White-Box Approaches to Cell Culture Media Optimization for Cultivated Meat

By

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Abstract

This work navigates some of the challenges in optimizing cell culture media for cultivated meat production, aiming to help overcome barriers hindering large-scale commercialization. Beginning with a review of the current state of the science of animal cell culture media, it draws concepts from conventional biomedical applications and microbial fermentation processes for application to cultivated meat. The subsequent experimental studies delve into the intricacies of tailoring media for diverse cell types, exploring the impact of serum-free culture on cellular nutrient requirements, and assessing plant and yeast hydrolysates as alternative nitrogen sources for scalable and cost-effective media formulations. Collectively, the research advocates for tailored and rigorous “white box” media optimization approaches to be used alongside innovative and efficient black box methods. It emphasizes the continued need for nuanced strategies to optimize media for varied cell types, reconcile challenges transitioning to animal component-free formulations, and identify alternative nutrient sources for economically viable cultivated meat production.

CHAPTER 1: Introduction

The burgeoning field of alternative protein production stands at the intersection of innovation and necessity, offering a promising solution to the global challenge of providing sustainable dietary protein for a growing population[1]. Over the past decade, significant strides have been made in addressing the technical obstacles hindering the large-scale commercialization of cultivated meat—real meat products grown directly from animal stem cells. One of the pivotal remaining challenges lies in the design and optimization of cell culture media, which plays a crucial role in cultivating muscle, fat, and connective tissue cells.

A journey towards understanding and refining cell culture media for cultivated meat is presented in the following major chapters of this dissertation. The first of these provides a frame of context for the work and delves into the foundations, providing an overview of cell culture media in the context of cultivated meat. Drawing comparisons with applications in therapeutic monoclonal antibody production and large-scale microbial fermentation processes, this literature review highlights the considerations for developing food-grade, cost-effective media capable of regulating cell proliferation and differentiation, possessing acceptable sensory qualities, and free from animal ingredients.

The first experimental study, presented in Chapter 3, confronts a critical question: Can media formulations be universally applied across different cell types and species? Through a comprehensive analysis of nutrient utilization in primary embryonic chicken muscle precursor cells, chicken fibroblasts, and murine C2C12 myoblasts, the study reveals significant differences in consumption patterns, underscoring the challenges of finding a one-size-fits-all solution. The

results emphasize the necessity for novel approaches to streamline media optimization efforts for the diverse landscape of cultivated meat production.

The study described in Chapter 4 builds upon the first spent media analysis by comparing serum-free and serum-containing media—exploring their respective impacts on cell growth behavior and nutrient requirements. The findings shed light on the intricate effects of serum and serum replacements, challenging assumptions about cell metabolism derived from historical studies using serum-containing media. Projected biomass yield data is also a key outcome of this study, as it provides critical data for accurate technoeconomic analyses in the space. Overall, the findings lay the groundwork for further development of efficient and inexpensive serum-free cell culture media—an imperative for the successful commercialization of cultivated meat.

The dissertation culminates with a forward-looking exploration of cost-effective and scalable cell culture media ingredients for the cultivated meat industry. Chapter 5 investigates plant and yeast hydrolysates as potential alternatives to conventional amino acid sources, presenting a meticulous analysis of their chemical compositions and their impacts on cell cultures. The findings provide the groundwork for the ongoing effort to create simplified and economically viable media formulations, paving the way for the scalable production of cultivated meat.

Embarking on this journey through the intricacies of cultivated meat media optimization, it is important to note that the narrative extends beyond the written pages. The pursuit of knowledge in this field is a collaborative effort, with the anticipation of future contributions from ongoing experiments both within and beyond our laboratory group, further enriching our understanding and advancing the prospects of cultivated meat production.

CHAPTER 2: Considerations for the development of cost-effective cell culture media for cultivated meat production

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Innovation in cultivated meat development has been rapidly accelerating in recent years because it holds the potential to help attenuate issues facing production of dietary protein for a growing world population. There are technical obstacles still hindering large-scale commercialization of cultivated meat, of which many are related to the media that is used to culture the muscle, fat, and connective tissue cells. While animal cell culture media has been used and refined for roughly a century, it has not been specifically designed with the requirements of cultivated meat in mind. Perhaps the most common industrial use of animal cell culture is currently the production of therapeutic monoclonal antibodies, which sell for orders of magnitude more than meat. Successful production of cultivated meat requires media that is food-grade with minimal cost, can regulate large scale cell proliferation and differentiation, has acceptable sensory qualities, and is animal ingredient-free. Much insight into strategies for achieving media formulations with these qualities can be obtained from knowledge of conventional culture media applications and from the metabolic pathways involved in myogenesis and protein synthesis. In addition, application of principles used to optimize media for large-scale microbial fermentation processes producing lower value commodity chemicals and food ingredients can also be instructive. As such, the present review shall provide an overview of the current understanding of cell culture media as it relates to cultivated meat.

Introduction

Consumers are becoming increasingly aware of the broader impacts of their choices and behaviors. Common activities such as air travel, internet usage, and fashion textiles consumption have long been taken for granted, but their environmental, ethical, and socioeconomic implications are starting to become more widely understood [2–4]. Similarly, consciousness of the impacts of the global food production system and personal food consumption choices is reaching the mainstream. With the global population expanding rapidly and expected to reach almost 10 billion by 2050 [5], innovation and expansion of the food system will need to occur to feed significantly more people over time.

The current food system has a sizable influence on the environment, with livestock production alone contributing a disproportionately large portion of global land use and greenhouse gas emissions [6–9]. Besides the ethical animal welfare considerations regarding industrial animal agriculture, there are significant public health concerns associated with this practice as well. The large-scale rearing of animals in close quarters with commonplace prophylactic antibiotic administration provides prime breeding grounds for the emergence of transmittable antibiotic resistant bacteria or other disease-causing agents [10,11]. Moreover, risks of significant zoonotic disease epidemics, such as influenzas, HIV/AIDS, ebola, and COVID-19, can be associated with the use of animals for food [12–17]. Perhaps most importantly, traditional animal agriculture is unlikely to meet the entire increased demand for meat and dietary protein as the population increases [18]. Therefore, innovation in the field of cultivated meat (and cellular agriculture, in general) has accelerated over the past decade, as it has the potential to provide true meat products without these environmental, ethical, and technical issues.

Cultivated meat products will inevitably be composed of multiple types of cells. This adds to the complexity of the target product, as well as the technical challenges associated with

its production. The cell types most likely to be important for industrial production include muscle satellite cells (or more generally, muscle precursor cells (MPC)), myoblasts, myocytes (also known as myotubes or myofibers), adipose-derived stem cells (ADSC), adipocytes, and fibroblasts. Satellite cells/MPCs, myoblasts, primary and secondary myocytes represent a range of muscle cells in order from the most stem-like to the most differentiated/mature. Adipocytes and their ADSC precursors are fat cells that could be useful for producing the fat component of cultivated meat products, while fibroblasts are connective tissue cells that could help provide texture and structure to the products. A major remaining technical hurdle for cultivated meat production is how to grow and manage all these cell types simultaneously. The term "muscle tissue cells" in the present review will be used in cases that refer to all these cell types collectively, while the more accurate and specific terms will be used when discussing a particular cell type.

There are several specific technical challenges still hindering low cost scale-up and commercialization of cultivated meat products; many of them are related to the design of the media that is used to culture the animal muscle tissue cells [19,20]. In most other animal cell culture applications, expensive and/or animal-derived ingredients play important roles in media formulations. Many of these components are commonly supplied by fetal bovine serum (FBS), which is the serum fraction of blood taken from a fetus within a slaughtered pregnant cow. Clearly, there are ethical concerns with this practice, but also FBS is inherently variable from batch to batch and is mostly undefined and expensive [21]. We have previously demonstrated that these differences in FBS can directly lead to changes in muscle phenotype that could dramatically alter meat quality [22]. This leads to technical challenges when serum is used even in mainstream applications. As such, replacing serum or other animal-derived ingredients remains a

primary challenge for cultivated meat media formulation, including for cell proliferation and differentiation. Other questions remain such as how media can be used to influence the organoleptic and nutritional qualities of cultivated meat products. In addition, with the large number of media components typically present in commercial media, sophisticated methods for experimental optimization of the components and their concentrations will likely be necessary to develop new media matched to the desired characteristics. Therefore, the present review seeks to provide insight into the myriad design considerations involved in cultivated meat media.

The Expected Modes of Cultivated Meat Production

In thinking about developing cell culture media for cultivated meat production, it is important to consider how media will influence product processing. Certainly, the main uses of media will be to grow stem cells and differentiate them into muscle, fat, and connective tissues. However, the way this is accomplished will depend on the desired product—either an unstructured product (for example, a burger or sausage product) or a structured product (such as a chicken breast or beefsteak). Therefore, it is instructive to think about these two cases separately.

It is likely that the first products to market will be unstructured products. While various scenarios could be imagined for production of the necessary cells, perhaps the most likely would be to proliferate embryonic or induced pluripotent stem cells through all inoculum stages to the final growth and differentiation scale [23]. If a cultivated meat cell culture scale were 25,000 L, as an example, which would be considered large-scale for mammalian-cell-based biotherapeutic production, approximately 10 successive cell inoculum stages would be needed (assuming a 20% inoculum at each stage, as would be common in biotherapeutics) [24]. With each one of these stages representing approximately three to four doublings, cells will need to survive in growth

media, without differentiation, for 40-50 generations. In this scenario, it would only be in the last stage, largest scale bioreactor that the medium would be changed to promote differentiation to the required cell types (muscle, fat, and connective tissue cells), probably in separate bioreactors and then blended together to achieve the desired mix for the final product formulation [25]. While this would be easiest for true suspension culture, it is at least possible that growth of these typically adherent cells might need to be in conjunction with edible microcarriers, which could further complicate media optimization [25,26].

An alternative approach to creating unstructured products would be to scale up growth of each constituent type of cell (muscle, fat, and connective tissue). This could be accomplished using more differentiated muscle tissue stem cells. Proliferation for a large enough number of generations to get to scale; however, could be problematic for these cell types. Their culture systems could be manipulated to enhance cell stemness and proliferation [27], but much is still unknown about this possibility and it is outside the scope of the present review to discuss the complexities that would be implicated.

It is the ultimate goal of the cultivated meat industry to produce structured products as well. It is possible that all cells will be grown and differentiated on an edible scaffold, though supplying oxygen and nutrients throughout this structure will not be an easy task, nor will differentiation into more specialized tissue types *in situ* [1]. Another possibility would be to take the same approach as for unstructured products, except to take differentiating or differentiated cells from the last-stage production bioreactor as described above and print them onto an edible scaffold in the desired pattern. This could be followed by one to two generations of growth *in situ* to solidify the form of the tissue.

For either of these scenarios, using different sources of cells (e.g. beef, pork, chicken, turkey, fish, etc.) will likely necessitate re-optimizing unique formulations for both growth and differentiation media. While it is not yet clear just how different these optimized media will be for each desired production stage, scheme, and species, they will likely involve varying concentrations of the signaling substances that regulate proliferation and myogenesis. Regardless, all media will need to be food grade, animal product-free, and inexpensive in order to develop and sell a viable cultivated meat product.

The last point is that the media cost is critical to product viability. Therefore, it is important to think about the expected costs for media used for cell proliferation and differentiation at scale when considering feasibility of commercial processes for producing unstructured or structured products. Others have delved into the theoretical cost reduction potential of media for cultivated meat. It has been suggested that the media required to produce one kilogram of cultivated meat can feasibly achieve a cost as low as around 5.00 USD (assuming around 23 L will be needed), which is below the average cost per kilogram of most conventional meat [23]. This estimate is also far below current prices for commercial culture media, pointing to the critical importance of further development, even when accounting for economies of scale. These figures take into account that cultivated meat will be measured in terms of wet cell (product) weight, while most existing industrial cell culture products are measured in terms of dry cell weight.

Mainstream Media Formulations and Uses

Animal cell culture is a well-established technique used in several industries, such as biomedical research and biopharmaceutical production [24]. Unlike in the food industry, these industries operate with a very high final product value, which therefore justifies large production

expenditures and minimal cost optimization effort. Nevertheless, cell culture's long history has yielded an understanding of the growth requirements of cells *in vitro* and an array of well-established media formulations [28,29]. Animal cell culture media consists of everything cells need to survive, as well as any additional components needed to elicit desired cellular behaviors like proliferation, attachment, or hypertrophy. Fundamentally, basic media is composed of carbon and nitrogen sources such as glucose, glutamine, and other amino acids; vitamins and inorganic salts; signaling molecules like growth factors; and buffers [29]. There have been numerous published studies and reviews discussing general and particular aspects of conventional culture media (cited throughout the present review); therefore, the goal of this section is not to detail every basic aspect of culture media, but rather to describe how conventional media formulations can inform media development for cultivated meat applications.

In the biological and medical research fields, cell culture media is often taken for granted. To facilitate easier reproducibility and sound scientific control, researchers often use the same media formulations across a wide variety of studies with different cell types and culture protocols. A range of basal media are commercially available; they generally supply most, but not all, of the basic components needed for cell survival and growth, including glucose, amino acids, and vitamins. Traditionally, basal media formulations are supplemented with complex animal-derived components (namely serum) that supply many trace nutrients and signaling molecules [29]. Basal media formulations have evolved over time and have several permutations for various applications; their history and uses have been well-documented. Among them, Eagle's Minimal Essential Medium (MEM) [30], Dulbecco's Modified Eagle Medium (DMEM) [31], and Ham's F-12 [32]—whose formulations are compared in **Table 1**—have emerged as the gold standards due to their versatility and widespread use, despite being originally developed more

than 60 years ago for human HeLa or specific rodent cell lines. DMEM, noted by its excess of amino acids, is clearly not optimized for minimum cost, which is an example of the fundamental difference in approach to media formulation between the biomedical and cultivated meat industries. Furthermore, commercial media formulations like DMEM do not generally attempt to provide nutrient concentrations that are similar to the nutrient supplies that cells would be exposed to *in vivo*. This has been shown to alter the transcriptome and metabolic profile of cells grown *in vitro* compared to their counterparts *in vivo* [33]. Nevertheless, derivatives of DMEM are in widespread use in current laboratory-scale cultivated meat research due to their familiarity, availability, and convenience [26,27,34,35].

The prophylactic use of antibiotics is also common in cell culture for biomedical research, even with inherent undesirable side effects on the cultured cells [36,37]. Despite an increased challenge in maintaining cell cultures free of bacterial contamination, the absence of antibiotics has been shown to allow for more facile serum-free media adaptation [34]. Moreover, the use of antibiotics in cultivated meat production could provide a problem for consumers sensitive to specific antibiotics and potentially contribute to the rise of antibiotic-resistant bacteria—both possible hinderances to consumer acceptance of the products. Standard use of antibiotics is therefore precluded from cultivated meat process design, as is typically the case in existing industrial-scale animal cell culture processes such as for monoclonal antibody production.

Balanced salt and buffer solutions form the basis of defined media formulations [29]. They are important in the regulation of pH and osmolarity and may secondarily be a source of some nutritional minerals. The pH and ion balances in culture media need to emulate physiological conditions (typically around pH 7.4) for optimal cell growth. Historically, phosphate buffered

saline (PBS) has served as an important salt solution for cell culture [38]. Bicarbonate buffer systems, in conjunction with ambient CO_2, are frequently employed in animal cell culture at bench scale, but this requires careful monitoring of the CO_2 concentration in the culture environment [30,31]. HEPES, one of the classical zwitterionic buffers described by Good *et al.* [39], is commonly used to supplement the bicarbonate system's buffering capacity; it displays quality buffering performance and relatively minimal cytotoxicity. However, it is an expensive ingredient and is often a primary cost driver of basal media formulations [23]. As such, less expensive buffering agents, such as TES or complex ingredients (see below), could be tested in cultivated meat media where buffering capacity is a concern. However, given that industrial culture processes allow for stricter control of CO_2 concentration compared to research settings, the less expensive bicarbonate buffer system alone is likely sufficient to control pH.

The stability and consumption rates of media components must also be considered. Whether due to light or heat exposure, mutual interactions, or variable consumption rates at different stages of the production process, the concentration and activity of media components will vary [29,40]. Many of the common media components can be adversely affected by excessive light and heat exposure [41]. This means that conventional media for muscle tissue cell culture generally cannot be heat sterilized; instead, they are usually filtered or irradiated to ideally eliminate all microorganisms. Because heat sterilization is likely a more efficient process at industrial scale, formulation of cultivated meat media using more heat stable ingredients should be considered, though this idea has not yet been directly tested. Heat stable, defined serum-free media have been developed for other cell types and applications [42], which lends credibility to the notion that this may be possible for cultivated meat.

It should be noted that the culture media is likely to influence the sensory qualities of harvested muscle/meat. Logically, residues of the media on or in the cells may impart flavor, texture, or color. For example, glutamic acid and asparagine are the well-known contributors to the umami flavor component of meat, and these amino acids are ingredients in some cell culture media [43]. As such, this introduces a new dimension to media formulation that must be considered. Some recent published studies with preliminary sensory tests indicate that laboratory-scale cultivated meat prototypes have acceptable organoleptic properties [35,44,45], and thus the sensory qualities of culture media do not appear to be of immediate concern. However, to date there have been no studies investigating sensory effects of media specifically, these sensory aspects of the media will become more important as the industry develops.

Nutrient Sources in Culture Media

Over the roughly 100-year history of cell culture, knowledge of the nutritional requirements of animal cells grown *in vitro* has developed [28,29]. While different cell types within and between species understandably have different nutritional and culture requirements, there are several common requirements that general basal culture media formulations can easily satisfy. Muscle cells, like all cells, utilize extraneous substances as sources of energy and as building blocks for cellular structures. The important macro-scale ingredients in culture media that satisfy these basic cell requirements can broadly be classified into two groups: carbon sources and nitrogen sources. This concept is commonly applied in industrial microbial and animal cell culture. The rates and modes of carbon and nitrogen utilization are regulated by availability, extracellular signaling, and the proliferative status of the cells [46]. Necessary micro-scale ingredients for cultivated meat media meet the cells' vitamin and inorganic ion requirements, but

these are less likely to significantly contribute to culture media costs at scale, so they require less discussion and investigation [23]. Accordingly, the present section will discuss the function and cost reduction potential for carbon and nitrogen sources as they relate to cultivated meat.

Carbohydrates, amino acids, and lipids are the fundamental classes of carbon sources for metabolism of muscle tissue cells. While a variety of carbon-based molecules can be used as nutrition by microbes and by animals at the organismal level, most cultured animal cells lack the biochemical ability at the fundamental level to efficiently utilize anything but glucose, glutamine, and a limited number of other amino acids and fatty acids [46,47]. Animal cells may make use of alternate sugar substrates, such as fructose, pyruvate, maltose, and sucrose, but generally these are metabolized significantly less efficiently than glucose and do not appear to be optimal for cell growth [47–49]. Likewise, while providing some fatty acids for cells in the culture medium may reduce the metabolic load involved in *de novo* fatty acid synthesis, most animal cells do not require extraneous fatty acids [50]. The different cell types of potential importance in cultivated meat—myotubes, satellite cells/myoblasts, fibroblasts, adipocytes, and more stem-like precursors of these cells—have differing metabolic rates and needs, as do cells from the different species of interest [51]. This creates the potential need for media formulations specific for the cell type of interest in a particular meat product production line; however, the more subtle metabolic differences between the cell types require further elucidation.

Cells utilize a variety of carbon source uptake mechanisms, such as transporter-mediated uptake, receptor-mediated endocytosis, and nonselective macropinocytosis [46]. After uptake and phosphorylation, glucose is used as the substrate for several metabolic pathways to generate adenosine triphosphate (ATP) for energy and to synthesize biomolecules like nucleotides, fatty acids, and amino acids. The major catabolic glucose metabolism pathways include glycolysis

(and subsequent fermentation or oxidative phosphorylation) and the pentose phosphate pathway [52]. Through glutaminolysis, glutamine functions as a carbon source by yielding α-ketoglutarate that is then used in a number of biosynthetic pathways [53,54].

Oxygen is important as a final electron acceptor in oxidative phosphorylation of carbon sources, and it is very important that cultivated meat media be constantly supplied with an appropriate concentration of oxygen to ensure efficient carbon metabolism [55]. There are challenges associated with this oxygen requirement at industrial scale. While bioreactor process design is outside the scope of the present review, it is worth noting that efficient oxygen control techniques (such as cascade control format) have been established for existing industrial animal cell culture applications. When oxygen gas is added to bioreactors, the physical forces that the gas bubbles may create (especially as they reach the liquid/gas interface at the surface of the media working volume), and their effects on the cells, must be considered. Industrial cell culture media now commonly make use of synthetic block copolymer surfactant additives such as poloxamers (e.g. Pluronic®) to reduce cell damage caused by shear forces due to gas sparging and agitation [56,57]. Poloxamer additives are likely usable for cultivated meat media since they have been approved by the US Food and Drug Administration for various food and drug applications. Moreover, protein components of cultivated meat media, such as plant hydrolysates or yeast extracts, could have surfactant properties that may reduce the need for synthetic additives like poloxamers.

Glutamine—and other amino acids—are perhaps most significant in their roles as nitrogen sources. Nitrogen is an integral component of functional and structural cell molecules, namely proteins and nucleotides. Heightened nitrogen anabolism is the driver of the increased nitrogen demand in proliferating cells [58]. Different animal species require trophic sources of

different amino acids (essential amino acids), usually at least histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine [59]. Some cell types have additionally been reported to be able to utilize the nitrogen in ammonia [60,61], but glutamine still carries great importance since it can be metabolized into many other amino acids used for protein assembly [58]. Glutaminolysis is also a mode of NADPH regeneration—the reducing agent necessary for many anabolic pathways—further highlighting glutamine's importance in cell proliferation [52]. Because of problems with ammonia accumulation in the media due to glutaminolysis, animal cells in industrial cell culture are often genetically modified to produce their own glutamine from glutamate; however, genetic manipulation of cells for cultivated meat production may not be desirable or possible, but such a modification could be explored. Therefore, based on the present understanding of carbon and nitrogen source metabolism by animal cells, the availability of free glucose and glutamine in the media for proliferating cultivated muscle tissue cells seems essential, as well as a sufficient to supply the essential amino acids specific to the animal species in question.

Actively proliferating muscle cells (myoblasts), as are necessary in cultivated meat production, understandably have unique metabolic activities and needs. Much of the current understanding of myoblast metabolism is derived from studies on cancer cells, since cancer cells are likewise rapidly proliferating cells. The Warburg effect describes the preference of cancer cells to derive most of their ATP from glycolysis, and allow most pyruvate to be converted to lactate, even in the presence of sufficient oxygen [62]. This effect is not limited to cancer cells; however, it is a hallmark of all rapidly proliferating cells. Glucose metabolism via the Warburg effect as opposed to oxidative phosphorylation yields several metabolites that are valuable to proliferating cells undergoing high rates of biosynthesis and division [62–64]. It would follow that

media for cultivated meat production could potentially be formulated to stimulate and support the Warburg effect to promote muscle cell proliferation. The Warburg effect has successfully been induced *in vitro* by genetic manipulation and by other intrinsic means such as stimulation of hypoxia inducible factors (HIF) and inhibition of pyruvate kinase M2 isoform by tyrosine phosphorylation [65–67]. These findings suggest additional methods of promoting *in vitro* muscle cell proliferation, such as modulating the oxygen concentration in culture media. However, many of the stimulatory mechanisms controlling the Warburg effect continue to be elusive, and its potential application to cultivated meat remains to be investigated.

Common animal cell lines used in biopharmaceutical production, such as Chinese hamster ovary (CHO) and murine myeloma lines (Sp2/0, NS0), have been selected or modified for highly efficient growth and metabolic behaviors that are also desirable in cultivated meat applications [24,68]. Future research should investigate whether aspects of the culture media formulations used for these biotechnological cell lines can be translated to cultivated meat media, keeping in mind that the cells themselves (functional or not) are not the desired final product in existing biotechnological processes, whereas the cells are the end product in cultivated meat processes. Media formulations for existing cell lines are often proprietary, as are the media currently being developed in the commercial cultivated meat industry, meaning that translating knowledge between the two industries could prove difficult.

Vitamin requirements differ between *in vivo* and *in vitro* contexts, due to differential metabolic needs and synthetic capacities of various cell types. For example, some fat-soluble vitamins are important for specific cell types in certain contexts, and may in fact help promote myogenesis [69], but in general they are likely not necessary in cultivated meat cell culture applications. The water-soluble (B and C) vitamins are considered to be the most important for

inclusion in basal animal cell culture media, and they have been relatively well characterized in this application [70]. Importantly, vitamin C is essential for the production of collagen protein, by fibroblasts, that will be necessary for the structural integrity of cultivated meat. The water-soluble vitamins are not necessarily stable in final media solution for extended durations, so care must be taken to ensure a sufficient supply of fresh and active vitamins in cultivated meat media [70]. Production of vitamins via industrial microbe fermentation or chemical synthesis is relatively easy and inexpensive [71], making vitamin sourcing costs a less pressing issue in cultivated meat cost reduction. However, vitamin supply by minimally processed plant extracts has yet to be explored fully.

The requirements of inorganic ions (minerals) that help control cell growth, constitute cellular structures, and regulate media osmolarity, are similarly varied depending on cell type and context [52,72]. Minerals having a relatively high importance in general cell culture media include bicarbonate, calcium, iron, magnesium, phosphate, potassium, sodium, and sulfate. Minerals required in trace amounts that have roles in the molecular regulation of metabolism and cell function include chromium, cobalt, copper, iodine, manganese, molybdenum, selenium, and zinc [28,52]. The major and trace minerals needed for muscle cell culture can generally be obtained relatively inexpensively [23]. A few important trace minerals, such as selenium, are usually directly supplemented in commercial defined media [29], but other typical media components, as well as simple plant extract ingredients, should be studied regarding their ability to supply the required minerals. As an example, Primatone RL, a proprietary tryptic digest of meat, has been used as a complex low-cost supplement for industrial animal cell culture, satisfying several growth requirements (although it could be unsuitable for cultivated meat due to its animal origin) [73].

Ingredients serving multiple functions in cultivated meat media could help to control final media costs.

Regulation of Proliferation, Differentiation, and Protein Synthesis

As discussed above, at some point in the cultivated meat process, stem cells will need to be differentiated into muscle and other types of cells at the large scale. The media requirements for this step will almost certainly be distinct from those mainly supporting cell proliferation. The complex interplay of extracellular signaling molecules, intracellular signaling pathways, and transcription factor activity ultimately determines muscle cell behavior [74]. A thorough understanding of the signaling molecules and molecular pathways implicated in myogenesis benefits the cultivated meat researcher by providing insight into the functions that cell culture media need to recapitulate. By providing molecules that can activate the same complement of signaling pathways necessary for myogenesis or other types of differentiation, different cell culture media formulations can regulate the proliferation and differentiation of muscle cells. Because a primary goal of cultivated meat is to provide high-quality dietary protein, an understanding of the molecular pathways involved in cellular protein synthesis is important as well.

Myogenesis is the development of muscle tissue with the goal of repair, homeostasis, or *de novo* formation. *In vivo* it is a complex suite of events occurring in both embryonic development and adult muscle regeneration, involving several stages with various molecular mechanisms and cell types. The proliferating cells are muscle precursor cells (MPCs; known as myoblasts in the embryo and satellite cells in the adult) that can differentiate and fuse to form *de novo* myotubes

or join existing myofibers. The processes of proliferation, differentiation and fusion are associated with the activity of several known growth factors, transcription factors, and signaling pathways. These signaling factors have been studied in model systems from rodent to human, and for this review we assumed that the general molecular pathways are shared between all of these species for the sake of discussing our general understanding of what is necessary for cultivated meat production. **Figure 1** provides a simplified graphical overview of the myogenic process.

