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Cell-free expression and characterization of multivalent rhamnose-binding lectins using bio-layer interferometry

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Lectins are important biological tools for binding glycans, but recombinant protein expression poses challenges for some lectin classes, limiting the pace of discovery and characterization. To discover and engineer lectins with new functions, workflows amenable to rapid expression and subsequent characterization are needed. Here, we present bacterial cell-free expression as a means for efficient, small-scale expression of multivalent, disulfide bond-rich, rhamnose-binding lectins. Furthermore, we demonstrate that the cell-free expressed lectins can be directly coupled with bio-layer interferometry analysis, either in solution or immobilized on the sensor, to measure interaction with carbohydrate ligands without purification. This workflow enables the determination of lectin substrate specificity and estimation of binding affinity. Overall, we believe that this method will enable high-throughput expression, screening, and characterization of new and engineered multivalent lectins for applications in synthetic glycobiology.

Key words: lectin; bio-layer interferometry; cell-free production.

Introduction

Lectins are proteins that recognize and bind specific glycans, making them interesting scaffolds for therapeutics, diagnostics, and quality control reagents for glycoprotein products (Arnaud et al. 2013; Fernandez-Poza et al. 2021). However, recombinant expression of lectins can be challenging because of properties such as toxicity, size, or presence of disulfide bonds (Martínez-Alarcón et al. 2018). One strategy to overcome these challenges is cell-free expression (CFE), which harnesses biological machinery to enable high-yielding transcription and translation outside of the living cell (Carlson et al. 2012; Silverman et al. 2020). The modular and open CFE reaction environment allows for the manipulation of expression conditions, enabling production of complex products including proteins containing disulfide bonds (Goerke and Swartz 2008; Dopp and Reuel 2020), membrane proteins (Matthies et al. 2011; Kruyer et al. 2021), and glycosylated proteins (Kightlinger et al. 2019; Hershewe et al. 2021; Stark et al. 2021; Jaroentomeechai et al. 2022). CFE is also scalable from the nanoliter to liter scale (Zawada et al. 2011; Yin et al. 2012), allowing for small-scale parallel expression of many proteins simply by switching out the template DNA added to the reaction, and accelerating protein screening (Hunt et al. 2021, 2022).

To take advantage of CFE for rapid lectin screening, it would be ideal to assess the functionality of produced lectins directly in the reaction without purification. Bio-layer interferometry (BLI) is a technique that has been recently used to characterize protein-binding parameters without a purification step, indicating possible compatibility with CFE reactions (Pogoutse et al. 2016). In addition, BLI has been used to study multivalent lectin-carbohydrate interactions in purified systems (Laigre et al. 2018; Picault et al. 2022). Notably, this technique requires less material than surface plasmon resonance or isothermal titration calorimetry, is label free, and can be run in a plate-based format, making it more compatible with small-scale CFE (Concepcion et al. 2009).

To demonstrate our cell-free workflow, we selected two model eukaryotic lectins, SUL-I (PDB-ID 5H4S) from *Toxopneustes pileolus* (sea urchin) venom and CSL3 (PDB-ID 2ZX2) from *Oncorhynchus keta* (chum salmon) eggs, that are characterized, but are difficult to isolate naturally or to produce in reasonable quantities in *Escherichia coli* (Shiina et al. 2002; Hatakeyama et al. 2015). These dimeric lectins are of interest because of their binding affinity to the glycosphingolipid globotriaosylceramide (Gb3), a tumor associated glycolipid, as well as to rhamnose-containing bacterial O-antigens (Shiina et al. 2002; Siukstaite et al. 2021). Here, we show that these rhamnose-binding lectins are soluble and active when expressed in a bacterial cell-free system. Furthermore, we show that cell-free expressed lectins are compatible with BLI-binding assays in crude reactions without purification, with lectins either immobilized or in solution. We believe that this demonstration will enable future high-throughput screening and characterization of the specificity and affinity of predicted and engineered lectins.

