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ESCOLA SUPERIOR DE BIOTECNOLOGIA
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VALORIZATION OF YEAST-BASED MANNANS AS SOURCES OF BIOACTIVE INGREDIENTS FOR NUTRACEUTICAL APPLICATIONS

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Microbiology

Ana Margarida Massa Faustino

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Supervisor: **Ana Paula Taboada Costa Santos Carvalho, PhD**

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"*The scientist is not the man who provides the real answers; is the one who asks the real questions.*"

Claude Lévi-Strauss

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Resumo

A Amyris, Inc. é uma empresa dedicada a biologia sintética que procura minimizar o impacto ambiental dos processos industriais. Assim, este trabalho foca-se na valorização de subprodutos da levedura, especialmente *Saccharomyces cerevisiae*, para obtenção de compostos bioativos como mananas e oligossacarídeos de mananas (MOS), para diversas aplicações nas indústrias alimentares (humana e animal) e de produtos nutracêuticos.

Diferentes processos de extração (físico, químico e enzimático) foram explorados para a extração de mananas da biomassa de levedura, e os produtos finais foram caracterizados quanto às suas propriedades estruturais, físico-químicas e biológicas. A hidrólise enzimática apresentou o maior rendimento de sólidos, apesar de ser o processo mais dispendioso. A hidrólise alcalina com 0.25 M de hidróxido de sódio resultou num maior rendimento de mananas, mas apresentou problemas de contaminação de sais. A hidrólise térmica com água foi selecionada como o método preferencial devido ao alto teor de mananas obtido e aos custos mínimos associados.

O passo seguinte visou a produção de MOS a partir das mananas extraídas utilizando duas metodologias: produção hidrotérmica em reator Parr (MOS Parr) e hidrólise com ácido fosfórico (MOS H_3PO_4). Além da caracterização estrutural e físico-química, os MOS produzidos foram avaliados quanto ao seu potencial biológico. Entre as propriedades anteriores, o extrato hidrotérmico apresentou populações de oligómeros com maior peso molecular. Ambos os extratos foram submetidos a uma simulação da digestão gastrointestinal, e suas respostas imunomoduladoras foram avaliadas. Além de não apresentarem propriedades anti-inflamatórias, os extratos atuaram como imunoestimulantes ao aumentar os níveis de expressão da interleucina 6 (IL-6), uma citocina pleiotrópica que desempenha um papel central na resposta imunológica.

Quanto às suas potenciais aplicações, os extratos de MOS foram avaliados quanto à sua capacidade de inibir a adesão da *Escherichia coli* uropatogénica (UPEC) às células da bexiga, com o objetivo de prevenir infeções do trato urinário (ITUs). Ambos os extratos de MOS foram avaliados em ensaios de profilaxia e competição, mostrando uma inibição significativa da UPEC no ensaio de competição (MOS H_3PO_4 89,6 ± 1,4% e MOS Parr 79,8 ± 1,1%) quando comparados com a D-manose, uma das referências comerciais mais utilizadas em terapias não-antibióticas contra a UPEC.

Além disso, os extratos mostraram potencial para serem utilizados no controlo da candidíase vulvovaginal (CVV). Quando em fluido vaginal simulado (FVS), e embora tenha sido observada uma pequena diminuição no crescimento de *Candida albicans* apenas nos ensaios profiláticos, ambos os extratos promoveram o crescimento de lactobacilos (um aumento de 4 ciclos logarítmicos na viabilidade celular). Ademais, estudos de competição *in vitro* demonstraram que o extrato MOS Parr, em combinação com *Lactobacillus crispatus*, inibiu a adesão de *C. albicans* às células vaginais (redução da adesão de ~69%), enquanto a avaliação profilática revelou que ambos os extratos, em sinergia com a população de *L. crispatus*, preveniram efetivamente a adesão de *C. albicans* (redução da adesão de ~56% e ~60% para MOS H_3PO_4 e MOS Parr, respetivamente).

Adicionalmente, a modificação química de mananas por carboximetilação foi investigada para aumentar o seu potencial biológico. A carboximetilação afetou a composição, aparência física e capacidade antioxidante das mananas, e as mananas modificadas demonstraram maior eficácia na inibição do crescimento de bactérias e fungos patogênicos, nomeadamente UPEC e *C. albicans*.

No geral, esta dissertação contribui para o objetivo mais amplo de valorizar os subprodutos da levedura e aproveitar seus compostos bioativos para aplicações nas indústrias alimentares e indústria nutracêutica, enquanto fornece as bases para o desenvolvimento de suplementos de mananas e MOS para a gestão de desafios específicos relacionados à UPEC e *C. albicans*.

Palavras-chave: Extração de Mananas, extratos de Oligossacarídeos de mananas (MOS), Caracterização, Inibição da adesão de uropatogénicos, Carboximetilação de mananas

Abstract

Amyris, Inc., a company dedicated to synthetic biotechnology, seeks to minimize the environmental impact of its industrial processes. Accordingly, the work hereby presented focuses on the valorization of their yeast by-products (*Saccharomyces cerevisiae*) to obtain bioactive compounds such as mannans and mannan oligosaccharides (MOS) for various applications in food, animal feed, and nutraceutical industries.

To extract mannans from yeast biomass, different extraction processes (physical, chemical, and enzymatic) were explored, and final products were thoroughly characterized regarding their structural, physicochemical, and biological properties. Enzymatic hydrolysis provided the highest solid yield but was costly. Alkaline hydrolysis with 0.25 M sodium hydroxide resulted in a higher mannan yield but presented salt contamination issues. Thermal hydrolysis with water was chosen as the preferred method due to its favourable characteristics, including a high mannan content and minimal associated costs.

The next step aimed at the production of MOS from the extracted mannans using two methodologies: hydrothermal production in a Parr reactor (MOS Parr), and hydrolysis using phosphoric acid (MOS H_3PO_4). In addition to their structural and physicochemical characterization, the produced MOS were also evaluated for their biological potential. Among the former properties, the hydrothermal extract showed higher molecular weight populations of oligomers. Both extracts were subjected to a simulation of the gastrointestinal tract digestion, and their immunomodulatory responses were assessed. Besides not showing anti-inflammatory properties, the extracts acted as immunostimulants by increasing the expression levels of the interleukin 6 (IL-6), a pleiotropic cytokine that plays a central role in the immune response.

Regarding their potential applications, the MOS extracts were evaluated for their ability to inhibit the adhesion of uropathogenic *Escherichia coli* (UPEC) to bladder cells, aiming to prevent urinary tract infections (UTIs). Both extracts were assessed in prophylaxis and competition mode assays, showing a significant inhibition of UPEC in the competition assay (MOS H_3PO_4 89.6 ± 1.4% and MOS Parr 79.8 ± 1.1%) when compared to D-mannose, one of the commercial benchmarks most widely used in non-antibiotic UPEC therapies.

Additionally, the extracts showed potential to be used in the management of vulvovaginal candidiasis (VVC). When in simulated vaginal fluid (SVF), and although a small decrease in *Candida albicans* growth was observed only in prophylaxis assays, both

extracts promoted lactobacilli growth (a 4 log cycles increase in cell viability). Furthermore, *in vitro* competition studies demonstrated that MOS Parr extract, in combination with *Lactobacillus crispatus*, effectively inhibited the adhesion of *C. albicans* to vaginal cells (adhesion reduction of ~69%), while prophylactic evaluation revealed that both extracts, in synergy with the population of *L. crispatus*, effectively prevented the adhesion of *C. albicans* (adhesion reduction of ~56% and ~60% for MOS H_3PO_4 and MOS Parr, respectively).

Furthermore, the chemical modification of mannans through carboxymethylation was investigated to enhance their biological potential. Carboxymethylation affected the composition, physical appearance, and antioxidant capacity of mannans, and the modified mannans demonstrated increased efficacy in inhibiting the growth of pathogenic bacteria and fungi, namely UPEC and *C. albicans*.

Overall, this dissertation contributes to the broader objective of valorizing yeast by-products and harnessing their bioactive compounds for applications in food and nutraceutical industries, while providing the basis for the development of mannans and MOS supplements for the management of specific health-related challenges associated with UPEC and *Candida albicans*.

Keywords: Mannans extracts, Mannan oligosaccharides extracts, Characterization, Inhibition of uropathogens adhesion, Carboxymethylation of mannans

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Thank you very much Everyone!

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List of abbreviations

A

ABTS – 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt)

ABTS$^{\bullet+}$ – 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) radical cation

ACS – American Chemical Society

AGP – Antibiotic Growth Promoters

ANOVA – Analysis of variance

AOAC – Association of Official Analytical Chemists

AOSs – Agaro-oligosaccharides

APPH – 2,2'-Azobis(2-amidino-propane) dihydrochloride

ATL – Autolysis

ATL+TH – Autolysis + Thermal hydrolysis with water

ATR-FT-IR– Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

ATCC – American Type Culture Collection

B

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

C

CFU – Colony-formity unit

CIELAB – Commission Internationale de I'Eclairage

CM – Commercial mannan

CMM – Carboxymethyl mannans

CMTH – Carboxymethyl mannans of Thermal hydrolysis extraction

D

Da – Dalton

DAMPs – Damage-associated molecular patterns

DMEM – Dulbecco's Modified Eagle medium

DMSO – Dimethyl sulfoxide

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen

DP – Degree of polymerization

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

DPPH• – 2,2-Diphenyl-1-picrylhydrazyl radical

DSC – Differential Scanning Calorimetry

E

ECACC – European Collection of Authenticated Cell Culture

EH – Enzymatic hydrolysis

ELISA – Enzyme-linked immunosorbent assay

EP – European Pharmacopoeia

ET – Electron transfer

EU – European Union

F

FBS – Fetal bovine serum

FD – Freeze drying

FDM – Freeze dry mannans extract

FOS – Fruto-oligosaccharides

G

g – gram

g – Gravitational force

GC – Gas Chromatography

GC-FID – Gas Chromatography-flame Ionization Detection

GIT – Gastrointestinal Tract

GlcNac – Glycosidic linked

GOS – Galacto-oligosaccharides

GRAS – Generally Recognized as Safe

H

h – hours

HAT – Hydrogen atom transfer

^{1}H NMR – Hydrogen Nuclear Magnetic Resonance

HPLC – High Performance Liquid Chromatography

I

IBD – Inflammatory bowel disease

IL-1β – Interleukin 1 beta

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-17 – Interleukin 17

INFOGEST – International network of food digestion and colonic fermentation models

ISO – International Organization for Standardization

IUB-IUPAC – International Union of Biochemistry and International Union of Pure and Applied Chemistry

L

L – Litre

LAB – Lactic acid bacteria

LDLs – Low density lipoproteins

LPS – Lipopolysaccharides

K

kDa – kilodalton

kWh – kilowatt-hour

M

Man – Mannose

mg – milligram

MH – Mueller Hinton medium

min – minutes

mL – militers

mM – millimolar

MOI – Multiplicity of infection

MOS – Mannan oligosaccharides

MOS Parr – Mannan oligosaccharides from hydrothermal process

MOS H_3PO_4 – Mannan oligosaccharides from phosphoric acid process

MRS – De Man, Rogosa and Sharpe medium

MW – Molecular weight

N

n.a. – non applicable

ns – non-significant statistical differences

O

OD – Optical density

ORAC – Oxygen Radical Absorbance Capacity

P

p – Probability value in statistical significance testes

PAMPs – Pathogen-associated molecular patterns

PB – PrestoBlue

PBS – Phosphate buffered saline

PCA – Plate Count Agar medium

PEF – Pulsed electric field

PXRD – Powder X-ray Diffraction

R

RID – Refractive index detector

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute medium

S

SD – Spray drying

SDM – Spray dry mannans extract

SDS – Sodium dodecyl sulphate

SEM – Scanning Electron Microscopy

SGLT1 – Sodium-dependent glucose transporter I

SPSS - Statistical Package for Social Sciences software

T

TE – Trolox equivalent

TEAC – Trolox equivalent antioxidant capacity

TEER – Transepithelial electrical resistance

TLR – Toll-like receptor

TH – Thermal hydrolysis with water

TH NaOH 0.25M – Thermal hydrolysis with 0.25 M of NaOH

TH NaOH 1.5M – Thermal hydrolysis with 1.5 M of NaOH

Trolox – (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

U

UPEC – Uropathogenic *Escherichia coli*

UP1a – Uroplakin 1a

UTI – Urinary tract infection

US – Ultrasounds

UV – Ultraviolet

V

VVC – Vulvovaginal candidiasis

W

w/w – Weight by weight

w/v – Weight by volume

X

XOS – Xylo oligosaccharides

Y

YM – Yeast & Mould broth

€ – Euro

® – Registered Trademark

µg – micrograms

™ – Trademark

% – Percentage

Chapter 1

Introduction

This chapter describes the current knowledge about yeast mannans and mannan oligosaccharides (MOS), and the main objective of the present thesis.

Adapted from the following scientific publication:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pintado, Manuela E.; Carvalho, Ana P.; 2021. Mannans and mannan oligosaccharides (MOS) from *Saccharomyces cerevisiae* – A sustainable source of functional ingredients. Carbohydrate Polymers **272**:118467. **https://doi.org/10.1016/j.carbpol.2021.118467**

Ana Paula Carvalho hereby authorize the PhD student Ana Margarida Faustino from the CBQF/ESBUCP to include the following publications in her doctoral thesis entitled "Valorization of yeast-based mannans as sources of bioactive ingredients for functional applications", as a result of her activity: Mannans and mannan oligosaccharides (MOS) from *Saccharomyces cerevisiae* – A sustainable source of functional ingredients

Margarida Faustino [a], Joana Durão [a,b], Carla F. Pereira [a] , Manuela E. Pintado [a], Ana P. Carvalho [a]

1.1. Framework

Yeasts, especially *Saccharomyces cerevisiae* and related species, have been used by humans since ancient times, for different purposes. In the past centuries, the increased demand in yeast-derived products have forced the development and optimization of industrial yeast biomass production processes, which currently generate large amounts of wastes, causing a tremendous environmental impact.

Currently, there is an increasing general concern and awareness to topics such as the preservation of the environment and its biodiversity. Therefore, alternatives are being developed to minimize or even eliminate industrial wastes, turning them into by-products. Within this path, their application and transformation into new products with high added value may be part of the solution.

The main objective of the Amyris Company is the development of competitive, sustainable, and economically viable products through biotechnological processes, as an alternative to traditional processes based on resource depletion and with considerable environmental impact. Spent yeast, *S. cerevisiae*, is the second most abundant by-product of fermentation at Amyris.

High unused volumes of spent yeast led to increasing economic burdens, related to the cost of their elimination. To avoid such costs and simultaneously increase the process sustainability, this doctoral project aims to contribute to the valorization of yeast by-products with novel studies that will enable to obtain bioactive compounds such as mannans and mannan oligosaccharides (MOS) for food and nutraceutical industries.

1.2. State of the art

Mannans and mannan oligosaccharides (MOS) from Saccharomyces cerevisiae – A sustainable source of functional ingredients

1.2.1. Introduction

1.2.1.1. Circular Economy

In recent years, there has been a growing global emphasis on adopting circular economy models as viable alternatives to the linear economic ones. The traditional linear model, known as "take, make, and dispose," relies on unlimited resources and unrestricted waste disposal for economic growth. In contrast, the circular economy presents a sustainable and efficient system that aims to minimize resource consumption and waste generation, offering a promising solution to address these challenges (Ness, 2008). Despite, unfortunately, most of the industrial current practices still contribute to resource depletion and the use of disposable products, the circular economy concept has increasingly been adopted, thus promoting an economic system in which the value of products, materials and resources is maintained for as long as possible. The main objective is to minimize the generation of waste, ensuring that products and resources remain in circulation and are reused, or recycled instead of being discarded. By adopting this approach, the circular economy aims to achieve a sustainable balance and reduce waste generation (European Commission, 2018).

The theoretical basis of circular economics focuses on the principles of industrial ecology. Industrial ecology aims to achieve two main objectives: closing the loop of materials and substances and minimizing resource consumption and environmental pollution. Within the field of industrial ecology, the concept of industrial metabolism highlights the notion that industrial systems can operate similarly to natural ecosystems (Ayres et al., 1989). These principles of nature, including utilizing waste for production, promoting resilience through diversity, harnessing renewable energy sources, adopting systemic thinking, and facilitating cascading flows of materials and energy, are applied within the context of industrial ecology (Ellen MacArthur Foundation, 2013).

For this reason, and to increase industrial ecology, the valorization and reuse of spent yeast to extract bioactive compounds and develop innovative products is an excellent

circularity of the industry, in the perspective of the food-health relationship, as well as in the protection of the environment and waste management. *S. cerevisiae* (Brewer's yeast) is described as a Generally Recognized as Safe (GRAS) microorganism (Rakowska et al., 2017) and, apart from its nutritional value, it also presents some interesting bioactive properties.

The development of competitive, sustainable, and economically viable biotechnological processes, as an alternative to traditional processes based on resource depletion and with considerable environmental impact, represents a core objective for Amyris activity. To successfully achieve this ambitious goal, Amyris has developed an innovative process to promote the conversion of plant sugars into stable, alternative, and low-cost sources of biomolecules with high commercial interest, whose field of application covers industries as diverse as food, pharmaceutical, cosmetic, agrochemical, energy, or materials.

The large-scale production of these biomolecules generates at least 2,200 tons of spent yeast per year which represents 20% of Amyris produced waste, making spent yeast (*S. cerevisiae*) the second fermentation by-product at Amyris, which is also the case in the brewing industry. Currently, their treatment expresses an additional cost for the company (about 30,000 dollars in 2016) (internal information from the company).

The cell wall of *S. cerevisiae* has high levels of mannans, a polysaccharide with several functional and bioactive properties, including modulation of the intestinal microbiota, due to its role as a substrate for beneficial bacteria and its ability to agglutinate pathogenic bacteria such as *Salmonella* sp. and *E. coli*, promoting intestinal health. They make this microorganism a very attractive ingredient for the food, feed, and nutraceutical industries.

1.2.1.2. Spent Yeast Industry

Besides the growing use of *S. cerevisiae* as a "live factory" for synthetic biology industry, its application in "older" industries is also raising annual worldwide production of beer accounted for 193 billion liters in 2017, enough to fill 77,000 Olympic swimming pools! This production generated 46.26 billion tons of by-products, among which spent yeast corresponds to 12.7%. It is estimated that for every 100 liters of beer produced, 1.5 – 3 kg of spent yeast is disposed (*Brewers By-Products Market Outlook, Share, Size, Forecast, Trends, Report*, 2020; *World Beer Production | Statista*, 2020; Nanyang Techological University, 2017). Indeed, Brewer's spent yeast (*Saccharomyces cerevisiae*, *Saccharomyces*

pastorianus and *Saccharomyces carlsbergensis* species) is the second major by-product from the brewing and wine industry (Vieira et al., 2016).

The valorization and reuse of spent yeast to extract functional compounds and develop innovative products is an excellent circularity of the industry, in the perspective of the food-health relationship, as well as in the protection of the environment and waste management.

The nutritional and bioactive composition of *S. cerevisiae* has been increasingly attracting the cosmetic, pharmaceutical, and food industries. Indeed, yeast residue is currently an important ingredient for the food (Butylina et al., 2007) or even cosmetic industries, as it is used, for instance, to integrate hydration products formulations (Gaspar et al., 2008).

Mannans are polysaccharides widely present in nature, such as in plants, bacteria, yeasts, and other organisms (Olaniyi et al., 2013). Although commonly used as a hardener ingredient and as an emulsion stabilizer (Singh et al., 2018), mannans have several commercial applications, derived from their physicochemical properties (water solubility, viscosity, and stability). Additionally, mannans also exhibit relevant health benefits, such as inhibition of pathogen adherence, modulation of bacterial growth (Smith et al., 2020) and improvement of the immune response (Lee & Dugoua, 2011; Onitake et al., 2015). In general, polysaccharides with immunostimulant properties interact both directly and indirectly with various parts of the immune system, subsequently stimulating different immunological mechanisms (Yin et al., 2019). Due to the above-mentioned properties, yeast mannans are commonly used in animal feed as antibiotic replacers (Smith et al., 2020; Spring et al., 2015).

As above described, mannans have been increasingly reported as presenting very promising activities, which tailor their use in various fields of application. The present study intends to review the current knowledge on yeast mannans, focusing on their extraction processes, mannans hydrolysis to produce MOS, and its chemical modification to enhance bioactive properties, a field of research still in its infancy. Furthermore, described bioactivities and potential applications of mannans, MOS and mannose are also revised, as well as an overview on the main products in the market.

1.2.2. Mannans

1.2.2.1. Sources and composition

Mannans are long-chain carbohydrates mainly composed of mannose residues (Tester & Al-Ghazzewi, 2013), which can be found in the most diverse sources, such as vegetables, microorganisms and seeds (**Figure 1**). Structurally, mannans and heteromannans (composed of two or more different monomers) are polysaccharides distributed in nature as part of hemicelluloses in plant tissue (Capek et al., 2000; Scheller & Ulvskov, 2010) or as a constituent of glycoproteins in yeast (e.g., *S. cerevisiae*) or bacteria cell walls (e.g., *Helicobacter pylori*) (Ballou, 1974, 1976; Singh et al., 2018).

In plants, mannans and heteromannans are an essential component of the hemicellulose family and can be classified into four subfamilies, according to their monosaccharide composition: pure mannans (containing only mannose); glucomannans; galactomannans and galacto-glucomannans (Libjaková et al., 2007; Schröder et al., 2009; Singh et al., 2018). Some of the plant-derived mannans are well known and widely used as thickeners (Millane & Hendrixson, 1994), stabilizers (Mikkonen et al., 2009), and gelling agents (Maekaji, 1974) in the food industry, e.g., locust bean gum galactomannan or konjac glucomannan. Mannans can also be found in the cell wall of various fungi, as summarized in **Table 1.**

Table 1. Sources of mannans from Fungi, main species, and monosaccharide composition (type).

Source	Species	Type	Reference
Fungi	*Aspergillus fumigatus*	Galactomannans	(Stynen et al., 1992)
	Penicillium oxalicum		(Kurakake et al., 2006)
	Trichophyton fermentans		(Gorin et al., 1969; Ikuta et al., 1997)
	Torulopsis gropengiesseri		(Gorin et al., 1969)
	Candida albicans		(Vazquez-Reyna et al., 1999)
	Candida lipolytica		(Ikuta et al., 1997)
	Saccharomyces cerevisiae	Mannans*	(Orlean, 2012)
	Candida utilis	Glucomannans	(Miadoková et al., 2006)

*Mannans from *S. cerevisiae* also contain a residual fraction of glucose which is part of the linkage between the polysaccharide and the protein.

The cell wall composition of yeast and filamentous fungi varies between species, although there are structural similarity (Abbott et al., 2015). The three major components of *S. cerevisiae* cell wall are mannoproteins (mannans bonded to protein), glucans, and chitin (Orlean, 2012; Pinto et al., 2015). These three components assign approximately 90% of the entire cell wall, with proportions varying significantly based on stress, environment, and growth stage (Orlean, 2012). Mannans are described as being present both in the yeast cell wall and periplasmic space, always associated with proteins (Kath & Kulicke, 1999; Orlean, 2012).

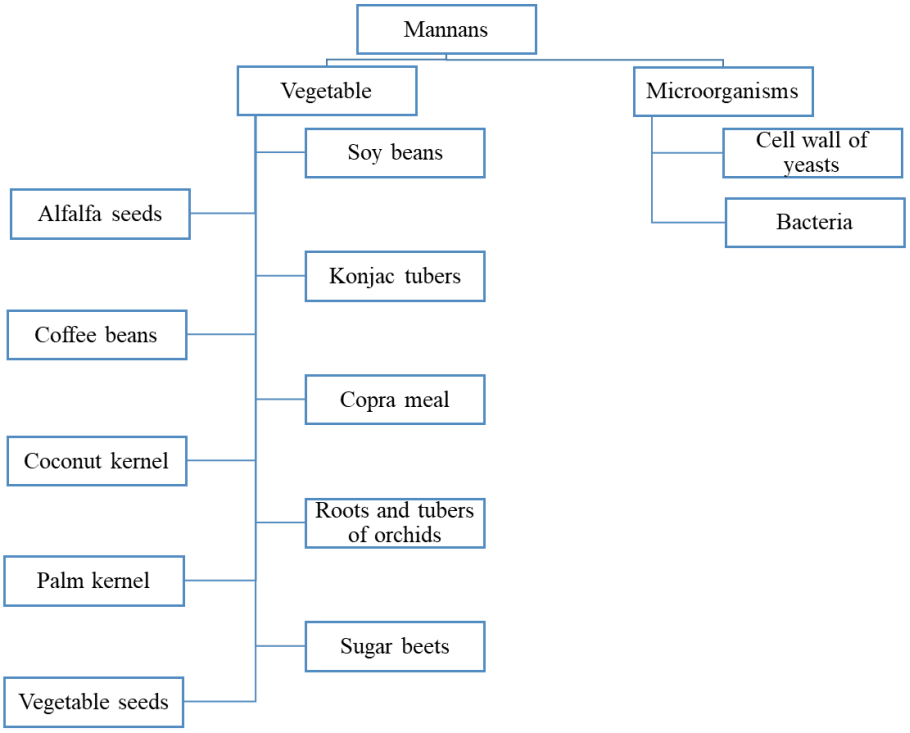


Figure 1. Sources of mannans (Olaniyi et al., 2013; Singh et al., 2018).

1.2.2.2. Mannans' relevance in Saccharomyces cerevisiae cell wall

Mannans are an important structural component of the yeast cell wall (Kath & Kulicke, 1999), with a composition directly related with the chemical and environmental conditions prevailing during culture growth (Bonaly et al., 1971). Yeast presents a cell with an oval to round shape, normally having an average diameter of approximately 5-13 µm (Kath & Kulicke, 1999). A schematic representation of the cell is shown in **Figure 2**, as described in the literature.

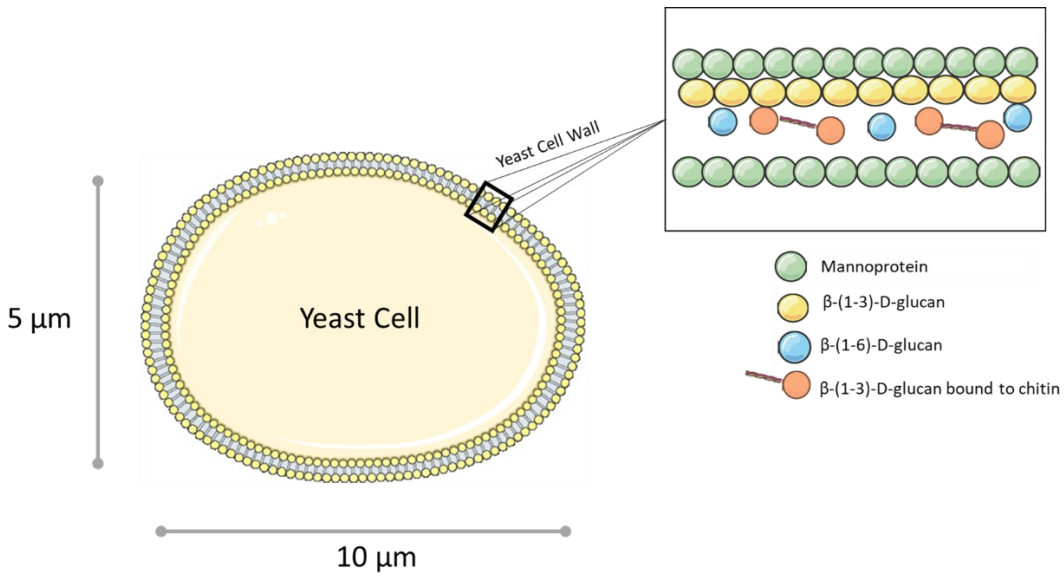


Figure 2. Yeast cell wall structure consisting of two layers: a mannoproteins complex located on the outside, and glucans (chains) as a structural polymer on the inside. Adapted from Kath & Kulicke (1999). The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

The cell wall is estimated to have ca. 70 nm of thickness with a layered structure, and represents 15-30% of the cell dry weight (Bacon et al., 1969; Kath & Kulicke, 1999; Klis et al., 2006; Latgé, 2007). Its chemical composition consists of 3% ash; 13% protein; 8.5% lipid (mostly neutral fat) and two major polysaccharides, glucans and mannans; the latter are associated with protein present in the wall, forming mannoproteins complexes (Northcote & Horne, 1977). More specifically, mannoproteins range 35%, and glucans are divided into β-(1-3)-D-glucan (25%); β-(1-3)-D-glucan bound to chitin (35%) and β-(1-6)-D-glucan (5%) (**Figure 2**) (Ikuta et al., 1997). It is also possible to observe a small amount of chitin (1-2%) (Feuillat, 2003; Orlean, 2012).

While mannans are the second major polysaccharides of the yeast cell wall, glucans are established as the main components responsible for the structural integrity of the cell wall (Eggensperger, 1997; Kath & Kulicke, 1999), playing a key role in the cross-linking of its components (Kollár et al., 1997). Chitin is a linear polymer of 1-4 linked *N*-acetyl glucosamine units with relatively minor abundance. Still, it is an important constituent in the performance of the role of maintaining the rigidity and the morphology of the cell (Matsumoto et al., 1980). The relative quantities and localization of individual wall components vary according to the cell cycle or developmental stage, growth phase,

nutritional conditions, and wall stresses imposed by hypo-osmolarity, mutational loss of wall bio- synthetic activities or wall proteins, or drug treatment (Orlean, 2012).

1.2.2.3. Structure

Mannans are generally associated with proteins in the outer and inner membrane. Such structure (mannoprotein complex) can be represented in three different sections: the peptide chain; the core region (central region) and in the external chain, in which the polymeric fraction is located (**Figure 3**). The polymeric region consists of an α-(1-6)-glycosidic basic chain that carries α-(1-2)- and α-(1-3)-glycosidically linked oligomannans as side groups. The peptide region contains oligomeric mannans directly linked to amino acids.

Characteristically, *S. cerevisiae*'s mannans are composed of long D-mannose chains linked by α-(1-6) bonds (backbone), with short side chains in the α-(1-2) and α-(1-3) linkages (Ballou, 1974; Peat, Whelan, et al., 1961); these macromolecules are linked to asparagine residues (Ballou, 1974; Sentandreu & Northcote, 1968) (**Figure 3a)**. In addition, mannans are also present as small oligomannosidic units (mannan oligosaccharides – MOS) with α-(1-2) and α-(1-3) linkages that are linked to serine or threonine residues (**Figure 3b**).

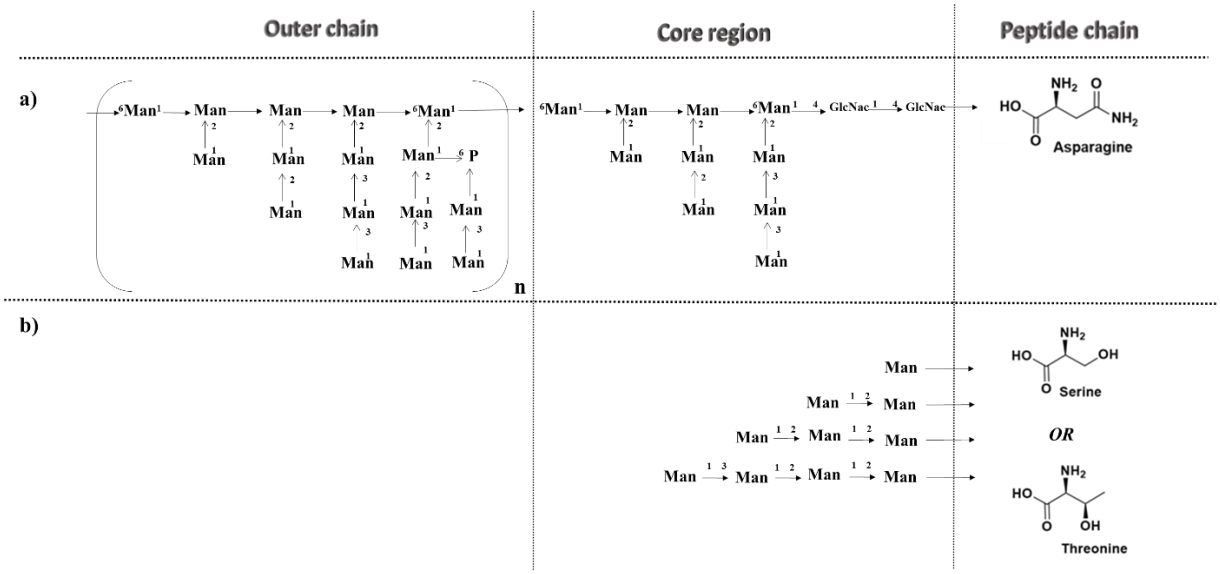


Figure 3. Representation of the mannoproteins complex, adapted from Kath & Kulicke (1999), and Ballou (1976). Man represent Mannose, P represent Phosphorus and GlcNac represent glycosidic linked.

1.2.2.4. Extraction Methods

Mannans extraction from cell wall usually includes several steps, namely: yeast cell lysis; separation of the released mannoproteins from the remaining cell components; fractionation of mannoproteins and purification of the resulting mannans fraction. Depending on the purity requirements of the final extract, some of the above-mentioned steps can be circumvented or extended. Cell lysis is the most studied step in the process, as it will dictate the final yield. The yeast cell lysis can be performed through chemical (usually using alkaline reagents or detergents); enzymatic (taking advantage of the existing internal enzymes – autolysis – or by addition of external enzymatic cocktails); physical (relying on temperature and pressure) or mechanical processes (e.g., by grinding cells with glass beads).

In early studies, mannans were isolated by extracting either whole yeast cells or isolated cell walls with alkali, followed by precipitation of the solubilized mannans, as copper complex, with Fehling's solution (Fritsche, 1971; Haworth et al., 1937; Orlean, 2012; Phaff, 1963). These processes also employed high temperature and pressure, typically by autoclaving whole cells or cell walls in a neutral or alkaline solution (Fleet, 1985).

Mannans can also be extracted by cell wall enzymatic hydrolysis with proteases (Cawley et al., 1972; Kath & Kulicke, 1999; Russell et al., 1973) and glucanases (Fleet & Manners, 1977; Kath & Kulicke, 1999; Shibata et al., 1983; Valentin et al., 1984).

Although the recovery of mannans from solution can be performed by the above-mentioned mannan-copper complex, solvent precipitation (e.g., with ethanol, methanol or acetone) has become more conventional. Other commonly used purification methodologies include chromatographic methods (e.g., affinity chromatography, size exclusion chromatography) and ultrafiltration.

More recently, other technologies, such as ultrasounds (US) and pulsed electric field (PEF) have shown some encouraging results (Martínez et al., 2016; Snyman et al., 2021). The use of US for mannoprotein extraction was explored by Snyman et al., (2021); in this study mannoprotein was extracted by US, enzymatic hydrolysis or a combination of both procedures and the yields were compared. Authors suggest that the extraction conditions that result in the best yield would involve subjecting the yeast cells to US, followed by an enzymatic hydrolysis – exact conditions presented in **Table 2**.

Other comparison studies have also been published analysing different methodologies for the extraction of mannoprotein, such as the publication by Li et al., (2020), in which three approaches under scrutiny were thermal, sodium dodecyl sulphate (SDS) and enzymatic

(Zymolyase®) treatments. The methodology which resulted in the highest mannoprotein yield was the enzymatic treatment (98% and 78% in Baker's and Brewer's yeast, respectively). Both studies used the same enzymatic treatment (Zymolyase®) and both point to better yields resulting from those treatment when compared to other methodologies. However, the use of different analytical techniques for the quantification of mannans and the lack of standardized information does not allow for an unambiguously withdrawal of conclusions.

In an effort to compare results from a myriad of publications, a compilation of the different methodologies, the mannan/mannoprotein purity and a calculation of the yield, whenever possible, is provided in **Table 2.**

Table 2. Methods for extraction of yeast mannans.

Yeast Source	Solvent	Ratio *	Cell lysis Process	Separation Process I	Separation Process II	Purification Process	Yield of Process (%) **	Extract Purity (%)	References
Baker's Yeast	Citrate buffer 0.19 mM; pH 7.0	n.a.	Autoclave 2 h; 120 °C	Ethanol precipitation	Copper complex with Benedict's solution	Purification resin (to eliminate the residues of copper complexes formed)	0.77%	n.a.	(Stewart et al., 1968)
Baker's Yeast	6% NaOH	n.a.	Boiling temperature, 8 h	Acidification (acetic acid). Centrifugation and neutralization; Ethanol precipitation	Complex with Fehling's solution	Washing with warm water and ethanol 60%	First step 2.1%; second step 65%	n.a.	(Haworth et al., 1937)
Baker's Yeast	Citrate buffer 0.19 mM; pH 7.0	n.a.	Autoclave 2 h; 140 °C	Centrifugation and washing of solid with acetic acid. Ethanol precipitation	Solubilisation of pellet in water and alkalinisation. Precipitation with Fehling's solution	Washing with warm water, acidification, and precipitation with ethanol	First step 0.99%; second step 0.54%	n.a.	(Peat, Whelan, et al., 1961)
Candida atmosférica Candida diddensii C parapsilosis Debaryomyces globosus Hansenula angusta Pichia bispora Saccharomyces lactis (NRRL Y1140 and NRRL Y1250)	Citrate buffer 0.02 M; pH 7.0	50%	Two cycles of autoclave for 90 min; 125 °C	Centrifugation. Alkalinisation of supernatant.	Complex with Fehling's solution	Purification in cationic resin	After two precipitations (Fehling's solution) 1.7 – 0.42%	n.a.	(Kocourek & Ballou, 1969)

Saccharomycodes ludwigii Lodderomyces elongasporus									
Saccharomyces cerevisiae	1% NaOH	10%	2 h; 100 °C	Centrifugation and filtration of the supernatant	Ethanol precipitation	Isoelectric point (to eliminate protein)	18%	96 %	(Liu & Huang, 2018)
Baker's Yeast	50 mM phosphate buffer (pH 5,7,9)	5%	Autoclave 4 h; 120 °C	Centrifugation and collection of the supernatant	n.a	Enzymatic isolation using Zymolyase	12%	n.a	(Li & Karboune, 2018)
Baker's Yeast	water	1-20%	90 °C-100 °C; 1-3 h	Centrifugation and collection of supernatants	n.a.	α-mannanase at 3 -4% for 8-12 h at 50-70 °C, pH 4.0-6.0. Proteases at 0.5-3% for 6-10 h at 50-70 °C, pH 7.0-8.0	n.a.	40-44%	(Yu et al., 2008)
Baker's Yeast	0.1M potassium buffer solution	20%	Autoclave for 3 h; 120-121 °C	Centrifugation and collection of supernatants	Precipitation with 3 volumes of 95% ethanol containing 1% (v/v) acetic acid	Ultrafiltration	18%	n.a.	(Cameron et al., 1988)
Baker's yeast	Tris buffer; pH 7.5	4%	Mechanical disruption with glass beads diameter (0.25-1.5 mm) for 12 min	Enzyme cocktails (mixture of proteases and glucanases) EC1- an enzyme cocktail from *Helix pomatia*	n.a.	Insoluble glucan was separated by centrifugation and mannans were purified by dialysis	n.a.	n.a.	(Kath & Kulicke, 1999)

				Conditions: pH=5.0, 37 °C EC2- an enzyme cocktail from *Cytophaga* sp. Conditions: pH=7.5, 37 °C EC3- lyticase					
Brewer's Yeast	n.a.	n.a.	Autolysis 16 h; 55 °C	Protease (Protex 6L) at pH=9.5, 55°C for 16 h	n.a.	Ultrafiltration (10 kDa)	n.a.	n.a.	(Sedmark, 2014)[a]
Brewer's Yeast	n.a.	n.a.	Autolysis 14 h; 55 °C	Protease, Amylase and/or Lipase (Protex 6L and Glucoamylase); pH=9.5, 55°C, 14 h + 4 h with Glucoamylase	n.a.	Ultrafiltration (10 kDa)	n.a.	n.a.	(Sedmark, 2014)[a]
Baker's yeast and brewer's yeast	Sodium phosphate buffer (50 mM, pH 7.5)	5%	n.a.	Enzymatic hydrolysis (167U/g DC;35ºC;4h)	n.a.	Affinity chromatography	27%-31% (Mannan Recovery Yield: 98%-75%)	37%- 38%	(Li et al., 2020)
***Saccharomyces boulardii* SB62**	0.1 M phosphate buffer, pH 6.5	n.a.	Ultrasounds (4min; 80% amplitude; 50% duty cycle)	Enzymatic hydrolysis with a glucanase (4000U lyticase/g DC; 37ºC;20h)	Precipitation with 3 volumes of 100% Acetone	n.a.	n.a.	n.a.	(Snyman et al., 2021)

[*]Yeast weight per volume of solvent; [**] The yield was obtained from the weight of extract divided by the weight of original yeast (wet or dried) and multiplied by 100.

