Metabolic flux analysis during galactose and lactate co-consumption reveals enhanced energy metabolism in continuous CHO cell cultures

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1 **Research article**

2 **Metabolic flux analysis during galactose and**
3 **lactate co-consumption reveals enhanced energy**
4 **metabolism in continuous CHO cell cultures**

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17 Metabolic flux analysis, Lactate.

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21 Abstract

22 Chinese Hamster Ovary (CHO) cells are the main expression system for production of therapeutic
23 recombinant (r-) proteins. The increased demand for these therapeutics has boosted the
24 development of more robust production processes. Use of optimised feed/media has dramatically
25 improved the performance of CHO cells in culture. However, such progress in biomass synthesis
26 and r-protein production often come with an increased accumulation of lactate. In this study, we
27 present a combined feeding strategy that uses galactose and lactate to replace glucose in CHO cell
28 cultures. Replacement of glucose by galactose and lactate sustained cell growth and r-protein
29 production in CHO cells. This strategy supported a better-balanced and more efficient metabolism,
30 observed by an overall decreased consumption of carbon sources and amino acids, associated with
31 an increased ATP production per C-mol consumed. Our results provide new insights of CHO cell
32 metabolism in glucose-free media based on galactose and lactate.

1. Introduction

Chinese hamster ovary (CHO) cells are currently the predominant host to manufacture of recombinant (r-) therapeutic proteins for clinical trials and commercial sales. Their capacity of performing human-like post-translational modifications (PTMs) enables the synthesis of proteins with the appropriate critical quality attributes (CQAs) (e.g., glycosylation) that impact upon the potency and immunogenicity of therapeutic proteins (Wurm, 2004). In recent years, substantive improvements in final product titres and viable cell densities have been achieved in CHO-based culture processes (Dickson, 2014). However, one of the disadvantages of the cultivation of CHO cells is their deregulated metabolism, which is characterised by a large consumption of glucose with a consequent greater production of growth inhibitory products such as lactate (Hartley et al., 2018; Torres et al., 2018a). High concentration of lactate limits cell growth and negatively impacts productivity and CQAs of the r-proteins. In pH-controlled bioreactors, a drop in medium pH due to lactate accumulation is usually fixed by addition of base throughout the time course of culture process. Nevertheless, this leads to an increase in osmolarity in culture medium that, itself, can adversely impact the culture performance (decreased cell growth and viability) (Buchsteiner et al., 2018; Cruz et al., 2000; Ma et al., 2009). Therefore, development of low lactate-producing cell culture processes remains a major challenge for improvement of process robustness in the field of therapeutic r-protein production.

Different strategies have been proposed (with different degrees of success) to alleviate production and accumulation of lactate in cultures. Replacement of glucose with an alternative, slowly-consumed carbon source (Altamirano et al., 2006; C. Altamirano et al., 2000) or alteration of the balance of nutrients in media (Camps et al., 2018; Ivarsson et al., 2015; Li et al., 2012) have proven successful at limiting production of lactate in culture. Previously, our group demonstrated that substitution of the primary carbon and nitrogen sources (i.e., glucose and glutamine) with galactose and glutamate, respectively, led to a significantly decreased rate of lactate production (C. Altamirano et al., 2000). However, with the implementation of this strategy, cell growth was dramatically decreased. In a subsequent study, a biphasic culture strategy using glucose and

galactose as primary and secondary carbon sources was designed to overcome the growth limitations observed in wholly galactose-based cultures. While the initial phase was characterised by a rapid cell growth with high rates of glucose consumption and lactate production, the second phase, when glucose was depleted, was characterised by the consumption of both galactose and lactate (produced in the initial phase) (Altamirano et al., 2006; Sun et al., 2013). To understand this phenomenon, Wilkens et al., (2011) studied lactate metabolism in CHO cell batch cultures growing in glucose and galactose using a metabolic flux analysis approach. These authors suggested that the lactate switch (i.e., from production to consumption) occurred when cytosolic pyruvate concentration was insufficient for cells to support their energy requirements, and that lactate uptake emerges as an alternative to incorporate carbon into the TCA cycle and improve energy metabolism (Wilkens et al., 2011). Similar observations were made in two different CHO cell lines (Gray et al., 2012; Sun et al., 2013) and in a mouse myeloma cell line (NS0) (Mulukutla et al., 2012) that underwent a lactate switch. Whilst galactose supplementation had little impact on r-protein production in CHO cell cultures, it did alter r-protein glycosylation (Karst et al., 2017; Liu et al., 2015; Sun et al., 2013; Zhang et al., 2018). Liu et al. (2015) observed that galactose addition (more than 20 mM) increased the total sialic acid content (44 %) of a monoclonal antibody (mAb) in fed-batch cultures. Similar observations have been made in batch, fed-batch and perfusion (operated at steady state) cultures of CHO cells expressing different r-proteins (e.g., interferon- γ and mAbs) as a result of galactose supplementation (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018). However, the impact of galactose on r-protein quality seems to be cell-line dependent, since other CHO cell lines have not shown any improvements in glycosylation (at least when galactose is below an optimal concentration) (Liu et al., 2015) or even an increased potential for desialylation (Clark et al., 2005).

Rational design of feed media has also considered lactate by itself as a carbon source. Li et al. (2012) designed a lactate-containing feed medium that supplies energy to cells and controls culture pH. This study demonstrated that lactate can be effectively used as a carbon source during certain stages of culture (in the presence of glucose) with comparable results regarding cell growth, productivity and CQAs to glucose-fed cultures (Li et al., 2012). Although feeding lactate can tip the

balance towards the lactate consumption phenotype and effectively decrease lactate production, the success of this strategy greatly relies on the cell capacity of consuming lactate. Freund and Croughan (2018) showed that lactate supplementation (at 35 mM) was able to eliminate the net production of lactate in CHO cells. Additionally, when CHO cells were cultured in lactate-supplemented medium for several passages (over 40 days), they presented a significantly decrease in lactate production in batch and fed-batch cultures (Freund and Croughan, 2018). The capacity of mammalian cells to consume lactate has captured great attention for researchers and companies, since it has been strongly correlated with an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Despite evidence of reported benefits of feeding galactose and/or lactate in CHO-based cultures, there is no available study, to our knowledge, using a combination of both components as a feeding strategy for improving culture performance.

Modification in the media/feeds composition, such as the supplementation of galactose or lactate, drives large changes in the metabolic pathways in CHO cells (Altamirano et al., 2006; Luo et al., 2012). The application of metabolic models has enabled to quantitatively estimate the flux distributions and deeply understand the mechanism behind metabolic changes (Sha et al., 2018). Metabolic flux analysis (MFA) has been extensively used for gaining understanding of CHO cell bioprocessing, such as changes in metabolic behaviour during different growth phases (Ahn and Antoniewicz, 2011), operational modes (Templeton et al., 2017b) and media composition (Wilkens et al., 2011). It has been also used for the identification of productivity traits of CHO cells. Templeton et al. (2013) observed that the peak r-protein production was associated with high oxidative metabolism in high producing cell lines. In a subsequent study, Templeton et al. (2017) recognized a higher activity of energy metabolism in recombinant CHO cell lines compared to their parental cell lines. These results indicate that an enhanced energy metabolism is required in cell factories for high production processes. In the present study, we combined the use of chemostat cultures and metabolic flux analysis to investigate and characterise the impact of substituting glucose with galactose and lactate on the modulation of culture performance (i.e., growth, r-protein production and quality) and cell metabolism of a hr-tPA producing CHO cell line. To achieve this, we relied on the capacity of chemostat culture to set the specific growth rate to a desired value and

116 study culture performance and metabolic changes at steady state. Meanwhile, the calculation of the
117 intracellular fluxes enabled to explore in-depth the metabolic impact of using galactose and lactate
118 as major carbon sources.

2. Material and methods

2.1. Cell line and medium

The rh-tPA-producing cell line (CHO TF 70R) was obtained from Pharmacia & Upjohn S.A. (Sweden) (a kind gift of Torsten Björlig). Glucose- and glutamine-free SFM4CHO cell culture medium (HyClone, Logan, USA) supplemented with glucose (G7021, Sigma, USA), galactose (G5388, Sigma, USA) and lactate (L1875, Sigma, USA) to define three different culture conditions. Culture medium and feed at each experimental condition were supplemented with 6 mM glutamate (G8415, Sigma, USA).

2.2. Chemostat cultures

Before chemostat culture experiments, cells were routinely sub-cultured every 48 h in batch systems using T-75 flasks and scaled up in spinner flasks (Techne, UK) by reseeding at 0.25×10^6 cells/ mL in fresh growth medium (glucose-based medium). Chemostat cultures were performed in 250 mL spinner flasks (Techne, UK) specially conditioned for continuous operation with a working volume of 150 mL. Cultures were initially operated in batch mode for 48 h and were then supplied with sterile feed throughout the period of operation (Berrios et al., 2011). All cultures were incubated in a Forma™ Series II 3110 Water-Jacketed CO₂ Incubator (Thermo Fisher Scientific, USA) at 50 rpm with 96% humidity and 5% CO₂ enriched atmosphere.

To evaluate the effect of glucose substitution with galactose and lactate in the feed, two different medium composition were evaluated: low concentration of galactose (10 mM) and lactate (4 mM) (G/L-Low) and high concentration of galactose (20 mM) and lactate (8 mM) (G/L-High). Control cultures were performed by feeding 10 mM glucose (GLC). All chemostat culture experiments were designed by maintaining a dilution rate at 0.015 1/h, controlled by a low-flow peristaltic pump (Ismatec, Germany) (Berrios et al., 2011). Sampling was carried out every 24 h for determining cell number, viability and pH. After cell counting, supernatant was frozen at -20°C until further analysis. Cultures were considered to reach steady state when, after at least five residence times, both the number of viable cells and residual substrate (i.e., glucose, galactose and lactate) concentration were constant for two consecutive samples (Altamirano et al., 2001).

2.3. Analytical techniques

Cell density and viability were determined by the trypan blue exclusion method (T8154, Sigma, USA) (1:1 mixture of 0.2% (w/v) trypan blue in saline and cell sample) using a hemocytometer (Neubauer, Germany). Glucose, galactose and lactate were measured with an automatic biochemistry analyser (YSI 2700, Yellow Springs Inc., USA). Ammonium ion and amino acid concentrations were measured with a PerkinElmer Series 200 HPLC (PerkinElmer, USA) using a C-18 reversed-phase column (AccQ-Tag Column, 3.9mm×150mm, Waters) and AccuTag kit (Waters, USA) according to the manufacturer's instructions.

Extracellular rh-tPA was quantified by enzyme immunoassay (Trinilize tPA-antigen kit, Tcoag Ltd., Ireland). For the analysis of the sialic acid content, rh-tPA was purified by affinity chromatography in fast protein liquid chromatography system (FPLC, Pharmacia, Sweden) with erythrin-trypsin-inhibitor immobilised in CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences, UK) (Heussen et al., 1984). The purification process was checked by SDS-PAGE with 10–15% gradient gels (PhastSystem, Pharmacia, Sweden). The sialic acid was removed from the glycan of purified rh-tPA according to Fu and O'Neill (1995). Each sample was incubated at 37°C for one hour with 0.5 U mL⁻¹ of each enzyme (neuraminidase and neuraminic acid aldolase) simultaneously. Manosamine was subsequently quantified by PerkinElmer Series 200 HPLC (PerkinElmer, USA) using fluorescence detection (excitation 360 nm, emission 425 nm) and a C-18 reversed-phase column (Waters, Ireland). Sample derivatisation was carried out with anthranilic acid according to Anumula and Du (1999).

2.4. Cell-specific rate calculations

Specific growth rate (μ) (Eq1.) was calculated from the mass balance within the bioreactor:

$$\mu = D(N_t/N_v) \quad \text{Eq1.}$$

where N_v is the concentration of viable cells (10^6 /mL), N_t is the concentration of total cells (10^6 /mL), and D is the dilution rate of the culture (1/h) (Vergara et al., 2015, 2014).

Specific rates of production or consumption (Eq2.) of metabolite i (q_i) were calculated from the mass balance within the reactor:

$$q_i = D((C_i^i - C_i^0)/N_v) \times 10^9 \quad \text{Eq2.}$$

where C_i^i is the concentration of i in the inlet (mmol/L), C_i^0 is the concentration of i in the outlet (mmol/L), N_v is the concentration of viable cells ($10^6/\text{mL}$), and D is the dilution rate of the culture (1/h).

2.5. Metabolic flux analysis

Metabolic flux analysis (MFA) was performed using a reduced metabolic network model from a *Mus musculus* genome-scale metabolic reconstruction, which adequately represents core mammalian metabolism and can be directly applied to cell culture flux experiments (Quek and Nielsen, 2008). The model included the elemental biomass composition and the rh-tPA production reaction (v64 and v71, Table A.1., Supplementary file) obtained from our previous study (Berrios et al., 2011). The intracellular fluxes (v) were calculated using the metabolite balancing constraints $S \cdot v = 0$, whereby S is the stoichiometric matrix derived from the metabolic model. For the determination of intracellular fluxes, the underdetermined system was solved by linear programming using the measured extracellular metabolite concentrations, the biomass synthesis rate and the specific rh-tPA productivity as constraints (Quek et al., 2010). The constraint $v \geq 0$ was imposed on all irreversible reactions, while the lower and upper boundary values of measured fluxes were specified using the measured cell-specific consumption or production rates and the estimated standard error ($v_{\text{measured}} \pm \text{SE}_{\text{measured}}$). Metabolic flux calculation was performed using MATLAB (The Mathworks) as previously described by Quek et al., (2010) (Quek et al., 2010). Estimated fluxes were also used to estimate the ATP production (Gray et al., 2012). The metabolic model is fully detailed in Table S1.

2.6. Statistical analysis

Kinetic and stoichiometric parameters were calculated from at least two independent experiments and are expressed as the mean $\pm$ SE. All statistical analyses were performed with R software

(version 3.1.)(R Core Team, 2014). The significant variation of the feeds among physiological parameters was evaluated by one-way ANOVA (using the feed composition – 3 levels–as factor) followed by multiple comparison tests (Tukey HSD test) with normally distributed data. The threshold for statistical significance was $p < 0.05$.

3. Results

To investigate the impact of glucose substitution for galactose and lactate in the feed on culture performance and cell metabolism, we performed steady state chemostat cultures in spinner flasks using a recombinant CHO cell line producing hr-tPA at low and high concentrations of galactose and lactate (G/L-Low and G/L-High, respectively), and glucose (GLC) as control. All chemostats at each experimental condition reached steady state after five residence times and cell viability remained above 95% for all cultures (Figure 1). From each cultivation, viable cell density (VCD), hr-tPA production, quality, and cell metabolism were evaluated. For a better understanding of the metabolic changes associated with the simultaneous galactose and lactate consumption, a metabolic flux analysis was performed in each culture condition using the experimental data at steady state.

3.1. Cell growth and protein production

Substitution of glucose for galactose and lactate in the feed did not impact significantly on viable cell densities (VCDs), cell viabilities and specific cell growth rates in cultures at steady state (One-way ANOVA, $p > 0.05$). Although chemostat cultures showed higher VCDs and cell viability using G/L-Low ($0.87 \pm 0.05 \cdot 10^6$ cells/mL and $97.9 \pm 1.2\%$) and G/L-High ($0.93 \pm 0.05 \cdot 10^6$ cells/mL and $99.5 \pm 0.4\%$) feeds compared to GLC ($0.79 \pm 0.04 \cdot 10^6$ cells/mL and $95 \pm 1.2\%$) (Figure 1), these differences were not statistically significant (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). Specific cell growth rate was calculated from the relationship between the dilution rate ($D = 0.015 \text{ 1/h}$) in which the chemostat cultures were set up and cell viability (Eq. 1) (Figure 1B). The specific cell growth rate was slightly decreased in G/L-Low and G/L-High in comparison with GLC cultures. However, as with VCDs and cell viability, no statistical significance

was observed among the culture feed conditions (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High).

