on the nutrient composition of hydrolysates (Johannes F. Buyel and Fischer 2014; J.F. Buyel and Fischer 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, Lobanov, and Buyel 2021), which has also been exploited to produce selective protein extracts used as replacements for albumin (Stout, Arnett, et al. 2023). 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 that can affect the growth and metabolism of animal cells (Ho 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 (Scherer, Syverton, and Gey 1953; Russell et al. 1977) but it could be relevant for CHO cells (Wurm and Wurm 2021) or immortalized CM/CSF cell lines (Stout, Arnett, et al. 2023; L. Pasitka et al. 2022).

Specific plant-derived compounds that were identified as toxic to animals at high concentrations include 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) (J.F. Buyel 2018). The median lethal dose (LD_{50}) of these compounds can range from ~30 to >5000 mg kg^{-1} body mass (Table 6). 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 anyway. Interestingly, all the compounds listed here have been extensively researched as nutraceuticals and 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 7).

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 and 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 and 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.

Some data indicate that 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 and 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.

Plant species	Compound	Concentration in biomass [mg/kg dry mass]	Reference	LD50 [mg/kg]	Organism	Route of administration	Reference
Sanguinaria canadensis	Sanguinarine	1400	(Campbell, Affolter, and Randle 2007)	1658	Rat	Oral	(Becci et al. 1987)
Sanguinaria canadensis	Sanguinarine	1400	(Campbell, Affolter, and Randle 2007)	29	Rat	Intravenous	(Becci et al. 1987)
Berberies vulgaris	Berberine sulfate	20000	(S. Ahmad et al. 2019)	205	Rat	Intraperitoneal	(Kulkarni, Dandiya, and Varandani 1972)
Berberies vulgaris	*Berberies vulgaris* root extract	20000	(S. Ahmad et al. 2019)	1280	Rat	Oral	(Kulkarni, Dandiya, and Varandani 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, Hood, and Griffiths 2004)	>5000	Rat	Oral	(Matulka, Hood, and Griffiths 2004)
Vitis vinifera	Resveratrol	50 - 700	(Langcake and Pryce 1976)	5	Human LX-2 hepatic stellate cells	In vitro	(Bechmann et al. 2009)
Vitis vinifera	Resveratrol	50 - 700	(Langcake and Pryce 1976)	>3000	Rat	Gavage	(Crowell 2004)

Table 6: 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**.

Using **plant extracts as sources for basic media components** in cultured meat applications has not been studied extensively, as most of the research was focused on substituting FBS and GFs, which so far showed limited success in CM field.

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 (Siemensma et al. 2008; Sung et al. 2004; Ballez 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.

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, Schneider, and Robinson 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, Erikci, and Kiran 2022); (Terada et al., n.d.; L. Liu et al. 2016)

Table 7: Substances / compounds promoting cell proliferation / differentiation.

The same effect was also anticipated for CM relevant cell lines, but as for now, only partial substitution of FBS was demonstrated, e.g. in a study by Kim et al. using fermented soybean meal hydrolysates (C. H. Kim et al. 2023). Also, for immortalized mouse myoblast cells C2C12 a complete substitution of FBS with chemical plant hydrolysates from **dried grass or cattle feed pellets** was not possible. Although growth was 1.5-fold higher than in a protein-free medium, this is still only 20% of the growth observed in the same medium supplemented with 10% FBS (Charlesworth, Jenner, and Le Coutre 2024). Further supplementation with ITS (insulin-transferrin-selenium) drastically improved the proliferation of this cell line to the level of approx. 50% of that in 10% FBS, which distinctly shows the dependence of immortalized C2C12 cells on components present in FBS.

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 in 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.

Thus, plant-derived hydrolysates can support 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.

The utilization of **hydrolysates from plant-based agro-industrial waste**, such as soybean meal or peanut meal, 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 nutrient source

Microalgae may provide a sustainable supplement source for cell culture media because they store large quantities of digestible proteins and lipids. The three most important classes of micro-algae in terms of abundance are the diatoms (*Bacillariophyceae*), the green algae (*Chlorophyceae*), and the golden algae (*Chrysophyceae*) (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, Fields, and Mayfield 2020). In addition to proteins, microalgae include carbohydrates, poly-unsaturated fatty acids, important minerals, and vitamins (Wells et al. 2017).