[a] Patent US8753668B2; n.a. – non applicable.

From the analysis of **Table 2** it can be observed that, from the several methods available for extracting/isolating yeast mannans, final yields are usually inversely related with purity, *i.e.,* the higher the purity of the final extract, the lower yield will be obtained, because of the increased number of steps required for purification. Nevertheless, there is no "perfect" extraction method since the targeted application of the extract will drive the choice of the method. The highest yields are usually obtained when using enzymes, but this may also increase costs, depending on its specificity and enzymatic hydrolysis conditions, such as temperature or time, when compared to other extraction methods. The high temperature procedures may present the additional advantage of decontamination from a possible microbial presence.

One important issue noticed from literature search is that many studies prefer to focus on glucans extraction from yeast cell wall, or the simultaneous recovery of mannans and glucans, which makes sense from a commercial point of view, since both molecules have shown biological activity. Patents that describe extraction methods to simultaneously obtain mannans and glucans (e.g. Patent US8753668B2 (Sedmark, 2014), Patent US9320291B2 (Juhani Saarinen et al., 2016), Patent EP0950716B1 (Lazzari, 1999), Patent US20110045545A1 (Yu et al., 2011)) involve the methodologies above described, such as autolysis, enzymatic hydrolysis, alkaline and acid hydrolysis. The fractionation and purification of these extracts involves a myriad of methodologies such as solvent precipitation, chromatographic methods, and ultrafiltration, depending on the characteristics and purity expectations of the final extract.

1.2.2.5. Chemical Modification

The biological functionalities of different polysaccharides could be tuned and improved by the introduction of specific chemical moieties in the polysaccharide native structure through derivatization (Li et al., 2016; Xie et al., 2020; Xu et al., 2019). Regarding the chemical modifications of mannans isolated from yeast strains, it is clear that this field is only in its infancy, with the majority of studies being focused on the carboxymethylation of mannans from pathogenic yeasts of *Candida* genus (Korcová, Machová, Filip, & Bystrick, 2015; Machová, Bystrický, et al., 2014; Machová, Čížová, et al., 2014; Oka et al., 1972).

The carboxymethylated yeast mannans have already demonstrated their higher performance over the underivatized mannans, namely regarding its antitumor activity, where the carboxymethylation demonstrated to be an effective pathway to remove the toxicity of

the original mannans without loss of antitumor activity (Oka et al., 1972), but also regarding antioxidant (Korcová, Machová, Filip, & Bystrick, 2015; Machová, Čížová, et al., 2014) and thrombolytic activities (Korcová, Machová, Filip, & Bystrick, 2015). Furthermore, it is also possible to find reports focused on the preparation of different oxidized forms of mannans from *Candida* genus used for the preparation of glycoconjugates vaccine precursors (Ďurana et al., 2006), and in the preparation of cationic and amphoteric mannans from *C. albicans* (Čížová et al., 2016, 2019).

Regarding the chemical modification of *S. cerevisiae,* the first report (dated from 2014) described the efforts in the optimization of the synthetic pathway in order to obtain carboxymethyl derivatives of yeast mannans, and the elucidation of their structure and properties through a myriad of characterization techniques (Attenuated Total Reflection Fourier-Transform Infrared spectroscopy (ATR-FT-IR), ultraviolet (UV) measurements, optical rotation, and potentiometric titration) (Machová, Bystrický, et al., 2014). Moreover, it is important to emphasize that while the carboxymethylation is well studied in the functionalization of insoluble (cellulose, starch) and soluble (dextran, hyaluronan) polysaccharides, in the specific case of mannans functionalization it is evident the demand for more information. The above-mentioned study constituted one of the few studies of mannans functionalization by carboxymethylation using three different procedures/media (water/alcohol system; non-aqueous dimethyl sulfoxide system and only in water). The derivatization of mannans in pure water under alkaline conditions was described as the most promising procedure after the optimization of the following set of reaction parameters: mannans and sodium monochloroacetate concentrations, reaction temperature and time, which led to the establishment of the best conditions to obtain the carboxymethylated product - 100 mg of mannan in 1 mL of 6 M sodium hydroxide (NaOH) at room temperature for 30 min, followed by addition of 300 mg of sodium monochloroacetate at 70 °C for 5 h, and neutralization with acetic acid to pH 7 followed by dialysis. Under these conditions, the carboxymethyl derivative of *S. cerevisiae* mannans was obtained with a good yield, with a degree of substitution of 0.43 (determined by potentiometric titration) and the strong alkaline conditions revealed to have a negligible effect in mannans' degradation. The successful carboxymethyl derivation of the original mannans was undoubtedly confirmed by ATR-FT-IR the assignment of a new vibration in the spectrum at 1587 cm^{-1} and 1408 cm^{-1}, indicating the substitution of $COO^{-}Na^{+}$ group on mannans chain. The UV measurements also corroborated the successful chemical modification by the UV absorbing carboxylate in the

carboxymethyl mannans product. The hydrogen nuclear magnetic resonance (^{1}H NMR) data showed to be complex due to the non-uniform carboxymethylation, which led to the impossibility in the assignment of all NMR resonance signals, while optical rotation reflected the structural pattern of the different compounds with α-glycosides exhibiting positive rotation (Machová, Bystrický, et al., 2014).

In the same year, the carboxymethyl derivatives from mannans *S. cerevisiae* obtained by the optimized conditions above-described (Machová, Bystrický, et al., 2014) were studied as potential antioxidants, along with the carboxymethyl derivatives from β-glucan and dextran (Machová, Čížová, et al., 2014). The antioxidant potential was ascribed by the analysis of hydroxyl radical capture, the 2,2 -Diphenyl-1-picrtylhydrazyl radical-scavenging (DPPH$^\bullet$) and by the Fe (II) chelating activity. All carboxymethyl derivatives proved to be stronger antioxidants against the hydroxyl radical and presented a higher Fe (II) chelating activity against the original mannans. Regarding the scavenging activity against DPPH, the carboxymethyl products exhibited a lower performance when compared with the underivatized ones. The properties and antioxidant performances for the carboxymethyl mannans extracted from *S. cerevisiae* are depicted in **Table 3**.

Table 3. Compilation of properties and antioxidant activities of *S. cerevisiae* mannans and functionalized mannans (Machová, Bystrický, et al., 2014; Machová, Čížová, et al., 2014).

Properties	Mannans	Carboxymethyl mannans	References
Molecular Weight (M_{peak} (kDa))	67	183	(Machová, Bystrický, et al., 2014)
UV spectrophotometric (A_{210nm})	0.06	0.39	
Optical rotation $[\alpha]_D^{20}$	+77.97°	+55.49°	
HO• (%)	11.8 ± 1.1	45.1 ± 1.4	(Machová, Čížová, et al., 2014)
DPPH• (%)	5.0 ± 0.6	4.1 ± 0.4	
Fe (II) chelating activity	10.9 ± 1.7	14.5 ± 1.5	

In the field of chemical modification of polysaccharides with a high demand to improve a variety of properties, such as solubility, robustness, and a myriad of biological activities, there is a clear contrast between what happens with mannans, where the information is very scarce, and other polysaccharides present in the yeast cell wall, namely glucans. In the last case, it is possible to find in the literature several studies reporting different chemical

modifications (carboxymethylation, sulfonylation, phosphorylation and acetylation) and their impact in the solubility and in the biological properties, namely the antioxidant, anticoagulant and antitumor activities (Kagimura et al., 2014; Theis et al., 2019).

The clear potential of the chemical modification of mannans in order to improve their properties demands deep studies in this niche that certainly will prompt new and exciting discoveries that may contribute to the prevention and treatment of countless diseases or even interesting properties to be used in nutraceutical and food industries.

1.2.3. Mannan oligosaccharides (MOS)

Mannan oligosaccharides (MOS) are non-digestible oligosaccharides derived *via* partial hydrolysis of the mannans polysaccharide (Tester & Al-Ghazzewi, 2013), which can be generally divided into two main groups: α- and β-MOS. While α-MOS are obtained by cleavage of α-(1-6) bonds from yeast cell wall mannans, β-MOS are commonly obtained from mannans-rich plants through cleavage of β-(1-4)-glycosidic bonds (Jana et al., 2020; Yamabhai et al., 2016).

MOS have gained significant interest as a prebiotic (Gibson et al., 2004). According to Gibson et al., (2017) the prebiotic concept has expanded, because of advances in tools for microbiome research, leading to the proposal of a new definition where a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit". According to this new definition fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) (Gibson et al., 2017), MOS (Al-Khalaifa et al., 2019; Gibson et al., 2017), xylo-oligosaccharides (XOS) (Charalampopoulos & Rastall, 2012; Gibson et al., 2017) and inulin (Gibson et al., 2017; Van Loo, 2004) are classified as prebiotics, apart from other non-carbohydrate matrices.

MOS are widely used as prebiotics in the animal industry with the aim of selectively stimulate the growth or metabolic activity of a limited number of internal microorganisms, suppressing enteric pathogens and improving the integrity of the intestinal mucosa (Baurhoo et al., 2007; Ghasemian & Jahanian, 2016; Jana et al., 2020; Spring et al., 2000, 2015).

1.2.3.1. Production methods

The different methods for obtaining MOS may be roughly divided into those using chemicals and those that apply enzymes. Among the former, selective acetolysis (Lee &

Ballou, 1965; Stewart et al., 1968; Young et al., 1998), acid or alkaline hydrolysis, combined or not with high temperatures, can be cited (Nakajima et al., 1974; Ogawa et al., 1994; Peat, Whelan, et al., 1961).

Chemical methods

Selective acetolysis

The bibliographic search conducted revealed a notable scarcity of information on large-scale processes to obtain MOS from *S. cerevisiae*'s mannans with chemical methods. One of the few exceptions is, perhaps, selective acetolysis, although this is a reaction only used in yeast mannans structural elucidation, due to the toxicity of some chemicals involved (Kocourek & Ballou, 1969). Nevertheless, this procedure has the ability to selectively cleave the backbone α-(1-6) linkages between mannose units, yielding the relatively stable α-(1-2) and α-(1-3) linked oligosaccharides (**Figure 4**) (Lee & Ballou, 1965).

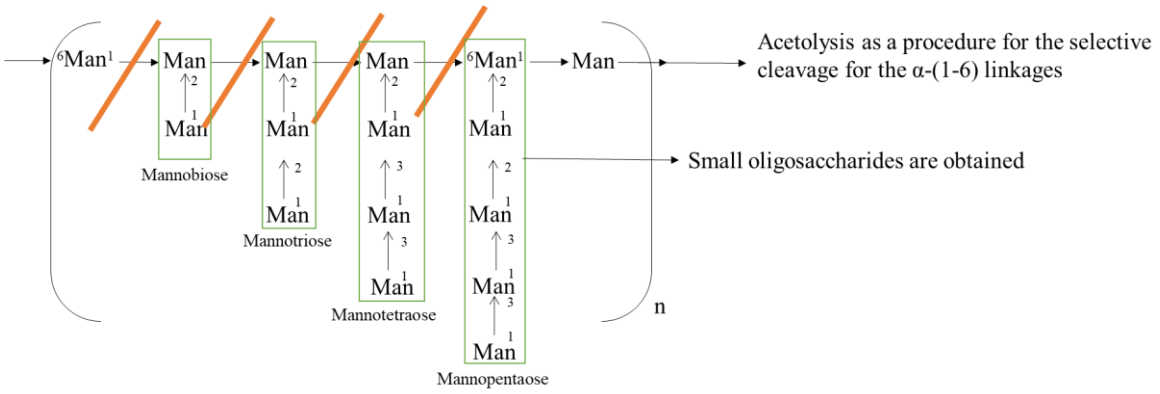


Figure 4. Acetolysis process of cleavage in the extracted mannan (orange lines indicate the cleavage locations).

Acetolysis reaction was applied for the first time to yeast mannans by Gorin & Perlin (1956), and the mechanism has been studied in more detail ever since (Ballou, 1976; Kocourek & Ballou, 1969; Lee & Ballou, 1965; Marzaioli et al., 2014; Matsumoto et al., 1982; Stewart et al., 1968; Tanimoto et al., 2002; Vinogradov et al., 1998). The process of acetolysis consists of dissolving yeast mannans in a mixture of acetic anhydride, glacial acetic acid and concentrated sulfuric acid. The reaction has been reported to proceed in a wide range of conditions, from room temperature to 40 °C and with a duration that may

extend from 2 h to 5 days. It is referred that the reaction is better controlled if mannans are firstly acetylated.

After acetolysis, the reaction must be stopped with a base, such as pyridine, and the solvents evaporated. The residue is dissolved by adding a chloroform:water solution. The organic solvent is evaporated, and the residue is treated with sodium methoxide, usually for 20 min at room temperature. The precipitate formed from the previous reaction may be washed with methanol and dissolved in water. Neutralization and separation may then proceed by a number of methods (Ballou, 1976; Kocourek & Ballou, 1969; Lee & Ballou, 1965; Rosenfeld & Ballou, 1974, 1975; Stewart et al., 1968; Tanimoto et al., 2002).

Acid

According to Peat et al., (1961), acid hydrolysis may cleave mannans preferentially in the side chains, since the α-(1 -6) -glycosidic bonds has been reported to be much more stable to acid hydrolysis than the α-(1- 2) -glycosidic bonds.

In the study of Ogawa et al., (1994), mannans extracts were treated with 0.33 N sulfuric acid in a boiling water-bath for 12 h, followed by neutralization with barium carbonate and left overnight at 4 °C. The resultant products were mannans oligomers with 1-4 degree of polymerization (DP). Since these acid hydrolysates from mannans of *S. cerevisiae* and *Trichosporon aceleatum* were still branched, this suggests that acid hydrolysis does not cause preferential cleavage of side chains.

Young et al., (1998), proposed a methodology that encompasses an acetolysis and a partial acid hydrolysis to break down the backbone and side chains. For the partial hydrolysis mannans were incubated in 0.4 M sulphuric acid at 100 °C for 60 min, followed by neutralization with barium carbonate. The precipitate and the supernatant were filtered using a Microcon (10 kDa) membrane in order to eliminate the salt created by neutralization. The results showed a predominance of the disaccharide (mannobiose) with bonds Manα1-3Man and smaller amounts of the trisaccharides (mannotriose), Manα1-3Manα2/6Man, Manα1-2[Manα1- 6] Man with these bonds. In conclusion, the resultant products from the acidic method were mostly mannan oligomers with 1-4 degree of polymerization, i.e., monosaccharides, disaccharides, trisaccharides and tetrasaccharides.

Alkaline

Another chemical procedure used to obtain mannans and MOS is alkaline hydrolysis under specific conditions: the β-elimination effects occurs in the replaced serine and threonine residues, releasing mannose, mannobiose, mannotriose, and mannotetraose (Nakajima et al., 1974). This alkaline extraction was described by the breakdown of the phosphodiester bonds or glycosyl-serine linkages (Ballou, 1974; Nakajima & Ballou, 1974). According to several studies (Nakajima & Ballou, 1974; Sentandreu & Northcote, 1968; Yen & Ballou, 1974; Young et al., 1998) the selective alkaline hydrolysis consists of mannans treatment with 0.05 - 0.1 M of NaOH or NaOH containing sodium borohydride (Yen & Ballou, 1974) at 25 °C-100 °C for 2-24 h. The reaction must be stopped by neutralization with acetic acid. By following this procedure, the authors (Nakajima & Ballou, 1974; Sentandreu & Northcote, 1968; Yen & Ballou, 1974; Young et al., 1998) claim to obtain di to tetra saccharides.

Enzymatic methods

As mannans are structurally complex, a sequential action of various enzymes may be required to hydrolyse them into MOS (Singh et al., 2018). **Table 4** summarizes different types of specific α-mannanases (for cleavage of the backbone of mannans into oligomers) and α-mannosidases (to hydrolyse mannans oligomers to mannose) (Dhawan & Kaur, 2007; Kommineni et al., 2019; Maruyama et al., 1994; Zordan et al., 2015; Zouchova et al., 1977).

According to Singh et al., (2018), enzymatic degradation is generally preferred in the food industry for partial degradation of commonly used food hydrocolloids (derived from plants and thus containing α-mannans). Since mannans derived from the yeast cell wall of *S. cerevisiae* are mainly composed of mannose structurally organized in α-(1-6) linked backbone and α-(1-2) and α-(1-3) linked branches, enzymes available for plant mannans cleavage are not predicted to be applicable to yeast mannans. Indeed, the industrial use of α-mannanase enzymes, cleaving alpha bonds, is practically inexistent, which leads to its very high price, thus leaving their use almost exclusively for research.

As an example, an endo-1,4 β-mannanase (from *Cellvibrio japonicus*) containing 10,000 units costs 173€ (Megazyme, Wicklow, Ireland) whereas an α-D-Mannosidase (from *Bacteroides thetaiotaomicron*) containing 10 units costs 152€ (Megazyme, Wicklow, Ireland). Thus, considering the units present, the cost is roughly 1000 times higher. As such,

the use of specific enzymes to catalyse the production of MOS is not an economically viable solution. Nevertheless, a potential solution could be the use of immobilized enzymes, which would allow their easier recovery and concomitant decrease in operational costs.

Table 4. Different types of enzymes and their action mechanism in the mannans structure.

Enzyme	Action Mechanism	Optimal Conditions	Reference
Endo α-1,6 mannanase	Random hydrolysis of (1-6)-α-D-mannosidic linkages in unbranched (1-6)-mannans	Temperature 37 °C pH= 7.0	(Zordan et al., 2015)
α-1,2,3,6-mannosidase	Hydrolysis of the terminal (1-2)-linked α-D-mannose residues in the oligo-mannose oligosaccharide Man9 (GlcNAc)2	Temperature 37 °C pH= 4.5	(Kommineni et al., 2019)
α-1,2,3 – mannosidase	Exoglycosidase that catalyzes the hydrolysis of α-(1-2) and α-(1-3) linked mannose residues from oligosaccharides	Temperature 37 °C pH= 5.5	(Wong-Madden & Landry, 1995)
α- 1,6 mannosidase isolated from *Xanthomonas manihotis*	removes unbranched α-(1-6) linked D-mannopyranosyl residues from oligosaccharides	Temperature 37 °C pH= 5.5	(Wong-Madden & Landry, 1995)

Summarizing, acetolysis of yeast mannan preferentially cleaves α-(1-6) linkages, and has been reported to originate mannose, mannobiose, mannotriose and mannotretraose, containing α-(1-2) and α-(1-3) linkages (Stewart & Ballou, 1968). This procedure is applied to help determine the polysaccharide structures, although its application for MOS production is hampered by the chemical agent employed (e.g., pyridine and sodium methoxide).

On the other hand, partial acid hydrolysis presents a lower specificity towards the linkages but remains an important technique to determine and elucidate the structure of the polysaccharides (Chiura et al., 1982). Despite not having a high selectivity as acetolysis, this procedure proves to be a simpler process. The alkaline hydrolysis was shown to cleave the linkages to serine and threonine residues in the oligomers (Nakajima et al., 1974), resulting

in the production of mannobiose, mannotriose and mannotretaose. This also proves to be a simple process without the use of highly toxic or environmentally harmful reagents. Finally, enzymatic methods for cleaving yeast mannan linkages, although very selective (depending on the chosen enzyme) and environmentally friendly, are at the present time particularly expensive.

In conclusion, although both acetolysis and enzymatic hydrolysis seem to be the most selective processes to obtain MOS from mannans hydrolysis, neither of them appears to be applicable at industrial scale, due to the above-mentioned reasons. Therefore, acid or alkaline hydrolysis processes remain applicable, despite being less effective choices. This may explain why in many commercial products claiming to be MOS, their compositional analysis shows a mixture of mannans and MOS (unpublished data from authors).

1.2.4. Bioactivities and potential applications of mannans, MOS and mannose residues

Mannans, MOS and mannose residues from yeast present specific bioactivities which tailor their potential use in pharmaceutical and animal feed industries (Singh et al., 2018). This section will cover some of the bioactivities present in these three compounds.

1.2.4.1. Mannans

In the literature, yeast mannans have mainly demonstrated prebiotic activity, when incorporated into animal diet, limiting gastrointestinal infections (Browne et al., 2019). Indeed, it has been reported (Oba et al., 2020) that mannans extracted from baker's yeast selectively increased the abundance of *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* in the fermentation of rat faeces *in vitro*, thus increasing the production of acetate, propionate and other beneficial short chain fatty acids, and improving intestinal environment. In the same context, mannans have been shown to limit gastrointestinal infection in susceptible animals, including pigs, broilers and cows, by blocking the mechanism by which pathogenic gram-negative bacteria adhere and invade intestine, an effect measured by a reduction in animal pro-inflammatory responses (Browne et al., 2019).

Concerning pathogenic control, *S. cerevisiae* mannans were also reported to enhance collateral sensitivity of *E. coli* to antibiotics, both in susceptible and antibiotic resistant strains, through modulation of bacterial metabolism (Smith et al., 2020).

According to Korcová et al., (2015) mannans could present immunomodulatory activity through the stimulation of macrophages (*via* interaction with their mannose receptors), which eliminate circulating atherogenic lipoproteins such as low density lipoproteins (LDLs). Additionally, mannans, as well as β-glucans, have shown antitumor and anti-metastatic effects (Joseph et al., 2013; Kogan et al., 2008).

Another activity reported was the ability to scavenge radicals, with mannans exhibiting a good scavenging ability to superoxide anions and hydroxyl radicals, suggesting a potential antioxidant effect (Liu et al., 2018); these properties enhance mannans possible applications in food, feed and cosmetic areas.

Despite its beneficial effects, there are also some reports on the undesirable responses observed after mannans ingestion. Khmaladze et al., (2014) found that intraperitoneal *S. cerevisiae* mannans injection resulted in the induction and exacerbation of psoriasis and psoriasis arthritis (production of interleukin 17 (IL-17), a pathway regulated by reactive oxygen species (ROS) in macrophages). Another study from Cuskin et al., (2015), in a group of patients with Crohn's disease, described a disturbed humoral and cellular immune response to yeast mannans. In older studies (Young et al., 1998) it has been shown that a fraction (Sc500) of mannans stimulates the production of T and B cells in patients with Crohn's disease.

1.2.4.2. MOS

MOS are reported to serve as prebiotics for beneficial bacteria in the gut, also showing certain biological effects such as enhancement of protection in intestinal mucosal layer, and boosting of the immunity, antimutagenic and antioxidant defences (Teng & Kim, 2018). According to Spring et al., (2015) and Spring et al., (2000), MOS are known for their ability to bind and limit the colonization of enteric pathogens such as *Salmonella*, *E. coli*, *Campylobacter*, and others (Koc et al., 2010). The α-(1-3) and α-(1-6) branched of MOS present in the cell wall of *S. cerevisiae* are particularly effective at binding pathogens Firon et al., (1987). According to Spring et al., (2015), when the attachment and colonization of the intestine by gram-negative pathogenic bacteria is blocked with MOS, the autogenous population can flourish, making the gut more efficient, and liberating more nutrients for lean tissue growth and improve immunity in the host animal. However, as reported on the studies of Baurhoo et al., (2007), Sims et al., (2004), and Spring et al., (2000) MOS effects on

promoting beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria* are more variable. The overall known different action modes of MOS are exemplified in **Figure 5**.

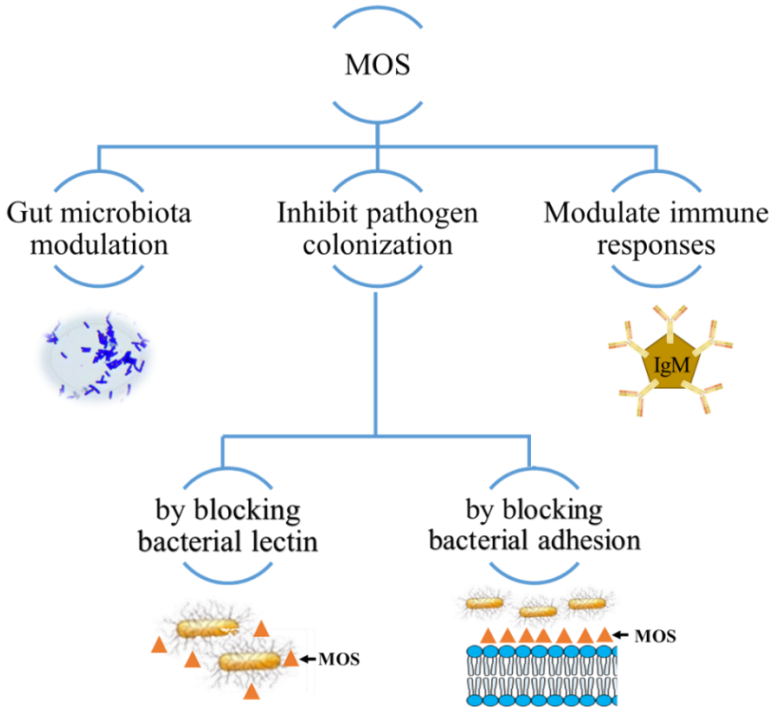


Figure 5. Potential mechanisms of action of MOS in pathogen colonization inhibition.

Apart from the effects on caecal microbiota, MOS also improved the microbial community in other intestine sections of broilers – jejunum, ileum, jejunal mucosa, ileal mucosa and ileocecal junction (Chee et al., 2010; Geier et al., 2009; Kim et al., 2011; Wang et al., 2016; Yang, Iji, Kocher, Thomson, et al., 2008). Studies by Corrigan et al., (2015) and Lee et al., (2016) reported that MOS increased caecal Bacteroidetes population in broilers, known for their strong metabolic activity in ferment indigestible saccharides (as MOS) to short-chain fatty acids and, consequently, improved nutrient absorption and protected the host from pathogen infection (Wexler, 2007).

Although not so clear in many studies, as previously mentioned, the effect of MOS on the promotion of increased prevalence of beneficial bacteria has also been reported by several authors. Indeed, ileal *Lactobacillus acetotolerans*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus sakei* subsp. *sakei*, and caecal *Lactobacillus ingluviei*, *Lactobacillus mucosae*, *Lactobacillus salivarius*, and *Lactobacillus crispatus* populations in broilers were reported to increase with the inclusion of MOS in their diet (Corrigan et al., 2011, 2015). Among these species, *L. crispatus* was reported in Chen et al., (2007) and Zhang et al.,

(2007) to have anti-*E. coli* and anti-*Salmonella* activities. In the same way, *L. salivarius* was mentioned by Chen et al., (2007) and Zhang et al., (2007) as limiting *Salmonella* colonization. The anti-pathogenic characteristics of *Lactobacillus* may be the reason why MOS indirectly reduced the numbers of *E. coli* or *Salmonella* in the intestine, ameliorating bacterial infection in pathogen-challenged broilers (Baurhoo et al., 2007, 2009; Spring et al., 2000). *In vitro* results have also been reported revealing that MOS are considered prebiotics that increase the efficiency of microbial elimination by phagocytosis through the agglutination process in various strains of *E. coli*, *Salmonella typhimurium* and *Salmonella enteritidis* (Spring et al., 2000).

MOS can also be used as substitutes for antibiotic growth promoters (AGP) (antibiotics in sub-therapeutical doses), widely used in animal feed due to their growth-promoting effects, but banned in European Union since 2006, due to concerns regarding the link between usage of antibiotics in animal feed and an the increase of bacterial antibiotic resistance (Al-Khalaifah, 2018). Furthermore, consumers prefer to reduce the use of antibiotics and other therapeutic chemicals in animal feed. In this perspective, MOS can be a greener approach to simultaneously prevent disease outbreaks and enhance animal health. When supplemented in animal feed, MOS contribute to mortality decrease and to the improvement of the levels of bactericidal (destroys bacteria) and lysozyme (destroys the protective layer of bacteria) activities (Chacher et al., 2017; Torrecillas et al., 2007; Torrecillas et al., 2014). In summary, there are countless advantages in using MOS in animal feed: they have the ability to modify microbiota composition, prevent pathogenic bacteria adhesion (as described above), can be associated with the proliferation of beneficial microorganisms such as lactic bacteria, and show positive effects in decreasing diarrhoea incidence (Agazzi et al., 2020; Al-Khalaifa et al., 2019; Leblebicier & Aydoğan, 2018; Valpotić et al., 2016; Zhao et al., 2012).

Nevertheless, despite the several benefits of MOS consumption and utilization reported, more systematic research on the mechanisms behind these beneficial effects on the human species, as well as the safety of its use, still needs to be carried out in depth. However, there is still a large ambiguity on the definition of MOS, mannans or yeast cell wall extracts, especially within market products, usually presented as MOS although being composed of cell wall extracts or mannans, most of the times. The clear characterization and labelling of these components are essential to better understand the relation between composition and activity.

1.2.4.3. Mannose

D-mannose is monosaccharide found in nature as a component of mannans from vegetable and microbial origin, hemicellulose, and cellulose in dietary fibre.

Mannose residues act in lock-and-key interactions with carbohydrate-binding proteins found on the surface of certain bacteria (Zopf & Roth, 1996). For example, D-mannose inhibits the adhesion of enteric pathogenic bacteria related to urinary tract infections (UTIs) to uroepithelial cells, thus reducing bacteriuria levels (Kranjčec et al., 2014). More specifically, the inhibition mechanism refers to the binding of free mannose in the bladder to the type 1 fimbriae (FimH) of pathogens such as uropathogenic *Escherichia coli* (UPEC) (Flores-Mireles et al., 2015; Pigrau & Escolà-Vergé, 2020; Ruggieri et al., 1985; Schilling et al., 2001; Scribano et al., 2020). Fimbriae structure presents lectins, glycoproteins which allow the bacteria to bind to the cellular receptors present on the surface of uroepithelial cells (Flores-Mireles et al., 2015; Pigrau and Escolà-Vergé, 2020; Scribano et al., 2020). By binding to free mannose molecules instead of the uroepithelial cells, pathogens are eliminated from the urinary tract by the urine flow, resulting in a reduction in the number of UTI, without the need to resort to antibiotics; this approach may even be applied to prevent UTI thus supporting the commercialization of D-mannose as a health supplement. Indeed, it has been described that after ingesting therapeutic amounts of D-mannose, only a fraction was metabolized by the body (Kyriakides et al., 2020; Lenger et al., 2020), and the remaining was excreted through the kidneys and bladder ureters, to eventually be eliminated from the system in urine. Such amounts of mannose in the bladder may promote the desired effect of preventing UTI. In a study by Kranjcec et al., (2014), D-mannose powder has shown to be effective in preventing UTI during 6-month trial of prophylaxis. The rate of recurrence of infection did not differ between patients taking standard medication (Nitrofurantion) and those taking D-mannose powder.

D-mannose is also reported to inhibit the binding of *S. typhimurium* to intestinal cells in chickens (Oyofo, DeLoach, et al., 1989; Oyofo, Droleskey, et al., 1989). This sugar, for now, is the most effective in blocking colonization to inhibit the growth of intestinal pathogens (Berge & Wierup, 2012; Galiş et al., 2013).

1.3. Thesis objectives and structure

Excess yeast production is a problem that affects most sectors that use fermentative processes, including those that produce ethanol, wine, beer, citric acid, or pharmaceuticals. In 2020 alone, it was estimated an annually production of 309,400 to 418,600 tonnes of brewer's spent yeast worldwide (Oliveira et al., 2022). For these reasons, the primary goal of this PhD program is an endeavour to valorize spent yeast from Amyris, Inc. through studies that will allow the production of mannans and MOS with unique bioactive properties, as they arise from proprietary genetically modified yeast strains, for food and specific nutraceutical industries. In addition to the modification of by-products with sustainable methods, studies also cover the synthesis of novel molecules, involving the chemical modification of *S. cerevisiae* yeast mannans, an area that has been underexplored in literature.

The specific objectives are: **i**) to develop an economically sustainable pipeline to extract mannans and MOS from fermentation spent yeast; **ii**) characterize the chemical and functional properties of the obtained extracts, the latter with *in vitro* assays; **iii**) evaluate the *in vitro* bio-accessibility and bioavailability of the extracts; **iv**) unveil the mechanisms of action of MOS aiming to establish relationships between the composition and activity; and **v**) obtain chemically functionalized mannans with enhanced bioactivity and assess their application.

From a general standpoint, (**Figure 6**) the present thesis can be divided into six major chapters: **1)** Survey of information reported in the literature concerning mannans, MOS and applications; **2)** selection of the most adequate extraction process to obtain mannans from spent yeast, and structural, physicochemical and biological characterization of the resulting extracts; **3)** selection of a suitable manufacture process to produce MOS from mannans extract, and subsequent structural, physicochemical and biological characterization; **4)** evaluation of MOS extracts in the prevention/reduction of uropathogenic adhesion, and in the maintenance of vaginal health, the later in a synergetic combination with vaginal probiotics; **5)** selection and optimization of methods for mannans carboxymethylation, and chemical characterization and evaluation of biological potential of the obtained molecules; **6)** conclusions and future perspectives.

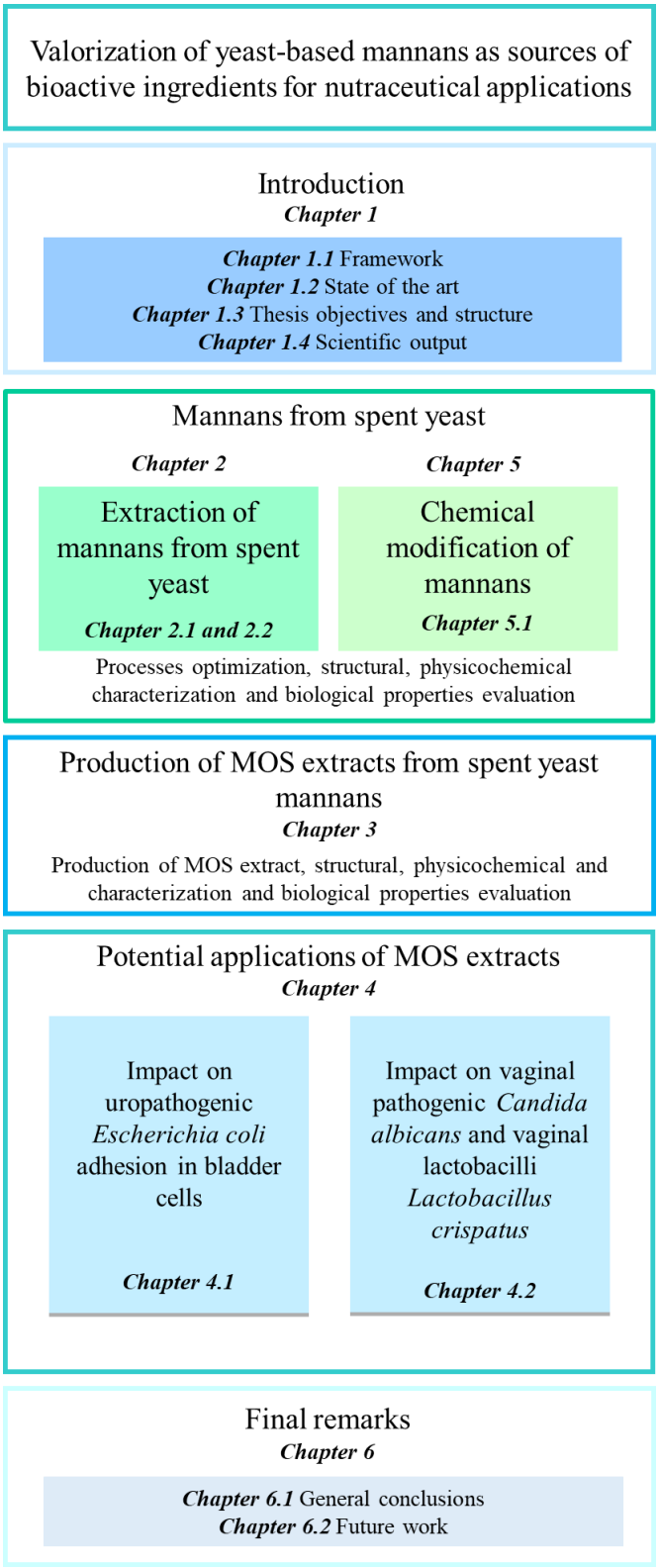


Figure 6. Schematic layout of the thesis structure.

Chapter 1 comprises a literature review on mannans and MOS from spent yeast (*S. cerevisiae*), including the extraction, production processes and structure characterization, their bioactivities, potential applications, and studies on chemical modification of mannans. This chapter also includes the scientific output of this dissertation thesis.

Chapter 2 analyses several described methodologies to extract mannans from residual yeast and selects the most adequate taking into consideration the sustainability of the process and economic viability. Obtained extracts were chemical and functionally characterized and the best performant methodology was selected to produce mannans for the subsequent studies. Additionally, preservation of mannans extracts was attempted through two different methods (spray drying and freeze drying), and the characteristics of the obtained dried extracts were evaluated.