In MPCs, the early myogenic regulatory factor (MRF) genes *Myf5* and *MyoD* drive proliferation [75]. These MRFs are essential for the proper formation of skeletal muscle, as their absence prevents the expansion of the myoblast pool [76]. During differentiation, a second group of MRFs, *MRF4* and *Myogenin* [77], are upregulated, driving differentiation and fusion, and helping to maintain the adult muscle structure. Beyond their biological functions, these genes serve as important markers for monitoring and optimizing media in cultivated meat applications. Extracellular signaling molecules, which in most cases would need to be supplied by the media in cultivated meat, can regulate the activity of MPCs. A number of growth factors and cytokines have been determined to play a role in MPC activation and proliferation [78], such as: fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β), and other cytokines like tumor necrosis factor-α (TNFα) and leukemia inhibitory factor (LIF). FGFs, especially FGF2, are known as potent activators of MPC proliferation and inhibitors of MPC differentiation [79]. HGF is predominantly important in the activation and chemotaxis of MPCs to promote muscle repair [80] and therefore is likely not important for cultivated meat applications. FGF is known to induce proliferation but block differentiation *in vitro* [81]. IGFs promote muscle development through the stimulation of both

MSC proliferation and differentiation [82], as well as through stimulation of protein synthesis within developing myotubes [83]. TNFα may promote myoblast differentiation [84], while LIF promotes myoblast proliferation but not differentiation [85]. Conversely, TGF-β ligands such as TGF-β1 and myostatin are known to inhibit both the proliferation, differentiation, and growth of MPCs and myotubes [86,87]. The presence of fibroblasts in coculture with MPCs has interestingly been shown to promote MPC proliferation, differentiation, and fusion [88].

Though adipogenesis is a highly ordered process, the extracellular factors modulating adipogenesis are arguably less complex than those that control myogenesis. When desired in a cultivated meat application, differentiation of pluripotent, or adipose-derived, stem cells into adipocytes can be stimulated by insulin and IGFs, and can be monitored by measuring levels of the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT enhancer-binding proteins (C/EBPs) [89]. Conversely, Wnt/β-catenin signaling can inhibit adipogenesis [90]. The differentiation and proliferation of fibroblasts is not a pressing concern in cultivated meat, since they are not the primary desired cell types in most cases, and they tend to be more robust to suboptimal conditions [91]. The fibroblast's role in cultivated meat is not to provide substantial bulk, but rather to support myocyte and adipocyte growth and to manufacture enough extracellular matrix to produce a more realistic meat product. A specific concern related to fibroblasts, however, is that vitamin C will be important to supplement in the media for efficient fibroblast production of collagen. Overall, cultivated meat media will need to provide some of the above important growth factors, or substitutes thereof, to allow proper proliferation and differentiation of the desired cell types.

The above signaling molecules function to stimulate myogenesis through a multitude of molecular pathways. Like many growth factor receptors, the IGF-1 receptor is a primary

activator of tyrosine kinase-linked signal transduction cascades such as the phosphatidylinositol-3 kinase (PI3K)-Akt, and extracellular signal-related kinase (MAPK/ERK) signaling pathways [92,93]. These pathways can activate transcription factors that promote proliferation and increase protein synthesis via the mechanistic target of rapamycin complex-1 (mTORC1), helping drive muscle tissue development [94,95]. Many cytokine receptors, such as the IL-6 receptor, invoke the JAK-STAT signaling pathway [96], while TGF-β receptors are serine/threonine kinase receptors and activate their own distinct signaling pathway involving SMAD protein phosphorylation [86,97]. The JNK/MAPK signaling pathway inhibits myogenesis, and it has been reported that several miRNAs function in muscle cells to repress this pathway while promoting the p38/MAPK pathway [98]. Inhibition of the p38 pathway (by a small molecule therapeutic) has been implicated in the retention of stem-like proliferation capacity in bovine primary satellite cells [27]. Multiple Wnt protein signaling pathways also play a role in myogenesis through the Frizzled family of G-protein coupled receptors. The canonical β-catenin mediated Wnt3a pathway has been shown to have stimulatory effects on myogenic proliferation and differentiation by antagonizing Notch signaling [99,100], while Wnt7a can activate the noncanonical Wnt/planar cell polarity (PCP) pathway to induce MPC proliferation [101]. Taken together, cultivated meat media needs to facilitate coordinated stimulation of a variety of types of intracellular signaling pathways, and future research should investigate the most inexpensive and efficient ways of doing so (discussed below).

Protein synthesis is an energetically-expensive cell function [102], but myoblasts have the means to supply large amounts of energy for this purpose [103]. Protein synthetic activity is regulated through mTOR-mediated signaling, and this has been reviewed extensively [104,105]. Amino acids can serve as regulators of protein metabolism, in addition to being the building

blocks of proteins themselves. Leucine and its metabolites are known to play important roles in promoting protein synthesis in myocytes, through activation of mTORC1 [106–108]. The leucine metabolite, β-hydroxy-β-methylbutyrate (HMB), has been shown to enhance muscle protein synthesis and promote muscle tissue hypertrophy *in vivo* [107,109]. Leucine and HMB, therefore, may be useful targets for enhancing *in vitro* muscle cell culture media; however, this has yet to be fully investigated.

Due to the cost associated with many of the purified growth factors and signaling molecules used for *in vitro* myogenesis and protein synthesis, less expensive alternative methods of stimulating the required cellular signaling pathways are likely necessary. One such method to achieve this could be to screen homology between amino acid sequences for cell signaling molecules and common plants or fungi. If significant sequence homology is identified in certain plants/fungi, those can then be tested for use in muscle cell culture media. One example of how plant extracts have been used as agonists for mammalian cell growth factor receptors is the use of cowpea (*Vigna unguiculata*) peptide extracts to stimulate insulin-associated cell signaling pathways [110,111]. More generally, wheat and cotton peptones have shown promise in acting as substitutes for bovine serum albumin in the culture of bovine embryos [112].

Strategies for Serum-Free Media Formulation

Due to the cost, variability, contamination, and ethical issues mentioned earlier, the animal serum that is used in conventional biomedical cell culture media needs to be efficiently replaced if cultivated meat is going to meet the ethical and cost goals set by the market. The multifaceted problems associated with FBS, and other animal sera, are of significance to the biomedical realm as well, and have thus been reviewed extensively [21,113–116]. The present section

therefore focuses on the existing and possible strategies to replace animal sera in the context of cultivated meat.

Serum supplies not only the growth factors and hormones that stimulate proper cell growth *in vitro*, but also many nutrients [29]. Many of the major functional constituents of serum have been revealed, but their concentrations are highly variable and there are still many unknown components [117]. For reference, **Table 2** lists approximate concentrations of many of the known components of animal serum. For the above reasons, formulating serum-free media is not always as simple as supplementing basal media with a few growth factors. Like serum-containing basal media preparations, there is no single serum-free formulation strategy that works efficiently for every cell type in every situation; different cells have different requirements. As such, there are many serum-free formulations and supplementation protocols that have been described or made commercially available [115]. In almost all cases, however, these still contain animal- or human-derived ingredients, are not optimized for efficient muscle cell cultivation, and/or remain too expensive [115]. Commonly, serum-free media for biopharmaceutical production or medical research are supplemented with expensive growth factors (especially insulin, FGF2, and TGFβ) isolated from animal serum or produced recombinantly [118,119], since these industries are not encumbered by the same cost restraints as the food industry.

Essential 8™ media is one such defined animal-free media formulation (given in **Table 1**) that is commonly used in research settings and is designed to promote stem cell growth, especially that of human pluripotent stem cells (hPSC) [120]. As its name suggests, it consists of eight ingredients, one of which is normal DMEM/F12 basal medium. The remaining ingredients include insulin, FGF-2, and TGF-β as the primary recombinant growth factors, as well as additional vitamin, mineral, and buffer supplementation. Fibroblast Growth Medium™ is

another similar commercially available serum-free media optimized for human primary fibroblasts. These two types of media were recently shown to have promise in cultivated meat applications, as they were able to support the proliferation of primary bovine myoblasts for at least six days [34]. However, they achieved only roughly one doubling, compared to the roughly 3-fold proliferation achieved by 20% FBS and 10% horse serum medium. Further, the ability of these media to promote long-term and high-density proliferation of bovine myoblasts has not been assessed to date, nor has their ability to sustain myoblast markers or allow retention of differentiation capacity. These media would also be prohibitively expensive if used at production scale; nevertheless, they demonstrate the potential for animal myoblasts to be cultured in serum-free media and provide useful insight.

Clearly, much more innovation and optimization need to be performed to achieve efficient and low-cost serum-free media for cultivated meat. Further optimization of recombinant growth factor production holds promise as a solution for cultivated meat supplementation, potentially through genetic modification of the cultivated cells themselves, so that they endogenously express the proteins [121]. However, much more research and optimization are required to achieve this at low cost. Additionally, this manipulation of the cultivated meat cells could ultimately deter potential consumers concerned about genetically modified foods. Another suggested method of replacing serum is the use of human blood platelet lysates, as a byproduct of conventional human blood donations [115,122]. Platelet lysates contain many of the growth factors, cytokines, and attachment factors that are useful for cultivated meat production. However, there are several drawbacks to this potential approach, including cross-species compatibility, cost, and supply logistics [123]. Conditioned media systems have also been suggested as a solution to replace serum—the growth factors and other useful products that the cultivated meat cells produce

themselves can be recycled and concentrated to support growth of other new cells in an interconnected system of bioreactors [124]. As will be discussed in more detail in a later section, there is the possibility that plant or fungal protein extracts can function as homologs for important growth factors and other regulatory proteins in cultivated meat media, but this remains to be investigated fully [111,125].

In any case, cell lines must be adapted slowly over time to new media formulations, especially when introducing serum-free media [126,127]. Usually, an adaptation protocol will involve reducing the serum concentration by around 50 percent with each subsequent culture passage until less than one percent of the original concentration remains. In its place, the serum replacement formulation can be gradually introduced until the serum is no longer needed. While this general procedure has been performed with many animal cell types, it has not been well characterized for primary muscle cell lines. Achieving both a muscle cell line adapted to have a reduced requirement for growth factors and other serum components, as well as animal-free media that can inexpensively replace the function of serum, remains a critical goal of the cultivated meat industry.

Media Formulation for Large-Scale Food and Commodity Chemical Fermentations

Fermentation is used as a means of producing a large variety of products, from food to commodity chemicals to therapeutic recombinant proteins. While some animal cell lines, such as myeloma and CHO cells, are used at large scale to produce therapeutic proteins [24], the price of these products (millions of dollars per kilogram) is such that process optimization and media cost reduction are not driving forces in commercialization. However, microbial food fermentations

producing products such as beer and yogurt, and specialty chemical production through fermentation, such as Vitamin C, citric acid, MSG, xanthan gum, and medicinal feed additives (antibiotics for livestock feed), necessarily need extensive media and process optimization to be competitive [128]. This is especially true of the latter category, in which media need to be optimized and inexpensive with multiple components supplying the required nutrients. In these cases, fermentations are generally large scale (>40,000 L) and product prices are low (generally less than $1-10/Kg), similar in scope to goal costs for cultivated meat.

In cases where media costs need to be kept especially low for profitability, the use of complex media ingredients is common. Generally, completely defined media are too expensive to make these processes viable. In many microbial fermentation systems, this means that complex ingredients (typically of agricultural origin) are substituted for defined sources of all key nutrients [128]. **Table 3**, adapted from [129], compares the compositions of several of these complex agricultural byproducts. Many times, these complex ingredients supply not only one key nutrient, but several, allowing for fewer overall media ingredients. For example, instead of using purified sugars such as glucose as a carbon source, these microbial fermentations will use less refined sources of sugar, such as molasses, corn starch, or malt powder/maltose [128]. Of course, usability of these more complex sugars sources for cultivated meat production will depend on the ability of animal cell lines to utilize or at least tolerate sugars other than glucose, as they often contain fructose, sucrose, maltose, and other more complex carbohydrates. Similarly, these large-scale microbial fermentations often use more complex nitrogen sources that are by-products of agricultural processing of grains. Typical examples of these include corn steep solids, corn steep liquor, soy flour, or peptones/enzymatic hydrolysates of plant- or microbe-based material such as yeast or soy extracts. The ability of animal cells to use these

types of nutrient sources has not been extensively tested, and the potential cytotoxicity of the impurities found in them remains a concern. However, peptones of plants and fungi have been used as nutrient and metabolite sources for animal cells in specific applications using specialized cells such as CHO [130,131], but their usefulness in cultivated meat media has not been thoroughly investigated. Commercial grades of these latter materials can be quite inexpensive but may not be as defined or consistent as their laboratory analogs. When needed, fatty acids are often supplied by the addition of vegetable oils or semi-purified components such as oleic or linoleic acids, often direct precursors of products such as polyketide antibiotics in large scale production [128].

Many of these complex ingredients supply more than just sugar or amino acids. Molasses and yeast extract, for example, are also rich in minerals (e.g. phosphorous or metals) and B-vitamins[132]. In **Figure 2**, the relative nutrient levels of two complex fermentation media are compared with those of Essential 8. The two media chosen are YPD, a common complex medium used for yeast growth, and an industrial medium (MFA) used to grow *Streptomyces* spp. for production of a medicinal feed additive (antibiotic for addition to animal feed). Media are compared for the broad categories of carbohydrates, free amino acids, minerals, and vitamins. While each production organism will likely have its own specific nutrient requirements within these broad categories, it can be seen from this figure that the complex ingredients that comprise the YPD and MFA media (the approximate nutrient profiles of which are provided in **Table 3**) can easily supply the general nutrition present in Essential 8 when supplied in some combination.

Because of the composition of these complex nutrients, they often preclude the need for specific buffers to be added to the media. They may also be able to substitute for specific proteins currently added to cell culture media if they contain analogs for the active domains of

these proteins, though this has not been thoroughly studied to date. As discussed above, this latter point would be important to investigate, since the specific protein components of cultivated meat media (such as growth factors) are some of the most expensive components of current media [23]. Finally, because of the complexity of these ingredients or limited solubility of their components, these ingredients can sometimes substitute for slow defined nutrient feeds that might otherwise be added in fed-batch fermentation processes [128]. This can simplify the processing but may lead to increased variability due to decreased control of nutrient additions.

Of course, there is a tradeoff between the use of complex and defined media ingredients. While complex ingredients can be considerably less expensive than their defined counterparts, they tend to introduce variability in processing [128,133]. This variability can be introduced because of seasonal differences in agricultural feedstocks or by processing differences between different suppliers of similar ingredients. Geographic differences of the agricultural feedstock origins can also contribute to variability if materials are sourced over a large area. Variability can also be a result of long-term storage of these materials at the production site. These agricultural materials will likely be stored at room temperature or outside temperatures in large quantities and may change over time. Some of them may be prone to chemical or microbiological degradation, or as in the case of molasses, may have solids that settle out over time causing variation in a non-stirred environment.

In addition to these potential media sources being nutrients for cell growth, they are all food grade and could potentially add desirable sensory qualities to the media, as mentioned above. Many of these ingredients are somewhat stable in high heat, allowing steam sterilization as the means for preparing for fermentation which is considerably less process intensive and expensive than filtration (typically used for cell culture media), especially as the fermentor tank itself still

needs to be steam sterilized, even when filtration is used for the media. While some component degradation may happen at high temperatures, especially if nitrogen and sugar sources are mixed, it is at least possible that this could have positive sensory implications for the cultivated meat product downstream.

Media Optimization Methods

Once media components of interest have been determined through experimentation and/or theory, their concentrations need to be optimized. Due to the large number of components likely to be used in cultured meat processes (DMEM has 30-52, for example) this is a combinatorically difficult and time-consuming process, especially as critical interaction effects can occur [134]. Additionally, due to physiological variability of cell lines, re-optimization may be needed as processes change and new components are identified, especially in an industry expected to work on a variety of different cell lines [135]. Therefore, efficient means of identifying and adjusting concentrations is necessary.

Traditionally, media design is initially conducted using a one-factor-at-a-time (OFAT) basis where a single component is considered for its effect on cell response. The drawback of this approach is that interaction effects can go unnoticed, leading to suboptimal media designs [136]. This is illustrated in the **Figure 3**a, where the nonlinear interactions mask the true optima if a simple OFAT method is used to follow the direction of steepest descent. *Design of Experiment* (DOE) techniques such as *Factorial*, *Plackett-Burman*, and *Central Composite* designs, where multiple nutrient concentrations are changed at once, allow for much faster optimization, and have successfully been used to characterize and optimize monoclonal antibody production and bacterial culture processes [137–141]. These experiments are typically conducted at the 'lower' and

'upper' extrema of the media design space as seen in **Figure 3**b. This arrangement allows first order 'effects' for each media component to be estimated without being convoluted with other media component's effects via analysis of variance methods. These methods can be combined with response surface models (**Figure 3**c), such as a linear or polynomial model to map inputs X to outputs Y, to sequentially improve mixtures by predicting optimal media component concentrations [142]. These methods can still require many experiments to be successful, and often become experimentally burdensome when optimizing more than 5 design variables, whereas typical industrial media may have 50-75 components [24,143].

Efficient media design has also been performed using stochastic optimization methods such as genetic algorithms, where media combinations are treated as chromosomes under natural selection pressures [144]. New media are computationally generated as stochastic combinations and mutations of previous media, with the goal of maximizing the fitness of the solution or objective function (maximizing biomass, for example) of each nutrient combination. More recently, stochastic methods have been augmented by mathematical surrogate models (neural networks, for example) that aid in prediction and store information about component effects and interactions through their parameters [141], as shown in **Figure 3**d. In this manner, as more experiments are conducted, the surrogate model enhanced stochastic optimizer can make better predictions about generating optimal media combinations. These methods can handle more nonlinear and interaction-heavy media design problems and have been successfully applied to designing microbial media with 19 components [139], resulting in more optimized media in half the experiments of traditional DOE. These efficient optimization methods continue to evolve and are increasing in robustness [145], hopefully to the point where they can be utilized effectively by practitioners not expert in artificial intelligence and numerical optimization methodology.

While these latter methods have proven to be much more efficient than random or OFAT optimization, it is still important to accumulate knowledge about the organism of interest and its nutrient requirements and molecular physiology in parallel to the more black-box approach to optimization. Therefore, it is advantageous to conduct experiments with each cell line fully characterizing the use and effects of carbon and nitrogen sources, in addition to other key nutrients, as well as the production of key positive or negative metabolites over time in a basal medium. These data will facilitate understanding of processes that can be used to help design new experiments (for example, choosing concentration ranges of key nutrients or feed compositions and rates) and to troubleshoot process issues during scaleup and manufacturing.

Conclusion

Clearly, the design of culture media to achieve scalable, low-cost, and high-quality cultivated meat products remains a complex challenge. Success will likely require careful media formulation combined with innovative adaptation of cells and culture systems to these new media. Continuing research should be focused on developing the understanding of how the molecular mechanisms controlling muscle cell growth and differentiation can be stimulated via more affordable and ethical means. This research can both inform and be informed by more mainstream biomedical research. However, media and processes are much more likely to resemble large scale microbial fermentation processes where commodity chemicals and food components are produced. Overall, the relatively rapid development and expansion in the cultivated meat space in recent years lends confidence to the notion that many of these remaining challenges can be overcome.

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Author Contributions

E. O'Neill and D. Block conceptualized the scope and sections of the review. Z. Cosenza drafted the section and figure about media optimization. D. Block drafted the sections about the modes of cultivated meat production and media for large-scale fermentation and provided expertise on industrial fermentation. K. Baar provided Figure 1 and expertise regarding muscle tissue growth and differentiation. E. O'Neill performed the literature review, drafting and compiling the remainder of the manuscript. All authors reviewed and edited the manuscript.

Table 1: Ingredient concentrations of common commercially available defined animal cell culture media used in biomedical research[a]

Component		Concentration (mg/L)				
		Minimal Essential Medium	**DMEM (high glucose, GlutaMAX, pyruvate)**	**Ham's F-12**	**DMEM/F-12**	**Essential 8**
Amino acids	Glycine		30	7.5	18.75	18.75
	L-Alanine			8.9	4.45	4.45
	L-Arginine hydrochloride	126	84	211	147.5	147.5
	L-Asparagine-H2O			15	7.5	7.5
	L-Aspartic acid			13.3	6.65	6.65
	L-Cysteine hydrochloride-H2O	31	63	35.12	17.56	17.56
	L-Cystine 2HCl				31.29	31.29
	L-Glutamic Acid			14.7	7.35	7.35
	L-Glutamine	292	862	146	365	365
	L-Histidine hydrochloride-H2O	42	42	21	31.48	31.48
	L-Isoleucine	52	105	4	54.47	54.47
	L-Leucine	52	105	13.1	59.05	59.05
	L-Lysine hydrochloride	73	146	36.5	91.25	91.25
	L-Methionine	15	30	4.5	17.24	17.24
	L-Phenylalanine	32	66	5	35.48	35.48
	L-Proline			34.5	17.25	17.25
	L-Serine		42	10.5	26.25	26.25
	L-Threonine	48	95	11.9	53.45	53.45
	L-Tryptophan	10	16	2.04	9.02	9.02
	L-Tyrosine	52	72	5.4	55.79	55.79
	L-Valine	46	94	11.7	52.85	52.85
Vitamins	Biotin			0.0073	0.0035	0.0035
	Choline chloride	1	4	14	8.98	8.98

[a] Formulations as supplied by Thermo Fisher Scientific, Waltham, MA, USA. Blank cells indicate the component is not included in the media formulation.

	D-Calcium pantothenate	1	4	0.5	2.24	2.24
	Folic Acid	1	4	1.3	2.65	2.65
	Niacinamide	1	4	0.036	2.02	2.02
	Pyridoxine hydrochloride	1	4	0.06	2.013	2.013
	Riboflavin	0.1	0.4	0.037	0.219	0.219
	Thiamine hydrochloride	1	4	0.3	2.17	2.17
	Vitamin B12			1.4	0.68	0.68
	Ascorbic acid 2-phosphate					64
	Vitamin A					
	i-Inositol	2	7.2	18	12.6	12.6
Inorganic Salts	Calcium Chloride (CaCl2-2H2O)	200	264	44	116.6	116.6
	Cupric sulfate (CuSO4-5H2O)			0.0025	0.0013	0.0013
	Ferric Nitrate (Fe(NO3)3"9H2O)				0.05	0.05
	Ferric sulfate (FeSO4-7H2O)		0.1	0.834	0.417	0.417
	Magnesium Chloride (MgCl2-6H2O)	97.67	200	122	28.64	28.64
	Magnesium Sulfate (MgSO4) (anhyd.)				48.84	48.84
	Potassium Chloride (KCl)	400	400	223.6	311.8	311.8
	Sodium Bicarbonate (NaHCO3)	2200	3700	1176	2438	2438
	Sodium Chloride (NaCl)	6800	6400	7599	6995.5	6995.5
	Sodium Phosphate dibasic (Na2HPO4) anhydrous	140	141	142	71.02	71.02
	Sodium Phosphate monobasic (NaH2PO4-H2O)				62.5	62.5
	Sodium Selenite (Na2SeO3)					0.014
	Zinc sulfate (ZnSO4-7H2O)			0.863	0.432	0.432
Carbohydrates	D-Glucose (Dextrose)	1000	4500	1802	3151	3151
	Sodium Pyruvate		110	110	55	55
Lipids	Linoleic Acid			0.084	0.042	0.042
	Lipoic Acid			0.21	0.105	0.105
Growth Factors / Hormones	Insulin					19.4
	FGF-2					0.1

	TGF-β					0.002
	Transferrin					10.7
Other	Phenol Red	10	15	1.2	8.1	8.1
	Putrescine 2HCl			0.161	0.081	0.081
	Thymidine			0.7	0.365	0.365
	Hypoxanthine			4	2.39	2.39

Table 2: Approximate nutrient and functional component concentrations in animal serum[b]

Proteins and Polypeptides	**40-80 g/L**
Albumin	20-50 g/L
Fetuin	10-20 g/L
Fibronectin	1-10 mg/L
Globulins	1-15 g/L
Protease inhibitors: α1-antitrypsin, α2-macroglobulin	0.5-2.5 g/L
Transferrin	2-4 g/L
Growth Factors	
EGF, PDGF, IGF-1 and 2, FGF, IL-1, IL-6	1-100 ug/L
Amino Acids	**0.01-1.0 uM**
Lipids	**2-10 g/L**
Cholesterol	3.867 mg/L
Linoleic acid	2.805-28.05 ug/L
Phospholipids	0.7-3.0 g/L
Carbohydrates	**1.0-2.0 g/L**
Glucose	0.6-1.0 g/L
Hexosamine	6-1.0 g/L
Lactic acid	0.5-2.0 g/L
Pyruvic acid	2-10 mg/L
Polyamines	
Putrescine, spermidine	8.815-88.15 ug/L
Urea	**170-300 mg/L**
Inorganic Ions	
Calcium	160.3-280.6mg/L
Chlorides	3.545 mg/L
Iron	0.559-2.793 mg/L
Potassium	195.5-586.5 mg/L
Phosphate	189.9-474.9 mg/L
Selenium	0.790 ug/L
Sodium	3.10-3.57 g/L
Zinc	6.538-65.38 ug/L
Hormones	
Hydrocortisone	3.625-72.49 ug/L
Insulin	5.778-577.8 mg/L
Triiodothyronine (T3)	13.02 ug/L
Thyroxine (T4)	77.69 ug/L
Vitamins	**10 ug - 10 mg/L**
Vitamin A	10-100 ug/L
Folate	5-20 ug/L

[b] Data are adapted from [117]. No specific species source is specified.

Table 3: Approximate average compositions of common agricultural byproducts used in industrial microbial fermentation[c]

Component		Beet Molasses[d]	Corn Steep Liquor[e]	Cottonseed Embryo	Bacto Peptone[f]	Yeast Extract
Amino Acids, %	Alanine	0.8			9.2	
	Arginine		3.3	2.9	5.8	0.78
	Aspartic Acid	1.5			5	5.1
	Cystine		1.9	1.52		
	Glutamic Acid	1.5			8.1	6.5
	Glycine	0.4	5.1	3.78	15.9	2.4
	Histidine		2.8	2.96	0.8	0.94
	Isoleucine		3.6	3.29	2.1	2.9
	Leucine	1.3	11.3	6.11	3.8	3.6
	Lysine		2.5	4.49	3.4	4
	Methionine		1.9	1.52	0.7	0.79
	Phenylalanine		4.4	5.92	2.8	2.2
	Proline				8.8	
	Serine				1.5	
	Threonine	0.6	4	3.31	1.1	3.4
	Tryptophan		0.2	0.95		0.88
	Tyrosine	0.7	5.8	3.42	0.6	0.6
	Valine	0.6	3.4	4.57	2.8	3.4
Vitamins, mg/100 g	Thiamine	0.01	0.5	0.399		0.32
	Riboflavin	1.1	0.1	0.482		1.9
	Nicotinic acid	8	1.6	8.33		
	Pantothenic acid	0.7	2.5	1.24		
	Folic acid	0.025	0.05			
	Pyridoxine-HCl		2	1.64		
	Biotin		0.01	0.152		0.14
Minerals, %	Potassium	6.4	4.5	1.72	0.2487	0.04
	Calcium	0.21		0.25		0.04
	Magnesium	0.12		0.74	0.0017	0.03
	Phosphorus	0.03		1.31		0.29
	Sodium	1.6	0.2		1.8127	0.32
	Iron	0.03	0.03		0.00078	
	SO3	0.74	0.25		0.32	
Sugars, %	Sucrose	48.9				
	Glucose	0.5	2.50		0.629	

[c] Most data adapted from [129]. Blank cells indicate no data available.
[d] Amino acid data additionally adapted from [226].
[e] Amino acid data additionally adapted from [227].
[f] All data for Bacto peptone adapted from the supplier product sheet (Thermo Fisher Scientific, Waltham, MA).

