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Media supplementation for targeted manipulation of monoclonal antibody galactosylation and fucosylation

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Abstract

Monoclonal antibodies are critically important biologics as the largest class of molecules used to treat cancers, rheumatoid arthritis, and other chronic diseases. Antibody glycosylation is a critical quality attribute that has ramifications for patient safety and physiological efficacy—one that can be modified by such factors as media formulation and process conditions during production. Using a design-of-experiments approach, we examined the effect of 2-F-peracetyl fucose (2FP), uridine, and galactose on cell growth and metabolism, titer, and gene expression of key glycosylation-related proteins, and report how the glycoform distribution changed from Days 4 to 7 in a batch process used for IgG1 production from Chinese hamster ovary cells. We observed major glycosylation changes upon supplement addition, where the addition of 2FP decreased antibody fucosylation by up to 48%, galactose addition increased galactosylation by up to 21%, and uridine addition decreased fucosylation and increased galactosylation by 6% and 2%, respectively. Despite having major effects on glycosylation, neither galactose nor 2FP significantly affected cell culture growth, metabolism, or titer. Uridine improved peak cell densities by 23% but also reduced titer by ~30%. The supplements caused significant changes in gene expression by Day 4 of the cultures where 2FP addition significantly reduced fucosyltransferase 8 and nucleotide sugar transporter gene expression (by ~2-fold), and uridine addition significantly increased expression of UDP-GlcNAcT (*SLC35A3*) and *B4GALT1–6* genes (by 1.5–3-fold). These gene expression data alongside glycosylation, metabolic, and growth data improve our understanding of the cellular mechanisms affected by media supplementation and suggest approaches for modifying antibody glycosylation in antibody production processes.

KEYWORDS
antibody, Chinese hamster ovary, design of experiments

1 | INTRODUCTION

Monoclonal antibodies (mAbs) have been very effective in treating numerous types of diseases and have become a driving force behind the growth of the biotherapeutic industry (Kunert & Reinhart, 2016). More than 86 mAbs and mAb derivatives have been approved by the US Food and Drug Administration (FDA) with ~90 more currently in clinical trials (Kaplon & Reichert, 2019). In 2019, for example, of the 20 FDA-approved biologics, nine of them are antibodies, antibody fragments, or antibody conjugates (Morrison, 2020). Commercially, the market for mAbs surpassed 98 billion USD in 2017 and is expected to expand even further to 128 billion USD by

2024 (Kaplon & Reichert, 2019; Morrison, 2020). The average cost to bring a biologic to market is ~$1.3–1.7 billion (Sertkaya, Wong, Jessup, & Beleche, 2016); as such, streamlining the development of new commercial products with improved cell line engineering, process design, and quality control could lower these development costs and improve overall accessibility. mAbs are typically produced by suspension-adapted Chinese hamster ovary (CHO) or murine myeloma cells in fed-batch processes, and titers of produced mAbs regularly reach between 3 and 8 g/L (Alt et al., 2016). Key product quality specifications for product release include protein glycosylation, charge variants, and other protein modifications (Alt et al., 2016).

All mAbs within the immunoglobulin G1 (IgG1) isotype share conserved regions in their protein structures and are N-glycosylated at the Asn297 residue located within the C_H2 domain of the Fc region (Zhang et al., 2016). N-glycosylation is a heterogeneous and nontemplate-directed posttranslational modification that begins with the addition and initial remodeling of an oligosaccharide at the Asn297 residue in the first compartment of the secretory pathway, the endoplasmic reticulum (Tejwani, Andersen, Nam, & Sharfstein, 2018). Further reactions occur in the second compartment—the Golgi apparatus—where enzymatic modifications to the initial oligosaccharide result in a base structure of two N-acetylglucosamine sugars and a biantennary trimannose core (Tejwani et al., 2018). Each antenna of the trimannose core may be further modified with additional N-acetylglucosamine, galactose, and sialic acid residues as the antibody traverses the Golgi before secretion (Raju, 2008; Tejwani et al., 2018). While other proteins can have far more diverse glycosylation patterns, mAbs have limited structural diversity because of steric interactions between the Fc region and glycosyltransferases (Zhang et al., 2016). Different terminal sugars affect antibody stability, serum half-life, and therapeutic functionality, and fucosylation of the core N-acetylglucosamine residue can significantly affect antibody-dependent cell-mediated cytotoxicity (ADCC; Raju & Lang, 2014; Zhang et al., 2016). Indeed, decreased core fucosylation can increase ADCC by 100-fold and increased terminal galactosylation can increase complement-dependent cytotoxicity (Raju, 2008; Zhang et al., 2016). The final secreted pool of antibodies varies by the abundance of specific glycoforms with certain glycan terminal groups being more prevalent than others.

Growth conditions, differences between mAb-producing CHO lines, and media formulation can all affect the final glycoform profiles of therapeutic antibodies. Changes in culture conditions such as temperature, pH, and pCO_2, can affect cellular growth rates and glycan profiles by altering enzyme levels in cell metabolism and glycosyltransferase activity (Nguyen Dang et al., 2019; Sou et al., 2015; Zheng et al., 2018). Variations observed in the glycoform profiles among mAb-producing CHO lines are likely caused by genetic and phenotypic changes that occur naturally during cell division (a phenomenon known as "genomic drift"). Indeed, these differences are further exacerbated through the process of generating cell lines that produce mAbs stably and by using drug selection to obtain high productivity clones (Dahodwala & Lee, 2019). CHO cells are routinely grown in chemically defined media that generally lack serum, dyes, or animal-derived components. Because of cost and to protect proprietary advantage, biopharmaceutical companies with both in-house and outsourced media formulations often do not disclose the final recipes of their commercial media products. However, certain supplements have been identified that affect culture growth rates, mAb titers, and final product quality, and these can be used with varying degrees of success to control desired characteristics in differently formulated media (Graham, Bhatia, & Yoon, 2019; Radhakrishnan, Wells, & Robinson, 2018). For instance, 2-F-peracetyl fucose (2FP) supplementation has been shown to lower fucosylation in mAbs by up to 75% by inhibiting fucosyltransferase 8 (FUT8; Ehret, Zimmermann, Eichhorn, & Zimmer, 2019; Mishra, Spearman, Donald, Perreault, & Butler, 2020); galactose addition has raised overall antibody galactosylation (St. Amand, Radhakrishnan, Robinson, & Ogunnaike, 2014); and the presence of uridine has raised titer, integrated viable cell density (below 10 mM concentrations), and galactosylation (Ehret et al., 2019; Grainger & James, 2013; Gramer et al., 2011). The use of design of experiments strategies with these supplements and other small molecules to explore the effects on cell growth and glycan profiles has been discussed in previous reports (Ehret et al., 2019; Grainger & James, 2013). Indeed, combinations of uridine (1–24 mM) and galactose (5–120 mM) have been demonstrated to induce concentration-dependent changes in antibody galactosylation (Ehret et al., 2019; Gramer et al., 2011). However, the focus of these previous studies often involves merely manipulating the galactosylation of mAbs with only a few reports employing supplementation strategies for the purpose of controlling antibody fucosylation (Ehret et al., 2019; Wang, Chung, Chough, & Betenbaugh, 2018). Furthermore, these studies do not investigate the effects of supplementation on cellular physiology to connect supplement inputs to bioprocess outputs. Some studies do examine the physiological effects of supplementation on glycan profiles throughout batch and fed-batch bioprocesses (Kotidis et al., 2019; St. Amand et al., 2014; Villiger et al., 2016), but additional comprehensive investigations like these are still necessary to understand fully the effects of media supplementation on bioprocess outcomes. Characterizing mAb-producing CHO cell cultures from the process level to the molecular level, such as we describe in this study, provides mechanistic insight into how small molecule supplementation can change cell growth and behavior, antibody yield, and antibody glycoform distributions. Metabolic information and mechanisms inferred from these bioprocesses, instead of just process performance and product characterization, should provide additional options for improving cost- and time-efficient cell line selection, process development, and media formulation.