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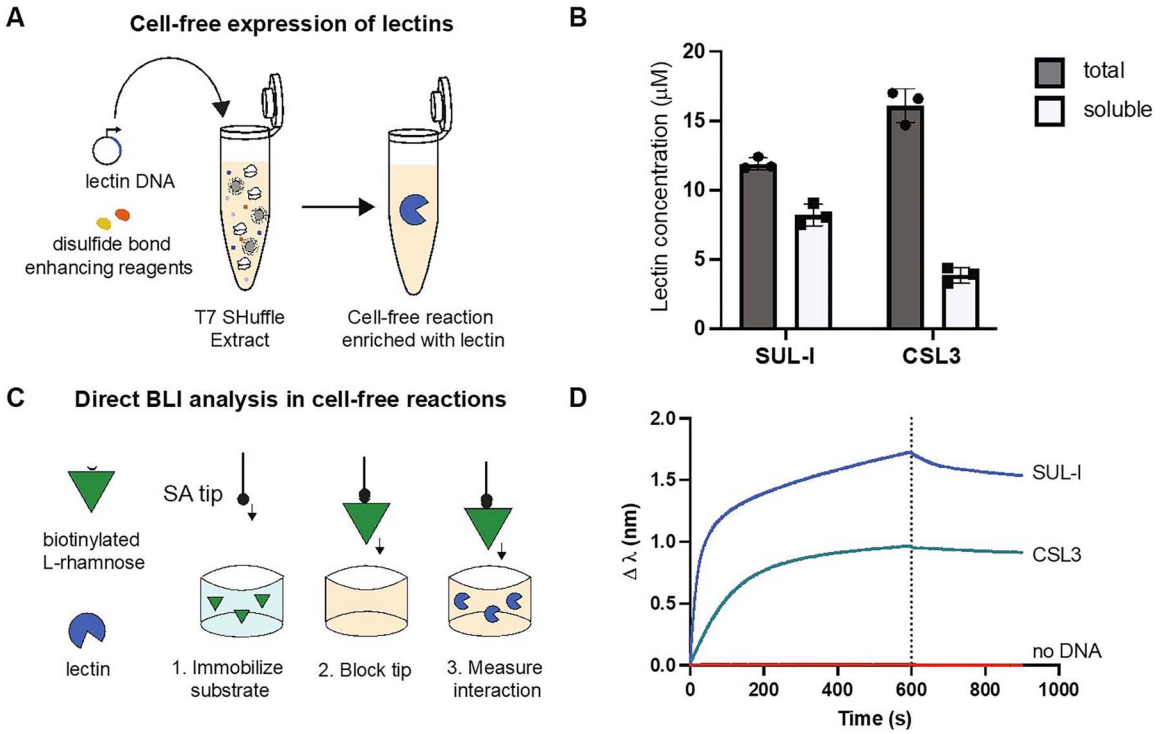

Fig. 1. Cell-free expressed rhamnose-binding lectins are directly compatible with BLI analysis. a) Schematic representation of CFE system for disulfide bond containing lectins. b) Average total and soluble yields of SUL-I and CSL3 expressed in 15 μL cell-free reactions at 16°C determined by radiolabeled amino acid (^{14}C-Leucine) incorporation. Error bars represent the standard deviation for $n = 3$ CFE reactions. c) Schematic of BLI assay where a biotinylated rhamnose monosaccharide is immobilized on the tip, followed by blocking with a cell-free reaction with no DNA template, and then measuring the interaction with a lectin synthesized in the cell-free reaction. d) BLI sensorgram of the interaction between a rhamnose monosaccharide immobilized on the tip and a 10-fold dilution in PBS of either negative control (no DNA template), SUL-I (~800 nM), or CSL3 (~400 nM) enriched cell-free reaction.

Materials and methods

Cell-free expression

CFE and ^{14}C-Leucine quantification were performed as described previously using a modified PANOx-SP (PEP) formulation in an extract prepared from SHuffle T7 Express (NEB) cells (Warfel et al. 2022). Extract was preincubated with 50 μM IAM at room temperature for 30 min. Reactions were supplemented with 10 μM DsbC, 1 mM GSH, and 4 mM GSSG and incubated at 16°C for 20 h for lectin expression. Detailed methods can be found in the Supplementary Information.

Bio-layer interferometry

All BLI experiments were performed using an Octet RED96 Instrument with data collected with ForteBio DataAcquisition9, analyzed and fit with ForteBio DataAnalysis9, and plotted with Graphpad PRISM. Detailed methods can be found in the Supplementary Information.

