Metabolism-Guided Strategies for Media Optimization in Growth and Productivity Applications

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Abstract

Cell culture media optimization has long been central to process development for biologics production in the pharmaceutical industry. More recently, the cultivated meat industry has begun adapting these strategies for food-relevant cell lines, where the key challenges are maintaining robust cell growth while substantially reducing input costs. Here, we present a metabolomics-based approach to guide rational media design strategies that both address the economic constraints of cellular agriculture and highlight overlooked media components important for productivity in CHO cell monoclonal antibody (mAb) production.

Metabolomic analysis of a chicken fibroblast cell line revealed that excess phenylalanine supplementation leads to accumulation of phenyllactic acid, a growth-inhibitory metabolite. Lowering phenylalanine levels alleviated this inhibition, improved proliferation, and, importantly, reduced media costs—underscoring the importance of tailoring amino acid balance to individual cell types in cultivated meat applications. In parallel, refinement of a chicken genome-scale metabolic model (GEM) through reduction of dead-end metabolites and blocked reactions improved predictive accuracy in comparison to experimental growth. This established a computational, metabolism-based framework for investigating the relationship between macromolecules and culture performance, accelerating future efforts in avian media design.

While metabolomics has previously been applied to biologics manufacturing, most emphasis has focused on macronutrients such as amino acids. However, less studied additives, including vitamins, also play an important role in culture performance,

particularly in relation to mAb productivity. In CHO cells, metabolomics differentiated high- and low-producing monoclonal antibody cell lines, identifying disrupted vitamin B6 metabolism as an indicator of reduced productivity. Complementary amino acid analysis revealed significant amino acid utilization patterns between the two cell lines. Together, these findings can inform metabolic engineering or supplementation strategies to enhance productivity through vitamin B6-related pathways.

Collectively, these studies demonstrate how metabolomics-guided insights can simultaneously improve growth, lower cost, and enhance productivity. The approaches presented establish a mechanistically informed framework for media optimization across process cost centered and product quality centered industries.

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Chapter 1. Introduction: Cell Culture Media Optimization Tools and Their Impact on Culture Performance

1.1 The role of media components in cell culture

Cell culture media are arguably one of the most critical components of in vitro cell cultivation technology, given their direct influence on proliferation, viability and productivity. Typically, animal cell culture media are composed of seven core components: amino acids, carbohydrates (often as the primary energy sources), vitamins, inorganic salts, lipids/fatty acids, buffering agents, and protein growth factors. Depending on the application, additional additives may be included. For example, antibiotics and antimycotics are often used to minimize microbial contamination, while non-ionic surfactant compounds like Pluronic F-68—a shear protectant—are commonly added during bioreactor-based cultivation to enhance cell viability under mechanical stress.

The development of commercially available cell culture media began in the 1950s when Harry Eagle identified the essential nutrients for cell proliferation of mouse fibroblast and HeLa cell lines. This led to the formulation of Minimal Essential Medium (MEM), which became the foundation for many modern basal media [1]. Since then, modifications to MEM have been made to better suit the needs of various cell types, resulting in a range of commonly used media in both research and industry. For example, Dulbecco’s Modified Eagle Medium (DMEM) has four times the concentration of amino acids and vitamins in MEM and was originally used to study polyoma virus plaque formation in mouse cells [2]. Iscove's Modified DMEM (IMDM) supplements DMEM with non-

essential amino acids and additional nutrients and protein factors, including transferrin, albumin, and soybean lipids, to support rapid cell growth and secretion of desired protein synthesis products such as immunoglobulins like IgM and IgG [3]. Other basal media formulations, such as RPMI-1640, Ham's F-10, Ham's F-12, and MCDB, were developed to meet specific cellular requirements. These media are widely used today as starting points for media optimization.

Amino acids are essential components of cell culture media. In addition to serving as the basic building blocks for proteins, peptides, nucleotides, and other biomolecules that comprise the cellular biomass, amino acids also directly influence the productivity of cell-based bioprocesses. Amino acids can be classified as essential or non-essential, depending on if they may be synthesized by cell *de novo*. Essential amino acids for most vertebrae include lysine, methionine, tryptophan, threonine, arginine, isoleucine, leucine, histidine, phenylalanine and valine. Essential amino acids must be supplied exogenously. While non-essential amino acids can in principle be synthesized by the cell, supplementation of non-essential amino acids in culture media can further enhance cell growth and performance.

Some non-essential amino acids can be further classified as semi-essential or conditionally essential. These amino acids can be synthesized by the cell but may become limiting in specific culture conditions, such as during rapid proliferation or high metabolic demand. A notable example is glutamine. While glutamine can be synthesized from glutamate, it is still commonly added to cell culture media due to its critical role in supporting cell growth, particularly for immortalized cell lines. Glutamine

acts as a key nitrogen donor in the synthesis of proteins, nucleotides, and other amino acids, and serves as an energy source by contributing carbon to the citric acid cycle [4].

Amino acid essentiality can vary by species, with most vertebrates sharing a common set of essential amino acids. However, cells of avian species exhibit variations in their amino acid requirements compared to mammalian species [5], [6], [7]. It is important to note that amino acid requirements of cells *in vivo* can differ from requirements of cells grown *in vitro*. In multicellular organisms, different cell types can synthesize and distribute amino acids systemically, whereas isolated cell cultures lack this capability. For instance, in Chinese Hamster Ovary (CHO) cells, which are widely used in biomanufacturing, proline is often included in the media due to the cell line's limited ability to synthesize sufficient pyrroline-5-carboxylate, a precursor for proline biosynthesis. However, this is not the case for the whole hamster organism, which can convert glutamate to proline via metabolic pathways present in other tissues [8].

Since the development of minimal essential media and classical basal formulations such as MEM, DMEM, and Ham's F-12, medium optimization for industrially relevant cell lines has advanced significantly. Yet, the emergence of cultivated meat—requiring large-scale and cost-efficient cultures of entirely new cell types—and the growing complexity of medium requirements for advanced biologics such as bispecific antibodies continue to present new challenges. For cultivated meat production, medium formulations must be carefully tailored to the distinct metabolic demands of food relevant cell types. When developing a new cell line for advanced biologics production, formulations often need to be further fine-tuned to maximize production of complex non-natural proteins even when the cell line is derived from a host already in use for

biologics production. Within this context, amino acids are particularly important, both for their essential roles in cell growth and protein synthesis and for practical considerations, including their high contribution to media cost and the chemical property related challenges of some amino acids in scale-up. For example, some amino acids suffer from limited solubility or instability, which can lead to precipitation or degradation during large-scale production [9], [10]. It is therefore imperative to design media that meets the amino acids demands to sustain growth, while avoiding degradation. Over-supplementation of amino acids can be detrimental as well. Excessive levels of certain amino acids—including tryptophan, phenylalanine, tyrosine, and methionine—have been shown in CHO cell cultures to reduce viable cell density and productivity by promoting accumulation of inhibitory metabolic byproducts [11], [12]. Optimized amino acid formulations can support higher cell densities, promote metabolic flux toward protein production, reduce raw material cost, and help establish scalable, robust processes suitable for industrial-scale production.

1.2 Economic considerations and bioprocess related optimization strategies

Media optimization can generally be divided into two categories: strategies aimed at improving cellular performance and those aimed at process efficiency, with some approaches influencing both. Amino acids are a primary target for optimization because of their significant impact on both cellular function and process economics. Although input costs are not trivial in pharmaceutical bioprocesses, the significance of input cost is much more pronounced in cultivated meat applications due to the expectation of meeting price parity with farmed products. A technoeconomic analysis on cultivated

meat production found that the largest contributors to the cost of cell mass production where amino acids until extremely large production capacities were reached (>1000 kTA) [13]. In contrast to traditional bioprocesses, which have historically emphasized maximizing product yield and quality, cell culture for cultivated meat production shifts the focus of optimization toward the need to balance input costs with growth requirements. In this regard, optimization strategies for cultivated meat production needs to consider both productivity and affordability.

Process-related optimization strategies often focus either on reducing reliance on amino acids with unfavorable chemical properties or on streamlining development timelines. For example, glutamine and cysteine are unstable in solution, while tyrosine is limited in aqueous solubility. One potential solution is replacing certain free amino acids with dipeptides, in which the target amino acid is linked to a supporting amino acid. Dipeptides can improve solubility and stability, though their metabolism in specific cell types requires further investigation [14]. Dipeptide supplementation also better aligns with the use of protein hydrolysates, which provide a mix of free amino acids and small peptides [15]. Hydrolysate supplementation has been suggested for cultivated meat applications as a potential method to reduce cost, however, would not be suitable for biopharmaceutical applications that require chemically defined media. Metabolic engineering techniques have been used to divert amino acid flux in such a way that reduces need for chemically problematic amino acids in the culture media. For example, CHO cells were engineered to overexpress enzymes for endogenous production of cysteine and tyrosine [16], [17]. Although metabolic engineering is widely applied in biotechnology, including animal cell-based processes, its adoption in cultivated meat

production is met with greater hesitation, largely due to consumer concerns surrounding genetically modified foods.

Strategies that streamline bioprocess development are valuable to cultivated meat and biologic production. One such strategy is platform media design—developing a basal medium that can be slightly modified to accommodate multiple cell lines derived from the same host. Platform media can reduce costs through bulk procurement of inputs and enable more efficient workflows for cell line development and scale-up. Pharmaceutical manufacturing has demonstrated that platform media can be used across development and production stages, replacing multiple stage-specific media types. This reduces adaptation times, simplifies processing, and minimizes batch-to-batch variability [18]. For example, a study with CHO cells producing monoclonal antibodies developed a platform medium in fed-batch culture by tuning nutrient feed rates relative to glucose metabolism. Using a high-performing clone as a model for optimal nutrient use, the researchers demonstrated that the medium also supported growth and productivity in three other cell lines [19]. Although cultivated meat cell types are currently in the early stages and require cell type-specific optimization, as new lines are developed and production scales increase, integrating platform-based technologies adapted from pharmaceutical processes could streamline development and reduce costs while meeting nutritional needs.

Over several decades, high-density and high-performing bioprocesses have been developed for a handful of cell types used for biologics (e.g., therapeutic antibody) production, by improving the cells' metabolic characteristics (e.g., reducing lactate production) using computational and -omics based approaches. In contrast, growth

optimized bioreactor processes are currently unavailable for many cell types relevant to cellular agriculture products. Computational tools that can be used to enhance the metabolic efficiency of cultures relevant to cellular agriculture have been of increasing interest for media optimization and process development. One common statistical modeling strategy is the use of DOE (Design of Experiments). DOE is valuable for understanding how factor interactions influence response in a high-throughput manner [20]. It has been widely applied in pharmaceutical bioprocessing to optimize media formulations or additives and assess their impact on product quality, cell growth, and titer [21], [22]. This success has inspired its use in cultivated meat media optimization. For instance, Cosenza and colleagues applied a hybrid nonlinear DOE method and conventional DOE to optimize 30 media components for C2C12 mouse myoblasts, a model system for cultivate meat. They achieved better cell growth efficiency characterized by cell number normalized to FBS utilized for both DOE methods compared to standard DMEM. However, the study noted that long-term growth was lower in the DOE-optimized media versus conventional media, highlighting a limitation of the DOE optimization approach [23]. To better capture long-term culture performance, the same group implemented Bayesian optimization to adjust 14 media components for long term growth. This effort resulted in a 181% increase in cell count over four passages compared to both DOE-optimized media and standard DMEM [24]. DOE is most useful for optimizing a limited number of parameters—for example, it can be effectively used to identify the optimal combination of growth factors that support cell proliferation in development of chemically defined media [25]—but it becomes less effective when addressing the full range of components in cell culture media and does

not account for the complex interactions between media components and cellular metabolism important for both cultivated meat and pharmaceutical applications.

In contrast, metabolic modeling approaches can offer mechanistic insights. Genome-scale metabolic models (GEMs) use gene–protein–reaction associations to reconstruct whole-organism metabolic networks. When combined with methods like flux balance analysis (FBA) or metabolic flux analysis (MFA), GEMs are powerful tools for understanding metabolite flow [26]. For example, a CHO cell GEM was used to simulate how varying asparagine and aspartate ratios affect metabolism, leading to an optimized amino acid ratio that improved viable cell density and IgG1 titer [27]. Combining metabolic modeling with DOE has been shown to be an effective strategy for assessing metabolic responses to media additives and subsequently identifying the optimal concentrations of the most beneficial supplements in CHO cell cultures [28]. Several biomanufacturing companies now integrate GEMs into digital twins to enhance predictive accuracy of cell culture processes. Insilico Biotechnology based in Germany is one such example [29]. However, building GEMs for cultivated meat-relevant organisms requires significant effort, as few curated models currently exist. To date, only a few draft GEMs have been published for cellular agriculture relevant species — domestic cattle [30], salmon [31], and chicken [32] – with additional efforts underway for swine. These modeling efforts represent a critical first step, but the draft models still require extensive curation and experimental validation. Additionally, most current cultivated meat relevant models are generic to species, not tailored to specific cell types or lines. Further, GEMs are ultimately more useful for cell type-specific predictions if they are appropriately trained on high-quality data linking cell growth and metabolite

utilization collected under a broad range of culture conditions. Here, metabolomics emerges as a powerful tool, capable of guiding media optimization for both cultivated meat—where cost-effective growth is critical—and biopharmaceutical production—where high protein productivity is the goal—while also providing data to refine GEM reconstruction.

1.3 Scientific Context and Prior Advances in the Field

Metabolomics, the study of the concentrations and dynamics of small molecules to provide a direct snapshot of cellular metabolism, has demonstrated success as a tool to optimize media formulation by uncovering molecular mechanisms that underlie specific phenotypic patterns. For example, metabolomics has been used to identify metabolic states that restrict desirable outcomes and has proven broadly applicable to improving both growth and productivity. In previous work from our lab, metabolomics revealed that 5-hydroxyindoleacetaldehyde, a catabolite of tryptophan metabolism, inhibited cell growth in CHO cell lines [11]. This finding suggested that cell growth could be enhanced by reducing tryptophan levels in culture media. In another study, our lab used metabolomics to identify citrate supplementation as a strategy to boost monoclonal antibody productivity, leading to a 490% increase in specific productivity without significantly affecting viable cell density [33]. Together, these examples highlight metabolomics as a powerful tool for enhancing growth and/or productivity through media interventions.

However, most applications to date have been demonstrated in the context of pharmaceutical bioprocesses, whereas cultivated meat production presents distinct

challenges, particularly the critical barrier of cost. The present study builds upon prior work by applying metabolomics in this new context, where optimization must balance growth efficiency with process economics. In addition to informing media formulation directly, the resulting metabolomic profiles revealed important gaps in an existing avian genome-scale metabolic model, motivating its refinement to more accurately capture cellular activity and enhance its utility for rapidly exploring metabolic trends to guide media design. Finally, this work demonstrates that beyond major macromolecules such as amino acids—historically the primary targets of metabolomics-driven media optimization—lesser-studied small molecules, including enzymatic cofactors, can significantly influence process performance and warrant equally rigorous investigation. Taken together, this research establishes untargeted metabolomics as a robust and broadly applicable tool for both traditional bioprocessing and emerging domains such as cellular agriculture.

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Chapter 2. Metabolomics-Guided Reduction of Phenylalanine Improves Chicken Cell Growth for Cost-Effective Cultivated Meat Media

2.1 Abstract

The high cost and inefficiency of cell culture media remain major barriers to scaling cultivated meat production, particularly for avian cell types that are underexplored compared to mammalian systems. In this study, we investigated the relationship between amino acid media composition, metabolite accumulation, and growth performance in suspension-adapted DF-1 chicken fibroblasts. Untargeted metabolomics revealed significant accumulation of phenylalanine- and tyrosine-derived byproducts, most notably phenyllactic acid, during late-stage culture. Targeted LC-MS confirmed dose-dependent accumulation of phenyllactic acid that correlated with declining viable cell density. Add-back experiments demonstrated a direct inhibitory effect of phenyllactic acid on cell growth rate and integral viable cell density (IVCD), while hydroxyphenyllactic acid exhibited only minor effects. Media reformulation experiments further showed that reducing starting phenylalanine concentration improved growth performance and mitigated inhibitory byproduct formation with the added benefit of reducing media costs. Together, these findings identify phenylalanine over supplementation as a driver of growth inhibition in chicken cell culture and establish phenyllactic acid as a key metabolic byproduct negatively impacting proliferation. This work underscores the importance of cell type–specific media design with a focus on amino acids as a critical attribute to improve performance, reduce costs, and advance the scalability of cultivated poultry production.

2.2 Introduction

Global meat consumption continues to rise especially resulting from growing populations and incomes in middle to low-income countries. Poultry, which accounts for nearly 41% of all meat consumption, is projected to increase in consumption by 15% by 2032 [1]. With growing demand for poultry consumption there also exists a growing risk of avian disease and rapid disease spread. In 2025, egg laying flocks were devastated by 44 outbreaks of highly pathogenic influenza leading to a loss of 36.3 million birds representing 11.8% of the caged population and 14.6% of the cage-free conventional bird population [2]. Animal-borne diseases not only threaten food security but also pose a major risk to human health, with an estimated 75% of emerging infectious diseases in humans originating from animals. [3], [4]. Increasing demand for meat production, coupled with the rising risk of zoonotic diseases and growing environmental and animal welfare concerns, underscores the need for alternative protein sources.