Figures

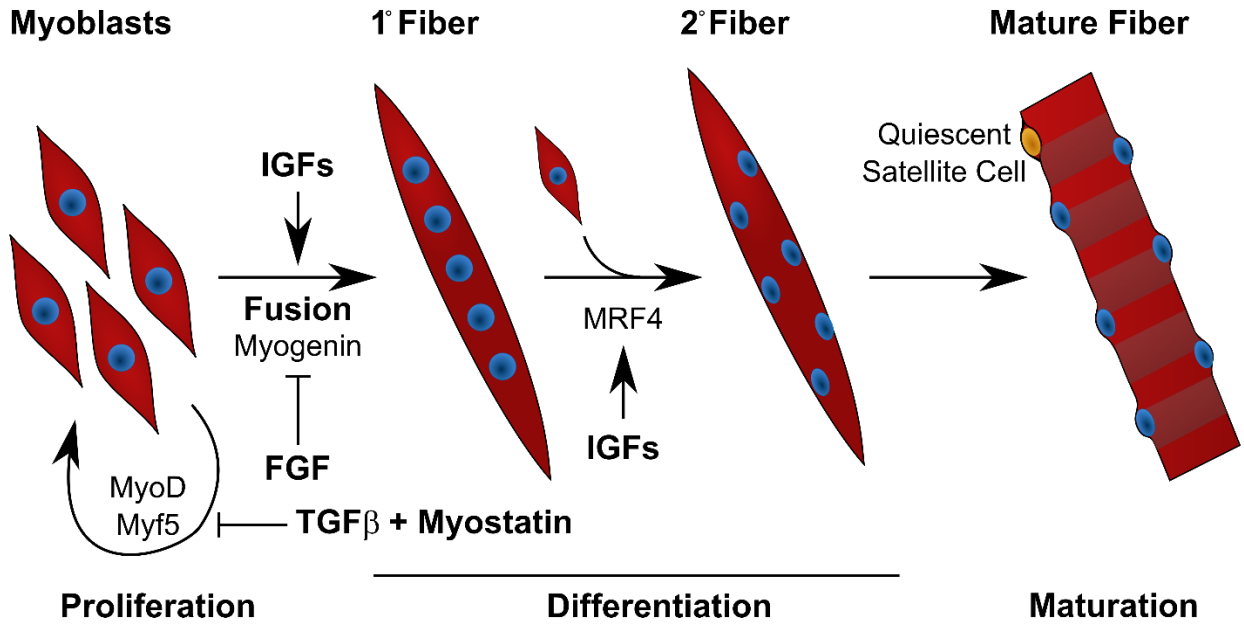

Figure 1: Overview of the myogenic regulation process relevant to cultivated meat media

Upon activation of a quiescent satellite cell, actively proliferating muscle precursor cells are known as myoblasts. The myogenic regulatory factors (MRFs) *MyoD* and *Myf*5 drive this proliferation. Fibroblast growth factor (FGF) supports proliferation, while transforming growth factor β (TGF-β) and myostatin inhibit proliferation. Insulin-like growth factors (IGFs) support proliferation as well as differentiation. IGFs, along with the MRF myogenin, drive fusion of myoblasts into primary myofibers. Continued fusion and differentiation are supported by IGFs and MRF4, yielding secondary fibers, which ultimately develop into mature fibers (myotubes) complete with associated quiescent satellite cells.

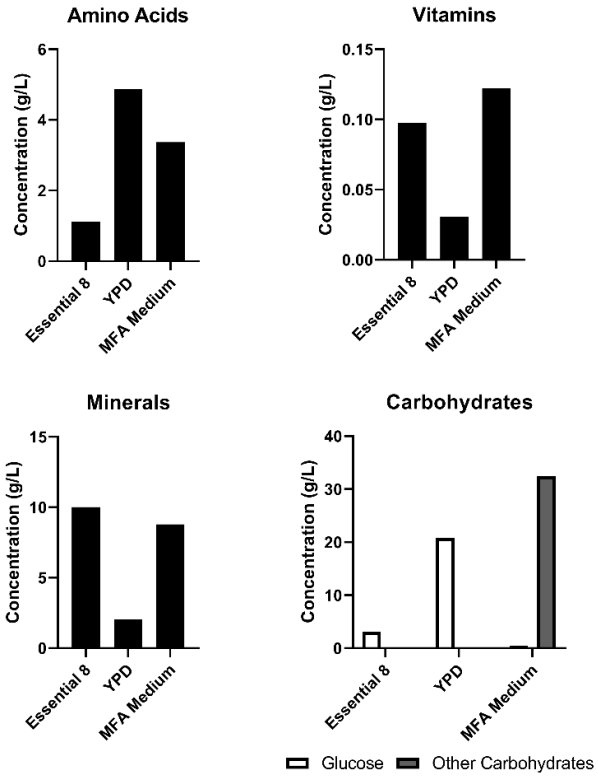

Figure 2: Nutrient profiles of example complex media

Approximate concentrations of four broad classes of nutrients between two examples of cell culture media containing complex and undefined ingredients. Yeast extract/peptone/dextrose (YPD) media is formulated for yeast cell culture and is composed of 1% yeast extract, 2% Bacto peptone, and 2% dextrose in water; the former two complex ingredients are described in more detail in **Table 3**. The example medicinal feed additive (MFA) medium to culture bacteria for the production of the antibiotic frenolicin is described in [146] and consists of corn oil, dextrin, corn steep solids, molasses, sodium formate, yeast extract, magnesium sulphate heptahydrate, monosodium phosphate heptahydrate, and calcium carbonate. The approximate nutrient profiles of the complex ingredients in MFA medium are given in **Table 3**. Essential 8 is a defined medium for animal cell culture and is included here for comparison; its exact composition is given in **Table 1**.

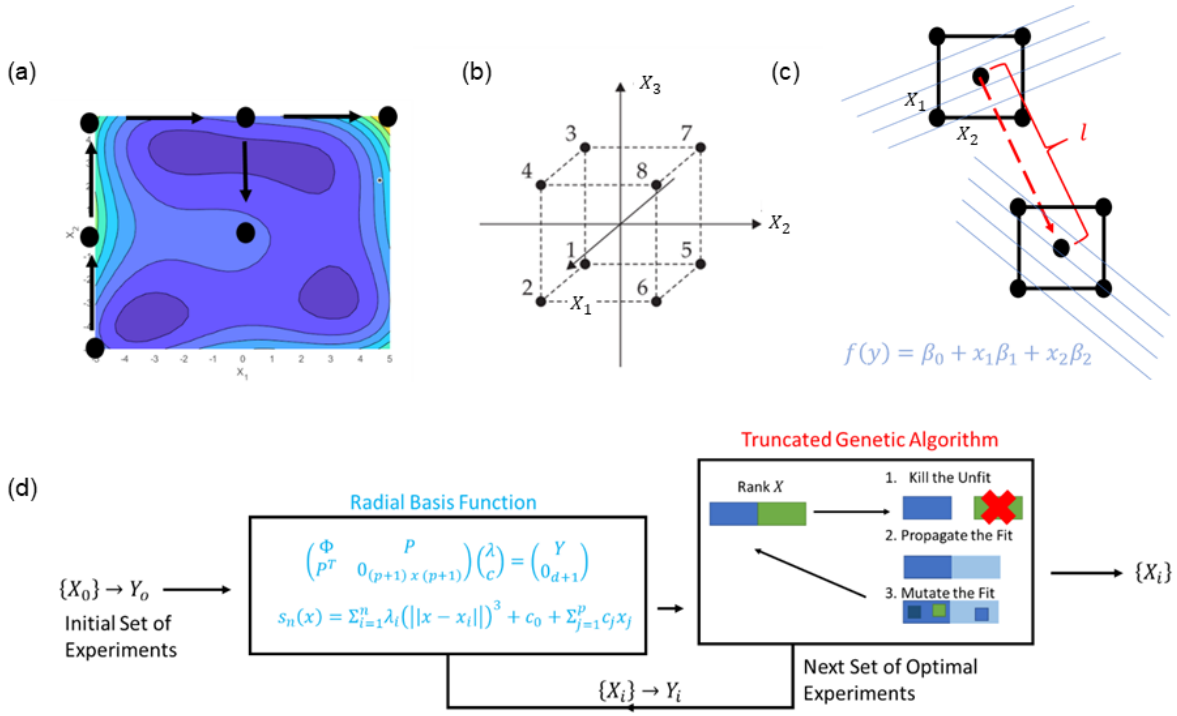

Figure 3: Experimental optimization methods

(a) one-factor-at-a-time for local minimization problems where darker blue is more optimal response and X_1 and X_2 are media components, (b) full factorial design for three media components X_1, X_2, X_3 and two levels to estimate effects of each factor across each level, (c) response surface methodology with full factorial design for two factors and two levels (in black) combined with a linear model (in blue) to suggest an optimal direction of search (in red) for iterative component optimization, (d) a nonlinear radial basis transformation of inputs X models an approximation of the medium components effects on the response Y, this is used by a genetic algorithm with a truncation stopping criteria (stops when experiments get close to one another) to suggest new optimal media component concentrations based on the principle of natural selection.

CHAPTER 3: Spent media analysis suggests cultivated meat media will require species and cell type optimization

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Cell culture media design is perhaps the most significant hurdle currently facing the commercialization of cultivated meat as an alternative source of dietary protein. Since media optimization for a specific culture system requires a significant amount of effort and investment, a major question remaining is whether media formulations can be easily shared across multiple production schemes for cells of different species and lineages. Here, we perform spent medium analysis to compare the specific nutrient utilization of primary embryonic chicken muscle precursor cells and fibroblasts to the murine C2C12 myoblast cell line. We demonstrate that these related cell types have significantly different nutrient utilization patterns collectively and on a per-cell basis, and that many components of conventional media do not appear to be depleted by the cells. Namely, glucose was not consumed as rapidly nor as completely by the chicken muscle precursors compared to other cells overall, and there were significant differences in specific consumption rates for several other key nutrients over the first day of culture. Ultimately, our results indicate that no one medium is likely ideal and cost effective to culture multiple cell types and that novel methods to streamline media optimization efforts will be important for the industry to develop.

Introduction

Faced with a proliferation of climate-related disasters, zoonotic disease outbreaks, and human population growth in recent decades, society is now at a crossroads determining how to satisfy the ever-growing demand for quality dietary protein in a way that is responsible and sustainable[18]. Cultivated meat (CM) is gaining notable traction and investment in recent years as a potential solution to this issue[147,148] and as a way to improve on the food safety, organoleptic, and nutritional attributes of conventional meat products[149–151]. However, presently, there are numerous technical hurdles to overcome before this food technology can be commercially viable at large scales[152]. As described in our recent review, the leading cost driver and challenge facing CM is the media used to culture the cells, since it is currently comprised of numerous indispensable and expensive components[153].

In many avenues of published CM research, media is often taken for granted. Using traditional and commercially available biomedical cell culture media like Dulbecco's Modified Eagle's Medium (DMEM)—without regard to its cost or scalability—to develop cell lines and investigate the use of scaffolding for CM is common. Therefore, few advances have been made in the academic realm toward overcoming the myriad CM media challenges. While several academic researchers are now beginning to develop less expensive media that promotes better cell growth[154], even with formulations that are animal product-free[34,155,156], no studies have been performed to our knowledge that investigate the fundamental media requirements of CM-relevant cell types specifically. Compared to the standard approach today of trying to improve existing conventional biomedical media formulations, understanding these cells' specific nutrient utilization rates will enable a much more directed approach to generating optimal media formulations for CM.

Spent media analysis (SMA) is a commonly used and fundamentally simple strategy for cell culture media optimization. Assuming one has the infrastructure, capabilities, or access to outside analytical services, SMA involves collecting samples of media and performing standard chemical analyses to measure the concentrations of important components and determine their rates of utilization. This approach is most suitable for understanding which media components are directly utilized by cells and should be supplied in greater quantities, those not consumed over time, and how waste products may accumulate with the potential to inhibit cell growth. SMA has been used successfully for a variety of industrial microbial and animal cell culture applications to reduce costs and improve scalability[157–159]; however, this approach has yet to be applied to CM media. SMA will have to be done in parallel with other approaches to media optimization since many components may be important to include in the media even if they are not consumed by the cells[74].

In this study, we present the most comprehensive analysis that has been published to date on the metabolic nutrient utilization rates of three CM-relevant cell types. Primary embryonic chicken muscle precursors (cMPCs) and primary chicken muscle fibroblasts (cMFBs) were cultured alongside the commonly used murine C2C12 myoblast cell line in order to perform subsequent SMA to reveal species- and cell type-dependent variations in media requirements. Muscle precursor cells are characterized by their expression of early myogenesis-related genes as well as their capacity to differentiate and fuse into mature multinucleated muscle fibers; they are otherwise known as myoblasts in the embryo and satellite cells in the adult[153]. We decided to use chicken cells in this study due to the longstanding history of chicken primary cells as a model system in muscle physiology research and their relative ease of use[160]. Perhaps more importantly, there appears to be a general lack of information in the literature on cultivated chicken, despite

conventional chicken being the fastest growing agricultural subsector today[161]. We were ultimately able to measure glucose, lactate, amino acids, water soluble vitamins, trace elements and minerals, and certain growth factors in the spent media samples over the course of cell proliferation and differentiation in a small scale 2D multi-well plate format.

Materials and Methods

Chicken cell isolation and storage

We developed and tested a method based on existing published procedures[162–164] and empirical observations to isolate cMPC from 19-day-old Hy-Line chicken embryos. Briefly, fertile eggs were sanitized with 70% ethanol before opening and euthanizing the embryos via decapitation. Pectoral muscles were extracted and placed in 20-30 mL sterile phosphate buffered saline (PBS) with 1x penicillin, streptomycin, and amphotericin B solution. Enzymatic and mechanical tissue dissociation was performed using a gentleMACS skeletal muscle tissue dissociation kit (Miltenyi Biotec, Auburn, CA). A short pre-plating step of 25 minutes on uncoated plastic culture flasks was used to remove many of the rapidly adhering non-MPC cell types in order to enrich the MPC population of the primary cell culture. Cells were seeded at a density of 17,000 cells per cm^2 of Matrigel-coated surface area (Corning, Corning, NY) during the pre-experiment expansion phase. The cells' ability to readily differentiate into long, well-developed myotubes after reaching confluence confirmed their identity as muscle precursors. cMFBs were derived by culturing a subset of the obtained cells on uncoated TC-treated plastic, which selected for the fibroblastic cell types that could better adhere to the plastic surface and synthesize their own extracellular matrix. The cMFBs and cMPCs were passaged (or media were changed) every two to three days during initial expansion. They were allowed to expand as much

as possible from passage 0 through 1 without allowing surfaces to become more than about 70% confluent. After the first passage, all cells were collected and frozen at $3.5x10^6$ cells per 1 mL aliquot of freezing medium (70% DMEM, 20% fetal bovine serum, 10% dimethyl sulfoxide) and stored in liquid nitrogen using conventional techniques.

General cell culture

The three types of cells (cMFB, cMPC, and C2C12) were each expanded in the experimental medium to generate enough cells to set up the SMA experiment (at least 10^7 cells). This medium consisted of 40% DMEM (containing high glucose, pyruvate, and l-glutamine), 40% Ham's F10 nutrient mix, 20% fetal bovine serum (FBS), and an additional 2.5 ng/mL of recombinant human basic fibroblast growth factor (hFGF2) (Cell Signaling Technology, Danvers, MA). The basal media and FBS were all obtained from Gibco (ThermoFisher Scientific, Waltham, MA). During the pre-experiment cell culture phase, the cells were grown on Matrigel-coated 15 cm polystyrene Petri dishes. During passaging, they were dissociated using TrypLE Express reagent (Gibco) and counted using a hemacytometer with trypan blue. Cells were kept in humidified incubators at 37 °C and 5% CO_2.

Experimental media collection phase

The three cell types were seeded onto Matrigel-coated 6-well plates at 10^5 cells per well. cMFBs and cMPCs were seeded at passage 3, while C2C12s were seeded at passage 17. Thirty wells were seeded per cell type to allow for a parallel culture setup for multiple collection time points in triplicate and to yield sufficient statistical power. All the spent media were collected from three wells per cell type at each of the predetermined time points, while the remaining wells

were left to continue culturing, without media changes, until their planned collection time. The collected spent media volumes were recorded (to account for evaporation during culture), filtered through 0.22 µm membrane filters, and stored at -30°C for later analysis. Directly after media collection at each time point, the cells remaining in the wells were imaged using an ImageXpress Pico automated microscope (Molecular Devices, San Jose, CA), dissociated, and manually counted via hemocytometer.

Glucose and lactic acid analysis

An Agilent 1260 Infinity II high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) coupled to a G7162A refractive index (RI) detector was used with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and a conventional method for measuring carbohydrates and organic acids according to the manufacturer's suggestions. The mobile phase was 5 mM sulfuric acid and the HPLC was operated at an isocratic 0.6 mL/min flow rate with the column kept at 50ºC. Samples were centrifuged at 10,000 x g for 10 mins and filtered through 0.22 µm polyvinylidene difluoride membrane filters before injection. Injection volume was 20 µL and run time was 20 mins. Standards of D-(+)-glucose and sodium L-lactate were obtained from Sigma-Aldrich (St. Louis, MO) and were prepared fresh prior to analysis to generate standard calibration curves with five points that captured the range of sample concentrations.

Amino acid analysis

Amino acid analysis was performed by the UC Davis Molecular Structure Facility using a Hitachi L-8900 amino acid analyzer (Hitachi High Tech, Tokyo, Japan). Samples were

acidified to 2% sulfosalicylic acid (SSA) and incubated at room temperature (23ºC) for 15 min before being frozen (-20ºC) overnight. They were then diluted with 100 nmol/mL AE-Cys Li. 20 µL of sample was injected into the system, and free amino acids were separated using ion-exchange chromatography with a post-column ninhydrin reaction. Column and buffers were obtained from Hitachi, ninhydrin was supplied by Wako (Richmond, VA), and amino acid standards were obtained from Sigma-Aldrich (St. Louis, MO). Absorbance was recorded at both 570 nm and 440 nm after the reaction with ninhydrin to determine the response factor for each individual amino acid and to quantify levels relative to the known amino acid standards. AE-Cys was used to correct for injection volume variance. Certain amino acids such as tryptophan could not be measured using this method.

Vitamin analysis

To measure water-soluble vitamins, an Agilent 1200 Infinity HPLC system coupled to a diode array detector was used with an Agilent Poroshell 120 EC-C18 column at 4.6 x 100 mm with particle size of 2.7 µm. The HPLC method was developed by the UC Davis Department of Viticulture and Enology analytical laboratory based on manufacturer suggestions. Briefly, mobile phases consisted of 25 mM HK_2PO_4 (pH 7.0) (A) and acetonitrile (B). The 20.1 min run time was set up with a constant flow rate of 0.5 mL/min. The flow consisted of 1% B at 0 to 5 mins, a gradient increase to 30% B from 5 to 15 mins, 30% B from 15 to 20 mins, a gradient decrease to 1% B from 20 to 20.1 mins, followed by 5 mins post run time at 1% B. Data were acquired at 205, 214, 232, 266, and 280 nm. The column was kept at 35ºC, and injection volume was 20 µL. Vitamin standards were obtained from Sigma and were prepared fresh and shielded from light prior to analysis.

Mineral analysis

A 7850 inductively coupled plasma mass spectrometer (ICP-MS) (Agilent Technologies, Santa Clara CA), which includes the Octopole Reaction System ORS^4 collision cell and Ultra High Matrix Introduction (UHMI) aerosol dilution system, was used for the analysis. Sampling was performed using an SPS 4 autosampler (Agilent Technologies). The ICP-MS was configured with the standard sample introduction system consisting of a MicroMist glass concentric nebulizer, temperature-controlled quartz spray chamber, and quartz torch with a 2.5 mm id injector. The interface consisted of a nickel-plated copper sampling cone and nickel skimmer cones.

ICP-MS uses an (ORS^4) cell operating in helium (He) collision cell mode with Kinetic Energy Discrimination (KED). This combination provides the optimum configuration to control common polyatomic interferences. This ICP-MS has a wide linear dynamic range (10 or 11 orders of magnitude), so major and trace elements in spent media samples were measured in a single run. The calibration standards were prepared in 2% nitric acid (HNO3) and 0.5% hydrochloric acid (HCl). HCl is routinely added to samples for analysis, as it ensures that chemically unstable elements such as Hg are retained in the solution. The Calibration standards were prepared from environmental calibration standard, p/n 5183-4688 (Agilent Technologies), and 1000 µg/mL single calibration standard for Hg, p/n 5190-8485 (Agilent Technologies). Most elements were calibrated from 0.1 to 100 ppb. Major Minerals were calibrated from 10 to 10000 ppb. Hg was calibrated from 0.01 to 2.0 ppb. National Institute of Standards and Technology (NIST, Gaithersburg, US) SRM 1643f was used to check the ICP-MS calibration as an Initial

calibration verification (ICV). Continuing calibration verification (CCV) standards were prepared at the midpoint of the curve (most elements 50 ppb, majors 5000 ppb, and Hg 1ppb).

The internal standard (ISTD) solution containing 2 ppm Sc, Ge, Rh, In, Bi, and Lu, was prepared in 2% HNO3, 0.5% HCl, and 10% isopropanol (IPA). The ISTD solution was automatically added at a flow rate approximately 16 times lower than the sample flow.

Growth factor analysis

A panel of 30 bovine cytokines was assessed using a multiplex enzyme-linked immunosorbent assay (ELISA) (Cat.# GSB-CAA-30, RayBiotech, Peachtree Corners, GA). Only a subset of the collected spent media samples and replicates was analyzed using this array due to sample number and volume limitations. This analysis served to provide a general indication of the relative abundance of these proteins in the samples and whether they may be changing significantly in the media over the culture period. Bovine FGF2 was then measured more precisely in more samples with a standard sandwich ELISA kit according to manufacturer instructions (ThermoFisher Cat.# EB2RB), using a SpectraMax iD3 multimode plate reader to measure absorbance at 450 nm (Molecular Devices, San Jose, CA).

Data and statistical analysis

Data obtained from the various analytical techniques were converted into mass per volume ratios and plotted versus the day of cell culture. For the multiplex cytokine screen, statistical analysis was performed using two-way analysis of variance (ANOVA) followed by the Holm-Šídák post-test for multiple comparisons where $\alpha=0.05$. For the calculation of the specific cellular rates of nutrient utilization, numerical derivatives were obtained between the

concentration values at a given time point and the point preceding it. These backward derivative approximation values were divided by the cell numbers at each respective time point to give specific utilization or production rates and plotted versus time in culture. Statistical significance was assessed using two-way ANOVA with the Holm-Šídák post-test for multiple comparisons where $\alpha=0.05$. Prism 9 (GraphPad Software, San Diego, CA) was used to create all charts and perform the statistical analyses.

Results

Evaluating sugar utilization patterns during cell growth and differentiation

After all media samples were collected from all cell types and time points, the samples were analyzed via a high-performance liquid chromatography (HPLC) method optimized for carbohydrate and organic acid analysis. The chromatogram peaks for glucose and lactic acid were the only two that displayed noticeable changes over the time course of spent media samples for any of the cell types. The integrated peak areas and heights of other distinguishable peaks in the chromatograms essentially remained constant across media samples for each cell type (Supplementary Figure 1). Analysis of glucose concentration revealed a notable difference in the utilization patterns between the cMPCs and the other two cell types (**Figure 4**a). The cMPCs exhibited a significantly lower rate of glucose utilization that appeared mostly linear over the observed culture period, while the cMFBs and C2C12s used glucose more rapidly and almost completely by D10, which also corresponds to the cell count plots leveling off. Lactate accumulated in the media following roughly the inverse path of glucose (**Figure 4**b).

To assess how the cell types differed in their overall growth characteristics and help explain the patterns of nutrient utilization, we counted the cells that remained in the culture

plates directly after collecting the spent media samples. All three cell types proliferated well throughout the observed culture period. The C2C12s grew exponentially for about 2 days after seeding (indicated by the roughly straight line on the semi-log cell count plot in **Figure 4**c) and continued some growth for an additional 4 days. The cMFBs proliferated exponentially for 7 days, and the cMPCs for 4 days. While C2C12s appeared to proliferate slightly faster than the chicken cells during the first 5 days, all three cell types reached a fairly similar maximum cell concentration around 7 days, which corresponded to complete confluence in the 6-well plates (**Figure 4**c). The cMPCs started differentiating into myotubes by day 3, even before the proliferation phase was complete, while the C2C12s started differentiating at day 5, around when the proliferation ended. The cMFBs did not display myotube formation at any point during the three-week culture period. Overall, there was a gradual decline in the measurable cell numbers at the time points after 7 days. This could be attributable to cell death and/or fusion into multinucleated myotubes prior to cell counting.

Assessment of nitrogen utilization during cell growth and differentiation

To assess relative nitrogen utilization between the three cell types, we measured free amino acid concentrations in all of the spent media samples collected. This analysis was meant to evaluate the relative importance of specific amino acids in CM media. The data in **Figure 5** indicate that the concentrations of several amino acids do not significantly decrease in the media over time. Although, many other amino acids do in fact appear to be partially depleted by the cells—most notably four essential amino acids (arginine, isoleucine, leucine, and methionine) and two non-essential amino acids (glutamine and serine). On an absolute scale, glutamine is the amino acid used the most, followed by arginine and serine. The serine in the C2C12 culture was

essentially the only amino acid that appeared to approach complete depletion—this occurred by day 7, which is also when the cell count began to slightly decrease (due to death and/or fusion). Other amino acids continued to be utilized after the end of exponential cell growth. Interestingly, proline in the media increased only in the cMFB culture after day 7. These data suggest that the cMFBs are differentiating and increasing the production and subsequent breakdown of collagen protein (roughly a fifth of all amino acids in collagen are proline). However, there was no significant difference in the consumption rates of any amino acids between the cell types during their proliferation phase.

Vitamins and other micronutrients are not utilized significantly

As seen in **Figure 6**, there is no appreciable decrease in the concentration of any of the water-soluble vitamins over time. Variations in the starting vitamin concentrations between the three cell types could potentially be explained by lot variation in the FBS that was used in the media, or by vitamin degradation in the samples during the handling and storage before analysis. Our recent review on cultivated meat media discusses these concerns in more detail[153]. Similarly, **Figure 7** displays no significant decrease in the elemental mineral concentrations measured by inductively coupled plasma-mass spectrometry (ICP-MS), nor any major difference in the concentrations between the three cell types. Additional elements analyzed included beryllium, aluminum, sulfur, vanadium, chromium, manganese, arsenic, selenium, strontium, molybdenum, silver, tin, antimony, barium, mercury, titanium, and lead; however, these elements were not present in the media at concentrations detectable by our ICP-MS methods (data not shown).

Screening of cytokines and growth factors

The initial screening of cytokine concentrations (**Figure 8**a-c) did not reveal a significant difference in their depletion rates between the three cell types. It is important to bear in mind that only two biological replicates were able to be measured per cell type per time point, so the experiment was statistically underpowered. However, significant differences were observed in a few cases, such as with basic fibroblast growth factor (FGF2, bFGF), interferon γ-induced protein 10 (IP-10), neural cell adhesion molecule 1 (NCAM-1), and decorin. FGF2, the most relevant of these to CM media formulation, displayed a general decreasing trend over time in all three cell types, although the decrease was statistically significant for the cMPCs only. IP-10 and NCAM-1, in general, increased over time in all three cell types, whereas decorin decreased for cMPC, increased for cMFB, and decreased then increased for C2C12.

Due to the general decrease in FGF2 observed over time in all three cell types, and due to its known importance in the promotion of myoblast proliferation and inhibition of differentiation, we performed a more focused analysis on the changes in FGF2 over time in the spent media samples. This analysis included three biological replicates per time point tested per cell type, but we only focused on key time points due to assay sample number limitations. **Figure 8**d shows the overall relative FGF2 concentrations in the media over time between the three cell types, as well as the observed concentrations in freshly prepared medium and Matrigel working solution for reference. All three cell types displayed a marked exponential decrease in FGF2 until around 5 days, which generally corresponded with the increase in cell number. However, the cMFB culture tended to deplete FGF2 more rapidly, while its cell proliferation rate did not seem to be significantly influenced by the roughly 10-fold reduction in the concentration of the growth factor.

Determination of specific utilization rates for key nutrients

To obtain a better understanding of the potential differences between the nutrient utilization behaviors of the three cell types on a cellular basis rather than on a collective culture basis, we determined the specific utilization rates of the key nutrients that were identified in our spent media analyses. This analysis helped us to determine whether the utilization differences were due solely to the relative cell concentration or were a function of the differences in metabolic activities of individual cells. In this regard, **Figure 9**a-c are intended to provide a better picture of the differences in cell proliferation between the cell types over the course of their exponential growth phase (roughly the first four days). **Figure 9**a presents the total cell count per well on a linear scale for the first seven days of culture, while **Figure 9**b shows the calculated specific proliferation rates in terms of cells produced per existing cell per day. These calculations are backward approximations of the derivatives using data between days 0 and 10 of culture. **Figure 9**c is a different representation of the same data shown in 6b and indicates that there are statistically significant differences between the three cell types which are dependent on the time in culture. **Figure 9**d includes the calculated specific utilization rates of glucose, glutamine, arginine, isoleucine, and leucine, which were the components that were found to be the most significantly depleted by the cells over time among all the media components we analyzed. The figure also includes the calculated specific production rates of lactic acid. Overall, **Figure 9**d indicates that these calculated rates were significantly different between the three cell types only during the first day of culture (as well as at day 2 for glutamine). This finding highlights the more fundamental metabolic differences in the cells themselves and indicates that the variations in nutrient utilization are not simply due to differences in total cell numbers.