Chapter 3 describes the evaluation of the production of MOS from mannans extract, in a sustainable and viable approach (without the use of chemicals that could compromise its utilization in food or nutraceutical areas). The functional and chemical characterization of MOS extracts has also been conducted.

Chapter 4 focuses on the characterization of some biological properties of the MOS extracts (*Chapter 4.1 and Chapter 4.2).* The ability of rich-MOS extracts to prevent uropathogenic *Escherichia coli* (UPEC) adhesion in bladder cell line (HTB-9) is described in *Chapter 4.1. Chapter 4.2* contemplates the potential of MOS extracts as antimicrobial agents against common vaginal pathogens (*Candida albicans*, *Candida glabrata*, and *Candida tropicalis*) and the ability to enhance beneficial vaginal biota (*Lactobacillus crispatus*, *Lactobacillus gasseri* and *Lactobacillus jenesenii*). Studies on vaginal microbiota were firstly conducted in simulated vaginal fluid, and after that, assays were performed in vaginal cells (HeLa).

Chapter 5 was dedicated to the chemical modification of the α-mannans through optimization of carboxymethylation. Structural, physicochemical, and biological characterization of modified mannans have been included in this chapter.

Finally, **Chapter 6** provide a general conclusion of the present thesis (*Chapter 6.1*) as well as the future perspectives *(Chapter 6.2)*.

The schematic representation of the overall doctoral dissertation work is illustrated in **Figure 7**.

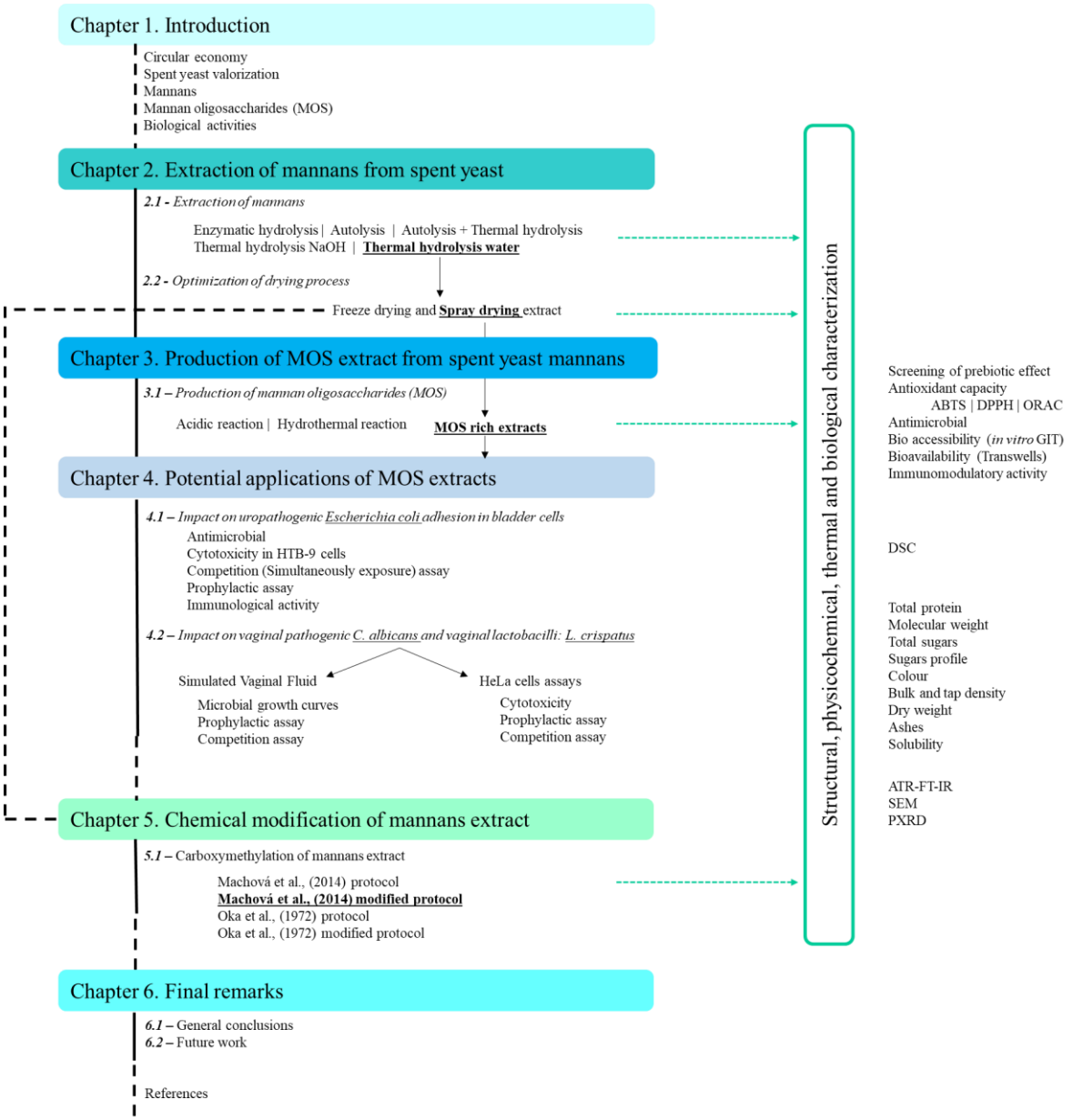


Figure 7. Detailed scheme of work dissertation.

1.4. Scientific output

Through the execution of the several stages of this PhD thesis, several publications were generated, as follows:

Published works:

Papers in international peer reviewed journals:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pintado, Manuela; Carvalho, Ana P.; 2021. Mannans and mannan oligosaccharides (MOS) from *Saccharomyces cerevisiae* – A sustainable source of functional ingredients. Carbohydrate Polymers 272:118467 **https://doi.org/10.1016/j.carbpol.2021.118467**

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Oliveira, Ana Sofia; Pereira, Joana Odila; Pereira, Ana M.; Ferreira., Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2022. Comparative Analysis of Mannans Extraction Processes from Spent Yeast *Saccharomyces cerevisiae.* FOODS 11:3753. **https://doi.org/10.3390/foods11233753**

Faustino, Margarida; Pereira, Carla F.; Durão, Joana; Oliveira, Ana Sofia; Pereira, Joana Odila; Ferreira., Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Effect of drying technology in *Saccharomyces cerevisiae* mannans: Structural, physicochemical, and functional properties. Food Chemistry 412:135545. **https://doi.org/10.1016/j.foodchem.2023.135545**

Faustino, Margarida; Silva, Sara; Costa, Eduardo; Pereira, Ana Margarida; Pereira, Joana Odila; Oliveira, Ana Sofia; Ferreira, Carlos; Pereira, Carla F.; Durão, Joana; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Effect of mannan oligosaccharides extracts in uropathogenic *Escherichia coli* adhesion in human bladder cells. Pathogens 12(7), 885. **https://doi.org/10.3390/pathogens12070885**

Poster communications:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pintado, Manuela E.; Carvalho, Ana P.; Effects of drying technology on the physical and functional properties of mannans from *S. cerevisiae*. XV Encontro de Química dos Alimentos, September 2021, Madeira, Portugal.

Faustino, Margarida; Carvalho, Nelson; Durão, Joana; Pintado, Manuela E.; Madureira, Raquel; Carvalho, Ana P.; Evaluation of mannans from *Saccharomyces cerevisiae* as potential human microbiota modulators. Microbiotec 21, November 2021, Lisbon, Portugal.

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pintado, Manuela E.; Carvalho, Ana P.; Optimization of mannans extraction from spent yeast *Saccharomyces cerevisiae*. 5 th Food Structure and Functionality Symposium, September 2022, Cork, Ireland.

Faustino, Margarida; Silva, Sara; Costa, Eduardo; Pereira, Joana Odila; Pereira, Ana Margarida; Pereira, Carla F.; Durão, Joana; Pintado, Manuela E.; Carvalho, Ana P.; Effect of mannan oligosaccharides extracts in uropathogenic *Escherichia coli* adhesion in human bladder cells. 1st International Congress on Food, Nutrition & Public Health, November 2022, Lisbon, Portugal.

Unpublished works

Papers submitted (or in preparation) to international peer-reviewed journals:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pereira, Ana Margarida; Oliveira, Ana Sofia; Sousa, Sérgio; Braga Ribeiro, Alessandra; Pereira, Joana Odila; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Unveiling the potential of yeast Mannan oligosaccharides (MOS): production, characterization, and bioavailability studies. Submitted to Food Research International.

Faustino, Margarida; Pereira, Joana Odila; Pereira, Ana Margarida; Oliveira, Ana Sofia; Ferreira, Carlos; Pereira, Carla F.; Durão, Joana; Pintado, Manuela E.; Carvalho, Ana P.; 2023 Alternative strategies to prevent *Candida albicans* proliferation in vaginal environment: synergistic effect of lactobacilli and mannan oligosaccharides (MOS). Submitted to Applied Microbiology and Biotechnology.

Faustino, Margarida; Pereira, Carla F.; Durão, Joana; Pereira, Ana Margarida; Oliveira, Ana Sofia; Pereira, Joana Odila; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Carboxymethylation of *Saccharomyces cerevisiae* mannans: preparation and properties evaluation. Manuscript in preparation.

Chapter 2

Extraction of mannans from spent yeast

This chapter aims to evaluate the different extraction methods and characterize structural, physicochemical, and biological properties of the mannans extracts.

Chapter 2.1 *Extraction of mannans*

Chapter 2.2 *Optimization of drying process*

Adapted from the following scientific publications:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Oliveira, Ana Sofia; Pereira, Joana Odila; Pereira, Ana M.; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2022. Comparative Analysis of Mannans Extraction Processes from Spent Yeast *Saccharomyces cerevisiae*. FOODS 11:3753. **htpps://doi.org/10.3390/foods11233753**

Faustino, Margarida; Pereira, Carla F.; Durão, Joana; Oliveira, Ana Sofia; Pereira, Joana Odila; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Effect of drying technology in *Saccharomyces cerevisiae* mannans: Structural, physicochemical, and functional properties. Food Chemistry 412:135545. **htpps://doi.org/10.1016/j.foodchem.2023.135545**

2.1. Extraction of Mannans

Comparative analysis of Mannans extraction processes from spent yeast *Saccharomyces cerevisiae*

Abstract: Mannans are outstanding polysaccharides that have gained exponential interest over the years. These polysaccharides may be extracted from the cell wall of Saccharomyces cerevisiae, and recovered from the brewing or synthetic biology industries, among others. In this work, several extraction processes - physical, chemical, and enzymatic - were studied, all aiming to obtain mannans from spent yeast *Saccharomyces cerevisiae*. Their performance was evaluated in terms of yield, mannose content and cost. The resultant extracts were characterized in terms of their structure (ATR-FT-IR, PXRD and SEM), physicochemical properties (colour, molecular weight distribution, sugars, protein, ash, and water content) and thermal stability (DSC). The biological properties were assessed through the screening of prebiotic activity in *Lactobacillus plantarum* and *Bifidobacterium animalis*. The highest yield (58.82%) was achieved by using an alkaline thermal process, though the correspondent mannose content was low. The extract obtained by autolysis followed by a hydrothermal step resulted in the highest mannose content (59.19%). On the other hand, the extract obtained through the enzymatic hydrolysis displayed the highest prebiotic activity. This comparative study is expected to lay the scientific foundation for the obtention of well-characterized mannans from yeast, which will pave the way for their application in various fields.

Keywords: Mannans; Thermal hydrolysis; Autolysis; Enzymatic hydrolysis; Structural characterization; Physicochemical characterization; Cost assessment

2.1.1. Introduction

Large amounts of yeast cell wall are available as a by-product in the bakery, fermentation, winemaking, and synthetic biology industries. Its recovery and reuse are excellent examples of a circular economy approach (Rakowska et al., 2017). The low cost, non-toxicity and compositional properties of this by-product provides an opportunity for its effective conversion into value-added functional ingredients, which in turn re-duces the environmental impact of the original processes.

S. cerevisiae has been well-characterized over the years and is considered a valuable model for studying the cell wall of other yeasts (Lee et al., 2001). The three main components of its cell wall are i) glucans (1,3-β-D-glucan and 1,6-β-D-glucan), ii) mannoproteins – mannans connected to protein, which comprise around 35 to 40% of cell dry mass (Orlean, 2012) and iii) chitin (Kath & Kulicke, 1999; Kwiatkowski et al., 2009; Orlean, 2012; Pinto et al., 2015).

The mannoproteins can be divided into three groups, non-covalently and covalently bound to the structural glucan and disulphide bound to other proteins that are covalently bound to the glucan of the cell wall (Li & Karboune, 2018). According to Abbott et al., (2015) mannans consist of a highly branched complex carbohydrate, with the α-1,6 backbone as the main chain and α-1,2- and α-1,3-linked mannose side chains. It has been reported that mannans possess several biological properties such as inhibition of pathogen adherence, modulation of bacterial growth (Smith et al., 2020) and improvement of the immune response (Lee & Dugoua, 2011; Onitake et al., 2015), thus being commonly used in animal feed as antibiotic replacers (Smith et al., 2020; Spring et al., 2015). Additionally, the ability to scavenge radicals such as superoxide anions and hydroxyl radicals provides a potential antioxidant effect (Liu et al., 2018). Furthermore, mannans exhibit many techno-functional properties that make them attractive for food applications derived from their physicochemical properties (water solubility, viscosity, and stability); typical applications are based on their use as a hardener ingredient and emulsion stabilizer (Singh et al., 2018). All these properties reveal the potential applications of mannans in different areas that range, but are not limited to, food (Butylina et al., 2007; Singh et al., 2018), feed (Shurson, 2018; Singh et al., 2018), cosmetics (Gaspar et al., 2008; Yoon et al., 2019) and drug delivery (Yu et al., 2010).

The extraction of mannans usually includes the following steps: cell lysis, fractionation, and purification (Faustino et al., 2021). The step of cell lysis can be performed by different processes, namely by chemical (e.g., alkaline reagents such as NaOH (Haworth et al., 1937; Liu & Huang, 2018) or buffer solutions such as sodium phosphate, citrate or Tris (Kath & Kulicke, 1999; Kocourek & Ballou, 1969; Li & Karboune, 2018; Snyman et al., 2021), physical (using temperature and pressure), enzymatic (e.g., proteases (Cawley et al., 1972; Kath & Kulicke, 1999; Russell et al., 1973), glucanases (Fleet & Manners, 1977; Kath & Kulicke, 1999; Shibata et al., 1983; Valentin et al., 1984) and carbohydrases (Li et al., 2020; Li & Karboune, 2018, 2019), or mechanical processes (e.g., glass beads) (Kath & Kulicke, 1999); a combination of processes is also widely employed. Their recovery from the solution (fractionation and/or purification) can be easily performed by precipitation – ethanol, methanol, or acetone are the solvents commonly reported in the literature for this purpose (Cameron et al., 1988; Peat, Whelan, et al., 1961; Snyman et al., 2021). Additionally, chromatographic methods (Li et al., 2020) and ultrafiltration (Cameron et al., 1988; Sedmark, 2014) are also reported in the literature as methodologies to purify mannans. These different conditions and strategies were previously reviewed and critically compared elsewhere (Faustino et al., 2021).

Overall, this study aimed to perform an integrative comparison of the isolation of mannans from the spent yeast *S. cerevisiae* through different processes (heat treatment in aqueous neutral and alkaline medium, autolysis, and enzymatic hydrolysis with Zymolyase®-20T), in terms of yield and mannose content of the obtained extracts. Structural features of all mannans extracts were unveiled by solid-state characterization techniques (ATR-FT-IR, PXRD and SEM), and their physicochemical properties (colour, total protein, neutral sugars, molecular weight distribution, solubility, dry weight, and ashes), along with the thermal stability were studied. Additionally, an evaluation of the extracts' cytotoxicity and prebiotic activities is also presented and properly compared. The structural and physiochemical characterization is highly demanded since the functional properties of mannans are dependent on these properties. To complement the comparison of the different processes, a cost assessment was performed. This comparative study is expected to lay the scientific foundation for the recovery of well-characterized mannans from yeast, which will pave the way for their application in various fields.

2.1.2. Materials and methods

2.1.2.1. Materials and equipment

Genetically modified spent yeast (*Saccharomyces cerevisiae*) was kindly provided by Amyris Company (Emeryville, California, USA), and bacteria *Lactobacillus plantarum* and *Bifidobacterium animalis* spp. *lactis* BB12® were purchased from Chr. Hansen (Hørsholm, Denmark). Zymolyase®-20T from *Arthrobacter luteus* was purchased from Amsbio (Cambridge, Massachusetts, USA). For reagents: Ethanol (>99%) was purchased from CHEM-LAB (Zedelgem, Belgium); sulphuric acid (H_2SO_4) was purchased from Honeywell (Charlotte, North Carolina, USA); NaOH was purchased from LabChem (Johannesburg, South Africa); and acid chloride (HCl) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All reagents were used as received without further purification. Equipment used in this study were: centrifuge, from ThermoFisher Scientific (Waltham, MA, USA); autoclave, from Prohs (Porto, Portugal); orbital system, from Eppendorf (Madrid, Spain); water bath (SW22, Julabo GmbH, Seelbach, Germany); and lyophilizer, from Christ, Osterode am Harz (model Alpha 2-4 LSCplus, Germany).

2.1.2.2. Isolation and purification of mannoproteins

Preparation of spent yeast

Spent yeast was centrifuged at 12 000 x *g* for 10 min, the supernatant was discarded, and the pellet was washed twice with water.

Thermal hydrolysis (TH)

The process was carried out according to Freimund et al., (2003) with slight modifications. In short, the yeast pellet was suspended in deionized water (10% w/v), and the pH adjusted to 7.0 (NaOH 6 M). The suspension was placed in an autoclave programmed to 120 °C for 3 h. The resulting suspension was centrifuged (4700 x *g*, 10 min), and the supernatant was collected, precipitated with twice its volume of cold (4 °C) absolute ethanol and left overnight at 4 °C. The resulting precipitate was collected by centrifugation (4700 x *g*, 10 min) and lyophilized.

Thermal hydrolysis with NaOH (TH NaOH 0.25 M and TH NaOH 1.5 M)

The process was carried out according to Liu & Huang (2018), and Lee & Ballou (1965) with slight modifications. Yeast pellet (10% w/v) was suspended in NaOH solutions at 0.25 M and 1.5 M. The suspensions were heated to 120 °C in an autoclave for 3 h and then centrifuged (4700 x *g*, 10 min) and the supernatants collected. The pH was adjusted to 7.0 (HCl 6 M) and then precipitated with twice its volume of cold (4 °C) absolute ethanol, and left overnight at 4 °C. The extracts were collected by centrifugation (4700 x *g*, 10 min) and lyophilized.

Autolysis (ATL) and Thermal hydrolysis (ATL + TH)

Autolysis was performed according to Milić et al., (2007) and Jacob et al., (2019), with slight modifications. Briefly, the yeast pellet was suspended in deionized water (50% w/v), with pH adjusted to 5.2 (NaOH 10 M). The suspension was heated at 52 °C in an orbital system for 16 h under 120 rpm and then inactivated by heating to 100 °C for 10 min in a water bath. The resulting products (pellet and supernatant) were thus separated by centrifugation (3290 x *g*, 10 min). The supernatant was precipitated with ethanol as described in TH, thus obtaining the extract ATL. The pellet fraction was submitted to a thermal hydrolysis treatment, as previously described. In brief, the pellet was suspended in water (10% w/v), the pH adjusted to 7.0, heated to 120 °C in an autoclave for 3 h and the supernatant collected by centrifugation (4700 x *g*, 10 min). The resulting supernatant was precipitated with cold ethanol, as previously described in the above treatments, and thus the extract ATL+TH was obtained.

Enzymatic hydrolysis (EH)

The enzymatic hydrolysis was performed according to Li et al., (2020) and Li & Karboune, (2018, 2019) with some modifications. The yeast pellet was suspended in sodium phosphate buffer 0.5 M, pH 7.5 (5% w/v), and the enzyme was added at a concentration of 167 units of β-1,3-glucan laminaripentaohydrolase (one of the components of Zymolyase®) per 1 g of yeast. The suspension was heated to 35 °C in an orbital system for 4 h under 130 rpm agitation and then inactivated by heating to 60 °C for 10 min, in a water bath. The supernatant was collected by centrifugation (4700 x *g*, 10 min) and treated as described in the previous treatments, being thus obtained the EH product.

2.1.2.3. Structural characterization

Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy—ATR-FT-IR

The ATR-FT-IR analyses were performed using the Frontier™ MIR/FIR spectrometer from PerkinElmer in a scanning range of 550–4000 cm^{-1} for 16 scans at a spectral resolution of 4 cm^{-1}.

Powder X-ray Diffraction—PXRD

The Powder X-Ray Diffraction (PXRD) analyses were performed on Rigaku MiniFlex 600 diffractometer with Cu kα radiation, with a voltage of 40 kV and a current of 15 mA ($3° < 2\theta > 90°$; a step of 0.01 and speed rate of 3.0°/min).

Scanning Electron Microscopy—SEM

The morphology of all mannans extracts was evaluated by Scanning Electron Microscopy on a Thermo Scientific™ Pro Scanning Electron Microscope. Before the analysis, the samples were placed into observation stubs covered with double-sided adhesive carbon tape (NEM tape, Nisshin, Japan), and coated with Au/Pd (target SC510-314B from ANAME, S.L., Madrid) using a Sputter Coater (Polaron, Bad Schwalbach, Germany). All observations were performed in high vacuum mode with an acceleration voltage of 5 kV. The specimen was observed using a 4000× magnification, and all images are representative of the morphology of each extract.

2.1.2.4. Physicochemical properties

Colour

To determine the colour point, a portable CR-410 Chroma meter (from Minolta Chroma, Osaka, Japan) was used. The CIELAB (Commission Internationale de I'Eclairage) colour system (L*, a*, b*) was used to determine the colour point, in which the L* corresponds to the luminosity coordinate (L* value of 100 for a white object and 0 for a black object); the a* sets the green-red coordinate, and the b* sets the blue-yellow colour coordinate (Ordóñez-Santos et al., 2017). Colour was measured on the surface of the

mannans samples in a petri dish placed on a white standard plate (L*= 93.22, a* = -0.08 and b*= 4.04). The total colour difference (ΔE*) was calculated according to Equation (1) against a mannan commercial product from *S. cerevisiae* by Sigma-Aldrich.

$$\Delta E^* = ((L2^* - L1^*)^2 + (a2^* - a1^*)^2 + (b2^* - b1^*)^2)^{1/2} \quad (1)$$

Total protein

For the evaluation of total protein, a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Bonn, Germany) was used, according to the manufacturer's instructions, in 96-well microplates (25 µL sample/200 µL BCA working reagent; 37 °C/30 min; 562 nm). A stock solution of bovine serum albumin (BSA) (Sigma, Munich, Germany) was prepared before carrying out the experiments. BSA dilution curves were made accordingly, and aliquots were stored to ensure an identical BSA content for each experiment. For each microplate prepared, a BSA dilution calibration curve was added, consisting of 8 points up to 1000 µg/mL BSA.

Neutral sugars

The quantification of neutral sugars was performed according to what has been reported by Pinto et al., (2015). Mannans extracts were hydrolysed using 72% H_2SO_4 (3 h, room temperature) followed by H_2SO_4 1 M (1 h, 120 °C) (Selvendran et al., 1979). Neutral sugars were derivatized to their alditol acetates, as described by Blakeney et al., (1983), 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). The carrier gas was nitrogen at constant flow (1 mL/min), and hydrogen (30 mL/min) and compressed air (400 mL/min) were also used in the analysis. A split ratio of 1:60 was also used, and the injector and detector temperatures were set at 220 °C and 230 °C, respectively. The oven temperature was first set to 200 °C and kept at that temperature for 2 min, then raised to 220 °C at 40 °C/min, with a hold time of 7 min, followed by a second linear increase to 230 ºC at 20 °C/min holding 5 min. The total run time is 15 min.

Molecular weight distribution

The Agilent 1260 Infinity II HPLC system was used to determine the molecular weight 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. 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 used for separation. A calibration curve of pullulan standards was used to estimate molecular weight.

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 under isocratic conditions. The columns were kept at 50.0 °C, and the RID was set to 35.0 °C.

Dry weight and ashes

To evaluate the moisture content according to the Association of Official Analytical chemists (AOAC, 2005), mannans samples were placed at 105 °C in a convection oven for 24 h. To determine the ash content, samples were placed at 550 °C in a muffle for 36 h for incineration. The ash was weighed after equilibration at room temperature.

Solubility test

The solubility tests were performed according to the European Pharmacopoeia (Pharmacopoeia, 2017): 1.0 g of the finely ground compound was dissolved in increasing volumes of solvent (water), and its classification in terms of solubility was directly related to the volume of solvent necessary for complete solubilization. Substances that dissolve in 10-30 mL, 30-100 mL, 100-1000 mL or 1000-10000 mL of solvent are classified as soluble, sparingly soluble, slightly soluble or very slightly soluble, respectively. If the substance does not dissolve, it is classified as insoluble in this solvent.

Differential scanning calorimetry—DSC

Differential scanning calorimetry (DSC) measurements were performed under a nitrogen atmosphere using DSC 204 F1 Phoenix equipment from Netzsch, calibrated using an indium standard. The samples (3–6 mg) were placed into aluminium DSC pans with a

pinhole, and an empty pan was used as a reference. Heating from 20 – 300 °C was carried out at a heating rate of 10 °C/min.

2.1.2.5. Cytotoxicity Evaluation

Cell line growth conditions

Human colon carcinoma (CaCo-2) cells were obtained from the American Type Culture Collection. They were 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 100x (Gibco, Milan, Italy) and 1% (v/v) of non-essential amino acids 100x (Sigma, Germany). Cells were used between passages 55 and 56.

Cytotoxicity assay

Cytotoxicity of the samples was assessed in the CaCo-2 cell line in conformity with ISO (International Organization for Standardization) 10993-5 (2009), using the PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, MA, USA) according to the instructions of the manufacturer. The cells in suspension were seeded at 1×10^4 cells/well in a 96-well microtiter plate and maintained in culture for 24 h to form a semi-confluent monolayer. Following this incubation period, the cell culture medium was removed and replaced with the samples. Samples were previously dissolved in phosphate buffered saline (PBS) solution, pH 7.4 to a final concentration 2-fold higher than the desired, and twofold diluted in antibiotic containing DMEM in the range of 10.0 – 0.31 mg/mL. Medium without the samples in each incubation period was used as positive control, whereas medium with a final concentration of 10% of DMSO was used as negative control. After an additional 24 h incubation period, PrestoBlue (PB) viable dye was added to the wells and changes in cell viability were detected using fluorescence spectroscopy (excitation 570 nm; emission 610 nm), (Synergy H1, BioTek, CA, USA) after incubation 2 h.

2.1.2.6. Screening of prebiotic effect

The potential prebiotic effect of the mannans extracts from the different extraction methods was determined by screening their impact upon the growth of two potential probiotic: *Lactobacillus plantarum* and *Bifidobacterium animalis* spp. *lactis* BB12®. All

strains were used as monoculture and were grown in MRS broth at 37 °C for 24 h, under aerobic conditions for *L. plantarum* and anaerobic conditions for *B. animalis* BB12. The mannans extracts and fructo-oligosaccharides (FOS) (Sigma-Aldrich Chemistry, St. Louis, MO, USA), were added to sterilized MRS broth without glucose to a final concentration of 2% (w/v). MRS broth with glucose (2%) was used as negative control (Control). From each MRS medium inoculated with *L. plantarum* and *B. animalis* BB12, 200 µL were transferred, in triplicate, to a 96-well microplate (Nunc, Denmark). Additionally, 50 µL of paraffin was added to ensure anaerobic conditions in the case of the *B. animalis* subsp *lactis* BB12. The microplates were incubated for 48 h at 37 °C, and absorbance was measured at 625 nm each hour with a multidetector plate reader (Epoch, BioTek, California, USA).

2.1.2.7. Cost assessment

The costs associated with the different processes were estimated considering two major components: energy and reagent consumption. Both parameters were calculated at laboratory scale, considering the average yields and consumptions of the triplicate experiments performed for each extraction process. The electricity price used for calculation purposes was 0.2067 €/kWh, which was the average household price in Portugal in 2022 (Pordata, n.d.). Energy consumption was estimated by considering the maximum power of each equipment and the time that same equipment was used. The water price used in the calculations was 1.8198 €/m^3 (Empresa de Águas e Energia do Porto - CMPEAE, n.d.).

2.1.2.8. Statistical analysis

All extractions and analyses were performed in triplicate (n = 3). Extraction yield, protein quantification, total sugar and glucose, dry weight, ashes content and main population distribution means were compared using one-way ANOVA followed by Tukey's Multiple Comparison Test, using a 95% confidence interval as criteria. The normality of the samples was evaluated using the Shapiro-Wilk's Test. All assays were performed using the Statistical Package for Social Sciences software (version 21, SPSS, Chicago, IL, USA).

2.1.3. Results and discussion

Several studies indicate that the cell wall and cytoplasm of yeast cells contain many nutritional components (Chae et al., 2001; Manners et al., 1973). However, to use its components effectively, it is necessary to overcome the difficulty of breaking through the thick and rigid cellular walls (Magnani et al., 2009) of the yeast. Various methods/processes for cell wall rupture have been developed through chemical, biological, physical, and mechanical approaches.

In this study, after a review of the most used conditions to extract mannans from *S. cerevisiae* yeast in the literature, some conditions of physical (temperature and pressure), chemical (alkaline and neutral solvents), and enzymatic (autolysis and enzymatic hydrolysis) extraction processes were selected. The efficiency of each of these extraction methods in terms of yield (considering the whole extract – solid yield, or just the mannose fraction – mannose yield) and mannose content were evaluated, as described in **Table 5**.

The highest production yield (mannose yield, 58.82%) was achieved by thermal hydrolysis with 0.25 M of NaOH (TH NaOH 0.25 M), although the correspondent mannose content was low (32.91%). On the other hand, autolysis followed by a hydro-thermal step (ATL + TH) allowed to reach the highest mannose content (59.19%). Thermal hydrolysis in neutral conditions (TH) presents the second highest mannose content (53.46%), but more than twice the yield obtained for ATL + TH. The use of enzymes poses a significant economic burden (Faustino et al., 2021), and in this process and looking to the parameters of **Table 5**, its use may not be justified, as the yield and content values obtained were not as appealing as with other processes. On the other hand, the use of high concentrations of NaOH in processes TH NaOH 0.25 M and TH NaOH 1.5 M, results in extracts with a high level of salt (NaCl), as discussed subsequently, which may imply additional steps of downstream processing.

Table 5. Solid and mannose yields and mannose content of mannan extracts obtained by different extraction methods (TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH).

	TH	TH NaOH 0.25 M	TH NaOH 1.5 M	ATL	ATL + TH	EH
Solid Yield (%)	8.25 ± 0.22 ^{c}	20.22 ± 1.23 ^{ab}	16.66 ± 3.43 ^{b}	10.18 ± 1.04 ^{c}	3.58 ± 1.30 ^{d}	23.88 ± 1.16 ^{a}
Mannose Yield (%)	39.04 ± 0.76 ^{b}	58.82 ± 1.57 ^{a}	28.65 ± 3.05 ^{c}	22.65 ± 2.66 ^{cd}	18.75 ± 6.84 ^{d}	45.80 ± 2.16 ^{b}
Mannose Content (%)	53.46 ± 0.44 ^{b}	32.91 ± 1.64 ^{c}	19.67 ± 1.82 ^{e}	25.14 ± 1.72 ^{d}	59.19 ± 1.35 ^{a}	21.72 ± 2.02 ^{de}

Superscript different letters in the same row represent statistically different values ($p < 0.05$). TH—Thermal hydrolysis (H_2O), TH NaOH 0.25 M—Thermal hydrolysis NaOH 0.25 M, TH NaOH 1.5 M—Thermal hydrolysis NaOH 1.5 M, ATL—Autolysis, ATL + TH—Autolysis + Thermal hydrolysis and EH—Enzymatic hydrolysis.

2.1.3.1. Structural characterization

The structural analysis of the mannans extracts from *Saccharomyces cerevisiae* obtained by different extraction methods was attained by the following set of solid-state techniques: ATR- FT-IR; PXRD; and SEM. The normalized ATR-FT-IR spectra of all products are depicted in **Figure 8**.

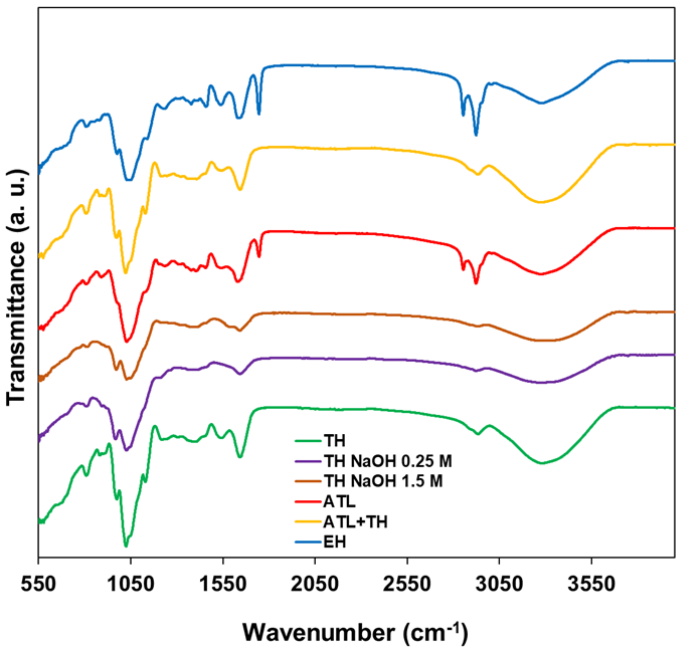


Figure 8. ATR-FT-IR spectra of the mannans extracts correspondent to the following extraction methods: pH-neutral Thermal hydrolysis (TH); alkaline Thermal hydrolysis (TH NaOH 0.25 M and TH NaOH 1.5 M); Autolysis (ATL); Autolysis followed by pH neutral Thermal hydrolysis (ATL+ TH); and Enzymatic hydrolysis (EH).

The analysis of the spectra highlighted in **Figure 8**, allows to infer that all mannans extracts share similar spectral properties. All spectra exhibit a strong and broad vibration band at 3000 – 3687 cm^{-1} correspondent to the stretching of the hydroxyl groups (Liu et al., 2018; Zhao et al., 2022); a vibration at ca. 2943 cm^{-1} that can be attributed to the C–H stretching vibration, and a vibration at 1403 cm^{-1} that can be assigned to the to the C–H bending vibration. All ATR-FT-IR spectra also exhibit a sharp band at 1647 cm^{-1} that can be attributed to the C–O asymmetric stretching vibration (Liu et al., 2015); a vibration band at 1025 cm^{-1} assigned to the O–H variable angle vibrations and the characteristic absorption of the mannan α-chain appeared at 814 cm^{-1} (Liu et al., 2018; Zhao et al., 2022). The spectra

correspondent to the mannan extracts obtained by autolysis and enzymatic hydrolysis, ATL and EH, respectively, exhibit two strong vibrations at 2925 and 2854 cm^{-1} (lipid region) that can be assigned to the asymmetric and symmetric vibrational modes of CH_2 groups, respectively, and a sharp vibration at 1743 cm^{-1} that can be attributed to the C=O stretching, generally representing lipids (Bikmurzin et al., 2022).

The analysis of the crystallinity of the mannans extracts obtained from the various extraction methods was assessed by Powder X-Ray Diffraction (PXRD), as depicted in **Figure 9**.

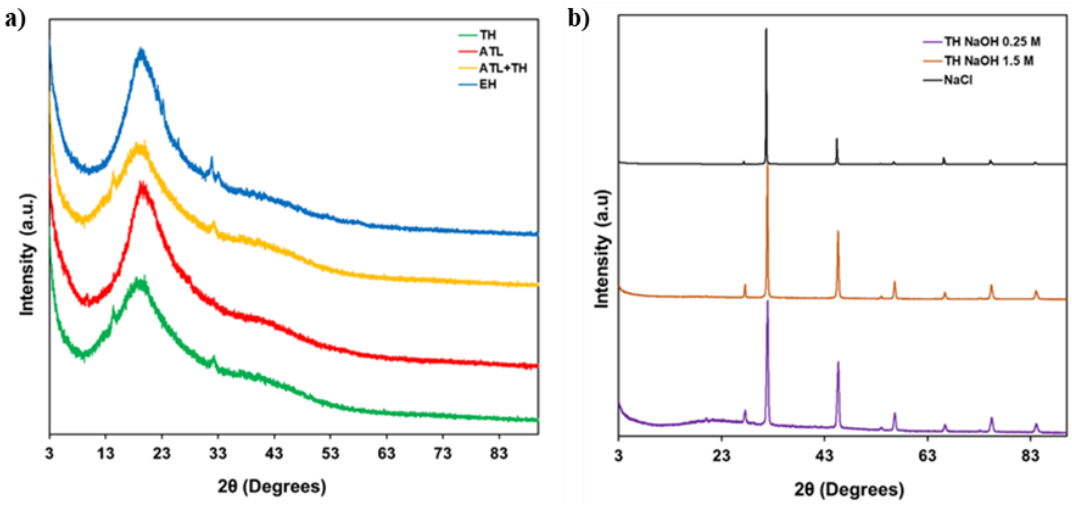


Figure 9. Powder X-Ray Diffraction analyses of the mannans extracts correspondent to the following extraction methods: (a) TH, ATL, ATL + TH and EH; and (b) TH NaOH 0.25 M and TH NaOH 1.5 M and commercial NaCl.

The PXRD patterns of the mannans extracts obtained by pH neutral hydrolysis (TH), autolysis (ATL), or even the product resulting from the combination of both previous methods (ATL + TH), along with the extract obtained by enzymatic hydrolysis (EH) exhibit a broad diffraction at 2θ = 19.7° consistent with the predominant amorphous character (**Figure 9a**) (Mary et al., 2019). However, the PXRD analysis of the products resultant from the alkaline thermal hydrolysis (**Figure 9b**), using different NaOH concentrations of 0.25 and 1.5 M, TH NaOH 0.25 M, and TH NaOH 1.5 M, respectively, revealed the presence of NaCl – produced in the neutralization reaction of NaOH and HCl – with both samples exhibiting the characteristic diffraction peaks of NaCl (27.5°, 31.7°, 45.4°, 53.7°, 56.6°, 66.2°, 75.5° and 84.1°) (Bao et al., 2017).These extracts resultant from the alkaline

extraction processes require further purification steps in to prompt the salt removal, namely by further washing with water or even by desalination.