Cultivated meat, produced by growing animal cells in tightly controlled bioreactor systems, offers a promising sustainable and ethical alternative to conventional meat. Life cycle analysis (LCA) studies on cultivated meat show significant reductions in global warming potential, land use, water consumption, and particulate matter formation compared to beef from conventional agriculture, though the improvements are less substantial when compared to poultry from farmed chicken [5]. Both LCA and techno-economic analyses identify culture media as a major bottleneck for scaling up cultivate meat production due to high costs and lack of optimization for relevant cell types [5], [6]. Despite recent approvals for sale of cultivate meat products—such as Good Meat in

Singapore and UPSIDE Foods in the U.S.—culture media optimization remains critical for industry growth [7], [8]. Advances include replacing pharmaceutical-grade components with food-grade alternatives, as demonstrated by Mosa Meat, which reported similar cell numbers and reduced costs after substituting 99.2% of its serum-free media components with food grade alternatives [9]. The use of protein hydrolysates has also shown promise as a serum replacement, though concerns remain over batch-to-batch variability [10], [11]. While these innovations are key to reducing costs, there is still limited understanding of how media composition—particularly amino acid requirements—impacts cell growth, posing an ongoing challenge to the scalability of cultivated meat production.

Media composition plays a critical role in cell culture performance, as extensively demonstrated in pharmaceutical production systems such as CHO cells. For instance, we have shown in CHO cells that the addition of specific metabolites, such as citrate, can enhance monoclonal antibody productivity by 490%, with only a slight reduction in cell growth—highlighting how media formulation can modulate cellular metabolism and redirect flux through desirable pathways [12]. In a separate study, our lab observed that over-supplementation of aromatic amino acids, particularly tryptophan, led to reduced viable cell density due to the accumulation of 5-hydroxyindoleacetylaldehyde, a growth-inhibitory metabolic byproduct [13]. While these studies in CHO cells underscore how media composition—particularly amino acid balance—can profoundly influence cell growth and productivity, it remains unclear whether similar metabolic vulnerabilities exist in avian cell lines used for cultivated meat. Given that amino acids contribute

substantially to media cost and complexity, understanding their impact on growth is critical for both performance optimization and economic feasibility. To address this, we investigated the effects of amino acid composition on DF-1 chicken cell growth and metabolism.

Here, we demonstrate enhanced DF-1 chicken cell growth using a reduced-aromatic-amino-acid medium formulation. Extracellular samples were collected during exponential, peak viable cell density (VCD), and decline phases for untargeted LC-MS analysis. Metabolic model-guided annotation and volcano plot analysis identified metabolites that significantly accumulated as VCD decreased. Targeted metabolomics confirmed an inverse relationship between cell growth and phenylalanine-derived metabolites and quantified the extent of metabolites' accumulation. Add-back experiments further validated the growth-inhibitory effect of a specific phenylalanine-derived metabolite, ultimately informing the development of a reduced-phenylalanine media formulation that better supports chicken cell proliferation.

2.3 Methods

2.3.1 DF-1 Cell Culture

Chicken embryonic fibroblast cell line (UMNSAH/DF-1; CRL-3586) were purchased from American Type Culture Collection (Manassas, VA, USA). DF-1 cells were adapted to suspension culture in Dr. David Kaplan's Lab. Suspension adapted DF-1 cells were gifted to our lab for use in this study.

Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM; 10569010) supplemented with 10% fetal bovine serum (FBS; 26140079), 1% 100X antibiotic-antimycotic (15240062), and 0.1% poloxamer 188 non-ionic surfactant (24040032) purchased from ThermoFisher, Waltham, MA, USA. DF-1 cells were seeded into Costar® 6-well ultra-low attachment plates (3471; Corning, Corning, NY, USA) at a density of 3 x 10^5 cells/mL. Suspension cultures were counted and diluted down to initial seeding density 2-3 times per week. Cell aggregates were broken down as needed using trypsin digestion (0.25%Trypsin-EDTA; 25200056; ThermoFisher, Waltham, MA, USA). Suspension adapted cells were cultured in a humidified incubator at 39 °C and 5% CO_2. Cell cultivation was carried out in static culture for 2 passages. Following the second passage cells were transitioned to dynamic culture at 125 RPM.

2.3.2 Shake Flask Cell Growth

DF-1 cells were seeded at a density of 1 x 10^5 cells/mL into 250mL baffled shaker flasks (Chemglass Life Sciences, Vineland, NJ, USA) coated with Sigmacote® (SL2; Sigma-Aldrich, St. Louis, MO, USA) and incubated as described previously with shaking at 125 RPM. Sample aliquots were taken at 24 h intervals. Sample aliquots were centrifuged at 25°C and 300xg then supernatants were transferred to separate vials for storage. Cell pellet and supernatant sample aliquots were flash frozen and stored at -80 °C. In addition, viable cell density and viability was measured using Countess™ 3 Automated Cell Counter (Invitrogen, Waltham, MA, USA). Glucose consumption was quantified offline using the Gluccell® Glucose Monitoring System (Chemglass Life Sciences,

Vineland, NJ, USA). Lactate was quantified according to lactate assay kit directions (Eton Biosciences, San Diego, CA, USA).

2.3.3 Untargeted Metabolomics

Supernatant samples collected on days 1-5 of shake flask batch growth experiments were diluted 1:8 using HPLC grade water. Samples were then analyzed for metabolite composition using information-dependent acquisition (IDA) experiments on a quadrupole time-of flight (TOF) mass spectrometer (TripleTOF 5600+, AB Sciex, Framingham, MA, USA) with an electrospray ionization source. IDA experiments include a single TOF MS scan corresponding to four dependent product ion (MS/MS) scans based on the highest intensity unique mass. Fragmentation was triggered when precursor ion counts rose quickly over several scans to ensure ions were selected near the top of their LC peaks. A total of four methods was applied to each sample using either a hydrophilic interaction chromatography (HILIC) column (Phenomenex Luna NH2, Torrance, CA, USA) or a reverse-phase column (Phenomenex Synergi Hydro-RP, Torrance, CA, USA) in both positive and negative ionization modes. The chromatographic separation was performed with a binary pump HPLC system (1260 Infinity, Agilent, Santa Clara, CA, USA). Chromatographic gradient methods described in supplementary data.

2.3.4 Feature Annotation

The LC-MS features obtained through untargeted analysis were annotated using the Biologically Consistent Annotation of Metabolomics Data computational tool (BioCAn).

Feature annotation using BioCAn has been previously described [15]. Briefly, BioCAn assesses potential annotations using mass to charge ratio (*m/z)* and product ion spectra (MS/MS) using the following annotation tools: CFM-ID [16], NIST17 [17], MONA (https://massbank.us/), and HMDB [18]. Features may be assigned multiple annotations either by a single annotation tool or by disagreement between assignments designated by different tools. Thus, to determine the most likely annotation for a feature, the feature was mapped to a metabolite in the *Gallus gallus* metabolic network and assigned an annotation score by BioCAn. Annotation scores were based on agreement of the annotation assignment by the integrated databases as well as confidence in annotation of neighboring metabolites within the metabolic network.

2.3.5 Data Analysis

The peak area corresponding to the integrated area under the curve (AUC) of each ion chromatograph was normalized by to the total ion chromatogram (TIC) for each sample per each method (4 total methods) followed by normalization by viable cell density. Each sample was blank subtracted to reduce noise. Features were filtered to ensure at least one annotation assigned by the annotation database tools. Day 1 corresponding to early exponential phase was compared to samples taken during peak VCD (day 3) and death phase (day 5). Fold changes were calculated between the AUC of the chosen time points and day 1 to find features with an AUC fold-change greater than 2. The subset of upregulated metabolites was manually inspected for confidence of annotation by both the integrated database as well as BioCAn.

2.3.6 Targeted Metabolomics

Targeted metabolomics was preformed to quantify accumulation of byproduct metabolites as well as consumption of amino acids. The same quadrupole time-of-flight mass spectrometer was used for targeted quantification as used for the untargeted metabolomic assays. High purity standards were used to optimize the following parameters: ionization mode, precursor ion, product ion, collision energy and delustering potential (Table 2-S1). Samples were diluted 1:8 with HPLC grade water then analyzed using the optimized parameters applied with the same RP method as described in the untargeted analysis. An example mirror plot may be found in supplementary data (Figure 2-S1). Amino acid quantification preformed using a similar technique to optimize parameters for each amino acid, however, analysis used a modified HILIC method with a positive ionization mode. Details of the amino acid quantification method gradient may also be found in supplementary data.

2.3.7 Addback Experiments

To determine potential inhibitory effects of phenyllactic acid and hydroxyphenyllactic acid, both metabolites were supplemented into DF-1 cell culture in two doses. DF-1 cells were seeded at 1 x 10^5 cells/mL in 6-well plates and allowed to grow in dynamic culture under normal cultivation conditions. After 24 h, 100 µL of well-mixed cell suspension was removed and replaced by a high or low dose of either metabolite dissolved in cell culture grade water. The high and low dose correspond to a concentration of 100 µM and 25 µM in each well respectively. Control wells received

only 100 µL of cell culture grade water. Viable cell density and viability measurements were taken at 24 h time increments following treatment.

2.3.8 Phenylalanine Adjusted Media Growth

To understand DF-1 cellular response to media concentrations of phenylalanine, cells were grown in reduced and excess phenylalanine concentrations. A minimal essential media (MEM; 41090036; ThermoFisher, Waltham, MA, USA) was used as a base media formulation containing 200 µM of L-phenylalanine in the formulation. The remaining amino acids (i.e. L-arginine, L-cystine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-threonine, L-tryptophan, L-tyrosine, L-valine) were added to the basal MEM media to match the concentration typically found in DMEM media. Two additional MEM formulations were similarly made with the addition of L-phenylalanine at a concentration of 400 µM and 800 µM. DF-1 cells were seeded at 1 x 10^5 cells/mL directly into one of the three adjusted MEM media formulation or a control DMEM media and grown in dynamic culture in 6-well plates. All media formulations additionally contained 10% FBS, 1% anti-anti, and 0.1% F-68. Viable cell density and viability measurements were taken over the course of cell growth. Cell pellets and supernatants were retained at day 4 for targeted metabolomic analysis.

2.4 Results

2.4.1 Untargeted metabolomics identifies metabolites accumulating in shake flask batch cell culture.

A suspension-adapted DF-1 G. gallus embryonic fibroblast cell line was cultured in a dynamic batch system to capture the complete cell growth profile in a commercially available off-the-shelf medium (Figure 2-1A). A peak viable cell density (VCD) of approximately 1 x 106 cells/mL was achieved after 72 hours of culture with an initial seeding density of 1 x 105 cells/mL Following this peak, VCD declined through 120 hours of culture, indicating the onset of the cell death, or decline, phase. Offline quantification of glucose and lactate concentrations was performed. Notably, glucose was not entirely consumed throughout the culture duration, indicating that cell death was not driven by glucose starvation. An accumulation of lactate was observed throughout the duration of culture duration, with the concentration reaching around 15 mM after 120 hours of culture. An anchorage independent immortalized cell line derived from Broiler Ross chicken displayed similar peak VCD, glucose consumption, and lactate accumulation profiles in serum free batch culture [14]

To compare extracellular metabolite profiles across cell growth phases – exponential, stationary, and decline – representative samples were analyzed using untargeted LC-MS assays. Specifically, we aimed to identify metabolites that accumulate as the cell culture transitions from growth-associated phases (i.e., exponential and stationary) to the death phase, potentially indicating growth inhibition by these metabolites. Volcano plot analysis using unpaired t-test (p-value< 0.05) was used to putatively identify a subset of metabolites upregulated by an AUC fold change of two or more at the end of culture (120h) in comparison to exponential growth phase (24h) (Figure 2-1B). A total of 167 features were found to accumulate at 120 hours of cell culture. Among these, 18

metabolites were annotated using an in-house developed automated tool (BioCAn) [15]. Since our primary focus was on metabolites closely associated with amino acids, particularly high-value essential amino acids, two metabolites were selected from the subset of upregulated and annotated metabolites. Namely, phenyllactic acid and hydroxyphenyllactic accumulated in cell culture as a product of phenylalanine and tyrosine metabolism, respectively.

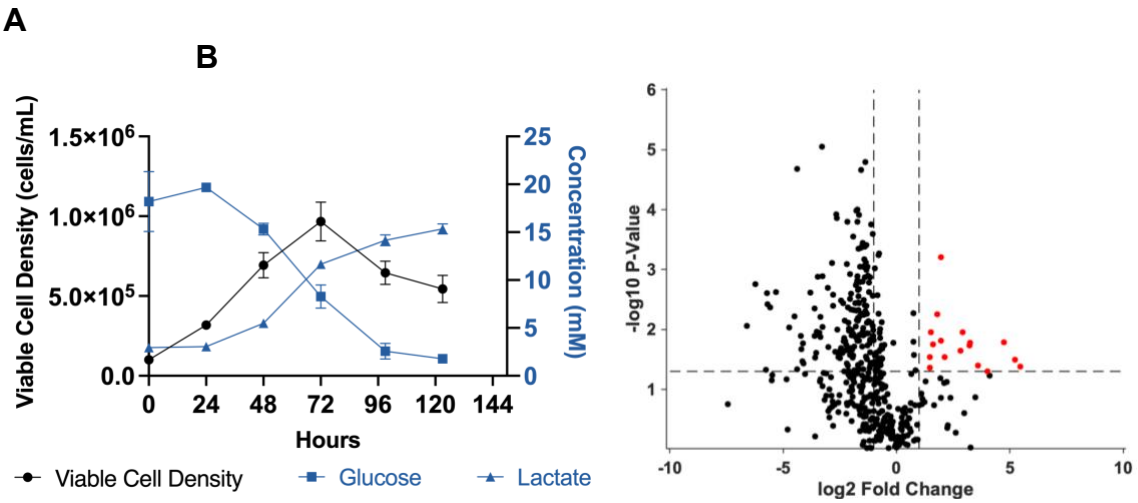

Figure 2-1 DF-1 cell growth and untargeted LC-MS analysis

Growth of suspension adapted DF-1 G. gallus fibroblast cell line in batch culture (A). Viable cell density. (▲) achieves peak at 72h. Glucose (●) does not indicate cell death due to starvation. Lactate (■) production shows only slight increase from 72-120h suggesting additional inhibitory metabolites formation. Significance and fold-change of annotated metabolites upregulated in spent media samples at death phase (120h) vs early exponential (24h) (B). Significantly upregulated metabolites determined by fold change greater than 2 and p-value <0.05 (red). Data representative of biological triplicate.

2.4.2 Targeted analysis confirms accumulation of phenylalanine and tyrosine derived metabolites, suggesting a potential link to growth inhibition.

Targeted LC-MS experiments were used to confirm putative metabolite annotations against chemical standards. The targeted method was adapted from the untargeted metabolomic analysis using HILIC chromatography with a negative ionization mode to quantify both phenyllactic acid as well as hydroxyphenyllactic acid. Extracellular supernatant samples were run alongside high-quality chemical standards to quantify phenyllactic acid and hydroxyphenyllactic acid concentrations over the duration of cell culture. A 2.5-fold increase in phenyllactic acid was observed at peak VCD (72h) compared to early exponential growth (24h), followed by a further 15-fold increase during the cell death phase (120h) relative to early exponential growth (Figure 2-2A). Similarly, hydroxyphenyllactic acid shows a 1.7-fold increase at peak VCD and a 5-fold increase during the cell death phase compared to the early exponential growth phase (Figure 2-2B).

Since phenyllactic acid is a product of the phenylalanine degradation pathway and hydroxyphenyllactic acid is a product of the tyrosine biosynthesis pathway, the consumption of both phenylalanine and tyrosine was quantified in DF-1 cell culture (Figure 2-2C & 2-2D). Both amino acids exhibited a decline in concentration over time; however, they appear to be supplied in excess of cellular growth requirements in the standard DMEM formulation. Based on these observations, we hypothesized that reducing phenylalanine or tyrosine in the initial media composition may reduce byproduct formation without compromising cellular growth.

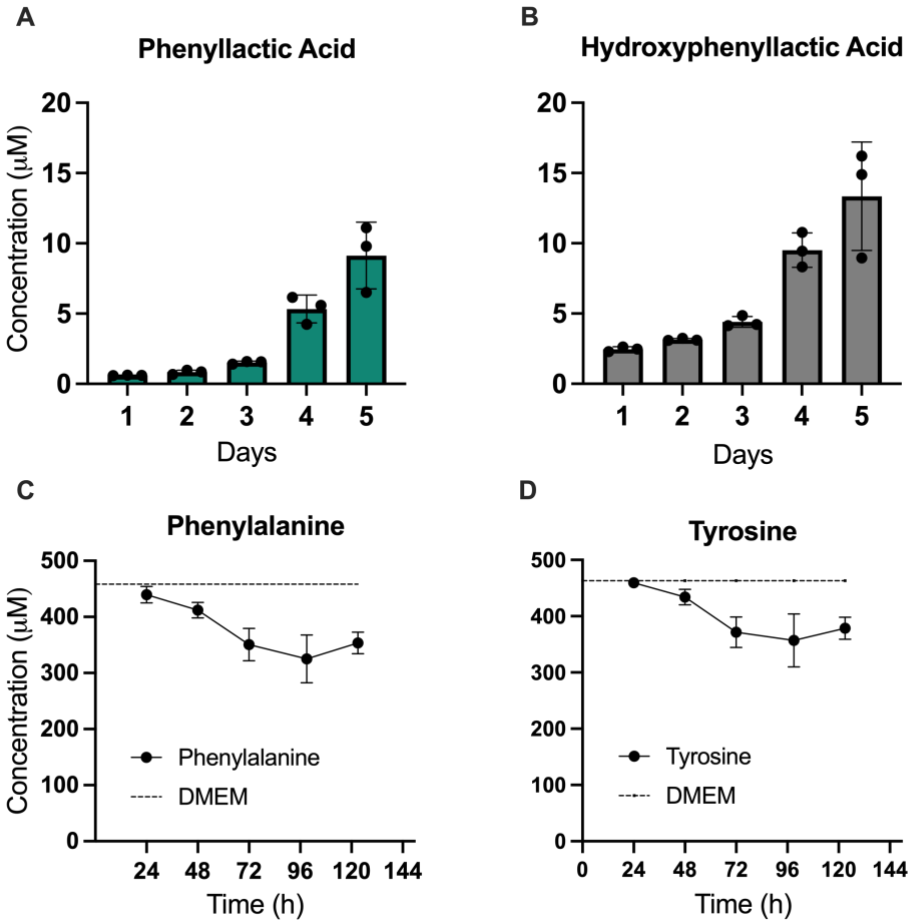

Figure 2-2 Quantification of metabolite accumulation and amino acid utilization using targeted LC-MS.