Figure 1. Performance of CHO cells in chemostat cultures at steady state. A, B, C and D correspond to the viable cell density (VCD), the cell viability and specific growth rate, the hr-tPA production and the specific hr-tPA productivity of cultures, respectively. (*Black*) glucose; (*Grey*) G/L-Low; (*White*) G/L-High. Experimental data was statistically analysed using one-way ANOVA (using the feed condition – 3 levels - as a factor) followed by a Tukey HSD test using normalised data. P values below 0.05 indicates statistically significant values among the culture conditions.

Production of hr-tPA was evaluated and compared among the different feeds through the final product titre and specific productivity (q_{hr-tPA}) at steady state (Figure 1C). A maximum hr-tPA titre of 509 ± 20 ng/mL and q_{hr-tPA} of 9.7 ± 0.1 ng/ 10^6 cells/h was achieved in the control (Figure 1D). Changes in the feed composition had a significant impact on the hr-tPA production at steady state (One-way ANOVA, $p < 0.05$). The G/L-Low cultures reduced hr-tPA titre by 62% (Tukey's post hoc test, $p < 0.05$) and q_{hr-tPA} by 51% ($p < 0.05$), while the G/L-High cultures decreased hr-tPA titre by 22% ($p < 0.05$) and q_{hr-tPA} by 16% ($p < 0.05$) when compared to control cultures. Additionally, variations in r-protein quality were evaluated by determining sialic acid (SA) content of hr-tPA (mol_{SA}/mol_{hr-tPA}) and calculating specific hr-tPA sialylation rate ($q_{SA\ on\ tPA}$) at each condition (Table 1). One-way ANOVA indicated that protein quality was significantly impacted by the feed composition ($p < 0.05$). The highest SA content of hr-tPA and $q_{SA\ on\ tPA}$ were found in the control cultures. The SA content of hr-tPA and $q_{SA\ on\ tPA}$ were decreased by 25% (Tukey's post hoc test, $p < 0.05$) and by 57% ($p < 0.05$) in G/L-Low cultures, respectively, while they were decreased by 15% ($p < 0.05$) and by 46% ($p < 0.05$) in G/L-High cultures when compared to GLC cultures. Therefore, the elimination of glucose in the medium negatively impacted on the hr-tPA production and quality.

Table 1. Sialic acid content of hr-tPA ($\text{mol}_{\text{SA}}/\text{mol}_{\text{hr-tPA}}$) and specific hr-tPA sialylation rates ($q_{\text{SA on tPA}}$) in CHO chemostat cultures at steady state.

Parameters	Units	GLC	G/L-Low	G/L-High
SA/t-PA	mol/mol	3.3 ± 0.2	2.5 ± 0.1	2.8 ± 0.2
$q_{\text{SA on tPA}}$	$\text{nmol}/10^9 \text{ cells/h}$	0.52 ± 0.05	0.22 ± 0.02	0.28 ± 0.01

3.2. Cell metabolism

To investigate the effect of glucose replacement for galactose and lactate in the feed on cell metabolism, we analysed the variations in consumption and/or production of glucose, galactose, lactate (Table 2) and amino acids (Figure 2) in the chemostat cultures at steady state. Control chemostat cultures consumed 57% of the fed glucose and reached a specific glucose consumption rate (q_{GLC}) of $110.9 \pm 2.8 \text{ nmol}/10^6 \text{ cells/h}$ at steady state. The replacement of glucose by galactose significantly decreased the consumption of the main carbon source (One-way ANOVA, $p < 0.05$), as expected. In G/L-Low cultures, cells only consumed 12% of the galactose and their specific galactose consumption rate (q_{GAL}) was 80% lower than the q_{GLC} in control cultures (Tukey's post hoc test, $p < 0.05$), while in G/L-High cultures, only 16.5% of the galactose was consumed and the q_{GAL} was decreased by 48% compared to the q_{GLC} in glucose-based cultures ($p < 0.05$). An increased concentration of galactose in the feed from 10 to 20 mM increased the q_{GAL} 2.6-fold ($p < 0.05$). Lactate metabolism is certainly a different matter for comparison since the conditions under study involve this metabolite as a substrate. Feeding lactate (simultaneously with galactose) in chemostat cultures effectively supported cells to consume lactate at both concentration in the study. An increased lactate concentration from 4 to 8 mM in the feed increased the specific lactate consumption rate (q_{LAC}) in 41% ($p < 0.05$), although feeding high concentration of lactate led to a similar residual lactate concentrations compared to the control (Table 2). Therefore, even though feeding lactate avoided its production, overfeeding lactate to the culture

(beyond the evaluated concentrations) might increase its accumulation in the medium at detrimental levels for cell growth and productivity. Residual medium pH was also measured and compared to the feeds to evaluate if differences in H⁺ gradient may induce a lactate consumption state in G/L cultures (Table 2). A slight decrease in pH was observed in all conditions, but no significant variations were observed between GLC and G/L (Low and High) cultures that link the lactate consuming phenotype with a dramatic decrease in the medium pH.

Table 2. Comparison of metabolic parameters of CHO cells in chemostat cultures at steady state.

	Units	GLC	G/L-Low	G/L-High
Hexose fed	mmol/L	10	10	20
Hexose res	mmol/L	4.3 ± 1.14	8.8 ± 2.22	16.7 ± 1.11
qHex	nmol/10 ⁶ cells/h	- 110.9 ± 2.8	- 22.2 ± 1.9	- 57.6 ± 3.5
Lactate fed	mmol/L	--	4	8
Lactate res	mmol/L	7.3 ± 0.2	3.5 ± 0.1	7.4 ± 0.3
qLac	nmol/10 ⁶ cells/h	142.0 ± 2.31	- 13.2 ± 1.11	- 18.7 ± 1.18
Y _{Lac/Hex}	mol/mol	1.29 ± 0.05	--	--
NH ₄ ⁺ res	mmol/L	0.44 ± 0.03	0.21 ± 0.01	0.22 ± 0.02
Y _{NH₄/GLU}	mol/mol	0.45 ± 0.03	0.35 ± 0.01	0.22 ± 0.03
pH fed	--	6.8	6.8	6.8
pH res	--	6.39 ± 0.04	6.55 ± 0.03	6.34 ± 0.05

Amino acid metabolism was analysed by determining their residual concentration and calculating their specific rates of consumption/production in relation to their concentrations in the feed. Chemostat cultures showed an overall consumption of amino acids in the feed, except for alanine, glycine and aspartate (Figure 2). This increased residual concentration of alanine, glycine and

aspartate has been previously observed in the hr-tPA producing CHO cell line (Altamirano et al., 2006). One-way ANOVA indicated that specific consumption and production rates of amino acids were significantly impacted by feeding galactose and lactate ($p < 0.05$). In response to addition of galactose and lactate, cells decreased the specific consumption rates in most of the amino acids. For instance, specific consumption rate of glutamate (qGLU) decreased by 25% in G/L-Low and by 17% in G/L-High cultures compared to the control. Another case is serine and glycine, of which their specific consumption and production rates (qSER and qGLY) decreased up to 80% and up to 98% respectively, as more galactose and lactate were consumed. The decrease in qSER and qGLY seems to be related to the lactate shift from production (GLC cultures) to consumption (G/L cultures). Serine and glycine metabolisms are closely related each other in CHO cells, where an increase in serine consumption results in an increase in glycine production (Narkewicz et al., 1996). The metabolism of both amino acids is also correlated to pyruvate metabolism, so the increase in lactate consumption in G/L cultures seems to increase intracellular pools of pyruvate and reduces the consumption of serine by cells, consequently impacting glycine production. The specific production rate of alanine (qALA) maintained similar levels for all conditions. Ammonium production was also decreased when cells were fed with galactose and lactate. Residual ammonium was 0.44 ± 0.03 mM in the control and was decreased by 52% in G/L-Low ($p < 0.05$) and by 48% in G/L-High ($p < 0.05$) cultures. The same was observed in specific ammonium production rate (qNH₄) which was decreased by 42% in G/L-Low ($p < 0.05$) and by 58% in G/L-High ($p < 0.05$) cultures. This is consistent with the overall decrease in amino acid consumption observed in G/L-Low and G/L-High, particularly glutamate. The ratio ammonium/glutamate (YNH₄/GLU) was also calculated for all cultures. While the control cultures achieved a YNH₄/GLU of 0.45 ± 0.03 mol/mol, these values decreased by 21% in G/L-Low ($p < 0.05$) and by 51% in G/L-High ($p < 0.05$) cultures.

Figure 2. Specific amino acid consumption/production and ammonia production rates (nmol/10⁶ cells/h) in CHO chemostat cultures at steady state. (Black) glucose; (Grey) G/L-Low;

(*White*) G/L-High. P values below 0.05 indicates statistically significant values between the conditions compared.

3.3. Metabolic flux analysis

To gain a better understanding of the metabolic status of CHO cells growing in galactose and lactate, we performed a metabolic flux analysis (MFA) using a constraint-based model of cell metabolism with 91 reactions and 68 metabolites (Table S1, Supplementary file). The experimental data detailed above (i.e., the specific metabolite consumption/production rates, the specific cell growth rate and the specific hr-tPA productivity) were used as inputs to the metabolic model to constraint the theoretical solution space. At first glance, the MFA revealed that glucose replacement for galactose and lactate significantly decreased glycolysis fluxes, while it increased fluxes through both tricarboxylic acid (TCA) cycle and energy metabolism, particularly in G/L-High cultures (Figure 3). Intracellular fluxes through pentose phosphate pathway (PPP) were very low compared to the other two main pathways (i.e., glycolysis and TCA cycle) for all conditions. However, this seems to be because MFA cannot correctly resolve fluxes in the oxidative PPP (Quek et al., 2010). Therefore, the estimated fluxes for this pathway were mainly associated with the flux to ribose 5-phosphate needed for nucleotide synthesis. Lactate dehydrogenase (LDH) showed a large carbon flux in the control cultures, indicating most of the consumed glucose seemed to be converted into lactate. This was reversed in G/L-Low and G/L-High cultures, which presented an increased influx of carbon from lactate to pyruvate. The switch in the LDH flux in G/L-Low and G/L-High cultures impacted largely pyruvate and oxidative metabolism (Figure 4A). For instance, a dramatic increase in the fluxes through pyruvate dehydrogenase complex (PDC) and the TCA cycle was observed when lactate was consumed (Figure 4A). Although no changes in malate and alanine metabolism were observed, the flux from serine to pyruvate were largely decreased and even inverted in G/L-High cultures (Figure 4A). This reverse of serine flux in G/L-High cultures was apparently associated with a higher lactate consumption and a higher NADH demand for this reaction. The increased TCA fluxes did not affect the carbon flux from glutamate to the α -ketoglutarate in all

cultures. However, it entailed a decrease in the activity anaplerotic pathways for G/L cultures (Figure 4B, Table S2).

Figure 3. Metabolic flux analysis in CHO chemostat cultures at steady state. The figure illustrates the flux distribution of the main metabolic pathways of central carbon metabolism of CHO cells (both in the cytosol and the mitochondria) cultured in chemostat fed with glucose (GLC) and galactose and lactate (G/L-Low and G/L-High). In GLC fed cultures, cells present large glycolytic fluxes which are mainly diverted to lactate production, whereas in G/L cultures, they present an overall decrease in glycolysis and lactate consumption. Mitochondria are more metabolically active in G/L cultures, particularly in G/L-High, with glutamate and lactate as main carbon sources for ATP aerobic respiration. Values represent intracellular fluxes ($\text{nmol}/10^6 \text{ cells/h}$) $\pm$ estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study. The fluxes with positive values are in the direction indicated by the arrow, while negative fluxes go in the opposite direction.

Figure 4. Zoom-in of metabolic fluxes through pyruvate (A), α -ketoglutarate (B) and glycosylation (C) metabolism in CHO chemostat cultures at steady state. The fluxes in the figure were normalised to the values of GLC fluxes to compare the variation between G/L (Low and High) and control cultures. Values in the boxes described the fraction of fluxes for G/L-Low or G/L-High compared to GLC cultures.

As glycolysis and TCA cycle were the metabolic pathways more affected by feed composition, we analysed in detail the energy metabolism among culture conditions evaluated in this study. Total ATP production rates were calculated by assuming theoretical P/O ratios of 2.5 and 1.5 for NADH and FADH_2 , respectively, and adding ATP generated through substrate level phosphorylation (Gray et al., 2012; Ivarsson et al., 2015) (Figure 5). One-way ANOVA revealed that total ATP production

rates were significantly impacted by feeding galactose and lactate ($p < 0.05$). A total of 1795 ± 34 nmol of ATP/ 10^6 cells/h was produced in control cultures. This was reduced by 47% in G/L-Low cultures (Tukey's post hoc test, $p < 0.05$), whereas in G/L-High cultures, it was increased by 41% ($p < 0.05$). We also analysed the energy efficiency of cells by calculating the ATP production on a carbon mol basis (i.e., total ATP produced per total C-mol substrate consumed), considering a contribution of 6 carbons for the hexoses (i.e., glucose and galactose) and 3 carbons for lactate. The control cultures showed an energy efficiency of 2.7 mol ATP/C-mol of glucose, while G/L-Low and G/L-High cultures presented 5.5 and 6.5 mol ATP/C-mol of galactose and lactate. This means that cells produced 2 and 2.4 times more ATP on a carbon mol basis in G/L-Low and G/L-High cultures, respectively, when compared to glucose culture. Therefore, CHO cells were more energy efficient when consuming galactose and lactate.

Figure 5. Balance of ATP produced and consumed in CHO chemostat cultures at steady state. A represent the specific ATP production in glucose (*Black*), G/L-Low (*Grey*) and G/L-High (*White*) cultures. For calculation of ATP generated by ATP synthetase, P/O ratios of 2.5 and 1.5 for NADH and FADH₂, respectively, were assumed. B and C represent the break-down of ATP contribution/utilization in different pathways. In all cultures, the main ATP production source was oxidative phosphorylation (OXPHOS), while most of the energy produced was derived to maintenance energy. Main differences in ATP production and consumption between the control and G/L cultures were observed in glycolysis contribution/utilization. Values represent intracellular fluxes (nmol/ 10^6 cells/h) $\pm$ estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study.

For all cultures, a major amount of ATP was produced in oxidative phosphorylation (OXPHOS) followed by glycolysis. The percentage of ATP produced in glycolysis in G/L-Low (6.3%) and G/L-High (8.2%) was significantly lower compared to the control (23.3%) (Tukey's post hoc test, $p < 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). This was consistent with the decreased glycolytic

fluxes observed in these cultures. Considering that glycolysis did not contribute largely to ATP production and lactate was mostly metabolised by TCA cycle in G/L-Low and G/L-High cultures, the amount of lactate in the feed had a considerable impact on the energetic status of cells. Differences in ATP production were also reflected in theoretical specific oxygen consumption rates (qO_2 , reaction v74 Table A.1., A.2., Supplementary file) in cultures. The qO_2 was 223 ± 20 nmol/ 10^6 cells/h in the control cultures, while it was decreased by 41% in G/L-Low ($p < 0.05$) and increased by 75% in G/L-High ($p < 0.05$). However, no statistical variations were observed in the respiratory quotient ($RQ = Y_{CO_2/O_2}$) and the oxygen consumption to ATP production ratio ($Y_{O_2/ATP}$).