The morphology of all mannans extracts was evaluated by Scanning Electron Microscopy (SEM), as highlighted in **Figure 10**.

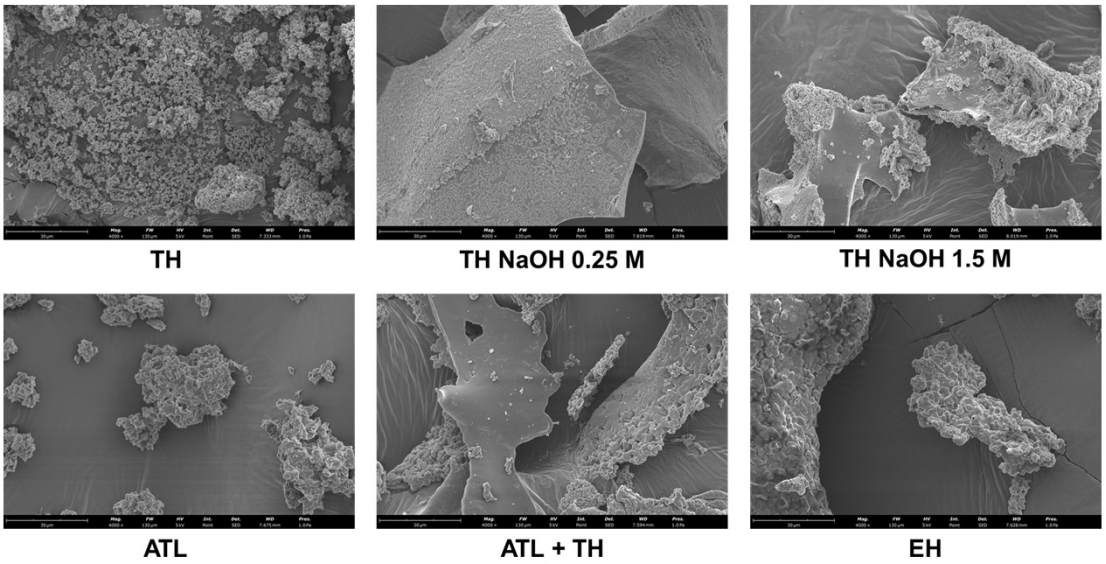


Figure 10. Scanning Electron Microscopy Analyses of the mannans extracts correspondent to the following extraction methods: TH; TH NaOH 0.25 M and TH NaOH 1.5 M; ATL; ATL + TH; and EH.

The study of morphology/size is highly relevant, since these properties may affect physicochemical parameters, namely wettability and solubility. The TH and EH extracts are constituted by rough spherical particles. However, while the particles corresponding to the extract obtained by thermal hydrolysis are the smallest, the EH extract exhibits large aggregates. The product resulting from the autolysis process (ATL) was revealed to be constituted of small aggregates of plates and rough spherical particles, and the extract obtained by the combination of extraction processes (ATL + TH) showed the most heterogeneous nature, constituted by large smooth plates and large aggregates of small plates and rough spherical particles. The products obtained from the alkaline hydrolysis processes are characterized by large plates coated with rough spherical particles and large plates with spherical particles for TH NaOH 0.25 M and TH NaOH 1.5 M, respectively.

2.1.3.2. Physicochemical characterization

The integrated and comparative analysis of the physical appearance of all mannans products (TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH), along with their physicochemical properties and thermal analysis, is presented in this section.

Physical appearance

The physical aspect of the powders is significantly different, as shown in **Table 6**. All the mannans extracts appeared as a homogeneous fine powder. The mannans extracts of TH NaOH 0.25 M and TH NaOH 1.5 M appeared as a gold tone. The extracts ATL and EH are brownish, while the TH and ATL + TH extracts revealed an off-white colour. The colour of products constitutes an important criterion for their commercialization, as the colours accepted by the consumers are very scarce. The colour point of all mannans extracts was measured using the system CIELAB (L*, a*, b*) (Ordóñez-Santos et al., 2017).

The TH extract exhibits the highest L* (lightness) value (83.88), followed by the TH NaOH 1.5 M (81.06) and TH NaOH 0.25 M (76.92), ATL (76.36) and ATL + TH (76.74), respectively, and the EH (74.81) reveals the lowest L* value.

The total colour difference (ΔE*) was determined to allow the quantification of the colour change between the yeast mannans products after the various extracting processes and a commercial mannan product of *S. cerevisiae* by Sigma-Aldrich. The ΔE* ($1.50 < \Delta E^* > 3.0$) between the differences extracts and the benchmark was 6.43 for TH extract, 15.10 for TH NaOH 0.25 M extract, 10.76 for TH NaOH 1.5 M, 14.35 for ATL, 13.45 for ATL + TH and finally 15.81 for EH. Since the determined total colour difference were higher than 3.0, this corroborates the distinct visual perception among all mannans samples (Bellary et al., 2016).

Table 6. Results of the physical appearance and colour characteristics of the TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH extracts.

	TH	TH NaOH 0.25 M	TH NaOH 1.5 M	ATL	ATL + TH	EH
Physical Appearance						
Colour Characteristics						
L*	83.88 ± 0.01 [a]	76.92 ± 0.01 [c]	81.06 ± 0.01 [b]	76.36 ± 0.01 [f]	76.74 ± 0.01 [e]	74.81 ± 0.02 [d]
a*	1.36 ± 0.01 [f]	3.68 ± 0.01 [a]	3.22 ± 0.00 [b]	1.72 ± 0.01 [c]	1.46 ± 0.01 [e]	1.69 ± 0.01 [d]
b*	7.08 ± 0.01 [f]	14.03 ± 0.02 [a]	12.48 ± 0.01 [b]	11.45 ± 0.02 [c]	7.64 ± 0.01 [e]	11.37 ± 0.02 [d]
ΔE* [1]	6.43 ± 0.01 [f]	15.10 ± 0.02 [b]	10.76 ± 0.00 [e]	14.35 ± 0.02 [c]	13.45 ± 0.01 [d]	15.81 ± 0.03 [a]

L* is the lightness coordinate, a* defines the green-red coordinate, and b* the blue-yellow coordinate. Superscript different letters in the same row represent statistically different values ($p < 0.05$). 1 The calculation of ΔE* was performed against a commercial mannan product to make the comparison.

Physicochemical properties

The physicochemical analyses performed for the mannans (TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH) extracts are summarized in **Table 7**. All mannans extracts were evaluated regarding their composition — protein and total sugars (mannose and glucose) — and molecular weight distribution (evaluated by HP-SEC). The moisture and ash content, along with solubility, were also studied for all products.

The yeast cell wall is made up of about 85 – 90% polysaccharides and 10 – 15% protein (Nguyen et al., 1998). In the cell wall, most of the proteins are covalently linked to mannans, forming mannoproteins, which are in the outer layer of the wall and have ‘‘gel-like’’ properties to protect the yeast cells.

To break down the yeast cell wall to extract the mannans different extraction methods were used, from alkaline methods (0.25 M and 1.5 M NaOH), neutral, enzymatic (autolysis and enzymatic hydrolysis) and combining with physical methods (pressure, time, and high temperature). In theory, by breaking down the cell wall of yeast, mannoproteins and mannans are released into the aqueous medium, which can be separated from the remaining insoluble components of the cell wall, such as glucans, by centrifugation. This is followed by the precipitation with absolute ethanol at low temperature, which will allow the separation of mannans and mannoproteins from other soluble components present in the supernatant. These extraction methods followed by absolute ethanol purification were not found to be sufficiently effective to eliminate the bound protein. Initial reports on mannans extraction from yeast cell wall used a thermal treatment under alkaline conditions (Haworth et al., 1937, 1941). Later studies revealed that heat treatment at neutral pH obtained similar extracts (Peat, Whelan, et al., 1961), having the advantage of reducing the number of steps required in the process. Both alkaline extraction processes presented the best results in term of the elimination of proteins from the mannoprotein complex. The glycosyl-serine and glycosyl-threonine bonds, phosphodiester bonds, some peptide bonds and disulphide and acyl ester bonds may be broken in these conditions, particularly in the most alkaline environment (Ballou, 1976). Even though the extraction using water as a solvent (TH) potentially generates material with intact phosphodiester bonds and, presumably, intact peptide bonds, the protein is likely denatured (Peat, Turvey, et al., 1961).

Table 7. Results of the physicochemical characterization of mannans extracts obtained by different extraction methods: TH; TH NaOH 0.25 M and TH NaOH 1.5 M; ATL; ATL + TH; and EH.

	TH		TH NaOH 0.25 M		TH NaOH 1.5 M		ATL		ATL + TH		EH	
Protein Content (% w/w)	13.00 ± 1.00 ^{b}		1.67 ± 0.58 ^{d}		0.33 ± 0.58 ^{d}		19.33 ± 2.08 ^{a}		7.00 ± 1.00 ^{c}		21.67 ± 2.08 ^{a}	
Total Sugars (% w/w) *	58.28 ± 0.79 ^{b}		36.65 ± 2.20 ^{c}		24.21 ± 2.32 ^{d}		39.94 ± 2.05 ^{c}		65.75 ± 2.30 ^{a}		27.76 ± 2.77 ^{d}	
Glucose (% w/w)	4.82 ± 0.44 ^{cb}		3.73 ± 0.63 ^{c}		4.54 ± 0.57 ^{cb}		14.81 ± 1.43 ^{a}		6.56 ± 1.24 ^{b}		6.04 ± 0.75 ^{b}	
Molecular Weight (MW, kDa)–Most Significant Populations	MW	Area %	MW	Area %	MW	Area %	MW	Area %	MW	Area %	MW	Area %
	237 ± 1 ^{a}	82 ± 2	192 ± 0 ^{e}	29 ± 1	192 ± 0 ^{e}	10 ± 1	221 ± 1 ^{c}	65 ± 1	231 ± 5 ^{b}	84 ± 1	214 ± 3 ^{d}	67 ± 5
	MW	Area %	MW	Area %	MW	Area %	MW	Area %	MW	Area %	MW	Area %
	129 ± 2 ^{c}	18 ± 2	135 ± 4 ^{cb}	23 ± 9	144 ± 1 ^{a}	8 ± 1	132 ± 0 ^{cd}	18 ± 1	133 ± 3 ^{cb}	16 ±1	136 ± 2 ^{b}	16 ± 4

Dry Weight (% w/w)	5.96 ± 1.44 ^{ab}	5.39 ± 2.00 ^{ab}	3.79 ± 1.15 ^{cb}	1.89 ± 0.85 ^{cb}	8.62 ± 2.41 ^{a}	0.57 ± 1.03 ^{c}
Ashes (% w/w)	6.11 ± 0.33 ^{d}	46.14 ± 2.13 ^{b}	63.96 ± 0.27 ^{a}	5.10 ± 0.22 ^{e}	3.79 ± 0.25 ^{ed}	10.93 ± 0.50 ^{c}
Solubility	Very slightly soluble (approx. 1 mg/mL)	Practically insoluble	Slightly soluble (approx. 5 mg/mL)	Practically insoluble	Very slightly soluble (approx. 1 mg/mL)	Practically insoluble

*Total sugars are the sum of the glucose content (% w/w) plus the mannose content (% w/w, in Table 5). Superscript different letters in the same row represent statistically different values ($p < 0.05$).

The processes that result in the highest protein content are the enzymatic hydrolysis (EH) and autolysis (ATL), 21.67% and 19.33%, respectively. Their mild temperature conditions are favourable to the preservation of proteins.

Regarding the sugar content obtained by the different methods (**Table 7**), ATL + TH extract had the highest total sugars content (65.75% w/w), while TH NaOH 1.5 M extract had the smallest total sugars content (24.21% w/w), which also corresponds to the highest and lowest mannose content extracts, 59.19% w/w and 19.67% w/w, respectively (**Table 7**). In the ATL + TH extraction process, and initial autolysis releases part of the mannoproteins in the cell wall into the aqueous medium, which resulted, following the precipitation with ethanol, in a mannose content of 25.14% w/w (ATL). The subsequent extraction from the remaining pellet resulted in a significantly purified extract. This double step methodology may be further explored to obtain higher purity extracts.

MW has been reported to have a direct relationship with bioactivity (Liu et al., 2021). Molecular weight distribution analysis revealed significant differences not only in the MW, but also in the number of populations, obtained by the different extraction processes, as shown in **Table 7**. The MW of the most significant population, evaluated in terms of % area, is significantly decreased in alkaline methods. According to Liu et al., (2018) the alkaline environment would hydrolyse mannans to populations with smaller molecular weight, which is in accordance with our analysis where extracts from TH NaOH 0.25 M and 1.5 M present not only a reduced MW on the highest MW population, around 192 kDa (versus 214 kDa to 237 kDa in the other extraction processes), but also a reduced % area, 29 ± 1% and 10 ± 1%, respectively. The reduction may be due to the hydrolysis of mannans, as mentioned by Liu et al., (2018), but also because of the loss of protein bound to mannans in these extracts. A study by Galinari et al., (2018) reported that mannans extract from fungus (*Kluyveromyces marxianus*) presented 5 different fractions with MW from 10 – 203 kDa. Although the extraction processes are different, they reveal the same two populations with a molecular weight ranging from 190 – 240 kDa populations. These are the only populations present in extracts from TH and ATL + TH, but the remaining extracts present a larger variety of populations with high significance in the range of 4 – 240 kDa. The highest value is slightly larger than those reported in the literature, which can be due to the origin and genetic modification of the yeast used.

A significant difference was found between the extracts concerning ash content, as shown in **Table 7**. Extracts TH NaOH 0.25 M and TH NaOH 1.5 M showed the highest

percentage of minerals (46.14 and 63.96% w/w, respectively). This scenario was already expected since the sodium chloride by-product resultant from the neutralization process (by-product confirmation by PXRD analyses – **Figure 9**) prompts the increase of the mineral content. As previously mentioned, the higher level of impurities may require extra purification processes, such as desalination, to meet the desired purity requirements.

Regarding the solubility of the different extracts, TH NaOH 0.25 M, ATL and EH extracts proved to be insoluble, which is not desirable as it may pose difficulties in its use/incorporation into new products. In addition, the latter method showed other dis-advantages, such as the cost of using enzymes, which may not pay off (considering the low mannose content values). The low solubility of these extracts (TH NaOH 0.25 M, ATL and EH) may be an important limitation for different applications.

The extract TH NaOH 1.5 M showed the highest solubility – approx. 5 mg/ml, which may be due to the high salt content, which may facilitate its solubility. However, its mannose content (19.67% w/w) is low when compared to the extracts obtained by the other processes.

Thermal analysis

The thermal properties of the mannans extracts (TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH) were studied by differential scanning calorimetry (DSC), as depicted in **Figure 11**.

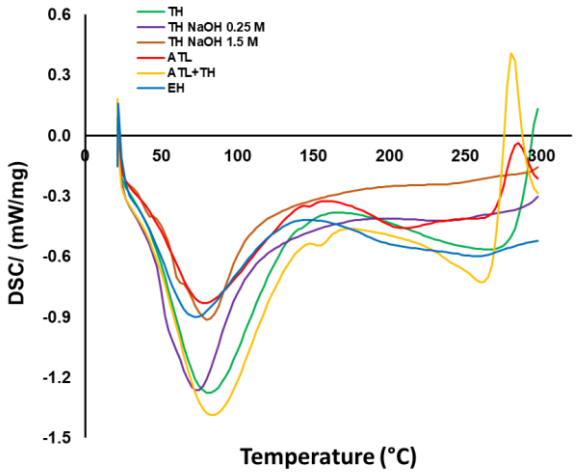


Figure 11. DSC curves resulting from heating the samples from 20 °C to 300 °C at a heating rate of 10 °C/min.

The DSC thermograms were very consistent with each other although some slight differences were observed. The first event, which was observed for all extracts was an endothermic event at around 70 to 80 °C was attributed to the evaporation of the water bound to the polysaccharides present in the sample (Harish Prashanth, 2002; Kittur et al., 2002). This endothermic event was observed in all the extracts.

Only the TH, ATL + TH and ATL reveals an exothermic event around 260 – 290 °C. This peak can be related to polysaccharide thermal decomposition. According to Ospina Álvarez et al., (2014) events close to or above 300 °C are typically observed during the degradation of the saccharide structure and may be involved in the dehydration of saccharide rings. Another study in fungi reveals that the exothermic event around 300 °C more specifically at 334 °C refers to the oxidation of the mannans (Ramos-Sanchez et al., 1991).

Another possibility to explain the position of the exothermic peak around 300 °C in TH, ATL + TH and ATL extracts was the presence of chitosan, a deacetylated derivative of chitin (Roca et al., 2012). The cell wall of spent yeast contains 1 – 2% of chitin (Feuillat, 2003; Orlean, 2012) and chitin bonded to β-(1-3)-D-glucan (Ikuta et al., 1997). Consequently, some residues of chitin bonded β-glucan may be present in the mannans ex-tracts after the extraction of the spent yeast.

The presence of the β-glucan on the mannans extracts is very possible because as shown above there is a percentage of glucose in the extracts which we can associate with the presence of β- glucan residues. The extracts ATL + TH and ATL presented a well-defined peak against the TH extract and according to the content of the glucose the extracts ATL + TH and ATL expose a higher content (6.56 and 14.81%, respectively) there is a possibility that this peak is not just degradation but the presence of β-glucan and, in turn, β-glucan linked to chitin. Thus, extracts such as TH NaOH 0.25 M, TH NaOH 1.5 M and EH reveals curves that indicate remarkable thermal stability, as they do not present this degradation or impurity peak.

Cytotoxicity

Cytotoxicity of samples was assayed against CaCo-2 cells, by evaluating their impact on cell metabolism using a resazurin-based dye as cell viability indicator. According to ISO 10993-5 (2009), a sample is cytotoxic when a metabolic inhibition percentage above 30% is observed.

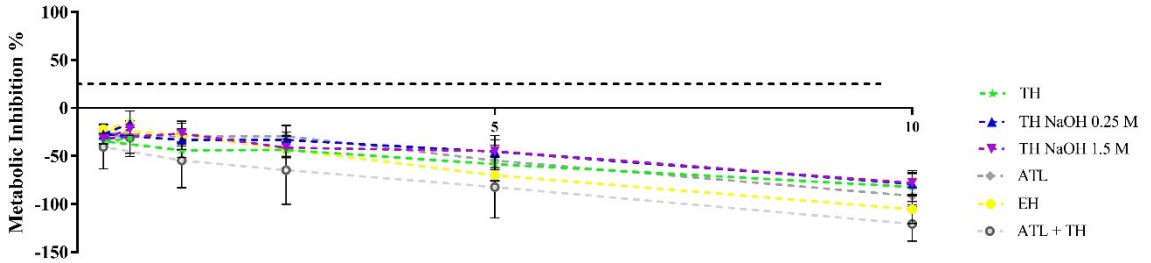


Figure 12. Results of the metabolic inhibition in % for the following concentrations: 10, 5, 2.5, 1.25, 0.63 and 0.31 mg/mL of the TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH mannans extracts.

All mannans extracts (TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH) exhibited no cytotoxicity up to 10 mg/mL (**Figure 12**). This indicates that the differences in the structural and physicochemical properties of the different products do not negatively impact their interactions with the CaCo-2 cells. Moreover, the highest metabolic stimuli were found in the products resulting from the neutral pH extract,TH, (-82.1%) and TH NaOH 0.25 M (-79.1%).

Screening of prebiotic effect

Nowadays, the prebiotic concept has expanded, in part due to the advances in the tools for microbiome research, which have improved our knowledge of the composition of the microbiota, and enabled the identification of additional substances influencing colonization (Gibson et al., 2017). To evaluate the prebiotic potential of mannans extracts, growth curves, obtained by optical density (OD), of the two probiotic strains *L. plantarum* and *B. animalis* subsp *lactis* BB12 were performed in microplates with MRS without glucose media, supplemented with the mannans extracts resulting from the different extraction processes. The 2% concentration of the carbon source was chosen based on the composition of the commercial MRS medium containing 2% (w/v) glucose. FOS was selected to compare with the tested mannans extracts, since it is a standard prebiotic.

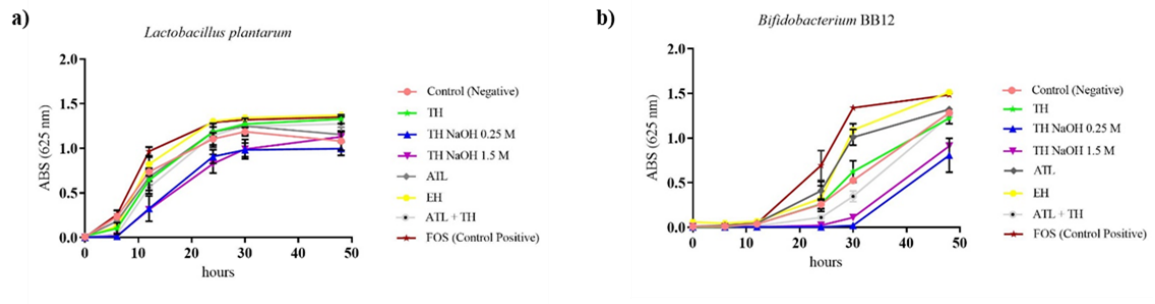


Figure 13. Growth curves of *Lactobacillus plantarum* (a) and *Bifidobacterium animalis* BB12 (b) in MRS broth supplemented with FOS, glucose or with mannans TH, TH NaOH 0.25 M, TH NaOH 1.5 M, ATL, ATL + TH and EH extracts, over 48 h incubation at 37 °C.

Analysis of the growth curves controls for *L. plantarum* controls (**Figure 13a**) revealed that the bacteria presented a reasonable growth rate in basal medium MRS with glucose (negative control) (1.081 ± 0.104), and a boosting growth when MRS medium was supplemented with FOS (positive control) (1.349 ± 0.024). Regarding the prebiotic potential of the mannans extracts, it was observed that the EH extract enabled the highest increase of OD absorbance over time, reaching a maximum value of 1.370 ± 0.024 at 48 h incubation, higher than the positive control (FOS). An additionally interesting result was obtained for the extract from the neutral pH (TH), with the growth promotion of the bacterium to an OD value of 1.328 ± 0.050.

Similar behaviour was reported for *B. animalis* BB12 (**Figure 13b**) in control in basal MRS medium supplemented with FOS revealing the highest growth (1.484 ± 0.010). When the prebiotic potential of the mannans extracts was assessed once again, the enzymatic hydrolysis extract (EH) revealed the highest increase in OD absorbance (1.514 ± 0.039). Differently, it is possible to observed that extracts TH NaOH 0.25 M (0.808 ± 0.189) and TH NaOH 1.5 M (0.911 ± 0.003) affect the growth of the probiotic strains, possibly due to the high concentration of salt in these extracts.

It was reported by Madadi et al., (2015) that extracts obtained from yeast, particularly α-mannans, significantly increase the number of lactic acid bacteria (LAB) in the host. Another work by Everard et al., (2014) illustrates how the α-mannans obtained from *S. boulardii*, increase the proportion of *Lactobacillus* sp. in the intestinal microbiota and have a beneficial effect on host metabolism. A study by Tang et al., (2022) reported the growth of 12 pure LAB cultures in the presence of three yeast α-mannans extracts and the obtained

results suggested that yeast α-mannans could be used as a new prebiotic in functional foods to improve the intestinal environment.

2.1.3.3. Cost assessment

Mannans extraction using the numerous methodologies presented within this study revealed how the different approaches resulted in such distinctive extracts, varying not only in composition, molecular weight, solubility, physical appearance, and morphology, but also in its biological performance. An additional and very relevant parameter is the assessment of the cost of each process.

In this estimate, the energy and reagent consumption were considered the major players contributing to the cost of the process. Both parameters were calculated at laboratory scale, considering the average yields and consumptions of the triplicate experiments performed for each extraction process. Reagents were American Chemical Society (ACS) grade, and the maximum power of each equipment was used. The analysis considered local energy and water prices (0.2067 €/kWh and 1.8198 €/m^3, respectively).

Since this assessment is performed with the goal of determining the most cost-effective process, the freeze-drying step was not considered, since this was the drying technology used for all extracts produced.

The results of cost, energy consumption and water consumption are presented in **Table 8**, both in relation to the total amount of extracts and the corresponding mannose fraction.

The cost assessment presented in **Table 8** revealed that the process with the lowest cost was autolysis (ATL), considering both the cost per gram of extract and per gram of mannose. This is essentially due to its lower water and ethanol consumption, since the initial pellet was suspended at 50% w/v, but also due to the lower energy consumption, as the process does not require such a high extraction temperature as the other procedures. This process is followed closely by the extract of TH NaOH 0.25 M, which owes its low cost to the high solid yield. Both these extracts are practically insoluble, which in some instances may limit its application.

ATL + TH is a double step process, with the sequential extraction via ATL and TH. This process presents the highest cost, as expected. It includes the high energy costs associated with the use of high temperature (autoclave) and the high water and ethanol consumption linked to the second step of the process (TH).

Table 8. Results of the Cost (€), Energy (kWh) and Water Consumption (L) per gram of extract and per gram of mannose of each mannans extract process.

	Cost		**Energy Consumption**		**Water Consumption**	
	(€/g of extract)	(€/g of mannose)	(kWh/g of extract)	(kWh/g of mannose)	(L/g of extract)	(L/g of mannose)
TH	15	24	32	51	0.4	0.7
TH NaOH 0.25 M	7	6	13	13	0.2	0.2
TH NaOH 1.5 M	8	9	16	17	0.2	0.2
ATL	2	5	15	33	0.1	0.2
ATL + TH	49	67	141	194	1.4	1.9
EH	31	103	4	13	0.3	0.1

The EH cost was relatively high, which is a direct consequence of the price associated with Zymolyase®. The immobilization of the enzyme could allow its reutilization, and the costs could be significantly reduced. In fact, the energy consumption of EH is the lowest, supporting the potential of this process for optimization. Even though the EH extract was determined to be practically insoluble, it showed great potential prebiotic activity.

Energy and water consumption (also called energy and water intensity, when normalized to the obtained product weight) are crucial factors in the evaluation of a process sustainability.

EH presents the lowest energy consumption, which is a consequence of the mild temperature conditions required for enzymatic hydrolysis to occur. Its water consumption was not particularly high. The fact that the extract resulting from this process was also bioactive shows the potential of enzymatic hydrolysis if the costs of enzyme can be circumvented by methods such as enzyme immobilization.

On the other hand, ATL presented the lowest water consumption and the lowest cost, which is also very appealing. One parameter that was not considered in the analysis is the time of processes, which in this case is particularly long. Additionally, the procedure used a pellet mass to volume ratio that was significantly different from the remaining procedures (50% w/v), which was a value based on literature. Ideally, the comparison should be

performed in the same starting conditions. Nonetheless, it is an interesting and promising result.

If we evaluate the sustainability of the process considering only these two parameters (water and energy consumption), TH NaOH 0.25 M presents good scores. However, the presence of salt in the extracts would likely require additional purification steps.

The estimation of the costs associated with the process is fundamental in decision making, particularly when planning the scaling up of processes. Additionally, the design of sustainable processes is paramount nowadays, and it is recommended to perform this analysis early on.

2.1.4. Conclusions

The present study reports the effect of different extraction methods on the structural, physicochemical, and biological characteristics of mannans. Additionally, the process yields and costs were evaluated. Interestingly, each extraction process was found to result in distinct characteristics, yields and costs.

The highest solid yield was obtained by enzymatic hydrolysis, which also presented the best prebiotic performance. Its cost is significantly high due to the cost of enzymes, but this could potentially be overcome with the use of immobilized enzymes. The most cost-effective extraction process was found to be autolysis, essentially because of the lower water and ethanol requirement, but also due to the mild extraction conditions which require less energy. Alkaline hydrolysis resulted in high yields, particularly using 0.25 M NaOH, although extracts become contaminated with NaCl, and may require additional purification steps depending on the purity requirements. TH is a good compromise, since it results in a high-purity extract without a major cost imposition. Potential improvement could result from increasing the mass to volume ratio, allowing the reduction of costs, energy, and water consumption.

This study provides key information to help design an extraction process that fits the requirements of the intended application.

2.2. Optimization of drying process

Effect of drying technology in Saccharomyces cerevisiae mannans: structural, physicochemical, and functional properties

Abstract: Mannans are polysaccharides whose physicochemical and biological properties render them commercialization in several products. Since these properties are strongly dependent on production conditions, the present study aims to assess the impact of different drying technologies – freeze (FDM) and spray drying (SDM) – on the structural, physicochemical, and biological properties of mannans from *Saccharomyces cerevisiae.* Structural analysis was assessed by ATR-FT-IR, PXRD and SEM, whereas physicochemical properties were evaluated based on sugars, protein, ash and water contents, solubility, and molecular weight distribution. Thermal behaviour was analysed by DSC, and antioxidant activity by DPPH and ABTS assays. The parameters which revealed major differences, in terms of structural and physicochemical properties regarded morphology (SEM), physical appearance (colour), moisture ($3.6 \pm 0.1\%$ and $11.9 \pm 0.6\%$ for FDM and SDM, respectively) and solubility (1 mg/mL for FDM and 25 mg/mL for SDM). Nevertheless, these differences were not translated into the antioxidant capacity.

Keywords: Yeast cell wall, Spray drying, Freeze drying, Structural characterization, DPPH, ABTS

2.2.1. Introduction

Polysaccharides are a very important class of biopolymers structurally characterized by the nature of monomer units, chain length, glycosidic linkages, and degree of branching.

Plant-derived polysaccharides have been extensively studied due to their numerous physiological benefits such as antioxidant, immunomodulatory, antitumor, anti-inflammatory, antidiabetic, and antiviral properties (Chen et al., 2018).

Mannans, long-chain carbohydrates mainly composed of mannose units, can be obtained from vegetables, microorganisms and seeds (Tester & Al-Ghazzewi, 2013). One important source is the cell wall of yeasts, particularly the baker's and brewer's yeast *S. cerevisiae* (Orlean, 2012). Data from 2017 concerning the annual world production of beer pointed out to values near 193 billion litres; this production generated 46.26 billion tons of by-products, among which spent yeast corresponds to ca. 12.7% therefore, 1.5 – 3 kg of spent yeast are generated for each 100 litres of beer produced (*Brewers By-Products Market Outlook, Share, Size, Forecast, Trends, Report*, 2020). The recovery and reuse of yeast is an excellent example of circular economy, as this by-product may be used to extract functional compounds and develop new innovative products, whereas waste management and environmental impacts are improved (Rakowska et al., 2017).

Mannans present several physicochemical properties, which render them the property to be used as a hardening ingredient or emulsion stabilizer (Singh et al., 2018). They are also claimed to be used as antibiotic replacers in animal feed (Smith et al., 2020), limiting gastrointestinal infections. Additionally, the ability to scavenge radicals such as superoxide anions and hydroxyl radicals provides a potential antioxidant effect (Liu et al., 2018). All these properties reveal the potential applications of mannans in the areas of food, feed, and cosmetics.

Mannan’s extraction from spent yeast usually includes several steps such as cell lysis, fractionation, and purification; in addition to the extraction methods, drying steps are also included, as they increase the shelf life of mannans extracts. Drying technologies are a critical step as they can affect the structural, physicochemical, and biological properties of the final product. Among the drying technologies, freeze drying (FD) and spray drying (SD) stand up as the most usually employed. SD was used at industrial scale since the 1920’s (Serra Costa et al., 2015). It presents the advantage of including only one processing step as opposite to FD (which requires a previous freezing of the extract), with yields ranging 50 –

70% and a final extract with homogeneity in terms of size and morphology (Emami et al., 2018). The main drawback pointed out to this technology is that it can cause heat stress on extract's constituents, thus promoting undesirable physicochemical and/or functional changes. On the other hand, FD processing increases the stability of the extracts and their resistance to microorganism propagation (Shukla, 2011). However, this technology requires expensive equipment, being a time-consuming method and, as above-mentioned, requiring additional processing (sample pre-freezing).

Although the processes pertaining to mannans extraction from yeast wall cells have been extensively reported in literature, the effect of drying step in the obtained extracts is usually poorly documented. Thus, the goal of the present work was to characterize the structural, physicochemical, and biological properties of mannans extracted from spent yeast *S. cerevisiae* and dried by FD or SD technologies. Additionally, those properties were compared to the ones exhibited by commercial pure mannans and duly discussed.

2.2.2. Materials and methods

2.2.2.1. Materials

Genetically modified spent yeast (*Saccharomyces cerevisiae*) was kindly provided by Amyris Inc. (Emeryville, CA, USA), and commercial *S. cerevisiae* mannan (M7504) was purchased from Sigma-Aldrich (USA). Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, ethanol, methanol, and ascorbic acid (> 99%) were also purchased from Sigma-Aldrich. DPPH (2,2-Diphenyl-1-picrylhydrazyl) was purchased from Thermo-Fisher Scientific (USA). All reagents were used as received without further purification.

2.2.2.2. Isolation and purification of mannans

Preparation of spent yeast

Spent yeast was centrifuged at 12 000 x *g* for 10 min (ThermoFisher Scientific, Massachusetts, USA), and the supernatant was removed. The pellet was washed twice with deionized water and centrifuged using the above-mentioned conditions.

Thermal hydrolysis

Subsequentially, pellet was resuspended in deionized water (10% w/v) and the pH was adjusted to 7.0. This solution was then heated to 120 °C in autoclave (Prohs, Porto, Portugal) for 3 h. After cooling down, the solution was centrifuged (4700 x *g*, 10 min) and the supernatant (rich in mannoproteins) was collected, precipitated with twice its volume with cold ethanol, and left overnight at 4 °C. The solid was collected by centrifugation and dried by freeze drying for approximately 72 h (Alpha 2-4 LSCplus, Germany) or spray drying (Mini Spray Dryer B-290, Buchi, Switzerland) with the condition's inlet temperature 110 ºC, aspirator 65%, pump 13%. The complete process is depicted in **Figure 14.**

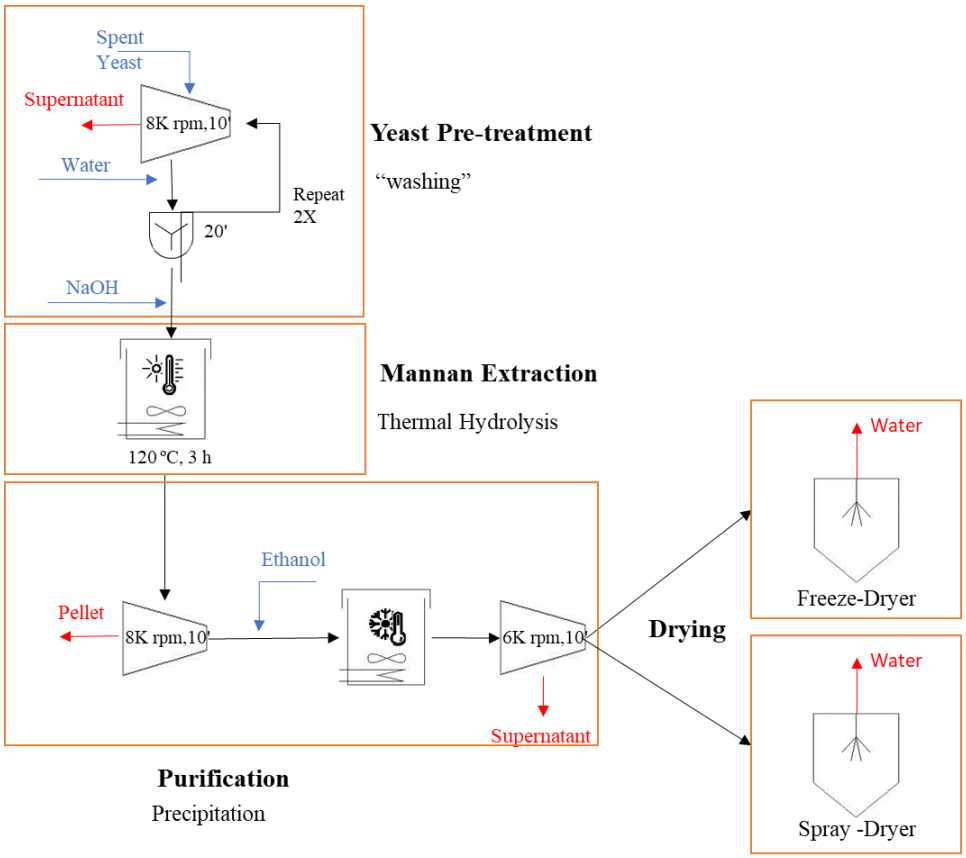


Figure 14. Extraction process of mannans extracts from spent yeast *S. cerevisiae*.

2.2.2.3. Structural characterization

ATR-FT-IR

The Attenuated Total Reflection Fourier-transform infrared spectroscopy (ATR-FT-IR) was determined using the Frontier™ MIR/FIR spectrometer as described in **section 2.1.2.3. of Chapter 2.1**.

PXRD

Powder X-Ray Diffraction Analysis (PXRD) was performed on Rigaku MiniFlex 600 diffractometer according to as described in **section 2.1.2.3. of Chapter 2.1**.

SEM

The morphology of all samples was evaluated by Scanning Electron Microscopy (SEM) on JSM-5600 LV Scanning Electron Microscope from JEOL, Japan. Prior to analysis, the mannans powdered samples were placed into observation stubs (covered with double-sided adhesive carbon tape (NEM tape, Nisshin, Japan) and coated with Aug/Pd using a Sputter Coater (Polaron, Bad Schwalbach, Germany). All observations were performed in the high-vacuum mode with an acceleration voltage of 20 kV, at a working distance of 9-10 mm and a spot-size of 27. The images presented are representative images of the morphology of each sample.

2.2.2.4. Physicochemical properties

Colour

The colour point was determined using a portable CR-410 Chroma meter according was described in **section 2.1.2.4. of Chapter 2.1**.

Bulk and tapped densities

The bulking properties and the tapped density of the powder mannan extracts was determined according to the European Pharmacopeia 7.0 (2010). Briefly, the bulk densities were determined using 10 mL graduated cylinders, where mannans' powders were gently introduced, without compacting, until the graduation of 10 mL. The bulk density of each

sample was then obtained using the formula m/V0 (m – sample mass in grams and V0 – unsettled apparent volume in mL). After observing the initial powder volume (V0), the cylinders in the support were submitted to 20 continuous taps (no difference observed with taps increment) and the tapped density was obtained using the formula m/Vf (m – mass of each powder sample and Vf – final tapped volume in mL). All analyses were performed in triplicate. The Compressibility index and the Hausner Ratio are considered simple methods of predicting powder flow characteristics and can be calculated according to Equations 2 and 3, respectively.

Compressibility Index = 100 x (tapped density – bulk density) / tapped density (2)

Hausner ratio= tapped density/ bulk density (3)

Total protein

Total protein content was assessed with the BCA Protein Assay Kit (Pierce, Bonn, Germany), used according to the manufacturer's instructions as was described in **section 2.1.2.4. of Chapter 2.1**.