Results

Cell-free expressed multivalent lectins are compatible with direct in-solution BLI analysis

Each SUL-I monomer presents 3 binding sites with 13 disulfide bonds, whereas each CSL3 monomer has 2 binding sites with 8 disulfide bonds and both of them are dimeric in solution (Fig. S1a and b; Shirai et al. 2009; Hatakeyama et al. 2017). To support proper protein folding, SUL-I and CSL3 were produced in an *E. coli* CFE system using an extract derived from T7 SHuffle cells (Dopp and Reuel 2020) and additives optimized for disulfide bond expression (Fig. 1a and Table S1). We produced 8.2 μM (274 μg/mL) and 3.9 μM (94 μg/mL) full-length, soluble SUL-I and CSL3, respectively, as determined by radiolabeled amino acid (^{14}C-Leucine) incorporation and quantification at a 15 μL reaction scale (Figs 1b and S2a–c). Importantly, lowering the expression temperature to 16°C was necessary to increase the solubility and yields of both lectins (Fig. S2a and b).

We first confirmed that cell-free expressed SUL-I and CSL3 were active by isolating them using either D-lactose or D-galactose affinity resin, respectively (Fig. S2d). Next, we determined if BLI analysis was directly compatible with unpurified lectins in cell-free reactions. We loaded biotinylated L-rhamnose monosaccharide (L-Rhaα-sp3-biot) onto a streptavidin BLI tip. We then accounted for background interaction from the cell-free reaction mixture by adding a "blocking" step in a negative cell-free reaction (containing water instead of plasmid DNA encoding the lectin) following rhamnose substrate immobilization on the tip and prior to the interaction assay (Figs 1c and S3). We observe a rapid and strong association phase for both lectins, with almost no dissociation phase, when cell-free reactions expressing either SUL-I or CSL3 are present in solution (Fig. 1d). No interaction occurs in reactions that did not contain lectin DNA expression template (no DNA), confirming the specificity of the method (Fig. 1d). The signal variation was different for the two lectins at increased dilution, indicating better binding from SUL-I. This may be related to the higher expression levels for SUL-I in the cell-free reaction and by the larger number of binding sites

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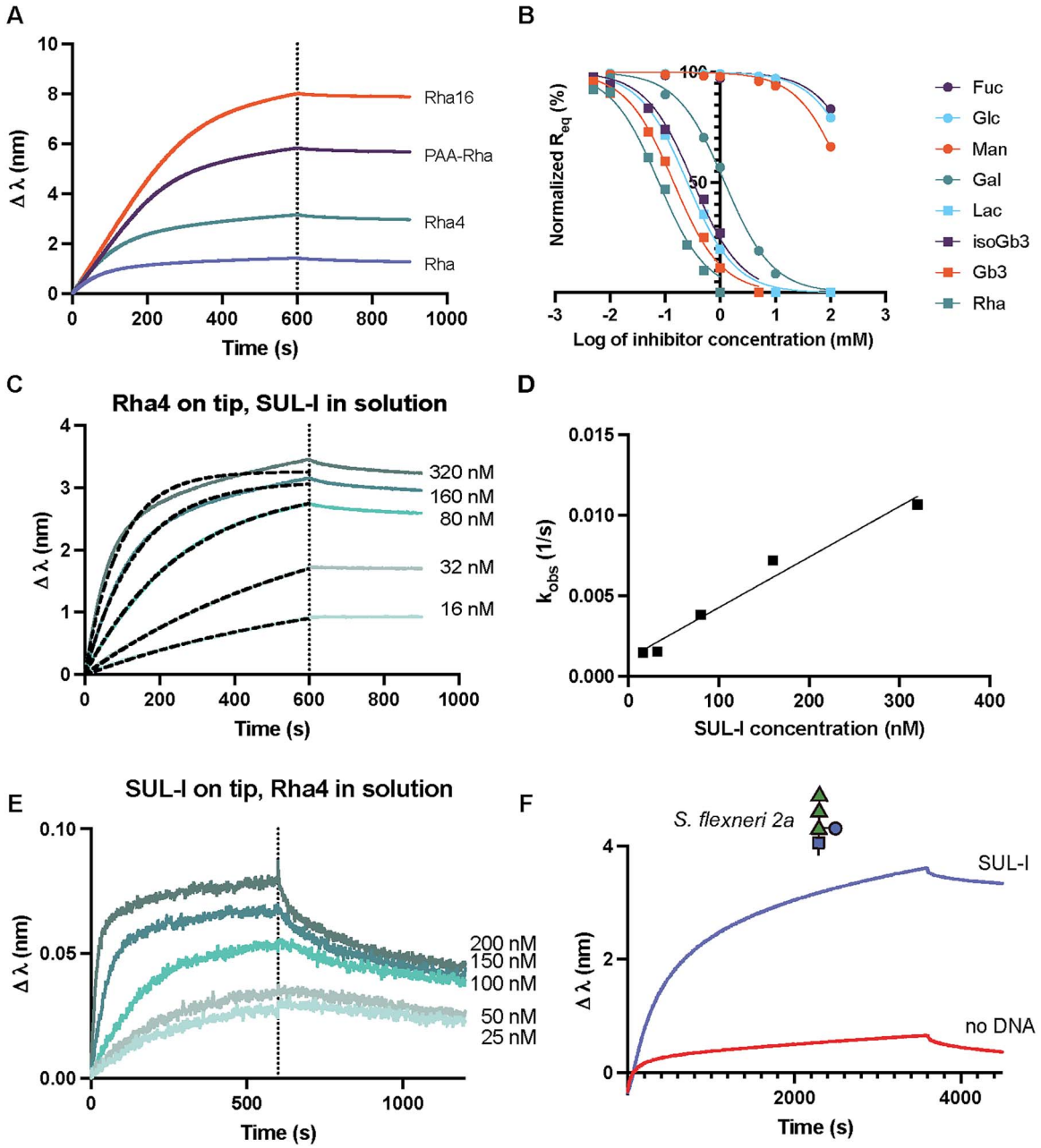