Most of the produced energy was used for maintenance in all culture conditions. Although cell maintenance energy is not a concept precisely defined, we considered it as the ATP that is not directly consumed by biomass synthesis (i.e., synthesis of DNA, RNA, cell protein, fatty acids and polysaccharides) and glycolysis reactions (i.e., hexokinase and phosphofructokinase) in our metabolic model. Therefore, maintenance energy included ATP involved in turnover of macromolecules, re-establishment of ion gradients across the cell membrane and synthesis of the r-protein and nucleotide sugars (glycosylation)(Varma and Palsson, 1995), among other things. We observed that a decrease in maintenance ATP flux negatively impacted on hr-tPA production and quality. However, when increasing this flux beyond a certain threshold, no significant variation was observed in hr-tPA production and quality of protein was even impaired (Figure 6). The replacement of glucose for galactose and lactate not only entailed a decrease in the availability of energy, but also a decrease in the carbon resources for nucleotide sugars, that may explain the impairment of hr-tPA quality in G/L-Low and G/L-High cultures (Table 1). An overall decrease in the fluxes for nucleotide sugars (i.e., UDP glucose, GDP mannose, UDP galactose and UDP- GlcNAc) and glycosylation of the hr-tPA in G/L cultures compared to the control (Figure 4C, Table S2). Moreover, total ATP used for biomass synthesis was higher in the control cultures (135 ± 3 nmol of ATP/ 10^6 cells/h) than G/L-Low (116 ± 1.6 nmol of ATP/ 10^6 cells/h) and G/L-High (102 ± 2 nmol of ATP/ 10^6 cells/h) cultures. This correlates with the overall decrease in metabolic fluxes through pathways involved in biomass synthesis, which showed a significant decrease in G/L-Low and G/L-High compared to the control.

419

420 **Figure 6. Relationship between ATP derived to maintenance energy and the hr-tPA**
421 **production (hr-tPA flux), and molar ratio of Sialic acid and hr-tPA (SA:rh-tPA ratio).** The
422 corresponding culture conditions are detailed in the figure using a segmented vertical line.

423

424 4. Discussion

425 Cultivation of recombinant CHO cells usually presents high rates of glucose consumption beyond
426 cellular needs that inevitably drive to large amounts of metabolic waste products, such as lactate
427 (C. Altamirano et al., 2000). Since lactate accumulation in culture negatively impacts cell growth, r-
428 protein production and quality (Buchsteiner et al., 2018; Ma et al., 2009), it comes as no surprise,
429 therefore, that many strategies are focused on the modulation of cell metabolism for reducing
430 lactate production. Utilisation of galactose has been extensively studied together with glucose in
431 biphasic culture strategies to increase biomass and productivity while attenuating lactate
432 accumulation (Altamirano et al., 2006; Karst et al., 2017; Liu et al., 2015; Sun et al., 2013).
433 Compared to glucose-based feeds, this strategy has often improved cell growth and r-protein
434 production. Such improvements have been associated with a metabolic shift to lactate utilisation
435 when glucose is depleted and galactose is co-consumed with the produced lactate (Altamirano et
436 al., 2006). In the present study, we aimed to study the impact of a combined feeding strategy of
437 galactose and lactate (glucose-free) on culture performance and cell metabolism in CHO cells using
438 chemostat cultures. As in our previous studies, the use of chemostat cultures operated at steady
439 state offers the opportunity to better understand the metabolic effects of culture environment
440 changes in a defined physiological state (by setting the dilution rate). Additionally, even though this
441 modality is not very representative of industrial cell cultures processes (mostly high density fed-
442 batch or perfusion cultures), it enables evaluation of the isolated effect of feeding strategies without
443 the consequent changes in specific cell growth and excessive accumulation of products (e.g., waste
444 metabolites, HCP or r-protein) (Berrios et al., 2011; Vergara et al., 2018, 2014).

While the use of a galactose and lactate-based feeding strategy sustained cell growth and viability in our experiments, r-protein production and quality were significantly compromised. In our previous study with this cell line, replacing glucose and glutamine by galactose and glutamate in batch cultures resulted in a significant decrease in maximum viable cell density and cell growth rate (Altamirano et al., 2000). However, when galactose was supplemented in biphasic culture strategies, it supported cell growth (at lower rates) and maintained viability in batch and fed-batch cultures after glucose depletion in the medium (Altamirano et al., 2006; Liu et al., 2015; Sun et al., 2013). Similar results were shown in perfusion cultures (at steady state) in which addition of galactose (10 mM) at late culture stages did not impact growth kinetics (Karst et al., 2017). CHO cells may consume galactose as a primary carbon source, but as its consumption is at a very low rate, they need an additional carbon source (in this case lactate) to fulfil their carbon and energy requirements.

This was not the case for r-protein production and quality for our chemostat cultures, which unexpectedly showed decreased productivity and sialic acid content when feeding galactose and lactate (Figure 1, Table 1). Mammalian cell lines that were able to consume lactate (due to a metabolic shift) were strongly correlated to an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Several studies have associated this increase in r-protein production under a lactate consuming state with an enhanced oxidative metabolism and metabolic efficiency (Charaniya et al., 2010; Luo et al., 2012; Mulukutla et al., 2012; Sun et al., 2013; Templeton et al., 2013). In a recent work reviewing the molecular aspects of lactate metabolism, the authors suggested that lactate consuming state increases ATP yield from oxidative metabolism and better equipped cells to maintain their redox balance, thus allowing cells to improve overall cell health and meet the energy requirements for protein synthesis (Hartley et al., 2018). Nevertheless, this study has shown that an increased energy availability and efficiency did not lead to an increase in r-protein production in CHO cells (Figure 6). Moreover, supplementation of galactose has proven to improve glycosylation of r-protein at different degrees in CHO cell cultures (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018), by supporting production of more complex glycans with higher content of galactose and sialic acids moieties as terminal units (Karst et al., 2017). However, all cases were evaluated with a previous presence or in presence of glucose

in medium (both at low or high concentration) that impacted on the availability of precursors for glycosylation. When eliminating glucose from feeds in our chemostat cultures, we observed an overall decrease in carbon fluxes through glycolysis and synthesis of nucleotide sugar (Figure 3, 4C). Similarly, Sou et al. (2015) observed decreased amounts of nucleotide sugars (i.e., UDP-Glc, UDP-Gal and UDP- GlcNAc) when glucose consumption was reduced due to a temperature down-shift (Sou et al., 2015). Therefore, a feeding strategy based on galactose and lactate decreased the availability of metabolic intermediaries for glycans and showed that culture strategies only focused on either channelling more carbon into the TCA or improving mitochondrial activity seem to be not enough to improve the overall performance of a CHO cell line. Future process or metabolic engineering strategies should balance glycolysis and TCA cycle activities in order to provide adequate amounts of intermediaries for biomass, r-protein and glycans biosynthesis.

In this study, we have reported that co-consumption of galactose and lactate led to a better-balanced and efficient metabolism in CHO cells. This was observed on one hand, by decreased use of carbon sources and amino acids consumption (without affecting cell growth and viability) (Figure 2, Table 2) and, on the other hand, by higher production of ATP on a carbon mol basis. Most of the mammalian cells that underwent a lactate metabolic shift presented a decrease in consumption of glucose (when it is still available on culture medium) and amino acids (Liste-Calleja et al., 2015; Mulukutla et al., 2012; Wilkens et al., 2011). This is often accompanied by decreased glycolytic fluxes and a drop in specific cell growth rate (Gray et al., 2012; Mulukutla et al., 2012). Similar metabolic behaviour has been observed in biphasic culture strategies using glucose and galactose during the co-consumption phase of galactose and lactate (Altamirano et al., 2006; Sun et al., 2013; Wilkens et al., 2011). Several studies have shown that lactate consuming state (after a metabolic shift induced either by pH, temperature or change in media composition) presented a more energy efficient metabolic state compared to lactate producing state (Gray et al., 2012; Ivarsson et al., 2015; Mulukutla et al., 2012). A metabolome analysis of CHO cells indicated that lactate consuming cells presented an increased TCA cycle capacity and a more robust mitochondrial function (Luo et al., 2012). Such a difference in energy production lies in the fact that large proportion of lactate is metabolised in the TCA cycle during the lactate consumption phase, while most of the pyruvate

during the lactate production phase (growth based on glucose) is converted into lactate (Gray et al., 2012; Mulukutla et al., 2017). In a theoretical calculation, cells may produce up to 14 times more energy in a lactate consuming state compared to a producing state if it is completely metabolised in the TCA cycle (Gray et al., 2012). Hence, this naturally brings us to the question of defining the main reasons driving this metabolic behaviour (lactate consumption).

The answer to this question, unfortunately, is not a simple one. Various process events have been reported to trigger a shift from lactate production to utilisation including depletion of carbon/energy sources (i.e., glucose and/or glutamine) in medium (Altamirano et al., 2006, 2004; Wahrheit et al., 2014; Zagari et al., 2013), cell growth cessation after reaching maximal cell numbers (Carinhas et al., 2013; Ma et al., 2009), temperature shift (Torres et al., 2018b), increased oxidative capacity by addition of copper (Luo et al., 2012; Yuk et al., 2015), and unbalanced lactate or H^+ concentrations (Li et al., 2012; Liste-Calleja et al., 2015). In our experiments, increasing extracellular lactate concentration led CHO cells to consume lactate, although doubling lactate concentration of the feed did not result in an equivalent increase in lactate consumption (Table 1). It was noteworthy that, when comparing the control and G/L-High cultures, they presented the same lactate residual concentration and pH (H^+ concentrations), but completely different metabolic behaviours. One of several hypotheses explaining this phenomenon is related to the carbon flux through glycolysis. High glycolytic flux (usually observed under glucose-based growth) results in large accumulation of pyruvate that cannot be processed by mitochondria. This leads to production of large amount of alanine and lactate (Lu et al., 2005; Ma et al., 2009). When the glycolytic flux decreases, a drop in pyruvate pools occurs and lactate consumption emerges as an alternative for supplying the required carbon (Hartley et al., 2018). A similar scenario was observed in our chemostat cultures fed with galactose and lactate, in which fluxes through glycolysis were dramatically reduced compared to the control (Figure 5).

The main novelty of this study is the evaluation of a combined feeding strategy of galactose and lactate as a means to decrease lactate accumulation in CHO cell cultures. To our knowledge, this is the first study showing that a free-glucose feeding strategy based on lactate may support cell

growth and enhance energy metabolism. On the other hand, the use of constrained-based MFA provided a deeper understanding of the physiology of CHO cells in cultures. We presented evidence that suggest that improving efficiency of metabolism and increasing ATP yield do not always lead to an improved culture performance. Further applications of the MFA in combination with transcriptome and metabolome data can be used to guide engineering efforts to improve r-protein production.

5. Conclusion

The principal aim of this study was to study the impact of simultaneous feeding galactose and lactate on the culture performance and cell metabolism of a rh-tPA producing CHO cell line using chemostats and **metabolic flux analysis**. Our results indicated that a combined feeding strategy of galactose and lactate did not significantly impact on cell growth and viability, but it did negatively impact on rh-tPA productivity, quality and cell metabolism. Increasing galactose and lactate concentrations in the feed led to a high rh-tPA production, although it did not improve rh-tPA quality (compared to glucose and G/L-Low). This feeding strategy also dramatically decrease consumption of the primary carbon source (i.e., galactose) and led to very low glycolytic fluxes in G/L-Low and G/L-High cultures. Feeding lactate promoted lactate consumption in CHO cells and drove to high fluxes through TCA cycle and an improved energy metabolism. The present study provides a better understanding of CHO cell metabolism in galactose and lactate. However, the success of this feeding strategy in industrial culture processes, such as fed-batch or perfusion, is a question that arises for future research on this topic.

6. Appendices

Table A.1 Metabolic network used for the estimation of intracellular fluxes.

Table A.2 Intracellular fluxes [nmol/10⁶cells/h] of CHO cell chemostat cultures at steady-state.

554 Conflict of interest statement

555 Nothing declared.

556

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1 **Research article**

2 **Metabolic flux analysis during galactose and**
3 **lactate co-consumption reveals enhanced energy**
4 **metabolism in continuous CHO cell cultures**

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21 Abstract

22 Chinese Hamster Ovary (CHO) cells are the main expression system for production of therapeutic
23 recombinant (r-) proteins. The increased demand for these therapeutics has boosted the
24 development of more robust production processes. Use of optimised feed/media has dramatically
25 improved the performance of CHO cells in culture. However, such progress in biomass synthesis
26 and r-protein production often come with an increased accumulation of lactate. In this study, we
27 present a combined feeding strategy that uses galactose and lactate to replace glucose in CHO cell
28 cultures. Replacement of glucose by galactose and lactate sustained cell growth and r-protein
29 production in CHO cells. This strategy supported a better-balanced and more efficient metabolism,
30 observed by an overall decreased consumption of carbon sources and amino acids, associated with
31 an increased ATP production per C-mol consumed. Our results provide new insights of CHO cell
32 metabolism in glucose-free media based on galactose and lactate.

1. Introduction

Chinese hamster ovary (CHO) cells are currently the predominant host to manufacture of recombinant (r-) therapeutic proteins for clinical trials and commercial sales. Their capacity of performing human-like post-translational modifications (PTMs) enables the synthesis of proteins with the appropriate critical quality attributes (CQAs) (e.g., glycosylation) that impact upon the potency and immunogenicity of therapeutic proteins (Wurm, 2004). In recent years, substantive improvements in final product titres and viable cell densities have been achieved in CHO-based culture processes (Dickson, 2014). However, one of the disadvantages of the cultivation of CHO cells is their deregulated metabolism, which is characterised by a large consumption of glucose with a consequent greater production of growth inhibitory products such as lactate (Hartley et al., 2018; Torres et al., 2018a). High concentration of lactate limits cell growth and negatively impacts productivity and CQAs of the r-proteins. In pH-controlled bioreactors, a drop in medium pH due to lactate accumulation is usually fixed by addition of base throughout the time course of culture process. Nevertheless, this leads to an increase in osmolarity in culture medium that, itself, can adversely impact the culture performance (decreased cell growth and viability) (Buchsteiner et al., 2018; Cruz et al., 2000; Ma et al., 2009). Therefore, development of low lactate-producing cell culture processes remains a major challenge for improvement of process robustness in the field of therapeutic r-protein production.

Different strategies have been proposed (with different degrees of success) to alleviate production and accumulation of lactate in cultures. Replacement of glucose with an alternative, slowly-consumed carbon source (Altamirano et al., 2006; C. Altamirano et al., 2000) or alteration of the balance of nutrients in media (Camps et al., 2018; Ivarsson et al., 2015; Li et al., 2012) have proven successful at limiting production of lactate in culture. Previously, our group demonstrated that substitution of the primary carbon and nitrogen sources (i.e., glucose and glutamine) with galactose and glutamate, respectively, led to a significantly decreased rate of lactate production (C. Altamirano et al., 2000). However, with the implementation of this strategy, cell growth was dramatically decreased. In a subsequent study, a biphasic culture strategy using glucose and

galactose as primary and secondary carbon sources was designed to overcome the growth limitations observed in wholly galactose-based cultures. While the initial phase was characterised by a rapid cell growth with high rates of glucose consumption and lactate production, the second phase, when glucose was depleted, was characterised by the consumption of both galactose and lactate (produced in the initial phase) (Altamirano et al., 2006; Sun et al., 2013). To understand this phenomenon, Wilkens et al., (2011) studied lactate metabolism in CHO cell batch cultures growing in glucose and galactose using a metabolic flux analysis approach. These authors suggested that the lactate switch (i.e., from production to consumption) occurred when cytosolic pyruvate concentration was insufficient for cells to support their energy requirements, and that lactate uptake emerges as an alternative to incorporate carbon into the TCA cycle and improve energy metabolism (Wilkens et al., 2011). Similar observations were made in two different CHO cell lines (Gray et al., 2012; Sun et al., 2013) and in a mouse myeloma cell line (NS0) (Mulukutla et al., 2012) that underwent a lactate switch. Whilst galactose supplementation had little impact on r-protein production in CHO cell cultures, it did alter r-protein glycosylation (Karst et al., 2017; Liu et al., 2015; Sun et al., 2013; Zhang et al., 2018). Liu et al. (2015) observed that galactose addition (more than 20 mM) increased the total sialic acid content (44 %) of a monoclonal antibody (mAb) in fed-batch cultures. Similar observations have been made in batch, fed-batch and perfusion (operated at steady state) cultures of CHO cells expressing different r-proteins (e.g., interferon- γ and mAbs) as a result of galactose supplementation (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018). However, the impact of galactose on r-protein quality seems to be cell-line dependent, since other CHO cell lines have not shown any improvements in glycosylation (at least when galactose is below an optimal concentration) (Liu et al., 2015) or even an increased potential for desialylation (Clark et al., 2005).