In this study, we supplemented batch cultures of mAb-producing CHO cells with 2FP, galactose, and uridine to characterize the effects of each supplement on cell growth, productivity, and gene expression. Specifically, we employed a statistically designed supplementation strategy using a design of experiments approach for targeted manipulation of fucosylation and galactosylation of mAbs using 2FP, uridine, and galactose, and we examined how cell culture

growth, productivity, and glycosylation change throughout the duration of a 7-day batch culture. The concentrations for 2FP, uridine, and galactose were chosen by canvassing media supplementation literature and selecting concentrations high enough to cause specific effects on core fucosylation, terminal galactosylation, and viable cell density (VCD)/titer (Ehret et al., 2019; Grainger & James, 2013; St. Amand et al., 2014). We also analyzed gene expression data of glycosylation pathway proteins, including glycosyltransferases and nucleotide sugar donor transporters, to determine how the machinery of cellular and protein modification was affected by the selected supplements.

2 | MATERIALS AND METHODS

2.1 | Cell culture, experimental design, and metabolite profiling

A CHO cell line producing an IgG1 antibody with only Fc glycosylation (a gift of Genentech) was cultured in suspension in CD OptiCHO media (Thermo Fisher Scientific) at a 30 ml working volume in 125 ml vented polycarbonate flasks in a shaking incubator at 37°C with a 5% CO_2 overlay at 120 rpm with a 25 mm throw. Stocks of 2FP (34 mM; Millipore-Sigma), uridine (200 mM; Sigma-Aldrich), and galactose (1M; Sigma-Aldrich) were prepared (Table S1). Cells were seeded at 0.5×10^6 cells/ml for each experimental condition, and supplements were distributed to each flask based on a 2^3 full factorial design (Ogunnaike, 2009) with working concentrations of each supplement chosen based on their expected effects on fucosylation, cell density, and galactosylation as follows: 50 μM for 2FP, 200 μM for uridine, and 100 mM for galactose (Table 1). Cells were counted daily using a trypan blue exclusion assay on an automated cell counter (Nexcelom Biosciences). Media were sampled (~3 ml) on Days 2, 4, and 7 for metabolite quantification, titer measurement, and gene expression analysis. Glucose, lactate, galactose, and ammonia concentrations from Days 2, 4, and 7 were quantified immediately using a CEDEX Bio Analyzer instrument (Roche) by comparison with an internal standard calibration per the manufacturer's instructions. On Day 7, cultures were pelleted and frozen at −20°C. The effects and associated *p*-values of each supplement on cell densities and metabolite profiles were determined using standard factorial analysis in Minitab 18 (Minitab, Inc.).

2.2 | Antibody purification and quantification

On Days 4 and 7, media from each flask were sampled for antibody isolation by pelleting cells and debris. A protein A column (Bio-Rad) was pre-equilibrated with 1× phosphate-buffered saline (PBS; NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 10 mM; KH_2PO_4, 1.8 mM; pH 7.4) on a BioLogic DuoFlow medium pressure chromatography instrument (Bio-Rad). Clarified conditioned media were injected directly onto the column at a 1.5 ml/min flow rate, and the column was subsequently washed with PBS until the A280 returned to baseline. Then, glycine (100 mM; pH 2.75) was used to elute the antibodies bound to the protein A resin, and antibodies were prepared for glycan analysis by concentration to between 2 and 5 mg/ml and buffer exchange to 1× PBS in 10 kDa molecular weight cutoff spin concentrators (Amicon, Millipore-Sigma). The mAb titer (mg/L) for each sample was determined using the same procedure described for purification, by injecting conditioned media through a 50 μl injection loop, eluting the antibody, and quantifying the area of the eluted peak. The concentration of antibody in the conditioned media was interpolated from a standard curve created by injecting antibody standards at 0.04–2.5 mg/ml (Waters). The effects and associated *p*-values of each supplement on antibody titer and productivity were determined using standard factorial analysis in Minitab 18 (Minitab Inc.).

2.3 | Glycosylation analysis

Antibodies purified from the conditioned media were prepared for released glycan analysis using a GlycoWorks RapiFluor-MS kit (Waters) per manufacturer's instructions. Briefly, the antibodies were denatured in a kit-provided surfactant at 95°C to enable PNGase F to access and cleave the glycans from the peptide chain. The mixture was incubated for 5 min at room temperature with the RapiFluor-MS fluorophore, and the reaction was quenched using pure acetonitrile. The entire mixture was loaded onto a solid-phase extraction plate included in the kit, washed, and eluted into a UPLC sampling plate (kit-provided). The solution-phase samples were then injected into an ACQUITY H-Class UPLC (Waters) and separated using the "universal glycan profiling" method outlined in the RapiFluor-MS kit protocol. Chromatograms generated by this method were integrated using Empower 3 software (Waters), and peaks were identified using in-house protein single-glycan standards (courtesy of Dr. Lai-Xi Wang). After identifying the peaks within each glycan distribution, they were grouped by terminal galactosylation (G0, G1, and G2) and core fucosylation (aFuc and Fuc). Estimates of the effects and associated *p*-values of each supplement on galactosylation and fucosylation were determined using standard factorial analysis in Minitab 18 (Minitab, Inc.).

TABLE 1 High and low values of concentrations for media supplements (mM) in full factorial 2^3 design of experiments

Supplement	Low	High
2-F-peracetyl fucose (2FP)	0	0.05
Uridine	0	0.20
Galactose	0	100

2.4 | Gene expression analysis

Total RNA was isolated from individual cell samples using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol, followed by the application of the RNA Clean and Concentrator Kit (Zymo; Song, Wang, Li, & Yang, 2016). Reverse transcription was carried out using 2 μg of total RNA and the High-Capacity Complementary DNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Primers used in the real-time polymerase chain reaction (PCR) for gene-specific quantification of N-glycosylation-related genes and 29 glycosylation enzyme transcripts (Table 2) were adapted from St. Amand et al. (2014). The β-actin gene was used as an endogenous control for normalization of expression levels. Real-time PCR reactions were performed on a ViiA™ 7 System using PowerUp SYBR Green Master Mix (Applied Biosystems). The amplification reactions were performed as follows: 15 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Fold-changes in gene expression were quantified by the comparative C_t method, which is based on the comparison of expression of the target gene (normalized to the endogenous control β-actin) among the compared samples (Livak & Schmittgen, 2001).