Significant extracellular accumulation of phenyllactic acid (A) and hydroxyphenyllactic acid (B) in DF-1 cell culture. Amino acid uptake of phenylalanine (C) and tyrosine (D), corresponding to the precursor metabolites for phenyllactic acid and hydroxyphenyllactic acid, respectively. Metabolites and amino acids quantified against chemical standards. Data represent biological triplicates.

2.4.3 Media supplementation with phenylalanine derived metabolites negatively impacts cell growth.

To understand the direct impact of amino acid derived catabolites in DF-1 cell culture, an add-back study was performed using phenyllactic acid and hydroxyphenyllactic acid based on their accumulation in later phases of batch cell culture. DF-1 cells were cultured in DMEM in six well plates using the same seeding density as the above-described growth experiment. After 24 hours of incubation, experimental wells were treated with either a high (100 μM) or low dose (25 μM) of either phenyllactic acid or hydroxyphenyllactic acid. Control wells received treatment with water. Incubation for 24 hours prior to metabolite addition allowed the cells to reach early exponential growth phase. The low dose was chosen as a biologically relevant concentration as determined by the targeted analysis and quantification of the metabolites in spent culture medium. The high dose was chosen to observe dose-dependent trends of metabolite supplementation. Viable cell density and viability measurements were taken at 24-hour time intervals following metabolite supplementation.

After 48 hours of incubation, the control condition reached early stationary phase whereas the cells treated with metabolites remain at mid exponential phase (Figure 2-3A). Despite differences in growth profile, no significant differences in viability were observed during the exponential growth phase between all treatment conditions (Figure 2-3B). A significant decrease was observed in the integral viable cell density (IVCD) of cells treated with phenyllactic acid in comparison to the control condition, indicating a lower overall culture productivity for cell. Addition of phenyllactic acid reduced IVCD by 25.8% and 23.7% for the high and low dose conditions, respectively (Figure 2-3C). Similarly, a significant decrease in growth rate during exponential growth phase of

31.6% for the high dose condition and 22.9% for the low dose was observed after supplementation with phneyllactic acid in comparison to the control (Figure 2-3D). Hydroxphenyllactic acid supplementation led to a slight decline in both IVCD and growth rate compared to the control; however, this decline was not significant.

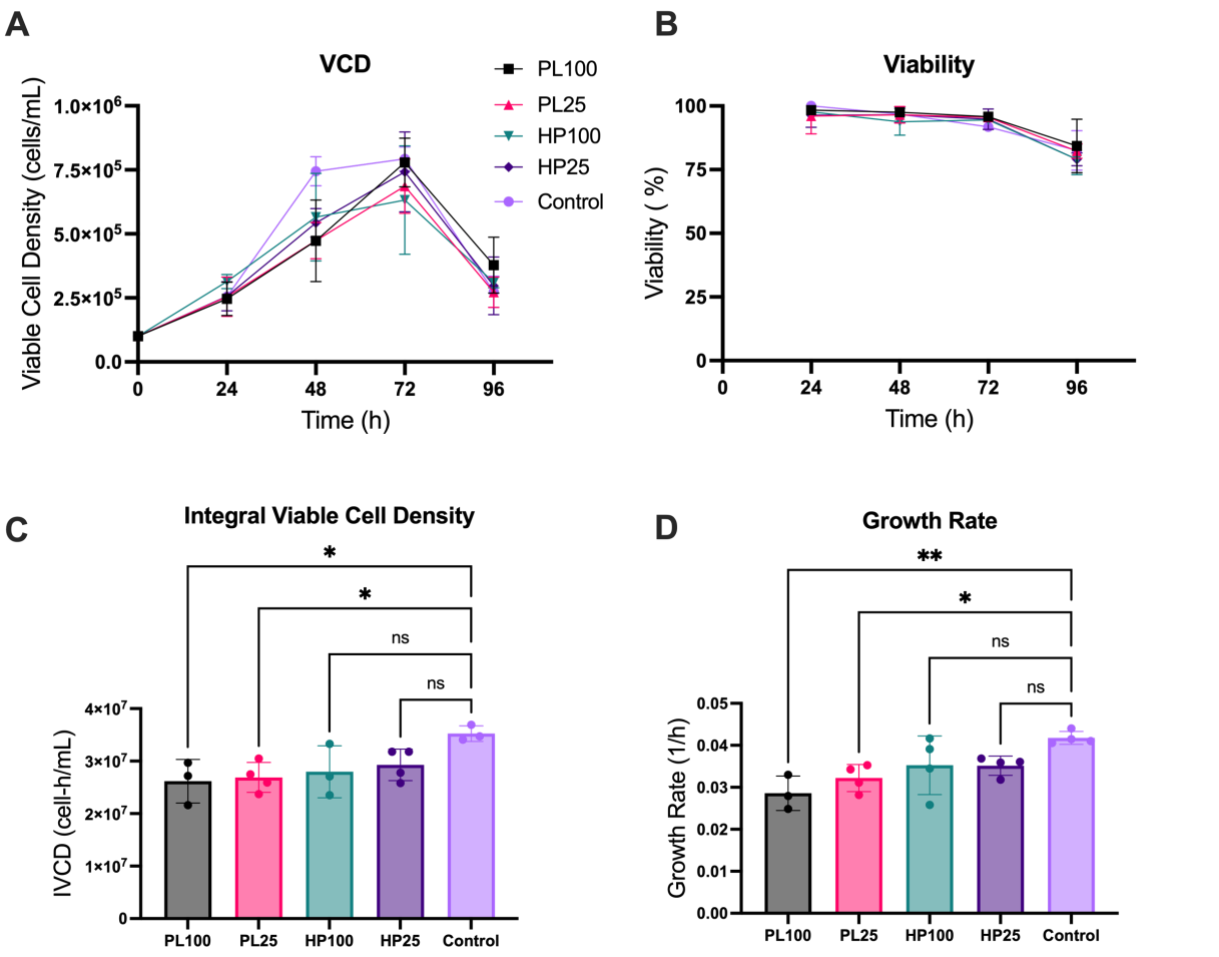

Figure 2-3 Media supplementation with phenylalanine and tyrosine derived catabolites.

Figure 2-3 (cont.) Viable cell density of DF-1 cell culture with supplementation at 24h of phenyllactic acid (PL) or hydroxyphenyllactic acid (HP) at 100μM or 25μM compared to control condition supplemented with water (A). Viability of cell culture following supplementation shows no significant differences during exponential growth phase between treatment conditions (B). A significant decrease in IVCD is noticed with supplementation of phenyllactic acid in comparison to the control (C). A significant and dose dependent decrease in growth rate calculated during exponential growth phase is observed with supplementation of phenyllactic acid (D). Hydroxyphenyllactic acid supplementation resulted in a slight decrease in IVCD and growth rate, however, this difference is not significant.

**P-value< 0.001; *P-value < 0.05

Trends based on 2 independent experiments (N = 2)

2.4.4 Phenylalanine concentration modulates growth profile of DF-1 cells in batch culture.

The relationship between phenylalanine concentration in the culture medium and cell growth was investigated to assess the potential inhibitory impact of excess amino acids. To isolate the effects of phenylalanine on cell growth, DF-1 cells were seeded in either a modified MEM formulation or standard DMEM. Off-the-shelf basal MEM was adjusted to match the amino acid concentrations of the control DMEM medium, except for phenylalanine. The modified MEM formulations contained low (200 μM), mid (400 μM), or high (800 μM) phenylalanine concentrations. It is important to note that basal MEM contains only one-fourth of the vitamin concentration of DMEM and also has slight differences in salt concentrations compared to DMEM. Since this study primarily

focused on the effects of phenylalanine on cell culture performance, a comprehensive analysis of MEM and DMEM differences was not conducted – instead, the DMEM control served as a reference point representing a commercial medium enriched in amino acids.

Viable cell density over time was inversely correlated with phenylalanine concentration in the modified MEM conditions (Figure 2-4A). The lowest phenylalanine concentration (200 μM) resulted in a growth profile comparable to the DMEM control, whereas higher concentrations (400 μM and 800 μM) led to reduced viable cell densities over time. The impact of phenylalanine concentration on cell culture performance is particularly evident when considering integral viable cell density (IVCD), which reflects the total accumulated number of viable cells over the culture duration. Compared to the 200 μM phenylalanine MEM condition, IVCD was significantly reduced by 42% in the 400 μM condition and 51% in the 800 μM condition (Figure 2-4C). Given that the modified MEM with 400 μM phenylalanine matched the amino acid levels of DMEM control, the differences in cell growth between the two media likely reflect their differences in vitamin and salt concentrations. Notably, lowering the phenylalanine concentration mitigated some of the growth limitations imposed by the reduced vitamin levels of MEM.

2.4.5 Phenylalanine impacts growth rate rather than imposes toxicity on cells through production of inhibitory catabolites

Although viable cell density noticeably depended on phenylalanine concentration, viability in contrast show limited differences across all media conditions. All cultures

maintained viability near 100% until 72 hours of growth. Cell culture viability was reduced by 5-10% in last two days of culture for all cultures, with no significant differences observed between different media (Figure 2-4B). These observations suggest that excess phenylalanine did not cause outright toxicity in DF-1 cells. To investigate potential causes for phenylalanine dependent reduction in VCD, metabolites identified from the untargeted analysis were quantified through targeted metabolomics after 96 hours of culture (Figure 2-4D & 2-4E). Phenyllactic acid dose dependently accumulated in culture in response to the concentration of phenylalanine present in the media. Phenyllactic acid accumulated to a significant greater extent in media with phenylalanine concentrations above 200 μM. In contrast, hydroxyphenyllactic acid showed no change in response to the phenylalanine concentration. Taken together, these observations suggested that accumulation of phenyllactic acid may contribute to the reduced growth observed at higher phenylalanine concentrations, whereas hydroxyphenyllactic acid did not directly affect cell growth.

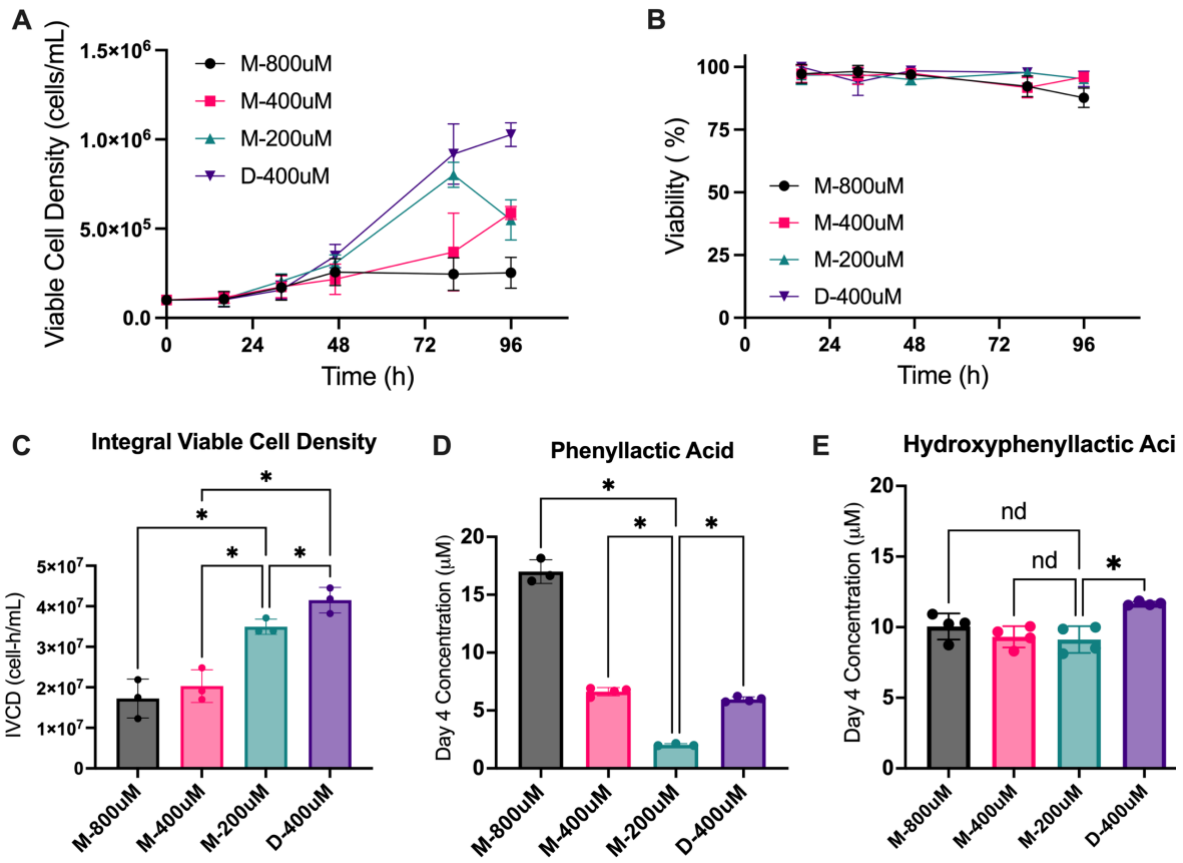

Figure 2-4 Modified phenylalanine media

Figure 2-4 (cont.) Viable cell density of DF-1 cells cultured in either MEM: M or DMEM: D with concentrations of phenylalanine in the media of 200μM, 400μM or 800μM (A). Media concentrations of other amino acids equivalent across all formulations. Viability is nearly identical in all media with the highest concentration of phenylalanine showing a slight reduction in later time points (B). Integral viable cell density (IVCD) indicates a significant reduction in cumulative viable cells over time with increasing concentrations of phenylalanine in MEM (C). Increasing concentrations of phenylalanine in the starting media formulation show a corresponding increase in phenyllactic acid after 4 days of culture (D). In contrast, increasing phenylalanine concentration in MEM base media did not affect hydroxyphenyllactic acid concentration after 4 days of culture (E).
*P-value <0.05; Trends based on 2 independent experiments (N = 2)

2.5 Discussion

Untargeted metabolomics was utilized to identify growth-inhibitory metabolites resulting from excess amino acid metabolism based on spent media accumulation patterns. Phenyllactic acid and hydroxyphenyllactic acid were selected and quantified through targeted LC-MS assays to assess accumulation in DF-1 cell culture. This study demonstrated a significant negative impact of phenyllactic acid on cell growth rate, suggesting that an overabundance of phenylalanine could lead to reduced growth rates and lower overall cell accumulation. This finding was further supported by a modified media experiment, which revealed an inverse correlation between IVCD and a direct correlation between phenyllactic acid production and initial phenylalanine media

concentration. Importantly, this study establishes a connection between the accumulation of growth-inhibitory metabolites and the over supplementation of amino acid media components emphasizing the need for cell type specific media formulations as new cell lines emerge for cultivated meat applications.

2.5.1 Accumulating metabolites and amino acid over supplementation in cell culture

In this study, we investigated the accumulation of extracellular metabolites and their potential inhibitory effects on avian cell growth. Our findings reveal that byproducts of aromatic amino acid metabolism, specifically those derived from phenylalanine, contribute to growth suppression—highlighting a previously underappreciated source of metabolic toxicity beyond the well-characterized effects of lactate and ammonia. Previous research linking byproduct accumulation in culture medium to growth inhibition primarily focus on lactate as the primary culprit. Over production of lactate is related to excess glucose abundance therefore there is a balance between meeting cellular substrate requirements for glycolytic growth and minimizing production of inhibitory bioproducts. To this end studies such as the HIPDOG glucose feeding strategy have been developed to enhance cellular growth by limiting glucose availability based on pH to mitigate lactic acid production [19]. However, similar to what was shown by this study, toxic byproducts can arise not only from glycolysis but also from inefficiencies in other cellular pathways, such as amino acid metabolism. A study on CHO cells grown in fed-batch culture found that the HIPDOG strategy was effective in extending cell viability up to a point, after which a significant decline in viable cell density was still observed. This suggests that growth inhibition was likely due to the accumulation of other inhibitory

metabolites. The study identified seven key byproducts of amino acid metabolism that contributed to limitations in cell growth. By reducing the concentrations of four or eight amino acids in the media, improved overall cell growth and titer was achieved. [20].