Rational design of feed media has also considered lactate by itself as a carbon source. Li et al. (2012) designed a lactate-containing feed medium that supplies energy to cells and controls culture pH. This study demonstrated that lactate can be effectively used as a carbon source during certain stages of culture (in the presence of glucose) with comparable results regarding cell growth, productivity and CQAs to glucose-fed cultures (Li et al., 2012). Although feeding lactate can tip the

balance towards the lactate consumption phenotype and effectively decrease lactate production, the success of this strategy greatly relies on the cell capacity of consuming lactate. Freund and Croughan (2018) showed that lactate supplementation (at 35 mM) was able to eliminate the net production of lactate in CHO cells. Additionally, when CHO cells were cultured in lactate-supplemented medium for several passages (over 40 days), they presented a significantly decrease in lactate production in batch and fed-batch cultures (Freund and Croughan, 2018). The capacity of mammalian cells to consume lactate has captured great attention for researchers and companies, since it has been strongly correlated with an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Despite evidence of reported benefits of feeding galactose and/or lactate in CHO-based cultures, there is no available study, to our knowledge, using a combination of both components as a feeding strategy for improving culture performance.

Modification in the media/feeds composition, such as the supplementation of galactose or lactate, drives large changes in the metabolic pathways in CHO cells (Altamirano et al., 2006; Luo et al., 2012). The application of metabolic models has enabled to quantitatively estimate the flux distributions and deeply understand the mechanism behind metabolic changes (Sha et al., 2018). Metabolic flux analysis (MFA) has been extensively used for gaining understanding of CHO cell bioprocessing, such as changes in metabolic behaviour during different growth phases (Ahn and Antoniewicz, 2011), operational modes (Templeton et al., 2017b) and media composition (Wilkens et al., 2011). It has been also used for the identification of productivity traits of CHO cells. Templeton et al. (2013) observed that the peak r-protein production was associated with high oxidative metabolism in high producing cell lines. In a subsequent study, Templeton et al. (2017) recognized a higher activity of energy metabolism in recombinant CHO cell lines compared to their parental cell lines. These results indicate that an enhanced energy metabolism is required in cell factories for high production processes. In the present study, we combined the use of chemostat cultures and metabolic flux analysis to investigate and characterise the impact of substituting glucose with galactose and lactate on the modulation of culture performance (i.e., growth, r-protein production and quality) and cell metabolism of a hr-tPA producing CHO cell line. To achieve this, we relied on the capacity of chemostat culture to set the specific growth rate to a desired value and

116 study culture performance and metabolic changes at steady state. Meanwhile, the calculation of the
117 intracellular fluxes enabled to explore in-depth the metabolic impact of using galactose and lactate
118 as major carbon sources.

2. Material and methods

2.1. Cell line and medium

The rh-tPA-producing cell line (CHO TF 70R) was obtained from Pharmacia & Upjohn S.A. (Sweden) (a kind gift of Torsten Björlig). Glucose- and glutamine-free SFM4CHO cell culture medium (HyClone, Logan, USA) supplemented with glucose (G7021, Sigma, USA), galactose (G5388, Sigma, USA) and lactate (L1875, Sigma, USA) to define three different culture conditions. Culture medium and feed at each experimental condition were supplemented with 6 mM glutamate (G8415, Sigma, USA).

2.2. Chemostat cultures

Before chemostat culture experiments, cells were routinely sub-cultured every 48 h in batch systems using T-75 flasks and scaled up in spinner flasks (Techne, UK) by reseeding at 0.25×10^6 cells/ mL in fresh growth medium (glucose-based medium). Chemostat cultures were performed in 250 mL spinner flasks (Techne, UK) specially conditioned for continuous operation with a working volume of 150 mL. Cultures were initially operated in batch mode for 48 h and were then supplied with sterile feed throughout the period of operation (Berrios et al., 2011). All cultures were incubated in a Forma™ Series II 3110 Water-Jacketed CO₂ Incubator (Thermo Fisher Scientific, USA) at 50 rpm with 96% humidity and 5% CO₂ enriched atmosphere.

To evaluate the effect of glucose substitution with galactose and lactate in the feed, two different medium composition were evaluated: low concentration of galactose (10 mM) and lactate (4 mM) (G/L-Low) and high concentration of galactose (20 mM) and lactate (8 mM) (G/L-High). Control cultures were performed by feeding 10 mM glucose (GLC). All chemostat culture experiments were designed by maintaining a dilution rate at 0.015 1/h, controlled by a low-flow peristaltic pump (Ismatec, Germany) (Berrios et al., 2011). Sampling was carried out every 24 h for determining cell number, viability and pH. After cell counting, supernatant was frozen at -20°C until further analysis. Cultures were considered to reach steady state when, after at least five residence times, both the number of viable cells and residual substrate (i.e., glucose, galactose and lactate) concentration were constant for two consecutive samples (Altamirano et al., 2001).

2.3. Analytical techniques

Cell density and viability were determined by the trypan blue exclusion method (T8154, Sigma, USA) (1:1 mixture of 0.2% (w/v) trypan blue in saline and cell sample) using a hemocytometer (Neubauer, Germany). Glucose, galactose and lactate were measured with an automatic biochemistry analyser (YSI 2700, Yellow Springs Inc., USA). Ammonium ion and amino acid concentrations were measured with a PerkinElmer Series 200 HPLC (PerkinElmer, USA) using a C-18 reversed-phase column (AccQ-Tag Column, 3.9mm×150mm, Waters) and AccuTag kit (Waters, USA) according to the manufacturer's instructions.

Extracellular rh-tPA was quantified by enzyme immunoassay (Trinilize tPA-antigen kit, Tcoag Ltd., Ireland). For the analysis of the sialic acid content, rh-tPA was purified by affinity chromatography in fast protein liquid chromatography system (FPLC, Pharmacia, Sweden) with erythrin-trypsin-inhibitor immobilised in CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences, UK) (Heussen et al., 1984). The purification process was checked by SDS-PAGE with 10–15% gradient gels (PhastSystem, Pharmacia, Sweden). The sialic acid was removed from the glycan of purified rh-tPA according to Fu and O'Neill (1995). Each sample was incubated at 37°C for one hour with 0.5 U mL⁻¹ of each enzyme (neuraminidase and neuraminic acid aldolase) simultaneously. Manosamine was subsequently quantified by PerkinElmer Series 200 HPLC (PerkinElmer, USA) using fluorescence detection (excitation 360 nm, emission 425 nm) and a C-18 reversed-phase column (Waters, Ireland). Sample derivatisation was carried out with anthranilic acid according to Anumula and Du (1999).

2.4. Cell-specific rate calculations

Specific growth rate (μ) (Eq1.) was calculated from the mass balance within the bioreactor:

$$\mu = D(N_t/N_v) \quad \text{Eq1.}$$

where N_v is the concentration of viable cells (10⁶/mL), N_t is the concentration of total cells (10⁶/mL), and D is the dilution rate of the culture (1/h) (Vergara et al., 2015, 2014).

Specific rates of production or consumption (Eq2.) of metabolite i (q_i) were calculated from the mass balance within the reactor:

$$q_i = D((C_i^i - C_i^0)/N_v) \times 10^9 \quad \text{Eq2.}$$

where C_i^i is the concentration of i in the inlet (mmol/L), C_i^0 is the concentration of i in the outlet (mmol/L), N_v is the concentration of viable cells ($10^6/\text{mL}$), and D is the dilution rate of the culture (1/h).

2.5. Metabolic flux analysis

Metabolic flux analysis (MFA) was performed using a reduced metabolic network model from a *Mus musculus* genome-scale metabolic reconstruction, which adequately represents core mammalian metabolism and can be directly applied to cell culture flux experiments (Quek and Nielsen, 2008). The model included the elemental biomass composition and the rh-tPA production reaction (v64 and v71, Table A.1., Supplementary file) obtained from our previous study (Berrios et al., 2011). The intracellular fluxes (v) were calculated using the metabolite balancing constraints $S \cdot v = 0$, whereby S is the stoichiometric matrix derived from the metabolic model. For the determination of intracellular fluxes, the underdetermined system was solved by linear programming using the measured extracellular metabolite concentrations, the biomass synthesis rate and the specific rh-tPA productivity as constraints (Quek et al., 2010). The constraint $v \geq 0$ was imposed on all irreversible reactions, while the lower and upper boundary values of measured fluxes were specified using the measured cell-specific consumption or production rates and the estimated standard error ($v_{\text{measured}} \pm \text{SE}_{\text{measured}}$). Metabolic flux calculation was performed using MATLAB (The Mathworks) as previously described by Quek et al., (2010) (Quek et al., 2010). Estimated fluxes were also used to estimate the ATP production (Gray et al., 2012). The metabolic model is fully detailed in Table S1.

2.6. Statistical analysis

Kinetic and stoichiometric parameters were calculated from at least two independent experiments and are expressed as the mean $\pm$ SE. All statistical analyses were performed with R software

(version 3.1.)(R Core Team, 2014). The significant variation of the feeds among physiological parameters was evaluated by one-way ANOVA (using the feed composition – 3 levels—as factor) followed by multiple comparison tests (Tukey HSD test) with normally distributed data. The threshold for statistical significance was $p < 0.05$.

3. Results

To investigate the impact of glucose substitution for galactose and lactate in the feed on culture performance and cell metabolism, we performed steady state chemostat cultures in spinner flasks using a recombinant CHO cell line producing hr-tPA at low and high concentrations of galactose and lactate (G/L-Low and G/L-High, respectively), and glucose (GLC) as control. All chemostats at each experimental condition reached steady state after five residence times and cell viability remained above 95% for all cultures (Figure 1). From each cultivation, viable cell density (VCD), hr-tPA production, quality, and cell metabolism were evaluated. For a better understanding of the metabolic changes associated with the simultaneous galactose and lactate consumption, a metabolic flux analysis was performed in each culture condition using the experimental data at steady state.

3.1. Cell growth and protein production

Substitution of glucose for galactose and lactate in the feed did not impact significantly on viable cell densities (VCDs), cell viabilities and specific cell growth rates in cultures at steady state (One-way ANOVA, $p > 0.05$). Although chemostat cultures showed higher VCDs and cell viability using G/L-Low ($0.87 \pm 0.05 \cdot 10^6$ cells/mL and $97.9 \pm 1.2\%$) and G/L-High ($0.93 \pm 0.05 \cdot 10^6$ cells/mL and $99.5 \pm 0.4\%$) feeds compared to GLC ($0.79 \pm 0.04 \cdot 10^6$ cells/mL and $95 \pm 1.2\%$) (Figure 1), these differences were not statistically significant (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). Specific cell growth rate was calculated from the relationship between the dilution rate ($D = 0.015 \text{ 1/h}$) in which the chemostat cultures were set up and cell viability (Eq. 1) (Figure 1B). The specific cell growth rate was slightly decreased in G/L-Low and G/L-High in comparison with GLC cultures. However, as with VCDs and cell viability, no statistical significance

was observed among the culture feed conditions (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High).

Figure 1. Performance of CHO cells in chemostat cultures at steady state. A, B, C and D correspond to the viable cell density (VCD), the cell viability and specific growth rate, the hr-tPA production and the specific hr-tPA productivity of cultures, respectively. (*Black*) glucose; (*Grey*) G/L-Low; (*White*) G/L-High. Experimental data was statistically analysed using one-way ANOVA (using the feed condition – 3 levels - as a factor) followed by a Tukey HSD test using normalised data. P values below 0.05 indicates statistically significant values among the culture conditions.

Production of hr-tPA was evaluated and compared among the different feeds through the final product titre and specific productivity (q_{hr-tPA}) at steady state (Figure 1C). A maximum hr-tPA titre of 509 ± 20 ng/mL and q_{hr-tPA} of 9.7 ± 0.1 ng/ 10^6 cells/h was achieved in the control (Figure 1D). Changes in the feed composition had a significant impact on the hr-tPA production at steady state (One-way ANOVA, $p < 0.05$). The G/L-Low cultures reduced hr-tPA titre by 62% (Tukey's post hoc test, $p < 0.05$) and q_{hr-tPA} by 51% ($p < 0.05$), while the G/L-High cultures decreased hr-tPA titre by 22% ($p < 0.05$) and q_{hr-tPA} by 16% ($p < 0.05$) when compared to control cultures. Additionally, variations in r-protein quality were evaluated by determining sialic acid (SA) content of hr-tPA (mol_{SA}/mol_{hr-tPA}) and calculating specific hr-tPA sialylation rate ($q_{SA\ on\ tPA}$) at each condition (Table 1). One-way ANOVA indicated that protein quality was significantly impacted by the feed composition ($p < 0.05$). The highest SA content of hr-tPA and $q_{SA\ on\ tPA}$ were found in the control cultures. The SA content of hr-tPA and $q_{SA\ on\ tPA}$ were decreased by 25% (Tukey's post hoc test, $p < 0.05$) and by 57% ($p < 0.05$) in G/L-Low cultures, respectively, while they were decreased by 15% ($p < 0.05$) and by 46% ($p < 0.05$) in G/L-High cultures when compared to GLC cultures. Therefore, the elimination of glucose in the medium negatively impacted on the hr-tPA production and quality.

Table 1. Sialic acid content of hr-tPA ($\text{mol}_{\text{SA}}/\text{mol}_{\text{hr-tPA}}$) and specific hr-tPA sialylation rates ($q_{\text{SA on tPA}}$) in CHO chemostat cultures at steady state.

Parameters	Units	GLC	G/L-Low	G/L-High
SA/t-PA	mol/mol	3.3 ± 0.2	2.5 ± 0.1	2.8 ± 0.2
$q_{\text{SA on tPA}}$	$\text{nmol}/10^9 \text{ cells/h}$	0.52 ± 0.05	0.22 ± 0.02	0.28 ± 0.01

3.2. Cell metabolism

To investigate the effect of glucose replacement for galactose and lactate in the feed on cell metabolism, we analysed the variations in consumption and/or production of glucose, galactose, lactate (Table 2) and amino acids (Figure 2) in the chemostat cultures at steady state. Control chemostat cultures consumed 57% of the fed glucose and reached a specific glucose consumption rate (q_{GLC}) of $110.9 \pm 2.8 \text{ nmol}/10^6 \text{ cells/h}$ at steady state. The replacement of glucose by galactose significantly decreased the consumption of the main carbon source (One-way ANOVA, $p < 0.05$), as expected. In G/L-Low cultures, cells only consumed 12% of the galactose and their specific galactose consumption rate (q_{GAL}) was 80% lower than the q_{GLC} in control cultures (Tukey's post hoc test, $p < 0.05$), while in G/L-High cultures, only 16.5% of the galactose was consumed and the q_{GAL} was decreased by 48% compared to the q_{GLC} in glucose-based cultures ($p < 0.05$). An increased concentration of galactose in the feed from 10 to 20 mM increased the q_{GAL} 2.6-fold ($p < 0.05$). Lactate metabolism is certainly a different matter for comparison since the conditions under study involve this metabolite as a substrate. Feeding lactate (simultaneously with galactose) in chemostat cultures effectively supported cells to consume lactate at both concentration in the study. An increased lactate concentration from 4 to 8 mM in the feed increased the specific lactate consumption rate (q_{LAC}) in 41% ($p < 0.05$), although feeding high concentration of lactate led to a similar residual lactate concentrations compared to the control (Table 2). Therefore, even though feeding lactate avoided its production, overfeeding lactate to the culture

(beyond the evaluated concentrations) might increase its accumulation in the medium at detrimental levels for cell growth and productivity. Residual medium pH was also measured and compared to the feeds to evaluate if differences in H^+ gradient may induce a lactate consumption state in G/L cultures (Table 2). A slight decrease in pH was observed in all conditions, but no significant variations were observed between GLC and G/L (Low and High) cultures that link the lactate consuming phenotype with a dramatic decrease in the medium pH.

Table 2. Comparison of metabolic parameters of CHO cells in chemostat cultures at steady state.

