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# Up-scalable approaches for yeast mannan oligosaccharides (MOS) production: characterization and immunomodulatory properties

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ABSTRACT

Mannan oligosaccharides (MOS) are widely used as feed additives due to their effects on gut microbiome modulation. Its application in the nutraceutical and functional food industries is also increasing. Numerous studies portraying MOS production from β-mannans can be found, but its production from yeast mannans is barely explored. Herein, two up-scalable approaches to produce yeast-derived MOS are presented: hydrothermal and acidic processing. Process efficiency was evaluated, and the resulting extracts were characterized (ATR-FT-IR, PXRD, SEM, MWD, DSC, sugar, and protein content). Extracts were also evaluated for their cytotoxicity and immunomodulatory effect; furthermore, a simulated gastrointestinal tract digestion was performed to confirm their biological potential. The hydrothermal process resulted in the highest yield, whereas the acidic process resulted in lower molecular weight populations. Immunomodulatory results indicate that MOS digested extracts do not hold anti-inflammatory activity but grant an immunostimulant effect, whereas the non-digested MOS acidic extract showed an anti-inflammatory potential. This work provides valuable contributions towards the industrial production of yeast-derived MOS extracts, highlighting their potential for applications in various fields while contributing to a circular economy approach.

## 1. Introduction

Mannans are long-chain carbohydrates obtained from such distinct origins as plants or microorganisms; among the later, a relevant source is the yeast *Saccharomyces cerevisiae* cell wall. Yeast extracted mannans primarily consist of mannose, structurally organized in long α-(1−6) linked backbones, with α-(1−2) and α-(1−3) linked branches (Ballou, 1974; Peat et al., 1961a). These large macromolecules are linked to asparagine via N-acetyl-glucosamine (Ballou, 1974; Sentandreu and Northcote, 1968), while oligosaccharides are linked to serine or threonine residues (Ballou, 1976).

Mannan oligosaccharides (MOS) are obtained by cleaving mannans polysaccharides through chemical, physical or enzymatical processes (Teng and Kim, 2018; Gibson et al., 2017; Kango et al., 2022). Oligosaccharides are usually defined as carbohydrates with a low degree of polymerization (DP) (Yun, 1996; Roberfroid and Slavin, 2000), typically ranging from 2 to 20 monosaccharide units (Roberfroid and Slavin, 2000). However, IUB-IUPAC (IUB-IUPAC, 1983) terminology specifies 10 monosaccharide units as the dividing point between oligo and polysaccharides, despite such distinction lacks a physiological or molecular basis (Cummings et al., 1997).

MOS are widely used in the animal feed industry, namely as prebiotics, with the aim of promoting the growth of beneficial gut bacteria, suppresssing enteric pathogens (*Salmonella* sp., *Escherichia coli*, *Campylobacter* sp., and others), and enhancing intestinal mucosal integrity (Baurhoo et al., 2007; Ghasemian and Jahanian, 2016; Jana et al., 2021; Spring et al., 2015, 2000). They have also shown other promising properties, such as improving immunity (Faustino et al.,

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2023), antimutagenic effect and antioxidant activity (Xie et al., 2017).

Interestingly, some products identified as MOS in some studies do not rigorously correspond to the definition of MOS, often regarding to yeast cell wall products with high protein and glucose levels. Additionally, their molecular weight is too high to classify them as oligosaccharides (supplementary material: Table S1). Commercially available MOS extracts, therefore, do not perfectly fit into the MOS definition, hampering the association between composition and structure investigation.

The large majority of publications regarding MOS production from yeast are part of structural elucidation studies. Those studies use acetolysis reactions (Lee and Ballou, 1965; Stewart et al., 1968; Young et al., 1998), acid or alkaline hydrolysis, sometimes in combination with high temperatures (Peat et al., 1961a; Nakajima et al., 1974; Ogawa et al., 1994), as well as other chemical (Lee and Ballou, 1965; Stewart et al., 1968; Young et al., 1998; Nakajima et al., 1974; Ogawa et al., 1994; Peat et al., 1961b) and enzymatic methodologies (Kommineni et al., 2019; Wong-Madden and Landry, 1995; Maruyama et al., 1994; Dhawan and Kaur, 2007). Despite this multitude of existent processes, most of them are not suitable for application in large-scale production, especially MOS targeted for nutraceutical applications, due to the toxicity of the reagents used.

Indeed, although acetolysis reactions selectively cleave backbone α-(1−6) linkages between mannose units, yielding stable α-(1−2) and α-(1−3) linked oligosaccharides (Lee and Ballou, 1965), its industrial application is hindered because it employs pyridine and sodium methoxide (Kocourek and Ballou, 1969).

Acid hydrolysis, on the other hand, preferentially cleaves side-chain mannans as α-(1−2)-glycosidic bonds (Peat et al., 1961a). Mild alkaline conditions (Sentandreu and Northcote, 1968) can promote β-elimination reactions, yielding mannose, mannobiose, mannotriose, and mannotetraose from O-glycosidic linkage to serine and threonine (Ballou, 1976; Nakajima et al., 1974).

This study aims to compare the production of MOS-rich extracts from yeast mannans by two different approaches – an acidic approach (MOS $H_3PO_4$) and a hydrothermal process (MOS Parr). The resulting extracts were characterized by Attenuated Total Reflection Fourier-transform Infrared Spectroscopy (ATR-FT-IR), Powder X-ray Diffraction (PXRD), Scanning Electron Microscopy (SEM) and thermal analysis (DSC). Molecular weight distribution (MWD) was determined by Size-Exclusion Chromatography (SEC), mannose content was quantified by GC-FID, and soluble protein content was assessed using a BCA Assay Kit.

To explore their potential as nutritional supplements, and as far as we know for the first time, MOS-rich extracts underwent simulated digestion in the human gastrointestinal tract, following the INFOGEST protocol (Brodkorb et al., 2019), with some modifications in intestinal absorption phase. The digested samples were evaluated for cytotoxicity against a human intestinal epithelial cell line (Caco-2), and their immunomodulatory activity was assessed by measuring expression levels of interleukins 6 (IL-6) and 8 (IL-8).

## 2. Materials and methods

### 2.1. Materials

Mannans extracts were produced from genetically modified spent yeast (*Saccharomyces cerevisiae*), kindly provided by Amyris Inc. (Emeryville, CA, USA), according to a previously described methodology (Faustino et al., 2022). Ohly-Go® MOS (used as benchmark for colour comparison) was obtained from Ohly GmbH. Recombinant human interleukin-1β (IL-1β) was acquired from Sigma-Aldrich (St. Louis, USA). Phosphoric acid (85%) was purchased from Honeywell-Fluka (Charlotte, USA), acetone (>99.8%) was obtained from VWR (Portugal), and calcium hydroxide ($Ca(OH)_2$) purchased from Merck (Darmstadt, Germany). All reagents were used as received, without further purification.