Neutral sugars

Neutral sugars were derivatized and analysed by gas-chromatography-flame ionization detection (GC-FID) according was described in **section 2.1.2.4. of Chapter 2.1**.

Molecular weight distribution

Molecular weight distribution determination was carried out on Agilent 1260 Infinity II HPLC system equipped with Vialsampler, quaternary pump, thermostatic oven and Agilent 1290 Infinity II ELSD – Evaporative Light Scattering Detector. Data acquisition and analysis were performed as described in **section 2.1.2.4. of Chapter 2.1** With modifications in: flow rate of 0.6 mL/min under isocratic conditions and ELSD Nebulizer temperature was set at 60 ºC, evaporator temperature 80 ºC and N_2 flow rate was kept at 1.25 SLM.

Dry weight and ashes

To evaluate the moisture content according to Association of Official Analytical chemists (AOAC, 2005) was performed as described in **section 2.1.2.4. of Chapter 2.1.**

Solubility test

Tests were performed according to the European Pharmacopoeia (Pharmacopoeia, 2017) according to the protocol shown in **section 2.1.2.4. of Chapter 2.1.**

DSC

Differential scanning calorimetry (DSC) measurements were performed corresponding was described in **section 2.1.2.4. of Chapter 2.1.**

2.2.2.5. Antioxidant capacity

ABTS scavenging assay

ABTS scavenging assay was performed in 96-well microplates, according to Gonçalves et al., (2009) with some modifications. This method can quantify antioxidants via direct production of the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) chromophore (blue/green) by reaction of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and potassium persulphate ($K_2S_2O_8$) (Merck, Darmstadt, Germany), after incubation at room temperature in the dark for 16 h. The $ABTS^{\bullet+}$ working solution (freshly prepared) was filtered with a 0.45 µm syringe filter and diluted with solvent to an absorbance of 0.70 ± 0.02, at 734 nm. $ABTS^{\bullet+}$ (200 µL) was added to 15 µL of sample (at concentrations of 5 mg/mL for mannans extracts, 10 mg/mL for commercial mannan, and 0.05 mg/mL for ascorbic acid), Trolox (in the range 50 – 560 µM) or solvent (blank assay). The mixture was incubated for 5 min at room temperature, and the absorbance at 734 nm was measured with a plate reader (Synergy H1, USA) controlled by Gen5 Biotek software (version 3.04). Scavenging activity was expressed as % reduction in absorbance as compared with the control. Regression equations between ABTS scavenging and Trolox concentration were calculated, and the results expressed as mmol TE (Trolox equivalent)/100 g of sample. The $ABTS^{\bullet+}$ scavenging effect percentage

was determined according to Equation (4):

$$ABTS^{\bullet+}\text{scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (4)$$

where $A_{control}$ and A_{sample} are the absorbances at 734 nm of control and sample, respectively. The assay was performed in triplicate.

DPPH scavenging assay

The DPPH$^{\bullet}$ free-radical scavenging activity was measured according to the method described by Schaich et al., (2015), with modifications for a 96-well microplate scale. The stock solution (600µM) was prepared in methanol and stored in the dark at -20 ºC. As for ABTS, a daily DPPH solution was prepared by dilution with methanol to 0.600 ± 0.100 at 515 nm. For the assay, 25 µL of sample (5 mg/mL for mannans extracts, 10 mg/mL for commercial mannan and 0.03 mg/mL for ascorbic acid), Trolox (7.5 – 240 µM) or solvent were added to 175 µL DPPH daily solution. The mixture was incubated for 30 min at room temperature, and the absorbance at 515 nm was measured with a plate reader (Synergy H1, USA). The scavenging activity was expressed as % reduction in absorbance related to the control. Regression equations between DPPH scavenging and Trolox concentration were calculated, and the results expressed as mmol TE (Trolox equivalent)/100 g of sample.
The DPPH scavenging effect percentage was determined according to Equation (5):

$$\text{DPPH scavenging effect (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (5)$$

where $A_{control}$ and A_{sample} are the absorbances at 515 nm of control and sample, respectively. The assay was performed in triplicate.

2.2.2.6. Cytotoxicity evaluation

Cell line growth conditions

Caco-2 cells were obtained from the American Type Culture Collection and were grown using high glucose (4.5 g/L) DMEM supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) penicillin–streptomycin–fungizone (Lonza, Verviers, Belgium), and 1% (v/v) of non-essential amino acids 100× (Sigma, Germany). Cells were used between passages 53 and 54.

Cytotoxicity assay by Presto Blue

CaCo-2 viability assay with PB reagent was performed according to ISO 10993-5 (2009) was described in **section 2.1.2.5. of Chapter 2.1**.

2.2.2.7. Statistical analysis

Data are expressed as the mean plus standard deviation of replicates. To analyse the differences between the different FDM, SDM and CM when a normal distribution was observed (Shapiro-Wilke normality tests), the one-way ANOVA test were carried out in association with Tukey's Multiple Comparison Test with a 95% confidence interval. All tests were realized with a significance level of 5% and using the Statistical Package for Social Sciences software (version 21, SPSS, Chicago, IL, USA).

2.2.3. Results and discussion

To extract functional compounds and develop innovative products in a circular economy concept, the recovery and reuse of spent yeast, namely *S. cerevisiae*, is an excellent opportunity. In this work we used a thermal extraction process with high temperature and the combination of pressure. The high temperatures used have the additional advantage of decontaminating a possible microbial presence.

In addition to the extraction methods, drying technologies are an important step to the final process since they can affect the structural, physicochemical, and biological properties of final products. The objective of the present work was to perform an integrated study on the structural, physiochemical, and biological (in terms of antioxidant capacity) characteristics of the freeze dry mannans extract (FDM) and spray dry mannans extract (SDM) obtained from spent *S. cerevisiae* of Amyris Company, as compared with commercial mannans.

2.2.3.1. Structural characterization

The structural analysis of the mannans extracts from *S. cerevisiae* obtained by different drying methods – freeze drying (FDM) and spray drying (SDM) – was attained by the following set of solid-state techniques: ATR-FT-IR, PXRD and SEM. The functional groups, crystallinity indexes and the morphological analyses of both samples were compared

with the mannan commercial sample, Mannan M7504 from Sigma (CM), as highlighted in **Figure 15**.

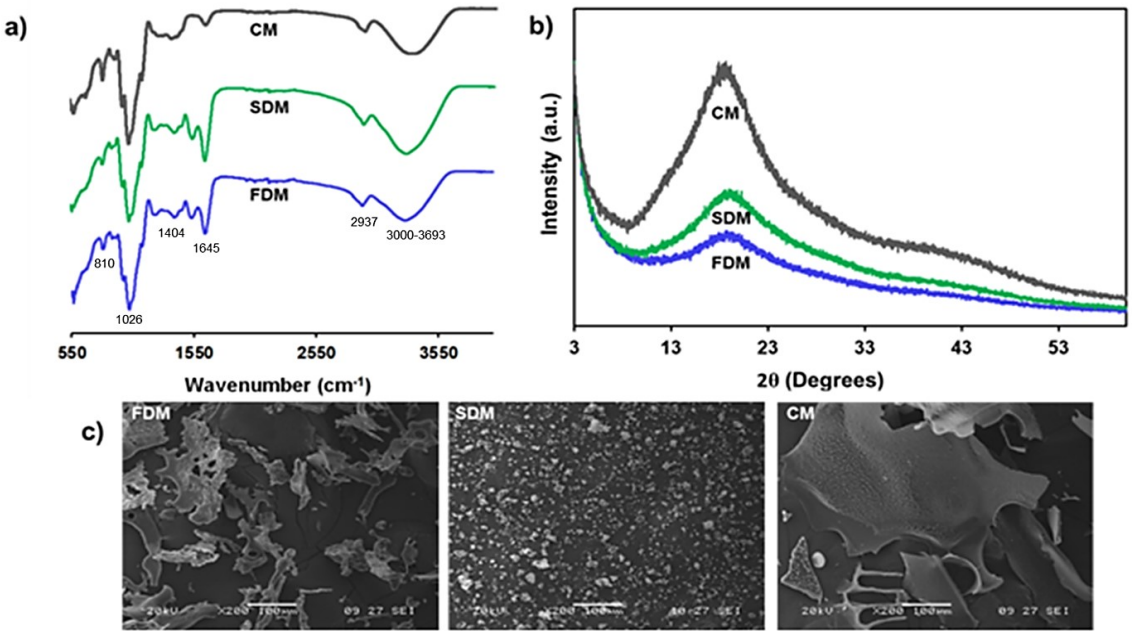


Figure 15. Structural characterization of the freeze (FDM) and spray dried (SDM) mannans extracts, and the commercial mannan (CM) by a) Attenuated Total Reflection Fourier- Transform Infrared Spectroscopy; b) Powder X-Ray Diffraction analysis and c) Scanning electron microscopy.

The normalized ATR-FT-IR spectra of the mannans extracts from *S. cerevisiae* (FDM and SDM) and the mannan benchmark (CM) are depicted in **Figure 15a**. All spectra exhibit a strong and broad vibration band at 3000–3693 cm^{-1} correspondent to the stretching of the hydroxyl groups (Liu & Huang, 2018). The weak absorption band at *ca.* 2937 cm^{-1} is attributed to the C–H stretching vibration, and the band at 1404 cm^{-1} corresponds to the C–H bending vibration. The sharp band at 1645 cm^{-1} is assigned to the C–O asymmetric stretching vibration. The absorption peak at 1026 cm^{-1} corresponds to the O–H variable angle vibrations and the characteristic absorption of the mannan α-chain appeared at 810 cm^{-1} (Liu & Huang, 2018).

In order to study the crystallinity differences resulting from the drying processes, both yeast mannans extracts (FDM and SDM) were analysed by PXRD. The crystallinity of the CM was also evaluated, since the crystallinity may affect physicochemical/biological properties, namely the solubility, being expected the decrease of solubility with an increase of crystallinity (Guo et al., 2017). All PXRD patterns highlighted in **Figure 15b** exhibits a single broad reflection centered at 2θ = 18.8º. Besides the predominant amorphous pattern

of all samples, the crystallinity index (CI in %) was calculated using the PDXL2 da Rigaku® (version 2.8.1.1) software. Briefly, the baseline and the diffractogram profile were properly adjusted for each analysis, followed by the modelling of the amorphous phase. The CI (%) is then obtained by the ratio of the crystalline area to the total area. The mannan benchmark exhibited the highest crystallinity (33.8% ± 1.0), followed by the spray dried mannans extract SDM (26.4% ± 0.9), and the freeze-dried product FDM (23.7% ± 0.8).

The different drying methods clearly affected the morphology and size of the products, as represented in **Figure 15c**. The morphology of the mannan benchmark was also studied, since morphology/size may affect physicochemical properties, such as wettability and solubility (Padma Ishwarya & Anandharamakrishnan, 2015). The spray dried product SDM exhibit a round shape with a smaller and more uniform size than the freeze-dried product FDM, or even the CM. The FDM is irregular in shape and exhibits larger particles than the SDM sample, while the CM sample is characterized by large plates along with smaller grains. These observations are in agreement with the morphology described in the literature for the spray (Emami et al., 2018) and freeze drying (Liapis & Bruttini, 2020) methods.

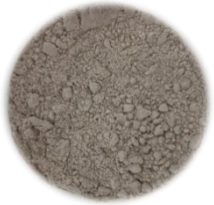
2.2.3.2. Physicochemical characterization

The integrated and comparative analysis of the physical appearance and powder flow properties of all mannans products (FDM, SDM and CM), along with their physicochemical properties and thermal analysis, is presented in this section.

Physical appearance

The powders' physical appearance is clearly different, as depicted in **Table 9**. While the SDM appeared as a homogeneous fine powder, the FDM product presents granules that difficult the maceration process after drying. The mannan benchmark CM appeared as little white sheets, while both yeast mannans extracts exhibit a brown colour. The colour of products constitutes a key criterion for their commercialization, since the colours accepted by the consumers are very scarce. The colour point of all mannans products was measured using the system CIELAB (L*, a*, b*) (Ordóñez-Santos et al., 2017). The benchmark product CM exhibits the highest L* value (90.08 ± 0.03), followed by the yeast mannans products SDM (83.67 ± 0.01) and FDM (75.15 ± 0.21), respectively.

Table 9. Results of physical appearance, colour characteristics, and powder flow properties of the FDM and SDM mannans extracts and the CM.

	Freeze Dry Mannans Extract (FDM)			**Spray Dry Mannans Extract (SDM)**			**Mannan M7054 from Sigma (CM)**		
Physical appearance									
Colour Characteristics	L*	a*	b*	L*	a*	b*	L*	a*	b*
	75.15±0.21	1.49±0.05	9.54± 0.09	83.67±0.01	1.54±0.00	7.12±0.04	90.08±0.03	-0.23 ±0.01	7.73±0.01
Bulk Density (g/ml)	0.09 ± 0.00 [a]			0.09 ± 0.01 [a]			0.03 ± 0.00 [b]		
Tapped Density (g/ml)	0.16 ± 0.01 [a]			0.21 ± 0.01 [a]			0.08 ± 0.01 [b]		
Hausner Ratio	1.89 ± 0.04 [b]			2.29 ± 0.03[a]			2.78 ± 0.08 [c]		
Compressibility (%)	47.00 ± 1.00 [b]			56.33 ± 0.58 [a]			64.00 ± 1.00 [c]		

[a,b,c] means within the same line, marked with the same letter, do not differ from each other ($p > 0.05$).

The total colour difference (ΔE*) was determined to allow the quantification of the colour change between the yeast mannans products and the mannan commercial product, and even between both *S. cerevisiae* mannans extracts.

The ΔE* (1.50 < ΔE* > 3.0) between the SDM and FDM samples corresponds to 8.86, while the ΔE* between the FDM or SDM samples and the benchmark CM was 15.13 and 6.68, respectively. Since the determined total colour difference were higher than 3.0, this corroborates the distinct visual perception among all mannans samples (Bellary et al., 2016).

The flow properties of all powder samples were studied using the compressibility index and the closely related Hausner ratio, calculated from the bulk and tapped densities using Equations 2 and 3, respectively. The different sizes and shapes of the mannans extracts FDM and SDM do not contribute to significant differences in the observed bulk (0.09 ± 0.01 g/mL) or tapped densities (0.21 ± 0.01 g/mL). The mannan benchmark CM revealed the lowest tapped and bulk densities of 0.03 ± 0.00 g/mL and 0.08 ± 0.01 g/mL, respectively. According to the European Pharmacopeia 7.0 (2010), all powder mannans samples exhibit a flow character of very, very poor, according to the generally accepted scale of flowability (Compressibility index > 38 and Hausner ratio > 1.60). The FDM extract has a larger particle size (**Figure 15**) and consequently presents an increase in interparticle void spaces with smaller contact surface areas per unit volume, that may have led to it having an apparently lower density (Caparino et al., 2012) than the SDM sample.

Physicochemical properties

The physicochemical analyses performed for the mannans extracts from *S. cerevisiae* (FDM and SDM) and for the mannan benchmark CM are summarized in **Table 10**. All mannans products were evaluated regarding to their composition – protein and total sugars (mannose and glucose); molecular weight by HP-SEC; moisture; ash content and solubility.

Both mannans extracts revealed to have a similar composition. The different drying processes revealed not to have a significant impact on the mannans content, since both extracts exhibit a mannans content of 42.7 ± 6.6% and 42.0 ± 0.0% for the FDM and SDM samples, respectively. The analyses of the FDM extract present higher standard deviations, which could be indicative of the higher heterogeneity of the powder. The CM sample

revealed the highest purity, since has the lowest protein content (0.5 ± 0.0%), no-glucose residue detected and a mannose content of 98.0 ± 0.0%.

The MW of samples usually has a direct relationship with their bioactivity (Liu et al., 2021). According to Hashim et al., (2018), mannans extracts with a MW of 12.9 kDa and 50% (w/w) of sugar demonstrated to stimulate immunity in broiler chicken. Galinari et al., (2018) extracted mannans from *K. marxianus* and obtained 5 different fractions with MW ranging from 10 – 203 kDa, all exhibiting antioxidant activity. **Table 10** shows the MW fractions present in both mannan extracts and CM. As observed, there are no significant differences between FDM and SDM (**Supplementary material Figure S 1**); on the other side, CM only exhibits one single population of 34 – 62.5 kDa, as expected due to its high purity.

According to the results obtained in **Table 10**, a significant difference found between FDM and SDM concerned the moisture content, although for the ash content this difference was not significant. It was also noted that SDM had a higher moisture content when compared with FDM and CM. For the inorganic matter, CM demonstrated a smaller quantity of matter in comparison to FDM and SDM, which may indicate that CM had a higher purity. A solution to achieve a more homogenous content between samples could be increase the purification process (e.g., through protein removal).

The solubility of polysaccharides plays a key role in their applications, since most of their functions are achieved in an aqueous medium, namely, emulsion capacity, drug delivery, among others. The FDM exhibited the lowest solubility limit (1 mg/mL), followed by the mannan benchmark CM (10 mg/mL) and the SDM sample (25 mg/mL). The morphological analyses showed in **Figure 15** corroborated the easiest solubility of the SDM product, since the lowest size may increase the surface area, and consequently the rate of solubility (Anandharamakrishnan et al., 2010). Besides the morphology, it was possible to verify that the crystallinity indexes of the different samples do not have a significant impact on the solubility. In fact, the FDM sample, which exhibits the highest amorphous nature (CI of 23.7%), was revealed to have the hardest sample to dissolve and not the easiest soluble character – compounds with higher amorphous content must exhibit higher solubility. Regarding to the sample composition, summarized in **Table 10**, it was possible to infer that the CM benchmark, exhibiting the lowest molecular weight of 69 kDa, was not the easiest product to solubilize, contrasting the tendency of polysaccharides solubility based on the promotion of solubility for low molecular weight molecules (Guo et al., 2017).

Table 10. Results of physicochemical characterization of same extract of mannans dried by different methods freeze drying (FDM) and spray drying (SDM) and compare with commercial mannan (CM).

	Freeze Dry Mannans Extract (FDM)				**Spray Dry Mannans Extract (SDM)**				**Mannan M7054 from Sigma (CM)**
Protein Content (% w/w)	23.4 ± 1.5 [a]				21.1 ± 1.4 [a]				0.52 ± 0.04 [b]
Total Sugars (% w/w) *	46.9 ± 6.1 [a]				44.9 ± 1.2 [a]				98.0 ± 0.0 [b]
Mannose (% w/w)	42.7 ± 6.6 [a]				42.0 ± 0.0 [a]				97.0 ± 0.0 [b]
Glucose (% w/w)	3.0 ± 0.8 [a]				2.0 ± 0.1 [a]				Not detected
Molecular Weight (kDa)	244 ± 3 [a]	119 ± 3 [b]	23 ± 0[a]	10 ± 0[a]	241 ±6[a]	105 ± 4[a]	24 ± 2[a]	10 ± 0[a]	34 - 62.5
Area %	79	17	2	3	81	16	1	2	100
Moisture (% w/w)	3.6 ± 0.1 [b]				11.9 ± 0.6 [a]				8.1 ± 0.4 [c]
Ash (% w/w)	8.0 ± 0.5 [a]				8.0 ± 0.5 [a]				0.4 ± 0.1 [b]
Solubility	Very slightly soluble (approx. 1 mg/mL)				Sparingly soluble (approx. 25 mg/ml)				Sparingly soluble (approx. 10 mg/ml)

*Total sugars are the sum of the glucose content (% w/w) plus the mannose content (% w/w). [a,b,c] means within the same line, marked with the same letter, do not differ from each other ($p > 0.05$). ** information provided by Sigma Aldrich.

Thermal analysis

The thermal properties of the mannans extract from *S. cerevisiae* dried by freeze (FDM) or spray drying (SDM), and the mannan benchmark (CM) were studied by differential scanning calorimetry (DSC), as depicted in **Figure 16**.

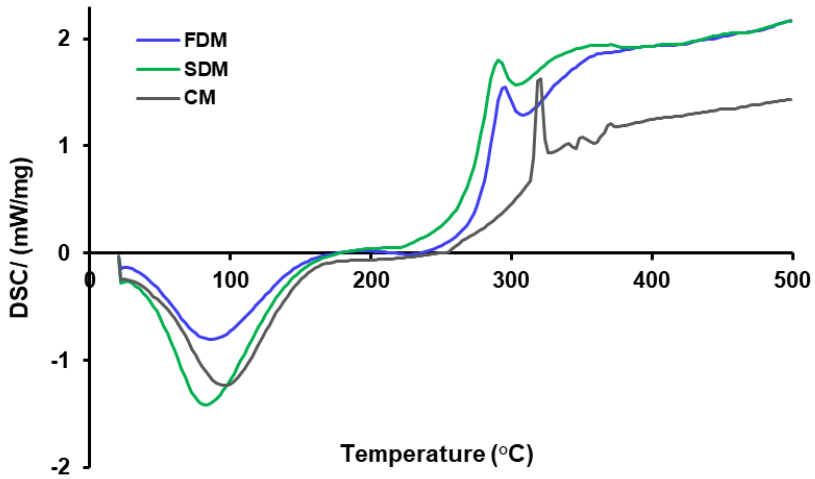


Figure 16. DSC thermograms of the freeze (FDM) and spray drying (SDM) mannan extracts, and the commercial mannan (CM) obtained at a heating rate of 10 °C/min under nitrogen atmosphere.

The DSC thermograms of the FDM and the SDM products exhibit a broad endothermic peak centered at 87.43 and 84.94 °C, respectively, which can be attributed to the evaporation of the residual water (Harish Prashanth, 2002). This endothermic event was also observed for the benchmark sample (CM) – peak centered at 99.89 °C. An exothermic event is observed at around 290 °C in the extracts FDM and SDM, which is also detected for CM, but in the latter, the event takes place at approximately 320 °C; this event also can be related with polysaccharide thermal decomposition. The FDM and SDM products are a mixture of different compounds, so it is likely to observe the contribution of the events of each single component. One possibility to explain the position of the exothermic peak at around 300 °C was typical for chitosan, a deacetylated derivative of chitin (Roca et al., 2012), spent yeast in the cell wall content 1 – 2% of chitin and chitin bonded to β-(1-3)-D-glucan, (Orlean, 2012), consequently, some residues of chitin bonded β-glucan may be present in the mannan extract after the extraction of the spent yeast. The presence of the β-glucan on the mannan extract is also possible, since as shown above in **Table 10**, there is a percentage of glucose in the extract which can be associated with the presence of β- glucan residues (Novák et al., 2012).

According to Ospina Álvarez et al., (2014) events close to or above 300 °C are typically observed during degradation of the saccharide structure and may be involved in the dehydration of saccharide rings. Even with the events not explained at molecular level, the DSC analysis allowed to infer about the close thermal stability of the mannans samples (FDM and SDM), and the mannan commercial sample (CM).

2.2.3.3. Cytotoxicity

Cytotoxicity of samples was assayed against CaCo-2 cells, by evaluating their impact upon cell metabolism using a viable dye. According to ISO 10993-5 (2009), a sample is cytotoxic when a metabolic inhibition percentage above 30% is observed. Both FDM (-31.8 ± 3.9%) and SDM (-67.3 ± 12.4%) exhibited no cytotoxicity up to 10 mg/mL (the highest concentration tested) with no metabolic inhibitions being observed. In fact, the negative values illustrate an increase in cell metabolism observed when in the presence of SDM and FDM. This indicates that the differences in drying process, while resulting in alterations in microstructure, do not negatively impact their interactions with CaCo-2 cells. Moreover, the highest metabolic stimuli of SDM could be a result of its better solubility. This possibility is substantiated by the fact that its metabolic inhibition percentages fall within the range of those observed for the benchmark CM (-79.4 ± 14.8%), which has similar solubility.

2.2.3.4. Antioxidant capacity

Polysaccharides from various sources have been reported to present strong antioxidant capacities (Machová & Bystrický, 2013), turning their incorporation in food products an interesting way to prevent oxidative damage in human body. However, processing conditions during their extraction, purification and drying may lead to structural/physicochemical modifications, thus interfering with their bioactivities. Therefore, the impact of the drying process on mannan extracts antioxidant capacity was assessed. Antioxidants play their beneficial role by deactivating reactive species through the transference of a hydrogen atom or an electron to those species. Therefore, the two mechanisms used to assess antioxidant capacity of compounds are Hydrogen Atom Transfer (HAT) and Electron Transfer (ET). ABTS (also known as Trolox equivalent antioxidant capacity - TEAC assay) and DPPH are mixed-mode assays, as both HAT and ET mechanisms occur (with H atom donors and reducing agents, respectively) depending on the

corresponding reaction conditions (such as pH and solvent) (Munteanu & Apetrei, 2021). Antioxidant ability was measured in the test compounds by ABTS and DPPH assays and compared with Ascorbic acid (vitamin C), as standard.

Table 11. $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity expressed as % scavenging activity and concentration mmol of Trolox/100 g in freeze drying (FDM) and spray drying (SDM) mannans extracts, commercial mannan (CM) and Ascorbic acid (Positive control).

	% Scavenging activity		**Concentration mmol of Trolox/100 g**	
	ABTS	DPPH	ABTS	DPPH
FDM	67 ± 0.77 [a]	13 ± 2.0 [a]	9.9 ± 0.11[a]	0.70 ± 0.15 [b]
SDM	66 ± 1.1[a]	19 ± 0.59 [a]	9.3 ± 0.15 [a]	2.2 ± 0.01[a]
CM	2.0 ± 1.0 [c]	8 ± 0.05 [c]	0.08 ± 0.17 [c]	0.30 ± 0.05 [d]
Ascorbic Acid	94 ± 0.16 [b]	92 ± 0.07 [b]	463 ± 25 [b]	424 ± 17 [c]

[a,b] and [c] means within the same column, labelled different subscripts, differ significantly ($p < 0.05$)

From **Table 11** and concerning ABTS, it can be observed that the use of distinct drying processes didn't promote significant variations in the antioxidant capacity of the extracts. Ascorbic acid presented a significantly higher radical scavenging capacity than SDM and FDM extracts, and the CM exhibited the lowest values. Regarding DPPH assay, the same trend is observed, although the difference in the radical scavenging activity was statistically significant within the two drying processes (13% for SDM and 19% for FDM, at a concentration of 5 mg/mL). Machová & Bystrický, (2013) also reported lower DPPH assay results for *S. cerevisiae* mannans than for ascorbic acid (2.9 – 6.5% for *S. cerevisiae* mannans, and 62-100% for ascorbic acid, for concentrations of 0.125 – 1 mg/mL, respectively). Although the relative scavenging inhibition percentage of our mannan extracts (i.e. normalized for an equivalent concentration of extract) was lower in our study, it has to be kept in mind that the results from Machová & Bystrický, (2013) were assessed in mannans prepared as mannan-cooper complexes, which may interfere with the determination.

The large difference found between the commercial mannan and the extracts in both antioxidant assays may be tentatively explained by their MW profile, as bioactivity is usually

inversely related with molecular size: whereas CM presents only one fraction of 34 – 62.5 kDa, SDM and FDM extracts present several fractions with MW ranging from 244 to 10 kDa. Nevertheless, this assumption must be validated with further studies. Regarding the difference found between SDM and FDM in DPPH assay, it may be tentatively explained by the differences found within the structural and/or physicochemical properties studied (namely differences in morphology, moisture, or solubility). Alternatively, we wonder if this difference could be due to the intrinsic characteristics of the assays, such as the reactant solvents; although we did not measure extracts solubility in methanol (solvent mixture used in DPPH assay), maybe a poor solubility of FDM could explain its lower performance in this assay.

2.2.4. Conclusions

The present study reports the effect of different drying technologies in the structural, physicochemical, and biological characteristics of mannans obtained from spent yeast *S. cerevisiae*. From the parameters evaluated it can be concluded that the structural analysis in FT-IR and PXRD showed no significant variations between the freeze drying and spray drying methods. However, these drying technologies clearly affected the morphology and size, apart from physical appearance in terms of colour (as measured by colour difference). Furthermore, differences in moisture content and solubility were also observed. These differences may dictate the choice of the drying process for mannans extracts, depending on their intended final application.

Supplementary material

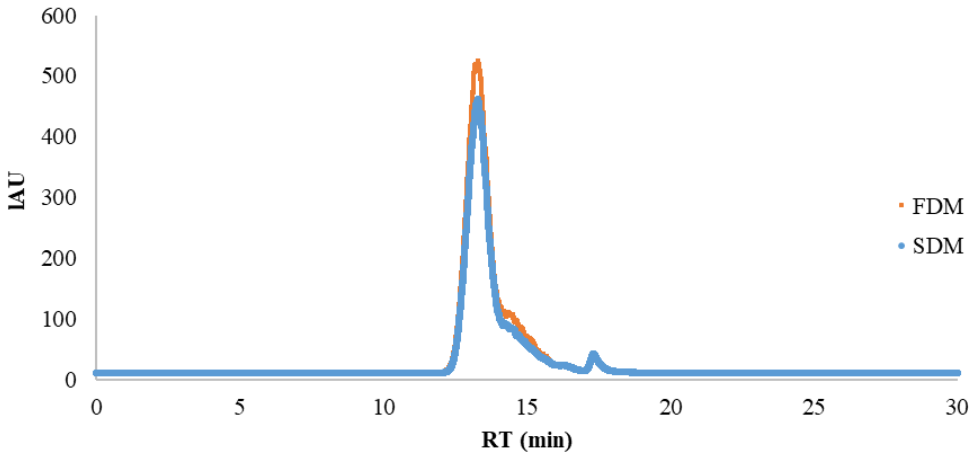


Figure S 1. Overlay of molecular weight distribution chromatograms of SDM and FDM extracts evaluated by HPGPC.

Chapter 3

Production of MOS extract from spent yeast mannans

This chapter aims to develop a sustainable and viable approach for producing MOS from the mannans extract, followed by the characterization of the functional and chemical properties of the MOS extracts.

Chapter 3.1 *Production of mannan oligosaccharides*

Adapted from the following submitted scientific publication:

Faustino, Margarida; Durão, Joana; Pereira, Carla F.; Pereira, Ana Margarida; Oliveira, Ana Sofia; Sousa, Sérgio; Braga Ribeiro, Alessandra; Pereira, Joana Odila; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Unveiling the potential of yeast Mannan oligosaccharides (MOS): production, characterization, and bioavailability studies. Submitted to Food Research International.

3.1. Production of mannan oligosaccharides (MOS)

Unveiling the potential of yeast Mannan oligosaccharides (MOS): production, characterization, and bioavailability studies

Abstract: Mannan oligosaccharides (MOS) are widely used in the animal industry as feed additives, with documented effects on the modulation of gut microbiome. Its numerous health benefits are also encouraging its application in the nutraceutical and functional food industries. While numerous studies portraying MOS production from β-Mannans can be found in the literature, its production from yeast mannans is barely explored. Herein, two approaches for the production of yeast-derived MOS are presented. Process efficiency was evaluated in terms of yield, and the resulting extracts were characterized through structural (ATR-FT-IR and PXRD), morphological (SEM), physicochemical (colour and molecular weight distribution), compositional (sugars and protein content), thermal (DSC) analysis and biological (immunomodulatory effect in CaCo-2 cells The extracts were submitted to a simulated gastrointestinal tract, and the resulting digested extracts were evaluated for cytotoxicity and immunomodulatory effect. Furthermore, a cell model consisting of a co-culture of CaCo-2 and HT-29-MTX cells was used to study the extracts *in vitro* intestinal absorption. The hydrothermal process resulted in the highest yield, whereas acidic reaction resulted in lower molecular weight populations. Expression levels of IL-6 and IL-8 by CaCo-2 cells stimulated by IL-1β indicated that MOS extracts do not hold anti-inflammatory activity but grant an immunostimulant effect. This work provides valuable contributions towards the production of yeast-derived MOS extracts, highlighting their potential for different applications in various fields.

Keywords: Mannan oligosaccharides, Hydrothermal reaction, Acidic extraction, Immunomodulation, GIT, Co-culture

3.1.1. Introduction

Mannans are long-chain carbohydrates that may be derived from plants and microorganisms, as the yeast cell wall of *Saccharomyces cerevisiae*. Regarding the latter source, mannans are mainly composed of mannose, structurally organized in α-(1-6) linked backbone, and α-(1-2) and α-(1-3) linked branches (Ballou, 1974; Peat, Whelan, et al., 1961). The large macromolecules are linked to asparagine via N-acetyl-glucosamine (Ballou, 1974; Sentandreu & Northcote, 1968), and oligosaccharides are linked to serine or threonine residues (Ballou, 1976).

Mannan oligosaccharides (MOS) are, by definition and as its name suggests, oligosaccharides composed essentially of mannose, essentially obtained by chemical, physical or enzymatic cleavage of mannans polysaccharides (Gibson et al., 2017; Kango et al., 2022; Teng & Kim, 2018).

MOS mainly serve as prebiotics for beneficial bacteria in the intestine, but have also shown some promising biological effects such as the improvement of the intestinal mucous layer protection, immunity, anti-mutagenic defences, and antioxidant activity (Teng & Kim, 2018). MOS are widely used in the animal industry with the aim of selectively stimulate the growth or metabolic activity of beneficial microorganisms, suppressing enteric pathogens (*Salmonella* sp., *E. coli*, *Campylobacter* sp., and others) and improving the integrity of the intestinal mucosa (Baurhoo et al., 2007; Ghasemian & Jahanian, 2016; Jana et al., 2021; Spring et al., 2000, 2015).

Interestingly, despite the fact that the products used in the above-mentioned studies are defined as MOS, they are indeed a mix of yeast cell wall products containing high levels of protein and glucose and presenting high molecular weights (data not shown). In fact, oligosaccharides are, by definition, carbohydrates that have a low degree of polymerization (DP) and thus a low molecular weight (Roberfroid & Slavin, 2000; Yun, 1996), being classified as having 2 to 20 monosaccharide units (Roberfroid & Slavin, 2000). While the IUB-IUPAC (1983) terminology specifies 10 monosaccharide units as the dividing point between oligo and polysaccharides, there is no physiological or molecular basis for this distinction (Cummings et al., 1997). Therefore, extracts commercially available as MOS do not strictly fit within MOS definition, which makes their relationship between composition and structure difficult to study.

Most of the reports to obtain MOS from the yeast cell wall refer chemical (Lee & Ballou, 1965; Nakajima et al., 1974; Ogawa et al., 1994; Peat, Turvey, et al., 1961; Stewart et al., 1968; Young et al., 1998) and enzymatic methodologies (Kommineni et al., 2019; Maruyama et al., 1994; Wong-Madden & Landry, 1995; Zordan et al., 2015). However, these approaches have been reported essentially in the context of structural elucidation studies, such as acetolysis (Lee & Ballou, 1965; Stewart et al., 1968; Young et al., 1998), acid or alkaline hydrolysis, associated or not with high temperatures (Nakajima et al., 1974; Ogawa et al., 1994; Peat, Whelan, et al., 1961).

Acetolysis reaction has the ability to preferentially cleave the backbone α-(1-6) linkages between mannose units, producing the relatively stable α-(1-2) and α-(1-3) linked oligosaccharides (Lee & Ballou, 1965). A study by Ballou (1974) regarding the degradation rates of various disaccharides from yeast mannan revealed that the relative rates of acetolysis of the mannose disaccharides at the α-(1-2), α-(1-3), and α-(1-6) are of 1, 13, and 280, while those of the corresponding glycosides are 1, 5, and 90, respectively. The relative rates for the same three linkages in the β-linked glucose series are 1, 0.8, and 25. Although the selectivity of acetolysis reaction makes it a very interesting approach to obtain smaller molecular weight products, the toxicity of some of the chemicals employed (e.g. pyridine and sodium methoxide) (Kocourek & Ballou, 1969) undermine its industrial use.

The acid hydrolysis can cleave mannans preferentially in the side chains, given that the α-(1-6)-glycosidic bonds have been described to be more stable to acid hydrolysis than the α-(1-2)-glycosidic bonds (Peat, Whelan, et al., 1961). Mild alkaline conditions have been reported by Sentandreu & Northcote (1968) to promote β-elimination reaction. This reaction originates oligosaccharides derived from O-glycosidic linkage to serine and threonine, releasing mannose, mannobiose, mannotriose, and mannotetraose (Ballou, 1976; Nakajima et al., 1974).

This study aimed to compare the production of MOS-rich extracts from yeast mannans by two different processes, both able to generate extracts suitable to be ingested. MOS-rich 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). Physicochemical properties such as molecular weight distribution (MWD) and mannose and protein content were also evaluated. Additionally, and aiming to take advantage on their bioactive properties as nutritional supplements, MOS-rich extracts underwent a simulated digestion process in the human

gastrointestinal tract, according to the INFOGEST protocol (Brodkorb et al., 2019), with some modifications in intestinal absorption phase. The digested samples were assessed for their cytotoxicity and immunomodulatory activity through the determination of expression levels of the pro-inflammatory interleukins 6 (IL-6) and 8 (IL-8), using a human intestinal epithelial cell line (CaCo-2). Additionally, the intestinal permeability was simulated with an *in vitro* model of the human intestinal epithelium, using CaCo-2 and HT-29-MTX cells (Hoffmann et al., 2021) seeded in a cell-permeable membrane, and the amount of mannose transported through the membrane was quantified over time.

3.1.2. Materials and methods

3.1.2.1. Materials

Mannans extract was produced from genetically modified spent yeast (*S. cerevisiae*) kindly provided by Amyris Inc. (Emeryville, CA, USA), according to a methodology previously described by Faustino et al., (2022). Ohly-Go® MOS (used as benchmark for colour comparison) was purchased from Ohly GmbH. Recombinant human 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.

3.1.2.2. Production of MOS from mannans extract

MOS H_3PO_4 - acidic reaction 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 then centrifuged at 4700 x *g* and 4° C for 20 min. The pellet was collected, and the previous precipitation step was repeated two more times 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).

MOS Parr - hydrothermal reaction with 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 the **section MOS H_3PO_4 - Acidic Reaction with Phosphoric Acid**

3.1.2.3. Structural characterization

ATR-FT-IR

The ATR-FT-IR analysis was carried out using a PerkinElmer Frontier™ MIR/FIR spectrometer according was described in **section 2.1.2.3. of Chapter 2.1.**

PXRD

PXRD analyses were carried out using a Rigaku MiniFlex 600 diffractometer as reported **in section 2.1.2.3. of Chapter 2.1**.

SEM

Scanning Electron Microscopy using a Thermo Scientific™ Pro Scanning Electron Microscope was used to examine the morphology of the MOS extracts, the methodology used was according with the methodology presented in **section 2.1.2.3. of Chapter 2.1**, with the amplification of 1000x.