	Units	GLC	G/L-Low	G/L-High
Hexose fed	mmol/L	10	10	20
Hexose res	mmol/L	4.3 ± 1.14	8.8 ± 2.22	16.7 ± 1.11
qHex	nmol/ 10^6 cells/h	-110.9 ± 2.8	-22.2 ± 1.9	-57.6 ± 3.5
Lactate fed	mmol/L	--	4	8
Lactate res	mmol/L	7.3 ± 0.2	3.5 ± 0.1	7.4 ± 0.3
qLac	nmol/ 10^6 cells/h	142.0 ± 2.31	-13.2 ± 1.11	-18.7 ± 1.18
$Y_{Lac/Hex}$	mol/mol	1.29 ± 0.05	--	--
NH ₄ ⁺ res	mmol/L	0.44 ± 0.03	0.21 ± 0.01	0.22 ± 0.02
$Y_{NH_4/GLU}$	mol/mol	0.45 ± 0.03	0.35 ± 0.01	0.22 ± 0.03
pH fed	--	6.8	6.8	6.8
pH res	--	6.39 ± 0.04	6.55 ± 0.03	6.34 ± 0.05

Amino acid metabolism was analysed by determining their residual concentration and calculating their specific rates of consumption/production in relation to their concentrations in the feed. Chemostat cultures showed an overall consumption of amino acids in the feed, except for alanine, glycine and aspartate (Figure 2). This increased residual concentration of alanine, glycine and

aspartate has been previously observed in the hr-tPA producing CHO cell line (Altamirano et al., 2006). One-way ANOVA indicated that specific consumption and production rates of amino acids were significantly impacted by feeding galactose and lactate ($p < 0.05$). In response to addition of galactose and lactate, cells decreased the specific consumption rates in most of the amino acids. For instance, specific consumption rate of glutamate (q_{GLU}) decreased by 25% in G/L-Low and by 17% in G/L-High cultures compared to the control. Another case is serine and glycine, of which their specific consumption and production rates (q_{SER} and q_{GLY}) decreased up to 80% and up to 98% respectively, as more galactose and lactate were consumed. The decrease in q_{SER} and q_{GLY} seems to be related to the lactate shift from production (GLC cultures) to consumption (G/L cultures). Serine and glycine metabolisms are closely related each other in CHO cells, where an increase in serine consumption results in an increase in glycine production (Narkewicz et al., 1996). The metabolism of both amino acids is also correlated to pyruvate metabolism, so the increase in lactate consumption in G/L cultures seems to increase intracellular pools of pyruvate and reduces the consumption of serine by cells, consequently impacting glycine production. The specific production rate of alanine (q_{ALA}) maintained similar levels for all conditions. Ammonium production was also decreased when cells were fed with galactose and lactate. Residual ammonium was 0.44 ± 0.03 mM in the control and was decreased by 52% in G/L-Low ($p < 0.05$) and by 48% in G/L-High ($p < 0.05$) cultures. The same was observed in specific ammonium production rate (q_{NH4}) which was decreased by 42% in G/L-Low ($p < 0.05$) and by 58% in G/L-High ($p < 0.05$) cultures. This is consistent with the overall decrease in amino acid consumption observed in G/L-Low and G/L-High, particularly glutamate. The ratio ammonium/glutamate ($Y_{NH4/GLU}$) was also calculated for all cultures. While the control cultures achieved a $Y_{NH4/GLU}$ of 0.45 ± 0.03 mol/mol, these values decreased by 21% in G/L-Low ($p < 0.05$) and by 51% in G/L-High ($p < 0.05$) cultures.

Figure 2. Specific amino acid consumption/production and ammonia production rates (nmol/ 10^6 cells/h) in CHO chemostat cultures at steady state. (Black) glucose; (Grey) G/L-Low;

(*White*) G/L-High. P values below 0.05 indicates statistically significant values between the conditions compared.

3.3. Metabolic flux analysis

To gain a better understanding of the metabolic status of CHO cells growing in galactose and lactate, we performed a metabolic flux analysis (MFA) using a constraint-based model of cell metabolism with 91 reactions and 68 metabolites (Table S1, Supplementary file). The experimental data detailed above (i.e., the specific metabolite consumption/production rates, the specific cell growth rate and the specific hr-tPA productivity) were used as inputs to the metabolic model to constraint the theoretical solution space. At first glance, the MFA revealed that glucose replacement for galactose and lactate significantly decreased glycolysis fluxes, while it increased fluxes through both tricarboxylic acid (TCA) cycle and energy metabolism, particularly in G/L-High cultures (Figure 3). Intracellular fluxes through pentose phosphate pathway (PPP) were very low compared to the other two main pathways (i.e., glycolysis and TCA cycle) for all conditions. However, this seems to be because MFA cannot correctly resolve fluxes in the oxidative PPP (Quek et al., 2010). Therefore, the estimated fluxes for this pathway were mainly associated with the flux to ribose 5-phosphate needed for nucleotide synthesis. Lactate dehydrogenase (LDH) showed a large carbon flux in the control cultures, indicating most of the consumed glucose seemed to be converted into lactate. This was reversed in G/L-Low and G/L-High cultures, which presented an increased influx of carbon from lactate to pyruvate. The switch in the LDH flux in G/L-Low and G/L-High cultures impacted largely pyruvate and oxidative metabolism (Figure 4A). For instance, a dramatic increase in the fluxes through pyruvate dehydrogenase complex (PDC) and the TCA cycle was observed when lactate was consumed (Figure 4A). Although no changes in malate and alanine metabolism were observed, the flux from serine to pyruvate were largely decreased and even inverted in G/L-High cultures (Figure 4A). This reverse of serine flux in G/L-High cultures was apparently associated with a higher lactate consumption and a higher NADH demand for this reaction. The increased TCA fluxes did not affect the carbon flux from glutamate to the α -ketoglutarate in all

cultures. However, it entailed a decrease in the activity anaplerotic pathways for G/L cultures (Figure 4B, Table S2).

Figure 3. Metabolic flux analysis in CHO chemostat cultures at steady state. The figure illustrates the flux distribution of the main metabolic pathways of central carbon metabolism of CHO cells (both in the cytosol and the mitochondria) cultured in chemostat fed with glucose (GLC) and galactose and lactate (G/L-Low and G/L-High). In GLC fed cultures, cells present large glycolytic fluxes which are mainly diverted to lactate production, whereas in G/L cultures, they present an overall decrease in glycolysis and lactate consumption. Mitochondria are more metabolically active in G/L cultures, particularly in G/L-High, with glutamate and lactate as main carbon sources for ATP aerobic respiration. Values represent intracellular fluxes ($\text{nmol}/10^6 \text{ cells/h}$) $\pm$ estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study. The fluxes with positive values are in the direction indicated by the arrow, while negative fluxes go in the opposite direction.

Figure 4. Zoom-in of metabolic fluxes through pyruvate (A), α -ketoglutarate (B) and glycosylation (C) metabolism in CHO chemostat cultures at steady state. The fluxes in the figure were normalised to the values of GLC fluxes to compare the variation between G/L (Low and High) and control cultures. Values in the boxes described the fraction of fluxes for G/L-Low or G/L-High compared to GLC cultures.

As glycolysis and TCA cycle were the metabolic pathways more affected by feed composition, we analysed in detail the energy metabolism among culture conditions evaluated in this study. Total ATP production rates were calculated by assuming theoretical P/O ratios of 2.5 and 1.5 for NADH and FADH_2 , respectively, and adding ATP generated through substrate level phosphorylation (Gray et al., 2012; Ivarsson et al., 2015) (Figure 5). One-way ANOVA revealed that total ATP production

rates were significantly impacted by feeding galactose and lactate ($p < 0.05$). A total of 1795 ± 34 nmol of ATP/ 10^6 cells/h was produced in control cultures. This was reduced by 47% in G/L-Low cultures (Tukey's post hoc test, $p < 0.05$), whereas in G/L-High cultures, it was increased by 41% ($p < 0.05$). We also analysed the energy efficiency of cells by calculating the ATP production on a carbon mol basis (i.e., total ATP produced per total C-mol substrate consumed), considering a contribution of 6 carbons for the hexoses (i.e., glucose and galactose) and 3 carbons for lactate. The control cultures showed an energy efficiency of 2.7 mol ATP/C-mol of glucose, while G/L-Low and G/L-High cultures presented 5.5 and 6.5 mol ATP/C-mol of galactose and lactate. This means that cells produced 2 and 2.4 times more ATP on a carbon mol basis in G/L-Low and G/L-High cultures, respectively, when compared to glucose culture. Therefore, CHO cells were more energy efficient when consuming galactose and lactate.

Figure 5. Balance of ATP produced and consumed in CHO chemostat cultures at steady state. A represent the specific ATP production in glucose (*Black*), G/L-Low (*Grey*) and G/L-High (*White*) cultures. For calculation of ATP generated by ATP synthetase, P/O ratios of 2.5 and 1.5 for NADH and FADH₂, respectively, were assumed. B and C represent the break-down of ATP contribution/utilization in different pathways. In all cultures, the main ATP production source was oxidative phosphorylation (OXPHOS), while most of the energy produced was derived to maintenance energy. Main differences in ATP production and consumption between the control and G/L cultures were observed in glycolysis contribution/utilization. Values represent intracellular fluxes (nmol/ 10^6 cells/h) $\pm$ estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study.

For all cultures, a major amount of ATP was produced in oxidative phosphorylation (OXPHOS) followed by glycolysis. The percentage of ATP produced in glycolysis in G/L-Low (6.3%) and G/L-High (8.2%) was significantly lower compared to the control (23.3%) (Tukey's post hoc test, $p < 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). This was consistent with the decreased glycolytic

fluxes observed in these cultures. Considering that glycolysis did not contribute largely to ATP production and lactate was mostly metabolised by TCA cycle in G/L-Low and G/L-High cultures, the amount of lactate in the feed had a considerable impact on the energetic status of cells. Differences in ATP production were also reflected in theoretical specific oxygen consumption rates (qO_2 , reaction v74 Table A.1., A.2., Supplementary file) in cultures. The qO_2 was 223 ± 20 nmol/ 10^6 cells/h in the control cultures, while it was decreased by 41% in G/L-Low ($p < 0.05$) and increased by 75% in G/L-High ($p < 0.05$). However, no statistical variations were observed in the respiratory quotient ($RQ = Y_{CO_2/O_2}$) and the oxygen consumption to ATP production ratio ($Y_{O_2/ATP}$).

Most of the produced energy was used for maintenance in all culture conditions. Although cell maintenance energy is not a concept precisely defined, we considered it as the ATP that is not directly consumed by biomass synthesis (i.e., synthesis of DNA, RNA, cell protein, fatty acids and polysaccharides) and glycolysis reactions (i.e., hexokinase and phosphofructokinase) in our metabolic model. Therefore, maintenance energy included ATP involved in turnover of macromolecules, re-establishment of ion gradients across the cell membrane and synthesis of the r-protein and nucleotide sugars (glycosylation)(Varma and Palsson, 1995), among other things. We observed that a decrease in maintenance ATP flux negatively impacted on hr-tPA production and quality. However, when increasing this flux beyond a certain threshold, no significant variation was observed in hr-tPA production and quality of protein was even impaired (Figure 6). The replacement of glucose for galactose and lactate not only entailed a decrease in the availability of energy, but also a decrease in the carbon resources for nucleotide sugars, that may explain the impairment of hr-tPA quality in G/L-Low and G/L-High cultures (Table 1). An overall decrease in the fluxes for nucleotide sugars (i.e., UDP glucose, GDP mannose, UDP galactose and UDP- GlcNAc) and glycosylation of the hr-tPA in G/L cultures compared to the control (Figure 4C, Table S2). Moreover, total ATP used for biomass synthesis was higher in the control cultures (135 ± 3 nmol of ATP/ 10^6 cells/h) than G/L-Low (116 ± 1.6 nmol of ATP/ 10^6 cells/h) and G/L-High (102 ± 2 nmol of ATP/ 10^6 cells/h) cultures. This correlates with the overall decrease in metabolic fluxes through pathways involved in biomass synthesis, which showed a significant decrease in G/L-Low and G/L-High compared to the control.

419

420 **Figure 6. Relationship between ATP derived to maintenance energy and the hr-tPA**
421 **production (hr-tPA flux), and molar ratio of Sialic acid and hr-tPA (SA:rh-tPA ratio).** The
422 corresponding culture conditions are detailed in the figure using a segmented vertical line.

423

424 4. Discussion

425 Cultivation of recombinant CHO cells usually presents high rates of glucose consumption beyond
426 cellular needs that inevitably drive to large amounts of metabolic waste products, such as lactate
427 (C. Altamirano et al., 2000). Since lactate accumulation in culture negatively impacts cell growth, r-
428 protein production and quality (Buchsteiner et al., 2018; Ma et al., 2009), it comes as no surprise,
429 therefore, that many strategies are focused on the modulation of cell metabolism for reducing
430 lactate production. Utilisation of galactose has been extensively studied together with glucose in
431 biphasic culture strategies to increase biomass and productivity while attenuating lactate
432 accumulation (Altamirano et al., 2006; Karst et al., 2017; Liu et al., 2015; Sun et al., 2013).
433 Compared to glucose-based feeds, this strategy has often improved cell growth and r-protein
434 production. Such improvements have been associated with a metabolic shift to lactate utilisation
435 when glucose is depleted and galactose is co-consumed with the produced lactate (Altamirano et
436 al., 2006). In the present study, we aimed to study the impact of a combined feeding strategy of
437 galactose and lactate (glucose-free) on culture performance and cell metabolism in CHO cells using
438 chemostat cultures. As in our previous studies, the use of chemostat cultures operated at steady
439 state offers the opportunity to better understand the metabolic effects of culture environment
440 changes in a defined physiological state (by setting the dilution rate). Additionally, even though this
441 modality is not very representative of industrial cell cultures processes (mostly high density fed-
442 batch or perfusion cultures), it enables evaluation of the isolated effect of feeding strategies without
443 the consequent changes in specific cell growth and excessive accumulation of products (e.g., waste
444 metabolites, HCP or r-protein) (Berrios et al., 2011; Vergara et al., 2018, 2014).

While the use of a galactose and lactate-based feeding strategy sustained cell growth and viability in our experiments, r-protein production and quality were significantly compromised. In our previous study with this cell line, replacing glucose and glutamine by galactose and glutamate in batch cultures resulted in a significant decrease in maximum viable cell density and cell growth rate (Altamirano et al., 2000). However, when galactose was supplemented in biphasic culture strategies, it supported cell growth (at lower rates) and maintained viability in batch and fed-batch cultures after glucose depletion in the medium (Altamirano et al., 2006; Liu et al., 2015; Sun et al., 2013). Similar results were shown in perfusion cultures (at steady state) in which addition of galactose (10 mM) at late culture stages did not impact growth kinetics (Karst et al., 2017). CHO cells may consume galactose as a primary carbon source, but as its consumption is at a very low rate, they need an additional carbon source (in this case lactate) to fulfil their carbon and energy requirements.

This was not the case for r-protein production and quality for our chemostat cultures, which unexpectedly showed decreased productivity and sialic acid content when feeding galactose and lactate (Figure 1, Table 1). Mammalian cell lines that were able to consume lactate (due to a metabolic shift) were strongly correlated to an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Several studies have associated this increase in r-protein production under a lactate consuming state with an enhanced oxidative metabolism and metabolic efficiency (Charaniya et al., 2010; Luo et al., 2012; Mulukutla et al., 2012; Sun et al., 2013; Templeton et al., 2013). In a recent work reviewing the molecular aspects of lactate metabolism, the authors suggested that lactate consuming state increases ATP yield from oxidative metabolism and better equipped cells to maintain their redox balance, thus allowing cells to improve overall cell health and meet the energy requirements for protein synthesis (Hartley et al., 2018). Nevertheless, this study has shown that an increased energy availability and efficiency did not lead to an increase in r-protein production in CHO cells (Figure 6). Moreover, supplementation of galactose has proven to improve glycosylation of r-protein at different degrees in CHO cell cultures (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018), by supporting production of more complex glycans with higher content of galactose and sialic acids moieties as terminal units (Karst et al., 2017). However, all cases were evaluated with a previous presence or in presence of glucose

in medium (both at low or high concentration) that impacted on the availability of precursors for glycosylation. When eliminating glucose from feeds in our chemostat cultures, we observed an overall decrease in carbon fluxes through glycolysis and synthesis of nucleotide sugar (Figure 3, 4C). Similarly, Sou et al. (2015) observed decreased amounts of nucleotide sugars (i.e., UDP-Glc, UDP-Gal and UDP- GlcNAc) when glucose consumption was reduced due to a temperature down-shift (Sou et al., 2015). Therefore, a feeding strategy based on galactose and lactate decreased the availability of metabolic intermediaries for glycans and showed that culture strategies only focused on either channelling more carbon into the TCA or improving mitochondrial activity seem to be not enough to improve the overall performance of a CHO cell line. Future process or metabolic engineering strategies should balance glycolysis and TCA cycle activities in order to provide adequate amounts of intermediaries for biomass, r-protein and glycans biosynthesis.