Discussion

One of the exciting aspects of CM is the potential to allow for the mass production of a greater diversity of types of meat than is typically eaten by humans today. Whether due to cultural taboos or technical impracticalities, there are many species of animals that are not widely exploited for food. Nevertheless, even in conventional cuisines, there is still a considerable variety of meat that is commonly consumed. Therefore, the CM industry will almost certainly be tasked with producing many different types of meat at large scales if it hopes to displace some amount of conventional meat production and help satisfy the growing global population's demand for nutritious and delicious dietary protein.

In the present paper, we present a straightforward characterization and comparison of three different cell types that are highly relevant to CM research and development. Murine C2C12s are not necessarily going to be useful in developing larger-scale CM bioprocesses in the longer term as the industry develops, although they are a useful model for bench-scale, early-stage CM research; indeed, they are used in many contemporary publications in the space [165–169]. Regardless, C2C12s, cMPCs, and cMFBs will not be the only cell types utilized in the CM industry, and the specific cellular behaviors that we observed in our study may not be generalizable to all other CM cell types. Moreover, the different bioprocesses and culture methods that could be used for CM will likely result in differing cell behaviors and nutrient requirements. Therefore, the aim of the present study is not to create a generalizable model for the growth characteristics of certain cell types, but rather to serve as a proof of concept that CM will require targeted media formulation and optimization efforts for each cell type, species, and bioprocess used for production. Further, the results suggest that several components found in conventional cell culture media may not be necessary to include in CM media, which could lead to significant cost savings, although more research needs to be performed in this regard.

The C2C12s we used in this study were seeded at passage 17, which is relatively high, especially compared to the primary chicken cells. C2C12s are an "immortalized" cell line: they are commonly used in studies at passage numbers upwards of 20 [170–172]. We observed extensive differentiation of our C2C12s which occurred to roughly the same extent as the cMPCs, suggesting that the two cultures could be comparable in this regard despite the difference in their passage numbers. However, as indicated in **Figure 4**c, the C2C12s began differentiating later (day 5) and at a higher cell density than the cMPCs (day 3), which could be due in part to the passage number difference, although it is more likely due to inherent physiological differences between the cell types themselves. The findings here in regards to passage number effects are not conclusive; it is certainly reasonable to assume that passage number will affect the metabolic activities and media nutrient requirements of cell populations. In fact, this subject has been extensively researched and proven over the years in many contexts, where studies generally demonstrate lower proliferation speeds, altered function, and different gene expression and phenotype patterns at higher passage numbers[171,173,174]. While the present study does not endeavor to reveal the effects of senescence and passage number on media requirements for cultivated meat, this is currently an active topic of research in our group because these will be important considerations for the large-scale proliferation of cells grown for CM over several doublings[153].

The complex and undefined nature of FBS is an inherent challenge in laboratory-scale CM research[116]. FBS batch variability likely had an effect on the variation in the concentrations of specific signaling molecules and trace nutrients, in addition to the general growth behavior that we observed between the three cell types we tested[22]. The unpredictability of a scaled culture system using FBS would be a reason to prefer a chemically defined medium. However, using

undefined and complex media ingredients derived from plants and agricultural waste streams is likely going to be important for producing CM at a competitive price compared to conventional meat[153]. Therefore, assessing the nutrient utilization rates of CM-relevant cell types grown in an FBS-containing medium can be justified here for the sake of understanding the major nutrient consumption trends that are observable in a complex medium.

Free amino acids have a variety of complex functions in cells, ranging from being the structural building blocks of proteins to participating in various metabolic and signaling pathways[175,176]. For instance, the interplay of serine and glutamate is implicated in the tetrahydrofolate pathway, influencing proliferation in a cell type-dependent fashion[177]. Based on the current understanding of amino acid metabolism, it is safe to assume that most amino acids will be required in CM cell culture media, even if they are not depleted by the cells over time. It is therefore challenging to predict and understand the effects of specific amino acid concentrations on the behaviors of all CM-relevant cell types, and more directed studies would need to be performed to elucidate these unknowns. However, based on our SMA, it is reasonable to imagine that primary cells should display a lower cellular metabolic rate than an immortalized cell line (like C2C12) that was selected for its ability to rapidly proliferate over many generations. This idea is reflected in the data shown in **Figure 9** for the first one or two days of the culture period. The figure also indicates that fibroblasts may not attain their highest specific growth rates as quickly as muscle cells after seeding. Further, the processes of cell differentiation, fusion, and hypertrophy in muscle cells and collagen production in cMFBs starting around days 5 to 7 complicate the understanding of per-cell utilization patterns. As the industry attempts to generate immortalized and optimized CM-relevant cell lines, it will be

important to consider and investigate the effects of genetic manipulation as well as differentiation state on cell metabolism and media requirements.

Through our analysis of carbohydrates, amino acids, water soluble vitamins, minerals, and cytokine proteins in our spent media samples, we found that only a select few of these components were appreciably decreasing in concentration over the cell culture period. These compounds included glucose, arginine, glutamine, isoleucine, leucine, methionine, serine, and FGF2. Decorin also displayed notable decreases and/or increases over time in the three cell culture groups. As a myokine proteoglycan that has been implicated in the regulation of both myogenesis and collagen synthesis, decorin likely plays a complex role in this context[178,179]. While decorin has not been extensively studied in the literature, it has been shown that it is secreted by contracting muscle cells[178]. The patterns of decorin abundance in our spent media samples could therefore be reflecting certain aspects of the overall health, differentiation status, and function of our cells, but this information itself is not enough to draw specific conclusions and ultimately may not be important in the context of cultivated meat media development.

The fact that the cMPC cultures overall did not appear to utilize glucose and potentially used the glucose more completely (less lactate production) when compared with C2C12s and the cMFBs is intriguing. This may be due in part to the fact that the cMPCs were smaller in size compared to C2C12s and did not reach the same cell density as the cMFBs at confluence; it may also point to more fundamental differences in the metabolic activities between the cells. The dissimilar differentiation behavior between the cMPCs and C2C12s, which did not appear to be influenced or explained by glucose or FGF2 concentrations, also suggests there are more intrinsic physiological differences between the cell types. It is also worth noting the possibility of the cell density itself to influence the metabolic activity of the cells due to cell-cell

interactions[180], and this should be investigated further in larger scale suspension culture systems for cultivated meat.

The specific rates of nutrient consumption in **Figure 9**d also clearly indicate that the cMFBs were using glucose and glutamine at a faster rate and producing less lactate at the beginning of the culture period compared to the other cell types. This suggests that the cMFBs may use glucose more completely (mitochondrial oxidative phosphorylation), whereas the cMPCs use glucose more in glycolysis and release lactate rather than completely metabolizing pyruvate in the mitochondria. It could be useful for future work to investigate the underlying cellular mechanisms that lead to these differential glucose and amino acid utilization rates. The main conclusion that can be drawn from **Figure 9**d, however, is that at the early phase of cell proliferation the specific cell lineage and species origin have a significant influence on the cellular rates of utilization of key media nutrients, and these differences may be less significant later in the cell culture period. This understanding could facilitate optimization for feed rates in batch fed bioprocesses that may be used in cultivated meat production.

It is already well-understood that many components of cell culture media are inherently unstable in solution [181–183]. It is certainly possible that chemical degradation played a major part in the concentration changes we observed in our spent media samples over time. However, since our study was intended simply to reveal whether component utilization rates vary between CM-relevant cell types, and not to create models for how these cells use the nutrients, the results reported here can still be considered valid in context of constant intrinsic rates of component degradation independent of cell metabolic activity. Also, in practice for CM production, cells would not likely be left in the same media for more than 5-7 days at maximum, suggesting that media instability would be even less of a concern.

In the present study, we confirm a foundational tenet of cultivated meat media formulation: the specific nutrient requirements of different CM-relevant cell types cannot be assumed to be similar. Future studies should examine whether chemically defined or complex serum free media formulation will also be so dependent on species and cell type, as well as how these media affect cellular metabolism compared to serum-containing media. It would also be interesting to investigate whether supplying excessive amounts of various nutrients could negatively affect cell growth. As the industry continues to develop, there will likely be an increasing need for more robust, streamlined, and accessible media optimization techniques[154]. If academic and industry researchers can rise to meet this demand, the path toward bringing CM products to market will become substantially clearer.

Acknowledgements

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Author Contributions

EO, KB, and DB conceptualized the methodology and scope of the work. EO, JA, GK, and MP performed cell culture, prepared samples, and analyzed data. JN performed the ICP-MS and drafted the corresponding part of the methods section. KB prepared chicks for cell isolation as well as provided physiological expertise for the data analysis. DB and KB acquired funding and provided overall supervision. EO oversaw all laboratory work, drafted the original manuscript, and completed formal data analysis and visualization. All authors reviewed and edited the manuscript.

Figures

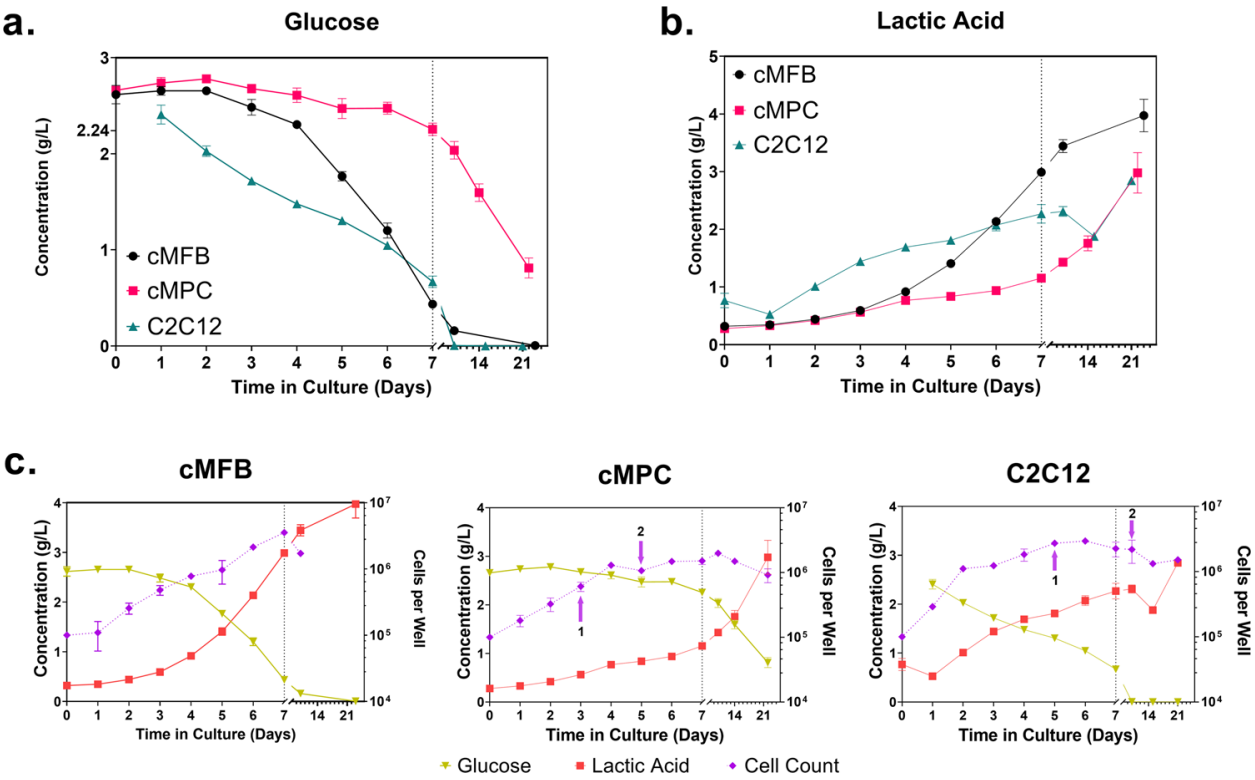


Figure 4: Glucose and LA + Cell count

Analysis of sugars and organic acids in spent media from cultures of three different cultivated meat-relevant cell types. The three cell types were plated at 100,000 cells per well in 6-well plates, and the media from three wells per cell type were collected at each predetermined timepoint for chemical analysis. The concentrations of glucose **(a)** and lactic acid **(b)** in the spent media samples are plotted separately with each cell type's trends overlaid. **(c)** The same data for the three cell types are each plotted separately with glucose and lactic acid concentrations overlaid. Cell counts per well in the 6-well plate experimental setup are overlaid in purple. Arrows pointing at cell count data points indicate differentiation status, with (1) indicating the time at which first signs of differentiation were apparent, and (2) indicating the time the maximum differentiation was reached. Data points represent the mean of three biological replicates ± standard deviation. cMFB, primary embryonic chicken muscle fibroblasts. cMPC,

primary embryonic chicken muscle precursor cells. C2C12, murine myoblast-like cell line. The expected concentration of glucose in fresh media (40% high glucose DMEM, 40% Ham's F10) is indicated in **(a)** at 2.24 g/L and is based on the manufacturer's formulation sheet but does not include the contribution from the 20% fetal bovine serum in the experimental medium. Vertical dotted lines highlight the break in the x-axis scales at day 7 in **(c)**.

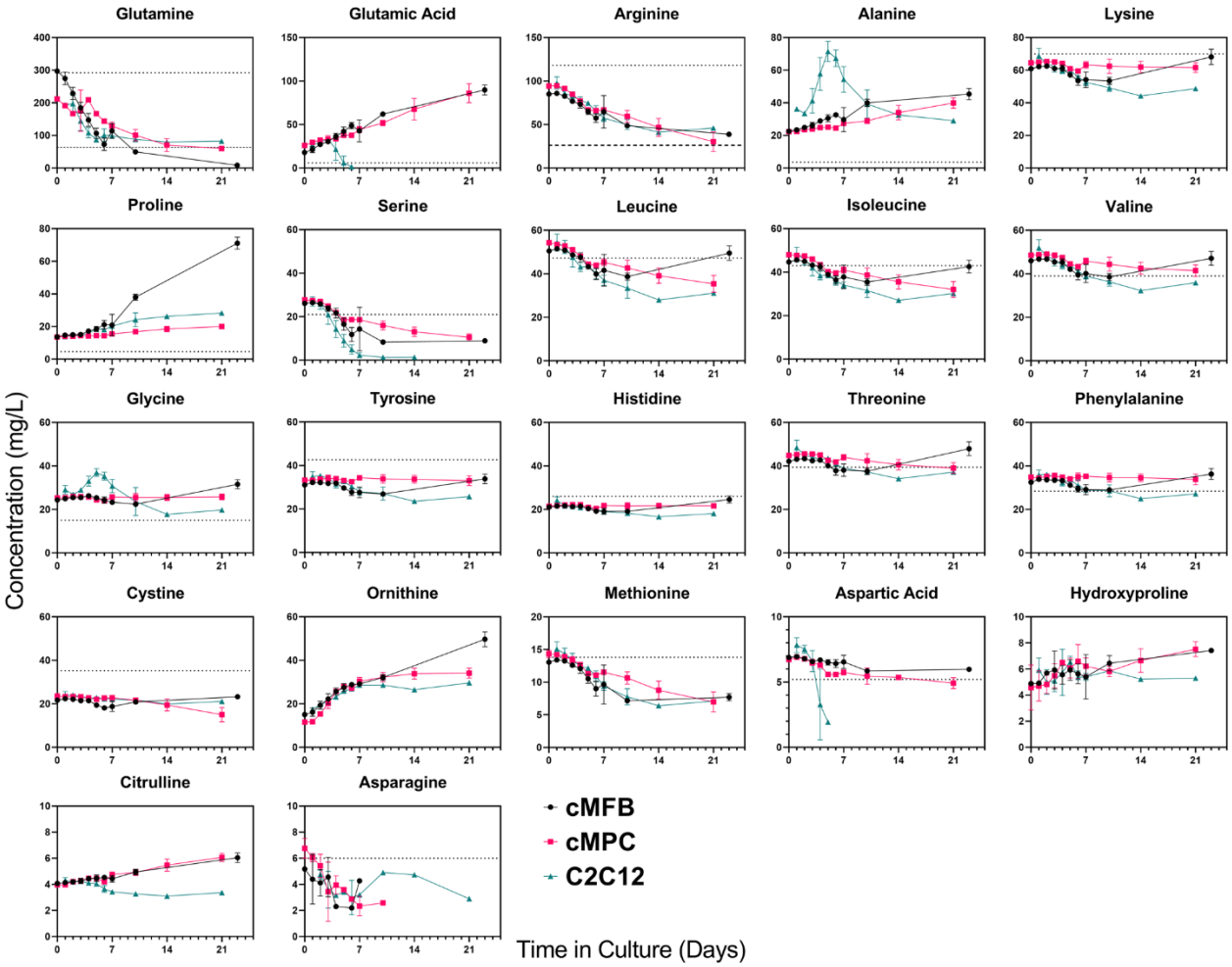


Figure 5: Amino acids

Analysis of amino acid concentrations in spent media from cultures of three cultivated meat-relevant cell types. Dotted lines represent expected concentrations of the amino acids based on the contributions from the basal media formulation consisting of 40% DMEM and 40% F10. The contribution from the 20% of fetal bovine serum in the media is not considered in the estimated expected starting concentrations. Data points represent the mean of three biological replicates ± standard deviation. The ranges of the y-axes for the graphs are not all the same. cMFB, primary embryonic chicken muscle fibroblasts. cMPC, primary embryonic chicken muscle precursor cells. C2C12, murine myoblast-like cell line.

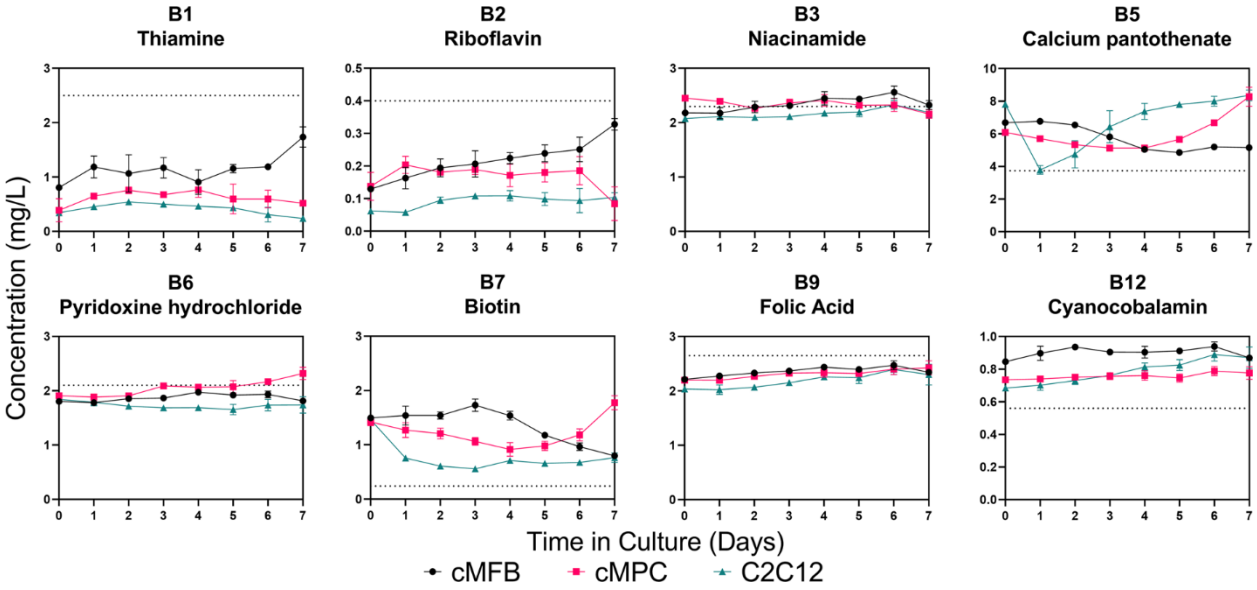


Figure 6: Water-soluble vitamins

Analysis of B-vitamin concentrations in spent media from cultures of three cultivated meat-relevant cell types, using high performance liquid chromatography. Dotted lines represent expected concentrations of the vitamin based on the contributions from the basal media formulation consisting of 40% DMEM and 40% F10. Data points represent the mean of three biological replicates ± standard deviation. Note that the y-axis scales are not all the same. cMFB, primary embryonic chicken muscle fibroblasts. cMPC, primary embryonic chicken muscle precursor cells. C2C12, murine myoblast-like cell line.

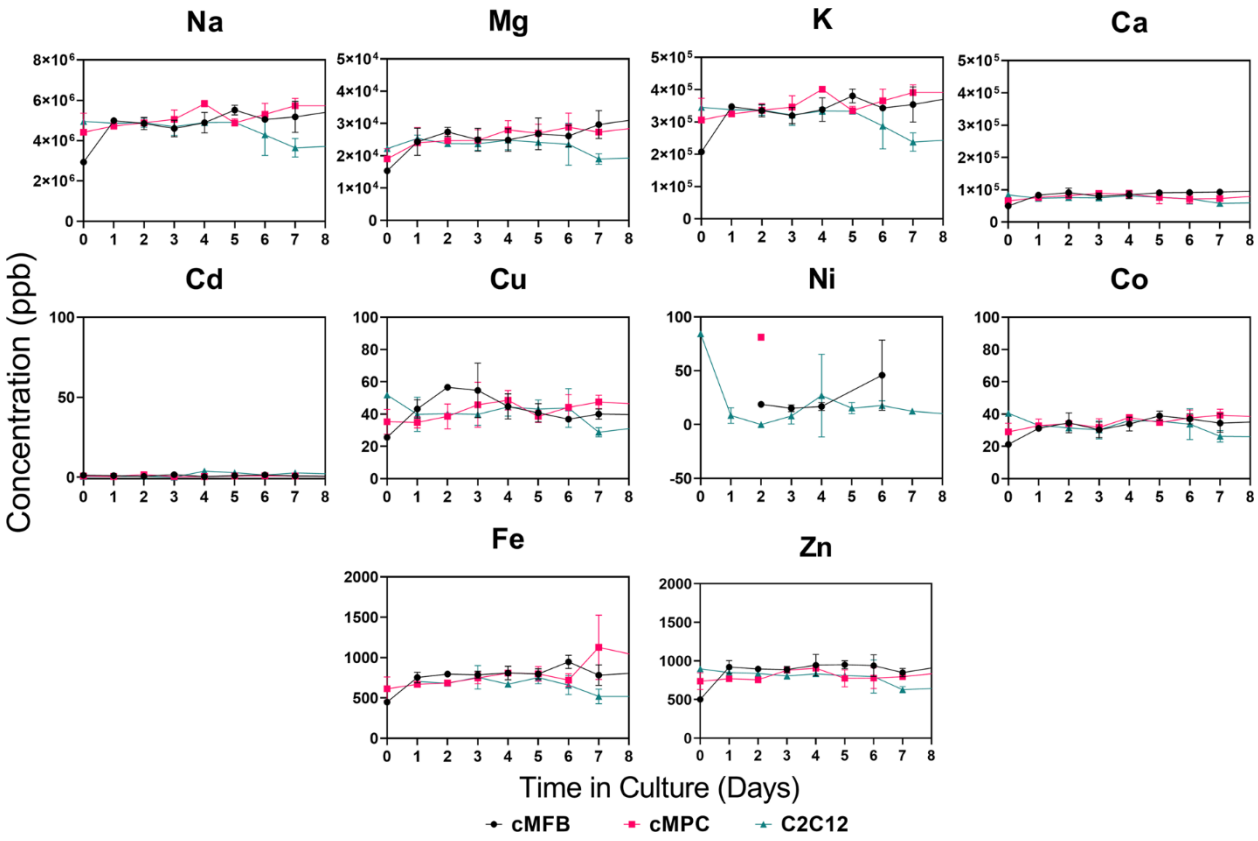


Figure 7: Minerals

Elemental analysis of spent media from cultures of three cultivated meat-relevant cell types, using inductively coupled plasma mass spectrometry. Data points represent the mean of three biological replicates ± standard deviation. The y-axis scales vary widely between the graphs, so the figure does not directly indicate relative abundances of the different elements in the spent media samples. Several other elements were targeted for analysis but their concentrations in the samples were too low for detection. cMFB, primary embryonic chicken muscle fibroblasts. cMPC, primary embryonic chicken muscle precursor cells. C2C12, murine myoblast-like cell line.

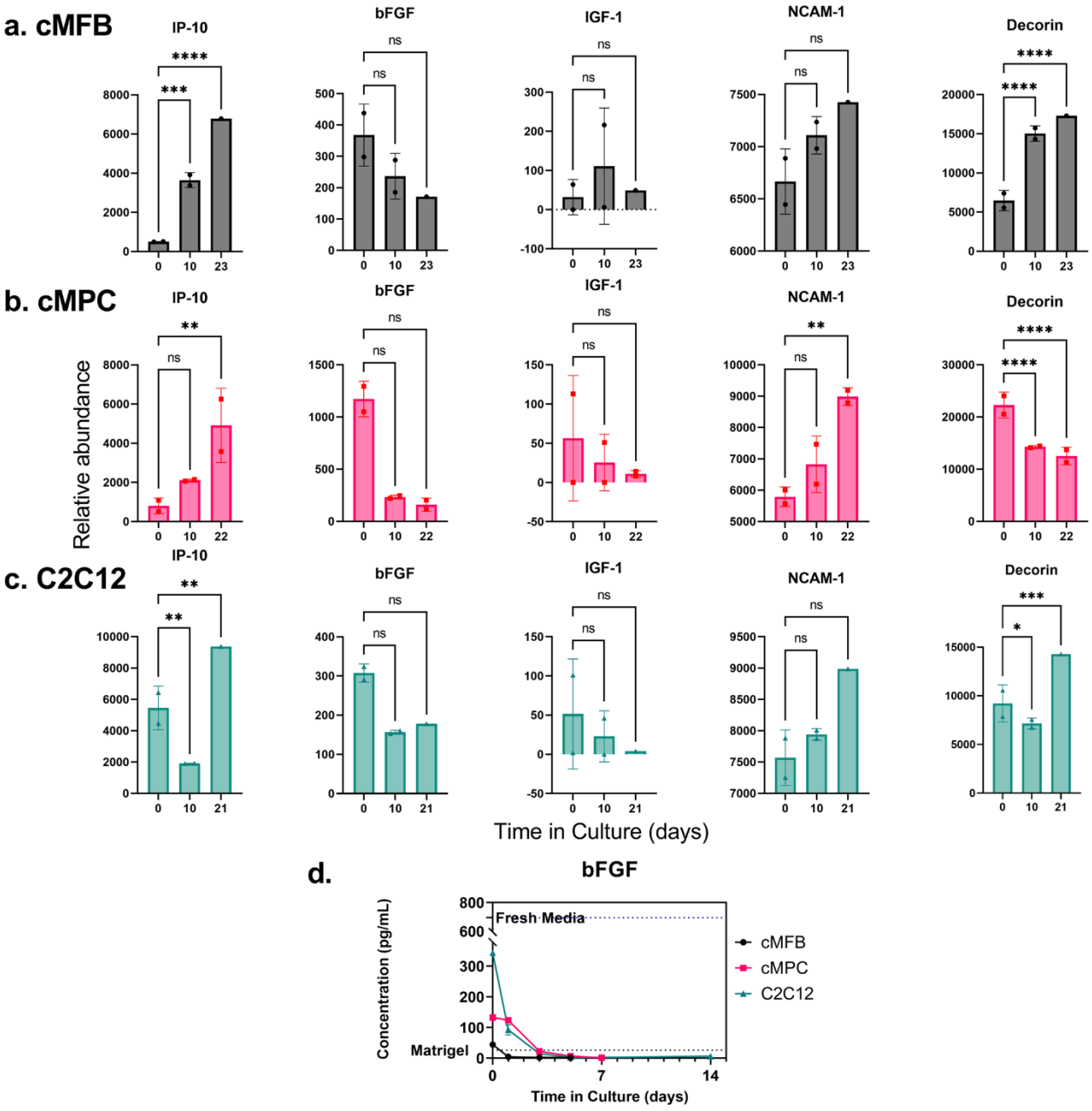


Figure 8: Cytokines & bFGF

Growth factor analysis of spent media from cultures of three cultivated meat-relevant cell types. A preliminary screening of 30 bovine cytokines was performed using a multiplex enzyme-linked immunosorbent assay (ELISA) to compare the concentrations in the spent media samples at three time points over three weeks. The five cytokines with the most significant changes over time were interferon gamma-induced protein 10 (IP-10), basic fibroblast growth factor (bFGF),

insulin-like growth factor 1 (IGF-10), neural cell adhesion molecule 1 (NCAM-1), and decorin. The data for these proteins are plotted for the samples from **(a)** primary embryonic chicken muscle fibroblasts (cMFB), **(b)** primary embryonic chicken muscle precursor cells (cMPC), and **(c)** the C2C12 murine myoblast-like cell line. Data points represent the mean of two biological replicates ± standard deviation. Statistical analysis for this preliminary screening was performed using two-way analysis of variance (ANOVA) followed by the Holm-Šídák post-test for multiple comparisons with $\alpha=0.05$ (ns, not significant. *, $p<0.05$. **, $p<0.01$. ****, $p<0.0001$). It should be noted that statistical power was limited by having up to only two biological replicates per time point, but overall trends can be discerned in many cases despite lack of statistical significance. **(d)** Further analysis of basic fibroblast growth factor (bFGF) was performed using a standard ELISA and concentrations are shown with each cell type overlaid. Dotted lines represent the measured concentrations of bFGF in fresh medium that was prepared immediately before analysis as well as in Matrigel stock solution.