Due to their high protein content and nutritional values, microalgae extracts were studied as an alternative to replace nutrients in basal 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 DMEM after 6 days in 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 some factor(s) that countered the positive effects of nutrients. Algal extracts obtained by acid hydrolysis contained glutamic acid, 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, salt concentrations in the microalgal culture medium are different from those in mammalian cell culture medium which 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 the conventional culture medium DMEM and may lead to rapid depletion of these basic nutrients (Okamoto et al. 2022).

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 (J. Y. Ng et al. 2020; Nickolay Rojas-Tavara and 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, bovine myoblasts cultured in such medium had reduced proliferative capacity indicating that this SF medium with GFs IGF-1 and IGF-2 and *C. vulgaris* extract could replace only some of the capabilities that FBS possesses (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 and 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.

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 have an environmental impact. 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** as a protein source is the large variability in protein contents in the raw material, which can vary from 3 to 47% (Kadam et al. 2017). Such variation relies on several parameters, such as algal species, harvesting season, and locations with different 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 serious drawback is their **rather low doubling rates under autotrophic conditions**, ranging from 8 hours to several days (Hidayati, Mubarak, and Sudarno 2020; Ma'mun, Wahyudi, and Raghdanesa 2022).

4.5.3. Microalgae and Circular Cell Culture for sustainable meat production

Solutions to recycle or treat the liquid waste, i.e. spent culture medium, generated during CM/CSF production must be sought as it cannot be simply discarded, as it would be inherently wasteful and will lead to a new environmental burden (Shimizu et al. 2021). After three days in culture, mouse myoblast cell line C2C12 have consumed 99% of glucose and 69% of glutamine initially present in DMEM. However, the amount of the 14 amino acids, excluding glutamine, decreased by only 26% (Haraguchi and Shimizu 2021). More than 80 % of the phosphorus in the culture medium was not consumed by the myoblasts after three days. The amounts of pyruvate and vitamin B1, B2, B6 and folic acid in the medium reduced insignificantly and levels of sodium, potassium, calcium and magnesium in the medium barely changed (Haraguchi and Shimizu 2021). Recycling these nutrients, and preferably also GFs from spent media would allow a resource-saving cultured meat production.

Further, the recycling of harmful substances such as ammonia and lactate generated as waste products by animal cells would reduce the requirements for regular media exchanges (Shimizu et al. 2021; Haraguchi et al. 2023; Yang et al. 2023). 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 and Shimizu 2021). In this system, algae supply oxygen to animal cells, while animal cells produce carbon dioxide and ammonia, which are necessary for algae growth (Haraguchi and Shimizu 2021; Haraguchi et al. 2017). However, animal muscle cells excreted much more lactate than ammonia, which is not a metabolite that microalgae naturally use. Therefore, a different approach was required for removing lactate from spent media by microalgae. 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, a metabolite that can be converted into glucose by cells (Kato et al. 2023). An advanced circular cell culture (CCC) system was developed in which the genetically modified *Synechococcus* microalgae and animal cells mutually exchanged substances via their waste media: (i) microalgae used lactate and ammonia excreted by animal muscle cells, (ii) the animal cells used pyruvate, and some amino acids excreted by the microalgae, and (iii) RL34 rat liver cells were used as animal muscle and growth factor-producing cells.

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** (Haraguchi et al. 2023). The use of microalgae is a characteristic feature of this CCC system, which makes it possible to use repeatedly culture medium throughout animal muscle cells and microalgal cultures, resulting in low-cost and low-environmental-load cultured food production (Haraguchi et al. 2023).

4.5.4. Brewer's Spent Yeast as nutrient source and potential for Circular Cell Culture

Yeast extracts (also known as yeast hydrolysate or yeastolate) are predominantly produced from *Saccharomyces cerevisiae* and are rich in nutrients, such as amino acids, polypeptides, carbohydrates, nucleosides, vitamins and minerals and are regarded as Generally Recognized as Safe (GRAS) by most food safety certification bodies around the world (Alim et al. 2019).