In this study, we have reported that co-consumption of galactose and lactate led to a better-balanced and efficient metabolism in CHO cells. This was observed on one hand, by decreased use of carbon sources and amino acids consumption (without affecting cell growth and viability) (Figure 2, Table 2) and, on the other hand, by higher production of ATP on a carbon mol basis. Most of the mammalian cells that underwent a lactate metabolic shift presented a decrease in consumption of glucose (when it is still available on culture medium) and amino acids (Liste-Calleja et al., 2015; Mulukutla et al., 2012; Wilkens et al., 2011). This is often accompanied by decreased glycolytic fluxes and a drop in specific cell growth rate (Gray et al., 2012; Mulukutla et al., 2012). Similar metabolic behaviour has been observed in biphasic culture strategies using glucose and galactose during the co-consumption phase of galactose and lactate (Altamirano et al., 2006; Sun et al., 2013; Wilkens et al., 2011). Several studies have shown that lactate consuming state (after a metabolic shift induced either by pH, temperature or change in media composition) presented a more energy efficient metabolic state compared to lactate producing state (Gray et al., 2012; Ivarsson et al., 2015; Mulukutla et al., 2012). A metabolome analysis of CHO cells indicated that lactate consuming cells presented an increased TCA cycle capacity and a more robust mitochondrial function (Luo et al., 2012). Such a difference in energy production lies in the fact that large proportion of lactate is metabolised in the TCA cycle during the lactate consumption phase, while most of the pyruvate

during the lactate production phase (growth based on glucose) is converted into lactate (Gray et al., 2012; Mulukutla et al., 2017). In a theoretical calculation, cells may produce up to 14 times more energy in a lactate consuming state compared to a producing state if it is completely metabolised in the TCA cycle (Gray et al., 2012). Hence, this naturally brings us to the question of defining the main reasons driving this metabolic behaviour (lactate consumption).

The answer to this question, unfortunately, is not a simple one. Various process events have been reported to trigger a shift from lactate production to utilisation including depletion of carbon/energy sources (i.e., glucose and/or glutamine) in medium (Altamirano et al., 2006, 2004; Wahrheit et al., 2014; Zagari et al., 2013), cell growth cessation after reaching maximal cell numbers (Carinhas et al., 2013; Ma et al., 2009), temperature shift (Torres et al., 2018b), increased oxidative capacity by addition of copper (Luo et al., 2012; Yuk et al., 2015), and unbalanced lactate or H^+ concentrations (Li et al., 2012; Liste-Calleja et al., 2015). In our experiments, increasing extracellular lactate concentration led CHO cells to consume lactate, although doubling lactate concentration of the feed did not result in an equivalent increase in lactate consumption (Table 1). It was noteworthy that, when comparing the control and G/L-High cultures, they presented the same lactate residual concentration and pH (H^+ concentrations), but completely different metabolic behaviours. One of several hypotheses explaining this phenomenon is related to the carbon flux through glycolysis. High glycolytic flux (usually observed under glucose-based growth) results in large accumulation of pyruvate that cannot be processed by mitochondria. This leads to production of large amount of alanine and lactate (Lu et al., 2005; Ma et al., 2009). When the glycolytic flux decreases, a drop in pyruvate pools occurs and lactate consumption emerges as an alternative for supplying the required carbon (Hartley et al., 2018). A similar scenario was observed in our chemostat cultures fed with galactose and lactate, in which fluxes through glycolysis were dramatically reduced compared to the control (Figure 5).

The main novelty of this study is the evaluation of a combined feeding strategy of galactose and lactate as a means to decrease lactate accumulation in CHO cell cultures. To our knowledge, this is the first study showing that a free-glucose feeding strategy based on lactate may support cell

growth and enhance energy metabolism. On the other hand, the use of constrained-based MFA provided a deeper understanding of the physiology of CHO cells in cultures. We presented evidence that suggest that improving efficiency of metabolism and increasing ATP yield do not always lead to an improved culture performance. Further applications of the MFA in combination with transcriptome and metabolome data can be used to guide engineering efforts to improve r-protein production.

5. Conclusion

The principal aim of this study was to study the impact of simultaneous feeding galactose and lactate on the culture performance and cell metabolism of a rh-tPA producing CHO cell line using chemostats and metabolic flux analysis. Our results indicated that a combined feeding strategy of galactose and lactate did not significantly impact on cell growth and viability, but it did negatively impact on rh-tPA productivity, quality and cell metabolism. Increasing galactose and lactate concentrations in the feed led to a high rh-tPA production, although it did not improve rh-tPA quality (compared to glucose and G/L-Low). This feeding strategy also dramatically decrease consumption of the primary carbon source (i.e., galactose) and led to very low glycolytic fluxes in G/L-Low and G/L-High cultures. Feeding lactate promoted lactate consumption in CHO cells and drove to high fluxes through TCA cycle and an improved energy metabolism. The present study provides a better understanding of CHO cell metabolism in galactose and lactate. However, the success of this feeding strategy in industrial culture processes, such as fed-batch or perfusion, is a question that arises for future research on this topic.

6. Appendices

Table A.1 Metabolic network used for the estimation of intracellular fluxes.

Table A.2 Intracellular fluxes [nmol/10⁶cells/h] of CHO cell chemostat cultures at steady-state.

554 Conflict of interest statement

555 Nothing declared.

556

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Research article

Metabolic flux analysis during galactose and lactate co-consumption reveals enhanced energy metabolism in continuous CHO cell cultures

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21 Abstract

22 Chinese Hamster Ovary (CHO) cells are the main expression system for production of therapeutic
23 recombinant (r-) proteins. The increased demand for these therapeutics has boosted the
24 development of more robust production processes. Use of optimised feed/media has dramatically
25 improved the performance of CHO cells in culture. However, such progress in biomass synthesis
26 and r-protein production often come with an increased accumulation of lactate. In this study, we
27 present a combined feeding strategy that uses galactose and lactate to replace glucose in CHO cell
28 cultures. Replacement of glucose by galactose and lactate sustained cell growth and r-protein
29 production in CHO cells. This strategy supported a better-balanced and more efficient metabolism,
30 observed by an overall decreased consumption of carbon sources and amino acids, associated with
31 an increased ATP production per C-mol consumed. Our results provide new insights of CHO cell
32 metabolism in glucose-free media based on galactose and lactate.

1. Introduction

Chinese hamster ovary (CHO) cells are currently the predominant host to manufacture of recombinant (r-) therapeutic proteins for clinical trials and commercial sales. Their capacity of performing human-like post-translational modifications (PTMs) enables the synthesis of proteins with the appropriate critical quality attributes (CQAs) (e.g., glycosylation) that impact upon the potency and immunogenicity of therapeutic proteins (Wurm, 2004). In recent years, substantive improvements in final product titres and viable cell densities have been achieved in CHO-based culture processes (Dickson, 2014). However, one of the disadvantages of the cultivation of CHO cells is their deregulated metabolism, which is characterised by a large consumption of glucose with a consequent greater production of growth inhibitory products such as lactate (Hartley et al., 2018; Torres et al., 2018a). High concentration of lactate limits cell growth and negatively impacts productivity and CQAs of the r-proteins. In pH-controlled bioreactors, a drop in medium pH due to lactate accumulation is usually fixed by addition of base throughout the time course of culture process. Nevertheless, this leads to an increase in osmolarity in culture medium that, itself, can adversely impact the culture performance (decreased cell growth and viability) (Buchsteiner et al., 2018; Cruz et al., 2000; Ma et al., 2009). Therefore, development of low lactate-producing cell culture processes remains a major challenge for improvement of process robustness in the field of therapeutic r-protein production.

Different strategies have been proposed (with different degrees of success) to alleviate production and accumulation of lactate in cultures. Replacement of glucose with an alternative, slowly-consumed carbon source (Altamirano et al., 2006; C. Altamirano et al., 2000) or alteration of the balance of nutrients in media (Camps et al., 2018; Ivarsson et al., 2015; Li et al., 2012) have proven successful at limiting production of lactate in culture. Previously, our group demonstrated that substitution of the primary carbon and nitrogen sources (i.e., glucose and glutamine) with galactose and glutamate, respectively, led to a significantly decreased rate of lactate production (C. Altamirano et al., 2000). However, with the implementation of this strategy, cell growth was dramatically decreased. In a subsequent study, a biphasic culture strategy using glucose and

galactose as primary and secondary carbon sources was designed to overcome the growth limitations observed in wholly galactose-based cultures. While the initial phase was characterised by a rapid cell growth with high rates of glucose consumption and lactate production, the second phase, when glucose was depleted, was characterised by the consumption of both galactose and lactate (produced in the initial phase) (Altamirano et al., 2006; Sun et al., 2013). To understand this phenomenon, Wilkens et al., (2011) studied lactate metabolism in CHO cell batch cultures growing in glucose and galactose using a metabolic flux analysis approach. These authors suggested that the lactate switch (i.e., from production to consumption) occurred when cytosolic pyruvate concentration was insufficient for cells to support their energy requirements, and that lactate uptake emerges as an alternative to incorporate carbon into the TCA cycle and improve energy metabolism (Wilkens et al., 2011). Similar observations were made in two different CHO cell lines (Gray et al., 2012; Sun et al., 2013) and in a mouse myeloma cell line (NS0) (Mulukutla et al., 2012) that underwent a lactate switch. Whilst galactose supplementation had little impact on r-protein production in CHO cell cultures, it did alter r-protein glycosylation (Karst et al., 2017; Liu et al., 2015; Sun et al., 2013; Zhang et al., 2018). Liu et al. (2015) observed that galactose addition (more than 20 mM) increased the total sialic acid content (44 %) of a monoclonal antibody (mAb) in fed-batch cultures. Similar observations have been made in batch, fed-batch and perfusion (operated at steady state) cultures of CHO cells expressing different r-proteins (e.g., interferon- γ and mAbs) as a result of galactose supplementation (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018). However, the impact of galactose on r-protein quality seems to be cell-line dependent, since other CHO cell lines have not shown any improvements in glycosylation (at least when galactose is below an optimal concentration) (Liu et al., 2015) or even an increased potential for desialylation (Clark et al., 2005).

Rational design of feed media has also considered lactate by itself as a carbon source. Li et al. (2012) designed a lactate-containing feed medium that supplies energy to cells and controls culture pH. This study demonstrated that lactate can be effectively used as a carbon source during certain stages of culture (in the presence of glucose) with comparable results regarding cell growth, productivity and CQAs to glucose-fed cultures (Li et al., 2012). Although feeding lactate can tip the

balance towards the lactate consumption phenotype and effectively decrease lactate production, the success of this strategy greatly relies on the cell capacity of consuming lactate. Freund and Croughan (2018) showed that lactate supplementation (at 35 mM) was able to eliminate the net production of lactate in CHO cells. Additionally, when CHO cells were cultured in lactate-supplemented medium for several passages (over 40 days), they presented a significantly decrease in lactate production in batch and fed-batch cultures (Freund and Croughan, 2018). The capacity of mammalian cells to consume lactate has captured great attention for researchers and companies, since it has been strongly correlated with an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Despite evidence of reported benefits of feeding galactose and/or lactate in CHO-based cultures, there is no available study, to our knowledge, using a combination of both components as a feeding strategy for improving culture performance.

In the present study, we aim to investigate and characterise the impact of substituting glucose with galactose and lactate on the modulation of culture performance and cell metabolism of a hr-tPA producing CHO cell line. To achieve this, we relied on the capacity of chemostat culture to set the specific growth rate to a desired value and study culture performance and metabolic changes at steady state. The response of growth, r-protein production, quality and cell metabolism were evaluated in chemostat cultures fed with galactose and lactate at two different concentrations (low and high concentration: G/L-Low and G/L-High, respectively) and compared to a glucose-fed culture as control. Additionally, we performed a metabolic flux analysis to explore in-depth the metabolic impact of using galactose and lactate as the major carbon sources.

2. Material and methods

2.1. Cell line and medium

The rh-tPA-producing cell line (CHO TF 70R) was obtained from Pharmacia & Upjohn S.A. (Sweden) (a kind gift of Torsten Björlig). Glucose- and glutamine-free SFM4CHO cell culture medium (HyClone, Logan, USA) supplemented with glucose (G7021, Sigma, USA), galactose (G5388, Sigma, USA) and lactate (L1875, Sigma, USA) to define three different culture conditions. Culture medium and feed at each experimental condition were supplemented with 6 mM glutamate (G8415, Sigma, USA).

2.2. Chemostat cultures

Before chemostat culture experiments, cells were routinely sub-cultured every 48 h in batch systems using T-75 flasks and scaled up in spinner flasks (Techne, UK) by reseeding at 0.25×10^6 cells/ mL in fresh growth medium (glucose-based medium). Chemostat cultures were performed in 250 mL spinner flasks (Techne, UK) specially conditioned for continuous operation with a working volume of 150 mL. Cultures were initially operated in batch mode for 48 h and were then supplied with sterile feed throughout the period of operation (Berrios et al., 2011). All cultures were incubated in a Forma™ Series II 3110 Water-Jacketed CO₂ Incubator (Thermo Fisher Scientific, USA) at 50 rpm with 96% humidity and 5% CO₂ enriched atmosphere.

To evaluate the effect of glucose substitution with galactose and lactate in the feed, two different medium composition were evaluated: low concentration of galactose (10 mM) and lactate (4 mM) (G/L-Low) and high concentration of galactose (20 mM) and lactate (8 mM) (G/L-High). Control cultures were performed by feeding 10 mM glucose (GLC). All chemostat culture experiments were designed by maintaining a dilution rate at 0.015 1/h, controlled by a low-flow peristaltic pump (Ismatec, Germany) (Berrios et al., 2011). Sampling was carried out every 24 h for determining cell number, viability and pH. After cell counting, supernatant was frozen at -20°C until further analysis. Cultures were considered to reach steady state when, after at least five residence times, both the number of viable cells and residual substrate (i.e., glucose, galactose and lactate) concentration were constant for two consecutive samples (Altamirano et al., 2001).

2.3. Analytical techniques

Cell density and viability were determined by the trypan blue exclusion method (T8154, Sigma, USA) (1:1 mixture of 0.2% (w/v) trypan blue in saline and cell sample) using a hemocytometer (Neubauer, Germany). Glucose, galactose and lactate were measured with an automatic biochemistry analyser (YSI 2700, Yellow Springs Inc., USA). Ammonium ion and amino acid concentrations were measured with a PerkinElmer Series 200 HPLC (PerkinElmer, USA) using a C-18 reversed-phase column (AccQ-Tag Column, 3.9mm×150mm, Waters) and AccuTag kit (Waters, USA) according to the manufacturer's instructions.