3 | RESULTS

3.1 | Cell growth characterization and metabolite profiling

IgG1-producing suspension CHO cells were grown in batch at 37°C as described in Section 2. The cells followed typical growth behavior for each condition of the experimental design, and each culture reached a peak VCD between 2.9 and 4.3 million cells/ml by the end of the batch on Day 7 (Figures 1 and S1). Cells grown in the presence of either 50 μM 2FP or 100 mM galactose did not exhibit significantly different growth behavior relative to unsupplemented cultures (Figure 1a,b), but the 200 μM uridine supplementation resulted in

TABLE 2 Primers used for qPCR gene expression analysis

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
MGAT1	CTGGGTGTCATGGATGACCT	CTAATTCCAGCTAGGATC
MGAT2	GATGATTATAACTGGGACTGG	TGACTCAATTTGGGCACTCTG
MGAT3	GTGCCTCATCTCCTTCTTAC	GAAGCTGGATACAAGGTTAGG
MGAT4	AAGCTCTTCAACACTTCTGTG	CTTGTAGAAGGAGCCAATCTG
MGAT5	TTCATGCCACTGTTTACGGC	TTCCCGGAGAAGAAACTGCA
B4GALT1	GACCTGGAGCTTTTGGCAAA	GGGATAATGATGGCCACCTTG
B4GALT2	CCTTCTCTGCCTGCTGCACT	CTGGGCTTCGGATACTGAAGC
B4GALT3	AACTGCCATAATTGTGCCCC	TGCCATATGCAAGCTGCTG
B4GALT4	TAATCGAGCCAAGCTCCTGAA	CATTCTCAGGCACCAGGTCC
B4GALT5	TCTTCATGGTGCAGGCTCAA	CCTCTTGGCATAGGCGCTAC
B4GALT6	GTGTCTCCAATCGCTCCCTG	GTATGTGTTGGCGATGCCTG
GNTE	TGCCAGGAGGTCTTTGACAAGCTA	TAGTTGGCCTCTTCTCGAGCCAAA
ST3GALT3	CTTGGAAGTTGCACTTGCTCC	GCCCAGCCGATCATACTCTG
ST3GALT4	ATGAGGGAGATGTGGGCTCC	GACCAAGAGCGTGTCTGGGT
FUT8	TATGGCACCCAGCGAACACTC	TTCACCTGACCAGTGTCCAG
SLC35A1	ATGAAAGGCTTCTCAGCAGCTG	AGAGCTCCCATTGCAAAGGA
GLB1	CCTACATCTGTGCAGAGTGG	TTCATCTTGGGCAGAAGGAC
SLC35A2	ACACACTCAAGCTCGCGGT	TGTCACCTGGAAAGTGGCAG
SLC35A3	CAGGAGTTGCTTTTGTACAG	GCTGTGAGAACTGCCATGAG
SLC35C1	AGAAGGTGTTGCAGGCGA-TAGACA	AGTGGCAAGAAGGATCACA-CAGGT
GNE	GTCGCCCATGCTGGTTGTAT	CCTATCTGGCGTGTTCCCA
NEU1	GTGGCCTCTACCATGTTGGT	GGCTCCCGCTGTTTCTGAAT
NEU2	CCTGTCCTGCAGAAGGAGA	TTCCGCAAAGGCCAGCAG
GPI	CAACTTCTTGGCCCAGACAGAG	TACAAGGCAATCAAGGCTCCC
β-actin (*ACTB*)	GTACTCCGTGTGGATCGGCG	AAGCATTTGCGGTGGACGATGG

Abbreviation: qPCR, quantitative polymerase chain reaction.

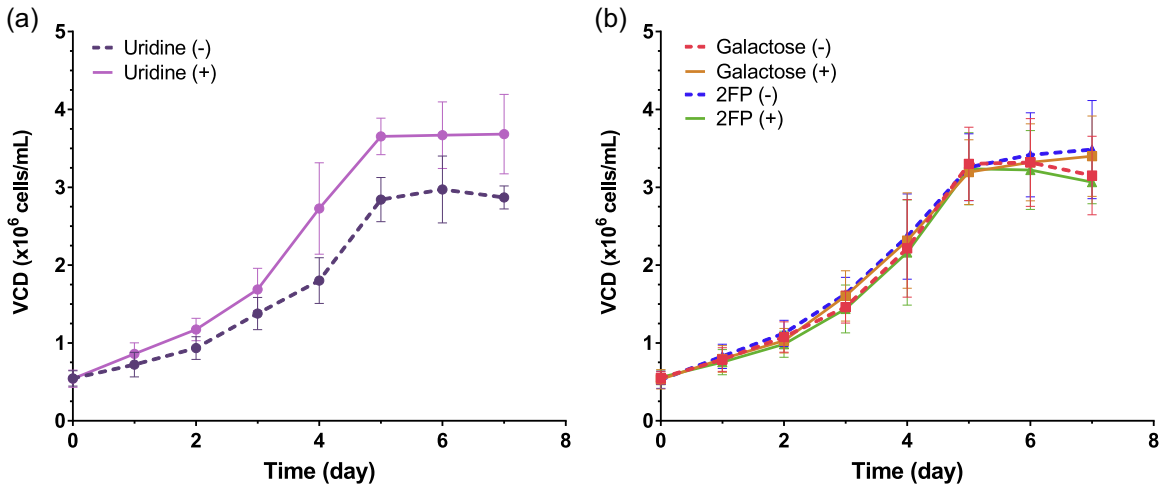

FIGURE 1 Main effects of media supplementation on viable cell density (VCD). (a) Uridine supplementation (pink solid) shows increase in VCD relative to the unsupplemented control (purple dashed); (b) galactose or 2FP addition (orange and green solid, respectively) has minimal effects on the VCD versus unsupplemented controls (red dashed and blue dashed). Symbols represent measurements, where error bars represent ±95% confidence interval ($n = 8$). Lines represent a guide to the eye. 2FP, 2-F-peracetyl fucose [Color figure can be viewed at wileyonlinelibrary.com]

