Highlights:

Oligosaccharides derived from lactulose (OsLu) were efficiently purified with yeast

OsLu treated with Saccharomyces cerevisiae introduced proteins reactive to Dectin-2

Proteomic analysis showed mannoproteins as main responsible for binding to Dectin-2

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Effect of purification of galactooligosaccharides derived from lactulose with *Saccharomyces cerevisiae* on their capacity to bind immune cell receptor Dectin-2

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ABSTRACT:

Lactulose-derived oligosaccharides (OsLu) are prebiotic galactooligosaccharides (GOS) beneficious for human health including immunomodulatory properties; however, the molecular mechanism is unclear. OsLu produced by enzymatic synthesis can be purified with *Saccharomyces cerevisiae* (OsLu-*Sc*). We show that this purification introduces yeast-derived proteins reactive to Dectin-2, an innate immune receptor for fungal polysaccharides. Using a cell-based bioassay, we tested the binding of OsLu and GOS samples to Dectin-2. While OsLu purified with active charcoal and commercial GOS failed to bind to Dectin-2, we found OsLu-*Sc* bound to this receptor. The carbohydrate-binding incompetent mutant of Dectin-2 failed to bind to OsLu-*Sc*. These data suggest that OsLu-*Sc* introduced carbohydrate ligands for Dectin-2. In accordance with this, proteomic analysis revealed OsLu-*Sc* contained *S. cerevisiae*-derived mannoproteins. Therefore, our data highlights the importance of the purification method for OsLu, which may positively affect the bioactivity of OsLu. Data are available via ProteomeXchange with identifier PXD010495.

Keywords: prebiotics; oligosaccharides; lactulose; mannoproteins; Dectin-2; *Saccharomyces*

1. Introduction

Galactooligosaccharides (GOS) are common prebiotic ingredients that are widely used in functional foods across the world and have emerged as a practical choice to positively modify the gut microbiota (Lamsal, 2012). Further, GOS are reported to have bioactive properties linked to health benefits including improved mineral absorption, antipathogenic activity, and immunomodulatory effects, among others (Lamsal, 2012). The main commercial strategy for synthesizing GOS involves the transgalactosylation activity of microbial β-galactosidases. Starting from lactose (β-D-galactose-(1→4)-D-glucose), the enzyme produces a complex mixture of prebiotic GOS with different β-linkages, 1→3, 1→4 and 1→6, depending on the enzyme source, as well as monosaccharides (primarily glucose), and unreacted lactose (Moreno, Corzo, Montilla, Villamiel, & Olano, 2017; Villamiel, Montilla, Olano, & Corzo, 2014).

Recently, it was reported that new lactulose (β-D-galactose-(1→4)-D-fructose)-derived oligosaccharides (OsLu) had lower digestibility against mammalian digestive enzymes both *in vitro* and *in vivo* (Ferreira-Lazarte et al., 2017; Hernández-Hernández, Marín-Manzano, Rubio, Moreno, Sanz, & Clemente, 2012), improved beneficial effects over GOS on the gut microbiota (Cardelle-Cobas et al., 2009; Cardelle-Cobas et al., 2012), and improved mineral absorption (Laparra, Diez-Municio, Herrero, & Moreno, 2014). OsLu is obtained enzymatically through use of β-galactosidases from *Aspergillus aculeatus* (Cardelle-Cobas, Martínez-Villaluenga, Villamiel, Olano, & Corzo, 2008)*, A. oryzae* (Cardelle-Cobas et al., 2016), and *Kluyveromyces lactis* (Cardelle-Cobas, Corzo, Martínez-Villaluenga, Olano, & Villamiel, 2011), which results in the synthesis of complex mixtures of oligosaccharides (with a degree of polymerization ≥ 2), along with lactulose, fructose and galactose.

In both, GOS and OsLu the presence of certain amounts of carbohydrates without relevant functionality, such as monosaccharides, may limit their potential applications. For example, residual glucose may not be suitable for individuals with diabetes Mellitus. Thus, different approaches have been developed for obtaining mixtures of bioactive oligosaccharides of high purity. For this end, from an industrial perspective, separation by use of membranes, namely nanofiltration, is a practical, although not selective procedure (Michelon, Manera, Carvalho, & Maugeri Filho, 2014). In this context, purification of oligosaccharides by fermentation with yeast, with subsequent removal of cells, has been also achieved for selective elimination of simple sugars (Michelon et al. 2014). Previously, a mixture of OsLu was obtained through the use of β-galactosidases from *A. oryzae* and subsequent use of *Saccharomyces cerevisiae* to remove monosaccharides. This OsLu sample showed a better anti-inflammatory activity than lactulose obtained by chemical synthesis in an ulcerative colitis model in rats (Algieri et al., 2014). These authors hypothesized that these effects may be ascribed to the enhanced immunomodulatory properties of OsLu purified with *S. cerevisiae*. However, it is unclear as to what molecular mechanism could be responsible.