### 2.2. MOS production from mannans extract

#### 2.2.1. MOS $H_3PO_4$ - acidic process with phosphoric acid

The reaction proceeded according to Liebert et al., (2008) with modifications. Briefly, 1 g of mannans extract was solubilized in 12 mL of phosphoric acid 85% (w/w) and placed in a water bath (Julabo TW20, Merck, Darmstadt, Germany) at 55 °C for 24 h. The suspension was poured into a glass container with 120 mL of cold (4 ºC) acetone to promote precipitation, and the resultant suspension was centrifuged at 4700 x *g* and 4 °C for 20 min. The pellet was collected, and the previous precipitation step was repeated twice with the supernatant. The collected pellets from the three precipitation steps were pooled and left to dry at room temperature overnight. The resultant solid was resuspended in 50 mL of deionized water and, after centrifugation (6600 x *g*, 20 min, 4 °C), the resulting supernatant was neutralized with a supersaturated solution of calcium hydroxide and centrifuged once more (6600 x *g*, 20 min, 4 °C). The pellet was discarded, and the MOS-enriched supernatant was filtered with a filter paper (Whatmann nº1) and then lyophilized (Freeze-dryer model Alpha 2–4 LSCplus, Christ, Osterode am Harz, Germany).

#### 2.2.2. MOS Parr - hydrothermal process in Parr reactor

Mannans extract, obtained as previously described, was solubilized in deionized water at a concentration of 10 mg/mL. The solution was placed in a reactor (Parr Instrument Company, Moline, Illinois) at 110 °C for 3 h. The resultant MOS-enriched suspension was collected, and then filtered and lyophilized, as detailed in section 2.2.1.

### 2.3. Structural and morphological characterization

#### 2.3.1. Attenuated Total Reflection Fourier-transform Infrared Spectroscopy (ATR-FT-IR)

ATR-FT-IR analysis was carried out using a PerkinElmer Frontier™ MIR/FIR spectrometer in a scanning range of 550–4000 $cm^{-1}$, scanning 16 times at a spectral resolution of 4 $cm^{-1}$.

#### 2.3.2. Powder X-ray Diffraction (PXRD)

PXRD analysis was performed using a Rigaku MiniFlex 600 diffractometer with Cu kα radiation, at a voltage of 40 kV and a current of 15 mA ($3° < 2\theta > 60°$; 0.01 step and 3.0°/min).

#### 2.3.3. Scanning Electron Microscopy (SEM)

SEM, using a Thermo Scientific™ Pro Scanning Electron Microscope, was used to examine the morphology of the MOS extracts. Extracts were coated with Au/Pd (target SC510–314B from ANAME, S.L., Madrid) using a Sputter Coater (Polaron, Bad Schwalbach, Germany), after being placed into observation stubs covered with double-sided adhesive carbon tape (NEM tape, Nisshin, Japan). All observations were carried out in a high vacuum with an acceleration voltage of 5 kV. The specimen was examined at a magnification of 1000x, and all micrographs are representative of the morphology of each extract.

### 2.4. Physicochemical properties and composition

#### 2.4.1. Colour

The colour point was determined using a portable CR-410 Chroma meter (from Minolta Chroma, Osaka, Japan). The CIELAB (L*, a*, b*) colour system was employed to determine the colour point, where L* is the lightness coordinate, a* defines the green-red coordinate, and b* the blue-yellow coordinate (Ordóñez-Santos et al., 2017). The total colour difference (ΔE * ) was calculated according to Equation (1) against Ohly-Go® MOS, benchmark product from *S. cerevisiae* by Ohly GmbH (Hamburg, Germany).

$$\Delta E^* = [(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2]^{1/2} \quad (1).$$

#### 2.4.2. Total protein

Total protein content was assessed using the microplate procedure of the BCA Protein Assay Kit (Pierce, Bonn, Germany), according to the manufacturer's instructions (25 μL sample/200 μL BCA working reagent; 37 °C/30 min; 562 nm). A BSA (Sigma, Munich, Germany) stock solution was prepared in bulk quantity prior to the experiments. BSA-dilution calibration curve was added, consisting of 8 points up to 1000 μg/mL BSA. Samples were measured in triplicate and averaged.

#### 2.4.3. Neutral sugars

Neutral sugars were previously hydrolysed and subsequently derivatized to their alditol acetates derivatives and analysed by Gas-chromatography-flame ionization detection (GC-FID) (Agilent Technologies, Inc., California, USA) in a 7890B GC System with a DB-225 capillary column (30 m length, 0.25 mm diameter, 0.15 μm thickness), according to the procedure previously described by Faustino et al (2022).

#### 2.4.4. Apparent Molecular Weight (MW) distribution

The Agilent 1260 Infinity II HPLC system was used to determine the apparent MW distribution, equipped with a vial sampler, quaternary pump, thermostatic oven, and refractive index (RID) detector. Agilent Technologies' OpenLAB CDS ChemStation was used for data acquisition and analysis. For separation, Agilent SEC, PL Aquagel-OH Mixed-M (250 ×4.6 mm, 8 μm), and PL Aquagel-OH 20 (300 ×7.5 mm, 5 μm) columns were utilized. To estimate molecular weight, a calibration curve of pullulan standards and mannan oligomers (DP2 to DP6) was employed (Supplementary material: Fig. S1). Under isocratic conditions, an aliquot of 10 μL of standards and test solutions was injected and eluted with the solvent (ammonium acetate 10 mM) at a flow rate of 0.5 mL/min. Columns were held at 50.0 °C, and RID was set to 35.0 °C. The mobile phase was filtered and degassed prior to use, and all samples and standards were filtered through 0.45 μm syringe filters.

#### 2.4.5. Differential scanning calorimetry (DSC)

DSC measurements were performed under a nitrogen atmosphere using Netzsch DSC 204 F1 Phoenix equipment calibrated with an indium standard. Samples (3–6 mg) were placed in aluminum DSC pans with a pinhole, with an empty pan serving as a control. Heating from 20 to 300 °C was done at a rate of 10 °C/min.

### 2.5. Evaluation of immunomodulatory potential in Caco-2 cells of non-digested and digested MOS extracts

#### 2.5.1. Cytotoxicity evaluation

*2.5.1.1. Cell line growth conditions.* Human colon carcinoma (Caco-2; HTB-37™) cells were obtained from the American Type Culture Collection (ATCC) and grown using high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS), 1% (v/v) antibiotic and antimycotic, and 1% (v/v) of non-essential amino acids 100 × (Gibco, Milan, Italy). Cells were used between passages 38 and 41.