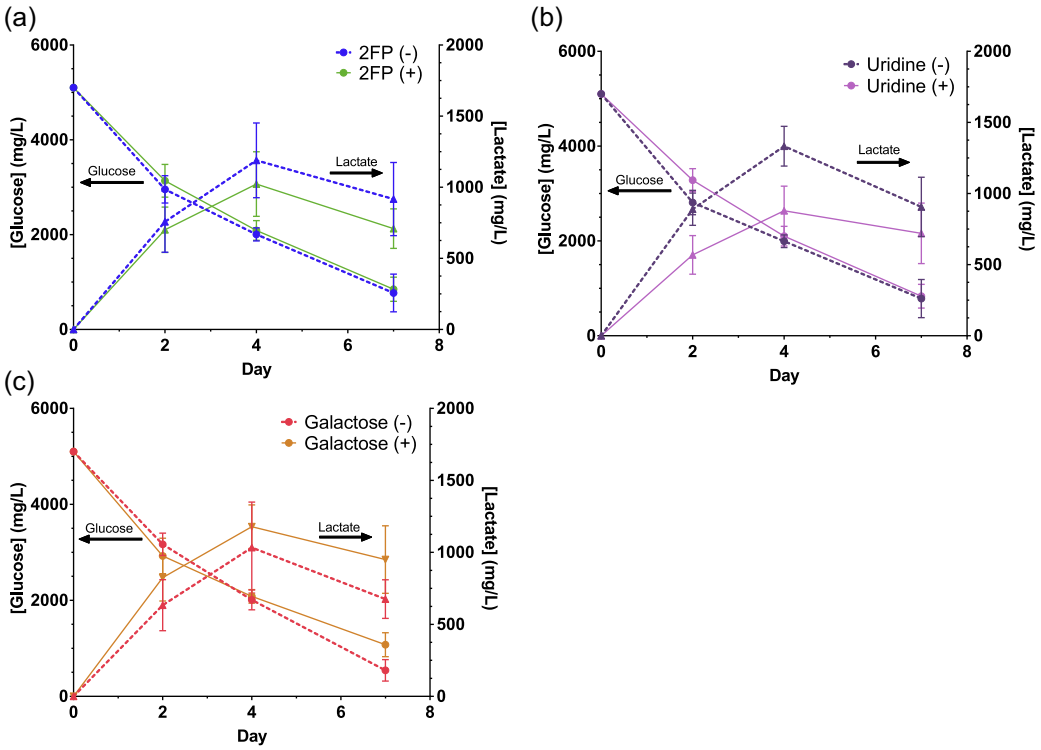

FIGURE 2 Main effects of supplements on glucose and lactate. (a) Minor differences in glucose and lactate concentrations are observed in 2FP-supplemented (green solid) versus unsupplemented control (blue dashed); (b) uridine supplementation (pink solid) has major changes on lactate levels versus unsupplemented (purple dashed), while glucose consumption is relatively unchanged; (c) minor differences in glucose and lactate concentrations are observed in galactose-supplemented (orange solid) versus unsupplemented control (red dashed). Symbols represent measurements, where error bars represent ±95% confidence interval ($n = 8$). Lines represent a guide to the eye. 2FP, 2-F-peracetyl fucose [Color figure can be viewed at wileyonlinelibrary.com]

significantly higher VCD starting on Day 4 and continuing until termination of the culture on Day 7 (Figure 1a; D4, $p = .017$; D7, $p = .003$). In addition, the uridine-supplemented cells reached 23% higher peak VCD relative to unsupplemented cultures.

Glucose and lactate concentrations were monitored throughout the batch for each experimental condition (Figure 2a–c). 2FP supplementation did not affect glucose or lactate profiles significantly relative to the nonsupplemented control. However, uridine-supplemented cultures showed a slightly reduced rate of glucose consumption relative to the nonsupplemented control from Day 0 to Day 2, and galactose-supplemented flasks showed a change in the glucose consumption rate from Day 4 to Day 7. Uridine-supplemented flasks had lactate concentrations that were significantly lower than those in the nonsupplemented control on Days 2 and 4 (D2, $p = .0007$; D4, $p = .0003$) and remained lower than those in the nonsupplemented control when the cultures were terminated on Day 7 ($p = .16$).

3.2 | Titer analysis and cell productivity

The titers of the cells grown in the presence of each supplement combination did not vary significantly from the nonsupplemented control on Days 4 or 7 (Figure 3a; D4, $p = .18$; D7, $p = .18$). However, specific productivity (pg/cell/day) on Day 7 of each of the uridine-supplemented cultures was significantly lower than the specific productivity of the nonsupplemented control (by about 50%; Figure 3b; $p < .05$). The main effects of the media supplementation on titer are as follows: In the presence of 50 μM 2FP or 100 mM galactose, the titer on Days 4 and 7 of the cultures did not vary significantly compared with unsupplemented controls (2FP: D4, $p = .65$; D7, $p = .24$; galactose: D4, $p = .44$; D7, $p = .59$). However, in the presence of 200 μM uridine, the titer decreased by 34% on Day 4 and by 30% on Day 7 compared with unsupplemented cultures (Uridine: D4, $p = .01$; D7, $p = .005$; Figure 3c,d).