3.1.2.4. Physicochemical properties

Colour

The colour point was determined using a portable CR-410 Chroma meter according to reported in **section 2.1.2.4. of Chapter 2.1**.

Total protein

Total protein content was assessed with the BCA Protein Assay Kit (Pierce, Bonn, Germany) according was performed in **section 2.1.2.4. of Chapter 2.1**.

Neutral sugars

Neutral sugars were 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 what was described in Faustino et al., (2022).

Molecular weight distribution

The Agilent 1260 Infinity II HPLC system was used to determine the molecular weight 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 x 4.6 mm, 8 µm), and PL Aquagel-OH 20 (300 x 7.5 mm, 5 µm) columns were utilized. The methodology used was described in **section 2.1.2.4. of Chapter 2.1**.

DSC

DSC measurements were performed in a nitrogen atmosphere using Netzsch DSC 204 F1 Phoenix equipment calibrated with an indium standard was performed according to **section 2.1.2.4. of Chapter 2.1.**

3.1.2.5. Simulation of gastrointestinal tract

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 about 1.5 g per day (Lenger et al., 2020). The amount of sample used in the GIT experiments was adjusted for the above-mentioned D-mannose quantity. The gastric phase and the intestinal phase of the GIT were performed according to what is described in 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, instead of being transferred to dialysis membranes and dialysed against a 10 mM sodium chloride solution to mimic intestinal absorption, 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. D-mannose content of all samples was determined by HPLC (see **section 3.1.2.8. Determination of D-mannose**) before being stored at -30 ºC until being used for cytotoxicity, immunomodulation, and absorption studies.

3.1.2.6. Cytotoxicity evaluation

Cell line growth conditions

Human colon carcinoma (CaCo-2; HTB-37^{TM}) 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.

Human Colorectal Adenocarcinoma (HT-29-MTX-E12, ECACC 12040401) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and grown using McCoy's 5A medium (Thermo Fisher Scientific, Massachusetts, USA) supplemented

with 10% (v/v) FBS and 1% (v/v) antibiotic and antimycotic (Gibco, Milan, Italy). Cells were used between passages 43 and 45.

Cytotoxicity assay of digested extracts

Cytotoxicity of the digested samples was assessed in both cell lines (HT-29-MTX and CaCo-2) according to ISO 10993-5 (2009), using PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, MA, USA) and according to the manufacturer's instructions (described **in section 2.1.2.5. of Chapter 2.1**). Samples were dissolved in phosphate-buffered saline, pH 7.4 solution (PBS) to a final concentration 2-fold higher than the desired one (mannose concentration determined after GIT simulation). After being filtered through a 0.22 µm filter, twofold dilutions of the solutions were prepared in antibiotic-containing DMEM medium, for CaCo-2 cells, and McCoy's 5A medium, for HT-29-MTX cells.

Cytotoxicity assay of non-digested extracts

Cytotoxicity of the non-digested extracts was performed only in CaCo-2 cells line according to the **section cytotoxicity assay of digested extracts**. Samples were dissolved in DMEM medium at the concentration of 10 mg/mL and sterilized by filter 0.22 µm. The commercial D-mannose was prepared at 20 mg/mL in PBS and sterilized at the conditions of 100 °C during 20 min in autoclave, after that, a two-fold dilution was performed to obtain the concentration of 10 mg/mL. The test concentrations (10 mg/mL to 0.31 mg/mL) were performed by serial dilutions of the stock concentration.

3.1.2.7. Immunomodulation of digested and non-digested extracts

Immunomodulatory assay was conducted according to Machado et al., (2022). CaCo-2 cells were seeded at $2.5x10^5$ 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. Interleukins 6 (IL-6) and 8 (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.

3.1.2.8. Transport studies in cells monolayers

Cell monolayers culture

CaCo-2 and HT-29-MTX cells were maintained as previously described. Polystyrene Transwells® cell culture filters (Corning, 0.4 µm, 12 mm diameter insert, 12 wells, USA) were used for permeability assays. Suspension of CaCo-2 and HT-29-MTX cells, in a 9:1 proportion, were seeded onto the filters at a concentration of 3×10^5 cells/cm^2. The cells were propagated at 37 °C in humidified air with 5% of CO_2, in standard culture medium, in the apical chamber for 21 days until the cells were confluent. The culture medium (DMEM) was replaced every two days.

Permeability studies

The permeability study with different samples was initiated by washing once both the basolateral and apical sides with PBS (1×) and by, then, adding 1.5 mL of transport buffer (recipe adapted from Rodríguez et al., (2005)) to the basolateral side (receiver side) and 0.5 mL of transport buffer with samples at non cytotoxic concentrations to the apical side (donor side). At different time points (2, 4, 6, 8, 12 and 24 h), 200 µL aliquots were withdrawn from the basolateral side and replaced with an equal volume of transport buffer. Transepithelial electrical resistance (TEER) was measured at each time-point and the plates were incubated with the samples at 37 °C in humidified atmosphere with 5% of CO_2.

Transepithelial electrical resistance

Transepithelial electrical resistance was measured at distinct time points of the permeability studies in cell monolayers (EVOM epithelial voltohmmeter equipped with chopstick electrodes, World Precision Instruments, Sarasota, FL, USA), as an indicator of

epithelial differentiation and epithelial tightness. At the end of the 21 days, as well as during the permeability experiment, the TEER values of the monolayers were evaluated. Caco-2 monolayer was considered valid for TEER values between 150 to 1600 Ω/cm^2, according to what was previously described by Shah et al., (2006).

Determination of D-mannose using HPLC

D-mannose concentration was quantified by HPLC according to the method described by de Carvalho et al., (2021), with slight modifications. An Agilent HPLC 1260 II system equipped with a refractive index detector (RID) and an Aminex HPX-87H column was used. A calibration curve was determined with D-mannose (0.02 – 10.18 mg/mL) resulting in a correlation coefficient of 1.00. The signal-to-noise ratio was determined for the lowest concentration, 0.02 mg/mL, according to European Pharmacopoeia (EP) (2010), and resulted in a value of 10.

The aliquots which were collected at each time point for the basolateral and apical sides were lyophilized. Dried samples were accurately weighed and hydrolyzed in sulfuric acid solution for quantification of free D-mannose. An aliquot of 10 µL of the standards and test solutions were injected and eluted with the mobile phase [5mM H_2SO_4 aqueous solution (Honeywell, Charlotte, NC, USA)] at a flow rate of 0.6 mL/min under isocratic conditions. The column was kept at 50.0 ºC and the optical unit (RID) was kept at 35.0 ºC.

3.1.2.9. Statistical analysis

The T-student tests were carried out in association with Tukey's Multiple Comparison Test and Two-way analysis of variance (ANOVA) with Tukey's post-test and 95% confidence level were carried out with GraphPad Prism 6 software (Dotmatics, Boston, MA, USA). The normality of the samples was evaluated using the Shapiro-Wilk's Test. Results are represented as mean values ± SD (standard deviation), and $p < 0.05$ was considered statistically significant. All experiments have been performed in triplicate and the MOS production processes were repeated at least three times in independent days.

3.1.3. Results and discussion

Different types of chemical and physical processes have been studied and used to generate MOS from mannans (Jana et al., 2021). Hydrothermal reaction and acid hydrolysis

are the most commonly used methods as they readily cleave mannans into oligomers (Pronyk et al., 2011). Despite the inconvenience of not being selective procedures, since the degree and extension of cleavage is not easily controlled, these processes do not employ toxic reagents, and thus the eventual presence of reagent residues does not compromise the use of MOS in food applications.

Since MOS chemical production processes require pH neutralization for removal of acids or bases, it is possible that the chemical nature of the produced MOS will change. On the other side, with physical methods, there is no such need for neutralization and therefore less likelihood of altering the nature of MOS (Bortoluzzi et al., 2018).

3.1.3.1. Structural, morphological and thermal characterization

The structural analysis of the MOS-rich extracts obtained was achieved by a set of solid-state techniques: ATR-FT-IR, SEM, PXRD and DSC, as illustrated in **Figure 17**.

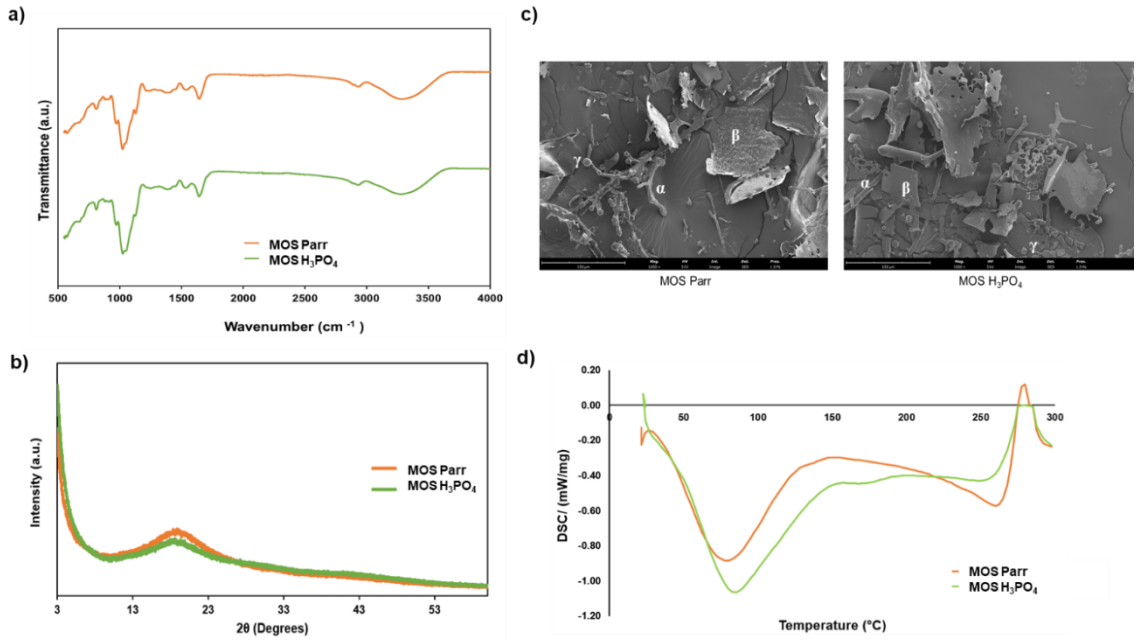


Figure 17. Structural characterization of the MOS extracts (MOS H_3PO_4 and MOS Parr) by a) Attenuated Total Reflection Fourier-transform Infrared Spectroscopy, b) Powder X-ray Diffraction analysis, c) Differential scanning calorimetry curves and d) Scanning electron microscopy.

Analysis of the IR spectra shown in **Figure 17a** indicates that MOS extracts exhibited similar spectral properties. Both spectra show a strong and broad vibration band at 3000 - 3285 cm^{-1}, corresponding to the stretching of the hydroxyl groups (Liu et al., 2018; Zhao et

al., 2022). Furthermore, according to Huang et al,. (2010), broad vibration band at 1637 – 1651 cm^{-1} demonstrated that there is residual protein in the mannan oligosaccharides samples. Also, the vibrations bands at 980 – 1120 cm^{-1} may be assigned to C-O and C-C stretching vibration of pyranose rings, commonly present in MOS (Jana & Kango, 2020).

To study potential crystallinity differences resulting from the different production processes, both MOS-rich extracts were analysed by PXRD (**Figure 17b**). PXRD patterns exhibit a single broad reflection centered at 2θ = 19.2°, which is consistent with the predominant amorphous character.

The morphology of MOS extracts was evaluated by SEM (**Figure 17c)**. The study of morphology/size is very relevant because it can affect certain physicochemical parameters. Considering that MOS H_3PO_4 and MOS Parr are constituted by a mix of structures, they can be classified as heterogeneous. Even though heterogenicity is observed, both reveal 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 made up 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 MOS Parr and MOS H_3PO_4 extracts were studied by DSC. The resulting thermograms are depicted in **Figure 17d**. The DSC thermograms of the extracts from the two production processes 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). An exothermic event is observed at around 260 – 290 °C and can be associated with polysaccharide thermal disintegration. 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).

3.1.3.2. Physicochemical characterization

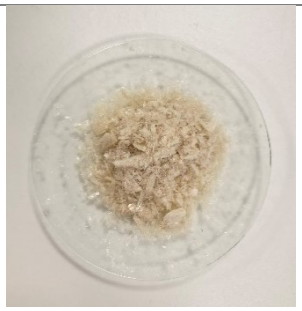
The integrated and comparative analysis of the physical appearance, along with some physicochemical properties is presented below.

Physical appearance

The powders' physical appearance is clearly different, as depicted in **Table 12**. Even though all MOS extracts appeared as a homogeneous fine powder, MOS Parr has a whiter tone when compared to MOS H_3PO_4, which presents a light brown colour.

The colour of the products for human consumption are a key criterion for their marketing, as only a limited number of colours is accepted by consumers. For this reason, information about the colour of the MOS extracts crucial for their commercialization for human consumption. The colour point of MOS extracts was evaluated by CIELAB (L*, a*, b*) system, according to Ordóñez-Santos et al., (2017), using a benchmark MOS product of *S. cerevisiae*, Ohly-GO® MOS, for comparation purposes. Results show that MOS Parr exhibits a highest lightness (L*) value (78.98 ± 0.04) than MOS H_3PO_4 (71.20 ± 0.74), that is comparable with the L* value of the benchmark product.

Table 12. 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- [b]
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.- non-applicable.

The total colour difference (ΔE*) measures the colour change between two samples. MOS extracts were compared the benchmark MOS product, as well as between themselves.

According to 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 benchmark MOS. 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 12**.

Physicochemical and composition

Bibliographic research for MOS production from *S. cerevisiae*'s mannans presents a notable scarcity of data on large-scale MOS production techniques. The methods found for MOS production from yeast mannans refer to their use essentially in the context of structural analysis.

In this study we tested a procedure previously applied to the hydrolysis of cellulose into cello oligosaccharides by using phosphoric acid. Phosphoric acid can be used as a swelling agent of cellulose which helps promote an effective hydrolysis. (Hsu & Penner, 1991; Zhang & Lynd, 2005). Additionally, it has been reported to benefit decrystalization, further facilitating hydrolysis (Liebert et al., 2008). To the best of our knowledge, there is no scientific article reporting the use of phosphoric acid to produce MOS from mannans. Nonetheless, it is expected that acidic conditions will break the bonds in the side chains, as previously described for other acids.

Other methods have also been reported, involving the production of MOS from guar gum (a galactomannan) using only hydrothermal conditions. According to Miyazawa & 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 below hydrothermal conditions (i.e., higher temperature and pressure) is an appealing reaction medium because its distinctive properties enable a variety of reactions without a catalyst (Akiya & Savage, 2002; Bröll et al., 1999; Katritzky et al., 1996). Water is an ecologically friendly reaction medium due to its non-toxicity, non-flammability, and high availability (Miyazawa & Funazukuri, 2005).

The hydrothermal process appears appealing because of the putative shorter reaction times and the absence of other reagents (Ehara & Saka, 2002; Matsushima et al., 2005). The

term “hydrothermal process” commonly refers to a process occurring under high-temperature and high-pressure water conditions, where hydrolysis occurs without the aid of a catalyst due to the high reactivity of water (Sato et al., 2003). Hydrothermal treatment allows for simple processes with low environmental impact. Hydrolysis of polysaccharides under hydrothermal conditions without additives is considered a benign method, as only water is needed for the process, consequently, neutralization and desalting processes can be eliminated (Ando et al., 2000; Bobleter, 1994; Kabel et al., 2002; Miyazawa et al., 2006; Miyazawa & Funazukuri, 2004; Mok & Antal, 1992; Nagamori & Funazukuri, 2004; Sasaki et al., 1998).

Indeed, 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 & Funazukuri, 2004) and starch (Nagamori & Funazukuri, 2004) under hydrothermal conditions, to generate mono and oligosaccharides has been significantly studied. However, optimization of the reaction conditions and reactor design is complicated because the desired products, for example, monosaccharides and oligosaccharides, are readily degraded and/or decomposed at elevated temperatures (Miyazawa & Funazukuri, 2006).

To assess the potential differences between MOS-rich extracts obtained from the two different production processes, the results of physicochemical analyses are summarized in **Table 13**.

The highest production yield (mannose yield, 85.7%) was achieved by MOS Parr, and the corresponding mannose content was higher (65.5%). MOS H_3PO_4 presents a lower mannose content (52.4%) and a correspondingly lower mannose yield (34.1%). The MOS Parr extract also reveals a higher solid yield in comparation with MOS H_3PO_4 having resulted in 75.8%. In sum, in terms of yield, the MOS Parr demonstrated the best yields.

The protein content, 10.9% and 14.0%, for MOS Parr and MOS H_3PO_4, respectively, was found to not be of statistical significance. So, the different extraction processes reveal approximately the same efficiency in terms of the elimination of proteins present in the *S. cerevisiae* mannans. It is possible that the conditions of MOS productions tested in this study were not sufficient to cleave the glycosyl-serine and glycosyl-threonine bonds, phosphodiester bonds, some peptide bond, disulphide, and acyl ester bonds present in mannans. Their mild temperature conditions are favourable to the preservation of proteins.

Regarding the sugar content obtained by the different methods (**Table 13**), MOS Parr had the highest total sugar content 74% w/w, while MOS H_3PO_4 had 55%.

Table 13. Results of physicochemical characterization of MOS extracts obtained by different extraction methods. All the values are the average of the different productions on different days.

	MOS Parr		**MOS H_3PO_4**	
Protein Content (% w/w)	10.85 ± 3.13[a]		14.03 ± 2.91[a]	
Mannose (% w/w)	65.51 ± 9.43 [a]		52.38 ± 3.25 [a]	
Glucose (% w/w)	8.10 ± 3.88 [a]		2.69± 1.40 [b]	
Total Sugars (% w/w) *	73.61 ± 10.27 [a]		55.08 ± 4.31 [b]	
Molecular Weight (MW, kDa) – Most Significant Population	MW	Area %	MW	Area %
	196 ± 44 [a]	73 ± 7	75 ± 5 [b]	33 ± 6
Population < 10 kDa (%A)	4 ± 3 [a]		30 ± 5 [b]	
Solid Yield (%) **	75.75 ± 7.68 [a]		37.03 ± 3.97 [b]	
Mannose Yield (%) ***	85.69 ± 12.57 [a]		34.14 ± 2.76 [b]	

[a,b,] means within the same line, labelled different subscripts, differ significantly ($p < 0.05$). *Total sugars are the sum of the glucose content (% w/w) plus the mannose content (% w/w). **Determined as the final weight of MOS extract, divided by the initial weight of mannans extract, multiplied by 100. *** Determined as the final mannose content weight in MOS extract, divided by the initial mannose content weight in mannans extract, multiplied by 100.

Molecular weight (MW) has been described to be directly associated to bioactivity (Liu et al., 2021). Analysis of the molecular weight distribution showed significant differences not only in the MW value (the MW of the most significant population, i.e., the one which presented the highest %A is shown in **Table 13**) but also in the number of populations achieved by the different extraction methods (**Supplementary material Figure S 2 and Figure S 3**).

The average MW of the most significant population of each of the extracts clearly emphasizes that the phosphoric acid hydrolysis process results in the production of components of lower molecular weight. This is also demonstrated by the range of MW populations resultant from phosphoric acid hydrolysis – 79 kDa to 196 Da. Extracts resultant from hydrothermal treatment presented fewer populations and of higher molecular weight.

The two most significant populations, accounting for a total of 93% Area, have a MW of 220 and 128 kDa. This heterogenicity of molecular weights can be attributed to the different experimental conditions. This diversity could be of great interest to study any potential bioactivities.

3.1.3.3. Evaluation of MOS extracts before simulated gastrointestinal tract

To assess the immunomodulatory potential of MOS extracts (MOS Parr and MOS H_3PO_4) and commercial D-mannose without the influence of the gastrointestinal tract process, we conducted an evaluation using CaCo-2 cells. This allowed us to study the behaviour of the extracts and D-mannose in relation to immunomodulation.

Cytotoxicity

To assess cytotoxicity, CaCo-2 cells were exposed to the samples (MOS Parr, MOS H_3PO_4 and D-mannose), and their impact on cell metabolism was measured using a resazurin-based dye to indicate cell viability (**Figure 18**). This assay was performed according to the guidelines of ISO 10993-5 (2009).

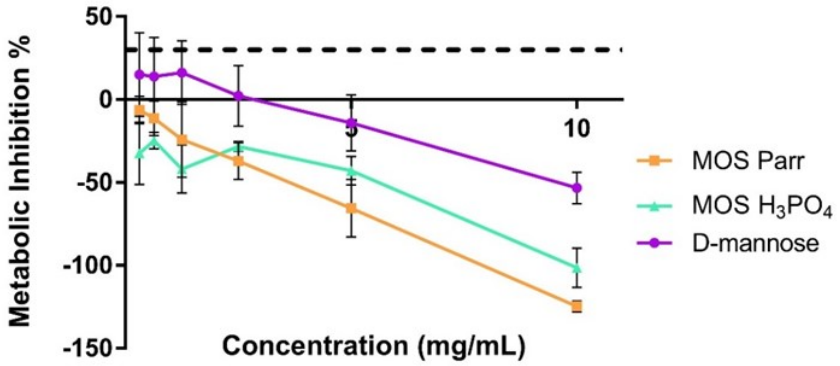


Figure 18. Results of the metabolic inhibition in % for the following concentrations: 0.31 to 10 mg/mL of the MOS Parr, MOS H_3PO_4 and D-mannose.

The two MOS extracts reveal no cytotoxicity up to 10 mg/mL. This suggests that despite the variations in structural and physicochemical properties but essentially in production methods among the MOS extracts, their interactions with CaCo-2 cells are not adversely affected (MOS Parr had a metabolic inhibition of -124.7 ± 3.4% and MOS H_3PO_4 presented -101.5 ± 9.5% at the concentration of 10 mg/mL of extract). For the commercial D-mannose

the graphic representation shows that the sample is also not cytotoxic at a concentration of 10 mg/mL (-53.2 ± 9.5%). The extract of MOS Parr had the highest metabolic stimuli comparative with the two other samples, which may indicate a better immunomodulatory potential in CaCo-2 cells.

Immunomodulatory assessment of extracts in CaCo-2 cell line

The study investigated the impact of yeast-derived MOS extracts on pro-inflammatory cytokines IL-6 and IL-8 secretion with and without an inflammatory stimulus (IL-1β) was assessed in CaCo-2 cells (**Figure 19**). In a non-stimulated cells 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 fortunately do not seem to differ from the levels presented by the basal condition ($p > 0.05$).

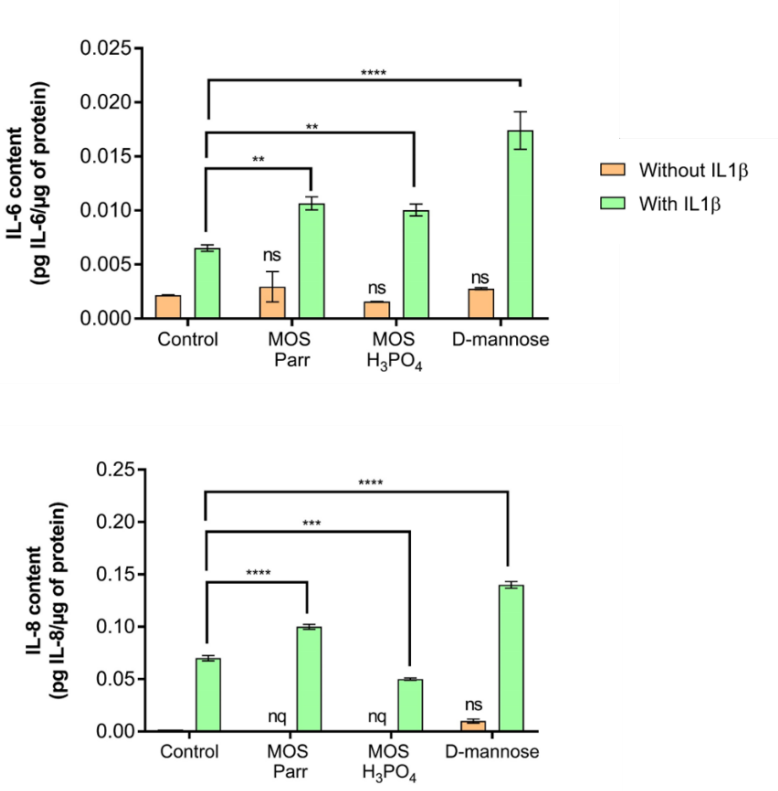


Figure 19. IL-6 and IL-8 production by CaCo-2 cells, with and without IL-1β stimulus. The statistical analysis revealed significant differences between the controls and the tested samples (MOS Parr, MOS H_3PO_4, and D-mannose) expressed by the symbol **** ($p < 0.0001$) or ** ($p < 0.01$). The symbol "ns" indicates non-significant statistical differences and nq – not quantified.

In IL-1β stimulated cells, the expression levels of IL-6 were higher in cells exposed to MOS Parr (0107 ± 0.0006 pg IL-6/µg of protein) and MOS H_3PO_4 extract (0.0100 ± 0.0006 pg IL-6/µg of protein) compared to their respective control (0.0022 ± 0.0000 pg IL-6/µg of protein) with statistical significance ($p < 0.01$). Notably, the highest difference was observed in cells exposed to D-mannose (0.0174 ± 0.0017 pg IL-6/µg of protein), which showed even more pronounced elevation ($p < 0.0001$).

These findings suggest that yeast-derived MOS extracts, particularly D-mannose, have the potential to modulate IL-6 expression levels in CaCo-2 cells under inflammatory conditions.

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

In an IL-1β stimulated model, the expression levels observed for MOS H_3PO_4 (0.0485 ± 0.0012 pg IL-8/µg of protein) extract were lower when compared to their respective basal control (0.0656 ± 0.0026 pg IL-8/µg of protein), revealing that this extract produces an anti-inflammatory response. IL-8 expression levels in cells exposed to MOS Parr (0.0961 ± 0.0023 pg IL-8/µg of protein) and D-mannose (0.1407 ± 0.0032 pg IL-8/µg of protein) were higher than those of the cells control with the stimulus ($p < 0.0001$).

These results suggest that yeast-derived MOS extracts, particularly D-mannose, 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.

3.1.3.4. Evaluation of MOS extracts after simulated gastrointestinal tract

Commercial supplements rich in polysaccharides (e.g., glucomannans) for human consumption are usually ingested orally in the form of capsules. However, little information is available in the literature about the impact of digestion process in the structural integrity of these molecules and, consequently, their biological properties. For this reason, and for a closer reproduction of *in vivo* conditions, it is essential to evaluate their bioactive potential after being subjected to conditions that simulate the gastrointestinal tract. This *in vitro* assay has been a valuable tool to assess the digestibility and bio accessibility of compounds, is less expensive and relatively precise than *in vivo* models, and also raises fewer ethical concerns,

which are in line with European (EU) guidelines (Ribeiro et al., 2020). The final mannose concentration of the samples after the GIT simulation (from samples with an initial amount of 1.5 g of mannose) are presented in **Table 14**.

Cytotoxicity

Cytotoxicity of digested samples was assayed against CaCo-2 and HT-29-MTX cells, by evaluating their impact on cell metabolism using a resazurin-based dye as cell viability indicator (a sample is considered cytotoxic when the observed percentage of metabolic inhibition is above 30%). This cytotoxicity assessment considered not the digested extract concentration but the mannose concentration of the corresponding extract. This assessment is critical to determine the maximum extract concentration that cells tolerate without significant metabolic inhibition. The concentration values determined in this assay were then used to perform the permeability experiments through cell membranes, which were composed of a monolayer of a co-culture of CaCo-2 and HT-29-MTX cells. The results are presented in **Table 14**.

Table 14. Mannose concentrations on samples after GIT simulation and non-cytotoxic for CaCo-2 and HT-29-MTX 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

* Concentration non-cytotoxic for both CaCo-2 and HT-29- MTX cells

As seen in **Table 14**, non-cytotoxic concentrations between samples are slightly different from each other. Considering that, to consume the previously mentioned daily dose of 1.5 g of D-mannose, higher quantities of extract were necessarily introduced in the GIT simulation. Thus, in the end, it was expected that due to dilution effects by the GIT matrix, the final mannose concentration would be higher in D-mannose sample and lower in the extracts. However, contrary to what was expected, mannose concentrations after GIT were similar for D-mannose and MOS Parr, but significantly higher for MOS H_3PO_4. This could be attributed to interactions occurring in the GIT, possibly a degradation of free mannose in

the gastric phase. Additional studies would be necessary to completely understand the mechanisms behind the unexpected values.

The solutions evaluated for its cytotoxic behaviour were a result of a serial-dilution of the ones originating from GIT and resulted in non-cytotoxic concentrations for the same dilution factor (16x). This suggests that cytotoxicity is primarily conferred by the components in the matrix from GIT and not necessarily from the extracts or D-mannose. This hypothesis is corroborated by the fact that the cytotoxicity assessment of a control consisting of the GIT without sample also resulted in non-cytotoxicity for the same dilution factor (data not shown).

Immunomodulatory assessment of digested samples in CaCo-2 cell line

Recently, D-mannose has been drawing some attention due to its immunoregulatory function (Zhang et al., 2017; Zhang et al., 2021). For this reason, it would be interesting to find if MOS extracts could also have a positively influence in the immune system, namely in expression of cytokines, a group of signalling proteins that play crucial roles in the control inflammation (Kany et al., 2019). Besides being synthesized by the innate immune system, cytokines can also be produced by epithelial, known to participate in immune responses (Stadnyk, 1994). Among these group of proteins, IL-1β is a major pro-inflammatory cytokine implicated in pain, inflammation and autoimmune diseases (Dinarello, 2011; Ren & Torres, 2009) that plays a central role in the intestinal inflammatory response leading to intestinal damage (Al-Sadi & Ma, 2007). *In vitro* studies regarding the impact of this proinflammatory mediator in inflammatory bowel diseases (IBDs) revealed an increased secretion of IL-6 and IL-8 (Van De Walle et al., 2010), two pro-inflammatory cytokines involved in chronic inflammation and produced by activated macrophages in response to inflammation (Harada et al., 1996; Tanaka et al., 2014), that have been widely assessed as biomarkers in IBDs.

Immunomodulatory activity evaluation of MOS often regards the effect of commercial or yeast-derived MOS-supplemented diet on animal gut health (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) and rarely contemplate CaCo-2 cells, the *in vitro* model of the human intestinal epithelial barrier (Hoving et al., 2018; Wang et al., 2016). Indeed, to the best of our knowledge, this is the first report of the evaluation of the immunomodulatory activity of digested yeast-derived MOS. Accordingly, the effect of MOS Parr and MOS

H_3PO_4 on pro-inflammatory cytokines IL-6 and IL-8 (**Figure 20**) 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 the supernatants of the cells exposed to the samples. On cells non-stimulated, IL-6 expression levels by cells exposed to both MOS extracts and D-mannose are not statistically significant ($p > 0.05$) than those from cells in DMEM. Similarly, exposure of cells to all samples does not seem to lead to expression levels different from those of cells in basal condition ($p > 0.05$).

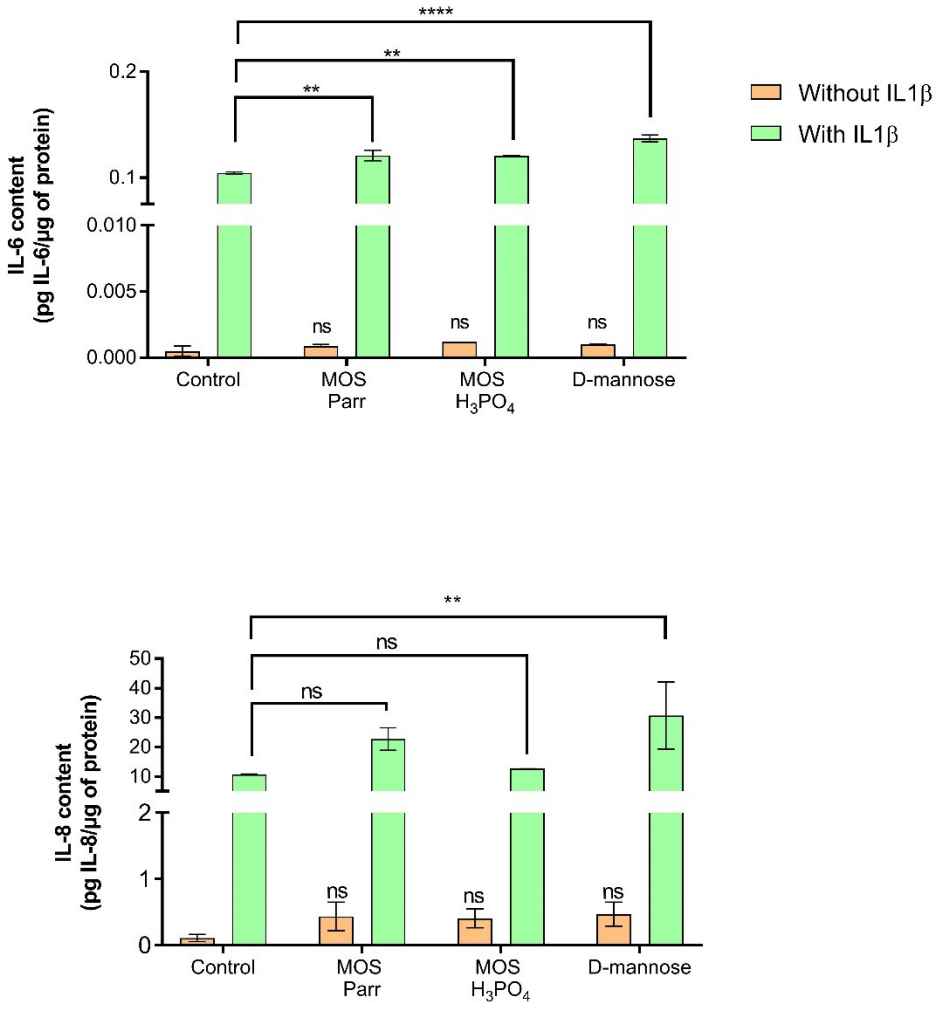


Figure 20. IL-6 and IL-8 production by CaCo-2 cells with and without stimulus (IL-1β). Results are expressed in relation to each control and bars represent means ± SD. Statistically significant differences between the controls and the tested samples (MOS Parr, MOS H_3PO_4 and D-mannose) are indicated $^{**}p < 0.01$ and $^{****}p < 0.0001$ (ns – non-significant statistical differences).

In cells stimulated IL-1β and as expected, expression levels of IL-6 exposed to MOS Parr (0.121 ± 0.001 pg IL-6/µg of protein) and MOS H_3PO_4 extract (0.121 ± 0.000 pg IL-6/µg of protein) are higher ($p < 0.01$) that those of the respective control (0.104 ± 0.001 pg IL-6/µg of protein). This difference is more significative ($p < 0.0001$) for cells exposed to D-mannose (0.138 ± 0.003 pg IL-6/µg of protein). Differently, no significant differences in IL-8 expression levels ($p > 0.05$) were observed in cells in DMEM (10.73 ± 0.15 pg IL-8/µg of protein) and cells exposed to MOS Parr (22.69 ± 3.81 pg IL-8/µg of protein) and MOS H_3PO_4 (12.69 ± 0.61 pg IL-8/µg of protein), although IL-8 expression levels in cells exposed to D-mannose (30.64 ± 11.43 pg IL-8/µg of protein) were slightly higher than those of the control. In addition, some differences were noticed between MOS extracts and D-mannose regarding IL-6 and IL-8 production ($p < 0.01$ and $p < 0.05$, respectively). 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 & Jones, 2015).

Transport studies in cell monolayers

Cell culture approaches that mimic the intestinal epithelial barrier are becoming widely used to assess nutrients absorption. CaCo-2 cells can be a well-established *in vitro* model of this barrier, considering that after some time in confluent culture, these cells acquire a phenotype that resembles enterocytes, not only in terms of morphology but also functionality (Panse & Gerk, 2022). However, given the complexity of the human intestinal epithelium, models based exclusively on these cells present multiple limitations. A more accurate representation can be achieved by a co-culture system consisting of enterocytes and mucous cells. In this work, and aiming to evaluate absorption of mannose through the intestine, transepithelial transport of the digested samples was assessed using a monolayer co-culture model of two intestinal cell lines: the enterocyte-like CaCo-2 cell and HT-29-MTX cells, a sub-strain of the colorectal carcinoma-derived cell line HT29 that can differentiate into mucus-secreting goblet cell-like cells during cultivation (Antoine et al., 2015; Antunes et al., 2013; Hilgendorf et al., 2000; Mahler et al., 2009; Panse & Gerk, 2022) To the best of our knowledge, transport of D-mannose across CaCo-2 cells/HT-29-MTX co-culture monolayers has not been reported yet. Nevertheless, CaCo-2 cells and small intestine epithelial cells have been reported to express a Na^+-independent (in the basolateral

membrane) and a Na^+-dependent (in the apical membrane) D-mannose transporter (Cano et al., 2001; De la Horra et al., 2001; Durán et al., 2004; Ogier-Denis et al., 1994).

However, D-mannose is mainly transported across cell membranes glucose transporters in several human cells, such as enterocytes (Rodríguez et al., 2005). Indeed, D-glucose and D-mannose share the sodium-dependent glucose transporter 1 (SGLT1) in the intestinal epithelial cells, with D-glucose uptake being higher than D-mannose uptake (Rodríguez et al., 2005). For this reason, in this assay, we used a transport medium without D-glucose so that the presence of glucose would not influence the transport of D-mannose by the intestinal cells. After cytotoxicity determination, D-mannose transport evaluation of the digested samples (MOS Parr and MOS H_3PO_4) was conducted to evaluate the differences between the absorption of these two samples in solution. Membrane integrity was assessed by transepithelial electric resistance (TEER) variations (**Figure 21**) and D-mannose was quantified in the basolateral chamber after 2, 4, 6, 8, 12 and 24 h (**Figure 22**).