Although several studies have investigated the relationship between media components and cellular metabolism for common cell types for pharmaceutical processes such as CHO cells, less is known for cell types relevant to cellular agriculture. Despite the importance of phenylalanine for chicken cell growth, our findings demonstrate that its overabundance inhibits cell growth, underscoring the need for cell type-specific media formulations. Current standard cell culture practices use generalized media formulations for cell growth despite non-standard nutrient requirements across various cell lines. For example, a comparison of the nutrient utilization pattern of proliferating primary chicken embryonic muscle precursors cells, primary chicken fibroblast cells and a C212 murine myoblast cell line showed significant differences in specific consumption rates of essential amino acids arginine, isoleucine, leucine, and non-essential amino acid glutamine in early stages of cell culture [21]. Tailoring media formulations to specific cell types is a key focus in cellular agriculture. A common strategy involves using Design of Experiments (DOE) to systematically evaluate the effects of media components and their interactions on cell culture performance [22], [23]. While DOE provides a robust framework for systematically evaluating media components, it often yields only a high-level view and offers limited insight into the molecular mechanisms driving cellular responses. In contrast, bottom-up metabolomics—such as the approach used in this study— provide mechanistic insights driving media dependent growth changes. A

deeper understanding of the causal relationships between media composition and cellular behavior is particularly critical in the context of cellular agriculture, where input costs must be tightly controlled to ensure economic feasibility of the final product. Ultimately, integrating both optimization strategies with omics approaches offers a powerful avenue for advancing media development in cultivated meat production [24], [25].

2.5.2 Phenylalanine metabolism

In this study, we found that elevated levels of phenylalanine led to the accumulation of 3-phenyllactate, which was significantly associated with reduced cell growth in chicken fibroblasts. While 4-hydroxyphenyllactate also accumulated in culture, its association with growth inhibition was not statistically significant, though it may contribute modestly to the overall reduced growth. Although phenylalanine is considered an essential amino acid for avian cells and serves as a precursor to tyrosine [26], our findings underscore the importance of balancing amino acid supplementation to avoid flux toward inhibitory metabolic byproducts. Similar phenylalanine-derived metabolites have been reported in later phases of CHO cell cultures, where 3-phenyllactate and 4-hydroxyphenyllactate were shown to suppress proliferation. In CHO systems, targeted metabolic engineering strategies overexpressing phenylalanine hydroxylase (PAH), pterin-4-alpha-carbinolamine dehydratase (PCBD1), 4-hydroxyphenylpyruvate dioxygenase (HPD), and homogentisate 1,2-dioxygenase (HGD) have been used to redirect flux from phenylalanine and tyrosine toward central carbon metabolism, alleviating this growth inhibition. Specifically, upregulation of PAH and PCBD1 enhanced conversion of

phenylalanine to tyrosine, while upregulation of HPD and HGD increased tyrosine conversion to TCA cycle intermediates [27]. These interventions reduced alternative reaction flux and accumulation of toxic intermediates. Additionally, upregulation of PAH and PCBD1 enabled growth in tyrosine-deficient conditions—a strategy potentially valuable in large-scale bioprocesses, where the limited aqueous solubility of tyrosine could be a challenge. Drawing from these examples, similar approaches might be considered to optimize phenylalanine flux in chicken cell cultures and improve media efficiency for cultivated meat applications.

In the present study, phenyllactic acid and hydroxyphenyllactic acid were identified as accumulating metabolites in DF-1 chicken fibroblast cultures. Both compounds are typically generated through enzymatic reduction of phenylpyruvate and 4-hydroxyphenylpyruvate, respectively. However, the specific enzymes mediating these reactions have not, to our knowledge, been annotated within the Gallus gallus genome, including in curated pathway databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [28]. The detection of these metabolites thus suggests a potentially incomplete annotation of aromatic amino acid metabolism in avian species and highlights the need for further investigation into candidate encoding genes responsible for these reactions in chicken cells. Enzymes have been shown to display substrate promiscuity referring to the ability to catalyze the same reaction using a range of substrates. To probe this as a possibility for phenyllactic acid production by chicken cells, the CoNCISE algorithm was used to investigate binding of the precursor metabolite, phenylpyruvate, to three candidate enzymes [29]. Lactate dehydrogenase A

(LDHA) was identified as a relatively strong binder to phenylpyruvate (Table 2-S2). A 3D structural prediction of LDH with a phenylpyruvate shows potential for the metabolite to serve as a non-native alternative substrate (Figure 2 - S2). However, further investigation is required to determine other potential promiscuous enzymes in the chicken genome with strong binding affinity to the parent metabolites as well as experimentally validate the catalysis capacity This gap in annotation is particularly relevant as the field increasingly turns to alternative cell types for cultivated meat production and representative computational modeling tools. Improved genomic and enzymatic annotation is essential for constructing accurate genome-scale metabolic models (GEMs), which are critical tools for predicting cellular phenotypes, optimizing media formulations, and guiding metabolic engineering strategies in a rational and efficient manner [30].

2.5.3 Amino Acid Reduction in the Context of Cultivated Meat

In this study, we demonstrate a viable approach for identifying the potential inhibitory effects of amino acid over supplementation in chicken cell culture, with the goal of informing the development of more efficient, tailored growth media. One of the major ongoing challenges in cultivated meat production is the high cost of inputs, particularly cell culture media. Reducing media-related costs is therefore essential to achieving price parity with conventionally farmed products. Current strategies to lower production costs include the development of less expensive recombinant growth factors, substitution of pharmaceutical-grade raw materials with food-grade alternatives or hydrolysates, and improved media utilization to extend the functional lifespan of media

components [11]. While each of these avenues contributes to overall cost reduction, amino acid optimization is especially critical, as amino acids represent a substantial portion of media costs. Technoeconomic analyses indicate that macronutrients—primarily amino acids—can account for over 50% of total media expenses and dominate cost contributions to cell biomass production, particularly at small to mid-scale production volumes [6], [31]. Among these, aromatic amino acids present unique challenges: while not the most expensive per kilogram, their concentrations must be carefully balanced to avoid growth-inhibitory byproduct formation [6], [13], [20]. In addition, as cultivated meat processes trend toward prospective industrial bioreactor volumes of 10,000L and beyond, the chemical properties of media components become increasingly important. Aromatic amino acids, in particular, have limited aqueous solubility and are most prone to precipitation under long-term storage conditions or fluctuations in pH [32], [33]. Thus, optimizing aromatic amino acid supplementation offers a several benefits—reducing input costs, improving cell growth performance, easier manufacturability—making it a crucial component for refinement in cultivated meat media design.

2.6 Future Directions

Future research should focus on adapting chicken cells to fully serum-free conditions and designing amino acid formulations that sustain robust growth without animal-derived supplements. The approaches applied in this study—untargeted and targeted metabolomics—can be extended to serum-free cultures to identify inhibitory byproducts and guide rational media optimization. Integrating these metabolomic insights with

genome-scale metabolic models (GEMs) will enable a deeper understanding of amino acid requirements and nutrient fluxes specific to avian cells. Defining these requirements not only supports the development of serum-free media but also informs opportunities to incorporate economically advantageous supplements, such as plant- or microbial-derived hydrolysates and cell lysates, as alternatives to purified amino acids or other media components. Together, these strategies will accelerate the creation of cost-effective, defined media that advance the scalability and productivity of cultivated poultry production.

2.7 Conclusion

This study demonstrates that phenylalanine over supplementation drives the accumulation of phenyllactic acid, a growth-inhibitory metabolite, in chicken cell cultures. By reducing phenylalanine levels in the medium, we alleviated inhibitory byproduct formation and improved cell growth, underscoring the importance of amino acid tuning for cellular agriculture processes. These findings highlight that cell type–specific metabolism must be considered when designing cost-effective media for cultivated meat to maximize growth and minimize input expenses.

2.8 Acknowledgements

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2.9 Supplementary Information

S1. LC-MS method development parameters

LC-MS untargeted methods

Untargeted analysis performed as described by Yao et al., 2021.

Reverse phase (RP) chromatography method

- **Column:** Phenomenex Synergi Hydro-RP
- **Solvents:**
  - A: 0.1% formic acid in water
  - B: 0.1% formic acid in methanol
- **Column temperature:** 15 °C
- **Flow rate:** 0.2 mL/min
- **Ion source:** Turbo spray (ESI)
- **Ion source Gas 1:** 35
- **Ion source Gas 2:** 45
- **Curtain Gas:** 25
- **Temperature:** 450 °C
- **IonSpray Voltage Floating:** ±4500 V

Gradient:

Time (min)	**%B**
0–8	3
8–38	3 → 95
38–45	95
45–47	95 → 3
47–55	3

Hydrophilic interaction chromatography (HILIC) method

- **Column:** Phenomenex Luna NH2
- **Solvents:**
  - A: 95:5 water:acetonitrile + 20 mM ammonium acetate, pH adjusted to 9.45
  - B: 100% acetonitrile
- **Column temperature:** 25 °C
- **Flow rate:** 0.3 mL/min
- **Ion source:** Turbo spray (ESI)
- **Ion source Gas 1:** 35
- **Ion source Gas 2:** 45
- **Curtain Gas:** 25

- **Temperature:** 450 °C
- **IonSpray Voltage Floating:** ±5500 V

Gradient:

Time (min)	%B
0–15	85 → 0
15–28	0
28–30	0 → 85
30–60	85

LC-MS targeted Amino acid method

Hydrophilic interaction chromatography (HILIC) method

- **Column:** Phenomenex Luna NH2
- **Solvents:**
  - A: 95:5 water:acetonitrile 20mM ammonium formate, pH adjusted to 3
  - B: 100% acetonitrile
- **Column temperature:** 25 °C
- **Flow rate:** 0.4 mL/min
- **Ion source:** Turbo spray (ESI)
- **Ion source Gas 1:** 35
- **Ion source Gas 2:** 45
- **Curtain Gas:** 25
- **Temperature:** 450 °C
- **IonSpray Voltage Floating:** ±5500 V

Gradient:

Time (min)	%B
0–2	92
2–11	92 → 70
11–19	70 → 0
19–29	0
29– 29.5	0 → 92
29.5–40	92

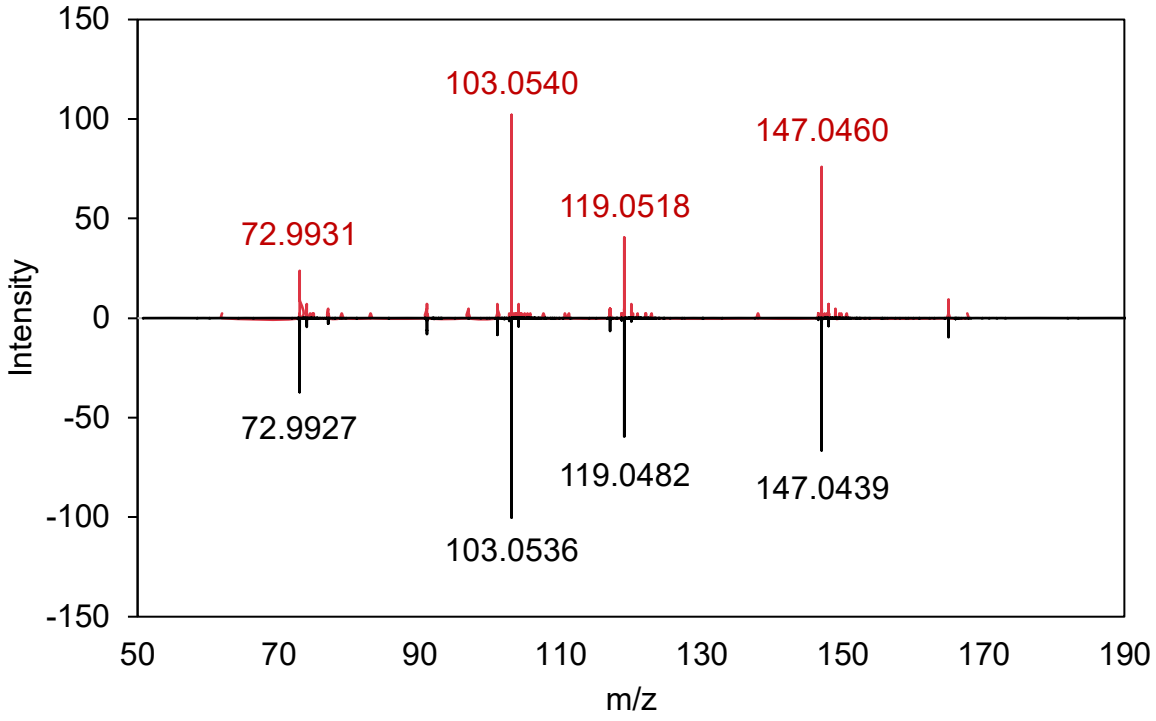

Figure 2-S 1 Mirror plot of phenyllactic acid quantified using targeted LC-MS. Sample spectra (red); standard spectra (black).

Table 2-S 1 Transition table and instrument parameters for targeted analysis.

Compound Name	Mass	Precursor	Product	Retention Time	CE	DP	Polarity
Phenyllactic Acid	166.06	165.06	103.06	33.3	25	30	Negative
Hydroxyphenyllactic Acid	182.06	181.05	135.05	27.8	25	20	Negative
Glycine	75.03	76.04	48.04	16.8	10	40	Positive
Arginine	174.11	175.12	70.07	14.6	25	40	Positive
Cystine	240.02	241.03	74.02	18.7	30	50	Positive
Histidine	155.07	156.08	110.07	16.2	20	40	Positive

Leucine	131.09	132.1	86.1	13.2	15	70	Positive
Lysine	146.11	147.11	84.08	15.2	20	30	Positive
Isoleucine	131.09	132.1	69.07	13.6	15	70	Positive
Methionine	149.05	150.06	104.05	13.9	15	50	Positive
Phenylalanine	165.08	166.09	120.08	12.9	20	40	Positive
Serine	105.04	106.05	60.04	17	20	40	Positive
Threonine	119.06	120.07	74.06	16.4	15	40	Positive
Tryptophan	204.09	205.1	188.07	13.5	10	30	Positive
Tyrosine	181.07	182.08	136.08	15.1	20	40	Positive
Valine	117.08	118.09	72.08	14.7	15	40	Positive
Glutamine	146.07	147.08	84.04	16.7	20	30	Positive
Alanine	89.05	90.05	44.05	16.4	15	40	Positive
Aspartic acid	133.04	134.04	74.02	27.1	20	30	Positive
Asparagine	132.05	133.06	87.06	16.9	25	50	Positive
Proline	115.06	116.07	70.07	15.4	20	40	Positive
Glutamic acid	147.05	148.06	84.04	23	25	40	Positive

S2. Potential promiscuous enzymatic activity

To investigate potential promiscuous enzymatic activity responsible for production of phenyllactic acid in chicken cell culture a small molecule binding predictive algorithm was used to determine possible binding of the parent and product metabolites for this study. The CoNCISE predictive algorithm uses a vector-quantized codebook approach through a residuals learning framework to query large datasets of ligands against protein targets in a extremely high throughput manner. Exact methods for CoNCISE based predictions described previously [29].

Table 2-S 2 Binding predictions to potential enzymatic candidates of phenyllactic acid precursor using CoNCISE algorithm

Enzymes	Phenylpyruvate
Lactate Dehydrogenase A (*Gallus gallus*)	0.78
Lactate Dehydrogenase B (*Gallus gallus)*	0.5
Malate Dehydrogenase (*Gallus gallus)*	0

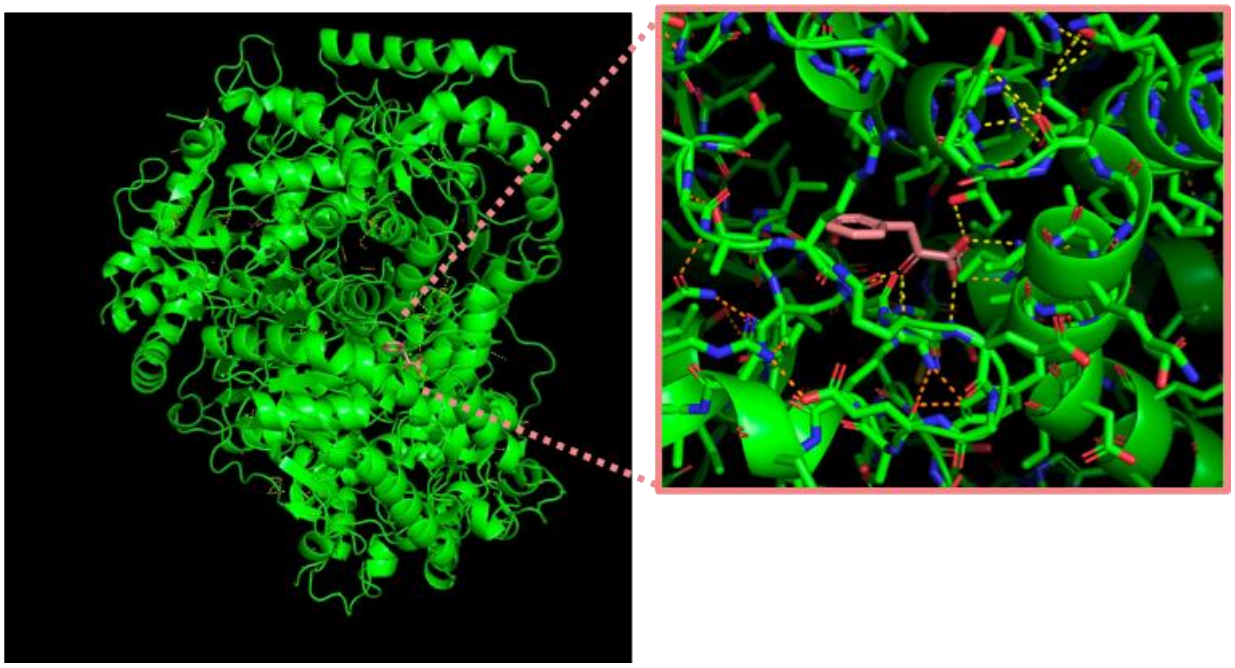

Figure 2-S 2 LDHA 3D structural binding prediction with phenylpyruvate as non-native ligand.