*2.5.1.2. Cytotoxicity assay.* Cytotoxicity of digested samples was evaluated using the Caco-2 cell line in accordance with ISO 10993–5 (2009), employing PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, MA, USA) per the manufacturer's instructions. For non-digested MOS extracts, a concentration of 10 mg/mL was prepared by dissolving them in DMEM medium and sterilizing through a 0.22 μm filter. Commercial D-mannose was prepared at 20 mg/mL in phosphate-buffered saline, pH 7.4 solution (PBS) and sterilized at 100 °C for 20 min in an autoclave, followed by a two-fold dilution to reach a concentration of 10 mg/mL. Test concentrations ranging from 10 to 0.31 mg/mL were established through serial dilutions of the stock concentration. Digested samples were dissolved in PBS to achieve a final concentration twice as high as the desired mannose concentration after gastrointestinal simulation. Following filtration through a 0.22 μm filter, two-fold dilutions were prepared in DMEM medium containing antibiotics for Caco-2 cells. Cells were seeded at $1 \times 10^4$ cells/well in 96-well microtiter plate after 24 h of incubation. The cell culture medium was then replaced with sample solutions. Media without samples served as the positive control, while media with a 10% DMSO concentration acted as the negative control. After an additional 24 h incubation period, PrestoBlue (PB) reagent (10 μL) was added to the wells, and changes in cell viability were measured using fluorescence spectroscopy by a multidetector plate reader (Synergy H1, BioTek, California, USA). Fluorescence (excitation 570 nm; emission 610 nm) was measured after a 2 h incubation with PB reagent.

#### 2.5.2. Immunomodulation assay

Immunomodulatory assay was conducted according to Machado et al., (2022). Caco-2 cells were seeded at $2.5 \times 10^5$ cells/well in a 24 wells microplate and incubated for 24 h at 37 °C in humidified atmosphere with 5% of $CO_2$. After the incubation period, the culture medium was carefully replaced with medium supplemented with the digested samples, at a non-cytotoxic mannose concentration or with the non-digested samples at non-cytotoxic extract concentration. The plates were incubated at 37 ºC for another 24 h. As an inflammation control, IL-1β (Sigma-Aldrich Chemistry, St. Louis, USA) was used at 0.01 μg/mL, while for basal activity control (negative control) plain DMEM medium was used. At the end of the assay, supernatants were collected, centrifuged to remove debris, and stored at −80 °C for further analysis. IL-6) and IL-8 detection was performed by enzyme-linked immunosorbent assay (ELISA) using the Human IL-6 Elisa Kit High Sensitivity (Abcam, Cambridge, UK) and the Legend Max Human Elisa Kit IL-8 (BioLegend, San Diego, USA) according to the manufacturer's instructions. Interleukin expression values were obtained in pg/ug protein. Protein content of samples was determined using the BCA Pierce Assay Kit.

#### 2.5.3. Simulation of gastrointestinal digestion

To mimic the potential modifications occurred in MOS-rich extracts during digestion process, MOS Parr, MOS $H_3PO_4$ and D-mannose (selected because MOS are mainly composed of mannose monomers) underwent an in vitro gastrointestinal tract (GIT) procedure, adapted from the model reported by Brodkorb et al., (2019) (INFOGEST protocol). The recommended daily dose of D-mannose in supplements for UTI prevention for adults is about 1.5 g per day (Lenger et al., 2020), and thus the amount of sample used in the GIT experiments was adjusted for the abovementioned D-mannose quantity. The gastric phase and the intestinal phase of the GIT were performed according to what is described in the INFOGEST protocol with the respective simulated fluids (simulated gastric fluid and simulated intestinal fluid). Pepsin at 101.06 mg/mL (Pepsin P7012, Sigma-Aldrich Chemistry, St. Louis, USA) was used in the gastric phase, and pancreatin at 186.05 mg/mL (Pancreatin P7545, Sigma-Aldrich Chemistry, St. Louis, USA) and bile at 90.39 mg/mL (Bile extract B3883, Sigma-Aldrich Chemistry, St. Louis, USA) were used in the intestinal phase. After the simulated GIT, samples were heated at 85 ºC for 10 min, to inactivate all the enzymes, and centrifuged for 1 h, 4 ºC at 4400 x *g* to collect the supernatant. These were stored at − 30 ºC until being used for cytotoxicity and immunomodulation evaluation in Caco-2 cells.

### 2.6. Statistical analysis

The characterization analysis employed T-student tests, while immunomodulatory assessment utilized Two-way ANOVA with Tukey's post-test, both conducted at a 95% confidence level using GraphPad Prism 6 software. Sample normality was evaluated with the Shapiro-Wilk's Test. Results are expressed as mean values ± SD (standard deviation), with significance set at $p < 0.05$. All experiments were

triplicated, and the MOS production processes were independently repeated at least three times on distinct days.

## 3. Results and discussion

Various methodologies, including enzymatic, alkaline and acid hydrolysis have been reported for MOS production from plant-derived mannans, which have mannose units linked by β-(1−4) glycosidic bonds (Jana et al., 2021). However, yeast mannans feature mannose moieties linked by α-(1−6), α-(1−2), and α-(1−3) glycosidic bonds, thus demanding the use of different α-mannanases for enzymatic hydrolysis, which are not cost-effective for industrial use. Therefore, hydrothermal, acid, and alkaline hydrolysis are the obvious options to efficiently break down carbohydrates into oligomers (Pronyk et al., 2011). Despite these procedures lack selectivity in cleavage extent and degree, they are cost-efficient and avoid the use of toxic reagents, making them suitable for food and nutraceutical applications. Unlike alkaline and acid hydrolysis, which require pH neutralization and salt removal, comprising extra processing steps and costs, hydrothermal treatment eliminates the need for these procedures.

The present study explores the production of MOS-rich extracts for nutraceutical area using two distinct methods: hydrothermal (MOS Parr) and acid hydrolysis (MOS $H_3PO_4$). The resulting extracts underwent comprehensive characterization and simulated gastrointestinal tract digestion. Cytotoxicity and immunomodulatory properties before and after digestion. were assessed.

### 3.1. Structural, morphological and thermal analysis

The structural, morphological, and thermal analysis of the MOS-rich extracts was achieved by a set of solid-state techniques: ATR-FT-IR, SEM, PXRD and DSC, as illustrated in Fig. 1.

Analysis of the ATR-FT-IR spectra (Fig. 1a) reveals similar spectral characteristics in both extracts: a strong and broad vibration band at 3000 - 3285 $cm^{-1}$, corresponding to the stretching of the hydroxyl groups (Liu et al., 2018a; Zhao et al., 2022); a vibration at 2925 $cm^{-1}$, which can be assigned to C-H stretching vibration (Zhao et al., 2022; Liu et al., 2018b); the vibrations at 1637 and 1651 $cm^{-1}$ can be assigned to the carbonyl group, thus suggesting the presence of residual protein in the samples (Huang et al., 2010). Furthermore, vibration bands at 1025 $cm^{-1}$, and 980 – 1120 $cm^{-1}$, may be attributed to the O-H angular vibrations, and C-O and C-C stretching vibration of pyranose rings (respectively), which are commonly present in MOS (Zhao et al., 2022; Liu et al., 2018b; Jana and Kango, 2020). The distinctive vibrations of mannan within the 1200–1400 $cm^{-1}$ and 1080 $cm^{-1}$ range correspond to the stretching vibrations of C-O and C-C bonds. According to Huang (2008), within this range, both mannan and mannan oligosaccharides exhibit absorption bands originating from the stretching vibrations of ν(CC) and ν(COC), along with the (OH) band.

The PXRD patterns for both extracts exhibit a single broad reflection centered at 2θ = 19.2° (Fig. 1b), which is consistent with the predominant amorphous character.