It has been postulated that the immunomodulatory function of oligosaccharides may stem from direct interaction with the host immune system (Jeurink, van Esch, Rijnierse, Garssen, & Knippels, 2013). In light of this, it is known that fungal cell-wall polysaccharides α-mannan and β-glucan are recognized by carbohydrate-binding immune cell receptors including C-type lectins, which regulate cell function (Geijtenbeek & Gringhuis, 2016; Gow, van de Veerdonk, Brown, & Netea, 2012). Among these receptors, Dectin-2 is a type II transmembrane receptor found on the cell surface of innate myeloid cells, such as monocytes and dendritic cells (Geijtenbeek & Gringhuis, 2016). Dectin-2 can directly bind to α-linked mannan structures on

glycoproteins and glycolipids, known as glycans, which are found in the cell walls of microbes including fungi, mycobacteria, and several gram-negative bacteria (Geijtenbeek & Gringhuis, 2016; Saijo 2010; Wittmann et al., 2016; Yonekawa et al., 2014). Binding of Dectin-2 with its glycan ligand mediates the host immune response (Geijtenbeek & Gringhuis, 2016). For example, Dectin-2 is responsible for the promotion of Th17 responses to fungi and is essential for resistance to infection with *Candida albicans* (Saijo et al., 2010; Vautier, Sousa, & Brown, 2010). α-mannans are polysaccharides found in abundance attached to fungal proteins on yeast species such as *C. albicans* and *S. cerevisiae* (Cabib & Arroyo, 2013; Gow et al. 2012; Saijo et al., 2010). The yeast cell wall contains glycoproteins with α-mannans, called mannoproteins, which are located on the outer surface providing both structural and functional support for the cell wall (Cabib & Arroyo, 2013; Gow et al. 2012; Saijo et al., 2010; Terashima et al., 2002). Such mannoproteins play important roles in cell adhesion, cell permeability, osmotic control, and morphology (Terashima et al., 2002). The α-mannan structures on the fungal cell wall in turn become a target of the mammalian immune system, through recognition by Dectin-2 (Saijo et al., 2010). Our group and others have shown that Dectin-2 recognizes *C. albicans* and *S. cerevisiae,* and mediate immune cell activation (McGreal, 2006; Robinson, Osorio, Rosas, Freitas, Schweighoffer, Groß et al., 2009).

In this study, we tested the impact of the different purification methods of OsLu samples on their ability to bind to immune cell receptor Dectin-2. We hypothesized that yeast-derived components were introduced into the OsLu samples through purification with *S. cerevisiae* that would bind to Dectin-2. Through proteomics techniques we have pointed out yeast mannoproteins introduced to OsLu samples when purified by *S. cerevisiae,* which are likely capable of binding to Dectin-2.

2. Materials and methods

2.1. Chemicals and Samples

Most of chemicals (ethanol, methanol, chloroform, acetonitrile, formic acid, activated charcoal, serum albumin, phenyl-β-D-glucoside, hydroxylamine chloride, pyridine, hexamethyldisilazane, trifluoroacetic acid, chlorophenol red-β-D-galactopyranoside, kifunensine, urea, thiourea, triethylammonium bicarbonate) were obtained from Sigma Chemical (St. Louis, MO), unless otherwise stated (Tris(2-carboxyethyl) phosphine (AB SCIEX), methyl methanethiosulfonate, (Pierce), trypsin (Sigma-Aldrich)). Vivinal®GOS syrup was kindly provided by Friesland Campina Domo (Hanzeplein, The Netherlands). Duphalac® (Abbott Biologicals B.V., Olst, The Netherlands) was purchased at a local pharmacy.

The study samples and the method of synthesis and purification are shown in Fig. 1. OsLu preparations were obtained at pilot scale by Innaves S.A. (Vigo, Spain) following the method described by Ferreira et al. (2017). OsLu was synthesized using a commercial lactulose preparation (Duphalac®) and a β-galactosidase from *A. oryzae* (16 U/mL; Sigma, St. Louis, MO). The mixture of oligosaccharides was either purified using activated charcoal (OsLu-ActC) or by fermentation with *S. cerevisiae* (OsLu-*Sc*). OsLu-ActC was purified following the method described by Morales, Sanz, Olano and Corzo (2006) with some modifications. In brief, a total of 2 g of carbohydrates were added to 250 mL of a 7:93 (v/v) ethanol/water solution and stirred for 30 min with 7.5 g of activated charcoal (Darco G60, 100 mesh, Sigma) to remove mono- and disaccharides. This mixture was filtered through Whatman no. 1 filter paper under vacuum, and the activated charcoal was washed with 100 mL of distilled water. The oligosaccharides adsorbed onto the activated charcoal were then extracted by stirring

the mixture with 250 mL of a 50:50 (v/v) ethanol/water solution. Activated charcoal was eliminated by filtering. Purification with fresh *S. cerevisiae* was carried out following the method described by Ferreira et al. (2017). A mixture with 20% [w/v] of carbohydrates was treated with 1.5% [w/v] of yeast (Levital, Paniberica de Levadura S.A., Valladolid, Spain) to remove monosaccharides (OsLu-*Sc*). All samples were dried at 40 ºC in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) to reach a dry matter (DM) of 80% (±1).