Binding affinity predicted ~0.586.

Chapter 3. Refinement of Genome-Scale Metabolic Modeling in Chicken Fibroblast Cells

3.1 Abstract

Cultivated meat has the potential to reduce environmental impact, improve animal welfare, and meet rising global demand for meat, but the high cost of cell culture media—particularly amino acids—remains a critical barrier to commercialization. Genome-scale metabolic models (GEMs) offer a powerful computational framework to predict cell growth, guide media formulation, and reduce reliance on costly experimentation. A recently published chicken GEM (iES1300) provided an important foundation but exhibited biological inconsistencies that limited its predictive accuracy. In this study, we refined the chicken GEM by incorporating experimental data from DF-1 fibroblast cultures and addressed gaps in the metabolic network. Key improvements included adjusting the biomass equation to reflect chicken-specific macromolecular composition, restoring balanced glycogen metabolism, and adding missing reactions in amino acid pathways. Reaction nomenclature was standardized and cross-referenced with well characterized models in the BiGG database to improve comparability and interoperability. The refined model successfully predicted DF-1 cell growth in DMEM medium within the correct order of magnitude of experimental measurements and demonstrated the ability to simulate growth under reduced phenylalanine availability. These results highlight the utility of GEMs for identifying amino acid sensitivities and informing rational media design in cultivated meat applications. By improving accuracy and standardization, this refined chicken GEM provides a robust framework for future

experimental validation and continued development of computational tools to optimize avian cell culture systems for cellular agriculture.

3.2 Introduction

Cultivated meat has emerged as a promising alternative to conventional livestock production, with the potential to reduce environmental impact, improve animal welfare, lower the risk of foodborne disease, and meet the growing global demand for meat. Efficient control of scale-up processes will be critical not only for producing consistent, high-quality products but also for lowering input costs. A recent technoeconomic analysis suggested that significant cost reductions in cultivated meat production will only be realized at very large scales (>10^5 kTA). In the same study, macronutrients—specifically amino acids—were identified as the main contributors to cell mass production costs [1]. Consequently, tools that enable the design of growth-optimized and cost-minimized bioprocesses for cell types used in cultivated meat are essential to achieving price parity and commercial viability. Traditional statistical and empirical modeling approaches, while useful, are insufficient on their own due to the inherent complexity of cellular metabolism. There is an urgent need for computational methods that explicitly capture the metabolic mechanisms underlying cell growth, thereby improving culture efficiency, reducing input costs, and supporting large-scale manufacturing.

Genome-scale metabolic models (GEMs) have proven highly valuable for process optimization in biopharmaceutical development, particularly in widely used mammalian host cell lines such as CHO and HEK used for biologics production. GEMs are reconstructed from genome annotation data by mapping gene-protein-reaction associations to form a network of mass- and charge-balanced metabolic reactions [2].

These models enable prediction of intracellular flux distributions using linear programming-based optimization approaches, such as flux balance analysis, under the pseudo–steady state assumption. CHO cell GEMs, for example, have been used to guide feed strategies that increase culture growth, raise product titers, and reduce accumulation of undesirable byproducts [3], [4]. In one case, a hybrid GEM derived from the CHO-K1 model, combined with empirical constraints, was used to design an optimized feed formulation that more than doubled viable cell density [3]. Despite these successes, GEM development requires significant curation and validation, which has slowed progress in creating models for species and cell types relevant to cultivated meat. To date, only a limited number of GEMs have been developed for food relevant organisms (i.e. bovine, salmon, shrimp, and duck cells) [5], [6], [7], [8].

Recently, a genome-scale metabolic model of chicken metabolism, iES1300, was published as the first avian GEM [9]. The model was manually reconstructed using genomic and biochemical data from KEGG [10], BiGG [11], CHEBI [12], UNIPROT [13], REACTOME [14], and MetaNetX [15] databases. This development represents an important first step toward computational tools tailored to avian cell lines, though the model still requires refinement and validation with experimental data. In this study, we build on the iES1300 GEM by incorporating experimental constraints derived from DF-1 fibroblast cultures to identify network gaps and resolve biological inconsistencies. Our refinements included redefining the biomass equation to more accurately represent chicken cells, rebalancing glycogen metabolism, adding missing reactions in amino acid pathways, and aligning reaction naming conventions with community standards (Figure

3-1). The updated model successfully recapitulated DF-1 growth in standard medium and predicted growth under reduced phenylalanine conditions consistent with our previous findings.

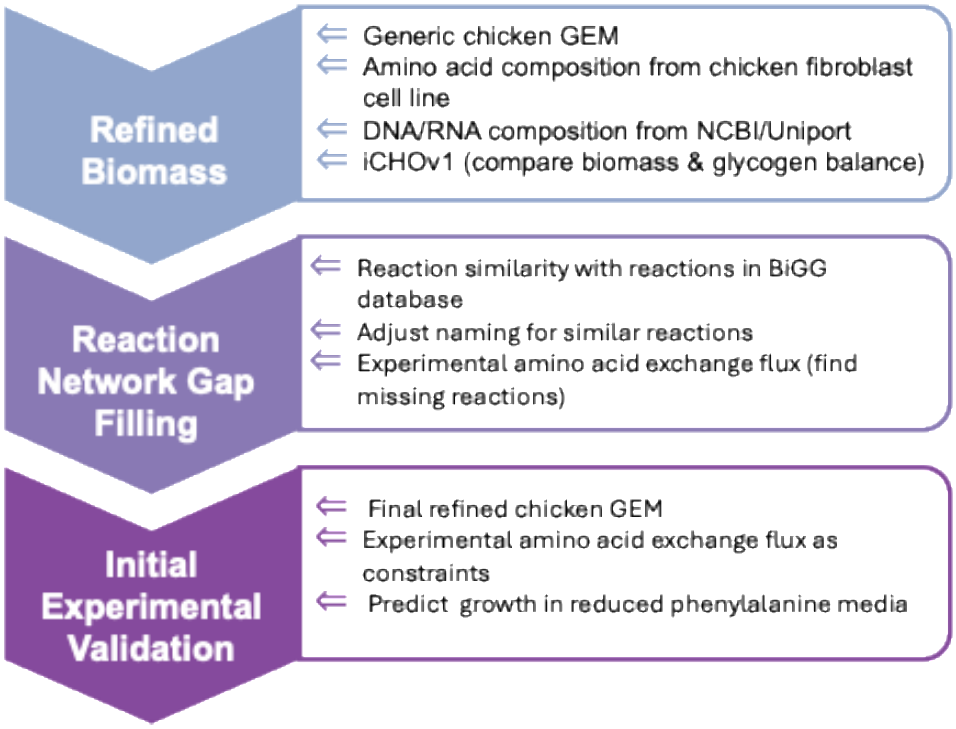

Figure 3-1 Overall schematic of chicken GEM refinement process including inputs per stage.

3.3 Methods

3.3.1 Experimental data

Experimental data for this study was utilized from the previous section detailing DF-1 chicken fibroblast cell growth in traditional DMEM media with 10% FBS. Amino acid utilization was quantified as detailed in the previous chapter. The amino acid consumption and production rates obtained from the previous study was used as flux constraints for the GEM. Experimental glucose, lactate, and growth rates measured in

the previous chapter were additionally compared against predicted values to determine model accuracy against experimental data.

3.3.2 Model curation

The original model was obtained from a recent publication describing a generic genome-scale metabolic model for chicken iES1300 [9]. Briefly, the published model was developed using gene, reaction, and metabolite data collected from various databases to construct a draft metabolic network. Initial flux balance analysis (FBA) calculations using the published iES1300 model revealed inconsistencies between model predictions and known nutrient (e.g., amino acid) requirements of chicken cells. Our lab then took a series of model curation steps to refine iES1300, generating a more biologically accurate GEM for chicken, as detailed in the following sections.

3.3.3 Adjustment of biomass equation based on experimental chicken fibroblast data

For the purpose of bioprocess optimization, GEMs are often utilized in conjunction with FBA, which optimizes an objective function typically defined as biomass production. While the reaction equations for biomass can be similar across related organisms in terms of their reactants, the stoichiometric coefficients of these reactants can vary significantly. To improve the accuracy of the biomass equation, data from a recent study on the spontaneous immortalization of a chicken fibroblast cell line were used to inform specific amino acid and lipid ratios [16]. The amino acid composition of the fibroblast cell line's dry cell matter was used to set the corresponding stoichiometric coefficients of the chicken cell GEM biomass equation. Cholesterol, cholesterol ether, and average

fatty acid chain length data from the same study were used to calculate lipid contributions and set the corresponding stoichiometric coefficients. The nucleotide contribution was subsequently determined by subtracting the total amino acid-derived protein content from the crude protein, with DNA composition obtained from NCBI [17] and noncoding RNA plus coding RNA (mRNA) derived from UniProt [13] and NCBI respectively. Finally, the resulting biomass equation was compared to iCHOv1, a well-curated GEM for CHO cells [18], to ensure completeness and fill any remaining gaps. The final biomass equation in the updated GEM has the following structure:

$$\text{Cellular dry weight} = a*\text{ATP} + b_i*\text{Amino Acids} + c_i*\text{dNTP} + d_i*\text{NTP} + e_i*\text{Lipids}$$

In the above equation, a, b_i, c_i, d_i, and e_i represent stoichiometric coefficients of corresponding biomass components.

3.3.4 Restoring glycogen balance

The original iES1300 model linked glycogen directly to protein production. However, glycogen typically serves as a carbon source or macromolecule for energy storage. Additionally, the glycogen cycle in the original GEM had an imbalance in glucose resulting from glycogen conversion. To address these errors, the linkage of glycogen to an amino acid was adjusted to a reversible reaction, serving as a proxy reaction to flexibly connect glycogen metabolism to protein synthesis. The overall glycogen cycle was then modified to accurately reflect balanced sugar metabolism, closely resembling the expected pathways observed in CHO cells.

3.3.5 Resolve real flux

During FBA calculations, several amino acid-related reactions were identified as preventing convergence to an optimal solution, specifically those involving proline, tryptophan, leucine, and isoleucine. Stepwise gap filling was performed for each amino acids metabolic pathway to identify potentially missing reactions. For proline, a spontaneous reaction involving glutamate-5-semialdehyde was missing. Both leucine and isoleucine pathways lacked reactions for transport of methyl-2-oxopentanoate from the cytosol to the mitochondria. Similarly, three reactions in the tryptophan metabolism pathway were absent, likely due to limited knowledge of the responsible genes. These pathways were subsequently added to the chicken GEM to improve the completeness of amino acid metabolism.

3.3.6 Model nomenclature consistency

Individual reactions in the chicken GEM were compared against well-characterized species to assess similarity with reactions in CHO or human models using the BiGG database [11]. Reactions were considered similar based on EC numbers and Kendall similarity scores. Similar reactions were cross-checked to ensure consistent naming conventions and proper integration into the overall metabolic network.

3.3.7 Flux balance analysis

Flux balance analysis (FBA) was performed using the COBRA Toolbox in MATLAB [19] assuming pseudo-steady state. The published chicken GEM was imported into the

COBRA environment. The updated biomass equation was designated as the objective function for prediction of cell growth rate, and linear programming was applied to maximize biomass flux using the optimizeCbModel function and the glpk solver. Exchange reactions were constrained to reflect actual DF-1 cell culture conditions, with glucose, and amino acids uptake rates constrained to reflect experimentally determined values. Other, intracellular reaction fluxes were kept at default values while paying careful attention to the reversibility of reactions. All simulations were conducted in MATLAB version R2021a with COBRA Toolbox version 3.5.

3.4 Results & Discussion

3.4.1 Refined model recapitulates experimental data

The previously published GEM as a starting point for modeling general chicken metabolism. However, when experimentally determined constraints for exchange fluxes were applied to this GEM, the model failed to converge to an optimal growth rate. Inspection of the GEM revealed that there were multiple disconnected sub-networks and pathways that were inconsistent with known biology of chicken cells. To resolve these issues, we performed several rounds of model curation and gap filling, which allowed the model to simulate biomass formation from required primary metabolites and enabled the model to better represent experimental culture conditions for chicken fibroblast growth. Details of these adjustments are provided in the Methods section. We next applied FBA to the refined model, constraining amino acid exchange fluxes with experimentally determined values, to predict the maximum specific growth rate of chicken cells with amino acid utilization representative of DMEM. The refined model

predicted a growth rate of 0.032 h^{-1}, a notable improvement over the original model, which could not predict growth in DMEM due to missing important reactions in amino acid metabolism (Figure 3-2A). Given that DF-1 cells indeed grow in DMEM, the original model was not in agreement with experimental data. In shake flask culture, DF-1 cells exhibited a maximum specific growth rate of 0.023 h^{-1}. Thus, the refined model captured growth within the correct order of magnitude. While the model overpredicted growth by ~39%, the updated GEM affords relative comparisons between different culture conditions through the associated exchange flux constraints, which can be determined experimentally.

Even for an accurate GEM, we expect overpredictions of growth, since the model does not account for regulatory mechanisms that prevent the cell from achieving the optimal growth rate. For example, the model does not capture growth impacts caused by lactate accumulation, which can alter pH, create osmotic stress, and inhibit metabolic reactions contributing to biomass formation. In the previous chapter, we demonstrated that over-supplementation of phenylalanine inhibited cell growth due to the buildup of growth-inhibitory metabolites. As a proof of concept for using GEMs in cultivated meat media optimization, we performed a sensitivity analysis by varying extracellular phenylalanine flux and predicting corresponding growth rates while keeping other fluxes constant (Figure 3-2B). The refined GEM predicted that extracellular phenylalanine flux could be reduced while maintaining biomass formation. In our earlier findings, lowering phenylalanine concentrations in DF-1 cultures improved growth by reducing inhibitory metabolites. Although the model did not capture this growth improvement, its ability to

predict growth at reduced phenylalanine levels—despite phenylalanine being essential—could be useful for systematic design of media that contain “just enough” levels of amino acids and other essential primary metabolites. Since amino acids represent one of the largest cost barriers in cultivated meat media, GEM-based tools could be valuable for approximating minimum amino acid requirements for cell cultivation, as well as quantifying culture sensitivity to individual amino acids, as shown in the presented example.

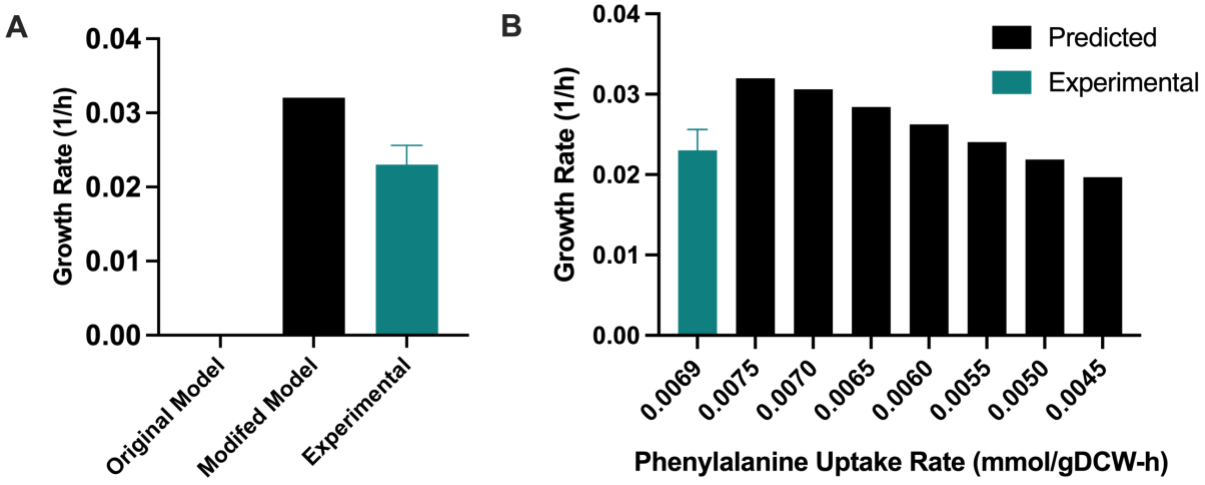

Figure 3-2 Comparison of original and refined GEM to experimental data

The Improved model was applied to simulate cell growth in DMEM media using experimentally determined amino acid data to constrain the solution space in comparison to simulated growth by the original model and experimentally determined growth rate (A). Sensitivity analysis of simulated phenylalanine flux predicts that chicken cell growth is possible when phenylalanine uptake is restricted (B).

3.4.2 Improved Reaction Naming and Model Consistency for broader applicability

Functional genes responsible for growth are highly conserved across species such as humans, pigs, and chickens [20]. Consequently, metabolic models based on gene–protein–reaction associations are also expected to share a large proportion of reactions across species. Refining reaction names within a GEM not only improves model functionality and network connectivity but also facilitates comparisons with models from other species and supports standardization. In the original chicken GEM, 36% of reactions appeared to be unique to chickens (Figure 3-3A). Following a stepwise analysis of these reactions and comparison with well-characterized models (e.g., human and CHO), the number of unique reactions was reduced to 22% (Figure 3-3B). Shared reactions were resolved by referencing EC numbers or calculating Kendall similarity scores, while reaction names were aligned with conventional naming practices used in the BiGG database. Standardized GEMs will be increasingly valuable in the cultivated meat field, where the high cost of experimentation is a major limitation for process optimization required to achieve production at scale. To ensure these models remain broadly useful, it is essential to incorporate standardization practices consistent with FAIR principles for digital tools [21].