The morphology of MOS extracts was also evaluated by SEM (Fig. 1c) and revealed a mixture of structures, thus classified as heterogeneous. Even though heterogenicity is observed, both extracts revealed similar tapered structures (highlighted with a α). MOS $H_3PO_4$ showed the most heterogeneous nature, demonstrated by the presence of large and small aggregates of plates (highlighted with a β) and rough spherical particles (highlighted with a γ) in greater density and quantity than MOS Parr, which was composed of large smooth plates and large aggregates of small plates and rough spherical particles (highlighted with a β and γ).

Finally, the thermal properties of extracts were assessed by DSC (Fig. 1d), and the corresponding thermograms are very similar: the first event shows an endothermic peak at around 70 °C, which can be attributed to water evaporation (Harish Prashanth, 2002; Kittur et al., 2002); the exothermic event observed at ca. 260–290 °C can be

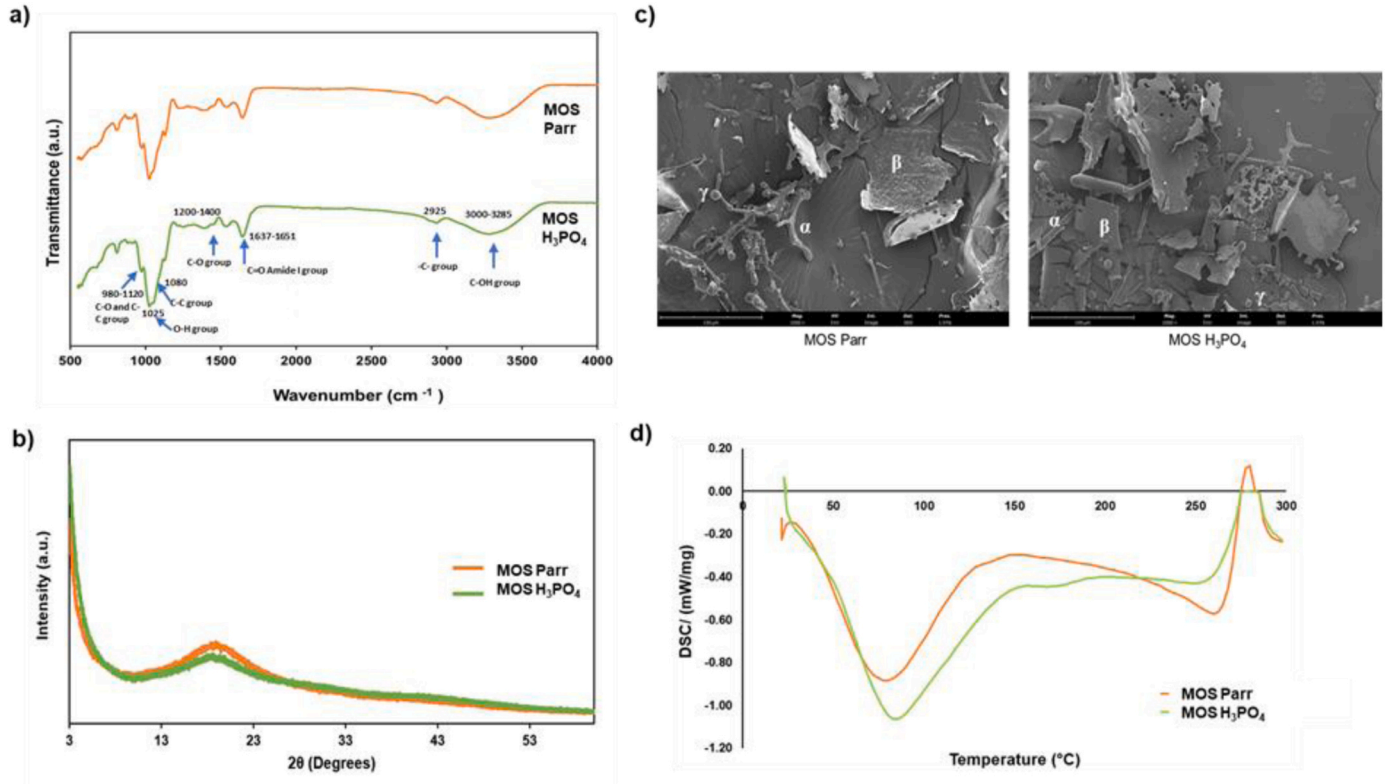


**Fig. 1.** Structural, morphological, and thermal behaviour characterization of the MOS extracts (MOS $H_3PO_4$ and MOS Parr) by a) Attenuated Total Reflection Fourier-transform Infrared Spectroscopy (ATR-FT-IR), b) Powder X-ray Diffraction (PXRD) analysis, c) Scanning electron microscopy (SEM) and d) Differential scanning calorimetry (DSC) curves.

associated with polysaccharide thermal disintegration, and the events near to or above 300 °C are generally observed during the degradation of the saccharide structure and may be implicated in the dehydration of saccharide rings (Ospina Álvarez et al., 2014).

In conclusion, the structural analysis of the extracts did not reveal significant differences resulting from the two production processes.

### 3.2. Physicochemical characterization

The integrated and comparative analysis of the physical appearance, along with some physicochemical properties, is presented below.

#### 3.2.1. Physical appearance

Powders' appearance is clearly different, as depicted in Table 1. Even though all MOS extracts appeared as homogeneous fine powders, MOS Parr has a whiter tone when compared to MOS $H_3PO_4$, which presents a light brown colour.

The colour of products for human consumption is a key criterion for their marketing, as only a limited number of colours is accepted by consumers. The colour point of MOS extracts was evaluated by CIELAB (L*, a*, b*) system, according to Ordóñez-Santos et al., (Ordóñez-Santos et al., 2017), using a MOS benchmark product of *S. cerevisiae*, Ohly-GO® MOS, for comparation purposes. Results show that MOS Parr exhibits a lightness (L*) value comparable to the benchmark and higher than MOS $H_3PO_4$.

According to Tiwari et al., (Tiwari et al., 2008) the value ΔE * represents the magnitude of the colour difference between the tested samples and can be classified analytically as very distinct (ΔE * >3), distinct (1.5 < ΔE * <3), and small (ΔE * <1.5). The resulting ΔE * was 9.21 for MOS Parr and 11.17 for MOS $H_3PO_4$, when compared to the MOS benchmark. The ΔE * that results from the direct comparison between MOS Parr and MOS $H_3PO_4$ extracts is 7.84 ± 0.54. Given these results, the colour differences between the samples are very distinct (above 3), further supporting the distinct visual perception that can be seen in Table 1.

#### 3.2.2. Physicochemical and composition

Regarding the MOS $H_3PO_4$ process, we employed a procedure previously applied to the hydrolysis of cellulose into cello oligosaccharides by using phosphoric acid (Hsu and Penner, 1991; Zhang and Lynd, 2005). Despite acidic conditions have already been described to preferentially break side chain bonds, to the best of our knowledge this is the first report on using phosphoric acid to produce MOS from yeast mannans.