OsLu mixture purified with *S. cerevisiae* (OsLu-*Sc*) was fractionated following two strategies: i) precipitation with ethanol, 20 g of OsLu-*Sc* was mixed with water (20 mL) and added ethanol (180 mL) to obtain a final ethanol concentration of 87%. This operation was repeated twice and a supernatant (OsLu-*Sc*-S) and a precipitate (OsLu-*Sc*-Pp) were obtained. ii) 20 g OsLu-*Sc* was diluted in ultrapure water (240 mL) and ultrafiltered through hydrophilic 10 kDa cut-off membranes (Amicon Ultra-15, Millipore Corp., Bedford, MA, USA) by centrifugation at 4,000*g* for 30 min at 30 ºC to obtain a enrichment fraction of higher molecular mass compounds. Finally, retentate (OsLu-*Sc*-R) and permeate (OsLu-*Sc*-P) were recovered, freeze-dried, and kept at −20 °C until analyzed.

2.2. Characterization of samples

DM content was gravimetrically determined in a conventional oven at 102 ºC until constant weight, using sand to increase the surface. Protein content in samples was determined by the Bradford's method using serum albumin as a standard.

The carbohydrate composition of samples was analyzed by GC-FID following the method of Montilla, Corzo, Olano and Jimeno (2009). Samples containing 5 mg of

carbohydrates were added to 400 μL of phenyl-β-D-glucoside (internal standard). The mixture was vacuum dried at 40 ºC in a rotary evaporator. Sugar oximes were formed by adding 250 μL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 ºC for 30 min. Subsequently, the oximes were silylated with hexamethyldisilazane (250 μL) and trifluoroacetic acid (25 μL) at 50 ºC for 30 min. Samples were centrifuged at 10,000 rpm for 2 min. Supernatants were injected in the GC or stored at 4 ºC prior to analysis. Injections were made in the split mode (1:20). The trimethyl silylated oximes were separated using a fused-silica capillary column (15 m x 0.32 mm i.d. x 0.1 μm film thickness) DB-5HT (J&W Scientific, Folson, California, USA). The oven initial temperature was 180 °C, increased at a rate of 3 °C/min to 380 °C and held for 10 min. The injector and detector temperatures were 280 and 380 °C, respectively. Nitrogen was used as carrier gas, 1 mL/min flow. Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for carbohydrates were calculated from FID peak areas relative to phenyl-β-D-glucoside. Mixtures of standard solutions of fructose, galactose, glucose, lactulose, lactose, raffinose and stachyose, over the expected concentration range, were prepared with 0.2 mg of the internal standard to calculate the response factor for each sugar. Data were expressed as percentage of carbohydrates.

2.3. Analysis of Dectin-2 binding to OsLu

The reporter cell assay was performed as described previously (Wittmann et al., 2016). Briefly, a flat-bottomed ELISA plate (MaxiSorp, Thermo Scientific) was incubated with the indicated carbohydrate samples. The BWZ cells express NFAT-*Lac*Z gene cassette and are commonly used to monitor receptor-ligand interaction by

185 monitoring *LacZ* activity (Sanderson & Shastri, 1994). Wild-type Dectin-2 (Dectin-
186 2^{WT}), carbohydrate-binding incompetent Dectin-2 mutant (Dectin-2^{QPD}) were cultured
187 in the carbohydrate-immobilized well. As a negative control, we used mock-transfectant
188 BWZ cells which do not express the lectin receptors. One day after incubation, β-
189 galactosidase activity was monitored using chlorophenol red-β-D-galactopyranoside
190 (CPRG) in a colorimetric assay (Wittmann et al., 2016).

191

192 *2.4 Proteomic analysis.*

193 *2.4.1. Protein digestion*

194 Total protein concentration was determined using Pierce 660 nm protein assay
195 (Thermo). Prior to digestion, protein was precipitated by methanol/chloroform method.
196 For digestion, protein pellets were resuspended and denatured in 20 μL 7 M Urea/2 M
197 Thiourea/100 mM triethylammonium bicarbonate (TEAB), pH 7.5, reduced with 2 μL
198 of 50 mM Tris(2-carboxyethyl) phosphine (TCEP, AB SCIEX), pH 8.0, at 37 °C for 60
199 min and followed by 2 μL of 200 mM cysteine-blocking reagent (methyl
200 methanethiosulfonate, MMTS, Pierce) for 10 min at 25 °C. Samples were diluted up to
201 120 μL to reduce urea concentration with 25 mM TEAB. Digestions were initiated by
202 adding 1 μL (1 μg/μL) sequence grade-modified trypsin (Sigma-Aldrich) to each
203 sample, which were then incubated overnight at 37 °C on a shaker. Sample digestions
204 were evaporated to dryness and then desalted onto SEP-PAK C_{18} cartridge (Waters)
205 until the MS analysis.