Fig. 2. CFE coupled with BLI enables the determination of lectin-binding specificity and affinity. a) Sensorgram of the interaction between different rhamnose substrate architectures (Rha16, PAA-Rha, Rha4, Rha monosaccharide) immobilized on the SA BLI tip with 50-fold diluted SUL-I (~160 nM) cell-free reaction in solution. b) Impact of different sugar inhibitors on the interaction between the rhamnose monosaccharide tip and SUL-I lectin in solution. The normalized equilibrium-binding signal (R_{eq}) as determined by a 1:1 association fit is plotted as a function of the log (inhibitor concentration) for each sugar inhibitor. Nonlinear curve fit using Graphpad PRISM log(inhibitor) vs normalized response is shown in the corresponding color for each inhibitor. c) Interaction of Rha4 substrate immobilized on the SA tip, with varying dilutions of cell-free reaction containing SUL-I (~16–320 nM) in solution. The 1:1 model fit used to determine k_{obs} is shown in a black dotted line for each concentration and estimated concentration of SUL-I in solution is indicated to the right of the corresponding curve. d) k_{obs} values plotted as a function of SUL-I concentration and fit with a simple linear regression in Graphpad PRISM for Rha4. e) Interaction of SUL-I substrate immobilized on the Ni-NTA tip from a cell-free reaction, with varying dilutions of Rha4 substrate (~25–200 nM) in solution. f) Detection of 100-fold dilution of crude extract enriched with *Shigella flexneri 2a* O-antigen in solution with SUL-I in cell-free reaction (blue) or control cell-free reaction with no DNA (red) immobilized on the Ni-NTA tip. The monomer structure of the O-antigen glycan is indicated above the sensorgram. All data are representative of at least two independent experiments.

available in SUL-I compared with CSL3 (Fig. S4). For SUL-I, the signal level remains unchanged with a 50-fold dilution, indicating that the dissociation constant (k_D) is lower than the lectin concentration at this dilution (160 nM). Whereas for CSL3, we observe a more rapid decrease of the signal with increasing dilution (80 nM), yielding an approximation of k_D that is higher than the concentration of CSL3 at this dilution (between 80 and 400 nM).