Concerning MOS Parr, several methods have been reported involving the production of MOS from guar gum (a galactomannan) using only hydrothermal conditions. According to Miyazawa & Funazukuri (Miyazawa and Funazukuri, 2006) polysaccharides such as agar, guar gum, starch, and xylan were hydrolyzed to produce mono and oligosaccharides under hydrothermal conditions with and without carbon dioxide in a small batch reactor. Water under hydrothermal conditions (i.e., higher temperature and pressure) is an appealing reaction medium because its distinctive properties enable a variety of reactions without the need to resort to a catalyst (Katritzky et al., 1996; Bröll et al., 1999; Akiya and Savage, 2002). Water is an eco-friendly reaction medium due to its non-toxicity, non-flammability, and availability (Miyazawa and Funazukuri, 2005). Furthermore, hydrolysis of polysaccharides under hydrothermal conditions does not require neutralization and desalting processes (Mok and Antal, 1992; Bobleter, 1994; Sasaki et al., 1998; Ando et al., 2000; Kabel et al., 2002; Nagamori and Funazukuri, 2004; Miyazawa and Funazukuri, 2004; Miyazawa et al., 2006). Despite the hydrolytic degradation of polysaccharides such as celluloses (Ando et al., 2000; Kim et al., 2001; Mok et al., 1992; Sasaki et al., 2004), hemicelluloses (Kabel et al., 2002), pectic acid (Miyazawa and Funazukuri, 2004) and starch (Nagamori and Funazukuri, 2004) under hydrothermal conditions has been significantly studied, optimizing the reaction conditions and reactor design is complicated because the desired products are readily degraded and/or decomposed at elevated temperatures (Miyazawa and Funazukuri, 2006).

To assess the potential differences between MOS-rich extracts obtained from the two different production processes, the physicochemical characteristics of the resulting extracts were studied and are summarized in Table 2.

The highest production yield was achieved by thermal hydrolysis, which also resulted in the extract with the highest mannose content (65.5%). Interestingly, this extract resulted in a lower mannose-to-glucose ratio than the acid hydrolysis extract.

The protein content from both extracts was statistically similar,

**Table 1**
Results of physical appearance and colour characteristics of the MOS Parr, MOS $H_3PO_4$ and benchmark Ohly-GO® MOS.

| | MOS Parr | MOS $H_3PO_4$ | Benchmark Ohly-Go® MOS |
|---|---|---|---|
| Physical appearance |  |  |  |
| Colour Characteristics | | | |
| L* | 78.98 ± 0.04[a] | 71.20 ± 0.74[b] | 79.04 ± 0.29[a] |
| a* | 2.22 ± 0.04[a] | 3.30 ± 0.11[b] | -0.87 ± 0.16[c] |
| b* | 10.51 ± 0.05[a] | 12.41 ± 0.56[b] | 19.18 ± 0.62[c] |
| ΔE *[1] | 9.21 ± 0.05[a] | 11.17 ± 0.23[b] | n.a. |

[a,b and c] means within the same line, labelled different subscripts, differ significantly ($p < 0.05$).[1]The calculation of ΔE * was performed against a commercial MOS product for comparison purposes.
L* is the lightness coordinate, a* defines the green-red coordinate, and b* the blue-yellow coordinate. n.a.- not-applicable.

**Table 2**
Results of physicochemical characterization of MOS extracts obtained by different extraction methods. The results are the average values of batches produced on different days.

| | MOS Parr | | MOS $H_3PO_4$ | |
|---|---|---|---|---|
| Protein Content (% w/w) | $10.85 \pm 3.13^a$ | | $14.03 \pm 2.91^a$ | |
| Mannose (% w/w) | $65.51 \pm 9.43^a$ | | $52.38 \pm 3.25^a$ | |
| Glucose (% w/w) | $8.10 \pm 3.88^a$ | | $2.69 \pm 1.40^b$ | |
| Molecular Weight * | MW (kDa) | Area % | MW (kDa) | Area % |
| | $196 \pm 44^a$ | $73 \pm 7$ | $75 \pm 5^b$ | $33 \pm 6$ |
| Solid Yield (%)** | $75.75 \pm 7.68^a$ | | $37.03 \pm 3.97^b$ | |

[a,b,] means within the same line, labelled different subscripts, differ significantly ($p < 0.05$) *Apparent molecular weight of the most significant population and its corresponding percentage area * *Determined as the final weight of MOS extract, divided by the initial weight of mannans extract, multiplied by 100.

implying that the different extraction processes present equivalent efficiencies regarding the elimination of proteins present in mannans. It is possible that the conditions tested in this study were insufficient to cleave the glycosyl-serine and glycosyl-threonine bonds, phosphodiester bonds, some peptide bonds, disulphide, and acyl ester bonds present in mannans.

Molecular weight (MW) has been described to be directly associated with bioactivity (Liu et al., 2021). Analysis of the apparent molecular weight distribution of MOS extracts revealed significantly different distribution profiles (Supplementary material: Fig. S1), as depicted in Fig. 2.

Whereas hydrothermal extract (Fig. 2a) presented one fundamental population (196 kDa), phosphoric acid hydrolysate exhibits a wide distribution (Fig. 2b), which has been determined to extend from around 79 kDa to 196 Da.

Table 2 also shows the average molecular weight (MW) of the most significant population, i.e., the one that presented the highest % area. These results clearly emphasize that the acid hydrolysis results in the production of components with lower MW, when compared to thermal hydrolysis, for this set of conditions. If the apparent MW corresponds solely to mannose units, then the degree of polymerization (DP) of the most significant population of MOS Parr and MOS $H_3PO_4$ could be estimated to 1209 and 462, respectively. As previously mentioned, the classification of oligosaccharides may implicate a DP below 20, which was only achieved in the acid hydrolysate.

In summary, the extracts resulting from hydrothermal treatment presented fewer populations of MW, whereas extracts from acid hydrolysis resulted in a wide range of populations, some of which fall under the classification of oligosaccharides.

This heterogenicity of the apparent MW can be attributed to the different experimental conditions. The diversity acquired could be of great interest in studying any potential bioactive effect and understanding any hypothetical correlations between function and molecular weight.