206

207

2.4.2. Liquid chromatography (LC) and MS analysis

Digested peptides of each sample were subjected to 1D-nano LC ESI-MS/MS analysis using a nanoLC system (Eksigent Technologies nanoLC Ultra 1D plus, SCIEX, Foster City, CA) coupled to high speed Triple TOF 5600 mass spectrometer (SCIEX, Foster City, CA) with a Nanospray III Source. The analytical column used was a silica-based reversed phase column C_{18} ChromXP 75 µm × 15 cm, 3 µm particle size and 120 Å pore size (Eksigent Technologies, SCIEX, Foster City, CA). The trap column was a C_{18} ChromXP (Eksigent Technologies, SCIEX, Foster City, CA), 3 µm particle diameter, 120 Å pore size, switched on-line with the analytical column. The loading pump delivered a solution of 0.1% formic acid in water at 2 µL/min. The nano-pump provided a flow-rate of 300 nL/min and was operated under gradient elution conditions. Peptides were separated using a 100-minute gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was 5 µL.

Data acquisition was performed with a TripleTOF 5600 System (SCIEX, Foster City, CA). Data were acquired using an ionspray voltage floating (ISVF) 2800 V, curtain gas (CUR) 20, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 20, declustering potential (DP) 85 V. All data were acquired using information-dependent acquisition (IDA) mode with Analyst TF 1.7 software (SCIEX, Foster City, CA). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 35 MS/MS scans of 100 ms in the mass range of 100–1800 (total cycle time: 3.8 s). Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1250 with charge state of 2–5 and an abundance threshold of more than 90 counts (cps). Former target ions were excluded for 20 s. IDA

232 rolling collision energy (CE) parameters script were used for automatically controlling
233 the CE.
234
235 *2.4.3. Data analysis.*
236 MS and MS/MS data obtained for individual samples were processed using
237 Analyst® TF 1.7 Software (SCIEX, Foster City, CA). Raw data file conversion tools
238 generated mgf files which were also searched against the *S. cerevisiae* protein database,
239 containing 70,639 protein coding genes and other common protein contaminants using
240 the Mascot Server v. 2.6 (Matrix Science, London, UK). Search parameters were set as
241 follows: Methylthio (C) as fixed modification and acetyl (Protein N-term), pyrolidone
242 from E, pyrolidone from Q and Oxidation (M) as variable modifications. Peptide mass
243 tolerance was set to 25 ppm and 0.05 Da for fragment masses, also 2 missed cleavages
244 were allowed. Only the peptides with an individual MOWSE score ≥ 20 were
245 considered correctly identified.
246 The mass spectrometry proteomics data have been deposited to the
247 ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository
248 with the dataset identifier PXD010495 and 10.6019/PXD010495. For the reviewer
249 account details: Username: reviewer59479@ebi.ac.uk and Password**:** FMDg292x
250
251 *2.5. Statistical analysis*
252 One-way ANOVA followed by Tukey's test was used for statistical analysis of
253 Dectin-2 reporter cell absorbance data using the Prism 6 software (GraphPad). Shown

data mean average values with error bars as standard deviation (S.D); p *value* < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. Characterization of samples

The study design is shown in Fig. 1. Briefly, we aimed to prepare different GOS samples and test their binding to Dectin-2. Previously, the physicochemical and compositional characterization of OsLu and Duphalac® was investigated showing they were primarily comprised of carbohydrates (López-Sanz, Montilla, Moreno, & Villamiel, 2015). The predominant glycosidic linkages of OsLu were β(1→6), whereas Vivinal®GOS and Duphalac® contained β(1→4) linkages. These structural differences could affect the digestibility by intestinal microbiota (Moreno, Montilla, Villamiel, Corzo, & Olano, 2014). The composition of protein and carbohydrates (fructose, glucose, galactose, lactose, lactulose, disaccharides different from lactose and lactulose, tri-, tetra- and pentasaccharides) in all prebiotic samples in this study are shown in Table 1. As expected, purification of OsLu resulted in a notable enrichment in prebiotic carbohydrates, regardless of the purification method used. Considering the protein content in all samples, the amount, in general, was very low. The only samples with appreciable protein content were the original OsLu purified with *S. cerevisiae* (OsLu-*Sc*), the corresponding retentate obtained from ultrafiltration (OsLu-*Sc*-R), and the precipitate from ethanol treatment (OsLu-*Sc*-Pp). As these samples were subjected to purification using *S. cerevisiae* and the other samples were not (e.g. OsLu-ActC), suggesting that the presence of protein in these fractions could be derived from *S. cerevisiae*. This implies that yeast-derived proteins were introduced into OsLu samples

278 by the purification step using *S. cerevisiae,* thus changing their composition and
279 possibly their bioactivity.