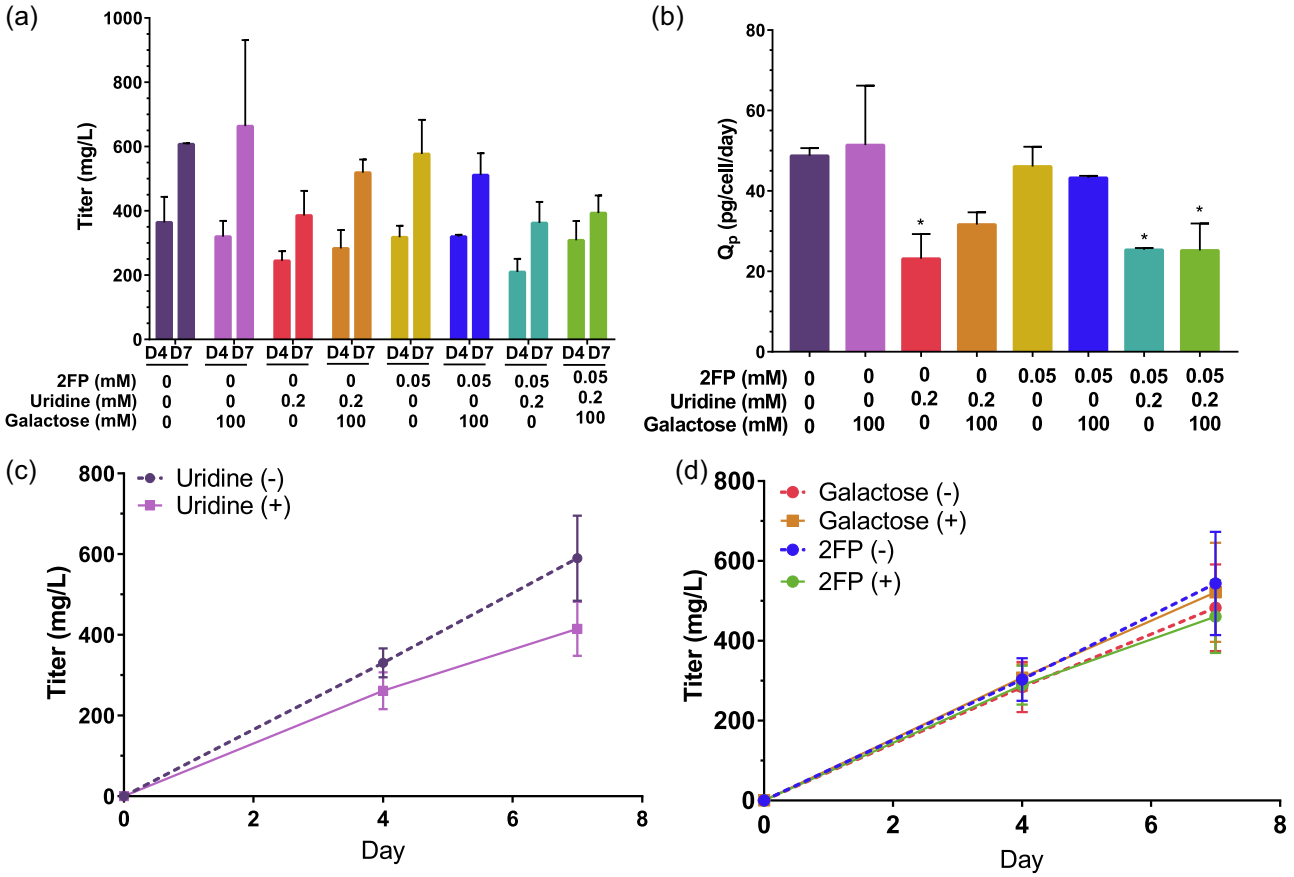

FIGURE 3 Titer and productivity analysis following media supplementation. (a) Titer of each combination of supplementation, measured by LC as described in Section 2, on Days 4 and 7. (b) Specific productivity (Qp, Titer/VCD/day) calculated for each supplementation condition described in Table 1. Significant differences (*$p < .05$) from control condition indicated above bar. (c) Main effects of uridine on titer between supplemented (pink solid) and unsupplemented (purple dashed); (d) main effects of galactose and 2FP on titer throughout the culture. Error bars for (a) and (b) represent the range of biological replicates ($n = 2$), and error bars in (c) and (d) indicate ±95% confidence interval ($n = 8$) in the presence of indicated supplement. 2FP, 2-F-peracetyl fucose; LC, liquid chromatography; VCD, viable cell density [Color figure can be viewed at wileyonlinelibrary.com]

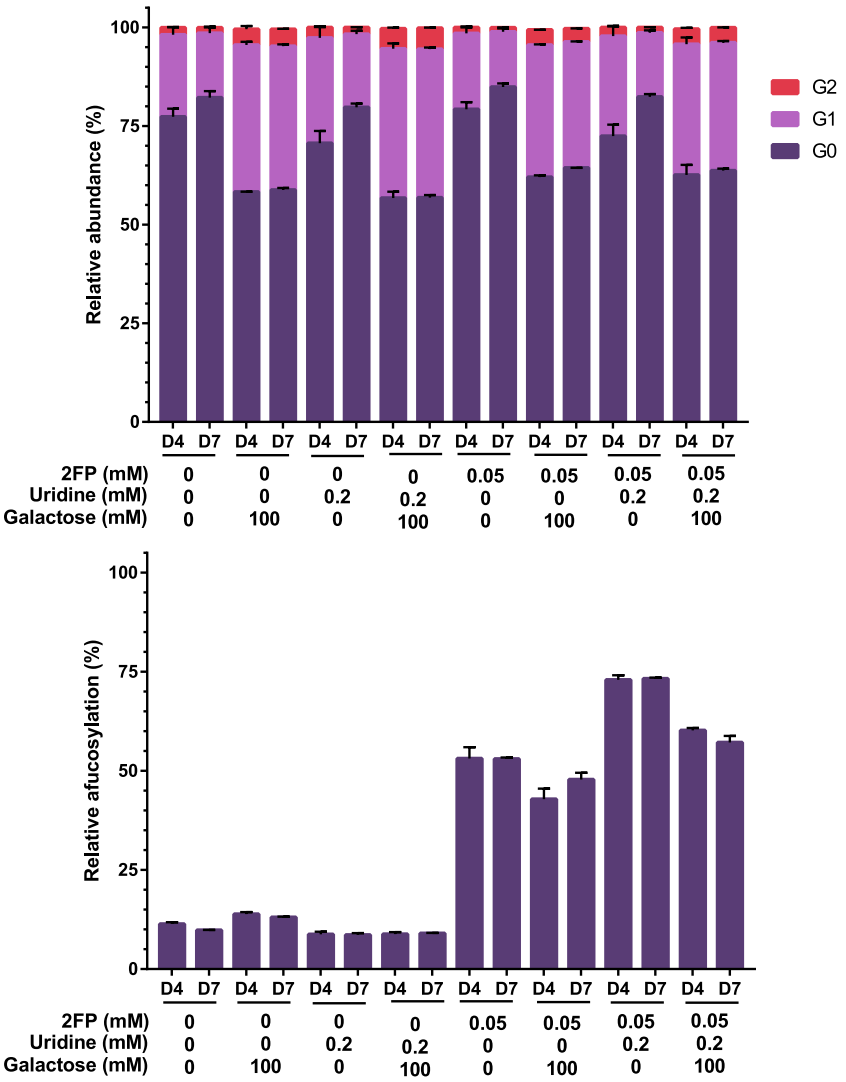

FIGURE 4 Galactosylation and fucosylation of antibodies obtained from all supplement combinations on Days 4 and 7 of the batch; (a) antibody galactosylation for each supplement combination on Days 4 and 7; (b) antibody fucosylation for each supplement combination on Days 4 and 7. Error bars indicate the range of biological replicates ($n = 2$). 2FP, 2-F-peracetyl fucose [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Glycan analysis: Galactosylation and fucosylation

The antibodies produced without supplementation were Fc-glycosylated with typical Day 7 glycoprofiles, as determined by cleaved glycan analysis and UPLC described in Section 2 (Figures 4 and S5). The glycan identities from these cultures included a G0F peak of about 74% abundance, 90% total fucosylation, 16% total galactosylation, and no detectable mannose-5 terminal sugars or sialic acids (Figure S3). Cultures supplemented with galactose produced antibodies with a 21% decrease in relative abundance of G0 glycans and concomitant increase of G1 or G2 glycans compared with control flasks on Day 7. In addition, between Day 4 and Day 7, the relative abundance of G0 glycans increased by an average of 5–10% in flasks that did not receive galactose supplements. In flasks supplemented with galactose, the relative abundance of G0 glycan remained mostly constant between Days 4 and 7. Both uridine and 2FP supplementation affected relative antibody galactosylation significantly, but their effects were far smaller than the effects of galactose supplementation; 2FP supplementation resulted in a 4% decrease in relative galactosylation and uridine a 2% increase. The interaction between 2FP and galactose also significantly affected D7 galactosylation ($p < .05$), but that effect was much smaller than the main effect of galactose alone.