Extracellular rh-tPA was quantified by enzyme immunoassay (Trinilize tPA-antigen kit, Tcoag Ltd., Ireland). For the analysis of the sialic acid content, rh-tPA was purified by affinity chromatography in fast protein liquid chromatography system (FPLC, Pharmacia, Sweden) with erythrin-trypsin-inhibitor immobilised in CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences, UK) (Heussen et al., 1984). The purification process was checked by SDS-PAGE with 10–15% gradient gels (PhastSystem, Pharmacia, Sweden). The sialic acid was removed from the glycan of purified rh-tPA according to Fu and O'Neill (1995). Each sample was incubated at 37°C for one hour with 0.5 U mL⁻¹ of each enzyme (neuraminidase and neuraminic acid aldolase) simultaneously. Manosamine was subsequently quantified by PerkinElmer Series 200 HPLC (PerkinElmer, USA) using fluorescence detection (excitation 360 nm, emission 425 nm) and a C-18 reversed-phase column (Waters, Ireland). Sample derivatisation was carried out with anthranilic acid according to Anumula and Du (1999).

2.4. Cell-specific rate calculations

Specific growth rate (μ) (Eq1.) was calculated from the mass balance within the bioreactor:

$$\mu = D(N_t/N_v) \quad \text{Eq1.}$$

where N_v is the concentration of viable cells (10^6 /mL), N_t is the concentration of total cells (10^6 /mL), and D is the dilution rate of the culture (1/h) (Vergara et al., 2015, 2014).

Specific rates of production or consumption (Eq2.) of metabolite i (q_i) were calculated from the mass balance within the reactor:

$$q_i = D((C_i^i - C_i^0)/N_v) \times 10^9 \quad \text{Eq2.}$$

where C_i^i is the concentration of i in the inlet (mmol/L), C_i^0 is the concentration of i in the outlet (mmol/L), N_v is the concentration of viable cells ($10^6/\text{mL}$), and D is the dilution rate of the culture (1/h).

2.5. Metabolic flux analysis

Metabolic flux analysis (MFA) was performed using a reduced metabolic network model from a *Mus musculus* genome-scale metabolic reconstruction, which adequately represents core mammalian metabolism and can be directly applied to cell culture flux experiments (Quek and Nielsen, 2008). The model included the elemental biomass composition and the rh-tPA production reaction obtained from our previous study (Berrios et al., 2011). The intracellular fluxes (v) were calculated using the metabolite balancing constraints $S \cdot v = 0$, whereby S is the stoichiometric matrix derived from the metabolic model. For the determination of intracellular fluxes, the underdetermined system was solved by linear programming using the measured extracellular metabolite concentrations, the biomass synthesis rate and the specific rh-tPA productivity as constraints (Quek et al., 2010). The constraint $v \geq 0$ was imposed on all irreversible reactions, while the lower and upper boundary values of measured fluxes were specified using the measured cell-specific consumption or production rates and the estimated standard error ($v_{\text{measured}} \pm \text{SE}_{\text{measured}}$). Metabolic flux calculation was performed using MATLAB (The Mathworks) as previously described by Quek et al., (2010) (Quek et al., 2010). Estimated fluxes were also used to estimate the ATP production (Gray et al., 2012). The metabolic model is fully detailed in Table S1.

2.6. Statistical analysis

Kinetic and stoichiometric parameters were calculated from at least two independent experiments and are expressed as the mean $\pm$ SE. All statistical analyses were performed with R software

(version 3.1.)(R Core Team, 2014). The significant variation of the feeds among physiological parameters was evaluated by one-way ANOVA (using the feed composition – 3 levels—as factor) followed by multiple comparison tests (Tukey HSD test) with normally distributed data. The threshold for statistical significance was $p < 0.05$.

3. Results

To investigate the impact of glucose substitution for galactose and lactate in the feed on culture performance and cell metabolism, we performed steady state chemostat cultures in spinner flasks using a recombinant CHO cell line producing hr-tPA at low and high concentrations of galactose and lactate (G/L-Low and G/L-High, respectively), and glucose (GLC) as control. All chemostats at each experimental condition reached steady state after five residence times and cell viability remained above 95% for all cultures (Figure 1). From each cultivation, viable cell density (VCD), hr-tPA production, quality, and cell metabolism were evaluated. For a better understanding of the metabolic changes associated with the simultaneous galactose and lactate consumption, a metabolic flux analysis was performed in each culture condition using the experimental data at steady state.

3.1. Cell growth and protein production

Substitution of glucose for galactose and lactate in the feed did not impact significantly on viable cell densities (VCDs), cell viabilities and specific cell growth rates in cultures at steady state (One-way ANOVA, $p > 0.05$). Although chemostat cultures showed higher VCDs and cell viability using G/L-Low ($0.87 \pm 0.05 \cdot 10^6$ cells/mL and $97.9 \pm 1.2\%$) and G/L-High ($0.93 \pm 0.05 \cdot 10^6$ cells/mL and $99.5 \pm 0.4\%$) feeds compared to GLC ($0.79 \pm 0.04 \cdot 10^6$ cells/mL and $95 \pm 1.2\%$) (Figure 1), these differences were not statistically significant (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). Specific cell growth rate was calculated from the relationship between the dilution rate ($D = 0.015 \text{ 1/h}$) in which the chemostat cultures were set up and cell viability (Eq. 1) (Figure 1B). The specific cell growth rate was slightly decreased in G/L-Low and G/L-High in comparison with GLC cultures. However, as with VCDs and cell viability, no statistical significance

was observed among the culture feed conditions (Tukey's post hoc test, $p > 0.05$ for GLC vs G/L-Low and GLC vs G/L-High).

Figure 1. Performance of CHO cells in chemostat cultures at steady state. A, B, C and D correspond to the viable cell density (VCD), the cell viability and specific growth rate, the hr-tPA production and the specific hr-tPA productivity of cultures, respectively. (*Black*) glucose; (*Grey*) G/L-Low; (*White*) G/L-High. Experimental data was statistically analysed using one-way ANOVA (using the feed condition – 3 levels - as a factor) followed by a Tukey HSD test using normalised data. P values below 0.05 indicates statistically significant values among the culture conditions.

Production of hr-tPA was evaluated and compared among the different feeds through the final product titre and specific productivity (q_{hr-tPA}) at steady state (Figure 1C). A maximum hr-tPA titre of 509 ± 20 ng/mL and q_{hr-tPA} of 9.7 ± 0.1 ng/ 10^6 cells/h was achieved in the control (Figure 1D). Changes in the feed composition had a significant impact on the hr-tPA production at steady state (One-way ANOVA, $p < 0.05$). The G/L-Low cultures reduced hr-tPA titre by 62% (Tukey's post hoc test, $p < 0.05$) and q_{hr-tPA} by 51% ($p < 0.05$), while the G/L-High cultures decreased hr-tPA titre by 22% ($p < 0.05$) and q_{hr-tPA} by 16% ($p < 0.05$) when compared to control cultures. Additionally, variations in r-protein quality were evaluated by determining sialic acid (SA) content of hr-tPA (mol_{SA}/mol_{hr-tPA}) and calculating specific hr-tPA sialylation rate ($q_{SA\ on\ tPA}$) at each condition (Table 1). One-way ANOVA indicated that protein quality was significantly impacted by the feed composition ($p < 0.05$). The highest SA content of hr-tPA and $q_{SA\ on\ tPA}$ were found in the control cultures. The SA content of hr-tPA and $q_{SA\ on\ tPA}$ were decreased by 25% (Tukey's post hoc test, $p < 0.05$) and by 57% ($p < 0.05$) in G/L-Low cultures, respectively, while they were decreased by 15% ($p < 0.05$) and by 46% ($p < 0.05$) in G/L-High cultures when compared to GLC cultures. Therefore, the elimination of glucose in the medium negatively impacted on the hr-tPA production and quality.

Table 1. Sialic acid content of hr-tPA ($\text{mol}_{\text{SA}}/\text{mol}_{\text{hr-tPA}}$) and specific hr-tPA sialylation rates ($q_{\text{SA on tPA}}$) in CHO chemostat cultures at steady state.

Parameters	Units	GLC	G/L-Low	G/L-High
SA/t-PA	mol/mol	3.3 ± 0.2	2.5 ± 0.1	2.8 ± 0.2
$q_{\text{SA on tPA}}$	$\text{nmol}/10^9 \text{ cells/h}$	0.52 ± 0.05	0.22 ± 0.02	0.28 ± 0.01

3.2. Cell metabolism

To investigate the effect of glucose replacement for galactose and lactate in the feed on cell metabolism, we analysed the variations in consumption and/or production of glucose, galactose, lactate (Table 2) and amino acids (Figure 2) in the chemostat cultures at steady state. Control chemostat cultures consumed 57% of the fed glucose and reached a specific glucose consumption rate (q_{GLC}) of $110.9 \pm 2.8 \text{ nmol}/10^6 \text{ cells/h}$ at steady state. The replacement of glucose by galactose significantly decreased the consumption of the main carbon source (One-way ANOVA, $p < 0.05$), as expected. In G/L-Low cultures, cells only consumed 12% of the galactose and their specific galactose consumption rate (q_{GAL}) was 80% lower than the q_{GLC} in control cultures (Tukey's post hoc test, $p < 0.05$), while in G/L-High cultures, only 16.5% of the galactose was consumed and the q_{GAL} was decreased by 48% compared to the q_{GLC} in glucose-based cultures ($p < 0.05$). An increased concentration of galactose in the feed from 10 to 20 mM increased the q_{GAL} 2.6- fold ($p < 0.05$). Lactate metabolism is certainly a different matter for comparison since the conditions under study involve this metabolite as a substrate. Feeding lactate (simultaneously with galactose) in chemostat cultures effectively supported cells to consume lactate at both concentration in the study. An increased lactate concentration from 4.2 to 8.5 mM in the feed increased the specific lactate consumption rate (q_{LAC}) in 41% ($p < 0.05$), although feeding high concentration of lactate led to a similar residual lactate concentrations compared to the control (Table 2). Therefore, even though feeding lactate avoided its production, overfeeding lactate to the

culture (beyond the evaluated concentrations) might increase its accumulation in the medium at detrimental levels for cell growth and productivity. Residual medium pH was also measured and compared to the feeds to evaluate if differences in H⁺ gradient may induce a lactate consumption state in G/L cultures (Table 2). A slight decrease in pH was observed in all conditions, but no significant variations were observed between GLC and G/L (Low and High) cultures that link the lactate consuming phenotype with a dramatic decrease in the medium pH.

Table 2. Comparison of metabolic parameters of CHO cells in chemostat cultures at steady state.

	Units	GLC	G/L-Low	G/L-High
Hexose fed	mmol/L	10	10	20
Hexose res	mmol/L	4.3 ± 1.14	8.8 ± 2.22	16.7 ± 1.11
qHex	nmol/10 ⁶ cells/h	- 110.9 ± 2.8	- 22.2 ± 1.9	- 57.6 ± 3.5
Lactate fed	mmol/L	--	4	8
Lactate res	mmol/L	7.3 ± 0.2	3.5 ± 0.1	7.4 ± 0.3
qLac	nmol/10 ⁶ cells/h	142.0 ± 2.31	- 13.2 ± 1.11	- 18.7 ± 1.18
Y _{Lac/Hex}	mol/mol	1.29 ± 0.05	--	--
NH ₄ ⁺ res	mmol/L	0.44 ± 0.03	0.21 ± 0.01	0.22 ± 0.02
Y _{NH₄/GLU}	mol/mol	0.45 ± 0.03	0.35 ± 0.01	0.22 ± 0.03
pH fed	--	6.8	6.8	6.8
pH res	--	6.39 ± 0.04	6.55 ± 0.03	6.34 ± 0.05

Amino acid metabolism was analysed by determining their residual concentration and calculating their specific rates of consumption/production in relation to their concentrations in the feed. Chemostat cultures showed an overall consumption of amino acids in the feed, except for alanine, glycine and aspartate (Figure 2). This increased residual concentration of alanine, glycine and

aspartate has been previously observed in the hr-tPA producing CHO cell line (Altamirano et al., 2006). One-way ANOVA indicated that specific consumption and production rates of amino acids were significantly impacted by feeding galactose and lactate ($p < 0.05$). In response to addition of galactose and lactate, cells decreased the specific consumption rates in most of the amino acids. For instance, specific consumption rate of glutamate (q_{GLU}) decreased by 25% in G/L-Low and by 17% in G/L-High cultures compared to the control. Another case is serine and glycine, of which their specific consumption and production rates (q_{SER} and q_{GLY}) decreased up to 80% and up to 98% respectively, as more galactose and lactate were consumed. The specific production rate of alanine (q_{ALA}) maintained similar levels for all conditions. Ammonium production was also decreased when cells were fed with galactose and lactate. Residual ammonium was 0.44 ± 0.03 mM in the control and was decreased by 52% in G/L-Low ($p < 0.05$) and by 48% in G/L-High ($p < 0.05$) cultures. The same was observed in specific ammonium production rate (q_{NH4}) which was decreased by 42% in G/L-Low ($p < 0.05$) and by 58% in G/L-High ($p < 0.05$) cultures. This is consistent with the overall decrease in amino acid consumption observed in G/L-Low and G/L-High, particularly glutamate. The ratio ammonium/glutamate ($Y_{NH4/GLU}$) was also calculated for all cultures. While the control cultures achieved a $Y_{NH4/GLU}$ of 0.45 ± 0.03 mol/mol, these values decreased by 21% in G/L-Low ($p < 0.05$) and by 51% in G/L-High ($p < 0.05$) cultures.

Figure 2. Specific amino acid consumption/production and ammonia production rates (nmol/ 10^6 cells/h) in CHO chemostat cultures at steady state. (*Black*) glucose; (*Grey*) G/L-Low; (*White*) G/L-High. P values below 0.05 indicates statistically significant values between the conditions compared.

3.3. Metabolic flux analysis

To gain a better understanding of the metabolic status of CHO cells growing in galactose and lactate, we performed a metabolic flux analysis (MFA) using a constraint-based model of cell

metabolism with 91 reactions and 68 metabolites (Table S1, Supplementary file). The experimental data detailed above (i.e., the specific metabolite consumption/production rates, the specific cell growth rate and the specific hr-tPA productivity) were used as inputs to the metabolic model to constraint the theoretical solution space. At first glance, the MFA revealed that glucose replacement for galactose and lactate significantly decreased glycolysis fluxes, while it increased fluxes through both tricarboxylic acid (TCA) cycle and energy metabolism, particularly in G/L-High cultures (Figure 3). Intracellular fluxes through pentose phosphate pathway (PPP) were very low compared to the other two main pathways (i.e., glycolysis and TCA cycle) for all conditions. However, this seems to be because MFA cannot correctly resolve fluxes in the oxidative PPP (Quek et al., 2010). Therefore, the estimated fluxes for this pathway were mainly associated with the flux to ribose 5-phosphate needed for nucleotide synthesis. Lactate dehydrogenase (LDH) showed a large carbon flux in the control cultures, indicating most of the consumed glucose seemed to be converted into lactate. This was reversed in G/L-Low and G/L-High cultures, which presented an increased influx of carbon from lactate to pyruvate. The switch in the LDH flux in G/L-Low and G/L-High cultures impacted largely pyruvate and oxidative metabolism (Figure 4A). For instance, a dramatic increase in the fluxes through pyruvate dehydrogenase complex (PDC) and the TCA cycle was observed when lactate was consumed (Figure 4A). Although no changes in malate and alanine metabolism were observed, the flux from serine to pyruvate were largely decreased and even inverted in G/L-High cultures (Figure 4A). This reverse of serine flux in G/L-High cultures was apparently associated with a higher lactate consumption and a higher NADH demand for this reaction. The increased TCA fluxes did not affect the carbon flux from glutamate to the α -ketoglutarate in all cultures. However, it entailed a decrease in the activity anaplerotic pathways for G/L cultures (Figure 4B, Table S2).

Figure 3. Metabolic flux analysis in CHO chemostat cultures at steady state. The figure illustrates the flux distribution of the main metabolic pathways of central carbon metabolism of CHO cells (both in the cytosol and the mitochondria) cultured in chemostat fed with glucose (GLC) and

galactose and lactate (G/L-Low and G/L-High). In GLC fed cultures, cells present large glycolytic fluxes which are mainly diverted to lactate production, whereas in G/L cultures, they present an overall decrease in glycolysis and lactate consumption. Mitochondria are more metabolically active in G/L cultures, particularly in G/L-High, with glutamate and lactate as main carbon sources for ATP aerobic respiration. Values represent intracellular fluxes ($\text{nmol}/10^6 \text{ cells/h}$) $\pm$ estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study. The fluxes with positive values are in the direction indicated by the arrow, while negative fluxes go in the opposite direction.