280

281 *3.2. Assessment of OsLu for binding to immune cell receptors*

282 Since Dectin-2 is known to bind to yeast-derived components such as
283 mannoproteins, we sought to determine if OsLu-*Sc*, which contains yeast-derived
284 proteins, bound to immune cell receptor Dectin-2. In order to test Dectin-2 binding to
285 the OsLu samples, we employed a cell-based assay using the Dectin-2-expressing
286 BWZ.36 reporter cell line (see Materials and Methods 2.3). With these reporter cells,
287 Dectin-2 binding to its ligand induces β-galactosidase production within in the cell
288 (Wittmann et al., 2016). We tested Dectin-2 binding to the OsLu samples prepared in
289 this study and other commercial prebiotics (Fig. 1). Using this assay, we found that
290 OsLu-*Sc* bound to Dectin-2, while the original OsLu and OsLu-ActC, which were not
291 purified using *S. cerevisiae,* failed to do so (Fig. 2A). Furthermore, OsLu-*Sc*-R and
292 OsLu-*Sc*-Pp, which contain yeast-derived proteins, bound to Dectin-2 whereas OsLu-
293 *Sc*-P, which was shown to contain no proteins, did not (Fig. 2A). Dectin-2 binding to
294 the OsLu samples was abolished by the amino-acid mutation in the carbohydrate-
295 recognition domain (CRD) of Dectin-2, which inactivates its carbohydrate-binding
296 ability (Fig. 2A, Dectin-2^{QPD}). This implies that the yeast-derived proteins introduced
297 into the OsLu samples bound to Dectin-2 via the CRD. We also found that
298 commercially available oligosaccharides Vivinal®GOS and Duphalac® were not
299 reactive to Dectin-2 (Fig. 2B). Overall, these data demonstrate that the purification of
300 OsLu using *S. cerevisiae* introduced yeast-derived proteins reactive to Dectin-2.

301

302 *3.3. Identification of proteins in OsLu-Sc.*

303 To confirm the introduction of yeast-derived proteins which potentially interact 304 with Dectin-2, the protein fractions of the OsLu-*Sc*-R and OsLu-*Sc*-Pp samples were 305 isolated and analyzed in LC-MS. The identified peptide sequences were compared with 306 the *S. cerevisiae* protein database, as this yeast strain was used to remove 307 monosaccharides from the original OsLu (see Materials and methods, section 2.1).

308 According to the results indicated in the Supplementary material (Tables 1S, 2S, 309 3S and 4S), 119 proteins were identified in the OsLu-*Sc*-R fraction, and 89 in the OsLu-310 *Sc*-Pp fraction. One of the proteins present was a cell wall mannoprotein, Hsp150, from 311 *S. cerevisiae* (strain RM11-1a), corresponding to the Uniprot access name B3LPW4, 312 with a Mw of 36925 Da and pI 5.13. But this protein was not the only one mannoprotein 313 since others were also present, for which the interaction with Dectin-2 is well known. In 314 fungi, mannan is the only carbohydrate structure attached to proteins and therefore, all 315 the glycoproteins have mannan. Having this in mind, we found invertase (Uniprot 316 access G5EKG9, Mw 60816 Da, pI 4.63), the most abundant, which is known as a 317 mannan-bearing glycoprotein[31]. In addition, from the amino acid sequence of the 318 proteins in the list, we can easily find mannan-type carbohydrate attachment sequence, 319 N-X-S/T. Therefore, it is likely that most of the proteins identified in the list bind to 320 Dectin-2 via the carbohydrate moiety.

321 It is known that the cell wall of *S. cerevisiae* consists of β-1,3-glucan, β-1,6-322 glucan, chitin and mannoproteins in ratios of approximately 55%, 5%, 2% and 40%, 323 respectively, in relation to the DM of the cell wall (Kapteyn et al., 2001). The cell wall 324 is a bilayer structure, with an inner layer primarily consisting of β-1,3-glucans, which 325 constitutes a skeletal layer that is fortified by hydrogen bonds and extended with

covalently-bound β-1,6-glucan and chitin chains. The outer layer mainly consists of mannoproteins covalently bound to β-1,6- or β-1,3-glucan (de Groot et al., 2004; Netea, Brown, Kullberg, & Gow, 2008). These mannoproteins determine the surface properties of the cell and play an important role in adhesion between yeasts, and to host cells and inert surfaces, such as human tissues, food matrices or medical equipment, which is a prerequisite for virulence, fungal morphogenesis, cell wall biogenesis and biofilm formation (Kapteyn, Van Den Ende, & Klis, 1999).

4. Conclusion

We found that the prebiotic OsLu mixture purified by *S. cerevisiae* bound to Dectin-2, a type II transmembrane receptor found on the cell surface of innate myeloid cells, in an immune cell-based assay, whereas those purified by charcoal not. Furthermore, the relevant commercial lactulose and GOS failed to bind to Dectin-2. Subsequent enrichment by UF or precipitation with ethanol and proteomic analysis enabled us to point out the protein fraction as responsible for binding to Dectin-2, suggesting the bioactive ligands are of *S. cerevisiae* origin. Although more research is needed, present results show the potential mechanisms related to modulation of immune response by OsLu-*Sc*, highlighting the importance of purification method for prebiotics. Thus, the utilization of *S. cerevisiae* for this purpose might increase the known benefits of oligosaccharides derived from lactulose.

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Abbreviations used

CWPs, cell wall proteins.