Each of the three supplements in this study affected antibody fucosylation significantly ($p < .05$). Cultures that received 50 μM 2FP supplementation had the largest shift in relative fucosylation, with reductions of up to 48% for mAbs sampled on both Days 4 and 7 between supplemented and control flasks. While not as large of a shift as that observed in the 2FP supplemented samples, the relative antibody fucosylation on Days 4 and 7 decreased by about 7% when produced in the presence of 200 μM uridine, and supplementing cultures with 100 mM galactose increased fucosylation by about 5%. In addition, the relative percentage of fucosylation did not change between Days 4 and 7 across all supplementation conditions. Each possible two- and three-way interaction among 2FP, uridine, and galactose significantly affected D7 fucosylation ($p < .05$), but the

interaction effects were small when compared with the main effect of 2FP alone.

3.4 | Gene expression analysis

Each supplement changed the messenger RNA (mRNA) expression levels of key glycosyltransferases and other glycosylation-related genes. Cell samples were obtained, and mRNA extracted and analyzed on Days 4 and 7 as described in Section 2. Uridine supplementation at 200 μM increased the expression of *MGAT1*, *B4GALT1*, *B4GALT2*, *B4GALT3*, *B4GALT4*, *B4GALT5*, *B4GALT6*, and *SLC35A3* (uridine diphosphate [UDP]–N-acetyl-D-glucosamine [GlcNAc] transporter) significantly ($p < .05$) compared with the nonsupplemented control on Day 4 (Figures 5 and S4). On Day 4, cells grown in the presence of 100 mM galactose showed significant ($p < .05$) changes in *MGAT2*, *MGAT5*, and *B4GALT3-4* expression. 2FP supplementation at 50 μM caused significant ($p < .05$) downregulation of MGAT5, B4GALT1, *B4GALT4*, *B4GALT5*, *B4GALT6*, and *FUT8* expression and multiple nucleotide sugar donor transporter genes including *SLC35A3*, *SLC35A2* (UDP–Gal transporter), and *SLC35C1* (guanosine diphosphate [GDP]–Fuc transporter) on Day 4. The effects of 2FP, uridine, and galactose on Day 4 gene expression are summarized in Table 3. On Day 7, the supplements affected gene expression less significantly relative to the nonsupplemented control (Figure S4), possibly because of the depletion of supplements and other culture nutrients since cell metabolism and productivity slow down as the batch nears termination.

4 | DISCUSSION

4.1 | Uridine

In this study, we describe the effects of three different media supplements—uridine, galactose, and 2FP—on cell growth, mAb titers, and glycan distribution. Uridine-supplemented cultures had significantly higher VCD (23%) and significantly lower titer (30%) than unsupplemented cultures, likely because cell proliferation was favored with fewer cellular resources available for expressing

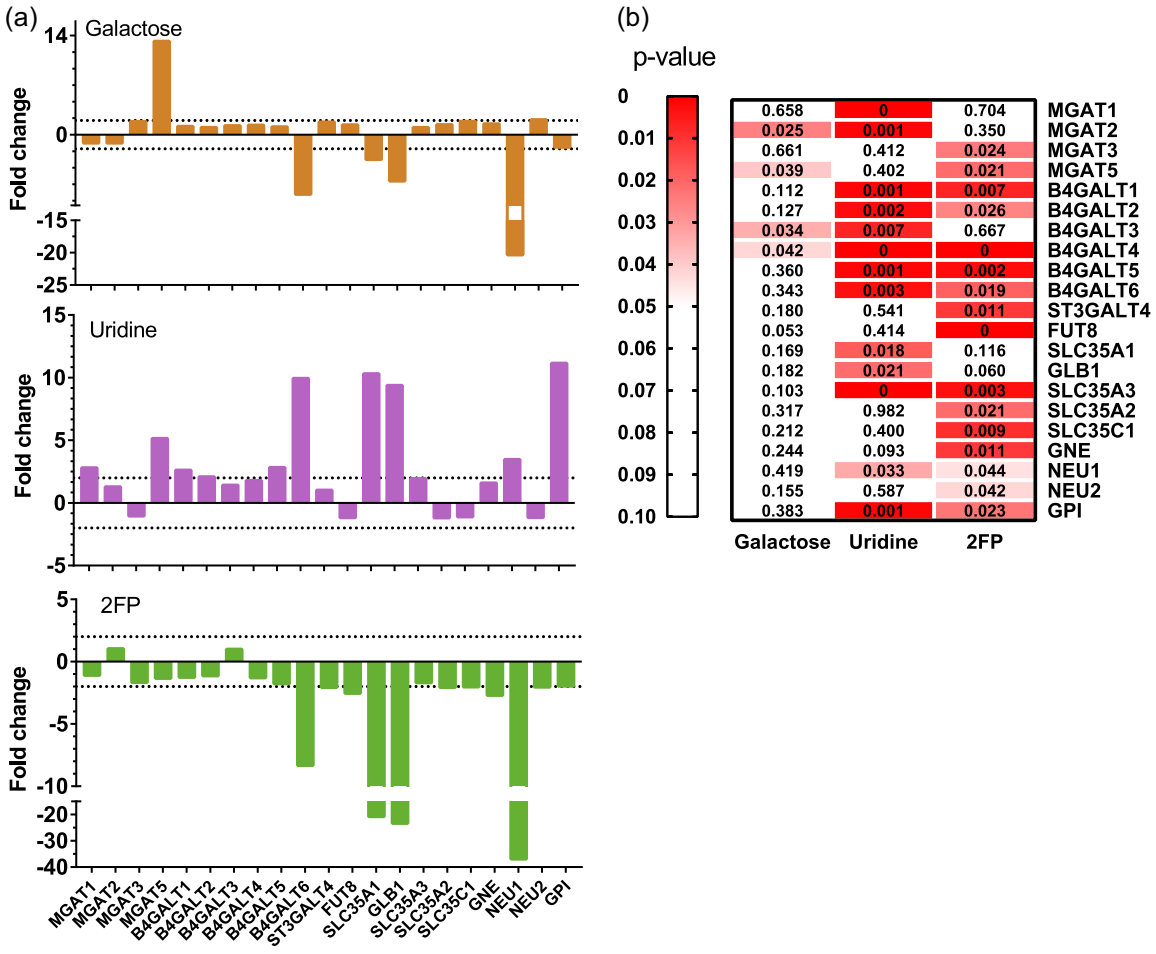

FIGURE 5 Individual supplement impacts on Day 4 gene expression; (a) fold change gene expression differences for galactose, uridine, and 2FP; (b) statistical significance of galactose, uridine, and 2FP on Day 4 expression of tested genes indicated by *p*-values from factorial analysis (white = $p > .05$, light to dark red = $p < .05$). 2FP, 2-F-peracetyl fucose [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Supplement effects on expression of key glycosyltransferase genes