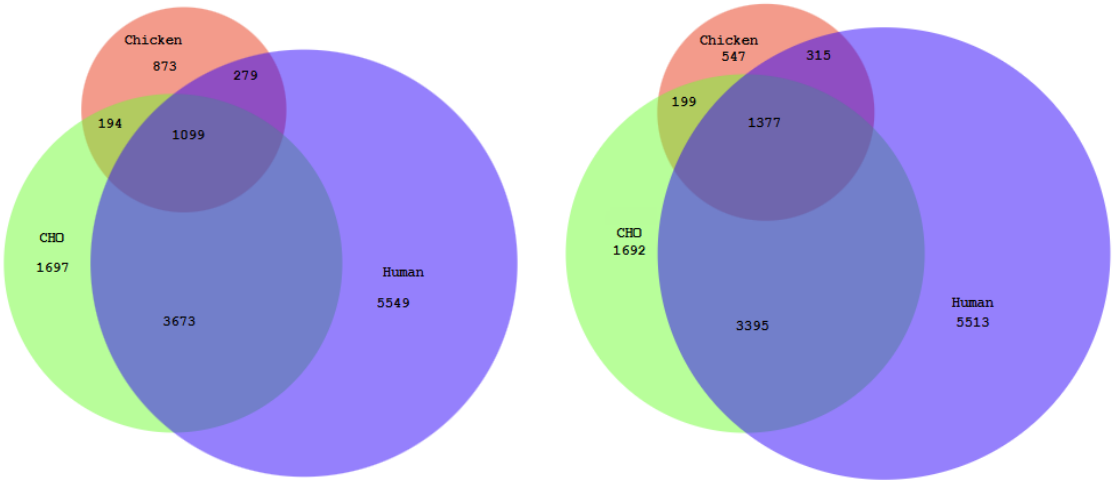

Figure 3-3 Resolving reaction naming

In the original published GEM, nearly half of the reactions were classified as unique compared to other species (A). After curation, in which individual reactions from the chicken GEM were cross-referenced with reactions in the BiGG database, the number of unique reactions was reduced by 37% (B). This reduction is likely due to inconsistencies in naming between the original published model and the standardized conventions used in GEM databases.

Following modification steps, the resulting refined model added 11 reactions and 3 metabolites (Table 3-1). In addition, dead-end metabolites, defined as metabolites participating in a single reaction (excluding extracellular metabolites) were reduced by 5 while blocked reactions defined as reactions unable to carry flux were reduced by 22

(Table 3-1). Taken together, the modifications to the model improved accuracy and usability.

Table 3-1 Comparison of metabolic network connectivity and functionality between the original and refined model.

Model	Original	Refined
Reactions	2427	2438
Metabolites	2569	2572
Dead-end Metabolites	1173	1168
Blocked Reactions	1662	1640
Subnetworks	60	60

3.5 Future Directions

In the context of cultivated meat, GEMs are most valuable for their ability to optimize media composition and computationally predict cellular responses to changes in nutrient availability. Future directions for this study should focus on rigorous experimental validation of the model, with particular emphasis on amino acid utilization and sensitivity. Ensuring accurate representation of amino acid production and consumption will be essential for all cultivated meat–related GEMs, as these models may ultimately guide the development of minimal amino acid media that supports robust cell growth while reducing input costs. Following thorough experimental qualification, cell type–specific transcriptional data could be integrated to represent the diverse cell types required to produce a complete cultivated meat product

3.6 Conclusion

This work presents a refined chicken GEM that incorporates critical structural and functional improvements. We adjusted the biomass equation to reflect chicken-specific macromolecular composition, restored balanced glycogen metabolism, and added crucial missing reactions in amino acid metabolic pathways. These modifications resolved biologic inconsistencies in the original model, improved network connectivity, and enabled the GEM to predict growth within the correct order of magnitude of experimental observations. In addition, standardizing reaction naming and harmonizing with existing GEMs reduced the number of reactions erroneously identified as unique to chicken, strengthening the model's comparability and interoperability. Collectively, these improvements establish a more biologically accurate and broadly applicable metabolic framework for use with cultivated meat chicken cell systems.

3.7 Acknowledgements

I would like to thank Karin Yanagi for her immense contribution towards the GEM curation process.

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Chapter 4. Metabolomics Links Vitamin B6 Metabolism to CHO Cell Culture Productivity

4.1 Abstract

Chinese hamster ovary (CHO) cells are the primary host for monoclonal antibody (mAb) production, limitations in productivity across cell lines remain a challenge for biomanufacturing. To investigate metabolic factors underlying these differences, we applied untargeted and targeted LC-MS metabolomics to high- and low- mAb producing CHO cell lines cultured in identical fed-batch processes. Untargeted analysis identified accumulation of vitamin B6 derivatives, including pyridoxamine and pyridoxic acid, in the low producer. Targeted quantification confirmed variation in vitamin B6 metabolism, suggesting possible differences in pyridoxal phosphate (PLP) dependent enzyme activity. Amino acid correlation analysis indicated that the high producer clustered flux supporting protein synthesis, while the low producer diverted amino acids toward the TCA cycle, potentially generating inhibitory byproducts. These results identify vitamin B6 metabolism as a possible determinant of productivity and provide targets for media optimization and metabolic engineering.

4.2 Introduction

Chinese hamster ovary (CHO) cells are the predominant host for biopharmaceutical manufacturing, particularly for the production of monoclonal antibodies (mAbs). As therapeutic antibody structures become increasingly complex and demand continues to grow, optimizing CHO cell performance is critical for achieving high titers at industrial scale. Antibody production imposes substantial metabolic stress due to the demands of protein folding, post-translational modifications, and secretion, often leading to imbalances in nutrient utilization and accumulation of inhibitory byproducts [1]. From an industrial perspective, it is desirable to develop platform production processes—such as standardized media multiple cell lines derived from the same host—that enhance productivity across cell lines and integrate into existing manufacturing workflows.

Metabolomics is a powerful tool to characterize metabolic changes in relationship to protein production, with previous studies revealing culture limitations due byproduct pathway flux as well as productivity correlated media additives [2], [3]. However, much of this work has focused broadly on central carbon metabolism and amino acid utilization, leaving gaps in our understanding of cofactor and vitamin-dependent pathways that also influence culture performance. In particular, the role of vitamin B6 metabolism, which underpins transamination reactions and amino acid interconversions, remains underexplored in the context of recombinant protein production.

In this study, we applied untargeted and targeted LC-MS assays to compare extracellular metabolite profiles between industrially relevant, high- and low-producing

CHO cell lines in fed-batch culture. Untargeted metabolomic revealed significant differences in vitamin B6 utilization patterns. By focusing on amino acid and vitamin B6–related metabolism, we identify metabolic signatures potentially linked to productivity differences and highlight nutrient pathways that may be leveraged for media optimization or metabolic engineering. These insights expand current knowledge of CHO cell metabolism and suggest new strategies to improve culture performance and protein yields.

4.3 Methods

4.3.1 Sample Preparation

Two CHO cell lines derived from the same host producing two different monoclonal antibodies in fed batch culture utilized for this experiment. Extracellular supernatant samples obtained from Amgen (Cambridge, MA, USA). Metabolite extraction performed in preparation for LC-MS experiments by first diluting samples in chilled HPLC grade methanol (3:1 v/v). Methanol containing samples were subsequently centrifuged at 15,000 xg at 4 ^{o}C for 15 minutes. Sample supernatants were then collected for drying using a SpeedVac evaporator without heat and stored at -80 ^{o}C prior to reconstitution in HPLC grade water for analysis.

4.3.2 Untargeted LCMS

Extracellular extracted samples were resuspended in HPLC grade water at two different dilutions 1:4 and 1:8 (v/v). Both dilutions were analyzed in the same run using the four untargeted LC-MS methods described in chapter 2 using a quadrupole time-of flight

(TOF) mass spectrometer (TripleTOF 5600+, AB Sciex, Framingham, MA, USA) with an electrospray ionization source. Feature annotation was performed using the BioCAn computational tool as described in chapter 2 [4].

4.3.3 Untargeted Metabolomics Data Analysis

The peak area, corresponding to the integrated area under the curve (AUC) of each ion chromatograph, was normalized to the total ion chromatogram (TIC) for each sample and dilution (two dilutions per sample) across all four methods. Each sample was blank-subtracted using HPLC-grade water to reduce background noise. The resulting values were then normalized to the normalized viable cell density (VCD), with both dilutions from the same sampling day normalized using the same VCD value. Features were screened against spectral databases (HMDB, NIST, MONA), and any feature lacking a match in at least one database was removed due to low annotation reliability. For the remaining features, fold changes were calculated both between the two CHO cell lines on the same day and within each cell line from early exponential phase (day 3) to peak VCD (day 8). Features with an AUC fold change greater than 2 were considered upregulated. Upregulated features were further cross-checked across dilutions to ensure that the direction of change was consistent for both dilutions; features showing conflicting trends between dilutions were removed to maintain stringency. Metabolites of interest were defined as those showing upregulation in the lower-producing cell line and accumulation over time in both cell lines, which may indicate potential metabolic inefficiencies of the low producer.

4.3.4 Targeted LCMS

Targeted metabolomics was performed to confirm and quantify metabolites of interest as determined through untargeted metabolomic analysis and to determine amino acid concentration profiles over the culture duration. The same quadrupole time-of-flight mass spectrometer was used for targeted quantification as the untargeted metabolomic assays. High purity chemical standards were used to optimize the following parameters: ionization mode, precursor ion, product ion, collision energy and delustering potential (Table 4-S1). Samples were diluted 1:16 with HPLC-grade water then analyzed using the optimized parameters. Quantification of Vitamin B6 metabolites used the same reverse phase method utilized for untargeted analysis in positive ionization mode. Amino acids were quantified using a HILIC method described previously in chapter 2 in positive ionization mode. Targeted quantification was based on three individual injections, which were randomized throughout the run. CHO producer concentrations were compared using unpaired T-test.

4.3.5 Correlation Analysis

Pairwise relationships between amino acid concentrations were assessed using Spearman's rank correlation coefficient to examine trends across culture timepoints for each CHO cell line. Absolute amino acid concentrations were first normalized to viable cell density (VCD) relative to day 0. Spearman correlation coefficients were calculated for all pairs of amino acids using R (version 2023.06.1). Results were visualized as a heatmap using the pheatmap R package, with clustering applied to highlight groups of amino acids showing similar correlation patterns.

4.4 Results

CHO cells are the leading workhorse of monoclonal antibody (mAb) production. As the portfolio of pharmaceutical biologics expands, there is an increasing demand for production platforms capable of generating complex mAbs such as bi-specific antibodies at industrially relevant titers. Antibody production imposes substantial metabolic stress on cellular functions due to the requirements of protein processing, quality control, secretion, and post-translational modifications. Metabolomics provides a powerful approach to uncover stress-driven differences in CHO cell metabolism that may underlie reduced productivity and growth, while also informing potential mitigation strategies.

4.4.1 Untargeted metabolomics reveals differences in vitamin B6 metabolism between CHO cell line

Two CHO cell lines derived from the same parental host but producing two different monoclonal antibodies were cultured using an identical fed-batch platform processes (Figure 1). To identify differences in metabolite profiles associated with growth and productivity, extracellular media samples were collected on days 3, 6, 8, 10, and 15 days of culture, corresponding to early exponential, mid-log, peak viable cell density (VCD), and decline phases. Samples were analyzed at two dilutions (4-fold and 8-fold) to minimize signal oversaturation effects common when analyzing rich, industrial media with LC-MS. Untargeted metabolomics identified 22,557 features across all assays, though these features do not represent unique metabolites as multiple combinations of

LC-MS and ionization methods were applied, and some compounds were likely detected more than one method. The untargeted method utilized data-dependent acquisition (DDA), which fragments the most abundant precursor ions. While DDA provides high-quality MS2 spectra, it can bias toward abundant metabolites, occasionally fragmenting the same metabolite multiple times despite the LC-MS methods being set up to minimize this occurrence. Additionally, adducts and isotopes may contribute to duplicate detection of the same compound.

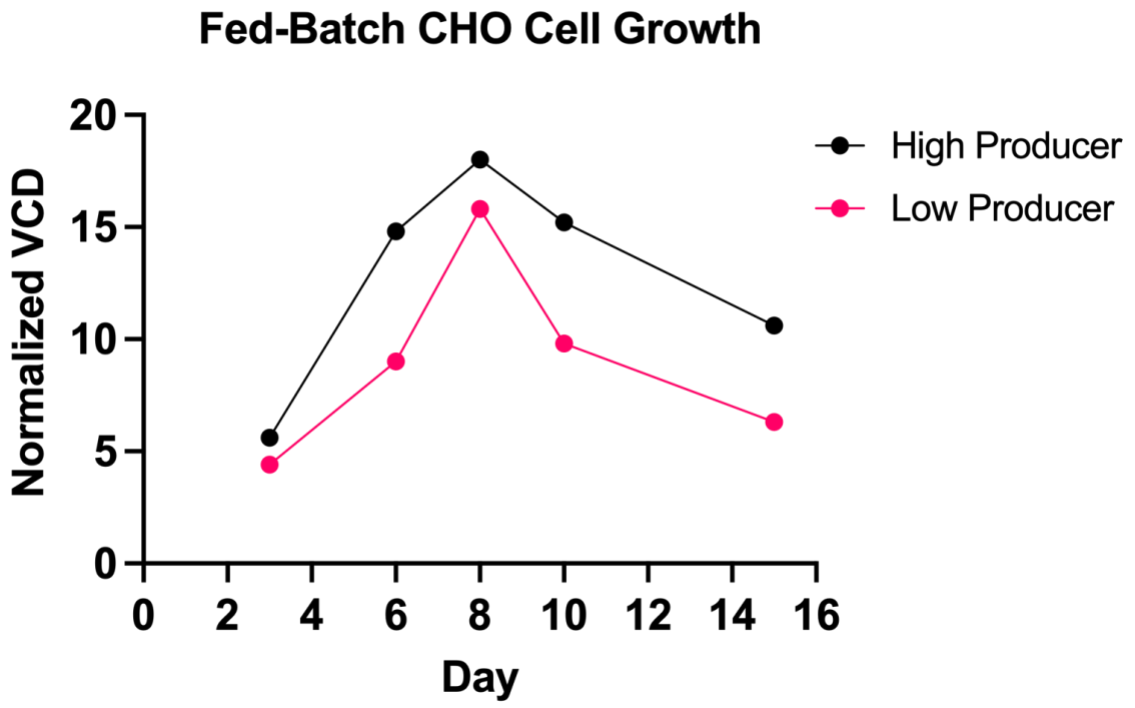

Figure 4-1 mAb producing CHO cell viable cell density grown in fed batch bioreactor

Viable cell density normalized to VCD at the start of culture (day 0; data not shown).

Following feature annotation using BioCAn, a successive filtering workflow (described in Methods) was used to identify metabolites upregulated in the low-producing cell line

(Table 4-2) and found to accumulate over the course of fed-batch culture (Table 4-1). At peak VCD (day 8), 29 features in the 4-fold dilution and 20 features in the 8-fold dilution accumulated by ≥2-fold in both cell lines relative to early exponential phase (day 3) with high annotation fidelity (Table 4-1). In the low-producing cell line, 34 features (4-fold dilution) and 39 features (8-fold) were identified as ≥2-fold upregulated at day 8, again with high annotation fidelity. Notably, vitamin B6 metabolites pyridoxamine and pyridoxic acid were consistently detected, highlighting vitamin B6 metabolism as a point of interest.