Dectin-2WT, wild-type Dectin-2

Dectin-2QPD, carbohydrate-binding incompetent Dectin-2 mutant

DM, dry matter

GOS, galactooligosaccharides

OsLu, Lactulose derived oligosaccharides

OsLu-*Sc*, OsLu purified using *Saccharomyces cerevisiae*

OsLu-*Sc*-S, supernatant of OsLu-*Sc* treated with ethanol

OsLu-*Sc*-Pp, precipitate of OsLu-*Sc* treated with ethanol

OsLu-*Sc*-R, retentate of OsLu-*Sc* ultrafiltrated

OsLu-*Sc*-P, permeate of OsLu-*Sc* ultrafiltrated

OsLu-ActC, OsLu purified with active charcoal

PSMs, peptide spectrum matches

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Figure captions

Fig. 1. Scheme and code of the different samples of assayed prebiotics. GOS samples tested for Dectin-2 binding is shown.

Fig. 2. OsLu-*Sc* binds to Dectin-2. BWZ cells expressing Dectin-2^{WT}, Dectin-2^{QPD}, and mock transfectant were cultured in the presence of 50 μg/mL of indicated OsLu samples (A) and 30, 100, 300 and 1000 μg/mL of OsLu-*Sc* and other GOSs (B). Mannan from *S. cerevisiae*, (Sigma-Aldrich) at 2 μg/mL was used as a positive control. The β-galactosidase expression was monitored by a substrate in a colorimetric assay. Data are representative of at least two independent experiments with similar results. Error bars, S.D. Statistical analyses were performed by one-way ANOVA followed by Tukey's test. ***, $p < 0.001$

Table 1. Content (%) of protein and carbohydrates (mono-, di- and oligosaccharides) found in the different prebiotic samples studied.

	Protein %*	Fructose %**	Galactose %	Glucose %	Lactulose %	Lactose %	Other disaccharides %	Trisaccharides %	Tetrasaccharides %	Pentasaccharides %
OsLu	0.05***	19.5	12.4	1.19	24.7	n.d.	13.6	22.6	5.42	0.65
	(0.02)	(0.01)	(0.00)	(0.00)	(0.02)		(0.01)	(0.02)	(0.55)	(0.20)
OsLu-ActC	<0.05	1.51	0.80	0.14	10.3	n.d.	7.00	52.1	25.2	2.99
		(0.12)	(0.09)	(0.08)	(1.20)		(0.29)	(0.15)	(0.02)	(0.05)
OsLu-*Sc*	0.44	0.63	14.1	n.d.	26.2	n.d.	21.1	25.6	9.67	2.80
	(0.10)	(0.20)	(1.04)		(1.20)		(1.14)	(0.74)	(0.75)	(0.60)
OsLu-*Sc*-R	0.46	0.25	10.4	0.06	24.2	n.d.	18.9	28.2	13.8	4.33
	(0.04)	(0.01)	(0.29)	(0.01)	(0.78)		(0.55)	(0.94)	(0.44)	(0.15)
OsLu-*Sc*-P	0.05	0.30	11.3	0.78	26.6	n.d.	20.0	25.9	11.7	3.50
	(0.03)	(0.01)	(0.42)	(0.19)	(0.98)		(0.38)	(0.82)	(0.95)	(0.01)
OsLu-*Sc*-Pp	0.53	0.35	9.63	0.08	23.6	n.d.	25.2	33.6	7.2	0.77
	(0.09)	(0.19)	(0.85)	(0.03)	(0.01)		(0.03)	(0.02)	(0.05)	(0.42)
OsLu-Sc-S	<0.05	0.94	25.8	0.07	39.0	n.d.	22.7	11.4	n.d.	n.d.
		(0.27)	(0.01)	(0.03)	(0.03)		(0.13)	(0.94)		
Duphalac®	<0.05		7.87	0.29	88.7	3.22				
			(0.79)	(0.05)	(0.69)	(0.25)				
Vivinal®GOS	<0.1****	-	1.41	20.7	-	18.0	20.5	21.0	13.1	5.45
			(0.11)	(2.14)		(0.29)	(0.69)	(0.79)	(0.89)	(0.66)

*% with respect to DM. ** % with respect to total carbohydrates. *** average (standard deviation). **** Data provided by the manufacturer.
n.d. no detected (quantification limit 10 mg/L)