Fold Change	Control	Uridine	Galactose	2FP
MGAT1	1.01 ±0.14	2.84 ±0.52	0.83 ±0.02	0.91 ±0.15
MGAT2	1.00 ±0.03	1.15 ±0.05	0.65 ±0.04	0.92 ±0.01
MGAT5	1.00 ±0.07	2.09 ±0.65	13.2 ±2.06	0.67 ±0.04
B4GALT1	1.00 ±0.08	2.49 ±0.26	1.16 ±0.08	0.77 ±0.03
B4GALT2	1.00 ±0.04	2.02 ±0.04	1.11 ±0.08	0.92 ±0.01
B4GALT3	1.01 ±0.22	1.51 ±0.06	1.16 ±0.00	1.16 ±0.16
B4GALT4	1.00 ±0.04	1.59 ±0.09	1.24 ±0.08	0.77 ±0.03
B4GALT5	1.00 ±0.10	2.71 ±0.21	1.09 ±0.16	0.56 ±0.05
B4GALT6	1.00 ±0.14	9.40 ±1.40	0.07 ±0.04	0.13 ±0.01
FUT8	1.02 ±0.27	0.85 ±0.08	1.41 ±0.37	0.39 ±0.39
SLC35A3	1.00 ±0.03	2.08 ±0.32	1.01 ±0.05	0.66 ±0.00
SLC35A2	1.00 ±0.01	0.78 ±0.34	1.46 ±1.35	0.38 ±0.01
SLC35C1	1.02 ±0.25	0.90 3±0.59	1.94 ±0.88	0.46 ±0.00

Note: Numbers indicate mean fold change ± *SD*. Gray squares indicate significant expression differences relative to control.
Abbreviations: 2FP, 2-F-peracetyl fucose; *SD*, standard deviation.

antibodies. These results differ from previous reports that showed increased titer alongside increased VCD (Grainger & James, 2013; Gramer et al., 2011). However, the uridine concentrations used in those studies were 2.5–100 times higher than the uridine concentrations examined here, which might account for the observed differences. Uridine-supplemented cells had a lower glucose consumption rate from Day 0 to Day 2 compared with control. The observed increase in VCD, decrease in titer, and differences in glucose metabolism are consistent with the direct availability of uridine as a substrate for de novo and salvage pathway biosynthesis of pyrimidines, enabling less energetically expensive cell division (Stryer, Berg, Tymoczko, & Gatto, 2019). Uridine is also a direct substrate for the synthesis of UDP-sugars that are synthesized and transported into the Golgi for building glycan structures on antibodies and other glycoproteins (Tejwani et al., 2018). Consistent with this knowledge, the addition of uridine significantly increased the expression of *MGAT1–2*, *B4GALT1–6*, and *SLC35A3* (Figure 6), which are all key enzymes for glycan maturation as proteins traverse the Golgi (Tejwani et al., 2018). *MGAT1* and *2* are critical enzymes for building the most common mAb glycan, G0 and G0F, each adding individual UDP-N-acetylglucosamine sugars terminally to a trimannose core. *B4GALT1–6* are critical for adding terminal galactose residues using UDP–Gal as the substrate. On Day 4, uridine-supplemented cells show upregulated expression of enzymes where UDP-sugars are substrates (*MGAT1–2* and *B4GALT1–6)*, perhaps indicating a positive feedback loop caused by a greater availability of UDP-sugars due to the presence of uridine (Figure 6). However, upregulating the expression of these enzymes changes the glycosylation profile only marginally compared with the glycan distribution shifts caused by galactose and 2FP supplementation. This result suggests that the availability of UDP for nucleotide sugar synthesis and expression of *MGAT1–2* and *B4GALT1–6* do not constitute significant bottlenecks for normal glycan maturation through the Golgi. Antibodies produced in cells grown in the presence of 200 μM uridine demonstrate a 6% decrease in antibody fucosylation and a 2% increase in galactosylation compared with those produced in non-supplemented cultures. Because of uridine's small effects on glycosylation, it could be used to effect slight changes to antibody fucosylation and galactosylation. Because uridine addition resulted in lower titers and had only small effects on the antibody glycan

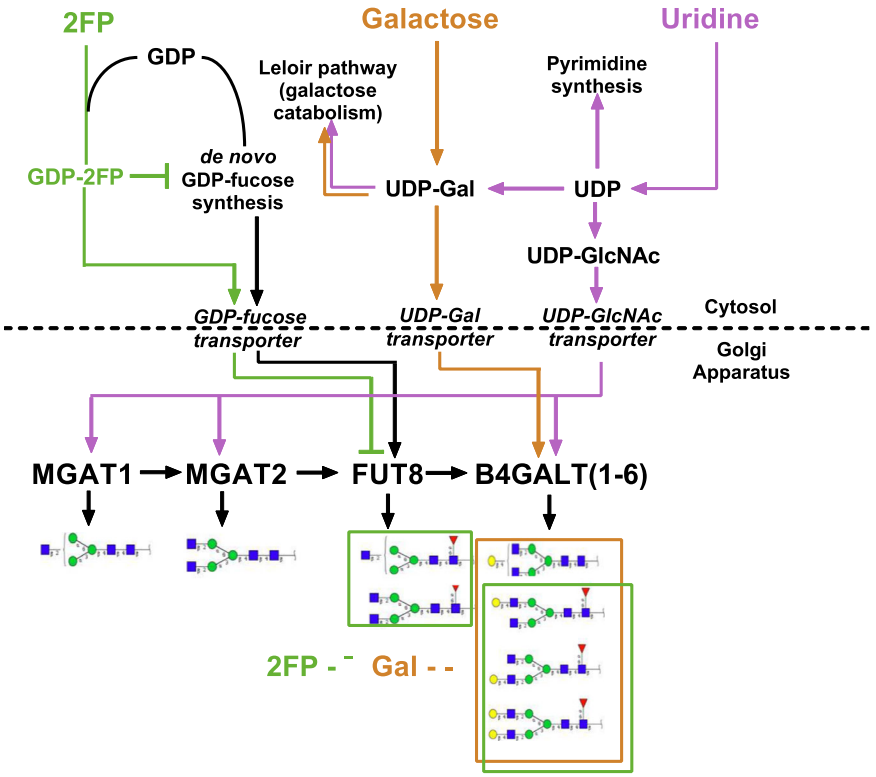

FIGURE 6 Schematic of 2FP, galactose, and uridine effects on glycosylation and other key pathways between the cytosol and Golgi apparatus. The presence of 50 μM 2FP (green) impacts de novo GDP–fucose synthesis, fucosyltransferase activity, and expression, and expression of GDP–fucose transporter. Cultures supplemented with 100 mM galactose (orange) increase substrate availability for antibody galactosylation and can also provide energy via the Leloir pathway. Supplementing with 200 μM uridine (pink) increases the expression of glycosyltransferases responsible for adding GlcNAc and Gal sugars to the antibody glycan and activates the sugars to allow usage in glycosylation and metabolism. In addition, uridine is a substrate for pyrimidine biosynthesis in preparing for cellular division. 2FP, 2-F-peracetyl fucose; GDP, guanosine diphosphate; GlcNAc, N-acetyl-D-glucosamine; UDP, uridine diphosphate [Color figure can be viewed at wileyonlinelibrary.com]

distribution, uridine at 200 μM is not considered a strong candidate for media supplementation unless its effect on VCD could be harnessed in a way that does not sacrifice antibody yield, perhaps by increasing the concentration of uridine used (Grainger & James, 2013; Gramer et al., 2011). However, simply increasing the uridine may have negative effects as cells grown in the presence of uridine concentrations at or above 10 mM have inhibited cell growth (Grainger & James, 2013; Kotidis et al., 2019).