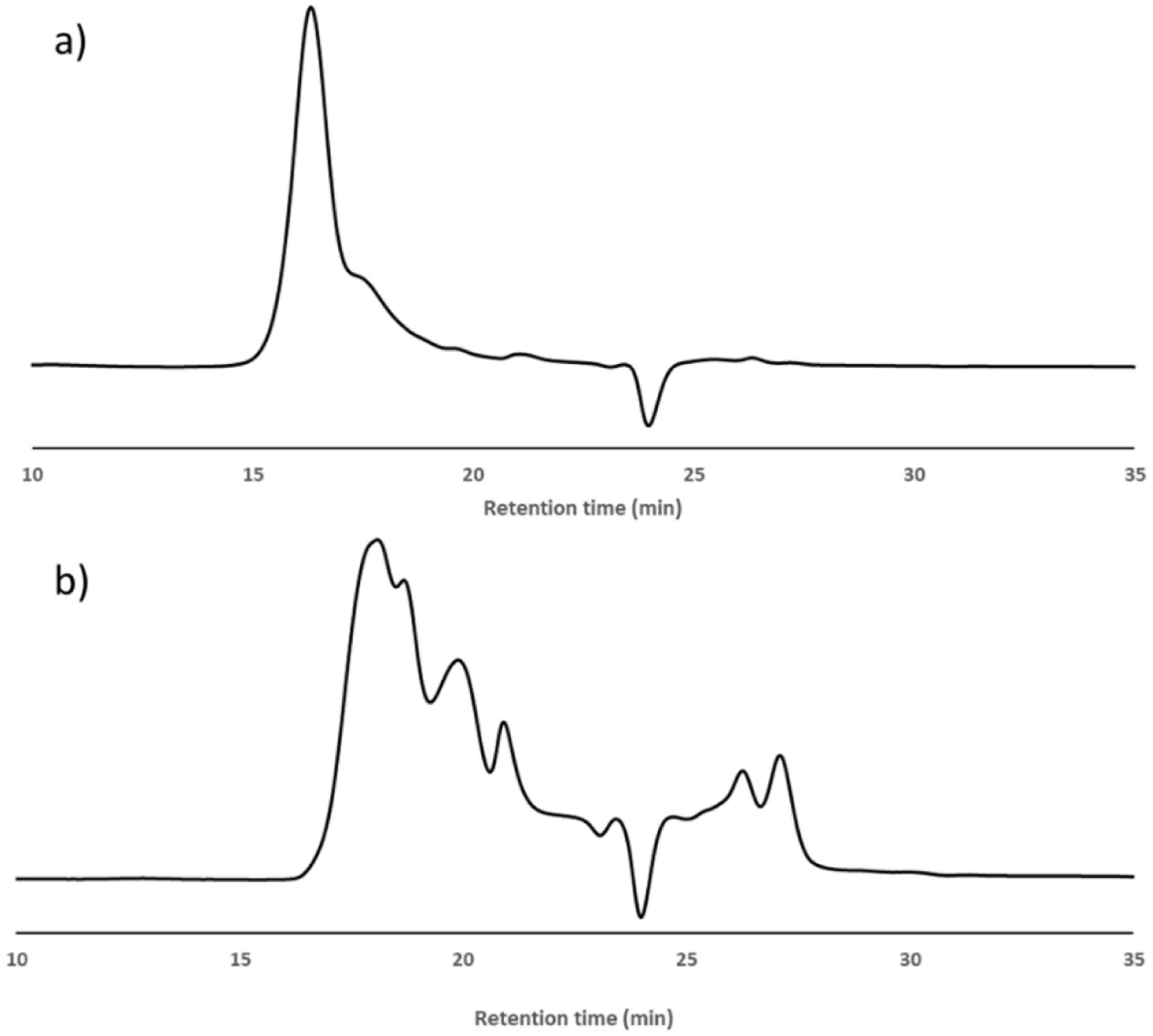


**Fig. 2.** Size exclusion chromatography elution profiles of a) MOS Parr extract and b) MOS $H_3PO_4$ extract.

### 3.3. Evaluation of MOS extracts before simulated gastrointestinal tract digestion

The immunomodulatory potential of MOS extracts was assessed before and after the digestion process. Since D-mannose has recently gained attention for its immunoregulatory function (Zhang et al., 2017, 2021), it is worth to assess whether MOS-rich extracts could positively influence the immune system, particularly cytokine expression. Cytokines, a group of signalling proteins crucial in inflammation control (Kany et al., 2019), are produced by both the innate system and epithelial cells involved in immune responses (Stadnyk, 1994). IL-1β, a major pro-inflammatory cytokine linked to pain, inflammation, and autoimmune diseases (Ren and Torres, 2009; Dinarello, 2011), plays a central role in intestinal inflammation leading to damage (Al-Sadi and Ma, 2007). In vitro studies of this proinflammatory mediator in inflammatory bowel diseases (IBDs) have shown increased secretion of IL-6 and IL-8 (Walle, 2010), two pro-inflammatory cytokines associated with chronic inflammation, produced by activated macrophages in response to inflammation (Tanaka et al., 2014; Harada et al., 1996), and widely used as biomarkers in IBDs.

Immunomodulatory MOS activity is often studied in the context of animal gut health with MOS-supplemented diets (Agazzi et al., 2020; Hoving et al., 2018; Kim et al., 2011; Lu et al., 2020; Torrecillas et al., 2007, 2014; Wang et al., 2016; Yang et al., 2008), but studies in Caco-2 cells, the in vitro model of the human intestinal epithelial barrier, are rarely considered. To the best of our knowledge, this is the first report evaluating the immunomodulatory activity of non-digested and digested yeast-derived MOS in Caco-2 cells.

#### 3.3.1. Cytotoxicity

Cytotoxicity assessment involved exposing Caco-2 cells to the samples (MOS Parr, MOS $H_3PO_4$ and D-mannose) and measuring their impact on cell metabolism (Fig. 3). According to ISO 10993–5 (ISO, 2009) guidelines, metabolic inhibition above 30% indicates cytotoxicity.

Both MOS extracts showed no cytotoxicity toward Caco-2 cells at concentrations up to 10 mg/mL (MOS Parr exhibited $-124.7 \pm 3.4\%$ metabolic inhibition, and MOS $H_3PO_4$ showed-$101.5 \pm 9.5\%$ at 10 mg/mL). D-mannose also did not present cytotoxic behaviour up to a concentration of 10 mg/mL ($-53.2 \pm 9.5\%$). MOS Parr demonstrated the highest metabolic stimulation among the samples, possibly indicating superior immunomodulatory potential in Caco-2 cells.

#### 3.3.2. Immunomodulatory assessment of extracts in Caco-2 cell line

The impact of MOS extracts on pro-inflammatory cytokines IL-6 and IL-8 secretion with and without an inflammatory stimulus (IL-1β) in Caco-2 cells is depicted in Fig. 4.

In the non-stimulated cell model, the expression levels of IL-6 by Caco-2 cells exposed to both MOS extracts and D-mannose were not statistically significant ($p > 0.05$) compared to the basal control (DMEM medium exposed to cells). The expression values present in all samples do not differ from the levels presented by the basal condition ($p > 0.05$).

In IL-1β stimulated cells, the expression levels of IL-6 were 1.6-fold higher in cells exposed to MOS Parr and 1.5-fold higher in cells exposed to MOS $H_3PO_4$ extract, when compared to their respective control ($p < 0.05$). Notably, the most significant difference was observed in cells exposed to D-mannose, which showed a 2.7-fold increase ($p < 0.01$).

Regarding the expression levels of the cytokine IL-8 on a non-stimulated model, no significant differences ($p > 0.05$) were observed. Indeed, MOS extracts could not be quantified as their levels were below the limit of quantification.

In an IL-1β stimulated model, the expression levels observed for MOS $H_3PO_4$ extract were lower (0.74-fold variation) when compared to their respective basal control, revealing that this extract produces an anti-inflammatory response. IL-8 expression levels in cells exposed to MOS Parr and D-mannose were, respectively, 1.5-fold and 2.1-fold higher than those of the cells control with the stimulus ($p < 0.001$).