Figure

Fig. 1

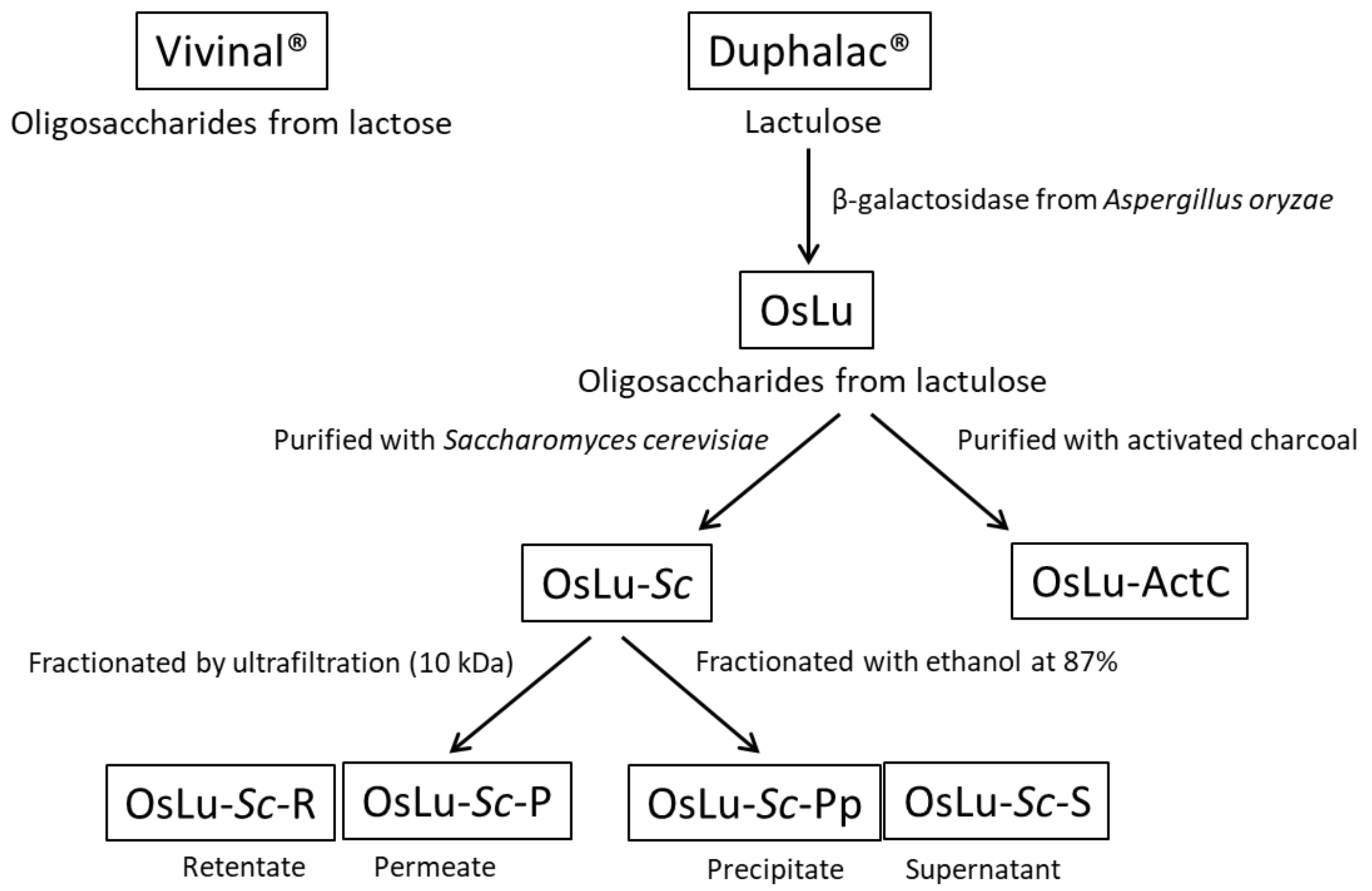
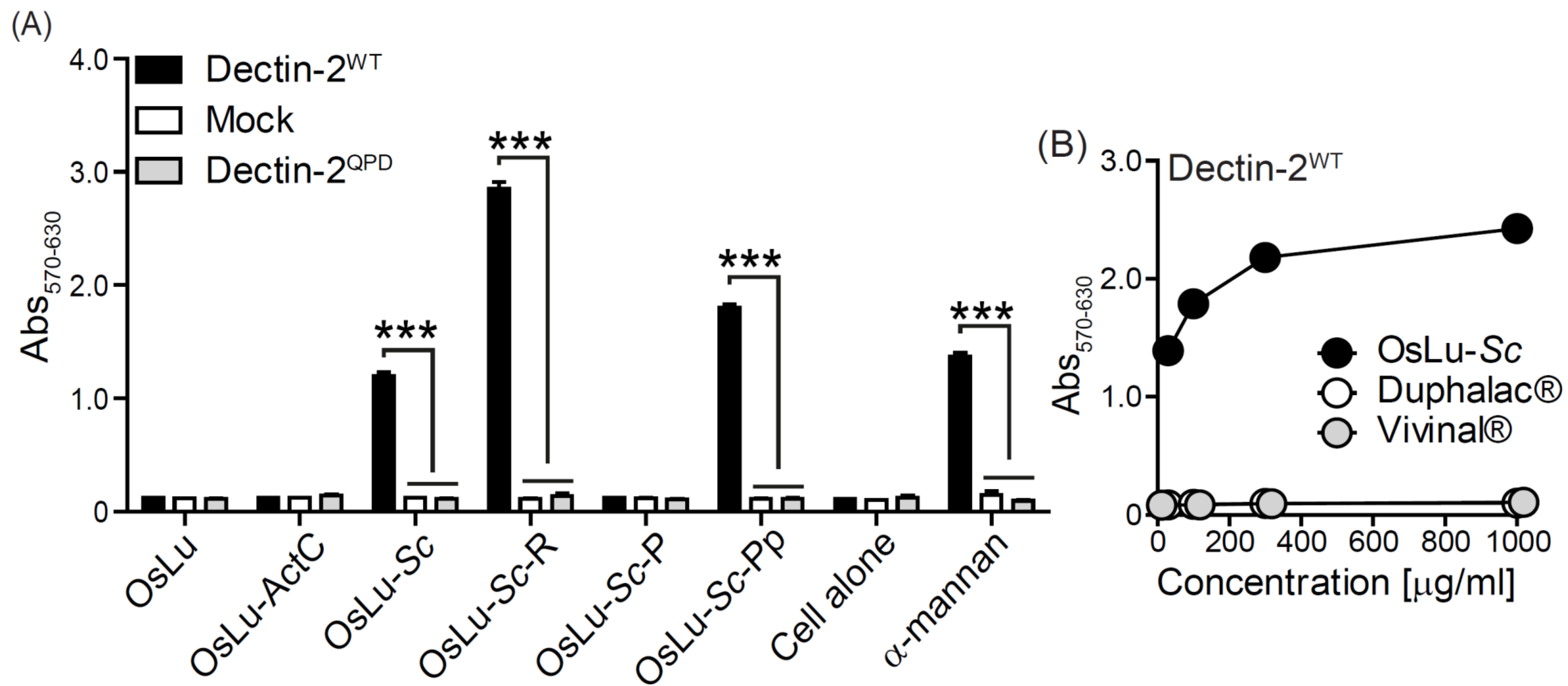