4.2 | Galactose

Cells supplemented with galactose at concentrations of 100 mM slowed their glucose consumption significantly from Day 4 to Day 7 compared with unsupplemented cultures. As glucose and lactate became less readily available, galactose, whose concentrations remained high throughout the batch in the supplemented cultures (Figure S2), likely served as an energy source for the supplemented cultures by means of epimerizing galactose into glucose-1-phosphate via the Leloir pathway (King, 2014). The shift in antibody galactosylation caused by galactose supplementation observed here (up to 21% more G1/G2 glycan in supplemented cells) has been reported previously (Grainger & James, 2013; St. Amand et al., 2014), as expected, since adding galactose has a direct effect on substrate availability for galactosyltransferases in the Golgi. Interestingly, antibody galactosylation in the galactose-supplemented flasks remained largely unchanged from Day 4 to 7. In contrast, the 5–10% increase in the G0 glycan content between Days 4 and 7 in flasks lacking galactose supplementation is likely because decreasing glucose levels leaves the cells with a limited substrate for making galactose; this decreased galactose availability for galactosyltransferases results in lower relative antibody galactosylation. The galactose-supplemented cells had an upregulated expression of *MGAT2* and *B4GALT3-6*, which has two effects: (a) Increased availability of glycans capable of accepting a terminal galactose residue and (b) Increased availability of enzymes for adding UDP–Gal to a maturing glycoform (Figure 6). Because antibody galactosylation remained unchanged from Days 4–7 in galactose-supplemented flasks, a Day 4 measurement could predictively inform end-of-run glycoform distributions early enough in a production batch to enable supplement-driven changes to antibody galactosylation without affecting yield, cell metabolism, or growth behavior.

4.3 | 2-F-peracetyl fucose

2FP supplementation did not affect cell growth, metabolism, or titer significantly, despite having major effects (up to 48% decrease) on antibody fucosylation. In addition, the fucosylation levels remained relatively constant between Days 4 and 7 in both supplemented and unsupplemented flasks, which demonstrates the robustness of antibody fucosylation to nutrient depletion throughout the duration of the culture. While 2FP addition caused changes in *MGAT* and *B4GALT* enzyme expression, most interestingly it lowered *FUT8* and transporter enzymes responsible for UDP–GlcNAc, UDP–Gal, and GDP–FucT (*SLC35A3*, *SLC35A1*, and *SLC35C1*; Figure 6). 2FP is a cell-permeable, fluorinated analog of fucose that is converted into GDP–2FP inside the cell. Similar to GDP–fucose, GDP–2FP is trafficked to the Golgi apparatus where it inhibits FUT8 directly, and this inhibition explains the decrease in antibody fucosylation of cultures treated with 2FP (Rillahan et al., 2013). In addition, because the cells cannot utilize the GDP–2FP, it accumulates, and its presence inhibits de novo synthesis of GDP–fucose. The limited GDP–fucose available as a substrate for the GDP–fucose transporter and FUT8 may cause the resulting downregulation of these genes on Day 4 (Figure 6). In the presence of 50 μM 2FP, the relative fucosylation is stable from Day 4 to Day 7, which suggests that de novo synthesis of GDP–fucose is not entirely inhibited at this concentration. In addition, it appears that antibody fucosylation is not affected significantly by nutrient depletion from Day 4 to Day 7 because the relative fucosylation for each sample remained stable with and without 2FP or the other supplements present.

The sensitive response to 2FP addition that we observed here may be different in other cell lines and media formulations. Indeed, Mishra et al. (2020) observed a 60% decrease in core fucosylation in antibodies produced in the presence of 50 μM 2FP and Ehret et al. (2019) observed a 75% decrease when supplementing with 800 μM 2FP. The variation in 2FP effects at different concentrations may be caused by media formulation differences and CHO cell heterogeneities. However, even if the overall magnitude of the effect varies between bioprocesses, the use of 2FP addition at a different concentration or with timed supplementation could be a powerful means to control antibody fucosylation a priori when paired with a well-understood process or predictive model without affecting cell growth, metabolism, or antibody yield. Because the presence of galactose has a direct effect on galactosylation and minimal effect on fucosylation, and vice versa for 2FP, these supplements offer a relatively inexpensive way to manipulate key glycosylation characteristics directly in a mAb-producing bioprocess.

While these experiments were performed in batch cultures, it would be straightforward to use galactose and 2FP to increase terminal galactosylation and decrease core fucosylation of antibodies produced in fed-batch processes. The 2FP, uridine, and galactose showed no growth inhibitory effects at the concentrations used, so post-inoculation supplementation with these supplements would only be necessary to adjust final glycan profiles and would increase the complexity of the feeding strategy. Even though cultures grown in the presence of 200 μM uridine decreased Day 7 titers, a longer run without post-inoculation uridine supplementation could boost cell densities early in the run and perhaps demonstrate increased later-day productivity in a 14-day fed-batch process. On Day 7, galactose was still abundant in cultures originally supplemented with 100 mM. Thus, we expect that with glucose feeding over the length of a fed-batch process, galactose will likely remain available through the end-of-run, and therefore, the effect of galactose on terminal galactosylation will still be anticipated. The core fucosylation of antibodies produced in the presence of 50 μM 2FP was constant from Days 4 to 7, and if 2FP is not degraded, its effect should remain present until process termination.

Combining gene expression analysis with standard measurements taken throughout the culture like VCD, titer, and glycan distribution provides physiological insight into media supplement effects. While we performed targeted analysis on key genes, a broader-omics approach would provide additional information about the interplay between the transcriptome, metabolome, and substrate availability as well as identify bottlenecks that affect culture growth, protein production, and glycan distribution (e.g., see Sumit et al., 2019). The main limitation of these approaches is the need for in-depth experimental probing after the identification of potentially important factors. An integrated approach involving targeted experimental probes like ours together with -omics approaches would accelerate the improvement of biopharmaceutical processes.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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