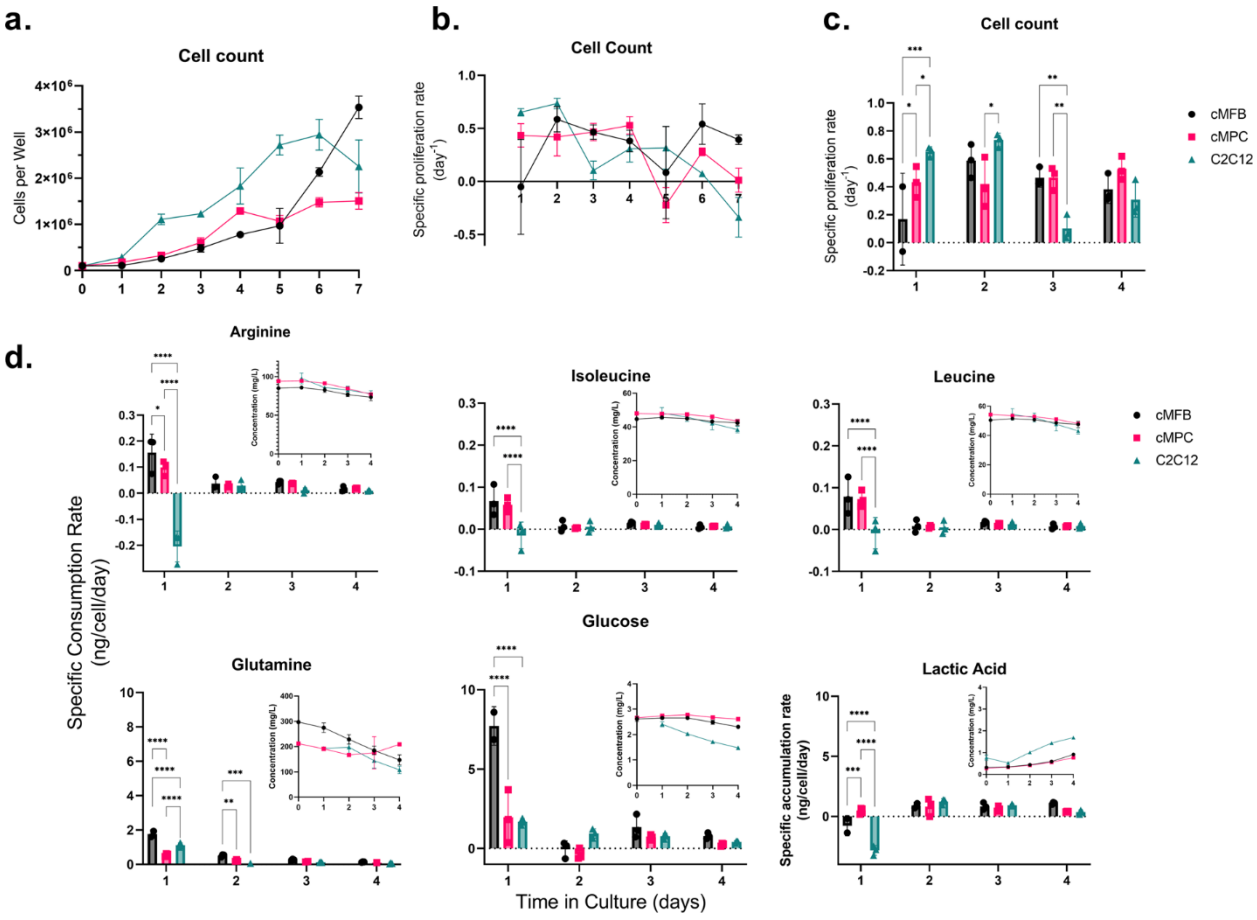
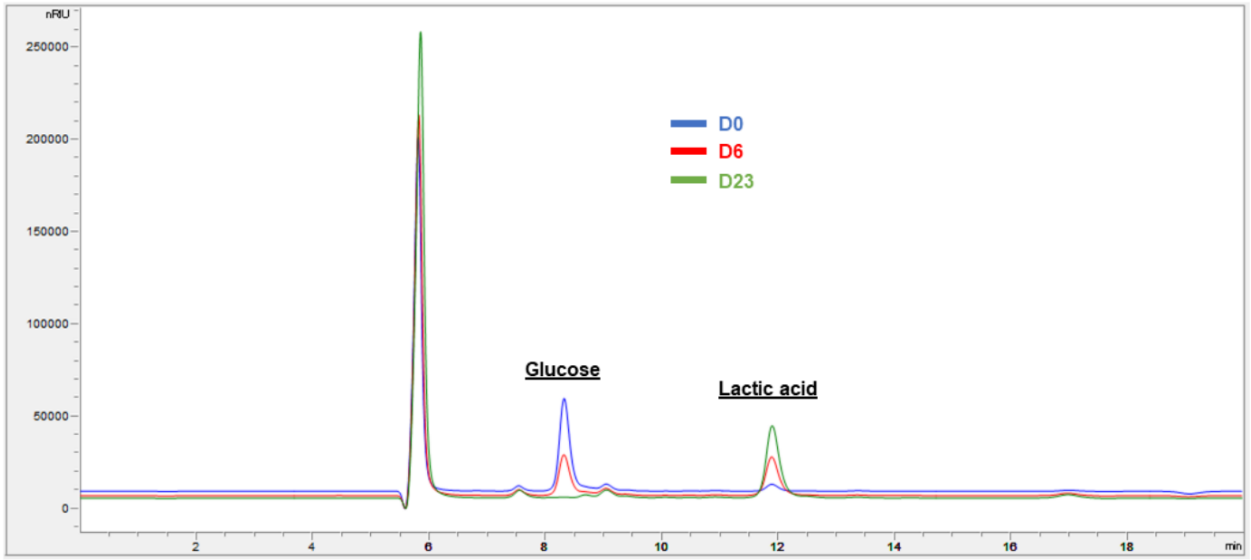


Figure 9: Specific growth and utilization rates

The specific cellular growth and nutrient utilization rates were calculated based on the spent media analysis data by finding the ratio of the approximate backward numerical derivatives for the cell count and nutrient concentration data to the cell count at the corresponding time point. **(a)** The total cell counts per well for the three cell types are plotted on a linear scale as a frame of reference. **(b)** The specific cellular proliferation rates are plotted as a line graph with the three cell types overlaid for the first seven days of culture. **(c)** The same specific cellular proliferation rate data are plotted as a bar graph for the first four days of culture, allowing for visualization of the statistical differences between the three cell types. **(d)** The specific consumption rates of five key nutrients are plotted separately in bar graphs to compare the three different cell types. The specific accumulation rate of lactic acid is also included. Arginine, isoleucine, and leucine are

plotted on y-axis scales up to 0.3 ng/cell/day, while glutamine, glucose, and lactic acid are plotted with scales ranging up to 10 ng/cell/day. Each bar graph includes an overlaid line graph showing the measured concentrations of the corresponding media component in mg/L as a reference. All error bars represent standard deviation. Statistical analysis for the specific proliferation rate and specific consumption rate data was performed using two-way analysis of variance (ANOVA) followed by the Holm-Šídák post-test for multiple comparisons with $\alpha=0.05$ (ns, not significant. *, $p<0.05$. **, $p<0.01$. ****, $p<0.0001$).



Supplementary figure 1: Chromatogram from glucose and lactic acid analysis

An overlaid example of three chromatograms obtained from the HPLC method used to measure glucose and lactate in our spent media samples. The three chromatograms are all from the C2C12 group, and represent media samples taken at day 0, 6, and 23 of culture according to the figure legend. The only peaks that significantly varied across the time point samples were those known to correspond to glucose and lactate. The other peaks visible in the chromatograms were more substantially similar across samples, and represent other components of the sample matrices that were not changing in concentration over the culture time period.

CHAPTER 4: The effect of serum-free media on the metabolic yields and growth rates of C2C12 cells in the context of cultivated meat production

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Successful commercialization of cultivated meat products as alternative sources of dietary protein for a growing global population will require development of efficient and inexpensive serum-free cell culture media. It is known that serum-free media elicit dissimilar cell growth behavior compared to serum-based media, but data comprehensively exploring the effects of serum-free culture on the nutrient requirements of cultivated meat-relevant cell types have yet to be reported. We performed spent media analysis of C2C12 cells growing in Essential 8 serum-free media and in conventional serum-containing media. Data indicated that although the cell growth rates were similar in the two media over seven days, there were significant differences in the utilization rates of some key nutrients such as glucose, glutamine, glycine, and cystine. We extrapolated from the data that roughly 250-275 g of amino acids and 1100-1500 g of glucose would be required to produce 1 kg of C2C12 cells, though these requirements may change when using more optimized cells, media, and feed strategies. Our results highlight the influence of serum and serum replacements on cell metabolism, and indicate that previous data from metabolic studies performed using serum-containing media may not directly translate to serum-free systems.

Introduction

Cell culture media formulation remains a key technical hurdle to successful commercialization of cultivated meat (CM) at scale [152,184]. There are several design challenges unique to CM media that are not present in other applications of cell culture media. Namely, the formulations should be animal product-free to align with consumers' ethical expectations. Most importantly, CM media will need to be substantially cheaper than all existing commercial animal cell culture media to permit price parity of CM products with conventional meat [185,186].

Animal sera—especially fetal bovine serum (FBS)—are frequently used in animal cell culture media as rich and relatively convenient sources of crucial growth factors and other trace nutrients necessary to support cell growth *in vitro*. However, the use of serum in CM media is antithetical to the overall goals of the CM industry: FBS has several important drawbacks in regard to consistency, economics, and ethics, which have been reviewed extensively by us and others [22,114–116,184]. Many companies and academics in the CM space still use FBS in early research and development work since cost effective serum-free media (SFM) do not exist. This fact highlights the continued pressing need for foundational research into how to inexpensively replace animal serum. It also raises the important question of whether the media and bioprocess design optimization work performed using serum-based formulations can be directly translated to the serum-free formulations that will ultimately be most relevant for CM [187–189].

A recent focus of our work has been to elucidate broad trends in the specific media requirements of cell types important to the CM industry via spent medium analysis (SMA). We have demonstrated using chicken muscle cells, chicken fibroblasts, and murine C2C12 muscle cells grown in serum-containing media that there are significant differences between the cell types in the cellular utilization rates of several key nutrients including glucose, arginine, glutamine, isoleucine, leucine, methionine, serine, and basic fibroblast growth factor (FGF2) [190].

The differences in these utilization rates appeared to be most significant in the earliest stages of the cell culture period. Interestingly, most other amino acids, vitamins, and minerals tested did not appear to be depleted by the cells over time, indicating possible targets for media optimization to reduce costs.

The main conclusion we have been able to draw from our work so far has been that there are indeed differences in the nutrient needs of CM-relevant cells from different species, as well as from different lineages within the same species. This would suggest that significant effort may be required to optimize media formulations specific to each of the cell types of interest for CM production. To build upon this basic assumption, though, it remains to be demonstrated whether the same key nutrient requirements will exist in a serum-free system. That is, does the presence or absence of serum influence nutrient metabolism of CM-relevant cells? In the present study we compared key nutrient utilization patterns and cell growth rates of C2C12 cells grown in a medium with 20% FBS versus Essential 8™ (E8) serum-free media.

Materials and Methods

General cell culture

C2C12 (CRL-1772™) (American Type Culture Collection, Manassas, VA) cells were cultured under the following general conditions. A period of expansion (proliferation) was first performed to obtain enough cells to inoculate the spent media collection experiments. The medium for this expansion (PCGM) consisted of 40% Dulbecco's Modified Eagle Medium (DMEM) (cat. # 11995073, Gibco, Waltham, MA), 40% Ham's F10 Nutrient Mix (Gibco cat. # 11550043), 20% FBS (Gibco), and additional 2.5 ng/mL recombinant human basic fibroblast growth factor (Cell Signaling Technology, Danvers, MA). Cells were maintained on tissue

culture-treated (TC) polystyrene dishes, seeded at 5,000 cells per cm^2 and passaged upon reaching about 75% confluence using TrypLE Express dissociation reagent (Gibco). Counting was performed via hemacytometer using trypan blue exclusion. Cultures were maintained at 37ºC and 5% CO_2 in humidified incubators throughout the experimental period.

Experimental media collection

Six-well TC-treated polystyrene plates were coated with Matrigel (growth factor-reduced, cat. # 356231, Corning, Corning, NY), using a conventional thin-coating protocol with a diluted solution at 0.15 mg/mL of protein. Sufficient wells were prepared to allow for three replicate wells per experimental media type per predetermined collection time points. C2C12 cells at passage 10 were resuspended in either serum-free Essential 8 (Gibco) or PCGM media and seeded in their respective sets of wells at 10^5 cells in a total volume of exactly 3 mL of media per well. **Table 4** provides a comparison of the chemical compositions of the two media formulations. 2 µL of a concentrated stock solution of Hoechst 33342 nuclear stain (ThermoFisher, Waltham, MA) was added to each well shortly thereafter to give a final concentration of 0.2 µg/mL in the wells. After cell seeding, the 6-well plates were left undisturbed in the cell culture incubator, with no media changes, until the predetermined collection times. At these time points, the corresponding set of three replicate wells from both media groups were imaged using an ImageXpress Pico high content screening microscope system (Molecular Devices, San Jose, CA). The instrument's software, CellReporterXpress v2.9, was used to obtain cell counts based on nuclear stain image segmentation. After imaging, all the media were collected from the three replicate wells from each media; the collected media volumes were also recorded to allow for normalization of later analyses to the potential degrees

of evaporation that took place during culture. However, the collected volumes were all within a few microliters of the 3 mL starting volumes, so normalization was not ultimately performed for the analysis. These media volumes were then filtered through 0.20 µm membrane filters into sterile tubes and stored at -80ºC for later analysis.

Glucose and lactate analysis

To measure carbohydrates and organic acids in the spent media samples, an HP 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) coupled with a refractive index (RI) detector (model # 1755, Bio-Rad Laboratories, Hercules, CA) was used with an Aminex HPX-87H column (Bio-Rad Laboratories). The mobile phase consisted of 5 mM sulfuric acid. Briefly, the method utilized an isocratic 0.6 mL/min flow rate for 25 min per injection, with the column kept at 50ºC. Standards of glucose and lactic acid were obtained from Sigma-Aldrich (St. Louis, MO) and dilutions were prepared fresh in mobile phase prior to analysis.

Amino acid analysis

Amino acid concentrations in fresh and spent media were measured using the REBEL analyzer (908 Devices, Boston, MA). The samples were prepared using a 0.20 µm sterile filter to remove cell debris, then diluted using REBEL diluent. Each sample was analyzed in triplicate via microfluidic capillary electrophoresis paired with high-pressure mass spectrometry (CE-HPMS). Analytes were automatically identified based on migration time and mass. Quantitation was achieved using calibration curves that were automatically generated from triplicate analysis of amino acid and vitamin standards.

Growth factor analysis

To measure the concentration of FGF2 in the spent media samples, an enzyme-linked immunosorbent assay (ELISA) kit for human FGF2 was used according to the manufacturer's instructions (ThermoFisher cat. # KHG0021). The E8 spent media samples were diluted 50-fold in sample diluent buffer while the PCGM samples were used undiluted in the assay. The final colorimetric absorbances in the 96-well assay plate were read at 450 nm using a SpectraMax iD3 multi-mode plate reader (Molecular Devices, San Jose, CA).

Calculation of yields of biomass on amino acids and on glucose

To calculate the amount of amino acid consumed to create 1 kg of wet cell mass, we focused on only the amino acids that decreased over time in either of the two media types. We first divided the mass of amino acids consumed per well from D0 to D4 by the number of new cells that were generated per well over that same time. We used an assumption of 3000 pg wet mass per cell [191] to convert this ratio to g of AA consumed per kg of wet cell mass produced. We used the same approach to calculate biomass yields on glucose use.

Results

Cell growth was largely similar between the two media but slightly higher in Essential 8

By recording cell counts per well every 24 hours during the culture period, we determined that the serum free E8 medium supported a similar degree of cell growth compared to the conventional serum-containing medium that we used. **Figure 10**A includes representative

micrographs of the two cell cultures at days 1 and 5 after plating, and shows their appearance before and after reaching confluence. **Figure 10**B is a graph of the cell count data obtained by nuclear stain image analysis, and shows that the cell counts between the two media were the same through the first two days of culture. The E8 cell count increased exponentially to day 3 and more quickly reached its maximum (which was higher than any cell count in the PCGM media) but then started to decline slightly and then dropped off significantly after day 6 (likely due to increased cell death and detachment). The PCGM cell count finished exponential growth after day 2 but continued gradually increasing to day 7, only reaching the E8 cell count by day 6.

Glucose utilization and lactate accumulation were similar between the two media

We next quantified the glucose and lactate concentrations in the spent media samples that were collected on each day of the cell culture period. **Figure 11**A compares the glucose utilization curves for both media types, while 11B overlays the lactate accumulation curves. The data indicate that glucose was used very similarly between the two media groups until around day 4, when the utilization slowed down for PCGM but continued for E8, despite continued cell growth in the PCGM group and decreasing cell number in the E8 group. While the initial lactate concentration in the PCGM was significantly higher than in the E8, the relative rates of lactate accumulation in the two media were similar over the seven-day culture period.

Basic fibroblast growth factor analysis

We measured the concentrations of FGF2 in our spent media samples to identify differences in utilization rates between the two media regimes, and determine whether there may be a correlation between a decline in the growth factor concentration and any particular cell

growth behaviors observed during the culture period. The primary consideration to note is that the E8 media contained several orders of magnitude more FGF2 than the PCGM at the early time points of culture; this reflects their respective formulations, with the extra FGF2 in E8 used as a primary ingredient intended to replace the function of FBS.

According to the data shown in **Figure 11**C, the FGF2 concentrations in both media began displaying significant reductions by around 3 or 4 days in culture, which is roughly the time the respective cell counts stopped increasing exponentially. While the FGF2 concentrations reached their minimum measurable values by around 5 or 6 days in culture for both media types, the cell numbers were maintained for at least a few additional days thereafter. Overall, there was not a significant difference in the relative FGF2 depletion patterns between the two media types.

Several key amino acids were utilized differently

According to our amino acid analysis in **Figure 11**E, the majority of initial (day 0) concentrations of each amino acid were similar between PCGM and E8, congruent to their basal media formulations. Discrepancies are likely due to the unknown amino acid profile of the FBS component in PCGM. Many of the amino acid concentrations did not display a significant decrease over time for either of the media, suggesting that they are not used or are metabolically cycled by the cells. For the amino acids that were depleted, the E8 media group seemed to decrease amino acids more rapidly and/or completely compared to the cells in PCGM. For example, glutamine and glycine were significantly more depleted in the E8 at 4-5 days in culture compared to their concentrations in PCGM. Serine was depleted almost completely in E8 by around day 3 which was the time at which exponential growth stopped. Otherwise, most amino acids showed similar general trends of utilization, except for a few, such as arginine.

Comparison of biomass yields on substrate utilization

Our data allowed us to calculate yield coefficients of biomass on amino acids and biomass on glucose. These numbers are critical for an effective technoeconomic analysis of a process like that of CM production. **Figure 11**D illustrates an extrapolation from our SMA, suggesting that 252.3 g of total amino acids (including 177.7 g of glutamine) and 1157.2 g of glucose might be required to produce a kilogram of C2C12 cells when cultured in E8. Conversely, 278.1 g of total amino acids (including 143.3 g of glutamine) and 1473.6 g of glucose may be required to produce a kilogram of cells cultured in PCGM. It should be noted that some of this glucose may be directed toward metabolic byproducts like lactic acid and not solely toward biomass.

Discussion

Important advances in serum-free cultivated meat media development are emerging in both academia and industry and studies investigating the most effective ways to minimize and replace expensive and animal-derived components of conventional media formulations have started to be published in recent years [34,156,192,193]. Because it was one of the best performing media tested with bovine myoblasts by Kolkmann et al. (2020), Essential 8™ has emerged as a model commercially available serum-free media for CM researchers, despite originally being developed to culture human pluripotent stem cells for biomedical research applications [194]. While E8 itself did not appear to yield bovine cell growth rates comparable to serum-containing media in these previous studies, our results indicate that the C2C12 myoblast cell line does perform at least as well in E8 (**Figure 10**). C2C12 is an immortalized muscle cell line that differs in

important physiological ways from adult stem cells that may be more relevant to CM. However, in order to reach better process efficiency, the CM industry may need to develop their own immortalized cell lines like C2C12. Our results nevertheless reinforce the fact that significant differences exist between the media requirements of CM-relevant cells from different species and lineages, and highlight the need to optimize media specifically for each cell type.

A recent study by Jang et al. (2022) characterized the metabolite profiles of proliferating and differentiated C2C12s growing in two commercially available SFM as well as a serum-containing medium. While the two mostly proprietary SFM formulations they compared contained complex additional proteins such as albumin (unlike the E8 media we tested), they found that the differentiation state of the cells had a more profound effect on the cell metabolic activity than the type of media that was used. Our more comprehensive SMA of similar cell culture paradigms complements these findings by elucidating how the presence or absence of serum affects the specific cellular nutrient requirements.

Our examination of glucose and lactate concentration kinetics (**Figure 11**A and B) suggest interesting influences of the media type on the metabolic behavior of the cells. The glucose utilization and lactate accumulation were largely similar between the serum- and serum-free media we tested, indicating the metabolic activity of the cells was not significantly different in the different media and suggesting that the absence of serum may not directly affect glucose metabolism. However, it is known that complex interactions of some of the many additional growth factors, cytokines, and other signaling molecules present in serum can regulate glycolysis and other metabolic activities [62]; these may be worth further investigation in the context of CM bioprocess optimization [62,195].

The initial presence of lactate in the PCGM, coming from the FBS, is one significant variable to consider when comparing serum- versus serum-free cell culture. Chronic lactate overload has been shown to inhibit myogenic activity [196], and it will thus be important to interpret previous myoblast physiology studies that used FBS-containing media with this consideration. The substantially higher lactate concentrations in our serum-containing culture may have had a subtle role in cell proliferation inhibition, and the lower lactate concentrations that we observed in E8 media may be another benefit of using serum-free media for CM production.

The analysis of cumulative substrate input required to produce a kilogram of cells (**Figure 11**D) reveals that there are not particularly large differences in glutamine, glucose, or total amino acid requirements between the two types of media. While the cells grown in PCGM appeared to require more of these substrates overall, the calculated requirements between the two media were still within 20% of each other. The actual average mass per cell at day 4 between the two media was likely different (although we were unable to directly measure that in this study and assumed a cell mass of 3000 pg in both media), and this could explain the differences in our calculated substrate requirements per kg of cells. The differences could also reflect the influence of the media formulations on the cellular metabolic activities.

Regardless, our finding that roughly 250-275 g of total amino acid would be required to produce 1 kg (wet weight) of C2C12 cells corresponds with estimates used in previous technoeconomic assessments regarding the amino acid requirement for CM cells [197]. Those estimates were derived from data on unrelated cell types because the specific metabolic requirements for CM-relevant cell types had not yet been evaluated. Similarly, our calculation of roughly 1100-1500 g of glucose needed to generate a kilogram of cells is congruent with these

previous estimates. However, it should be noted that there is potentially a great possibility of improving biomass yields on glucose utilization. Our culture system was essentially unoptimized for efficient bioproduction. For example, many industrial bioprocess feed strategies employ the use of lower-nutrient media during the initial culture periods to help mitigate lactate production and accumulation, before introducing more glucose-rich feeds to support greater cell growth [198]. Given that we observed substantial lactate production relatively early on, this is an indication that there is room for improvement (through cell line, media formulation, and/or feed strategy optimization) to shunt glucose metabolism to a process that produces less lactate and is more efficient for biomass production, requiring less glucose overall [62,199,200].

The amino acid kinetics analysis (**Figure 11**E) suggests that many amino acids are not significantly depleted by cells growing in either serum-containing or serum-free media and so are potentially not essential to include in optimized CM media, or at least in such substantial quantities. However, the analysis also reveals intriguing differences between the amino acid metabolism of cells growing in the two types of media. The difference in the rates of glutamine utilization suggest that cells in E8 media were undergoing higher rates of glutaminolysis until glutamine completely ran out by around day 4; this idea is supported by the low depletion rates of aspartate and alanine (metabolites of glutaminolysis) in E8 until day 4. However, the substantially higher glutamate concentration observed in PCGM through day 4 could suggest that glutaminolysis in the PCGM cells was occurring with a lesser degree of final deamination of glutamate to α-ketoglutarate. While these types of biochemical differences in amino acid metabolism between the serum- vs serum-free media are interesting and worth exploring further, the intention of this study was simply to highlight overall tendencies in amino acid consumption

rates and determine how the media formulations themselves may influence the nutrient requirements of C2C12 cells.

While the overall FGF2 utilization trends did not appear significantly different between the two media, the data in **Figure 11**C, compared with the growth curve data in 1B, suggest that around 700x more cells were generated in PCGM per mg of FGF2 utilized. FGF2 was utilized more by the cells growing in the serum-free E8 media compared to PCGM, which could be due to the lack of other growth-promoting factors normally found in serum, making the FGF2 even more important as a "substrate" for growth in SFM than it would otherwise be. The greater FGF2 in the E8 media could also explain the higher total cell number achieved in E8. FGF2 directly inhibits myoblast differentiation and promotes proliferation by altering the activity of the myogenic regulatory factor myogenin and preventing differentiation [201]. In this way, FGF2 could decrease contact inhibition, permitting more proliferation even as cell density increases. However, whether this degree of growth warrants the high levels of an expensive growth factor in CM media remains an open question.

Our results here provide important confirmation of general assumptions on media substrate requirements and yield expectations for CM production that will be highly useful for further technoeconomic analyses. Relatively little work has previously been completed on characterizing the metabolic requirements of the most CM-relevant cell types, so many of the more specific metabolic assumptions being made about them are derived from knowledge of how these cell types behaved in earlier studies using serum-containing media for other scientific purposes. Our study indicates that these assumptions may be disadvantageous to the overall ease and success of transitioning the cells to completely serum-free and chemically-defined media. A significant amount of time and effort will potentially be needed—utilizing SMA, black-box

machine learning, and other biological insights—to properly optimize serum-free media for these cell types to achieve the most efficient CM bioprocesses at lowest cost [154,165]. Perhaps, cell lines for CM could be created to be more robust to the effects of various SFM formulations to allow for greater versatility, and this may be a worthwhile pursuit for academic CM researchers in the future.