Fig. 2



Supplementary material for online publication only
Click here to download Supplementary material for online publication only: Table S1_List of protein OsLu_Sc_R.docx

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Click here to download Supplementary material for online publication only: Table S3_List of protein OsLu_Sc_Pp.docx

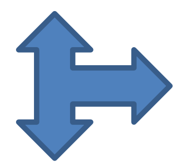
Supplementary material for online publication only
Click here to download Supplementary material for online publication only: Table S4_List of peptides OsLu_Sc_Pp.docx

*Graphical Abstract

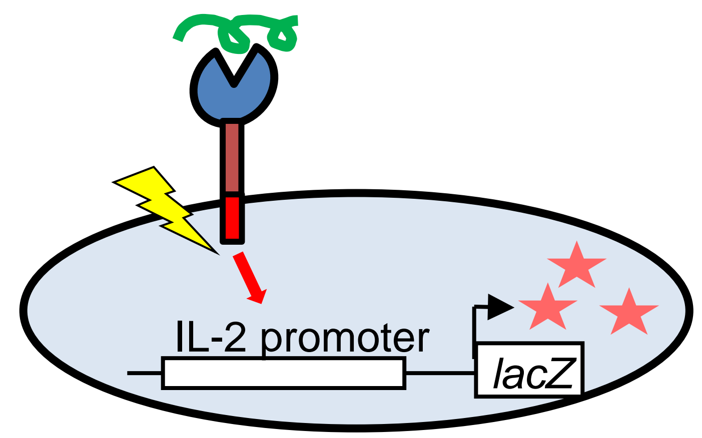
Oligosaccharides derived from lactulose (OsLu)

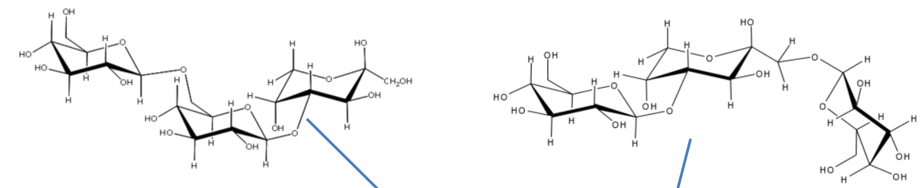
Baker's yeast (*Saccharomyces cerevisiae*) (Levital)

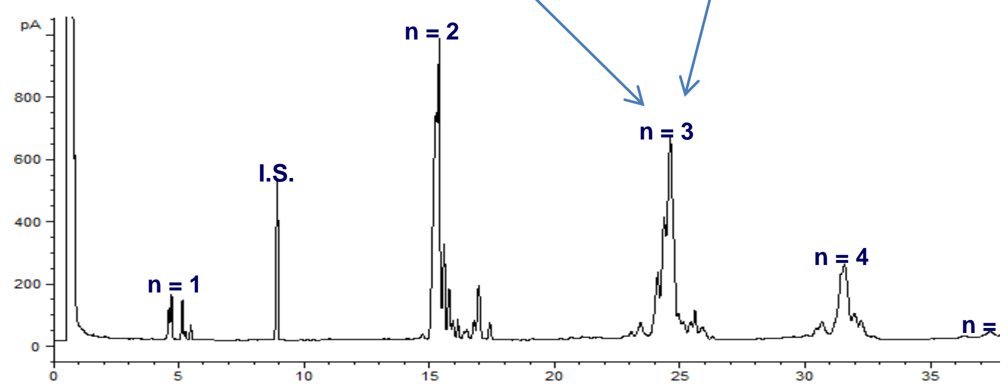
OsLu purified with *S. cerevisiae*



Mannoprotein (green) binding stimulates IL-2 promoter and lacZ gene to produce β-galactosidase.







Prebiotic syrups bind to Dectin-2