These findings suggest that yeast-derived MOS extracts have the potential to modulate IL-6 expression levels in Caco-2 cells under inflammatory conditions. However, further research is needed to elucidate the mechanisms involved in the modulation of IL-8 expression, and to better understand the anti-inflammatory properties demonstrated by MOS $H_3PO_4$ extract.

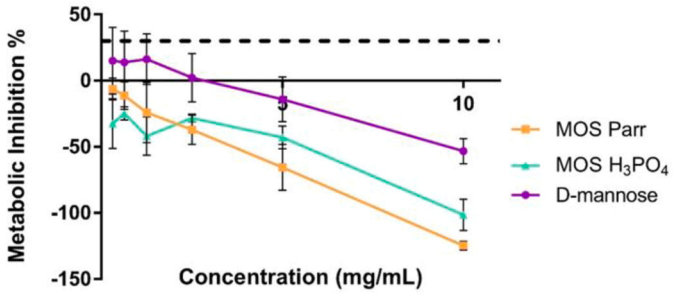


**Fig. 3.** Results of the metabolic inhibition in % for the following concentrations: 0.31 to 10 mg/mL of the MOS Parr, MOS $H_3PO_4$ and D-mannose. Dotted line indicates the threshold of cytotoxicity.

### 3.4. Evaluation of MOS-rich extracts after simulated gastrointestinal tract digestion

Commercial supplements containing polysaccharides (e.g., glucomannans) are typically consumed orally in capsule form. However, limited literature exists on how digestion affects their structural integrity and, consequently, their biological properties. To mimic in vivo conditions more closely and assess their bioactive potential, it is crucial to evaluate these compounds after exposure to simulated GIT conditions. In vitro assays, valuable for assessing compound digestibility and bio accessibility, offer a cost-effective and relatively precise alternative to in vivo models with fewer ethical concerns and aligning with European (EU) guidelines (Ribeiro et al., 2020). Table 3 presents the final mannose concentrations of samples after undergoing GIT simulation, starting with an initial dose of 1.5 g of D-mannose.

#### 3.4.1. Cytotoxicity

Cytotoxicity assessment results, presented in Table 3 as mannose concentrations, are crucial for determining the maximum concentration of digested extract that cells can tolerate without significant metabolic inhibition.

Despite starting GIT with equivalent amounts of mannose, samples concentrations after GIT were distinct, especially for MOS $H_3PO_4$, which presented significantly higher values. This difference may be attributed to interactions occurring in the GIT, potentially involving the degradation of free mannose in the gastric phase. In-depth studies are required to fully understand these mechanisms.

Cytotoxicity evaluations of solutions resulting from serial dilutions of GIT samples demonstrated non-cytotoxicity concentrations for the same dilution factor (16x), which suggests that cytotoxicity is primarily due to the components from GIT matrix rather than the extracts or D-mannose. This hypothesis was supported by the non-cytotoxicity observed in a control test involving GIT simulations without the sample (data not shown).

#### 3.4.2. Immunomodulatory assessment of digested samples in Caco-2 cell line

The effect of MOS Parr and MOS $H_3PO_4$ on pro-inflammatory cytokines IL-6 and IL-8 (Fig. 5) was evaluated in Caco-2 cells (both stimulated and non-stimulated with IL-1β to trigger an inflammatory response) by quantification of the expression levels of the cytokines on

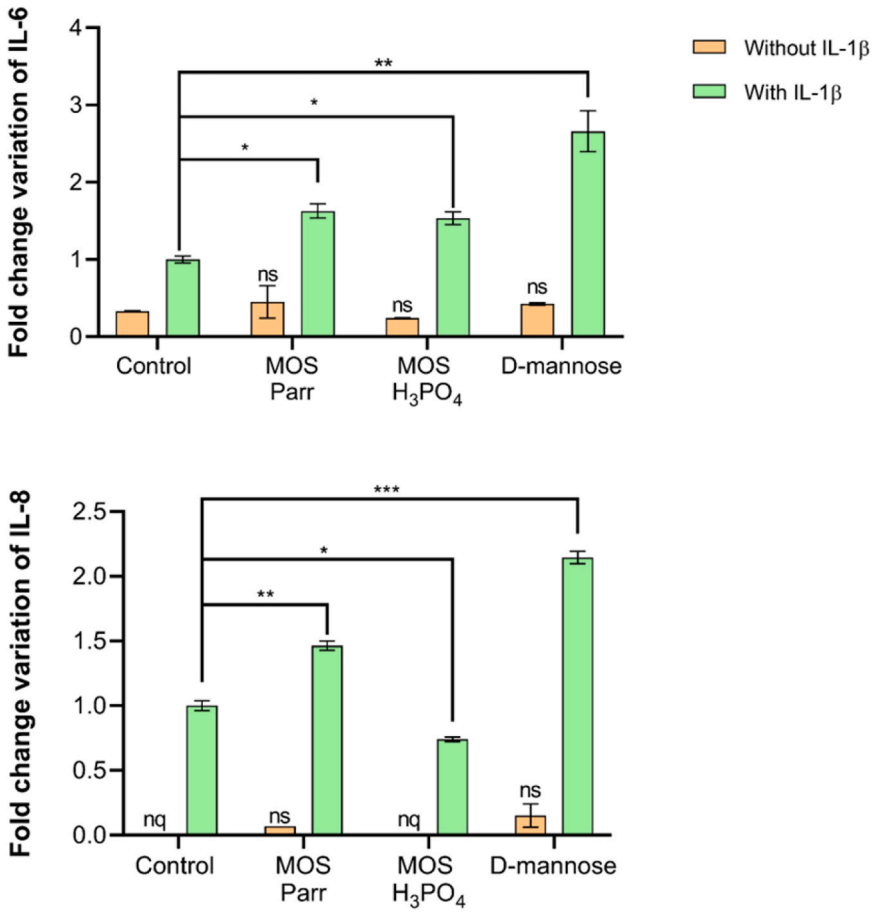


**Fig. 4.** Fold change variation of IL-6 and IL-8 production by Caco-2 cells, with and without IL-1β stimulus. The statistical analysis revealed significant differences between the controls and the non-digested tested samples (MOS Parr, MOS $H_3PO_4$, and D-mannose) expressed by the symbol * ** ($p < 0.001$), * * ($p < 0.01$) and * ($p < 0.05$). The symbol "ns" indicates non-significant statistical differences and nq – not quantified. Results were normalized against the control of stimulated cells.

**Table 3**
Mannose concentrations of samples after GIT simulation and non-cytotoxic mannose concentrations for Caco-2 cells.

| Mannose concentration | MOS Parr | MOS $H_3PO_4$ | D-mannose |
|---|---|---|---|
| After GIT | 6.51 mg/mL | 15.63 mg/mL | 9.30 mg/mL |
| Non-cytotoxic* | 0.41 mg/mL | 0.98 mg/mL | 0.58 mg/mL |

* Non-cytotoxic concentration for Caco-2 cells

the supernatants of the cells exposed to the samples.