Figure 4. Zoom-in of metabolic fluxes through pyruvate (A), α -ketoglutarate (B) and glycosylation (C) metabolism in CHO chemostat cultures at steady state. The fluxes in the figure were normalised to the values of GLC fluxes to compare the variation between G/L (Low and High) and control cultures. Values in the boxes described the fraction of fluxes for G/L-Low or G/L-High compared to GLC cultures.

As glycolysis and TCA cycle were the metabolic pathways more affected by feed composition, we analysed in detail the energy metabolism among culture conditions evaluated in this study. Total ATP production rates were calculated by assuming theoretical P/O ratios of 2.5 and 1.5 for NADH and FADH_2 , respectively, and adding ATP generated through substrate level phosphorylation (Gray et al., 2012; Ivarsson et al., 2015) (Figure 5). One-way ANOVA revealed that total ATP production rates were significantly impacted by feeding galactose and lactate ($p < 0.05$). A total of 1795 ± 34 nmol of $\text{ATP}/10^6 \text{ cells/h}$ was produced in control cultures. This was reduced by 47% in G/L-Low cultures (Tukey's post hoc test, $p < 0.05$), whereas in G/L-High cultures, it was increased by 41% ($p < 0.05$). We also analysed the energy efficiency of cells by calculating the ATP production on a carbon mol basis (i.e., total ATP produced per total C-mol substrate consumed), considering a contribution of 6 carbons for the hexoses (i.e., glucose and galactose) and 3 carbons for lactate. The control cultures showed an energy efficiency of 2.7 mol ATP/C-mol of glucose, while G/L-Low

and G/L-High cultures presented 5.5 and 6.5 mol ATP/C-mol of galactose and lactate. This means that cells produced 2 and 2.4 times more ATP on a carbon mol basis in G/L-Low and G/L-High cultures, respectively, when compared to glucose culture. Therefore, CHO cells were more energy efficient when consuming galactose and lactate.

Figure 5. Balance of ATP produced and consumed in CHO chemostat cultures at steady state. A represent the specific ATP production in glucose (*Black*), G/L-Low (*Grey*) and G/L-High (*White*) cultures. For calculation of ATP generated by ATP synthetase, P/O ratios of 2.5 and 1.5 for NADH and FADH₂, respectively, were assumed. B and C represent the break-down of ATP contribution/utilization in different pathways. In all cultures, the main ATP production source was oxidative phosphorylation (OXPHOS), while most of the energy produced was derived to maintenance energy. Main differences in ATP production and consumption between the control and G/L cultures were observed in glycolysis contribution/utilization. Values represent intracellular fluxes (nmol/10⁶ cells/h) ± estimated standard error in GLC and G/L cultures as determined by the MFA model developed within this study.

For all cultures, a major amount of ATP was produced in oxidative phosphorylation (OXPHOS) followed by glycolysis. The percentage of ATP produced in glycolysis in G/L-Low (6.3%) and G/L-High (8.2%) was significantly lower compared to the control (23.3%) (Tukey's post hoc test, $p < 0.05$ for GLC vs G/L-Low and GLC vs G/L-High). This was consistent with the decreased glycolytic fluxes observed in these cultures. Considering that glycolysis did not contribute largely to ATP production and lactate was mostly metabolised by TCA cycle in G/L-Low and G/L-High cultures, the amount of lactate in the feed had a considerable impact on the energetic status of cells. Differences in ATP production were also reflected in theoretical specific oxygen consumption rates (qO_2) in cultures. The qO_2 was 233 ± 7.6 nmol/10⁶cells/h in the control cultures, while it was decreased by 34% in G/L-Low ($p < 0.05$) and increased by 67% in G/L-High ($p < 0.05$). However, no statistical

variations were observed in the respiratory quotient ($RQ = Y_{CO_2/O_2}$) and the oxygen consumption to ATP production ratio ($Y_{O_2/ATP}$).

Most of the produced energy was used for maintenance in all culture conditions. Although cell maintenance energy is not a concept precisely defined, we considered it as the ATP that is not directly consumed by biomass synthesis (i.e., synthesis of DNA, RNA, cell protein, fatty acids and polysaccharides) and glycolysis reactions (i.e., hexokinase and phosphofructokinase) in our metabolic model. Therefore, maintenance energy included ATP involved in turnover of macromolecules, re-establishment of ion gradients across the cell membrane and synthesis of the r-protein and nucleotide sugars (glycosylation)(Varma and Palsson, 1995), among other things. We observed that a decrease in maintenance ATP flux negatively impacted on hr-tPA production and quality. However, when increasing this flux beyond a certain threshold, no significant variation was observed in hr-tPA production and quality of protein was even impaired (Figure 6). The replacement of glucose for galactose and lactate not only entailed a decrease in the availability of energy, but also a decrease in the carbon resources for nucleotide sugars, that may explain the impairment of hr-tPA quality in G/L-Low and G/L-High cultures (Table 1). An overall decrease in the fluxes for nucleotide sugars (i.e., UDP glucose, GDP mannose, UDP galactose and UDP- GlcNAc) and glycosylation of the hr-tPA in G/L cultures compared to the control (Figure 4C, Table S2). Moreover, total ATP used for biomass synthesis was higher in the control cultures (135 ± 3 nmol of ATP/ 10^6 cells/h) than G/L-Low (116 ± 1.6 nmol of ATP/ 10^6 cells/h) and G/L-High (102 ± 2 nmol of ATP/ 10^6 cells/h) cultures. This correlates with the overall decrease in metabolic fluxes through pathways involved in biomass synthesis, which showed a significant decrease in G/L-Low and G/L-High compared to the control.

Figure 6. Relationship between ATP derived to maintenance energy and the hr-tPA production (hr-tPA flux), and molar ratio of Sialic acid and hr-tPA (SA:rh-tPA ratio). The corresponding culture conditions are detailed in the figure using a segmented vertical line.

4. Discussion

Cultivation of recombinant CHO cells usually presents high rates of glucose consumption beyond cellular needs that inevitably drive to large amounts of metabolic waste products, such as lactate (C. Altamirano et al., 2000). Since lactate accumulation in culture negatively impacts cell growth, r-protein production and quality (Buchsteiner et al., 2018; Ma et al., 2009), it comes as no surprise, therefore, that many strategies are focused on the modulation of cell metabolism for reducing lactate production. Utilisation of galactose has been extensively studied together with glucose in biphasic culture strategies to increase biomass and productivity while attenuating lactate accumulation (Altamirano et al., 2006; Karst et al., 2017; Liu et al., 2015; Sun et al., 2013). Compared to glucose-based feeds, this strategy has often improved cell growth and r-protein production. Such improvements have been associated with a metabolic shift to lactate utilisation when glucose is depleted and galactose is co-consumed with the produced lactate (Altamirano et al., 2006). In the present study, we aimed to study the impact of a combined feeding strategy of galactose and lactate (glucose-free) on culture performance and cell metabolism in CHO cells using chemostat cultures. As in our previous studies, the use of chemostat cultures operated at steady state offers the opportunity to better understand the metabolic effects of culture environment changes in a defined physiological state (by setting the dilution rate). Additionally, even though this modality is not very representative of industrial cell cultures processes (mostly high density fed-batch or perfusion cultures), it enables evaluation of the isolated effect of feeding strategies without the consequent changes in specific cell growth and excessive accumulation of products (e.g., waste metabolites, HCP or r-protein) (Berrios et al., 2011; Vergara et al., 2018, 2014).

While the use of a galactose and lactate-based feeding strategy sustained cell growth and viability in our experiments, r-protein production and quality were significantly compromised. In our previous study with this cell line, replacing glucose and glutamine by galactose and glutamate in batch cultures resulted in a significant decrease in maximum viable cell density and cell growth rate (C Altamirano et al., 2000). However, when galactose was supplemented in biphasic culture strategies, it supported cell growth (at lower rates) and maintained viability in batch and fed-batch cultures after

glucose depletion in the medium (Altamirano et al., 2006; Liu et al., 2015; Sun et al., 2013). Similar results were shown in perfusion cultures (at steady state) in which addition of galactose (10 mM) at late culture stages did not impact growth kinetics (Karst et al., 2017). CHO cells may consume galactose as a primary carbon source, but as its consumption is at a very low rate, they need an additional carbon source (in this case lactate) to fulfil their carbon and energy requirements.

This was not the case for r-protein production and quality for our chemostat cultures, which unexpectedly showed decreased productivity and sialic acid content when feeding galactose and lactate (Figure 1, Table 1). Mammalian cell lines that were able to consume lactate (due to a metabolic shift) were strongly correlated to an increased r-protein production (Le et al., 2012; Torres et al., 2018b). Several studies have associated this increase in r-protein production under a lactate consuming state with an enhanced oxidative metabolism and metabolic efficiency (Charaniya et al., 2010; Luo et al., 2012; Mulukutla et al., 2012; Sun et al., 2013; Templeton et al., 2013). In a recent work reviewing the molecular aspects of lactate metabolism, the authors suggested that lactate consuming state increases ATP yield from oxidative metabolism and better equipped cells to maintain their redox balance, thus allowing cells to improve overall cell health and meet the energy requirements for protein synthesis (Hartley et al., 2018). Nevertheless, this study has shown that an increased energy availability and efficiency did not lead to an increase in r-protein production in CHO cells (Figure 6). Moreover, supplementation of galactose has proven to improve glycosylation of r-protein at different degrees in CHO cell cultures (Grainger and James, 2013; Gramer et al., 2011; Karst et al., 2017; Wong et al., 2010; Zhang et al., 2018), by supporting production of more complex glycans with higher content of galactose and sialic acids moieties as terminal units (Karst et al., 2017). However, all cases were evaluated with a previous presence or in presence of glucose in medium (both at low or high concentration) that impacted on the availability of precursors for glycosylation. When eliminating glucose from feeds in our chemostat cultures, we observed an overall decrease in carbon fluxes through glycolysis and synthesis of nucleotide sugar (Figure 3, 4C). Similarly, Sou et al. (2015) observed decreased amounts of nucleotide sugars (i.e., UDP-Glc, UDP-Gal and UDP- GlcNAc) when glucose consumption was reduced due to a temperature down-shift (Sou et al., 2015). Therefore, a feeding strategy based on galactose and lactate decreased the

availability of metabolic intermediaries for glycans and showed that culture strategies only focused on either channelling more carbon into the TCA or improving mitochondrial activity seem to be not enough to improve the overall performance of a CHO cell line. Future process or metabolic engineering strategies should balance glycolysis and TCA cycle activities in order to provide adequate amounts of intermediaries for biomass, r-protein and glycans biosynthesis.

In this study, we have reported that co-consumption of galactose and lactate led to a better-balanced and efficient metabolism in CHO cells. This was observed on one hand, by decreased use of carbon sources and amino acids consumption (without affecting cell growth and viability) (Figure 2, Table 2) and, on the other hand, by higher production of ATP on a carbon mol basis. Most of the mammalian cells that underwent a lactate metabolic shift presented a decrease in consumption of glucose (when it is still available on culture medium) and amino acids (Liste-Calleja et al., 2015; Mulukutla et al., 2012; Wilkens et al., 2011). This is often accompanied by decreased glycolytic fluxes and a drop in specific cell growth rate (Gray et al., 2012; Mulukutla et al., 2012). Similar metabolic behaviour has been observed in biphasic culture strategies using glucose and galactose during the co-consumption phase of galactose and lactate (Altamirano et al., 2006; Sun et al., 2013; Wilkens et al., 2011). Several studies have shown that lactate consuming state (after a metabolic shift induced either by pH, temperature or change in media composition) presented a more energy efficient metabolic state compared to lactate producing state (Gray et al., 2012; Ivarsson et al., 2015; Mulukutla et al., 2012). A metabolome analysis of CHO cells indicated that lactate consuming cells presented an increased TCA cycle capacity and a more robust mitochondrial function (Luo et al., 2012). Such a difference in energy production lies in the fact that large proportion of lactate is metabolised in the TCA cycle during the lactate consumption phase, while most of the pyruvate during the lactate production phase (growth based on glucose) is converted into lactate (Gray et al., 2012; Mulukutla et al., 2017). In a theoretical calculation, cells may produce up to 14 times more energy in a lactate consuming state compared to a producing state if it is completely metabolised in the TCA cycle (Gray et al., 2012). Hence, this naturally brings us to the question of defining the main reasons driving this metabolic behaviour (lactate consumption).

The answer to this question, unfortunately, is not a simple one. Various process events have been reported to trigger a shift from lactate production to utilisation including depletion of carbon/energy sources (i.e., glucose and/or glutamine) in medium (Altamirano et al., 2006, 2004; Wahrheit et al., 2014; Zagari et al., 2013), cell growth cessation after reaching maximal cell numbers (Carinhas et al., 2013; Ma et al., 2009), temperature shift (Torres et al., 2018b), increased oxidative capacity by addition of copper (Luo et al., 2012; Yuk et al., 2015), and unbalanced lactate or H^+ concentrations (Li et al., 2012; Liste-Calleja et al., 2015). In our experiments, increasing extracellular lactate concentration led CHO cells to consume lactate, although doubling lactate concentration of the feed did not result in an equivalent increase in lactate consumption (Table 1). It was noteworthy that, when comparing the control and G/L-High cultures, they presented the same lactate residual concentration and pH (H^+ concentrations), but completely different metabolic behaviours. One of several hypotheses explaining this phenomenon is related to the carbon flux through glycolysis. High glycolytic flux (usually observed under glucose-based growth) results in large accumulation of pyruvate that cannot be processed by mitochondria. This leads to production of large amount of alanine and lactate (Lu et al., 2005; Ma et al., 2009). When the glycolytic flux decreases, a drop in pyruvate pools occurs and lactate consumption emerges as an alternative for supplying the required carbon (Hartley et al., 2018). A similar scenario was observed in our chemostat cultures fed with galactose and lactate, in which fluxes through glycolysis were dramatically reduced compared to the control (Figure 5).

The main novelty of this study is the evaluation of a combined feeding strategy of galactose and lactate as a means to decrease lactate accumulation in CHO cell cultures. To our knowledge, this is the first study showing that a free-glucose feeding strategy based on lactate may support cell growth and enhance energy metabolism. On the other hand, the use of constrained-based MFA provided a deeper understanding of the physiology of CHO cells in cultures. We presented evidence that suggest that improving efficiency of metabolism and increasing ATP yield do not always lead to an improved culture performance. Further applications of the MFA in combination with transcriptome and metabolome data can be used to guide engineering efforts to improve r-protein production.

518

519 5. Conclusion

520 The principal aim of this study was to study the impact of simultaneous feeding galactose and
521 lactate on the culture performance and cell metabolism of a rh-tPA producing CHO cell line using
522 chemostats. Our results indicated that a combined feeding strategy of galactose and lactate did not
523 significantly impact cell growth and viability, but it did negatively impact on the rh-tPA productivity,
524 quality and cell metabolism. Increasing galactose and lactate concentrations in the feed led to a
525 high rh-tPA production, although it did not improve rh-tPA quality (compared to glucose and G/L-
526 Low). This feeding strategy also dramatically decrease consumption of the primary carbon source
527 (i.e., galactose) and led to very low glycolytic fluxes in G/L-Low and G/L-High cultures. Feeding
528 lactate promoted lactate consumption in CHO cells and drove to high fluxes through TCA cycle and
529 an improved energy metabolism. The present study provides a better understanding of CHO cell
530 metabolism in galactose and lactate. However, the success of this feeding strategy in industrial
531 culture processes, such as fed-batch or perfusion, is a question that arises for future research on
532 this topic.

533 6. Appendices

534 **Table A.1 Metabolic network used for the estimation of intracellular fluxes.**

535 **Table A.2 Intracellular fluxes [nmol/10⁶cells/h] of CHO cell chemostat cultures at steady-**
536 **state.**

537

538 Conflict of interest statement

539 Nothing declared.

540

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