CFE coupled with BLI enables the determination of SUL-I-binding specificity and affinity

To further validate this experimental setup, we tested the specificity of SUL-I for different rhamnose substrate architectures immobilized on the BLI tip (Figs 2a and S4). In addition to the monosaccharide, we also assessed two synthesized multivalent compounds, a tetravalent (Rha4)

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and a hexadecavalent (Rha16) cyclopeptide functionalized with L-rhamnose by triazole linkages, using architectures that have previously been used to assay multivalent lectin/substrate interactions (Picault et al. 2022). We also examined a commercial multivalent polyacrylamide polymer functionalized with rhamnose (PAA-rhamnose). We observed higher signal as the valency of the substrate on the tip increased, with the smallest change in wavelength for the monosaccharide and the largest change in wavelength (~8-fold higher) for the Rha16 substrate, indicating that more SUL-I was able to bind to the additional L-rhamnose immobilized on the tip (Fig. 2a). Very little dissociation was observed in all cases.

Additionally, we employed this strategy to determine the specificity of SUL-I by screening the ability of different mono- and oligo-saccharides (rhamnose, Gb3, isoGb3, lactose, galactose, glucose, fucose, and mannose) to inhibit the binding interaction with the rhamnose monosaccharide on the tip. We determined that a 50-fold dilution of cell-free reaction enriched with SUL-1 (~160 nM) still resulted in robust signal (Fig. S4) and incubated the diluted cell-free reaction with sugar competitors at varying concentrations for 1 h before the interaction step. For all experiments where the sugar acted as an inhibitor, the signal decreased with increased concentration of inhibitor (Fig. S5). Subsequently, we compared the equilibrium response R_{eq} (maximum equilibrium change in wavelength), as determined by fitting a 1:1 association curve for each condition, to estimate relative IC50 values for each sugar (Figs 2b and S5; Orthwein et al. 2021). Rhamnose had the lowest IC50 for this system (0.07 mM), followed by Gb3 (0.15 mM), whereas galactose had the highest observed IC50 (1.26 mM; Figs 2b and S5a and Table S2). Fucose, glucose, and mannose did not fully inhibit binding at the concentrations tested (Fig. 2b).

Finally, we demonstrated the utility of this method for estimating binding kinetics and affinity in the background of the crude cell-free reaction (Fig. 2c). As previously described for multivalent lectin-ligand systems with weak dissociation, we estimated the kinetic parameters k_{on}, k_{off}, and k_D from the linear relationship between k_{obs}, fit by a 1:1 association model, and the lectin concentration, determined by ^{14}C-Leucine incorporation (Figs 2c and d, and S6; Picault et al. 2022). Values of k_{on} were not affected much by the architecture of the substrates, with variations from 3.5×10^4 M^{-1} s^{-1} to 1.9×10^4 M^{-1} s^{-1} (Table S3). We obtained the highest k_D of ~80 nM for the monosaccharide and slightly higher affinity k_D (~30–40 nM) for the Rha4, Rha16, and PAA-Rha on the tip (Fig. S6 and Table S3). When the rhamnose ligands are immobilized, all sensors present rhamnose multivalently regardless of individual ligand structure, resulting in little difference in binding behavior between the architectures.

We also inverted the experiment and immobilized the lectins with N-terminal 6xHis tag from a crude cell-free reaction using Ni-NTA tips, to measure interaction with rhamnose ligands in solution (Figs 2e and S7a). This system did not follow the same kinetics as when the rhamnose ligand was on the tip, possibly because of different affinity when the lectin is immobilized, preventing a 1:1 binding analysis. However, using steady state analysis we were able to estimate a k_D of ~100 nM for Rha4 and ~50 nM for Rha16 when SUL-I on the tip (Fig. S8 and Table S4). Importantly, these k_D values are the same order of magnitude as our estimates for SUL-I-binding affinity when the rhamnose substrate was immobilized, although the Rha16 has higher affinity than Rha4 when SUL-I is immobilized.