Author Contributions

EO, KB, and DB conceptualized the scope and methodology of the work. EO, JA, and GK performed all cell culture, HPLC, and/or ELISA work. EO completed formal data visualization and analysis, and drafted the original manuscript. MF and NR (908 Devices) provided amino acid analysis and drafted the corresponding methods section. DB and KB assisted with data interpretation, acquired funding, and provided overall supervision. All authors reviewed and edited the manuscript.

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Table 4: A comparison of the formulations of the primary cell growth medium (PCGM) and Essential 8™ media

Component	Concentration (mg/L)	
	PCGM[g]	**E8[h]**
Amino Acids		
Glycine	15	18.75
L-Alanine	3.6	4.45
L-Arginine hydrochloride	118	147.5
L-Asparagine-H2O	6	7.5
L-Aspartic acid	5.2	6.65
L-Cysteine	35.2	17.56
L-Cystine 2HCl		31.29
L-Glutamic Acid	5.88	7.35
L-Glutamine	292	365
L-Histidine hydrochloride-H2O	26	31.48
L-Isoleucine	43.04	54.47
L-Leucine	47.2	59.05
L-Lysine hydrochloride	70	91.25
L-Methionine	13.8	17.24
L-Phenylalanine	28.4	35.48
L-Proline	4.6	17.25
L-Serine	21	26.25
L-Threonine	39.44	53.45
L-Tryptophan	6.64	9.02
L-Tyrosine disodium salt dihydrate	42.648	55.79
L-Valine	39	52.85
Vitamins		
Biotin	0.0096	0.0035
Choline chloride	1.88	8.98
D-Calcium pantothenate	1.88	2.24
Folic Acid	2.12	2.65
Niacinamide	1.84	2.02
Pyridoxine hydrochloride	1.68	2
Riboflavin	0.32	0.219
Thiamine hydrochloride	2	2.17

[g] The values for PCGM component concentrations are 80% of those in the conventional DMEM/F10 formulation and do not include the contribution from the 20% FBS in the medium.
[h] The E8 formulation is essentially a modification of conventional DMEM/F12.

Vitamin B12	0.56	0.68
i-Inositol	3.08	12.6
Inorganic Salts		
Calcium Chloride (CaCl2) (anhyd.)	93.32	116.6
Cupric sulfate (CuSO4-5H2O)	0.001	0.0013
Ferric sulfate (FeSO4-7H2O)	0.3336	0.417
Ferric Nitrate (Fe(NO3)3-9H2O)	0.04	0.05
Magnesium Chloride (anhydrous)		28.64
Magnesium Sulfate (MgSO4) (anhyd.)	68.916	48.84
Potassium Chloride (KCl)	274	311.8
Potassium Phosphate monobasic (KH2PO4)	83.2	
Sodium Bicarbonate (NaHCO3)	1960	1743
Sodium Chloride (NaCl)	5520	6995.5
Sodium Phosphate monobasic (NaH2PO4-H2O)		62.5
Sodium Phosphate dibasic (Na2HPO4) anhydrous	61.48	71.02
Zinc sulfate (ZnSO4-7H2O)	0.012	0.432
Other		
D-Glucose (Dextrose)	2240	3151
Hypoxanthine Na	1.88	2.39
Lipoic Acid	0.08	0.105
Linoleic Acid		0.042
Phenol Red	6.48	8.1
Sodium Pyruvate	88	55
Putrescine 2HCl		0.081
Thymidine	0.28	0.365
HEPES		3574.5
Ascorbic acid-2-phosphate		64
Sodium selenite		0.28
Insulin		19.4
Transferrin		10.7
FGF-2		0.1
TGF-b		0.002
Fetal Bovine Serum	(20%)	

Figures

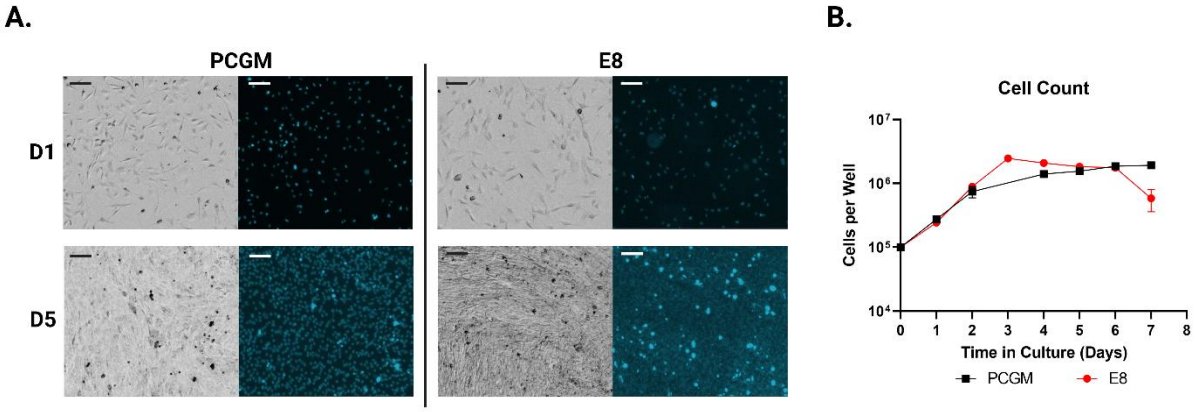

Figure 10: Comparison of the growth characteristics of C2C12 in serum and serum-free media

A, micrographs of live cells *in situ* captured at days 1 and 5 of culture for comparison of the morphology and degree of growth of the C2C12 cells in PCGM media (20% fetal bovine serum) and E8 media (serum-free). The brightfield image on the left is paired with its corresponding DAPI channel image on the right. Cell nuclei visible in the DAPI channel were stained with Hoechst 33342 and used to facilitate cell counting. Scale bars are 130 µm. **B**, cell count data of the same cells over 7 days, obtained by software image analysis using nuclear staining. Data points indicate the mean cell count of three biological replicate wells ± biological standard deviation.

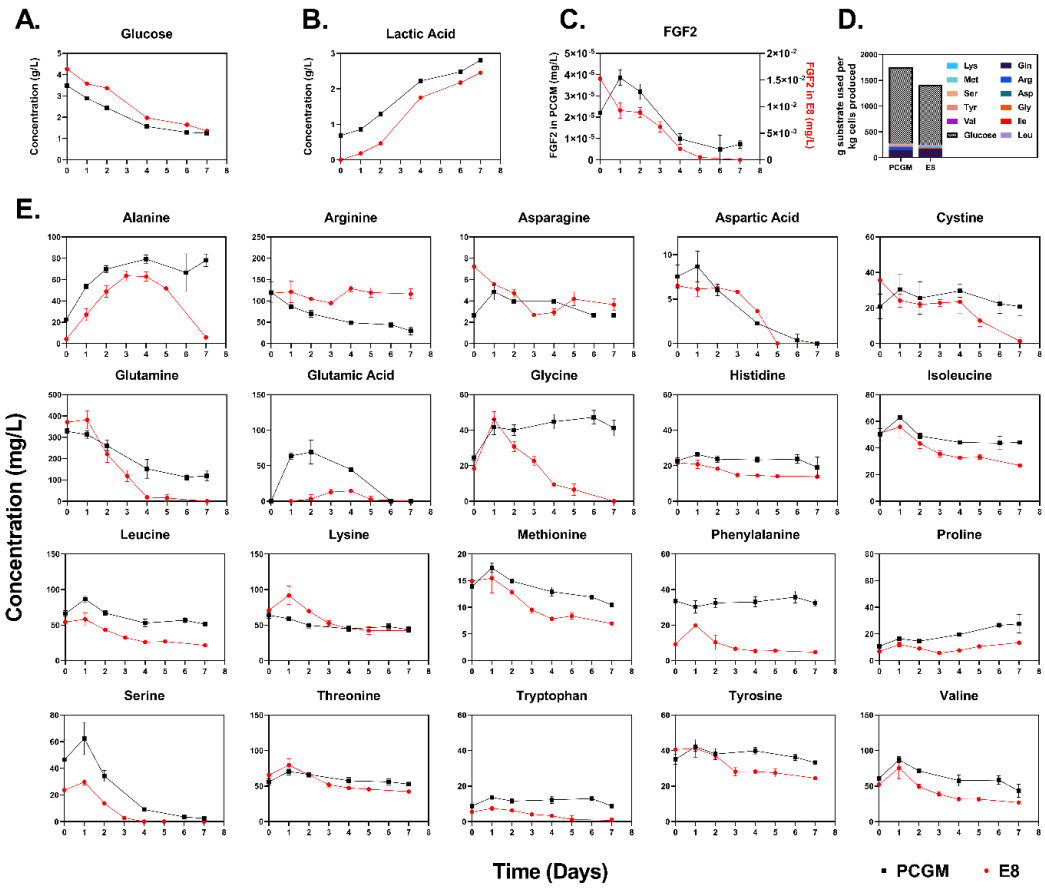

Figure 11: Comparison of the metabolite concentrations in spent media samples over time

Samples of spent media were collected at each day of C2C12 cell culture from the serum-containing PCGM group as well as the serum-free E8 group for chemical analysis. **A**, glucose concentration curve from HPLC analysis. **B**, lactic acid concentration curve from HPLC analysis. **C**, concentration curves of basic fibroblast growth factor (FGF2) from ELISA analysis, with the curve for each media overlaid on different linear y-axis scales. **D**, analysis of cumulative substrate consumption from D0 to D4 to produce 1 kg of wet cell mass (based on an assumption of a single-cell wet mass of 3000 pg). **E**, concentration curves of free amino acids from CE-HPMS-based REBEL analysis. Note that the y-axis scales are not all the same. The data points represent mean concentration values from three biological replicate wells ± biological standard deviation.

CHAPTER 5: Unraveling the complexity of hydrolysate-based media for cultivated meat production: A comprehensive analysis of hydrolysate chemical compositions and their impacts on cell growth

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This study addresses the imperative need for cost-effective and scalable cell culture media for the burgeoning cultivated meat industry. Focusing on plant and yeast hydrolysates provided by Kerry Group as potential alternatives to conventional media components, we scrutinized the variability and impact of their specific chemical compositions. We attempted to replace all purified amino acids with the hydrolysates in serum-free media for the murine C2C12 myoblast cell line. While none of the hydrolysates successfully replaced purified amino acids completely, certain hydrolysates exhibited better performance, with one wheat-derived hydrolysate named HyPep 4601N standing out. Extensive chemical analyses revealed low levels of free sugars, organic acids, and total phenolic compounds in the hydrolysates. Divalent cations, specifically calcium and magnesium, were identified as potential inhibitors for cell growth. Amino acid profiles varied widely among hydrolysates, with higher concentrations of some such as glutamate and alanine correlating with poorer performance. Endotoxin levels were low in all hydrolysates, suggesting limited impact on cell cultures. Multivariate statistical analyses, including principal component analysis and partial least squares regression, provided insights into the influence of hydrolysate source and processing methods on their chemical compositions, as well as the relative importance of the specific chemical components in the hydrolysates. The

findings contribute to the ongoing effort to develop simplified and cost-effective media formulations, laying the groundwork for scalable and economically viable cultivated meat production.

Introduction

The emergence of cultivated meat (CM) as an alternative to conventional meat production has garnered significant attention in recent years due to its potential to address global food system challenges related to sustainability, animal welfare, and food security[152]. The successful commercialization of CM requires the optimization of cell culture media to inexpensively and sustainably support the growth and differentiation of various cell types at large scales[202]. Media development represents a critical bottleneck, however, as existing conventional media are complex and often rely on animal-derived or expensive refined ingredients, limiting scalability and increasing production costs. Our previous review discusses and summarizes the breadth of key challenges and considerations for developing CM cell culture media[184].

Our recent research has shown that achieving consistent and efficient growth across cell types from different species—and different lineages within the same species—remains a key challenge, requiring media formulations tailored to the specific requirements of each cell type. However, our spent media analysis data have also demonstrated that several nutrient ingredients in conventional media do not appear to be appreciably consumed by CM cell types[190,203], although many of them are likely still necessary to be present in some amount for optimal cell growth. It may therefore be possible to focus attention on ensuring that the key cellular nutrient needs are being efficiently satisfied in the media, while placing less emphasis on optimizing every individual media component concentration.

Using plant and fungal protein hydrolysates holds promise as a cost-effective and sustainable way to satisfy the bulk of these identified nutrient requirements for CM cells[156]. Hydrolysates can be derived from protein fractions of plant or fungal biomass through enzymatic, chemical, or fermentative processes[204]. These preparations are known for their high nutritional content, including free amino acids, vitamins, minerals, and di-, tri-, and oligo-peptides. Conventionally, hydrolysates have found myriad applications in diverse industries, including pharmaceuticals, agriculture, and food and have been studied extensively for these purposes[204].

In the context of cell culture media, the use of hydrolysates has been investigated and successfully employed in large scale animal cell culture for biopharmaceutical production[73,130,205–207]. The promise of incorporating crude plant and yeast hydrolysates in media for a CM production bioprocess lies in their potential to inexpensively satisfy many of the nutrient requirements for cell growth and differentiation with only a few (or even just one) hydrolysate ingredients. This could substantially reduce the final media formulation complexity and the ultimate difficulty and cost of preparation. The diverse chemical profiles of different hydrolysates allow for a multitude of possibilities to complementarily combine them to create more optimal media.

A recent technoeconomic analysis (TEA)[208] predicts that media will account for approximately 85% of the cost of CM at large scale production, and further, about 60-70% of that media cost will be due to the need for amino acids. This suggests that the cost of amino acids will be even more important than that of specific signaling molecules and growth factor proteins which are traditionally thought to be leading cost-drivers of media. As such, identifying a very

inexpensive source of amino acids for CM media represents a potential way toward significantly reduced final production costs at scale.

However, little research has been published to date on using hydrolysates in CM media specifically. Essentially all published studies on CM have used media containing animal serum or chemically defined media enriched with multiple growth factors. There are many unknowns that need to be addressed toward the use of hydrolysates in CM media, such as the kinds of hydrolysates that would be most useful, the survival of different CM-relevant cell types in different hydrolysates, the ideal modes of preparation and utilization of the hydrolysates, and the potential effects of the hydrolysates on the final sensory and nutritional qualities of the CM products.

In the present study, we investigate the use of various crude plant and yeast hydrolysates provided by Kerry Group (Beloit, WI) in custom media formulations for CM cells. By building on the insights from our previous spent media analysis studies[190,203] and preliminary compositional data on the hydrolysates, we aim to lay foundational knowledge for the development of simplified inexpensive media formulations that effectively support the growth and differentiation of various CM cell lines.

We attempted to replace the purified free amino acid ingredients with the hydrolysates in serum-free media based on Essential 8™[120] for the murine C2C12 myoblast cell line. While none of the hydrolysates were able to completely replace the amino acids and function as the sole nitrogen source in the media, certain hydrolysates performed better than others. We then performed extensive chemical measurements of each hydrolysate combined with multivariate statistical analyses to determine whether particular chemical components in the hydrolysates are potentially inhibitory for C2C12 growth. The ultimate aim of the study was to inform ongoing

investigations into the selection and manufacture of hydrolysates that are more suitable to be used in CM media, which could enable significant media cost reductions at scale.

Materials and Methods

General cell culture

C2C12 (CRL-1722™) (American Type Culture Collection, Manassas, VA) cells at passage 13 were seeded onto 15 cm diameter tissue culture-treated (TC) polystyrene culture dishes at a density of approximately $5x10^3$ cells per cm^2 in 20 mL serum-free Essential 8™ (E8) media (Gibco, Waltham, MA) containing 50 µg/mL of Primocin® (InvivoGen, San Diego, CA), a broad spectrum anti-microbial formulation. The cells were allowed to proliferate for one to two days until reaching about 75 percent confluence before being used for experiments. Cultures were kept in humidified incubators at 37°C and 5% CO_2.

To detach cells from the culture dishes, the spent media was aspirated, cells were rinsed twice with Dulbecco's phosphate buffered saline (DPBS, no Ca or Mg) (Gibco), and 2 mL of TrypLE Express (Gibco) was added. The dishes were incubated at 37°C for 5 minutes before 4 mL of DPBS was added. The detached cells were then triturated in the dishes before being transferred to centrifuge tubes and centrifuged at 1500 x g for 5 minutes. The resulting cell pellets were then resuspended in DPBS and counted using a hemocytometer and trypan blue to assess viability.

Custom media formulation

Various media component stock solutions were prepared to accommodate specific parameters of the experimental designs used in the study. All containers and utensils used were

depyrogenated to minimize introduction of endotoxins, and all chemical components were obtained from Sigma-Aldrich (St. Louis, MO). Generally, a stock 2x concentrated solution of the standard E8 media formulation, omitting the amino acids, was created by combining specific volumes of individual chemical stock solutions that were dissolved in fresh 18.2 MΩ water. A separate concentrated stock solution of the amino acid mixture used in the E8 formulation was created, using L-alanyl-L-glutamine in place of L-glutamine to improve media stability. The osmolalities of these solutions were tested using a micro-osmometer (Advanced Instruments 3300, Norwood, MA) and brought to ~ 300 mOsm/kg with the addition of sodium chloride. All stock solutions were sterilized by passing through 0.2 µm pore size polyethersulfone (PES) membranes using syringes. The E8 growth factor supplement component (containing the seven other ingredients added to DMEM/F12 to make E8 (Table 1)) was kept aliquoted and stored at -20°C until needed. After addition of the supplement to the stock basal media solution, the solution was used for no more than one week (due to the short shelf life of the thawed supplement components).

Hydrolysate stock solution preparation

Eighteen dry powdered hydrolysate products were provided by Kerry and are described in **Table 5**. For cell culture experiments, stock solutions of each hydrolysate were prepared by dissolving in fresh 18.2 MΩ water at 40 g/L, then centrifuging at 15,000 x g for 10 mins at 4°C and sterile filtering the supernatant through 0.2 µm pore size PES filters (although no solid precipitates were apparent after centrifuging). These stock solutions were prepared fresh in this manner immediately before each cell culture experiment. For chemical analysis of the hydrolysates, stock solutions were prepared in the same manner, except at 10 g/L.

Table 5: Preliminary compositional data on the Kerry hydrolysates[ij]

Product Name	Source	Digestion	pH	AN (%)	TN (%)	AN/TN Ratio	Ash (%)	Moisture (%)	%>10 kDa	%5-10 kDa	%2-5 kDa	%1-2 kDa	%1k-500 Da	%<500 Da
HyPep 1510	Soy	Enzymatic	7.2	2	9.2	21.7	9.7	3.6	0	0.2	4.6	14.3	22.8	58.1
HyPea 7404	Pea	Enzymatic	4.7	3	13.2	23	6.1	4	0.1	2.6	16.8	27.1	23.6	29.8
HyPep 1512	Soy	Enzymatic	7.7	2.2	8.8	25	11.6	2.8	0	0.1	3.2	13.5	21.1	62.1
HyYest 412	Yeast	Enzymatic	5.6	4.9	10.9	44.6	13	2.7	0.2	0.1	3.6	14.4	17.2	64.4
HyPep 5603N	Rice	Enzymatic	6.3	2.3	12.8	18	3.9	3.8	0.5	1.6	9.7	19.7	24.6	43.9
HyYest 444	Yeast	Enzymatic	6.7	5.5	11.3	48.7	15.3	4.1	0	0.1	3.5	13.1	16.3	67.1
HyPep 7504	Cotton	Enzymatic	7.1	1.9	9.9	19.5	10.7	3.2	0.1	2	9.9	19.1	21.9	47
HyYest 504	Yeast	Enzymatic	5.2	4.1	10.6	39.1	11	3.8	0.3	0.6	6.2	14	18	60.9
Sheff CHO PF ACF	Various	Enzymatic	7.1	0	6.2	0	6.6	3	0	0.9	5.3	13.9	19.6	60.3
HyYest 466	Yeast	Enzymatic	5.6	5	11.9	42.4	14.1	4.2	0	0.1	5.5	15.8	15.1	63.5
UltraPep YE	Yeast	Enzymatic	6.7	4.4	9.6	45.8	13	2.9	0	0.6	6	14.7	19.3	59.4
Hy Soy	Soy	Enzymatic	7.1	1.9	9.1	21	10.7	2.5	0	0.3	5.3	16.4	23.5	54.6
HyPep YE	Yeast	Enzymatic	6.1	5	10	50	9	4.3	0	0.1	3.4	10.2	13.7	72.6
UltraPep Soy	Soy	Enzymatic	6.4	2.5	8	31.3	11	5.2	0.2	1.4	5.1	12	18.4	62.9
HyPep 4601N	Wheat	Enzymatic	6.5	2.7	13.4	20.1	2.4	4.2	0.17	1.25	5.5	13.1	21.1	58.1
HyPep 1511	Soy	Enzymatic	7	2.7	16.6	20	8.6	2.6	0.1	1.4	10.1	19.7	25	43.7
AmiSoy BF	Soy	Acid	5.9	6.7	9.3	72.1	14	5	0	0	0.9	7.3	16.5	75.3
AmiSoy	Soy	Acid	5.8	8.6	13	66	10.1	3.7	0	0	0.4	4.5	18.5	76.6

Cell proliferation experiments

The various media component stock solutions were combined in appropriate permutations directly in the wells of 96-well TC plates (Thermo Fisher, Waltham MA) according to experimental design requirements. For all experiments, the basal media nutrient mixture concentrations, osmolalities, final volumes, and fluorescent stain concentrations were kept constant across all wells and experimental groups. The amino acids and hydrolysates were the only components that were varied in the experiments. DPBS was used to normalize the final volumes and concentrations of the components in the wells. Each unique media formulation was tested in triplicate.

The wells of the 96-well plates all contained 200 uL total of experimental media with 500 ng/mL of Hoechst 33342 (Thermo Fisher) and 50 nM of SYTOX® Green stain (Thermo Fisher) to facilitate live cell counting. All experiments were seeded with about 3,000 C2C12 cells at passage 14 per well. The seeding density here is about 9400 cells/cm^2. Conventional protocols

[i] Data are adapted from Kerry Group's analyses and are available at https://www.kerry.com/products/pharma-and-biotechnology/cell-nutrition.html
[j] AN, available nitrogen; TN, total nitrogen

for C2C12 cell culture call for approximately 5000 cells/cm^2, but we have found that higher seeding densities improve overall viability in the serum-free context. An ImageXpress Pico automated high content screening microscope system (Molecular Devices, San Jose, CA) was used to image the experimental wells one hour after cell seeding, and daily thereafter for 4-7 days, depending on the experiment. The instrument's software, CellReporterXpress, was used to analyze the images to obtain live cell count data for each well at each time point.

Chemical analysis of Kerry hydrolysates

HPLC analysis of sugars and organic acids

To detect and quantify free sugars and organic acids in the hydrolysate solutions, an HP 1100 high performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA) coupled with a diode array detector (HP G1315A) and a refractive index detector (model 1755, Bio-Rad Laboratories, Hercules, CA) was used with an Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad) as previously described[190]. Briefly, the mobile phase consisted of 5 mM sulfuric acid with an isocratic flow rate of 0.6 mL/min for 25 min per injection, and the column kept at 50°C. Absorbance data were collected at 210 nm. Solutions of the Kerry hydrolysates were prepared at 10 g/L in mobile phase (to overcome potential limits of detection of particular compounds) and sterile filtered. 5 uL of each sample was injected into the HPLC.

Standards of the following sugars and organic acids were obtained from Sigma-Aldrich and prepared fresh at 10 g/L in mobile phase: maltose, citric acid, glucose, galactose, fructose, succinic acid, sodium L-lactate, pyroglutamic acid, pyruvic acid, α-ketoglutaric acid, formic acid, fumaric acid, gallic acid, isobutyric acid, maleic acid, malic acid, oxalic acid, phosphoric

acid, shikimic acid, adipic acid, benzoic acid, propionic acid, quinic acid, butyric acid, phytic acid, xylose, arabinose, and cellobiose.

Total phenolics quantification

To measure the total abundance of phenolic compounds present in the 10 g/L stock solutions of the Kerry hydrolysates, a colorimetric phenolic compounds assay kit (Sigma-Aldrich cat. # MAK365) was used according to the manufacturer's instructions. The assay utilizes the interaction of phenolic compounds with diazonium salts in alkaline conditions that create a diazo chromophore that can be detected by absorbance at 480 nm. Catechin was used as the standard for calibration curve preparation, and results are expressed in terms of mM catechin equivalents. Assay plates were read using a SpectraMax iD3 multimode plate reader (Molecular Devices).

HPLC-DAD analysis of phenolic compounds and water-soluble vitamins

To attempt to detect and quantify specific free phenolic compounds in the hydrolysate solutions, the same HPLC system as described above was used with an Agilent Poroshell 120 EC-C18 column (4.6 x 100 mm, 2.7 µm particle size). Mobile phases consisted of 25 mM HK_2PO_4 (pH 7.0) (A) and acetonitrile (B) at a constant flow rate of 0.5 mL/min. The 25 min method used an isocratic flow of 1% B from 0 to 5 mins, a gradient increase to 30% B from 5 to 15 mins, an isocratic flow at 30% B from 15 to 20 mins, a gradient decrease to 1% B from 20 to 20.1 mins, an isocratic flow at 1% B from 20.1 to 25 mins, followed by 5 mins post run time at 1% B. Absorbance data were acquired at 205, 214, 232, 266, and 280 nm with reference at 360 nm, as well as at 360 nm with reference wavelength of 400 nm. The Kerry hydrolysates were prepared at 10 g/L in mobile phase A and 20 µL were injected.

Standards of the following phenolic compounds were dissolved in dimethyl sulfoxide at 10 g/L: gallic acid, vanillic acid, sinapic acid, caffeic acid, ferulic acid, p-coumaric acid, m-coumaric acid, o-coumaric acid, chlorogenic acid, quinic acid, syringic acid, *trans*-cinnamic acid, hydroxycinnamic acid, salicylic acid, (+)-catechin hydrate, (-)-epicatechin, quercetin hydrate, naringenin, and rutin. Standards of the following water-soluble vitamins were prepared in the same manner: thiamine, riboflavin, niacinamide, calcium pantothenate, pyridoxine hydrochloride, biotin, folic acid, and cyanocobalamin. The standards were injected on the HPLC individually and as mixtures at various final concentrations. The chromatograms for the Kerry hydrolysates were searched for these phenolic and vitamin compounds via retention time and spectral library matching.

After determining that there were few free phenolic compounds detectable in the hydrolysates, we summed the A280 peak areas of the chromatograms from 1 to 9.5 mins, and from 9.5 to 23 mins. These ranges were selected after observing that a large number of unresolved peaks occurred in the latter time range, while fewer, more distinguishable peaks appeared in the earlier time range. We designated the earlier time range as generally corresponding to "monomeric" phenolics, and the second time range as "polymeric" phenolics. This approximate classification scheme, while not particularly informative on its own, allowed for additional insight into how the general chemical compositions of the hydrolysates are correlated with effects on cell culture through PLS regression.

Elemental analysis

Analysis of bulk and trace elements in the Kerry hydrolysates was performed via inductively coupled plasma-mass spectrometry (ICP-MS) as previously described[190]. Briefly, an

Agilent 7850 ICP-MS system including the Octopole Reaction System ORS^4 collision cell and Ultra High Matrix Introduction aerosol dilution system was used in helium collision cell mode with kinetic energy discrimination (KED). Calibrations and standards were performed in the same manner as previously described.

Amino acid analysis

Quantification of free amino acids in the 10 g/L stock solutions of Kerry hydrolysates was performed by the UC Davis Molecular Structure Facility using a Hitachi LA8080 amino acid analyzer (Hitachi High Tech, Tokyo, Japan), as previously described[190]. Briefly, samples were acidified with 2% sulfosalicylic acid (SSA) and incubated at room temperature (23 °C) for 15 minutes. Afterward, the samples were frozen overnight at -20 °C. To dilute the samples, they were mixed with 100 nmol/mL AE-Cys Li. The system was injected with 20 μL of the sample, and ion-exchange chromatography was employed to separate the free amino acids. A post-column ninhydrin reaction was used in conjunction with a column and buffers obtained from Hitachi, ninhydrin supplied by Wako (Richmond, VA), and amino acid standards acquired from Sigma-Aldrich. The absorbance was measured at 570 nm and 440 nm after the reaction with ninhydrin to determine the response factor for each amino acid and to quantify their levels relative to the known amino acid standards. AE-Cys was utilized to correct for any variation in injection volume. It should be noted that certain amino acids, like tryptophan, could not be measured using this particular method.