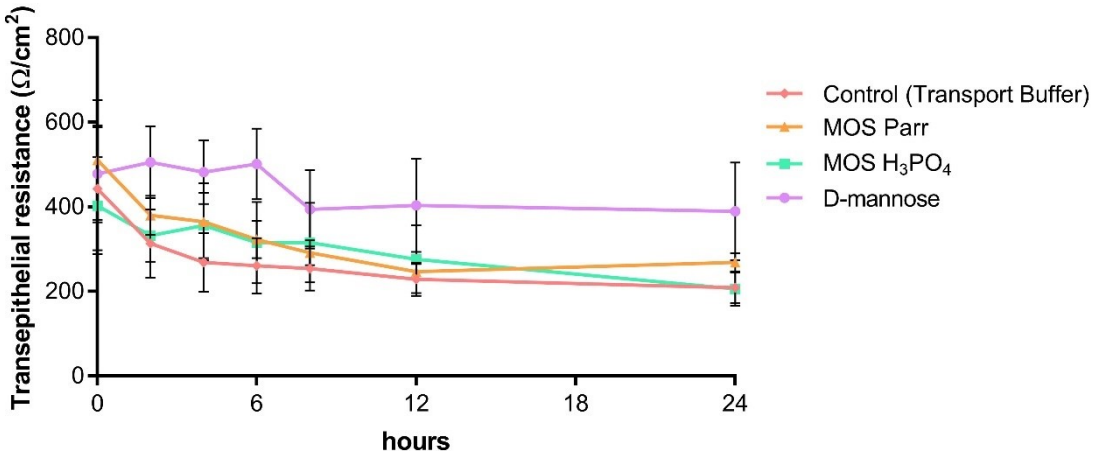


Figure 21. TEER measurements of cell monolayer across CaCo-2/HT-29-MTX co-culture seeded in Transwells® membranes exposed to MOS Parr, MOS H_3PO_4 and D-mannose digested samples. Results are represented as the mean value and error bars represent standard deviations from four replicates.

The representation, in **Figure 21**, of TEER measurements performed during the transport assay revealed that all samples in all time-points presented values above 150 Ω/cm^2, which has been previously reported as threshold for identification of membrane integrity (Shah et al., 2006). In the literature, permeation studies carried out in cellular models using CaCo-2 cells, show that TEER values tend to decrease over time, possibly

resulting in increased permeability of tight junctions (Silva et al., 2006). TEER values in the beginning of the assay were determined to be, average 1240.5 ± 55.0 Ω/cm^2, changing, in the end, to 269.3 ± 21.8 Ω/cm^2 for MOS Parr, 206.4 ± 39.7 Ω/cm^2 for MOS H_3PO_4, 389.5 ± 115.7 Ω/cm^2 for D-mannose and 208.9 ± 36.4 Ω/cm^2 for the control, thus revealing that the CaCo-2/HT-29-MTX cell monolayer retained its integrity throughout the experiments. Furthermore, the decrease in TEER values discloses an increase in the overall epithelial permeability for all samples.

The representation of mannose transported through the cell membrane is depicted in **Figure 22**. On the y axis is presented the cumulative percentage of mannose quantified in the basolateral chamber at the different time-points, in relation to the initial concentration placed in the apical chamber. For this evaluation, the cells were exposed at the maximum non-cytotoxic mannose concentration of digested samples, as previously presented in **Table 14**. For this reason, different initial concentrations were tested for each sample.

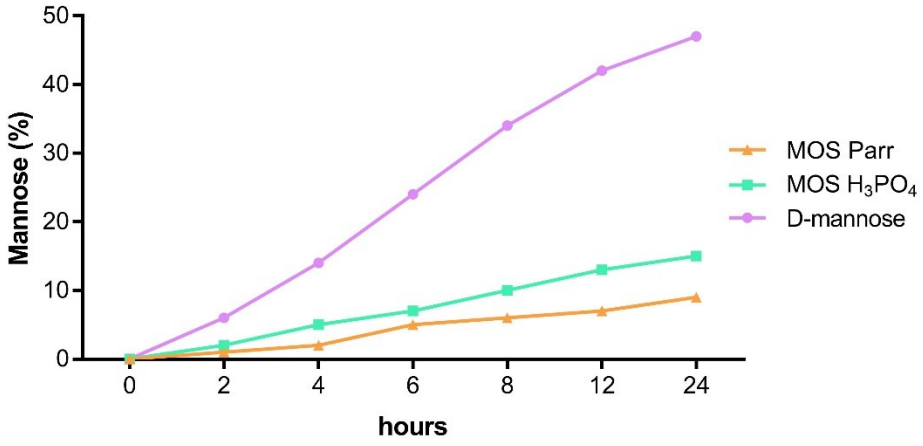


Figure 22. Evaluation of accumulative D-mannose transport percentage of the digested samples MOS Parr, MOS H_3PO_4 and D-mannose after the absorption in cell monolayer across CaCo-2/HT-29-MTX co-culture seeded in Transwells® membranes.

The transport of mannose increased up to the final time point of 24 h, for all samples tested, reaching 15% for MOS H_3PO_4, 9% for MOS Parr, and 47% for D-mannose, as depicted in **Figure 22**. In addition, it is possible to observe that digested D-mannose displays a higher transport rate compared to the digested extracts. Within the digested extracts, MOS H_3PO_4 was the one which presented the highest transport rate. Even if only a small fraction of the initial mannose content was transported, it is interesting to verify that mannose from

the digested hydrolysates was able to pass through the cell monolayer. The physicochemical characterization of the extracts has shown that MOS H_3PO_4 is composed of lower molecular weight populations, when compared to MOS Parr. The presence of oligomers could potentially decrease the transport restriction that a higher MW polymer, such as MOS Parr, may face, although this would only be the case if transport was also feasible through the paracellular pathway. It would be advantageous to characterize the molecular weight of the digested samples, since hydrolysis can occur, particularly in the acidic conditions of the simulated gastric phase, and this could be a relevant factor impacting permeability.

To the best of our knowledge, there are no reports on the transport of MOS through the intestinal epithelium. Nonetheless, the permeation of other oligosaccharides has been studied previously. *In vitro* and *in vivo* transport evaluation of agaro-oligosaccharides (AOSs), has shown a good correlation between permeation studies in CaCo-2 cell monolayer and AOSs present in the plasma of rats following its oral administration (Shirai et al., 2022), emphasizing the significance of *in vitro* studies.

The fact that in this case different initial concentrations of digested extracts were tested, as a direct consequence of cytotoxic results, undermined a thorough interpretation of the results obtained. Additional studies would be beneficial to understand both the mechanisms involved and the molecular weight of the molecules that were able to cross the CaCo-2/HT-29-MTX cell monolayer.

3.1.4. Conclusions

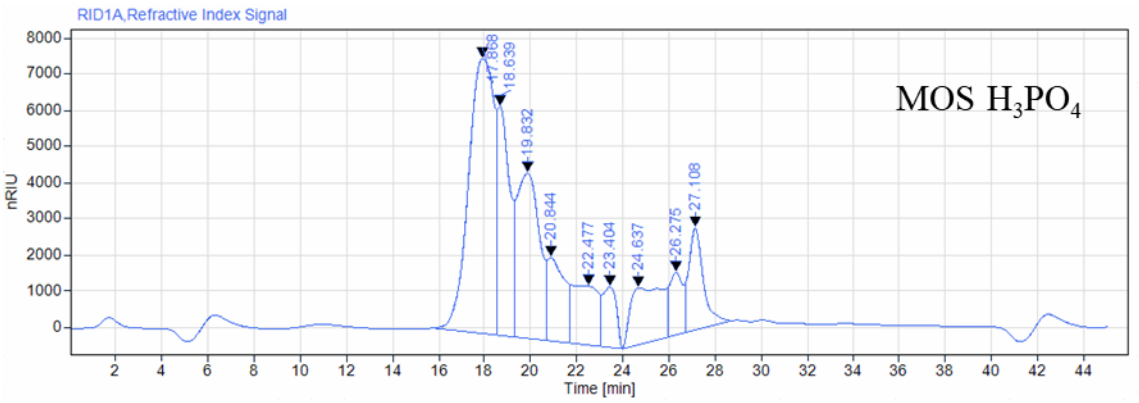
The present study reports the effects of two different MOS production methodologies on the structural, physicochemical, compositional, and biological properties of the resulting extracts. MOS was produced from a mannans extract obtained from spent yeast (a genetically modified strain of *S. cerevisiae*). Structural comparison disclosed that the two methodologies generated similar extracts. Significant differences were, however, found between the MOS extracts regarding solid and mannose yields, and molecular weight. MOS Parr extract showed the highest yields, and higher molecular weight populations, when compared to MOS H_3PO_4. The yeast-derived MOS extracts were evaluated for their immunomodulatory activity without the interference of GIT process. Based on the findings, it can be concluded that MOS extracts, especially D-mannose, show promise in regulating IL-6 expression levels in CaCo-2 cells during inflammatory conditions. However, additional research is required to investigate the mechanisms underlying the modulation of IL-8

expression and to gain a deeper understanding of the anti-inflammatory properties observed in the MOS H_3PO_4 extract.

In order to mimic what happens in the digestion process, the extracts were digested in a simulated gastrointestinal tract based on the INFOGEST methodology, and the resulting digested extracts were evaluated for their immunomodulatory activity and D-mannose transport through an intestinal epithelium cell model. The immunomodulatory activity of the MOS extracts was measured by quantification of IL-6 and IL-8 level in CaCo-2 cells stimulated with IL-1β and exposed to the digested sample, showing that, although not anti-inflammatory, MOS extracts can be immunostimulant, aiding in the immune response of cells by enhancing the expression levels of the IL-6, a cytokine also regarded as anti-inflammatory.

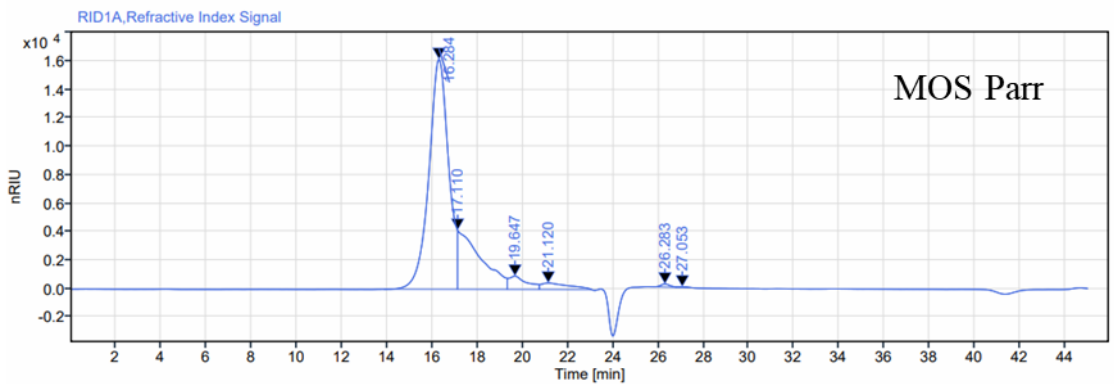
Regarding the permeability study of the digested extracts, the mannose present in the MOS extracts (MOS Parr and MOS H_3PO_4) can only be absorbed at low quantities, which limit its use if this absorption is necessary, in applications such as pathogenic adhesion inhibition in the urinary tract (Kranjčec et al., 2014). On the other hand, MOS extracts can have advantageous applications when its integrity remains intact after undergoing digestion, such as prebiotic in human microbiota (Carvalho et al., 2022).

Supplementary material



% Area	MW/ kDa	% Area	MW/ kDa	% Area	MW/ kDa	% Area	MW/ kDa	% Area	MW/ kDa	% Area	MW/ kDa	% Area	MW/ Da	% Area	MW/ Da	% Area	MW / Da
32	**79**	13	**48**	18	**22**	7	**11**	7	**4**	4	**2**	8	**978**	4	**337**	7	**196**

Figure S 2. An example of chromatograms resulting from molecular weight distribution analysis as per analytical method of the one production of MOS H_3PO_4 extract.



% Area	MW /kDa	% Area	MW /kDa	% Area	MW /kDa	% Area	MW /kDa	% Area	MW /kDa	% Area	MW /Da
71	**220**	22	**128**	4	**24**	3	**9**	0	**0.3**	0	**195**

Figure S 3. An example of chromatograms resulting from molecular weight distribution analysis as per analytical method of the one production of MOS Parr extract.

Chapter 4

Potential applications of MOS extracts

This chapter aims to evaluate the potential applications of MOS extracts as UPEC adhesion inhibitors and in synergy with vaginal lactobacilli adhesion inhibitors of *Candida albicans*.

Chapter 4.1 *Impact on uropathogenic Escherichia coli adhesion on bladder cells*

Chapter 4.2 *Impact on vaginal pathogenic Candida albicans and vaginal lactobacilli*

Adapted from the following submitted scientific publication:

Faustino, Margarida; Silva, Sara; Costa, Eduardo; Pereira, Ana Margarida; Pereira, Joana Odila; Oliveira, Ana Sofia; Ferreira, Carlos; Pereira, Carla F.; Durão, Joana; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Effect of mannan oligosaccharides extracts in uropathogenic *Escherichia coli* adhesion in human bladder cells. Pathogens 12(7), 885. **https://doi.org/10.3390/pathogens12070885**

Faustino, Margarida; Pereira, Joana Odila; Pereira, Ana Margarida; Oliveira, Ana Sofia; Ferreira, Carlos; Pereira, Carla F.; Durão, Joana; Pintado, Manuela E.; Carvalho, Ana P.; 2023 Alternative strategies to prevent *Candida albicans* proliferation in vaginal environment: synergistic effect of lactobacilli and mannan oligosaccharides (MOS). Submitted to Applied Microbiology and Biotechnology.

4.1. Impact on uropathogenic Escherichia coli adhesion in bladder cells

Effect of Mannan oligosaccharides extracts in uropathogenic Escherichia coli adhesion in human bladder cells

Abstract: Urinary tract infections (UTIs) are a common public health problem, mainly caused by uropathogenic *Escherichia coli* (UPEC). Patients with chronic UTIs are usually treated with long-acting prophylactic antibiotics, which promotes the development of antibiotic-resistant UPEC strains and may complicate their long-term management. D-mannose and extracts rich in D-mannose such as mannan oligosaccharides (MOS; D-mannose oligomers), are promising alternatives to antibiotic prophylaxis due to their ability to inhibit bacterial adhesion to urothelial cells and, therefore, infection. This approach could decrease the number of UTIs that rely on antibiotics. This highlights the therapeutic potential and commercial value of using them as health supplements. Aiming to evaluate the potential benefits of using MOS extracts in UTIs prophylaxis, their ability to inhibit the adhesion of UPEC to urothelial cells and its mechanism of action were assessed. Additionally, the expression levels of the pro-inflammatory marker interleukin 6 (IL-6) were also evaluated. After characterized for their cytotoxic profile preliminary results indicated that MOS extracts can have the potential to be used for the handling of UTIs and demonstrated that the mechanism through which they inhibit bacterial adhesion is by competitive inhibition of FimH adhesins through the action of mannose, validated by bacterial growth impact assessment.

Keywords: Uropathogenic *Escherichia coli* (UPEC); Mannan oligosaccharides (MOS); Adhesion; Bladder cells; D-mannose; Urinary tract infections (UTIs)

4.1.1. Introduction

Urinary tract infections (UTIs) represent a severe public health problem and are caused by a range of pathogens, most commonly by *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* (Flores-Mireles et al., 2015). About 150 million people worldwide each year are affected by UTIs, which are among the most common infectious diseases (Kalas et al., 2018; Terlizzi et al., 2017). UTIs are the second leading cause of infections in the general population, predominantly in adult females, and are the leading cause of infection in hospital environment (Magliano et al., 2012; Vyas et al., 2015). According to the European Association of Urology (EAU) Guidelines (Bonkat et al., 2023), they can be divided into uncomplicated (including lower UTIs (cystitis) and upper UTIs (pyelonephritis), typically affecting non-pregnant women, without any other health problem or anatomical/functional irregularities) and complicated (associated to individuals with increased probability of complications, namely as pregnant women, men, or patients with other health related problems such as renal anatomical/functional abnormalities, failure or transplantation, other diseases such as immunocompromised or diabetic individual). In most cases, UTIs are caused by the pathogenic uropathogenic *E. coli* (UPEC), that is capable to migrate from the intestinal microbiota to the perianal region and, thereafter, to the urinary tract (McLellan & Hunstad, 2016). This bacterium is the most prevalent in symptomatic and/or asymptomatic infections, representing up to 75% to 95% of the reported UTIs (Flores-Mireles et al., 2015; Forsyth et al., 2018; McLellan & Hunstad, 2016), with the ability to colonize all parts of the urinary tract (including the urethra, ureters, kidney and bladder), thus causing acute, chronic, persistent, and recurrent infections (Hannan et al., 2010; Schwartz et al., 2011). Amidst the multitude of well-studied virulence factors, the ability of UPEC to adhere to host epithelial cells is the crucial step for the establishment and progression of infection (Flores-Mireles et al., 2015; Forsyth et al., 2018; McLellan & Hunstad, 2016; Terlizzi et al., 2017). This adhesion ability is mainly conferred by the type 1 fimbriae D-mannose specific adhesin (FimH), located at the tip of UPEC's type 1 fimbriae, which specifically binds to terminal epitopes of high mannosylated glycans conjugated to uroplakin 1a (UP1a), a receptor specifically expressed on the surface of urothelial cells (Anderson et al., 2003; Mickiewicz et al.,

2019; Mydock-Mcgrane et al., 2016; Ribić et al., 2018; Snyder et al., 2006; Wu et al., 1996).

D-mannose is widely used in food, medicine, cosmetics, and food additives industries. Among its physiological health benefits, the most reported regard their positive effect on the immune system, diabetes mellitus (type 2), intestinal diseases, and UTIs (Wei et al., 2020; Wu et al., 2019). D-mannose, a C-2 epimer of D-Glucose, and D-mannose analogues can prevent FimH mediated bacterial adhesion through a competitive inhibition mechanism. This competition mechanism is based on the structural similarity between the D-mannose receptors and the urothelial mannosylated receptors exposed by the urinary tract epithelium (Cusumano et al., 2011; Firon et al., 1982; Klein et al., 2010; Mydock-Mcgrane et al., 2016; Ofek et al., 1977). When administered in sufficient amounts, D-mannose is rapidly absorbed and excreted through the urinary tract where it will saturate UPEC's FimH, thus blocking its binding and subsequent adhesion to the urothelium (O'Brien et al., 2016; Zacchè & Giarenis, 2016). According to Scribano et al., (2020), the D-mannose dosage range for UTI prevention determined in clinical trials is between 2 and 3 g per day and the normal urine volume is 800 to 2000 mL/day. When administered orally, the absorption of D-mannose is rapid and detectable in plasma approximately 30 min after taking it, being then excreted thought the urinary tract. In addition to binding to bacterial fimbriae, D-mannose acts on the activation of the Tamm-Horsfall protein, which plays a key role in the body's defense against UTIs (Pigrau & Escolà-Vergé, 2020; Serafini-Cessi et al., 2005).

In this work, the viability of using mannan oligosaccharides (MOS) as supplements for the management of UTIs was evaluated through the assessment of the ability of MOS extracts to inhibit the adhesion of UPEC to urothelial cells. Two MOS samples, produced from yeast-purified mannans fractions by two different methodologies, were assessed and commercial D-mannose was used as controls. Expression levels of the pro-inflammatory interleukin 6 (IL-6) and the potential antimicrobial activity of all samples using a time-growth inhibition curve, were also assessed.

4.1.2. Materials and methods

4.1.2.1. Materials

MOS extracts (MOS H_3PO_4 and MOS Parr) were obtained from genetically modified spent yeast (*S. cerevisiae*) from Amyris, Inc. (Emeryville, California, USA) according to methodologies previously described by the group (**section 3.1.2.2. of Chapter 3**). Mannose content of these extracts was determined by gas-chromatography-flame ionization detection (GC-FID) as previously described. D-mannose were purchased from Sigma-Aldrich, Munich, Germany.

4.1.2.2. Bacterial strain and cell line

The well characterized uropathogenic *E. coli* strain CFT073 (DSMZ, Braunschweig, Germany) was used in this study. Strain CFT073 was grown at 37 °C in Tryptic Soy Broth (TSB; Biokar Diagnostic, Beauvais, France) with 3 g/L of yeast extract (Sigma-Aldrich, Munich, Germany), from now on referred only as TSB. The human bladder epithelial cell line 5637, HTB-9 was obtained from the American Type Culture Collection 5637 (ATCC-LGC, Milan, Italy) and routinely cultured in T75 flasks at 37 °C in a humidified atmosphere with 5% CO_2 using Roswell Park Memorial Institute (RPMI 1640) medium (Invitrogen, Massachusetts, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), with the addition of 1% (v/v) antibiotic 100x (Invitrogen, MA, USA). Unless indicated otherwise, RPMI medium supplemented with FBS and without antibiotic will, from now one, be referred only as RPMI.

4.1.2.3. Cytotoxicity assay

Cytotoxic effect of the samples was assessed in HTB-9 cell line in conformity with the ISO 10993-5 guideline (2009), using the PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, MA, USA) according to the instructions of the manufacturer. A stock solution of 30.00 mg/mL of each sample in RPMI medium with antibiotic was prepared, and D-mannose was used for comparation purposes. Different preparations were implemented due to the nature of their constituents and the stability of these compounds. For MOS H_3PO_4 and MOS Parr extracts were directly dissolved in RPMI medium with antibiotic and sterilized using a sterile syringe filter with a 0.22 µm pore

size (Millipore, Billerica, MA, USA). Commercial D-mannose was dissolved in PBS solution at pH 7.4, to a final concentration 2-fold higher than the desired one. After being autoclaved for 20 min at 100 °C, a twofold dilution of the solution was prepared in antibiotic containing RPMI medium to achieve the final concentration of 30 mg/mL. For HTB-9 viability assay the protocol used was described in **section 2.1.2.5. of Chapter 2.1**.

4.1.2.4. Immunological response without stimulus

HTB-9 cells were seeded at a final concentration of 2.5×10^5 cells/mL in a 24-well microtiter plate and immediately incubated at 37 °C with 5% CO_2 in a humidified environment for 24 h. Following this incubation period, cells were washed twice with warm, sterile PBS to remove all the antibiotic-containing medium. HTB-9 cells were then exposed to the samples (MOS H_3PO_4, MOS Parr and D-mannose) diluted in RPMI medium at a non-cytotoxicity mannose concentration. Medium without the samples was used as positive control in each incubation period and cells were incubated at 37 °C with 5% CO_2 in a humidified environment for 1, 2 and 3 h. After that, the media was collected, transferred to a microcentrifuge tube, and kept at -20 °C until protein and IL-6 expression levels quantification (**section 4.1.2.7.**).

4.1.2.5. UPEC adhesion assays

Simultaneous exposure (Competition)

HTB-9 cells were seeded at a final concentration of 2.5×10^5 cells/mL in a 24-well microtiter plate and immediately incubated at 37 °C with 5% CO_2 in a humidified environment for 24 h. Following this incubation period, cells were washed twice with warm, sterile PBS to remove all the antibiotic-containing medium. UPEC previously grown in TBS were centrifuged (4700 x *g*, 5 min, 4 °C), washed twice with PBS and resuspended in the same buffer at a multiplicity of infection (MOI) of 10 (Scribano et al., 2020), while MOS H_3PO_4, MOS Parr and D-mannose samples were diluted in RPMI medium at a non-cytotoxic mannose concentration (2.5 mg/mL). HTB-9 cells were then simultaneously exposed to the samples and to UPEC suspension in PBS and incubated for 2 h at the same incubation conditions above-mentioned. HTB-9 cells in RPMI and infected with UPEC were used as positive control in each incubation period.

Prophylaxis assessment

Similar to the above-described study, HTB-9 cells were seeded at a final concentration of 2.5×10^5 cells/mL in a 24-well microtiter plate and incubated at 37 °C with 5% CO_2 in a humidified environment. After 24 h of incubation, medium was discarded, and cells were washed twice with warm, sterile PBS to remove any antibiotic traces. MOS H_3PO_4, MOS Parr and D-mannose were diluted in antibiotic-free RPMI medium at a mannose concentration of 2.5 mg/mL and added to the wells containing HTB-9 cells, that were then incubated at 37 °C with 5% CO_2 in a humidified environment during 1, 2, and 3 h before infection of the urothelial cells with UPEC. HTB-9 cells in RPMI and infected with UPEC were used as positive control in each incubation period. After incubation with extracts, bacterial cells grown in TSB were centrifuged (4700 x *g*, 5 min, 4 °C), washed twice with PBS, resuspended at MOI of 10 and added to the cells for 2 h (time for adhesion of the UPEC).

4.1.2.6. Total viable counts determination

At the end of the competition, and prophylaxis assessments, media conditioned by the samples were transferred to a microcentrifuge tube and processed for protein and IL-6 expression levels quantification as described in **section 4.1.2.7.** and **section 4.1.2.4**. Cell monolayers were carefully washed twice with sterile, warm PBS to remove unbound bacteria and, after detachment with trypsin (TrypLE™ Thermo Fisher Scientific, MA, USA), cells were resuspended in PBS. After serially dilutions in 0.1% sterile peptone water (w/v), 100 µL of the cell suspensions were plated by the drop method (Miles et al., 1938) on Plate Count Agar (PCA) plates and incubated at 37 ºC for 24 h before counting to determine the total viable counts (CFU/mL). Results were expressed as adhesion inhibition percentage (% Inhibition) calculated using Equation 6.

$$\%\ \text{Inhibition} = 100 - \left(\frac{\frac{CFU}{mL} of\ Sample}{\frac{CFU}{mL} of\ Control}\right) \text{x } 100 \qquad (6)$$

4.1.2.7. Protein determination and interleukin evaluation

Media conditioned by the samples and collected to microtubes at the end of each experiment were centrifuged (990 x *g*, 20 min, 4 °C) and the supernatants were used for protein and IL-6 expression levels quantification. Protein determination was performed in 96- well microtiter plates by the bicinchoninic acid (BCA) methodology, using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA) according to the instructions of the manufacturer. IL-6 expression levels were assessed by ELISA using the Human IL-6 Elisa Max™ Deluxe Kit (BioLegend, San Diego, USA) according to the instructions of the manufacturer and results were presented in pg/µg of protein.

4.1.2.8. Antimicrobial activity – growth inhibition curves

The potential antimicrobial activity of MOS H_3PO_4, MOS Parr and D-mannose was assessed resorting to a time-growth inhibition curve (CLSI, 2012). A solution of each sample in the growth medium Mueller Hinton (MH) (Biokar Diagnostic, Beauvais, France) was prepared to a final concentration of non-cytotoxic mannose concentration. Mannose content of each sample was previously determined by GC-FID. MOS H_3PO_4 and MOS Parr samples were diluted in MH medium and sterilized using a sterile 0.22 µm filter (Millipore, Billerica, MA, USA). D-mannose was dissolved in PBS solution to a final concentration 2-fold higher than the desired and sterilized using the autoclave (Prohs, Porto, Portugal) at 100 °C for 20 min. After sterilization, D-mannose solution was twofold diluted in MH medium to achieve the final a concentration, in mannose, of 2.5 mg/mL. The UPEC strain was used as a monoculture and, before the assay was grown in Tryptic Soy Agar (TSA; Biokar Diagnostic, Beauvais, France) with 3 g/L of yeast extract (Sigma-Aldrich, Munich, Germany) at 37 °C for 24 h under aerobic conditions. Afterwards, one colony was picked, resuspended in 10 mL of MH broth, and grown at 37 °C for 24 h under aerobic conditions. The bacteria inoculum was adjusted to an optical density (OD) at 625 nm of 0.1 - 0.08 (cell density of 1 x 10^8 cells/mL) and 10 times diluted to obtain the work inoculum. To evaluate bacterial growth inhibition, 980 µL of each sample were transferred to a sterile microtube and inoculated with 20 µL of the work UPEC inoculum. After mixing by vortexing, 200 µL of the suspensions were transferred to a 96-well microtiter plate (Nunc, Darmstadt, Germany) and the OD at 625 nm was assessed for a 24 h period at 37 °C (1 h intervals) using a microplate reader (Epoch,

Vermont, USA) with the increase in OD values being considered a consequence of bacterial growth. A positive control was drawn using inoculated MH medium without antimicrobial agent and sterile MH medium was used as a negative control. Blanks of the samples were performed to correct sample colour OD interference.

4.1.2.9. Statistical analysis

The normality of the samples was evaluated using the Shapiro-Wilk's Test. Two-way analysis of variance (ANOVA) with Tukey's post-test and 95% confidence level was carried out with GraphPad Prism 7.04 software (Dotmatics, Boston, MA, USA). Results were presented as mean values ± SD (standard deviation), and $p < 0.05$ was considered statistically significant. All experiments were performed in triplicate.

4.1.3. Results and discussion

Before assessing the ability of the MOS extracts to inhibit the adhesion of UPEC to the urothelial cells and given their intended purpose as dietary supplements for UTIs' prevention, it was necessary to establish the cytotoxic profile of the samples against urothelial cells. D-mannose was used as control and the immunomodulatory activity of the samples was also assessed.

4.1.3.1. Cytotoxicity

Cytotoxicity of the samples was assessed against HTB-9 cells, by evaluating their impact upon cell metabolism using a viable dye (**Figure 23**) and tacking into account the cytotoxicity threshold defined by ISO 10993-5 (2009), i.e. a sample is cytotoxic when a metabolic inhibition percentage above 30% is observed. Although a slight metabolic inhibition can be seen for D-mannose (14.79 ± 1%), according to the above-mentioned standard, none of the samples was cytotoxic to HTB-9 cells at a mannose concentration of 2.5 mg/mL. In fact, the negative values observed for MOS Parr (-98.59 ± 0.34%) and MOS H_3PO_4 (-73.87 ± 9.91%) indicate an increase in cell metabolism when in the presence of these two extracts. When considering the total weight of extract and not just mannose, the 2.5 mg/mL of mannose translates into a total extract concentration of 4.17 mg/mL for MOS Parr and 4.10 mg/mL for MOS H_3PO_4.

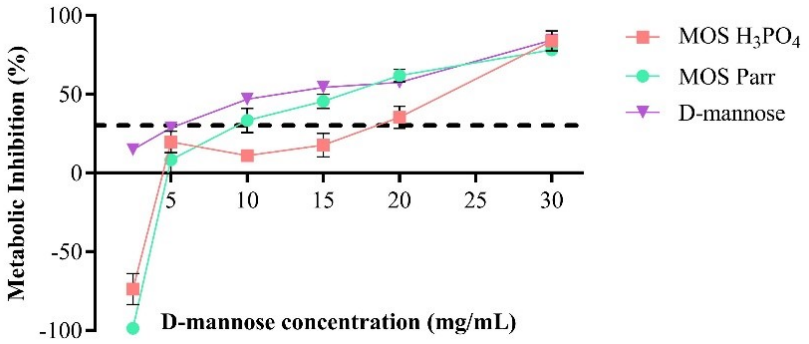


Figure 23. Cytotoxic effect on HTB-9 after 24 h of contact with the samples at different concentrations of D-mannose (mg/mL). Results are expressed in percentage of metabolic inhibition (%).

4.1.3.2. Impact upon UPEC growth

To ensure that the inhibitions observed for the MOS extracts and D-mannose were a result of an inhibition in adhesion and not a consequence of their impact upon bacterial growth and survival, the samples' influence on UPEC survival was studied by performing a growth inhibition assay, and results presented in **Figure 24**. As can be seen the samples had no impact on the bacterial growth, with UPEC following the typical bacterial growth curve. Furthermore, it is possible to observe that, except for D-mannose, after entering the stationary phase, the number of viable bacteria in culture starts to increase again, albeit at a much slower pace than in the exponential phase.

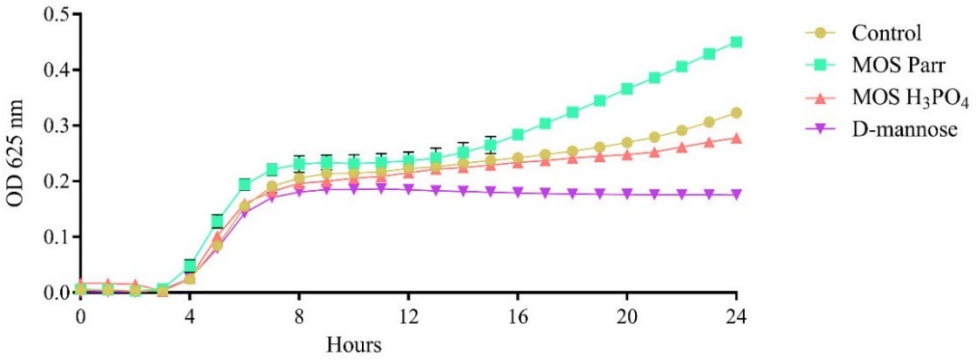


Figure 24. Effect upon the growth curves for UPEC exposed to the samples MOS extracts (Parr and H_3PO_4) and D-mannose.

Nevertheless, it was possible to demonstrate that none of the samples exerted any antimicrobial effect against UPEC.

4.1.3.3. Immunologic response (1, 2 and 3 h exposure to the samples)

Enhancing the innate immune properties of the urothelium (bladder cells) represents an attractive approach for the prevention and treatment of UTIs, as the urothelium secretes and responds to chemokines and cytokines as an important component of its response to UTIs (Svanborg et al., 1999). Produced by a myriad of cells such as T-cells, macrophages, fibroblasts, keratinocytes, glia cells, mesenchymal stem cells and endothelial cells, IL-6 is a pleiotropic cytokine that plays crucial roles in biological processes such as inflammation, immune response, and haematopoiesis (Kishimoto, 2005; Tanaka et al., 2014). IL-6 is expressed as a response to different stimulus molecules such as pathogen-associated molecular patterns (PAMPs) associated with pathogen infections or damage-associated molecular patterns (DAMPs) associated with damaged or dying cells due to trauma or burns (Moriyama & Nishida, 2021; Tanaka et al., 2014), thus acting as inflammation biomarkers. Thus MOS Parr, MOS H_3PO_4 and D-mannose samples were evaluated for their innate inflammatory profile and immunomodulatory assays were performed, focusing on this cytokine, using the supernatants collected after HTB-9 cells exposure to the samples.

The expression of IL-6 cytokine is depicted in **Figure 25**, revealing a tendency for increased IL-6 production by HTB-9 cells with longer incubation periods. Significant differences in cytokine expression were observed after 3 h of incubation with HTB-9 cells for MOS Parr ($p < 0.0001$), H_3PO_4 ($p < 0.01$), and D-mannose ($p < 0.001$). Except for specific time points, such as MOS Parr 1 h and 3 h, MOS H_3PO_4 1 h, and D-mannose 3 h ($p < 0.01$), no significant differences ($p > 0.05$) were observed between the samples and their controls. Moreover, except for MOS H_3PO_4 after 2 h of incubation ($p < 0.01$) no significant differences for IL-6 production levels were found between the extracts and D-mannose ($p > 0.05$). MOS has been commercially available as a feed additive and, therefore, information reported regarding its immunomodulatory effect in humans is, to our knowledge, non-existent.

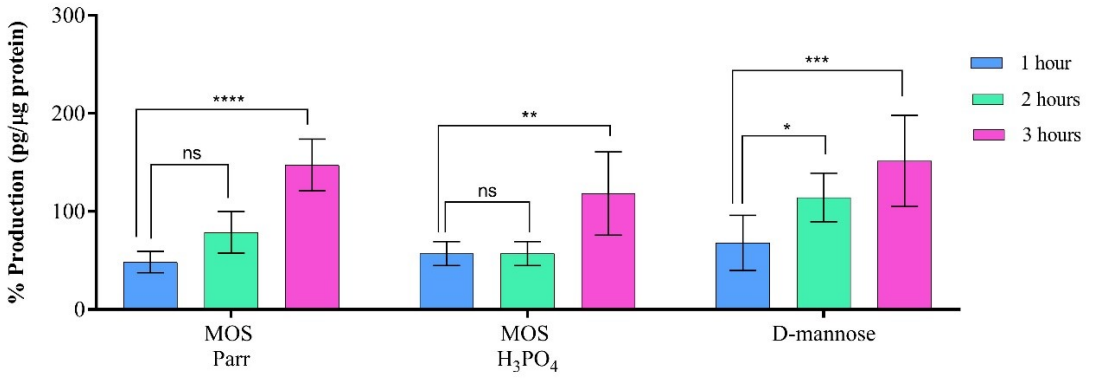


Figure 25. IL-6 produced values after HTB-9 cells exposure to MOS H_3PO_4, MOS Parr and D-mannose samples. Results are expressed in relation to the control in each incubation period and bars represent means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ indicate statistically significant differences observed for the different incubations periods within samples (ns – non-significant statistical differences).

4.1.3.4. MOS extracts inhibitory effect on UPEC adhesion to HTB-9

To assess the adhesion inhibition of the MOS extracts, HTB-9 cells were simultaneously exposed to the samples and to a UPEC suspension. As can be seen in **Figure 26**, adhesion inhibition values obtained for MOS H_3PO_4 (89.6 ± 1.4%), MOS Parr (79.8 ± 1.1%) and D-mannose (30.7 ± 5.9%) are significantly higher ($p < 0.001$) than those obtained for the control, demonstrating that all samples inhibited UPEC's adhesion to HTB-9 cells. Furthermore, it was possible to observe that although no significant difference in bacterial adhesion was found between MOS H_3PO_4 and MOS Parr ($p > 0.05$), both extracts display a higher adhesion inhibition effectiveness than D-mannose ($p < 0.001$).

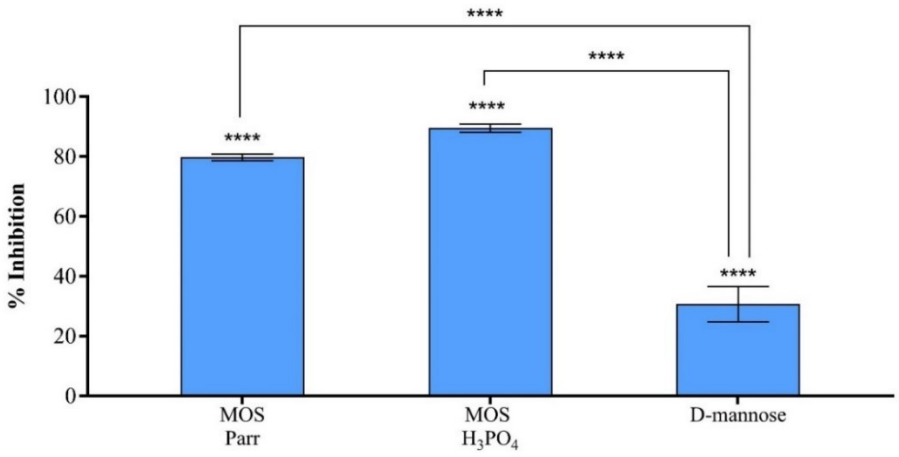


Figure 26. Percentage of adhesion inhibition of UPEC to HTB-9 after 2 h of simultaneous incubation with MOS H_3PO_4, MOS Parr and D-mannose Results are expressed in relation to the control and bars represent means ± SD. **** indicates statistically significant differences ($p < 0.0001$) between the MOS extracts and D-mannose, and between all samples and the control (HTB-9 cells in RPMI and infected with UPEC, used as reference for 100% survival).