While we could detect interaction with the Rha4 and Rha16 ligands in solution, this experimental setup is limited by the detectable ligand size, and we could not detect binding with the rhamnose monosaccharide in solution (Fig. S7b) as previously observed for other monosaccharides (Laigre et al. 2018). However, this method is also of interest for the detection of glycans in crude samples. Toward this goal, we show that SUL-I enables the detection of the *Shigella flexneri* O-antigen glycan, which contains terminal rhamnose residues (Anderson et al. 2016), in crude cell-free glycoprotein synthesis extracts (Jaroentomeechai et al. 2018; Kightlinger et al. 2019; Warfel et al. 2022) without purification (Fig. 2f). In these experiments, we accounted for background binding to the Ni-NTA tip with a reference sensor with immobilized cell-free reaction that did not contain the DNA expression template for the lectin (Figs S7 and S8), shown without subtraction in Fig. 2f. Nevertheless, as previously described for protein loading onto BLI tips from crude cell lysate, a stronger, more specific interaction such as biotin-streptavidin may result in more uniform and saturated binding of the lectin to the tip (Pogoutse et al. 2016).

Discussion

This work couples a bacterial CFE platform directly with BLI to create a workflow compatible with small-scale expression and screening of lectins in high-throughput. As a proof of concept, we expressed 2 multivalent rhamnose-binding lectins from marine organisms and demonstrated that lectins in crude cell-free reactions are compatible with BLI interaction assays when tips are functionalized with glycan ligands. Importantly, we demonstrated this method with 2 commercially available biotinylated rhamnose ligands as well as 2 synthesized rhamnose substrates of varying sizes, enabling wide use of this technique. This approach does not require the immobilization of the lectin and enables in-solution determination of binding specificity as well as estimation of affinity. Furthermore, the ability to quantify the cell-free expressed protein in the crude mixture enables kinetics measurements using this method. While the cell-free reaction matrix could impact the affinity constant, the main goal of this work is the rapid characterization of binding and estimation of affinity constants in a crude background rather than exact determination using purified proteins.

We also demonstrated that SUL-I expressed in a crude cell-free reaction can be immobilized on the BLI tip and used for the qualitative detection of glycan epitopes in solution. While the immobilization of the lectin has some drawbacks, such as size of glycoconjugate that can be detected or the impact of immobilization on binding activity, it can enable the detection of glycan substrates from crude mixtures. Here, we demonstrate the detection of the *S. flexneri* 2a O-antigen, of interest for vaccine development, directly in cell extract, aligning with previous reports that rhamnose-binding lectins can bind smooth LPS from other Shigella serotypes (Shiina et al. 2002).

In total, we have shown that unpurified lectins in CFE reactions can be used directly in BLI experiments, either in solution or immobilized on the sensor tip, for the qualitative detection of carbohydrate-lectin interactions. This work

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advances efforts to characterize binding interactions without a purification step, which could expedite discovery and screening of new binders (Khavrutskii et al. 2013; Pogoutse et al. 2016). While it was advantageous to express lectins at a small scale in this work, we expect that the concentration of lectins determined here are generalizable across scales, provided the surface area to volume ratio of the reaction vessel is adjusted appropriately (Zawada et al. 2011; Yin et al. 2012). We expect that this workflow can be coupled to tools like LectomeExplore (Bonnardel et al. 2021) to express and screen uncharacterized lectins, and will advance the field of synthetic glycobiology for lectin discovery and application.

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Author contributions

Katherine F. Warfel (Formal analysis-Equal, Investigation-Lead, Methodology-Equal, Visualization-Lead, Writing—original draft-Lead), Eugénie Laigre (Investigation-Equal, Methodology-Equal), Sarah E. Sobol (Investigation-Supporting), Emilie Gillon (Investigation-Supporting, Methodology-Supporting), Annabelle Varrot (Conceptualization-Supporting, Investigation-Supporting, Writing—review & editing-Supporting), Olivier Renaudet (Resources-Equal), Jérôme DEJEU (Formal analysis-Lead, Methodology-Lead, Writing—review & editing-Equal), Michael Jewett (Conceptualization-Equal, Funding acquisition-Equal, Supervision-Equal, Writing—review & editing-Equal), and Anne Imberty (Conceptualization-Equal, Formal analysis-Equal, Funding acquisition-Equal, Supervision-Equal, Writing—review & editing-Equal)

Supplementary data

Supplementary material is available at *GLYCOB Journal* online.

Conflict of interest statement

None declared.

Data availability statement

Data from replicate trials are provided in the supplementary material. Any other information pertaining to this study and is not included here will be made available from the corresponding author upon request.

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