Endotoxin quantification

To measure the abundance of endotoxins in freshly-prepared stock solutions of the Kerry hydrolysate products, a Pierce Chromogenic Endotoxin Quant Kit (Thermo Fisher cat. # A39552) was used according to the manufacturer's instructions. Briefly, stock solutions of the Kerry products were prepared at 10 g/L in fresh endotoxin-free water in sterile tubes, then centrifuged at 15,000 x g. The supernatant was sterile filtered with 0.2 um pore size PES filters into new sterile tubes before use in the assay. The assay plates were read using a SpectraMax iD3 multimode plate reader (Molecular Devices).

Data analysis

Cell culture experiment analysis

The number of live cells at each time point during the cell culture experiments was determined by subtracting the number of dead cells (stained with SYTOX) from the total number of cells (both live and dead, stained with Hoechst 33342) obtained from software analysis of the corresponding micrographs. The live cell counts per well were then plotted versus the time in culture. Fold changes in cell count on select days after seeding were calculated by dividing the number of live cells counted on a given day by the Day 0 live cell count for each well.

Multivariate statistical analysis

The chemical composition data for all the Kerry hydrolysates were analyzed by principal component analysis (PCA) using Prism 10 software (GraphPad Software, San Diego, CA). PCA was performed for all data collectively as well as individually for each major class of components that were measured. All data were centered and standardized for analysis, and principal components with the largest eigenvalues that together explain 75% of the total variance

in the data sets were selected. Loadings and PC score plots were prepared for the first two principal components from each analysis.

Partial least squares (PLS) regression analysis using JMP 17 software (SAS Institute, Cary, NC) was performed on the hydrolysate chemical composition data in relation to their effects on C2C12 cell growth, with the response variable being the fold change in live cell count from 0 to 3 days in culture. This time range was chosen due to the fact that no additional cell growth was observed past 3 days for the hydrolysate groups, even in pilot experiments observing similar cell culture conditions beyond 7 days. Data were scaled and centered, and the SIMPLS algorithm[209] was used with fast singular value decomposition (SVD). The holdback cross-validation method was employed using a proportion of 33 percent. The initial number of factors to search was 15, and the minimum root mean predicted residual error sum of squares (PRESS) was found using 7 factors. The root mean PRESS, van der Voet T^2 statistic, correlation coefficient (R^2), and cross-validated correlation coefficient (Q^2) were determined from 0 to 15 factors in order to assess the quality of the model. Chemical component (X) loadings and hydrolysate cell culture performance (Y) scores plots for selected factors were generated to assess their influence on the PLS model and identify chemical components that most likely influenced cell culture performance.

Results

Assessing cell growth when substituting hydrolysates for the amino acids in Essential 8 media

An initial screening of all 17 Kerry hydrolysates was performed to assess their ability to replace all free amino acids (AAs) in the E8 media formulation. Custom-made E8 media was

prepared by omitting all free AAs and substituting in the hydrolysates at 1 and 1.5 g/L in the final media in 96-well plates. These concentrations were chosen based on Kerry's suggestions and based on the fact that E8 contains approximately 1.03 g/L of free AA. Live cell counts were recorded daily (**Figure 13**A), and the Day 3 cell counts and fold changes in live cells per media group were plotted separately (**Figure 13**B). These figures show that none of the hydrolysate-containing media supported cell growth to nearly the same degree as the homemade 100% AA control media (same concentrations of all AAs as the standard E8 formulation). Generally, most hydrolysates either supported simple cell survival over the cell culture period with minimal to no proliferation or killed the cells in the same manner as the 0% AA control.

However, **Figure 13**B highlights the fact that certain hydrolysates functioned better than others, with HyPep 4601N generally being the best and AmiSoy performing the worst. The 1 g/L concentration performed slightly better than the 1.5 g/L concentration in this case, so 1 g/L was used for subsequent statistical data analyses and for follow-up cell culture experiments. HyPep 4601N had a mean Day 3 fold change in live cell count of 1.66 from the seeding density, while AmiSoy had 0.15.

Free sugars and organic acids were not detectable in the hydrolysates in significant quantities

HPLC analysis of the Kerry hydrolysate stock solutions did not reveal distinct peak matches with any of the 28 simple sugar or organic acid standards that were tested (data not shown). Spectral library and retention time comparison of the DAD traces of samples and standards did not reveal any matches above the match threshold of 800 for any peaks.

Hydrolysates contained low levels of total phenolic compounds

The assay for total phenolic compounds in 10 g/L stock solutions of the Kerry hydrolysates revealed that the concentrations were all relatively low. **Figure 14**A indicates that many of the hydrolysate solutions had concentrations below the lowest level of the standard curve for the assay. The remainder of the hydrolysates had concentrations between around 0.04 and 0.08 mM catechin equivalents. For context, brewed green tea may contain close to 4 mM catechin equivalents[210], which is 50- to 100-fold higher than the hydrolysate samples.

HPLC-DAD analysis suggested hydrolysates did not contain appreciable quantities of free phenolics or water-soluble vitamins

The spectral library searching of the Kerry hydrolysate sample chromatograms did not return matches with any of the phenolic or vitamin standards that were tested (data not shown). It is likely that any phenolic compounds present in the hydrolysates were complexed with other components such as peptides, thus complicating chemical analysis in this regard. Large clusters of unresolved peaks and increasing baselines in the chromatograms beginning after about 9.5 minutes suggested that polymeric phenolic complexes were eluting upon introduction of the acetonitrile mobile phase[211]. As such, the total peak areas of the first and second sections (1 to 9.5 and 9.5 to 23 min) were integrated and plotted separately.

Figure 14B shows the relative levels of integrated absorbance area at 280 nm of all eluates in the first and second summed sections of the hydrolysate chromatograms, labeled as “monomeric” and “polymeric”, respectively. In general, the polymeric peak areas were larger than the monomeric areas for most hydrolysates.

ICP-MS analysis revealed hydrolysates contained varying levels of bulk and trace minerals

Elemental analysis via ICP-MS indicated that total mineral content of the various Kerry hydrolysates ranged from approximately $1x10^5$ to $8x10^5$ ppb. **Figure 15**A indicates that HyPep 4601N contained the lowest quantity of minerals, while AmiSoy had the highest, with HyPep 4601N having approximately 20 percent of the amount of AmiSoy. Moreover, the primary minerals present in most hydrolysates were sodium and potassium, with the ratio of these two elements being different across different hydrolysates. The concentrations of calcium and magnesium made up a relatively small percentage of the total bulk minerals in all hydrolysates except for AmiSoy, which had calcium as its mineral of highest concentration.

The total trace mineral content plotted in **Figure 15**B varied across the hydrolysates almost 10-fold, ranging from approximately 1,300 to 13,000 ppb. Again, HyPep 4601N had one of the lowest amounts of total trace minerals, whereas HyYest 412, HyYest 466, HyYest 504, and HyPea 7404 contained the highest. The yeast-derived samples appeared to contain relatively high levels of zinc. More generally, zinc and/or iron seemed to be the minerals that comprised most of the measurable trace elements in the hydrolysates, although the ratios varied.

Amino acid profiles vary widely among the hydrolysates

Amino acid analysis revealed that the Kerry hydrolysates contained varying levels of total free amino acids, and that specific AA ratios also varied considerably.

Figure 16A shows that the total free AA content of the 10 g/L hydrolysate stock solutions ranged from about 400 mg/L on the low end (HyPea 7404) to about 5000 mg/L on the high end (AmiSoy). The graph includes the free amino acid profile of fresh E8 media for comparison.

The data in

Figure 16B and C show that the total free AA content of the 10 g/L hydrolysate stock solutions varied from about 0.4 to 5 times the total AA content of E8. HyPep 4601N (the best-performing hydrolysate) had a total free AA content very close to that of E8. AmiSoy (the worst-performing hydrolysate) had the highest free AA content out of all products tested. The yeast-derived hydrolysates all contained more free AA than E8, with a range from around 1800 to 3400 mg/L. The other plant-derived hydrolysate stock solutions, except for UltraPep Soy, contained less total free AA than E8. Given that the hydrolysates were ultimately used at 1.0 and 1.5 g/L final concentration in the experimental media for cell culture, these AA analysis data suggest that all hydrolysate-supplemented experimental media contained at least 2 times less free AA than the E8 media control in this study, although they presumably contained a significant quantity of small peptides.

The specific free AA ratios of the hydrolysates also varied substantially—most notably, E8 includes a significantly higher concentration of glutamine than was present in any of the hydrolysate products. Glutamate and alanine were also seen to vary greatly among the hydrolysates, with the yeast-derived products and AmiSoy (generally the worst-performing hydrolysates) containing much higher amounts of these two amino acids than the rest of the hydrolysates.

Endotoxin levels were low in all hydrolysates

The endotoxin quantification assay revealed that all hydrolysate products contained relatively low amounts of endotoxins. **Figure 17** shows that total measurable endotoxin content of the 10 g/L hydrolysate stock solutions were highest for the HyYest and HySoy products, at

about 0.5 to 0.6 EU/mL. For reference, cell culture-grade FBS used in biomedical research may contain endotoxin levels exceeding 10 EU/mL[212].

Principal component analysis confirmed the influence of the hydrolysates' source and processing method on their chemical composition

The PCA scores plot for the first two principal components in **Figure 18**A shows distinct groupings of hydrolysate products that broadly correspond to their respective production source and processing methods. All yeast-derived products are generally seen to cluster in the lower right quadrant of the plot, and AmiSoy (the only acid-hydrolyzed product among the tested products) is isolated in the upper right quadrant. Generally, the worst-performing hydrolysates grouped in the right quadrants. For this PCA of all measured chemical components, PC1 accounted for 36.27 percent of the variation, and PC2 accounted for 14.31 percent.

Figure 18B indicates that in PCA of only the amino acid components, PC1 accounted for 49.21 percent of the variation and PC2 accounted for 14.47 percent. In the PC scores plot, the yeast hydrolysates and AmiSoy were again clustered in similar locations as in the overall PCA scores plot in **Figure 18**A. **Figure 18**C shows results from PCA of just the mineral content of the hydrolysates. The first two PCs together accounted for only 40.46 percent of the total variation. The PC scores plot for PCA of the remaining chemical components in **Figure 18**D shows less distinct cluster separation of the yeast-based hydrolysates and AmiSoy from the rest of the hydrolysates, although the general trend of these products being plotted in the right quadrants remains consistent as in **Figure 18**A.

Partial least squares regression highlighted potentially beneficial and detrimental chemical components in the hydrolysates

Table 6 includes the results of the PLS regression holdback cross validation that was performed for the analysis. The lowest root mean PRESS was found when using 5 factors, and the corresponding cumulative Q^2 value was 0.599. The cumulative percent variation explained for X effects (chemical component concentrations) by the three factors used in the model was 77.47. The cumulative percent variation explained for the Y responses (fold change in cell count at day 3 for each hydrolysate culture experiment) was 99.93.

Table 6: PLS holdback cross validation with proportion=0.333 using SIMPLS with Fast SVD

Number of factors	Root Mean PRESS	van der Voet T²	Prob > van der Voet T²	Q²	Cumulative Q²	R²X	Cumulative R²X	R²Y	Cumulative R²Y
0	1.605421	2.675786	0.0300*	-0.218408	-0.218408	0.000000	0.000000	0.000000	0.000000
1	1.350895	0.679018	0.4190	0.137304	0.137304	0.351619	0.351619	0.583892	0.583892
2	1.402143	1.251320	0.3370	0.070607	0.198216	0.142087	0.493706	0.341520	0.925412
3	1.350517	1.003131	0.4270	0.137786	0.308691	0.162775	0.656480	0.045651	0.971062
4	1.270985	0.013594	0.7530	0.236347	0.472080	0.069628	0.726109	0.024043	0.995105
5	1.267697	0.000000	1.0000	0.240294	0.598936	0.048549	0.774658	0.004189	0.999294
6	1.307071	1.330048	0.0250*	0.192369	0.676088	0.057699	0.832357	0.000543	0.999837
7	1.362488	1.184164	0.1930	0.122433	0.715746	0.040576	0.872933	0.000155	0.999992
8	1.417560	1.127043	0.1560	0.050056	0.729974	0.039941	0.912874	0.000007	1.000000
9	1.473787	1.087234	0.1580	-0.026797	0.722739	0.046935	0.959808	0.000000	1.000000
10	1.547793	1.060987	0.1780	-0.132506	0.686000	0.039318	0.999126	0.000000	1.000000
11	1.547793	1.060987	0.1430	-0.132506	0.644393	0.000000	0.999126	0.000000	1.000000
12	1.547793	1.060987	0.1490	-0.132506	0.597273	0.000000	0.999126	0.000000	1.000000
13	1.547793	1.060987	0.1370	-0.132506	0.543909	0.000000	0.999126	0.000000	1.000000
14	1.547793	1.060987	0.1760	-0.132506	0.483474	0.000000	0.999126	0.000000	1.000000
15	1.547793	1.060987	0.1470	-0.132506	0.415031	0.000000	0.999126	0.000000	1.000000

Figure 19A displays the X effects loading plot for the first factor of the PLS model (which accounted for 37.10 percent of the variation). Chemical components with positive loadings values can generally be said to be potentially beneficial for cell growth according to the model, while components with negative values are potentially inhibitory for growth. 24 of the 75 total X effects in the model had positive loading values, indicating that the majority

(approximately two thirds) of the chemical components measured in the hydrolysates are negatively correlated with cell growth.

Figure 19B shows the scores plot of the Y responses for the eleven hydrolysates used to create the model. There is a generally monotonic trend of increasing cell culture performance of the hydrolysates in the plot from the bottom left to the top right quadrants, which is an indication of the quality of the model.

Figure 19C shows the variable importance plot (VIP) of the X effects in the model, with the conventional threshold value of 0.8 [213]. Of the 75 chemical components, 23 had variable importance values below the threshold level, indicating that they are perhaps less important in explaining the variability among the X effects. Some of the components and variables with the highest VIP values included sodium, silicon, barium, sarcosine, α-aminobutyric acid, moisture percentage, and polymeric/monomeric phenolic content ratio. Chemical components with both a low VIP value and a low PLS coefficient had minimal influence on explaining the variability among either the X effects or Y responses and are thus candidates for pruning from the model (although that was not performed in this case). A plot of the VIP values versus PLS coefficients for the chemical components is provided in Supplementary Figure 2.

Discussion

With cell culture media being the current leading cost driver behind cultivated meat scale up and commercialization, there is an urgent need to identify significantly less expensive ways to satisfy cellular nutrient needs. Existing technoeconomic analyses (TEA) assert that the amino acids in conventional basal media formulations account for the largest portion of the final media costs and are thus prime targets for cost reduction efforts. For example, at least 60% of the

projected cost of a model serum-free medium used in a recent TEA by Negulescu et al.[208] is attributed to the amino acid components. Further, the recent TEA completed by Humbird[197] suggests that approximately 50% of the overall cost of production for a fed-batch CM production process is contributed by the need for amino acids in the media. These TEAs argue that employing plant and fungal hydrolysates in media formulae in place of laboratory or pharmaceutical grade amino acids is one of the key potential routes toward development of cost-effective media due to the hypothetical ease of substituting expensive purified components in the media with crude agricultural byproducts that are rich sources of many of the required cellular nutrients.

The present study aimed to assess the feasibility of using hydrolysates as the nitrogen source in media to support muscle cell growth, and to gather evidence to help explain the inhibitory effects that were observed for many types and preparations of hydrolysates. Indeed, the hydrolysates (as used in this study) were unable to adequately substitute for the purified amino acids in the serum-free E8 formulation, as demonstrated by the 10-fold lower rate of proliferation in media made from hydrolysates relative to amino acid control media (**Figure 13**). However, the more significant impact of this work lies in the identification of potential targets for improving the use of these inexpensive nitrogen sources.

One classes of compounds in plant hydrolysates that can have a significant inhibitory impact on CM cell growth is phenolics. Phenolics are a broad class of diverse and often complex phenol group-containing molecules that are naturally abundant in most plant tissues and are important for a range of functions from cellular signaling to protection against pathogens and predators[214]. There has been a modest amount of work done investigating the influence of phenolic compounds on animal cell culture. Intriguingly, some studies have observed positive

effects of specific phenolics on certain animal cell lines, while others recorded negative effects[215,216]. These diverse results highlight the complexity and unknown nature of how this broad class of plant metabolites could affect CM cell growth.

Figure 14A shows that, while the absolute quantities of phenolic compounds in our hydrolysate preparations were low (relative to common plant products such as tea, berries, and chocolate[217,218]), they varied significantly according to their source and processing methods. A challenge facing the interpretation of the influence of phenolics in hydrolysates for CM is that there were few, if any, individual phenolic compounds identifiable in the hydrolysates that we tested. The results shown in **Figure 14**B and Supplementary Figure 2 indicate that the phenolic chemical constituents in the hydrolysates are exceedingly complex, with most phenolics likely being complexed with one another[219]. Of course, this leads to difficulty in analyzing and understanding details of hydrolysate compositions and their effects on cell cultures. Moreover, PLS regression analysis (**Figure 19**) suggested that total phenolics, and high levels of monomeric phenolics, may indeed have an important negative effect on cell culture performance, indicating that there is a need for more extensive investigation in this regard.

It is worth noting that the phenolics measurements were performed on individual stock solutions of the hydrolysates—not the final hydrolysate-supplemented experimental media. There are a multitude of potential interactions possible between phenolic compounds and the various components of the E8 media formulation which could influence their respective availability and functions when hydrolysates are supplemented in. For instance, it is plausible that phenolics could bind or interact with the important specific growth factor proteins in E8 like bFGF[219]. Moreover, the particular processing and preparation methods of hydrolysate-supplemented media likely have effects on phenolic interactions. The sterilization method used

may have an especially important influence; high temperature-short time (HTST) sterilization, as might be used for large scale CM production, may induce additional complex interactions among the phenolic components. These ideas will be important to explore further to ensure that the use of complex hydrolysates does not impair the expected functions of other important ingredients in otherwise-chemically defined media.

Another group of chemical components that was identified in the hydrolysates as being potentially more directly inhibitory for cell growth was the divalent cations, including calcium and magnesium. As seen in **Figure 15**, AmiSoy contained by far the highest concentrations of these two elements, and this stands out as the most dramatic compositional difference between AmiSoy and the other hydrolysates. As reflected in the PCA and PLS analyses (**Figure 18** and **Figure 19**), the relatively high levels of these divalent cations in AmiSoy are potentially correlated with the poor cell growth that was observed for this hydrolysate. Divalent cations, especially calcium and magnesium (and their interactions), have a variety of important functions in cell culture media[220–222]; however, their effects are highly dose dependent with high concentrations causingand they can cause detrimental effects including solubility issues as well as apoptosis. As such, hydrolysates for CM cell culture media should be selected and processed with careful attention to the concentrations of these ions.

The free amino acid content of the hydrolysates, originally hypothesized to be a beneficial inexpensive replacement for pharmaceutical grade media components, counterintuitively appeared to be negatively correlated with cell growth performance in this study.

Figure 16A indicates that AmiSoy, the worst performing hydrolysate, contained significantly higher concentrations of almost all measurable free amino acids than most of the

other hydrolysates. This is likely because it was the only acid-hydrolyzed product included in this study (the others being enzymatically hydrolyzed), and thus had a greater degree of hydrolysis. Molecular weight distribution analysis of the hydrolysate chemical constituents found that AmiSoy had a significantly lower average molecular weight than any of the other hydrolysates (Table 1). The other hydrolysates with relatively high concentrations of free amino acids and high percentages of low molecular weight components (the yeast-derived hydrolysates) were observed to also be among the poorer-performing products.

In regard to the cell growth correlations observable for specific amino acids, glutamate and alanine tended to be highest in the worse-performing hydrolysates (

Figure 16B). Conversely, leucine, isoleucine, arginine, and lysine all generally appeared to be at higher proportions in the better-performing hydrolysates. These trends are not reflected in the X effects loadings plot for the PLS regression analysis (**Figure 19**A) since the model dealt with overall absolute concentrations of all the measured chemical components, as opposed to the relative ratios of the specific free amino acids across the different hydrolysates.

Notwithstanding the general lack of measurable free glutamine in most of the hydrolysate solutions, the products with amino acid composition ratios that otherwise more closely resembled that of the E8 media formulation tended to be the better-performing hydrolysates. This may indicate that the relative ratios of free amino acids are an important consideration for media formulation and that conventional media like DMEM/F12 (the basis of E8) are designed with a generally satisfactory amino acid ratio for most animal cell cultures. It is also worth considering that the C2C12 cells used in this study may have been previously adapted to prefer the amino acid ratios supplied by DMEM/F12 and could not efficiently transition suddenly to different amino acid ratios in the serum-free context. Initial pilot experiments performed as part of this

study measured cell growth in the hydrolysate-containing experimental media for upwards of 7 days; however, no increase in viable cell count was observed past approximately 3 days in culture. It may then be possible that the cell growth may have benefited from a more gradual transition into the experimental media with hydrolysates as the sole nitrogen source. The cell culture format may have also played a role, and it may have been easier to transition the cells to hydolysate-based media over several passages at a larger (T-flask) scale.

The potential of cytotoxicity and bioprocess inhibition due to endotoxin contamination of hydrolysate products has been a cause for concern among some in the CM space[206]. Endotoxin, or lipopolysaccharide, is a major component of the outer cell membrane of Gram-negative bacteria and is known to be a potent pyrogen in humans. Cell culture studies testing the effects of endotoxins on various cell types *in vitro* have generally found that detrimental effects vary for different cell types. Regardless, the presence of endotoxins in media is assumed to be a potential source of variability for most cell cultures and is thus undesirable[223]. However, no studies have yet been published on the sensitivities of CM cells to endotoxin, or whether CM-relevant media are even likely to contain elevated endotoxin levels. The low levels of endotoxins measured in the Kerry hydrolysate stock solutions (**Figure 17**), as well as the results of the PLS regression (**Figure 19**A), indicate that endotoxin did not play a significant role in the outcome of the experiments reported here. This suggests that potential endotoxin contamination of plant and fungal hydrolysates does not need to be a significant cause for concern for CM media, although more directed studies should be performed in this regard.

The multivariate statistical analyses included in this study assisted the elucidation of which specific chemical components (and classes of components) of hydrolysates are most likely to be particularly influential in determining whether a hydrolysate will be compatible with CM

cell culture. From the PCA (**Figure 18**), some general conclusions can be drawn. The relatively large percentage of variation explained by the first two PCs of the amino acid PCA, as well as the more distinct separation of yeast hydrolysates and AmiSoy from the others in the PC scores plot, suggest that amino acid profiles have a relatively large influence on the overall variation observed in the chemical compositions of the hydrolysates. In contrast, there was less obvious clustering and less variation captured in the PC scores plot for the minerals, which may indicate that the specific species source of the hydrolysates may not influence the final hydrolysate mineral composition as significantly. However, the fact that AmiSoy (the only acid-hydrolyzed product) was plotted further in the negative direction on the PC2 axis than all the other hydrolysates could suggest that acid hydrolysis has a more significant effect on hydrolysate mineral content, and this could be an important consideration when selecting hydrolysates for other CM applications.

Broadly, the PLS regression analysis (**Figure 19**) suggests that there is an optimal degree of hydrolysis to maximize compatibility of plant and fungal hydrolysate products with CM media. Milder enzymatic processing methods that result in an enrichment of peptides in the 500-1000 Da range appear to be preferable based on the X loading plot in **Figure 19**A. More aggressive acid hydrolysis processing approaches (as was used for AmiSoy) result in greater liberation of individual amino acids and ions[224]. The effects of acid- versus enzymatic hydrolysis of media nitrogen sources have been compared for other cell culture applications and, indeed, the acid hydrolysis approaches tend to be more inhibitory for cell growth[225]. It is worth noting that the acid hydrolysis used for AmiSoy could have led to formation of greater amounts or different types of undesirable byproducts that were not detectable or targeted for analysis in this study. This would then suggest that it is possible that the higher concentrations of free amino acids, ions

like calcium and magnesium, and small molecular weight compounds that accompany AmiSoy are merely correlated with—and not causative factors for—the poor cell growth in this hydrolysate. As such, the results of the PLS regression should be interpreted with caution, and more direct investigation into the effects of these classes of chemical components on CM cell culture should be performed.

In summary, this work helps to unravel the complexity and diversity of the chemical compositions of hydrolysates that have been proposed for CM media. It is unsurprising that there are still many challenges to be overcome toward understanding the specific impacts of plant and fungal hydrolysate compositions on CM cells and identifying processing approaches that could be applied to remove inhibitory components. Further research is warranted to validate the findings in different CM cell lines and to explore the sensory and nutritional qualities of CM products produced using optimized media formulations. These findings lay the groundwork for future research aimed at developing simplified hydrolysate-based media and contribute to the goal of scalable and economically viable cultivated meat production.

Figures

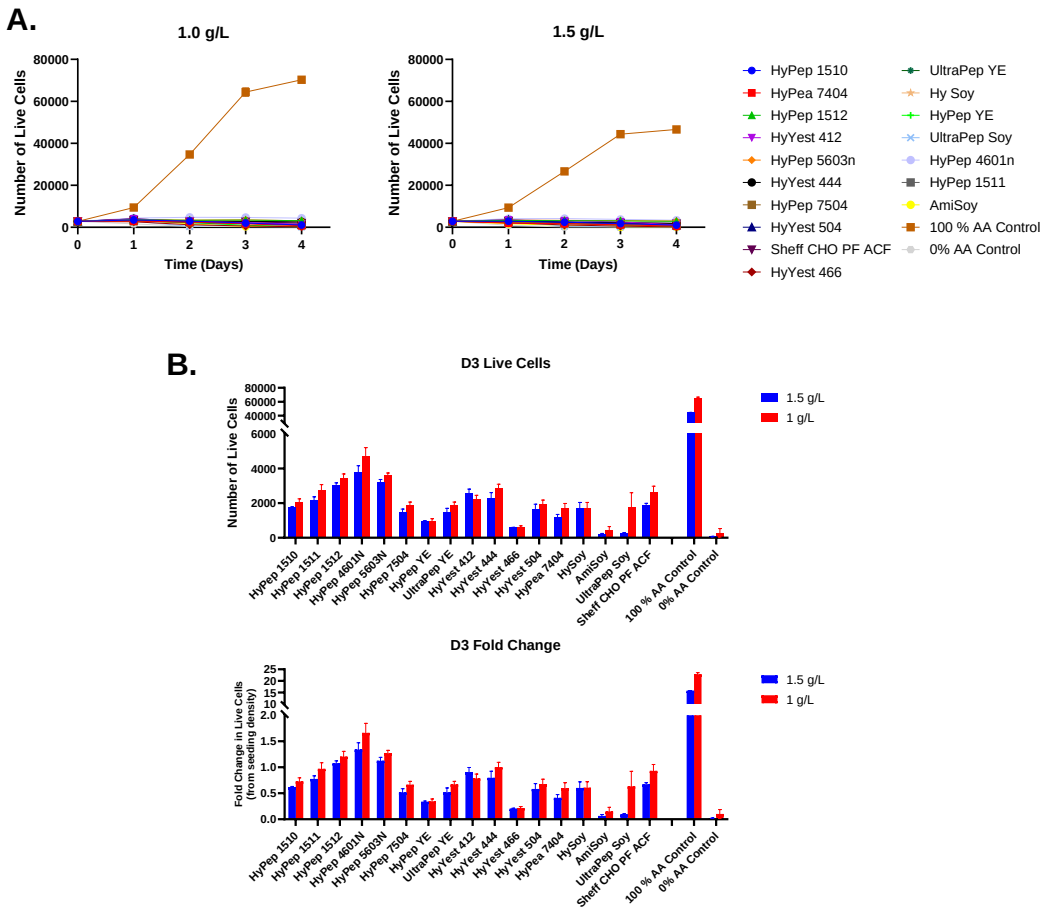


Figure 12: Results of the Kerry hydrolysate cell culture screening experiment

This experiment assessed the growth of C2C12 cells in serum-free media using hydrolysates in place of the free amino acids in the basal formulation. A, Growth curves of the C2C12 cells over 4 days in culture, comparing 17 different Kerry hydrolysates at both 1.0 and 1.5 g/L in the media. Also included are control media containing 100% and 0% of the standard free amino acid components in the Essential 8 media formulation. B, The same C2C12 cell count data for Day 3 only are plotted, both in terms of absolute number of live cells per well and in terms of the fold change in the number of live cells per well on Day 3 compared to the seeding density. This allows for better visualization of the differences in performance among the hydrolysates.