Table 4-1 Features identified through untargeted LC-MS analysis showing accumulation in both cell lines from day 3 to day 8 of *culture.*

	Dilution 1					Dilution 2				
	m/z	*Retention Time (s)*	*Method*	*Log_2FC High Producer*	*Log_2FC Low Producer*	*m/z*	*Retention Time (s)*	*Method*	*Log_2FC High Producer*	*Log_2FC Low Producer*
Accumulating features in both cell lines	117.02	1548.64	HilNeg	inf	inf	167.07	1996.33	SynPos	inf	inf
	137.07	571.54	HilPos	inf	inf	169.06	184.95	SynPos	inf	inf
	164.06	688.54	HilPos	inf	inf	183.06	1663.63	SynPos	inf	inf
	262.15	2446.40	SynNeg	inf	inf	224.09	1677.53	SynPos	inf	inf
	115.07	1560.89	SynPos	inf	inf	245.08	822.13	SynPos	inf	inf
	131.03	371.00	SynPos	inf	inf	150.01	662.49	HilPos	inf	2.85
	167.07	1996.33	SynPos	inf	inf	189.02	283.72	SynPos	inf	2.79
	169.06	184.95	SynPos	inf	inf	245.09	2041.05	SynNeg	inf	2.35
	175.09	243.24	SynPos	inf	inf	178.05	1524.12	SynPos	inf	2.16
	183.06	1663.63	SynPos	inf	inf	166.07	1428.54	SynPos	inf	1.80
	184.06	1286.46	SynPos	inf	inf	147.03	371.64	SynNeg	3.06	1.43
	224.09	1677.53	SynPos	inf	inf	190.07	558.65	SynPos	2.61	2.75
	245.08	822.13	SynPos	inf	inf	176.04	1531.59	SynNeg	2.44	1.42
	258.13	691.62	HilPos	inf	5.97	311.12	1084.95	HilPos	2.40	1.27
	126.07	503.28	SynPos	inf	4.80	182.05	1293.19	SynNeg	2.37	1.19
	291.13	1018.90	HilPos	inf	2.34	247.11	2038.75	SynPos	2.31	2.90
	164.06	1092.99	SynPos	8.91	3.96	409.18	1390.06	SynPos	2.30	2.53
	189.13	194.41	SynPos	4.12	1.27	259.13	936.48	HilNeg	1.72	1.49
	161.04	1547.35	HilNeg	3.34	inf	259.13	1678.98	SynNeg	1.41	2.01
	258.11	192.38	SynPos	2.39	1.20	189.13	194.41	SynPos	1.30	2.93
	409.18	1390.06	SynPos	2.36	2.76					
	133.05	783.66	HilNeg	2.23	inf					

243.06	847.25	SynNeg	2.18		3.63
189.02	283.72	SynPos	2.14		3.00
178.05	1524.12	SynPos	1.81	inf	
311.12	1084.95	HilPos	1.81	inf	
309.13	810.84	HilPos	1.76		1.53
247.11	2038.75	SynPos	1.74		1.62
259.13	936.48	HilNeg	1.37		1.34

Pyridoxamine (pink) and pyridoxic acid (grey)

HilNeg: HILIC Negative, HilPos: HILIC Positive, SynNeg: RP Negative, SynPos: RP Positive

Inf: Feature was detected below the limit of detection on day 3 of culture

Dilution 1: 4 - fold Dilution 2: 8 - fold

Table 4-2 Features identified through untargeted LC-MS analysis showing upregulation in the low producing cell line at peak VCD.

	Dilution 1				Dilution 2			
	m/z	*Retention Time (s)*	*Method*	*Log_2FC*	*m/z*	*Retention Time (s)*	*Method*	*Log_2FC*
Features upregulated in low producer	124.99	762.73	HilNeg	inf	131.05	181.58	SynNeg	inf
	243.10	240.09	HilPos	inf	145.05	1596.72	SynNeg	inf
	144.97	240.28	SynNeg	inf	147.03	615.33	SynNeg	inf
	127.05	1418.57	SynPos	inf	151.04	1791.91	SynNeg	inf
	175.13	2806.14	SynPos	inf	161.05	823.73	SynNeg	inf
	175.09	243.24	SynPos	-7.08	201.11	2330.94	SynNeg	inf
	158.06	554.36	HilNeg	-4.93	203.03	1668.28	SynNeg	inf
	146.05	972.34	HilNeg	-4.43	127.05	1418.57	SynPos	inf
	148.06	193.32	SynPos	-3.90	191.02	506.46	SynNeg	-7.35
	169.06	184.95	SynPos	-3.31	346.06	454.81	SynNeg	-5.86
	241.06	1407.27	SynNeg	-3.12	188.06	566.35	SynNeg	-4.70
	132.03	189.52	SynNeg	-2.35	164.07	1271.76	SynNeg	-4.70
	146.16	148.75	SynPos	-1.99	156.03	989.31	HilPos	-4.33
	175.12	1603.15	HilPos	-1.96	146.05	972.34	HilNeg	-4.31
	174.04	335.81	SynNeg	-1.94	132.03	189.52	SynNeg	-4.26
	131.05	181.58	SynNeg	-1.84	148.06	193.32	SynPos	-4.13
	156.03	989.31	HilPos	-1.62	175.12	1603.15	HilPos	-3.63
	188.06	566.35	SynNeg	-1.59	105.02	216.41	SynNeg	-3.63
	133.06	664.58	HilPos	-1.54	145.06	186.59	SynNeg	-3.05
	205.10	1630.36	SynPos	-1.44	218.10	1444.52	SynNeg	-2.63
	134.06	2012.48	SynPos	-1.36	203.08	1639.45	SynNeg	-2.56
	145.10	691.09	HilNeg	-1.33	245.09	2041.05	SynNeg	-2.53

150.06	461.84	SynPos	-1.31	167.07	1996.33	SynPos	-2.47
148.04	1843.91	SynPos	-1.30	182.05	1293.19	SynNeg	-2.45
307.14	1203.56	SynPos	-1.29	181.05	1670.39	SynNeg	-2.44
145.06	186.59	SynNeg	-1.28	123.05	642.19	SynPos	-2.27
147.08	645.84	HilPos	-1.19	165.06	1999.47	SynNeg	-2.23
184.06	1286.46	SynPos	-1.17	181.07	530.05	HilNeg	-2.15
190.07	558.65	SynPos	-1.08	243.06	323.65	SynNeg	-2.03
295.08	1529.00	SynNeg	-1.07	169.06	184.95	SynPos	-1.99
220.06	1403.74	SynPos	-1.07	176.04	1531.59	SynNeg	-1.84
162.05	2102.31	SynPos	-1.03	133.06	664.58	HilPos	-1.74
160.06	218.95	SynPos	-1.02	160.06	426.04	SynNeg	-1.42
117.06	1630.58	SynPos	-1.01	147.08	645.84	HilPos	-1.37
				89.03	303.68	SynNeg	-1.35
				123.05	204.93	HilPos	-1.31
				160.06	218.95	SynPos	-1.28
				147.08	189.91	SynPos	-1.27
				195.05	928.81	HilNeg	-1.07

Pyridoxamine (pink) and pyridoxic acid (grey)

HilNeg: HILIC Negative, HilPos: HILIC Positive, SynNeg: RP Negative, SynPos: RP Positive

Inf: Feature was detected below the limit of detection in the high producer

Dilution 1: 4 - fold Dilution 2: 8 - fold

4.4.2 Targeted analysis confirms significant differences in vitamin B6 metabolites and amino acid profiles

To validate these findings, a targeted LC-MS method was developed to quantify vitamin B6 metabolites identified in the untargeted analysis. Quantification confirmed significant differences in vitamin B6 utilization profiles between the high- and low-producing clones. Mammalian cells cannot synthesize vitamin B6 *de novo*; thus, culture media are supplemented with pyridoxine, which is a stable form of vitamin B6. Vitamin B6 derivatives in culture reflect cellular processing of this additive. Pyridoxamine levels were significantly higher in the low-producing clone during early to mid-log phases compared to the high producer (Figure 4-2A). Precursor metabolites pyridoxine and pyridoxal showed no significant differences between cell lines (Figures 4-2C and 4-2D). Pyridoxamine, pyridoxal, and pyridoxine can interconvert via pyridoxine-5-phosphate oxidase, and all forms are capable of passing through the cell membrane and becoming phosphorylated by intracellular enzymes. Phosphorylation retains vitamin B6 within cells, while dephosphorylation or conversion to other metabolites is required for export [5]. Pyridoxal phosphate (PLP) is the primary biologically active form of vitamin B6, supporting a wide range of cellular processes [6].

The elevated pyridoxamine in the low-producing clone may indicate reduced capacity to convert vitamin B6 to its active PLP form. Alternatively, increased pyridoxamine could reflect a mechanism to remove excess B6. Pyridoxic acid accumulated in both clones, but accumulation was significantly higher in the low producer during late culture (Figure 4-2B). Pyridoxic acid is formed via oxidation of pyridoxal by aldehyde oxidases and

represents a terminal metabolite. Since pyridoxal levels did not significantly differ on days 10 and 15, the higher pyridoxic acid accumulation suggests the low producer was exporting unused vitamin B6 metabolites, whereas the high producer preferentially retained active forms for metabolism.

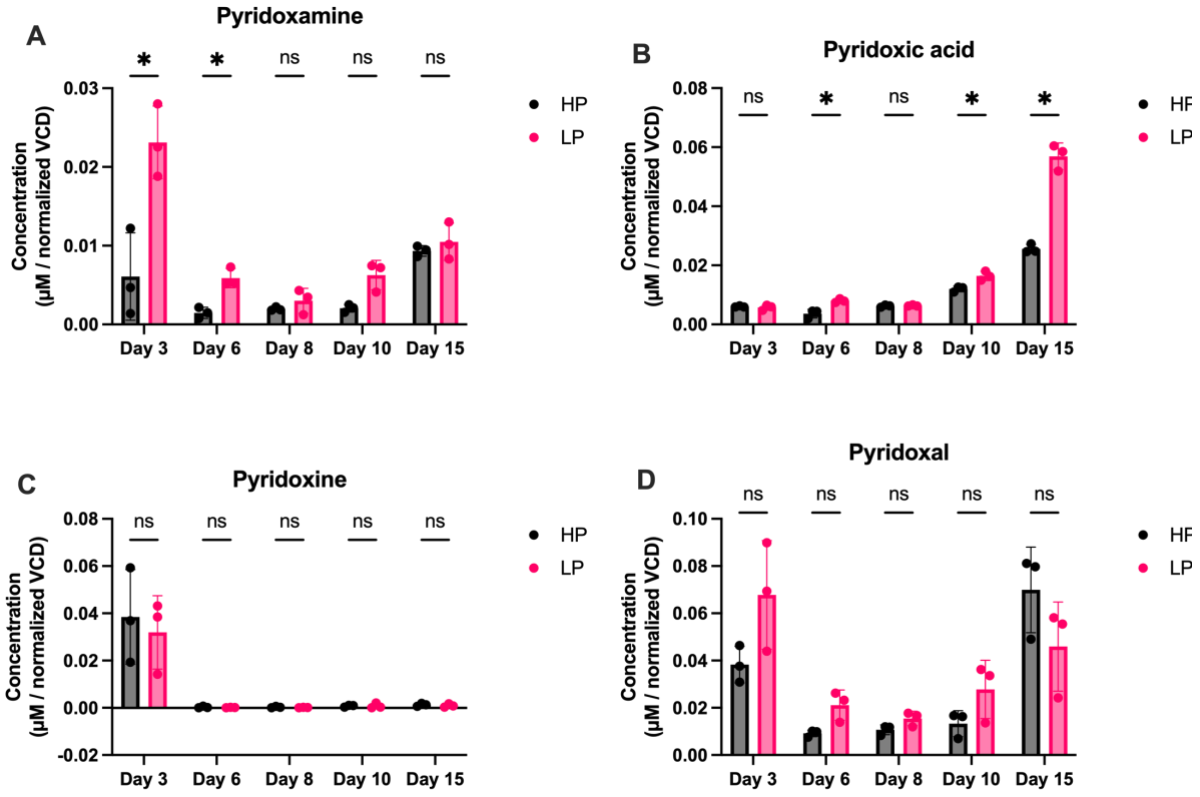

Figure 4-2 LC-MS Based quantification of vitamin B6 and metabolites in extracellular CHO cell culture samples.

Supernatant samples representative of early exponential growth phase through stationary/cell decline phase normalized to viable cell density data. Pyridoxamine (A) and pyridoxic acid (B) identified as accumulating metabolites in culture and upregulated in the CHO cell line with lower productivity. Pyridoxine (C) and pyridoxal (D) vitamin B6 parent metabolites. Pyridoxine is included in the basal and feed media formulations.

Data represents technical triplicate; *p-value < 0.05

4.4.3 Relationship between vitamin B6 and amino acid utilization

Vitamin B6, in its PLP form, functions as a cofactor in many metabolic reactions catalyzed by enzymes. These enzymes include aminotransferases, which catalyze reversible reactions between amino acids and α-keto acids [7]. Aminotransferases are central for balancing cellular metabolism through the interconversion of amino acids and central carbon metabolism intermediates according to cellular needs. To probe this relationship, amino acid concentrations were measured by targeted LC-MS. Spearman correlation analysis revealed distinct amino acid utilization patterns between high- and low-producing clones (Figure 4-3). In the high producer, strong correlations were observed among branched-chain amino acids (BCAAs) and essential amino acids (Figure 4-3A). This clustering could suggest a more specialized metabolism supported by a balanced vitamin B6 profile, with amino acid flux directed toward protein synthesis.

In contrast, the low producer showed correlations more consistent with transamination and TCA cycle utilization of amino acids (Figure 4-3B), suggesting preferential routing of amino acids toward energy generation. In the low producer, potentially channeling excess amino acids toward energy generation rather than protein synthesis may drive overflow metabolism and increase the accumulation of inhibitory byproducts. Prior studies in CHO fed-batch culture have shown that aminotransferase activity can generate such byproducts, including catabolites of leucine, isoleucine, valine, phenylalanine, and tyrosine. [8]. One mitigation strategy reported was BCAT1/2 knockout to reduce BCAA toxic byproduct formation and increase viable cell density [9], [10]. However, the same group also demonstrated reduced productivity due to lower availability of short chain fatty acids as a result of amino transferase knockout, which

could explain the lower productivity noticed by less utilization of active vitamin B6 in our system [16]. Taken together with previously reported studies, our results suggest that the low producer may be hindered by either limited PLP mediated aminotransferase activity reducing short chain fatty acids important for protein production or potentially suffering from excess metabolism resulting an accumulation of inhibitory metabolites downstream from PLP mediated enzymatic activity.

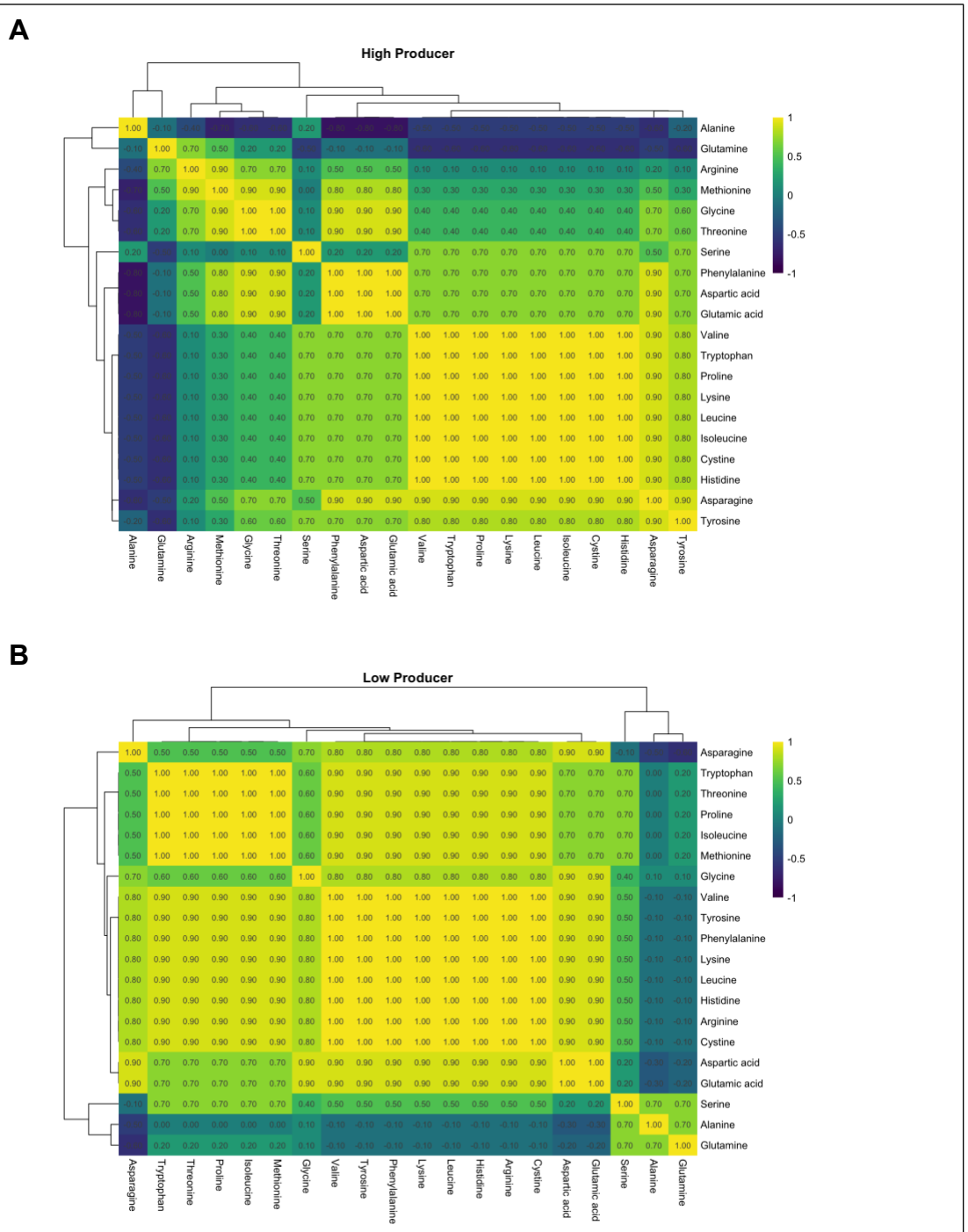

Figure 4-3 Correlation analysis of extracellular amino acid patterns from day 3 to day 15 of cell culture.

Figure 4-3 (cont.) High producing CHO cell line shows strong correlation of essential and branch chain amino acids (A). Low producing CHO cell line shows clustering of metabolites based on TCA cycle utilization. Extracellular amino acid concentrations quantified through targeted LC-MS and normalized to viable cell density. Amino acid correlations calculated using spearman correlation coefficient.