Due to their low production costs and **abundant raw material supply** from beer brewery wastes, yeast extracts have become economical sources of additional nutrients for animal component-free growth media of many cell lines (Spearman et al. 2016). Although most residual biomass streams from big breweries are already subjected to a cascading use, smaller-scale breweries frequently dispose of yeast slurries, as the quantities produced are not substantial enough to garner interest from the food industry, thereby 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. The variety of extraction processes and conditions that can be used to produce yeast extract allows its composition to be tailored to specific applications by maximizing the content of certain nutrients, bio-active compounds, or polysaccharides (Tao et al. 2023). The composition and characteristics of yeast extracts prepared by different processes have been compared and comprehensively reviewed recently by Tao et al. (Tao et al. 2023).

Generally, 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 (Vieira et al. 2016; Jaeger et al. 2020) but the choice of preparation method have an impact on the final composition of yeast extracts and consequently their impact on cell growth *in vitro*. When autolysis was carried out at 47°C, it was possible to obtain autolysates with varying free amino acid content and peptides of different molecular weights depending on autolysis time (Podpora et al. 2016). 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 media for muscle cells could provide an economical and sustainable option for cell culture media, thereby enhancing the circular economy within the agriculture sector.

Yeast-based hydrolysates have been also 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 cell proliferation with almost 50% as compared to medium without FBS. This effect was dose-dependent, with an upper limit of 1 g/L, while media supplementation with 10 g/L nearly depleted cell growth and was highly cytotoxic 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 4.4.4 above, 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, and able to support short term cell proliferation, a complementing strategy could work for a 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 substitute FBS completely, 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. In regard to the potential of yeast for circular bioeconomy, it is exciting to see that the search for identifying low-cost C-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).

Considering that **yeast cell lysates** could contribute constituents to **basal cell culture medium**, thereby reducing the overall costs, and can simultaneously allow the production of GFs independent of human-consumable carbon sources, this approach fulfils the essential requirements for the development of **yeast-based CCC systems**, similar to those utilizing microalgae, 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)) compared to microalgae (doubling time 8 hours to several days (Hidayati, Mubarak, and Sudarno 2020; Ma'mun, Wahyudi, and Raghdanesa 2022)).

5. KEY challenges that remain to be addressed (white spaces)

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.

6. 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 (UPSIDE Foods 2021; GOOD Meat 2022). 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 growth factors (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 (GOOD Meat 2022; Folmer et al. 2023).

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).

7. KPIs

Key performance indicators 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; Vow 2024; L. Pasitka et al. 2022; Laura Pasitka et al. 2024; SuperMeat 2024; UPSIDE Foods 2021), 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 growth factors 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.

8. 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. 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. 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.

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. 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.

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. In this regard, circulating cell culture (CCC) systems are promising. In a novel CCC system developed by Haraguchi et al., microalgae serve as nutrient supplier for mammalian cells and as a waste-medium recycler (Haraguchi, Okamoto, and Shimizu 2022). Mixed cell cultures have long been used for industrial applications (Goers, Freemont, and Polizzi 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 still the most common way of optimizing media formulations, but this strategy is very inefficient due to the high number of media components, e.g. the basal medium DMEM/F12 consists of 52 components (G. Chen et al. 2011) and is unable to consider interactions among media components (Cosenza, Block, and Baar 2021). The throughput capacity of medium optimization studies can be enhanced by the adoption of statistical experimental designs and the application of high-throughput systems (Z. Xiao et al. 2014). Design-of-Experiments (DoE) methods are better able to manage large numbers of components in fewer experiments and allow the evaluation of a wider design space to identify optimal conditions (Z. Xiao et al. 2014; Cosenza, Block, and Baar 2021). In general, DoE methods can optimize < 10 variables (Singh et al. 2017), and with the help of screening designs can solve problems > 25 variables (Weuster-Botz 2000). The DoE approach together with a Response Surface Methodology (RSM) 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 of stabilizer combination (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. ML approaches are generally used to develop predictive models based on the relationships among features of a given dataset. Its workflow commonly involves processing input data, training the underlying model, and predicting new data (Camacho et al. 2018). ML-based approaches have been already successfully applied for medium development for mammalian cell cultures (Hashizume and Ying 2024; Grzesik and Warth 2021; Hashizume, Ozawa, and Ying 2023). 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 culture media requires a deeper understanding of the physiological and nutritional needs of mammalian and fish cell lines and the collection of high throughput data from transcriptomic, proteomic, and metabolic analyses (Hashizume and 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 $\leq$ 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.

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Regina Leber: 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: Writing – original draft, Writing – review and editing, Visualization.

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