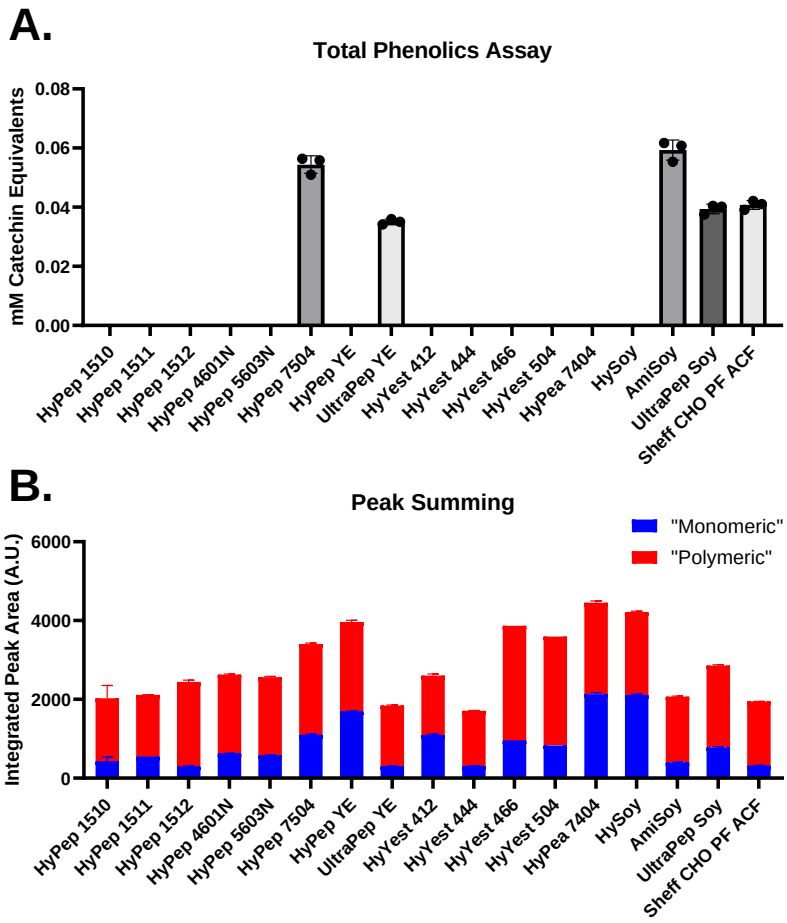


Figure 14: Analysis of the phenolic content of the Kerry hydrolysates when prepared at 10 g/L in water

A, Results of the total phenolic compounds assay performed on the hydrolysate stock solutions. The majority of the hydrolysates had phenolic levels below the lowest point on the assay standard curve and are therefore not plotted. The remaining five hydrolysates had phenolic catechin equivalent concentrations ranging from about 0.035 to 0.060 mM. **B**, Summed A280 peak areas of the HPLC-DAD chromatograms. For each hydrolysate, the entire A280 chromatogram range from 1 to 9.5 minutes was integrated together and plotted as representing monomeric phenolic compounds. Likewise, the range from 9.5 to 23 minutes was integrated and plotted to represent polymeric phenolics.

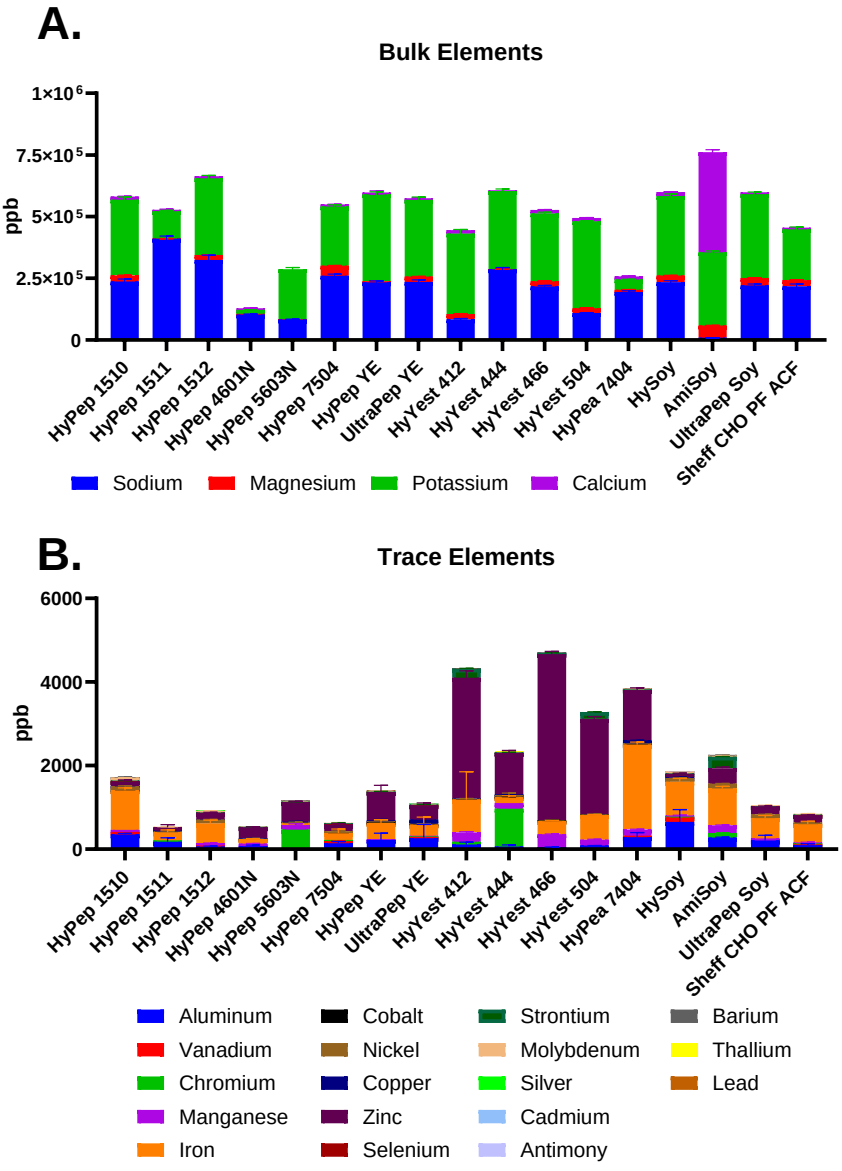


Figure 15: Elemental analysis of Kerry hydrolysate stock solutions

The hydrolysate stocks were prepared at 10 g/L in water and analyzed by ICP-MS. **A**, The bulk elements—those present at the highest concentrations—are plotted for each hydrolysate. **B**, The trace elements—the remaining detectable elements that were measured at lower concentrations—are plotted for each hydrolysate.

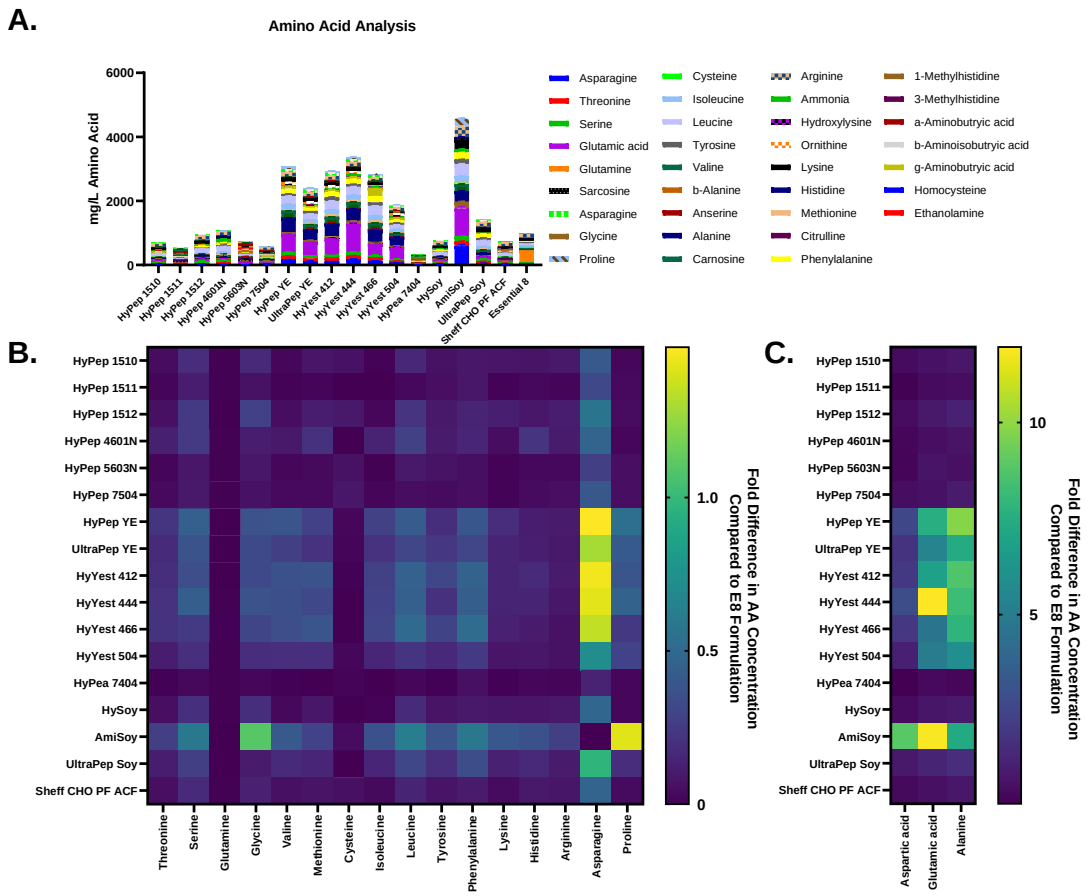


Figure 16: Amino acid analysis of Kerry hydrolysates

The 10 g/L stock solutions of Kerry hydrolysates were analyzed by HPLC with post-column ninhydrin derivatization to measure free amino acid content. **A**, The data for all identifiable and quantifiable amino acids are stacked in a bar chart to allow visualization of the relative contributions of each amino acid to the total amino acid content of each hydrolysate stock solution. The standard Essential 8 (E8) media formulation is included for reference. The fold difference in specific amino acid concentrations of the hydrolysate stock solutions to the standard E8 formulation are depicted in two heatmaps separated by generally low-abundance amino acids (**B**) and generally high-abundance amino acids (**C**).

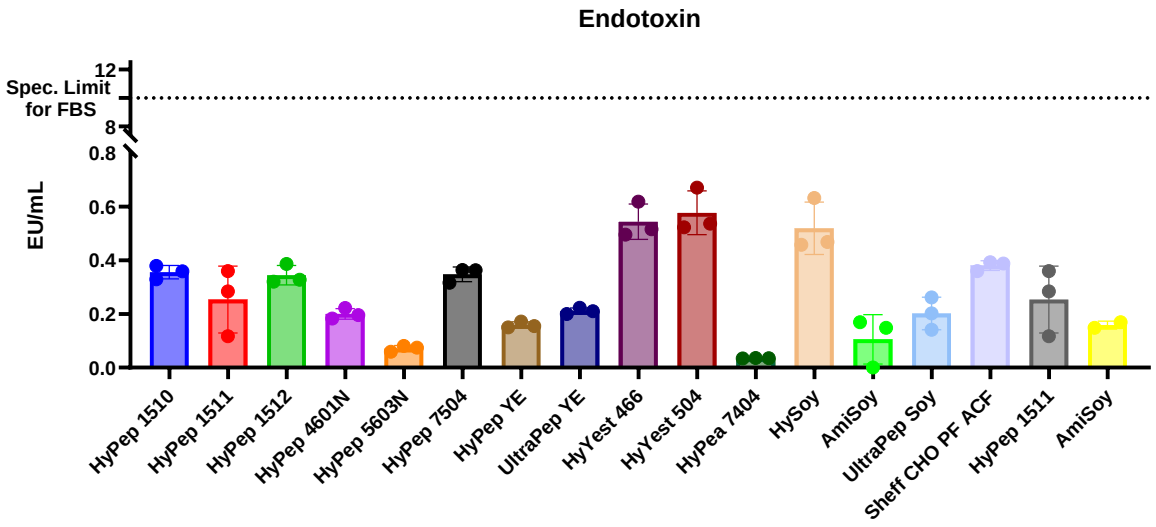


Figure 17: Endotoxin quantification

The presence of bacterial endotoxin, or lipopolysaccharide, in the 10 g/L stock solutions of Kerry hydrolysates was measured using a chromogenic endotoxin assay. The resulting data are plotted for each hydrolysate. The dotted line represents a typical upper specification limit for commercially available fetal bovine serum used for biomedical cell culture applications.

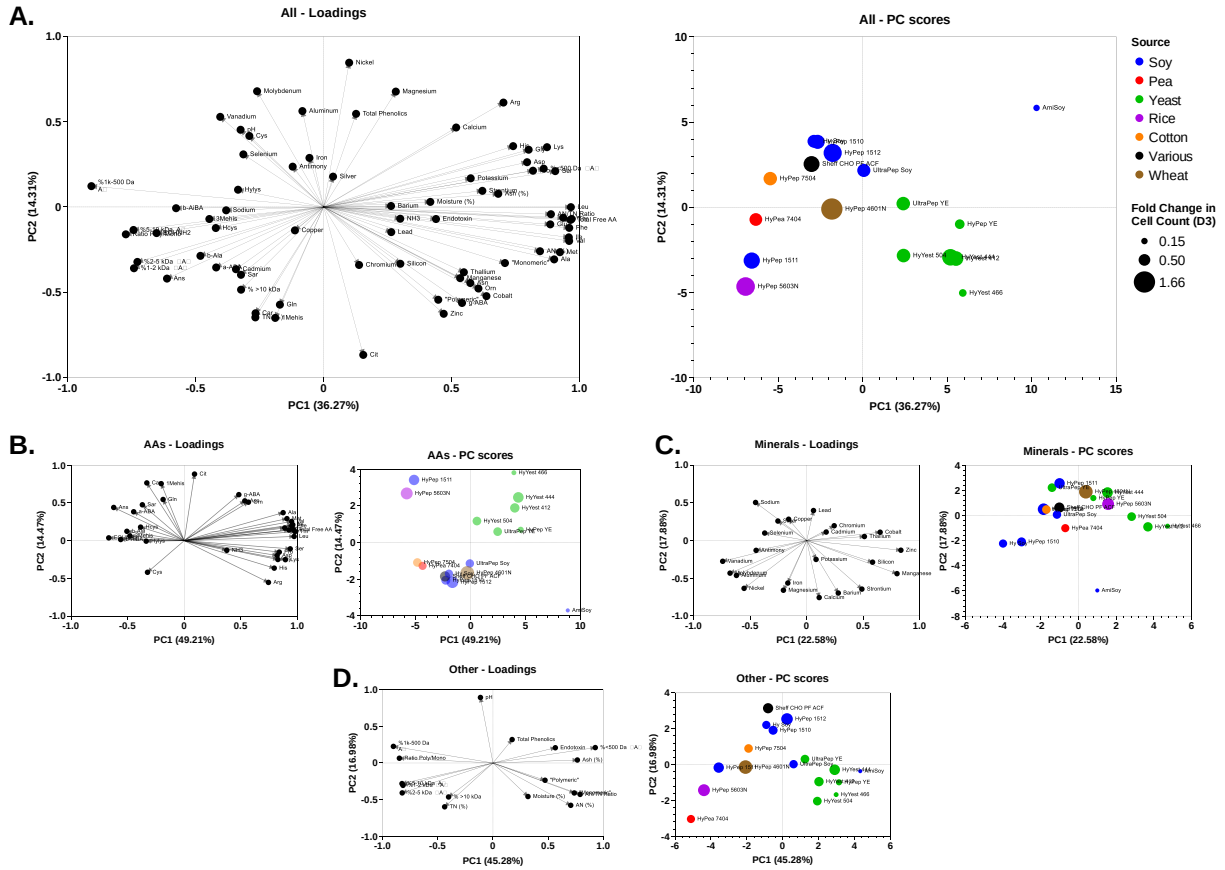


Figure 18: Principal component analysis of hydrolysate chemical compositions

The compositional data that were obtained for all 17 Kerry hydrolysate stock solutions were used to perform PCA to highlight the variation that may arise from the hydrolysate sources or processing methods. Loadings and PC scores plots are shown for PCA analysis on (**A**) all available compositional data, (**B**) amino acid data only, (**C**) mineral analysis data only, and (**D**) the data for components other than amino acids and minerals. The color of the points on the PC scores plots indicate the type of starting material that was used to manufacture the corresponding hydrolysates, while the sizes of the points indicate the relative performance of the hydrolysates in the cell culture screening experiment, in terms of the day 3 fold change in live cell count.

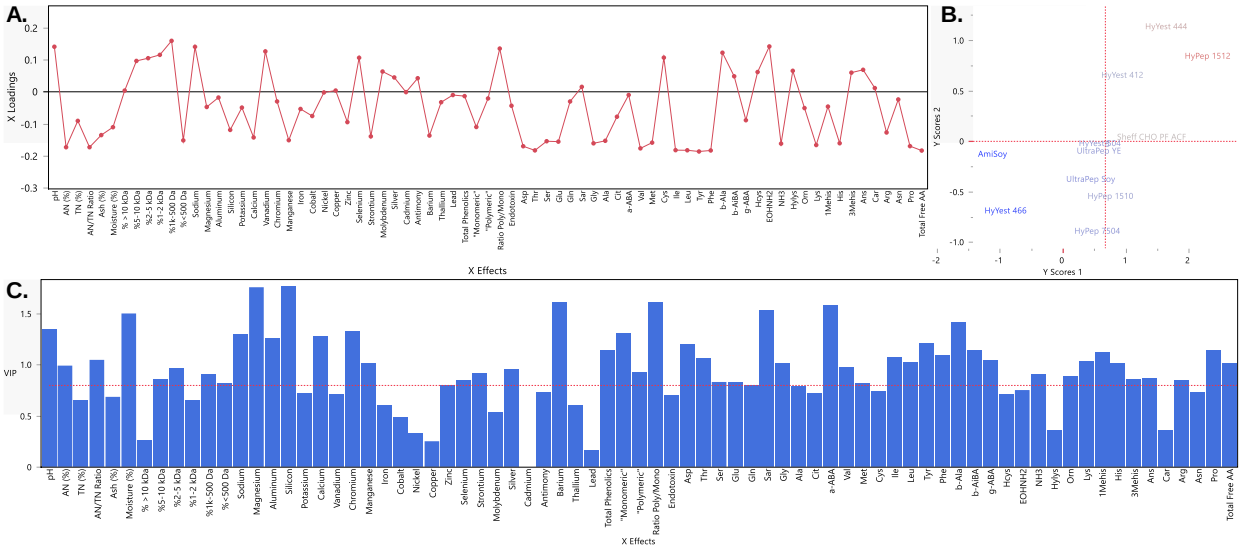
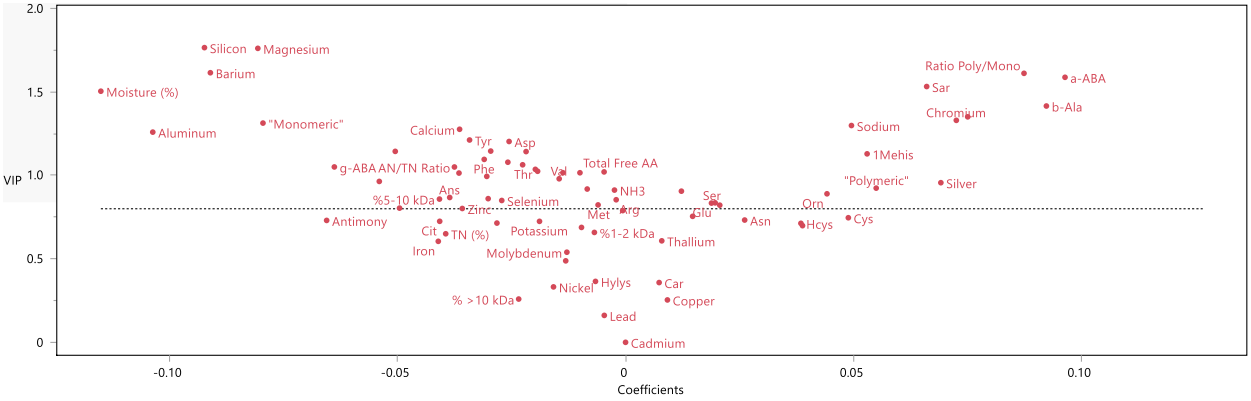


Figure 19: Partial least squares regression analysis of hydrolysate compositional data

The measured chemical composition data for the Kerry hydrolysate stock solutions were used to perform PLS to reveal potential correlations between chemical component concentrations and hydrolysate cell culture performance when used as the nitrogen source in serum-free media. **A**, X-effects loadings plot for the first factor in the model, indicating the relative correlation of each measured chemical component on hydrolysate cell culture performance, with points in the positive y-axis direction being positively correlated and points in the negative y-axis direction being negatively correlated. **B**, Y-response scores plot for the first two factors in the model, displaying the eleven hydrolysates used in the model with their labels on a color scale gradient from blue to red. Blue-colored labels indicate relatively poor cell culture performance, while red labels indicate good performance. The generally monotonic trend of increasing performance from the lower left to upper right quadrants is apparent, which is an indication of the quality of the model. **C**, The variable importance plot of the x-effects in the model. This indicates the relative importance of each hydrolysate chemical component in explaining the variability in hydrolysate cell culture performance. The dotted line denotes the conventional threshold of 0.8 which can be used to select x-effects to create a pruned model.



Supplementary Figure 2: Variable importance versus model coefficient value for hydrolysate chemical components

The calculated variable importance plot (VIP) statistics for each X-effect (chemical component) used in the partial least squares regression analysis (PLS) of the (centered and scaled) Kerry hydrolysate composition data are plotted versus their respective model coefficient values. Components with both a low VIP and coefficient value are candidates for pruning from the PLS model. Dotted line represents the VIP threshold value of 0.8.

CHAPTER 6: Conclusions and Future Directions

In the realm of cultivated meat development, this work has aimed to contribute meaningful insights into white-box approaches for optimizing cell culture media. The urgency of overcoming challenges related to large-scale CM commercialization to help address the growing global demand for quality dietary protein has been the driving force behind this research. Navigating the intricacies of media requirements for muscle and connective tissue cells, the findings presented here not only help to shape the trajectory of CM development, but also offer potential applications in broader areas of biotechnology and medicine.

Building upon the identification of diverse nutrient utilization patterns among different cell types, ongoing research should delve further into the metabolic nuances inherent in various cell species and lineages. A deeper understanding of variances and similarities in cell requirements will be highly beneficial in the development of adaptable media that can meet the specific needs of diverse cell types in the context of cellular agriculture. More extensive integration of multi-omics techniques—genomics, transcriptomics, proteomics, and metabolomics—becomes a pivotal next step in unraveling cellular responses to diverse media formulations. This holistic approach can allow for a deeper exploration of molecular intricacies, identifying key metabolic regulatory points and subtle media component interactions impacting growth and product quality for a range of CM applications. Further development of innovative analytical methods that go beyond traditional optimization approaches will be crucial to ensure flexibility and efficiency in the pursuit of ideal media formulations for various CM applications.

Machine learning algorithms present a promising avenue for accelerating the formulation discovery process. As discussed in Chapter 2, utilizing computational tools to predict optimal media compositions can hasten innovation in the field, merging computational efficiencies and

biological understandings. The data-driven nature of machine learning is particularly advantageous when dealing with the inherent complexity of cellular processes. As the intricate interplay between cells and their environment unfolds, machine learning algorithms can uncover hidden correlations and identify non-linear relationships that may be challenging to discern through traditional optimization methods. Future research should aim to develop artificial intelligence processes and models informed and trained by data from ongoing white-box characterizations of various CM cells and bioprocesses to streamline media formulation efforts going forward. Appling the metabolomic data presented in this work to a model-based optimization approach similar to those developed by others in our group could be an interesting next step towards this overall aim.

Regarding the quest towards the utilization of inexpensive hydrolysates as nitrogen sources in the media, the next phase of experimentation should focus on assessing the benefit of a gradual transition to these media over several passages. A major limitation of the study described in Chapter 5 was the lack of a sufficient transition period for the cells before comparing their performance in various hydrolysate-supplemented media. Furthermore, it will be important to directly assess the potential inhibitory effects of specific chemical components present in these hydrolysates. A systematic evaluation of the impact of individual hydrolysate components on cell culture performance would be highly useful. Selectively adding or removing specific constituents, such as divalent cations, phenolics, and free amino acids, can help elucidate the causal relationships between hydrolysate components and cellular responses. Exploring additional processing approaches, such as the use of specific enzymes, chemicals, physical separations, or microbial cultures to selectively modify hydrolysate constituents, presents a bioengineering whitespace. This strategy could allow for a targeted and controlled adjustment of

hydrolysate composition, potentially mitigating inhibitory factors while preserving the cost-effective nature of these raw materials.

More broadly, it will be important to characterize the specific effects of various hydrolysates (and their constituents) on cell phenotypes, other CM-relevant cell behaviors, and bioprocess metrics that were not directly assessed in this work. These could include cell protein accumulation, attachment ability, and overall biomass yield. Further downstream in CM production, hydrolysates in the media may have effects on final product nutrition and sensory qualities. Additionally, synergistic combinations of hydrolysates with other media components should be systematically investigated. By understanding the interactions and potential synergies between hydrolysates and other ingredients, optimized media formulations could be designed that leverage the strengths of each component while mitigating their individual limitations.

Considering the complexity of hydrolysates, another promising avenue involves leveraging machine learning algorithms to unravel the intricate relationships between hydrolysate composition and cell culture outcomes. By training models on a diverse dataset encompassing various hydrolysates, their chemical compositions, and their effects on cell growth and protein synthesis, predictive models can be developed. These models can then guide the use of hydrolysates to meet specific criteria, such as maximizing cell proliferation or protein yield.

In conclusion, the next set of experiments should revolve around a meticulous exploration of hydrolysate components, aiming to identify and mitigate inhibitory factors. Leveraging advanced analytical techniques, targeted modifications, and innovative approaches such as machine learning and bioengineering will pave the way for unlocking the full potential of inexpensive hydrolysates in cell culture media. This iterative process aligns with the overarching objective of refining media formulations for cultivated meat production, fostering cost-

effectiveness and scalability while expanding the knowledge base applicable to broader biotechnological applications.

The broader impact of this research extends beyond cultivated meat. The methodologies and insights gleaned serve as a model for media development and optimization in various cellular agriculture processes. The fusion of meticulous white-box optimization and cutting-edge black-box approaches not only transforms the landscape of sustainable protein production but also offers a paradigm for advancements in biotechnology and medicine. This research journey, grounded in precision and innovation, stands poised to revolutionize cell culture media development for global sustainability and health.

In conclusion, this dissertation on white-box approaches to cell culture media optimization addressed a key challenge still facing the field of cultivated meat. Drawing insights from conventional cell culture practices, metabolic pathways, and cell-nutrient interactions, this work provides a foundational understanding for ongoing media development. The challenges and opportunities highlighted in the exploration of using diverse cell types, serum-free conditions, and alternative ingredients like hydrolysates in the media point towards future research that should be undertaken. Beyond cultivated meat, the media optimization paradigms described here are poised to inform media formulation in diverse biotechnological applications, potentially leading to improved efficiencies and technological breakthroughs in other industries. Overall, this work represents a step toward understanding how best to approach media development for cultivated meat, harnessing the power of informed optimization to drive efficiency in sustainable protein production for the wellbeing of the planet, the people, and the animals.

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