On non-stimulated cells, IL-6 expression levels by cells exposed to both MOS extracts and D-mannose are not statistically different ($p > 0.05$) than those from cells in DMEM. Similarly, exposure of cells to all samples, does not seem to lead to expression levels that are different from those of cells in basal condition ($p > 0.05$).

As expected for cells stimulated IL-1β, expression levels of IL-6 exposed both to MOS Parr and MOS $H_3PO_4$ extract were 1.2-fold higher ($p < 0.05$) than those of the respective control. This difference is more significative ($p < 0.01$) for cells exposed to D-mannose (1.3-fold variation). Differently, and although IL-8 expression levels were 2.1-fold and 1.2-fold higher than the levels observed for cells in DMEM, this increase was not statistically significant ($p > 0.05$). When exposed to D-mannose, a 2.9-fold significant improvement in IL-8 expression levels was observed ($p < 0.05$). In addition, slightly differences were noticed between MOS extracts and D-mannose regarding the fold change variation of IL-6 expression levels ($p < 0.05$).

Overall, these results indicate that MOS samples, although not displaying anti-inflammatory properties or increasing the secretion of IL-8 levels, can still elicit an immune response, since, unlike IL-8, IL-6 has also anti-inflammatory activity and is pivotal in several processes implicated in the resolution of inflammation (Hunter and Jones, 2015).

## 4. Conclusions

This study explored two approaches to produce MOS from genetically modified yeast mannans, and their impact on extract properties. Structural, morphological, and thermal analysis using ATR-FT-IR, PXRD, SEM, and DSC revealed no significant differences between the extracts. However, physicochemical analysis revealed distinct distribution profiles. Hydrothermal process resulted in higher yields but low molecular weight conversion, while acid process resulted in lower yields and a higher conversion to oligosaccharides. Cytotoxicity assessments on Caco-2 cells revealed that concentrations up to 10 mg/mL of MOS extracts and D-mannose are non-cytotoxic.

Immunomodulation evaluations on Caco-2 cells demonstrated that the extracts, particularly MOS $H_3PO_4$, could modulate IL-6 and IL-8 expression under inflammatory conditions. After simulated gastrointestinal tract (GIT) digestion, significant differences in mannose concentration between the digested extracts and control (D-mannose) emerged. This suggests potential interactions of the extracts with GIT solutions and enzymes. Immunomodulation assessments on digested extracts indicated an immune response, with increased IL-6 expression under inflammatory conditions.

In conclusion, this study proposes two methods for producing MOS-rich extracts for nutraceutical applications and explores their impact. The most significant variation observed was in molecular weight

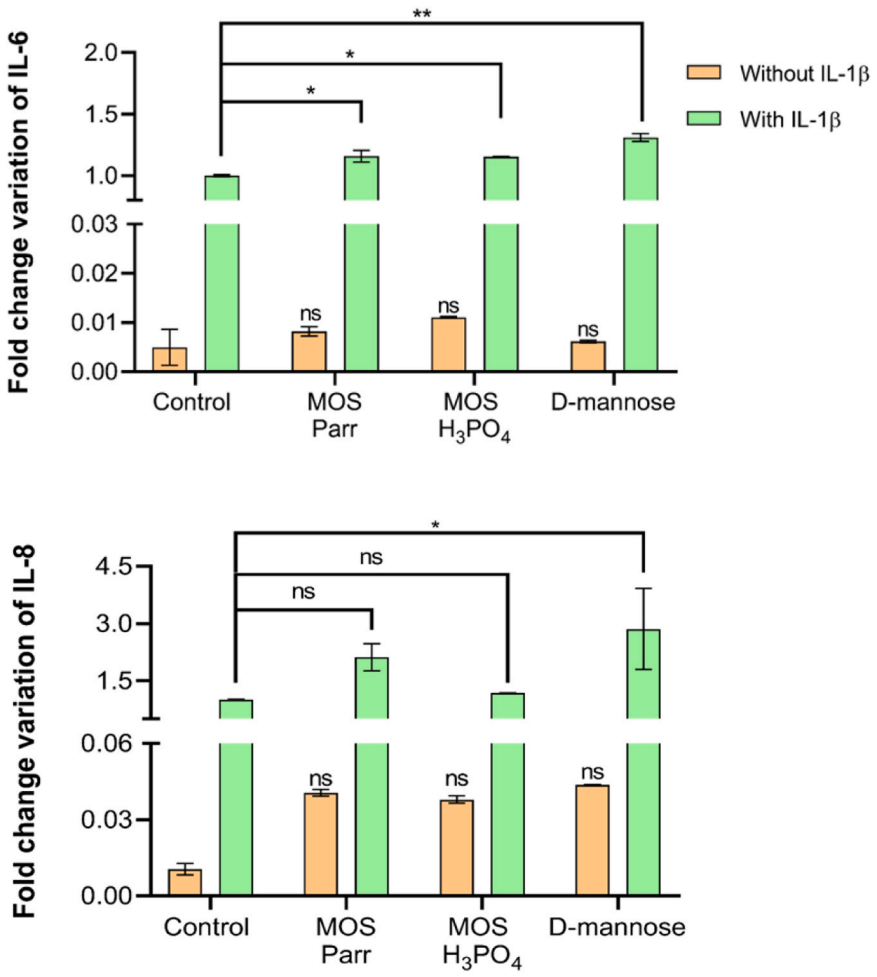


**Fig. 5.** Fold change variation of IL-6 and IL-8 production by Caco-2 cells with and without stimulus (IL-1β). Results are expressed in relation to each control and bars represent means ± SD. Statistically significant differences between the controls and the digested tested samples (MOS Parr, MOS $H_3PO_4$ and D-mannose) are indicated *$p < 0.05$ and * *$p < 0.01$ (ns – non-significant statistical differences).

distribution, with acid hydrolysis resulting in a more substantial conversion to lower molecular weight molecules. Interestingly, acid-derived MOS also exhibited the most pronounced immunomodulatory response, making it a potential candidate for further exploration and practical applications. Nevertheless, additional investigation is necessary to fully understand the mechanisms and potential benefits of using yeast derived MOS-rich extracts for human consumption in the food and healthcare industries.

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## Institutional review board statement

In this manuscript were used Caco-2 [Caco2] epithelial cells HTB-37™ from ATCC (https://www.atcc.org/products/htb-37).

## CRediT authorship contribution statement

Conceptualization, M.F., J.D., C.F.P. and A.P.C.; Methodology, M.F., J.D. and C.F.P.; Investigation, M.F., J.D., C.F.P., J.O.P., A.S.O., A.M.P., S. S., A.B.R. and C.F.; Writing–original draft preparation, M.F., J.D., C.F.P. and A.M.P; Writing–review and editing, J.D., C.F.P., A.M.P and A.P.C.; Supervision, A.P.C.; Project administration, M.E.P.; and Funding acquisition, M.E.P. All authors have read and agreed to the published version of the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2023.12.005.

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