4.4.4 Vitamin B6 linked to methionine metabolism and redox balance

Vitamin B6 also serves as an essential cofactor in enzymes linked to methionine metabolism. One example is the trans-sulfuration pathway, which converts methionine and serine into cysteine and ultimately feeds into glutathione synthesis (Figure 4-4). PLP is required for the activity of cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CTH), which catalyze the conversion of homocysteine to cystathionine and cystathionine to cysteine, respectively [6]. Cysteine is a critical precursor for glutathione, a major antioxidant that reduces intracellular oxidative stress. Recombinant protein production is known to elevate reactive oxygen species (ROS) due to increased energy demands and endoplasmic reticulum stress. Glutathione and related antioxidants reduce ROS, apoptosis, and lysosomal activity, thereby improving cell viability and titers [11], [12]. Amino acid profiles from the high-producing clone revealed significant decreases in glutamate, cysteine/cystine, and serine during late culture phases, when oxidative stress is expected to increase (Figure 4-5A, B, D). This pattern suggests increased flux toward glutathione production. However, a recent study on sulfur containing metabolites in DG44 CHO cells reported limited CBS and CTH activity in the trans-sulfuration pathway [13]. This previous study focused on growth potential in low-

cysteine media and did not directly evaluate productivity differences in cysteine-rich conditions. It is possible that the impact of redox balance and CBS/CTH enzymatic activity on culture productivity depend on the host cell type (e.g., CHO-DG44 vs. CHO-S cells) as well as the culture condition.

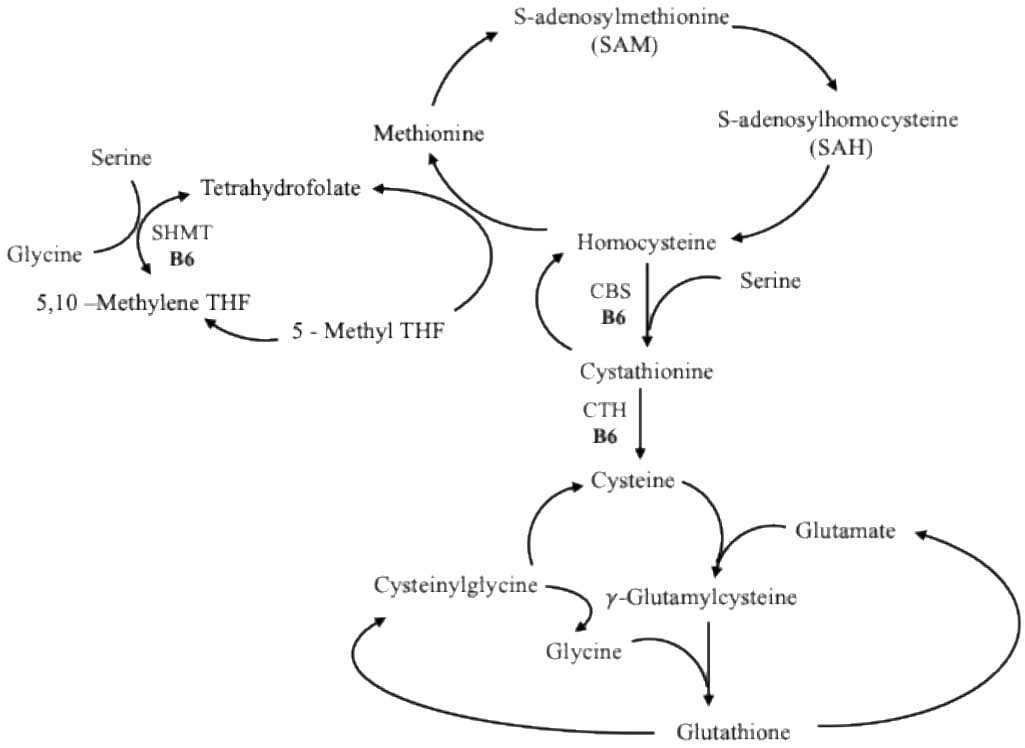

Figure 4-4 Link between methionine cycle, trans-sulfuration pathway and glutathione metabolism.

Pyridoxal-5-phosphate (B6) mediates conversion of homocysteine to cystathionine as well as the conversion from cystathionine to cysteine. Homocysteine represents branching point between methionine remethylation and the trans-sulfuration pathway. When trans-sulfuration pathway activity is low the folate cycle mediated in part by vitamin B6 is important for remethylation of homocysteine to methionine.

PLP also mediates serine hydroxymethyltransferase (SHMT) activity, which catalyzes the reversible conversion of tetrahydrofolate (THF) to 5,10-methylene-THF within the folate cycle [14]. This cycle supplies methyl groups for the remethylation of homocysteine to methionine (Figure 4-4), an essential reaction for preventing inhibitory metabolite accumulation as well as providing key methyl groups for epigenetic and gene regulation [6], [8], [15]. Extracellular amino acid analysis revealed significantly higher methionine and serine accumulation in the low-producing clone at late culture stages (Figure 4-5C, D). Since methionine is essential, its accumulation suggests reduced uptake or utilization. Similarly, higher serine levels in the low producer, combined with reduced PLP availability, may indicate impaired SHMT activity. Taken together, these findings suggest that the low-producing clone could exhibit reduced efficiency in both the methionine and folate cycles, potentially limiting S-adenosylmethionine (SAM) availability. SAM is required for methylation reactions essential to DNA, RNA, and protein synthesis, processes critical for cell productivity and regulation.

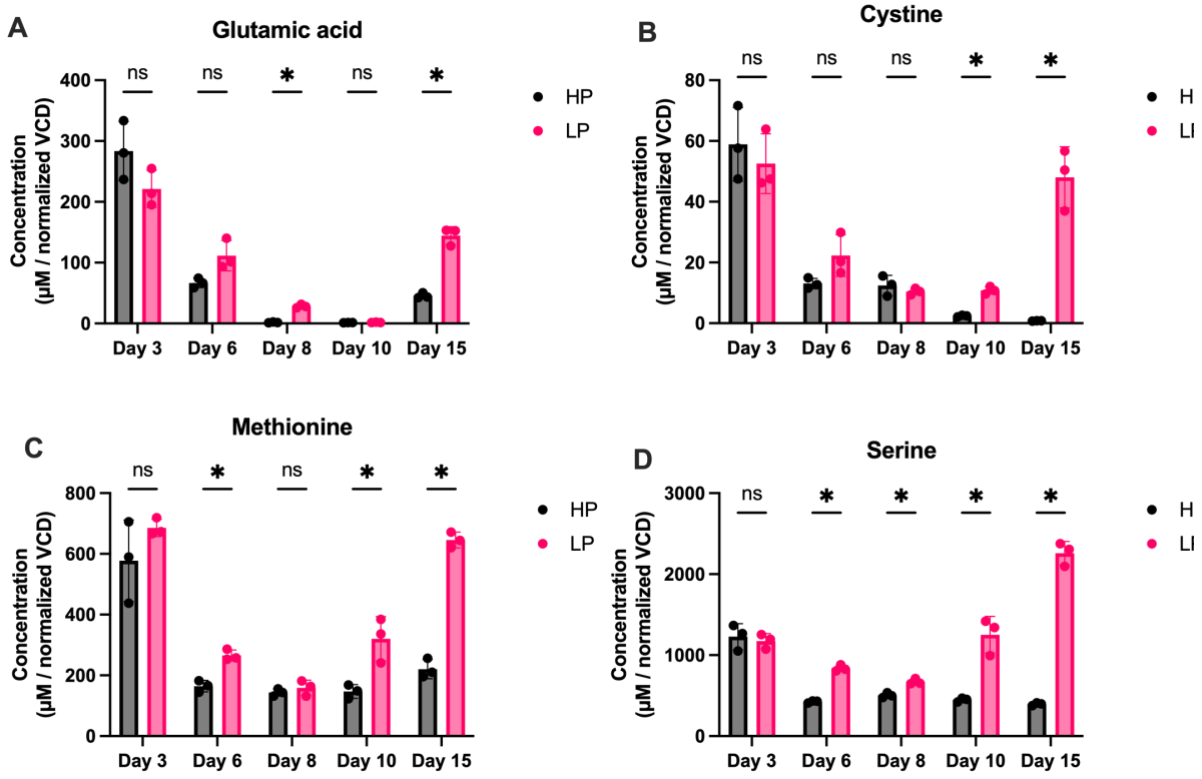

Figure 4-5 Select targeted LC-MS amino acid quantification

Quantification of glutamic acid (A), Cystine (B), Methionine (C), and Serine (D) as key amino acids in methionine and glutathione metabolism. Amino acid quantification from extracellular extracts from early exponential growth phase through stationary/decline phase normalized to viable cell density data. Cystine (B) assumed to be representative of extracellular cysteine and cystine due to spontaneous dimerization of cysteine. Amino acids shown are included in both the basal and feed media.

Data representative of technical triplicate; *p-value < 0.05

4.5 Discussion

4.5.1 Aminotransferase-related pathways and their implication in inhibitory metabolite accumulation

The observed differences in vitamin B6 utilization likely reflect broader metabolic dysregulation in the low-producing clone, rather than direct inhibition by vitamin B6 metabolites themselves. The accumulation of pyridoxic acid, combined with altered amino acid correlations, points to dysregulated PLP-mediated aminotransferase activity. Future studies should focus on profiling inhibitory BCAA- and aromatic amino acid–derived metabolites downstream of aminotransferase reactions, including 4-hydroxyphenyllactate, 3-phenyllactate, isovalerate, isobutyrate, and 2-methylbutyrate, which have been reported to inhibit CHO growth in fed-batch culture [8], [9], [15]. Complementary approaches could include proteomic analysis of aminotransferases such as BCAT1/BCAT2 and tyrosine aminotransferase (TAT), which catalyze upstream reactions producing inhibitory metabolites in excess metabolism as well as short chain fatty acids important for protein production. Previous studies have demonstrated that BCAT1 or BCAT2 knockouts can improve growth in producing CHO lines, while overexpression of phenylalanine hydroxylase (PAH) can redirect flux from phenylalanine toward tyrosine, reducing inhibitory byproduct accumulation [9], [10]. However, while such interventions can improve growth, trade-offs with productivity must be carefully considered. For example, BCAT1 knockout cells showed reduced specific productivity (~20%) but achieved higher overall titers, with productivity partially rescued by butyrate supplementation [16]. Thus, for a system interested in productivity

aminotransferase knockouts could be considered with additional supplementation strategies to rescue potential productivity losses.

4.5.2 Methionine cycle limitations and oxidative stress

Industrial CHO lines are particularly vulnerable to oxidative stress, which arises from the high metabolic demands of complex protein production. Our results suggest that the high-producing clone retains PLP in active forms and could direct amino acid flux toward glutathione synthesis, supporting improved ROS scavenging. This may provide a viability and productivity advantage compared to the low-producing clone. Additionally supporting this, cell free studies have shown vitamin B6 can directly quench ROS [17]. To further investigate, ROS levels could be quantified using assays such as DCFDA staining, or by directly measuring oxidative stress markers, including reduced/oxidized glutathione ratios, NADPH/NADP+ balance, or activities of antioxidant enzymes such as SOD and glutathione peroxidase [12]. Alternatively, proteomics could be performed to understand expression and protein quality of oxidative stress related enzymes specifically endoplasmic reticulum enzymes (e.g. ERO1) [18]. Proteomics could similarly be used to determine the activity level of CBS and CTH in the trans-sulfuration pathway in the CHO cell lines used for this analysis. Stable isotope tracing, e.g., with ^{13}C-labeled methionine, could map flux through the methionine and PLP-dependent folate cycles to understand differences in SAM generation and homocysteine flux. If methionine cycle limitations are confirmed, supplementation strategies could be explored. For instance, exogenous SAM supplementation has been shown to increase

specific productivity by up to 50% in CHO-DP12 producing anti–IL-8 [19], highlighting its potential for improving productivity in cell lines limited by methionine cycle activity.

4.5.3 Limitations in data availability

This project was conducted in collaboration with Amgen. Due to the proprietary nature of Amgen's bioprocesses, certain data could not be shared with the University. Access to this information would have enabled additional investigations into the impact of mAb type on productivity. For instance, knowledge of the amino acid composition of the mAb products could help clarify amino acid utilization needs, while titer data would provide insight into the onset of protein production and allow productivity related understanding metabolic changes over time. Additional process attributes, such as feed strategy and selection mechanisms, would further inform amino acid utilization and dependencies on specific media nutrients.

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4.7 Supplementary Information

Table 4-S 1 *Transition table for vitamin B6 and metabolites*

Compound Name	Mass	Precursor	Product	Retention Time	CE	DP	Polarity
Pyridoxamine	168.09	169.10	152.1	2.7	10	20	Positive
4-Pyridoxic acid	183.05	184.06	166	22.4	30	20	Positive
Pyridoxine	169.07	170.08	152.1	4.7	10	30	Positive
Pyridoxal	167.06	168.07	150.2	4.2	10	25	Positive

Chapter 5. Conclusion and Future Directions

In the first study, untargeted metabolomics was applied to identify, for the first time, phenylalanine-derived metabolites linked to growth inhibition in chicken cell culture. This work demonstrated that reformulating the cell culture medium based on metabolic insights could improve growth while also reducing input costs. While this approach focused on one essential amino acid, phenylalanine, it can inform a broader strategy for lowering input costs for *in vitro* cell cultivation and could be adapted to meet the metabolic demands of industrially relevant animal cells that need to be grown in low-cost, defined media. Currently, animal cells are typically maintained in serum-supplemented media, which poses challenges related to cost, ethics, safety, and batch-to-batch variability. In the case of the model chicken fibroblasts used in this study (DF-1 cells), adaptation to fully defined, serum-free media has not yet been achieved. However, defined media has been reported to support the growth of a spontaneously immortalized chicken fibroblast cell line in a research setting, and Good Meat has stated that its approved cultivated chicken product uses cells in serum-free culture processes [1], [2]. Ongoing efforts in our lab aim to adapt DF-1 cells to similar formulations. Given that animal cells cultured in serum-free media often exhibit reduced growth compared to serum-containing media, a metabolomics approach analogous to the approach described in this study could be used to identify metabolic inefficiencies of cells in serum-free culture in comparison to conventional, serum containing culture and inform low-cost supplementation strategies. Although use of chemically defined media is a standard requirement for regulatory qualification in the biopharmaceutical industry, the

cultivated meat industry does not have the same regulatory requirement. Moreover, the two industries have different considerations of input costs for cell culture due to the higher per unit market price of pharmaceutical products compared to alternative proteins such as cultivated meat. For cultivated meat production, undefined supplements such as plant-based hydrolysates and microbial lysates have been proposed as cost-effective serum alternatives. Future applications of metabolomics could provide molecular insights into why certain microbial lysates confer growth benefits, whereas other lysates cause toxicity. Another avenue of research for developing low-cost culture media that can effectively support *in vitro* cell growth is to better understand how to redirect flux away from harmful byproducts and into profitable biosynthesis pathways. For example, in CHO cells, a previous study that also identified phenyllactic acid as a growth-inhibitory metabolite formed from excess catabolism of phenylalanine engineered the cells to upregulate expression of phenylalanine hydroxylase (PAH), which catalyzes the hydroxylation of phenylalanine into tyrosine. This cell engineering effort redirected phenylalanine flux toward tyrosine biosynthesis and away from transamination and harmful catabolite production [3]. While genetic engineering may face regulatory and consumer resistance in cultivated meat, sequential adaptation to low-tyrosine media may provide a non-engineered route to redirect phenylalanine flux toward tyrosine and away from growth-inhibiting metabolites.

Related to DF-1 medium optimization, a genome-scale metabolic model (GEM) was developed in parallel through refinement of a previously published chicken GEM. Targeted metabolomics provided experimental constraints that helped identify and

resolve gaps in the metabolic network through the above-described refinement strategies. While the resulting GEM showed clear improvements in predictive capacity, further work is needed to test its performance in additional solution spaces, i.e., in a broader set of culture conditions. Once validated, the updated GEM could be used for computational medium design experiments. For example, a CHO cell GEM was previously used to identify optimal asparagine/aspartate ratios that improved culture performance [4]. Similarly, the refined chicken GEM could be used to optimize ratios of other problematic amino acids in culture media. The model could also be paired with flux variability analysis to identify amino acids most critical to growth, enabling selective reduction of less essential ones without substantially compromising performance. Since the main goal of cultivated meat media optimization is cost reduction, future studies could combine GEM-based growth predictions with machine learning or other modeling approaches to develop hybrid frameworks that explicitly integrate cost optimization with biological performance.

The third project contrasted the emerging application of cultivated meat with the established industrial use of CHO cells producing two distinct monoclonal antibodies. This study focused on the role of enzymatic cofactors in culture performance—factors often overshadowed by macronutrients in media optimization yet could play a critical role in culture productivity. Several potential directions for future work guided by our findings are discussed in Chapter 4. Additional research directions could include identifying a broader set of metabolites of interest from the metabolomics data by improving the annotation as many significantly accumulating LC-MS features remain

unexplored due to lack of knowledge regarding their chemical identity. Applying a lower threshold for upregulation or considering differences across all late-stage culture days could also reveal additional metabolites relevant for pathway analysis and culture optimization. Together, these studies demonstrate how integrating metabolomics, genome-scale modeling, and targeted media reformulation can uncover metabolic bottlenecks, guide nutrient balancing strategies, and reduce input costs. Whether applied to optimizing cultures for cultivated meat or improving productivity in CHO cell-based biomanufacturing, this combined systems-level approach highlights the power of mechanistic insight to advance both emerging and established cell culture applications.

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