Due to their exploitation in animal supplementation as prebiotics, scientific reports on MOS have been focusing on their impact on the gastrointestinal microflora and immune system of farm animals (Agazzi et al., 2020; Halas & Nochta, 2012; Hooge et al., 2003; Park et al., 2019; Zheng et al., 2021). Studies on the effect of MOS in UTIs are scarce (Brown et al., 2012), with most reports focusing on the benefit of using D-mannose as a therapeutic agent against UTIs (Brown et al., 2012; Kim & Lee, 2017; Porru et al., 2014; Ruggieri et al., 1985; Scribano et al., 2020; Taleb, 2018). One of the mechanisms through which UPEC is capable of adhering to cells is mediated by a bacterial ligand specific for D-mannose (FimH) located at the tip of type 1 pili anchored to UPEC's outer membrane (Crépin et al., 2012; Flores-Mireles et al., 2015, 2016; Ribić et al., 2018; Samuelsson et al., 2004; Schwan et al., 2018). D-mannose thus inhibits bacterial binding to the mannosylated proteins (UP1a) expressed on the surface of urothelial cells (Sarshar et al., 2020; Scribano et al., 2020), facilitating bacterial clearance though the urine flow.

4.1.3.5. Prophylactic potential of MOS extracts

To evaluate the prophylactic potential of MOS extracts supplements, HTB-9 cells were incubated with the samples for 1, 2 and 3 h before infection with UPEC. Incubation

periods were chosen considering that the majority of ingested mannose is filtered by the kidneys and excreted to the bladder via urine within 30 to 60 min (Scaglione et al., 2021), and that, although urinary frequency depends on a multitude of factors, it is commonly accepted that most people urinate over eight times in a 24 h period (up to 3 h interval between voiding) (Lukacz et al., 2009; McAchran et al., 2009). The infection was allowed to occur during 2 h before determination of the number of bacterial cells that were able to adhere to the urothelial cells (**Figure 27**). Results demonstrated that, within the first hour of incubation (**Figure 27a**), all samples limited UPEC's attachment to HTB-9 cells ($p < 0.0001$), with MOS Parr (59.3 ± 4.0%) and MOS H_3PO_4 (64.6 ± 0.9%) extracts inhibiting UPEC adhesion at significantly higher levels ($p < 0.0001$) than D-mannose (25.4 ± 5.6%). However, the inhibitory effect of the extracts significantly decreased (**Figure 27b and c**) after 2 h ($p < 0.001$ for MOS Parr and $p < 0.0001$ for H_3PO_4) and after 3 h ($p < 0.0001$ for both extracts) of incubation when compared with D-mannose, with this reduction being more substantial after 3 h. Indeed, the negative values observed for MOS Parr after 2 h (-5.19 ± 3.4%) and after 3 h of incubation (-42.3 ± 21.6%) and the negative values observed for MOS H_3PO_4 after 2 h (-24.6 ± 12.3%) and 3 h (-124.0 ± 41.0%) reveal that the extracts might be promoting bacterial adhesion to HTB-9 cells. A reasonable explanation for this observation may lay in the likely binding of cell proteins to the lipidic content of the MOS extracts, thus forming time-depending protein–lipid complexes that would act as sort of "adhesion bridges" between the urothelium and the UPEC's FimH binding sites saturated with mannose from the same MOS extracts. While not statistically significant ($p > 0.05$) for the MOS Parr extract after 2 h, this putative bacterial adhesion promotion can be slightly seen for MOS Parr after 3 h ($p < 0.01$) and is statistically evident for the MOS H_3PO_4 extracts after 2 h ($p < 0.001$) and after 3 h ($p < 0.0001$) of incubation with the urothelial cells. These results were in line with what has been previously reported by our group regarding the composition of MOS Parr and MOS H_3PO_4 extracts, when it was demonstrated that, although composed of the same percentage of protein, the latter possess a lower total sugar content, thus indicating a higher percentage, for instance, of lipids (among others) in MOS H_3PO_4, since MOS extracts are also composed of lipids (Faustino et al., 2021). Differently from what is observed with the MOS extracts, the inhibition efficacy of D-mannose was superior after 3 h of incubation with the HTB-9 cells ($p < 0.05$).

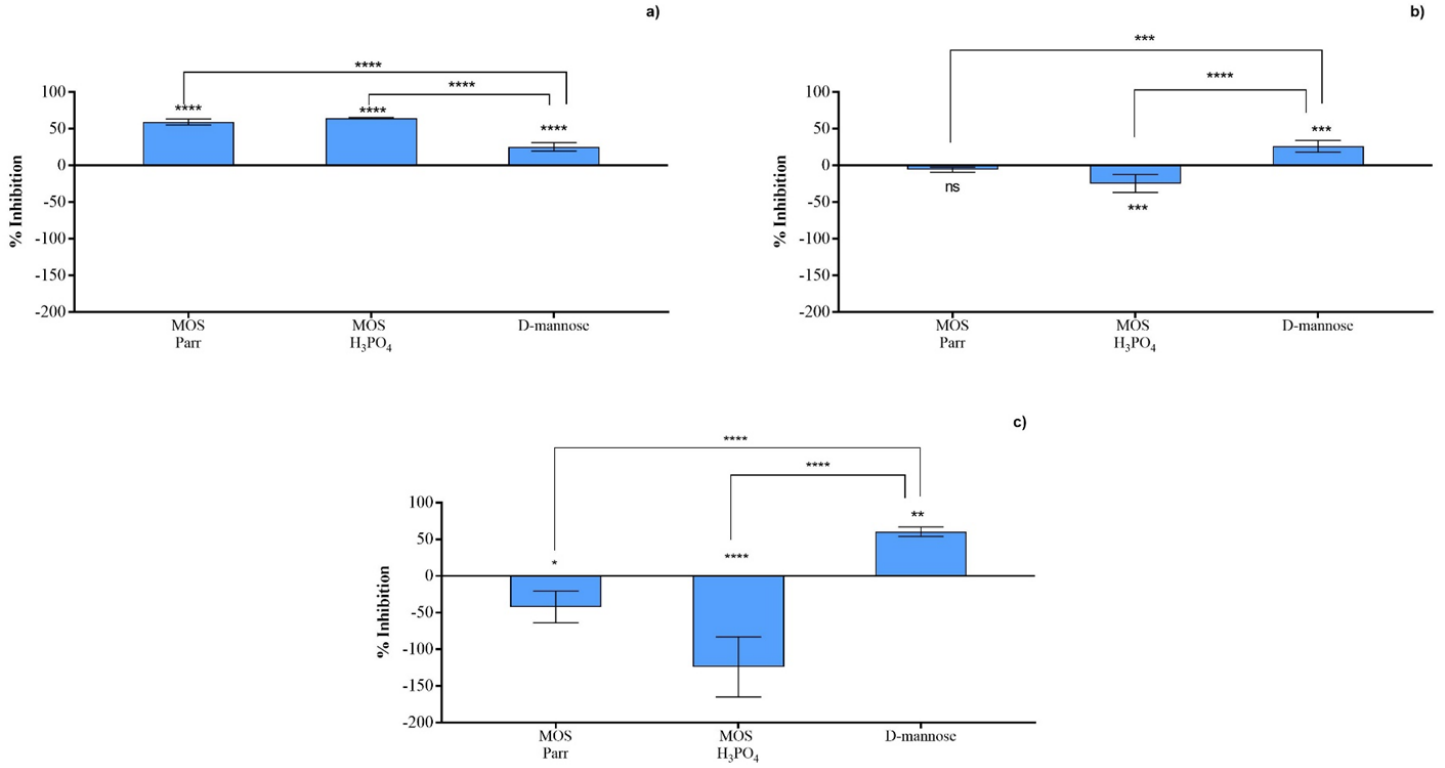


Figure 27. Percentage (%) of adhesion inhibition of UPEC to HTB-9 after 1 h (a), 2 h (b) and 3 h (c) of HTB-9 incubation with MOS H_3PO_4, MOS Parr and D-mannose. Bars represent means ± SD. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$ indicate statistically significant differences between the MOS extracts and D-mannose and between the different samples and the control (ns – non-significant statistical differences). Results are expressed in relation to the control (HTB-9 cells in RPMI and infected with UPEC, used as reference for 100% survival).

4.1.3.6. Cytokine production in the presence of UPEC

In addition to providing a physical barrier against invading microorganisms, epithelium bladder cells also act as first line of defense against urinary tract infections, with crucial roles such as pathogen recognition, recruitment of phagocytes, production of antimicrobial molecules or the release inflammatory mediators and cytokines (Spencer et al., 2014). IL-6 is rapidly excreted from urothelial cells after exposure to *E. coli* (Hedges et al., 1994; Samuelsson et al., 2004; Schilling et al., 2003). Studies by De Man et al., (1989), Wult et al., (2001) and Schiling et al., (2001) have implicated bacterial determinants such as lipopolysaccharide (LPS) and P-fimbriae in urothelial production of IL-6. In the bladder urothelium, LPS interacts with Toll-like receptor (TLR) 4 and triggers an intracellular signalling cascade, leading to IL-6 secretion (Samuelsson et al., 2004; Schilling et al., 2003; Schilling, Mulvey, Vincent, et al., 2001). Thus, and considering that the bladder is commonly a sterile site, UTI generates a local powerful inflammatory response, with neutrophils, IL-6 and IL-8 being found in the urine of affected individuals (Graham & Galloway, 2001; Jantausch et al., 2000). IL-6 expression levels can be an indicator of the severity of clinical UTIs (Hannan et al., 2010; Schwartz et al., 2015). Indeed, children with pyelonephritis and renal scarring have been reported to exhibit higher IL-6 levels in urine and serum than those of children with cystitis (Rodríguez et al., 2008; Sheu et al., 2006, 2009; Tramma et al., 2012). To assess the effect of the samples in the immune response of bladder cells, expression levels of the pro-inflammatory IL-6 were assessed in HTB-9 cells simultaneously exposed to the samples and subjected to UPEC infection at a MOI of 10 (**Figure 28**). This effect was also evaluated in HTB-9 cells infected with UPEC after pretreatment with the samples for 1 h, 2 h and 3 h (**Figure 29**).

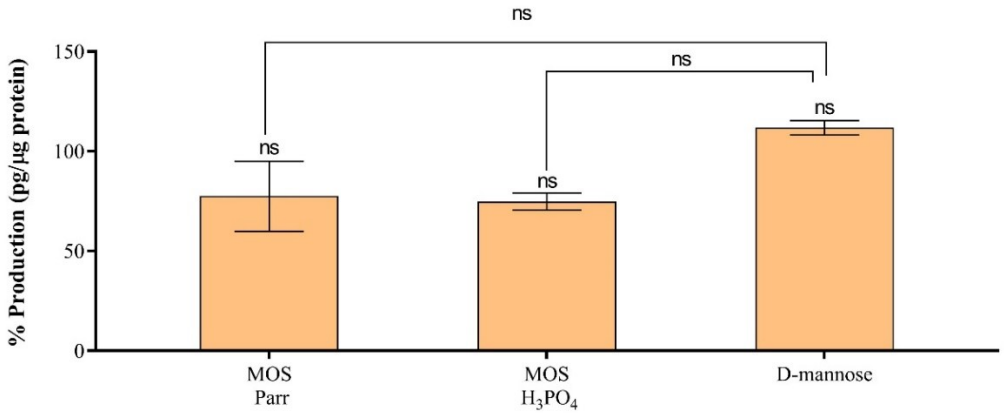


Figure 28. Quantification of IL-6 production levels by HTB-9 cells simultaneously exposed to UPEC, MOS extracts and D-mannose. No significant statistically differences (ns; $p < 0.05$) were found between MOS extracts and D-mannose nor between the different samples and the control. Bars represent means ± SD and results are expressed in relation to the control (HTB-9 cells in RPMI medium exposed UPEC, used as reference for 100% IL-6 expression).

Determination of the expression levels of IL-6 on the supernatants of HTB-9 cell cultures exposed simultaneously to the samples and to UPEC (**Figure 28**) revealed no significant alteration ($p > 0.05$) in cytokine secretion when compared to cells in RPMI infected with UPEC (control). Moreover, expression levels of IL-6 obtained for cells exposed to both MOS extracts and those obtained for cells exposed to D-mannose were not statistically different ($p > 0.05$). Given these results, it was reasonable to conclude that MOS extracts and D-mannose were not immunomodulatory and that only bacteria appeared to be contributing to the immune response of the urothelium cells observed. This conclusion was further supported by the results attained with HTB-9 cells pre-treated with the samples (**Figure 29**), where no synergetic effect was observed ($p > 0.05$) between the samples and the bacteria, except for cells pre-treated with MOS Parr for 1 h and 2 h ($p < 0.0001$), where is possible to see an exacerbation of the pro-inflammatory response (**Figure 29a and b)**. The observed behaviour of an initial response followed by a subsequent decrease in activity could potentially be attributed to the breakdown of a component that can be metabolized by the cells or the formation of complexes with certain components in the medium, leading to a loss of effectiveness. This hypothesis suggests that the component's activity may be compromised over time, resulting in the observed decrease in response.

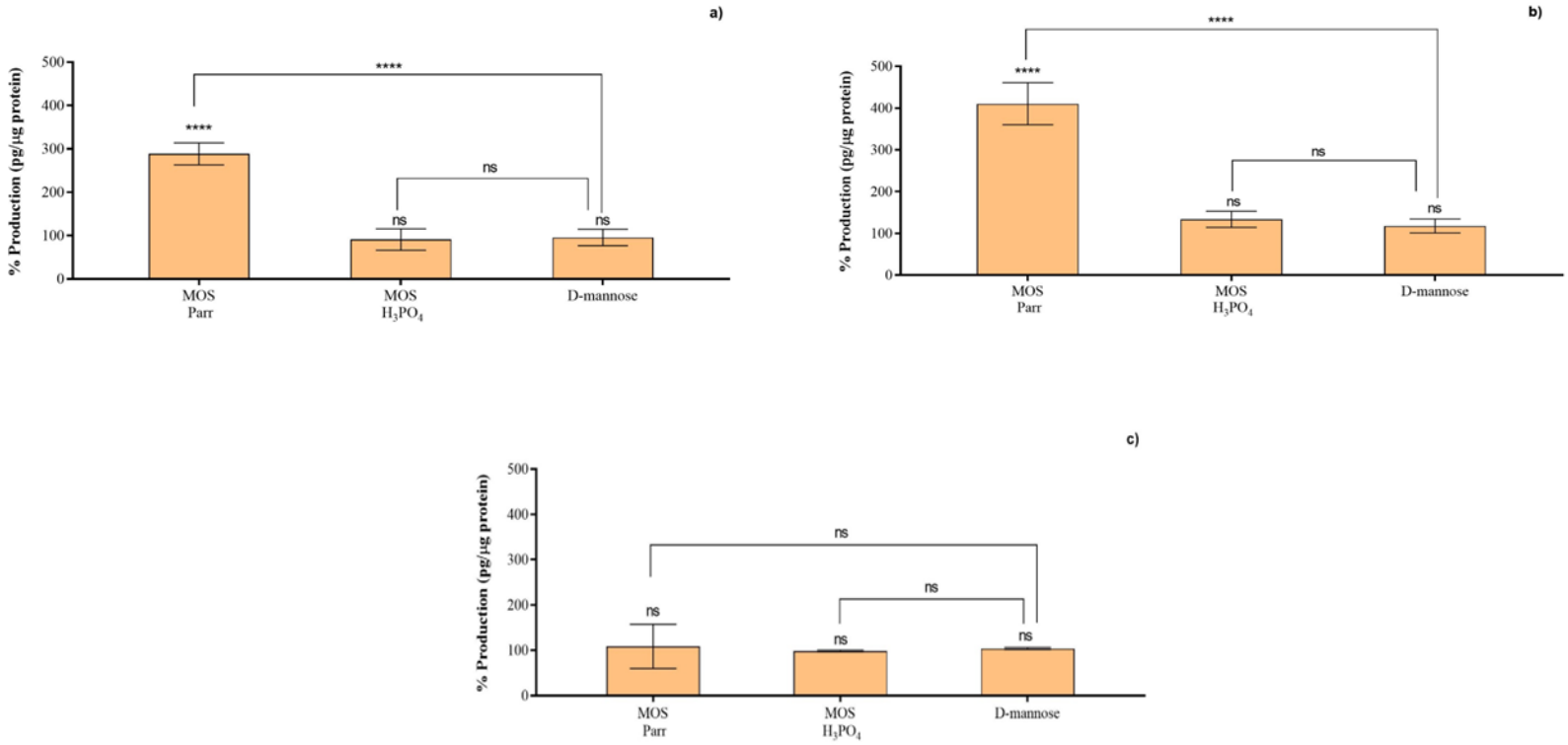


Figure 29. Quantification of IL-6 production levels by HTB-9 cells incubated with UPEC after exposition to MOS extracts and D-mannose for 1 h (a), 2 h (b) and 3 h (c). Bars represent means ± SD. **** indicate statistically significant differences ($p < 0.0001$) between MOS extracts and D-mannose (ns – non-significant statistical differences). Results are expressed in relation to the control (HTB-9 cells pretreated with RPMI medium before UPEC induced infection, used as reference for 100% survival at each incubation period).

4.1.4. Conclusions

In this study, feasibility of using MOS extracts to prevent UTIs was assessed and although their prophylactic capacity can be hindered by some unexpected interaction with the cells, they exhibit potential use as a dietary supplement aiming the handling of UTIs, due to its outstanding competitive inhibition of UPEC adherence to urothelial cells. Indeed, the competitive assay demonstrated that the effectiveness of bacterial adherence inhibition to bladder cells of the MOS was 2- to 3-fold higher than the one of D-mannose. Moreover, MOS extracts showed no significant immunomodulatory effect or evoke a cytotoxic response up to 2.5 mg/mL of mannose concentration. These promising results must, however, be validated with samples submitted to an *in vitro* simulation of the gastrointestinal tract (GIT) system, since it is known that, to reach their destination, bladder targeted food supplements must, firstly, go through a digestion process and mainly through an absorption process that can alter the structure of the molecules that compose them and, consequently, their activity with consequences in the amount absorbed that may not be sufficient to produce the desired effect.

4.2. Impact on vaginal pathogenic Candida albicans and vaginal lactobacilli: Lactobacillus crispatus

Alternative strategies to prevent Candida albicans proliferation in vaginal environment: synergistic effect of lactobacilli and mannan oligosaccharides (MOS)

Abstract: Vulvovaginal candidiasis (VVC) affects approximately 30-50% of women at least once during their lifetime. This infection causes several symptoms, promoting discomfort and limitations in their daily quality of life. Antifungal therapy is not very effective, does not prevent recurrencies and usually causes side effects. Therefore, alternative therapies are urgently needed. The purpose of this work was to investigate the potential benefits of using mannan oligosaccharides (MOS) extracts together with a *Lactobacillus* sp. pool, composed by the most significant species present in the vaginal environment, to prevent infections by *Candida albicans*. Microbial growth of isolated strains of the main vaginal lactobacilli and *Candida* strains was assessed with the presence of MOS, to screen their impact upon growth. A pool of the lactobacilli was then tested against *C. albicans* in competition and prophylaxis studies; bacterial and yeast cell numbers were quantified in specific time points, and the above-mentioned studies were performed in simulated vaginal fluid (SVF). Finally, adhesion to vaginal epithelial cells (HeLa) was also evaluated, once again resorting to simultaneous exposure (competition) or prophylaxis assays, aiming to measure the effect of MOS presence in pathogen adherence. Results demonstrated that MOS extracts have potential to prevent vaginal candidiasis in synergy with vaginal lactobacilli.

Keywords: Mannan oligosaccharides; *Lactobacillus crispatus*; *Candida albicans*; HeLa cells; Competition; Prophylaxis; Vaginal therapeutics

4.2.1. Introduction

Worldwide, 30-50% of women suffer from vulvovaginal candidiasis (VVC) at least once in their lifetime (Corsello et al., 2003; Foxman et al., 2013); in many situations their appearance is recurrent (Sobel, 2014). This infection causes several symptoms, from which white discharge, local itching, dyspareunia, vulvar erythema, and swelling are typically reported (Al-Ghazzewi & Tester, 2016; Falagas et al., 2006). An increased amount of studies highlights the significant impact of fungal infections on the quality of life of women, underscoring the importance of optimizing patient management and treatment for those diagnosed with VVC (Aballéa et al., 2013). Therefore, the study of alternative strategies to replace or to combine with standard therapies for more efficient prevention and/or treatment of VVC is of great relevance.

VVC is mainly caused by *Candida* species, mainly *Candida albicans*, *Candida glabrata*, or *Candida krusei* (Jacob et al., 2018). The currently available antifungal treatments and antifungal agents, result in suboptimal cure levels and high recurrence rates, due to acquired antimycotic resistance (van de Wijgert & Verwijs, 2020; Zangl et al., 2020). Alternative strategies may include substances that aim to re-establish the "healthy" physiologic vaginal environment such as probiotics, prebiotics, and acidifying agents, while improving the local immunity response.

The vaginal microbiota of healthy women consists typically of a diversity of anaerobic and aerobic microorganisms. Lactobacilli are the most prevalent and often numerically dominant microorganisms and are relevant as a barrier to infection. The capacity of lactobacilli to adhere and compete for the adhesion sites in the vaginal epithelium and the capacity to produce antimicrobial compounds (hydrogen peroxide, lactic acid, bacteriocin-like substances), are important traits in the impairment of colonization by pathogens (Borges et al., 2014). Six types of vaginal microbiota, named community state types (CSTs), have been described. The CST I is dominated by *Lactobacillus crispatus*, CST II by *Lactobacillus gasseri*, CST III by *Lactobacillus iners*, CST IV A and CST IV-B by different taxa composed by anaerobic bacteria, and CST V by *Lactobacillus jensenii* (Lacroix et al., 2020).

The pro- and prebiotic approaches have been widely studied in the treatment of gastrointestinal infectious diseases, inflammatory bowel disease (such as ulcerative colitis) (Lin et al., 2008), Crohn's disease (De Vrese & Schrezenmeir, 2008), and in prevention of colon cancer (Liong, 2008). The use of probiotics for vaginal health is predominantly

reported on both oral and topical routes (Coste et al., 2012; Hilton et al., 1992), supporting the possibility of vaginal microbiota restoration after oral administration of lactobacilli. Furthermore, many clinical studies have reported the efficacy of probiotics in treatment and prevention of vaginal infections such as bacterial vaginosis (Homayouni et al., 2014), vulvovaginal candidiasis (Falagas et al., 2006), and UTIs (Borchert et al., 2008) when administered topically. The utilization of vaginal probiotics is based on the importance of maintaining a healthy vaginal microbiota and the necessity to restore the microbial ecosystem following disturbances (Mastromarino et al., 2013).

A potential alternative to the probiotic treatments is the use of prebiotic compounds, owing to their possible local microflora stimulus effect. To the best of our knowledge there are only a few reports on the use of prebiotic carbohydrates for vaginal infections. Tester & Al-Ghazzewi (2018) reported the use of oligosaccharides or polysaccharides (derived from glucose, fructose, galactose, mannose and uronic acids) locally delivered in the form of pessaries to stimulate the selective growth of healthy vaginal bacteria. The introduction of these carbohydrates into the vagina aims to provide prebiotics that are selectively utilized by lactic acid bacteria, inhibiting the growth of pathogens (Tester & Al-Ghazzewi, 2018). A study by Sutherland et al., (2009) has also shown the preventive potential of topical application of prebiotics on vaginal infections, such as bacterial vaginosis and candidiasis. Rousseau et al., (2005) reported beneficial effect of fructo-oligosaccharides and gluco-oligosaccharides on the growth of vaginal lactobacilli in *in vitro* models. A clinical study by Coste et al. (2012) has shown the recovery of normal vaginal flora in patients treated with gels containing gluco-oligosaccharides. In a related research direction, Rousseau et al., (2005) and Bou-Antoun (2011) investigated the *in vitro* impact of fructo- and galacto-oligosaccharides on vaginal lactobacilli, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus fermentum*. These oligosaccharides were utilized to promote the growth of beneficial vaginal microflora and regulate the proliferation of pathogens.

In this work, the feasibility of using yeast mannan oligosaccharides (MOS) as supplements for the management of candidiasis was evaluated by assessing its effect on *Lactobacillus* sp. and *C. albicans*. Competition and prophylaxis assays were performed in simulated vaginal fluid (SVF). Additionally, MOS effect on the adhesion to HeLa vaginal cells of both *Lactobacillus* sp. and *C. albicans* was studied also in competition and prophylaxis simulation assay.

4.2.2. Materials and Methods

4.2.2.1. MOS extracts, commercial benchmark, culture media and microorganisms

MOS extracts (MOS H_3PO_4 and MOS Parr) were obtained from genetically modified spent yeast (*S. cerevisiae*) kindly provided by Amyris Company (Emeryville, California, USA), according to methodologies previously described in **section 3.1.2.1. of Chapter 3.1**, and D-mannose, was purchased from Sigma-Aldrich. All microorganisms (listed in **Supplementary material Table S 1)** were acquired from DSMZ (Braunschweig, Germany). Different culture media used were according to the assays requirements: (i) Broth and agar De Man, Rogosa and Sharpe (MRS, Biokar Diagnostics, Allone, France) for *Lactobacill*us sp.; (ii) Yeast & Mould broth (YM, Sigma-Aldrich, St. Louis, MO, USA) for growth of *Candida* sp.; (iii) Simulated vaginal fluid (SVF) pH 4.2 (reagents used are listed in **Supplementary material Table S 2)** according to the recipe described in Owen and Katz (1999); and (iv) Chromogenic Candida agar (CHROMagar Candida, Frilabo, Maia, Portugal).

4.2.2.2. Inoculum preparation

L. crispatus, *L. gasseri* and *L. jensenii* were grown at 37 °C in anaerobic conditions in modified MRS, with 5 g/L of glucose and 0.05% L-cysteine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). The amount of glucose was reduced from the original recipe to mimic the sugar content of SVF, whereas cysteine was added due to *L. crispatus* nutritional requirements, according to DSMZ recommendations. *C. albicans*, *C. glabrata* and *C. tropicalis* were grown at 30°C in aerobic conditions in YM broth.

4.2.2.3. MOS antifungal activity against Candida sp.

The study of the potential antifungal activity of the different samples (MOS H_3PO_4, MOS Parr and D-mannose) was performed in modified Muller Hinton (MH) (CLSI, 2012). Natamycin (Sigma- Aldrich, St. Louis, USA) was used as positive control, while MH medium was used as negative control. All samples and Natamycin were diluted at 2% (w/v) in MH medium and sterilized using a sterile 0.22 µm filter (Millipore, Billerica, MA, USA).

The above-mentioned *Candida* strains were grown as a monoculture before the assay in YM broth at 30 °C for 24 h under aerobic conditions, and subsequently cultivated in solid

media. Afterwards, one colony was picked, resuspended in 10 mL of MH broth, and grown at 37 °C for 24 h under aerobic conditions. The inoculum was then adjusted to an optical density (OD) at 625 nm of 0.1 - 0.08 (cell density of $1x10^8$ cells/mL) and diluted 10 times to obtain the work inoculum. To evaluate bacterial growth inhibition, 980 µL of each sample were transferred to a sterile microtube and inoculated with 20 µL of the work inoculums. Once transferred to 96-well microplate (Nunc, Darmstadt, Germany), bacteria were incubated for 48 h and growth was monitored at 625 nm and 37 °C (1 h intervals), using a microplate reader (Epoch, Vermont, USA). Blank of the samples were included to compensate any interference in sample colour and optical density (OD).

4.2.2.4. Assessing microbial growth in simulated vaginal fluid: competition and prophylaxis assays

Lactobacillus sp. pool preparation

Despite the referred prevalence of *L. crispatus*, *L. gasseri* and/or *L. jensenii* in vaginal environmental, this is not a consensual issue, since vaginal microbiome may change between individual women but also depend on age and ethnicity, among others (Zangl et al., 2020). Therefore, to simulates the predominant environment in the vaginal flora of the *Lactobacillus* sp. population, a mix of the three strains of vaginal lactobacilli (*L. crispatus*, *L. gasseri and L. jensenii*) in a ratio of 1:1:1 was used, with a final OD of 0.1 at 625 nm.

Simulated vaginal fluid

SVF was prepared according to the procedure from Owen & Katz (1999) with a final pH of 4.2. This pH value was selected to replicate the vaginal pH in normal conditions. The solution was sterilized at 121 °C for 20 min before adding 0.05% L-cysteine hydrochloride, previously sterilized through filtration using a 0.22 µm filter (Millipore, Billerica, MA, USA).

Microbial growth curves with isolated strains

The effect of the different MOS extracts on microbial growth was determined by screening their impact upon the growth curves of *Lactobacillus* sp. (*L. crispatus*, *L. gasseri and L. jensenii*) and vaginal pathogens (*C. glabrata, C. albicans* and *C. tropicalis*) in

modified SVF. This preliminary assay allowed to better design the subsequent prophylaxis and competition assays, in which both *Lactobacillus* sp. and *Candida* sp. species are used simultaneously.

The *Lactobacillus* strains, and *Candida* strains were grown as described in **section 4.2.2.2**. The inoculums were centrifuged after 24 h of growth (4700 x *g*, 5 min, 4 °C), washed twice with PBS, pH 7.4 and resuspended in the same initial volume with SVF. To obtain a standard concentration in each condition, inoculums were diluted to an OD of 0.1 at 625 nm, to which 2% (v/v) of each extract, previously prepared by dissolving MOS in SVF at 2% (w/v) and sterilizing with a 0.22 µm pore size filter, was added. The assay was performed in a 96-well microplate (Nunc, Darmstadt, Germany) to which 200 µL of each sample condition were transferred. Additionally, 50 µL of paraffin were added to ensure the anaerobic environment. The microplates were incubated for 48 h at 37 °C, and absorbance was measured at 625 nm, every hour, with a multidetector plate reader (Epoch, Vermont, USA). SVF medium was used as negative control. The D-mannose was used as a commercial benchmark against MOS extracts (since these are mainly composed by mannose oligomers), also at 2% (w/v) in SVF. The experiment was performed in triplicates.

Prophylaxis assay (sequential exposure)

Each of the three *Lactobacillus* sp. inoculums was adjusted to an OD (625 nm) of 0.1 and then mixed in a proportion of 1:1:1. This pool was allowed to grow in SVF for 16 h in anaerobiosis (selected time based on the results from **Supplementary material Figure S 4**) with the different extracts at a concentration of 2% (w/v). At the end of the 16 h, the *C. albicans* inoculum (OD of 0.1, at 625 nm) was added at 2% (v/v). Sampling points were carried out at 0 h and 16 h pre-infection, and then at 18 h, 20 h, 24 h and 40 h after infection with *C. albicans*. Sequential 10-fold dilutions were carried out in sterile peptone water (Sigma-Aldrich, St. Louis, USA) and plated (in quadruplicate), using the drop technique (Miles et al., 1938). MRS agar with 98 mg/L of fluconazole (to inhibit *Candida* growth) was used for *Lactobacillus* sp. counting, while CHROMagar was used for *C. albicans* counting, incubated at 37 °C under anaerobic conditions and 30 °C under aerobic conditions, respectively.

Competition assay (simultaneous exposure)

Except for adding the *Lactobacillus* sp. pool and *C. albicans* to SVF at the same time (both at 2% (v/v)) followed by incubation at 37 °C in anaerobic conditions, this assay was performed as described for the prophylaxis assay. Sampling points were carried out, at 0 h, 2 h, 4 h, 8 h, 24 h and 48 h. Microorganism counting methodology was the performed as described in **section 4.2.4.4. Prophylaxis assay (sequential exposure).**

4.2.2.5. Adhesion to cervical cells

Cell line growth conditions

Immortalized human cervical (HeLa; CCL-2™) cells from the American Type Culture Collection (ATCC, Manassas, VA) used in this study were kindly provided by Prof. José das Neves from Instituto de Investigação e Inovação em Saúde (i3S). HeLa cells were maintained in high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat inactivated Fetal Bovine Serum (FBS) and 1% (v/v) antibiotic and antimycotic (Invitrogen, MA, USA), using routine cultures conditions (37 °C in a humidified atmosphere with 5% CO_2). Cells were used between passages 29 and 49.

Cytotoxicity assay

Cytotoxicity of the samples was assessed in the HeLa cell line according to ISO 10993-5 (2009), using PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific, MA, USA) as per manufacturer's instructions, the protocol was described in **section 2.1.2.5. of Chapter 2.1**. The samples (MOS Parr, MOS H_3PO_4 and commercial D-mannose) were directly dissolved in DMEM medium and sterilized using a sterile filter with a 0.22 µm pore size (Millipore, Billerica, MA, USA) at a concentration of 20 mg/mL. Decimal dilutions were performed to test the concentration range of 10.0–0.31 mg/mL.

4.2.2.6. Adhesion assays and growth

Individual adhesion to vaginal epithelial cell-line

HeLa cells at a confluency of 80% were harvested and seeded at a final concentration of 2.0×10^4 cells/mL in a 24-well microtiter plate and immediately incubated at 37 °C with

5% CO_2 in a humidified environment for 24 h. After this incubation period, cells were washed twice with PBS to remove all the antibiotic-containing medium. *L. crispatus* and *C. albicans*, previously grown according to what was described in **section 4.2.2.2.**, were centrifuged (4700 x g, 5 min, 4 °C), washed twice with PBS and resuspended in the same buffer at a multiplicity of infection (MOI) of 100, while the samples (MOS H_3PO_4, MOS Parr and D-mannose) were diluted in DMEM medium at a non-cytotoxic concentration (2.5 mg/mL). HeLa cells were exposed to the samples and to *L. crispatus* or *C. albicans* suspension in PBS, and incubated for independent time-points: 15, 30, 60 and 120 min, at the same incubation conditions above-mentioned (Rizzo et al., 2013). DMEM was used as control in each incubation period.

Competition assay (simultaneous exposure)

Similarly, to the above-described protocols, HeLa confluent monolayers were trypsinized and then seeded at a final concentration of 2.0×10^4 cells/mL in a 24-well microtiter plate and incubated at 37 °C with 5% CO_2 in a humidified environment. After 24 h of incubation, medium was discarded, and cells were washed twice with PBS to remove any antibiotic traces. A detailed description of the preparation of *L. crispatus* and *C. albicans* inoculums for cell inoculation can be found in **section 4.2.2.6. Individual adhesion to vaginal epithelial cell line**. MOS H_3PO_4, MOS Parr and D-mannose were diluted in antibiotic-free DMEM medium to a final concentration of 2.5 mg/mL. HeLa cells were then simultaneously exposed to the samples and to *L. crispatus* and *C. albicans* suspension in PBS, incubated for the same time-points and incubation conditions above-mentioned. DMEM was used as control in each incubation period.

Prophylaxis assay (sequential exposure)

HeLa cells at a confluency of 80% were seeded according to the described in **section 4.2.2.6. Individual adhesion to vaginal epithelial cell line** and incubated at 37 °C with 5% CO_2 in a humidified environment. After 24 h of incubation, medium was discarded, and cells were washed twice with PBS to remove any antibiotic traces. A detailed description of the preparation of *L. crispatus* and *C. albicans* inoculums for cell inoculation can be found in **section 4.2.2.6. Individual adhesion to vaginal epithelial cell line**. Samples (MOS H_3PO_4, MOS Parr and D-mannose) were diluted in antibiotic-free DMEM medium to a

concentration of 2.5 mg/mL. HeLa cells, that were then incubated at 37 °C with 5% CO_2 in a humidified environment with the samples and the *L. crispatus* at MOI of 100 during 2 h before infection of the vaginal cells with *C. albicans*. DMEM was used as control in each incubation period. After incubation, *C. albicans* was added to the cells with samples and *L. crispatus* at MOI of 100, followed by an incubation for the same time-points and incubation conditions above-mentioned.

Total Viable Count Determination

At the end of the cellular assays cell monolayers were extensively washed twice with PBS to remove unbound bacteria and after detachment with trypsin (TrypLE™ Thermo Fisher Scientific, Massachusetts, USA), cells were resuspended in PBS. The same method for dilutions and plating is described in **section 4.2.2.4. Prophylaxis assay (sequential exposure** was used. Results were expressed as colony forming unit (CFU) per mL per HeLa cells in well, as described in Equation 7.

$$[(\text{CFU/mL})/\ (\text{HeLa cells in well})] = \left(\frac{\frac{CFU}{mL} of\ Sample}{HeLa\ cells\ in\ well}\right) \quad (7)$$

4.2.2.7. Statistical analysis

The normality of the samples was evaluated using the Shapiro-Wilk's Test. Two-way analysis of variance (ANOVA) was conducted followed by Tukey's post-test. Tests were carried out using GraphPad Prism 7.04 software (Dotmatics, Boston, MA, USA). Results were presented as mean values ± SD (standard deviation). Statistical significance was determined at a 95% confidence level. All experiments were performed in triplicate.

4.2.3. Results and Discussion

Candida albicans is the predominant species isolated in women with vulvovaginal candidiasis (VVC), accounting for 80-90% of cases. Other species such as *C. glabrata*, *C. tropicalis, C. krusei* and *Candida parapsilosis*, and are less commonly identified (Blostein et al., 2017; Ferrer, 2000; Gonçalves et al., 2015; Makanjuola et al., 2018; Sobel, 2007).

The effectiveness of using probiotics to prevent/treat yeast infections is highly variable in the literature (Falagas et al., 2006). According to Abad & Safdar (2013), efficacy evaluation of probiotics in the prevention or treatment of three urological illnesses (bacterial

vaginosis, VVC and UTI) revealed that lactobacilli might help cure bacterial vaginosis, but there was no evidence supporting their benefit against candidiasis or UTI.

Carbohydrates such as gluco-oligosaccharides, fructo-oligosaccharides, galacto-oligosaccharides and glucomannan hydrolysates were already reported in as beneficial in vaginal therapy (Bou-Antoun, 2011; Coste et al., 2012; Rousseau et al., 2005; Sutherland et al., 2009; Tester et al., 2012; Tester & Al-Ghazzewi, 2016). According to Rousseau et al., (2005) fructo-oligosaccharides and gluco-oligosaccharides selectively promote the growth of vaginal lactobacilli *in vitro*. Another report concludes that gels containing gluco-oligosaccharides promote the return of normal vaginal flora and maintenance of appropriate pH in women who had previously been treated with metronidazole (Coste et al., 2012).

The present study aims to assess the potential of yeast mannan oligosaccharides (MOS) as a synergetic therapy with probiotics, to promote *Lactobacillus* sp. (*L. crispatus*, *L. gasseri* and *L. jensenii*) population in vaginal flora and simultaneously regulate the uncontrolled growth of *C. albicans*, thus promoting a healthy vaginal microbiome.

4.2.3.1. Antimicrobial activity against C. albicans, C. tropicalis and C. glabrata

The antimicrobial potential of the MOS extracts on several *Candida* strains (*C. albicans, C. glabrata* and *C. tropicalis)* was evaluated, and results are shown in **Figure 30**.

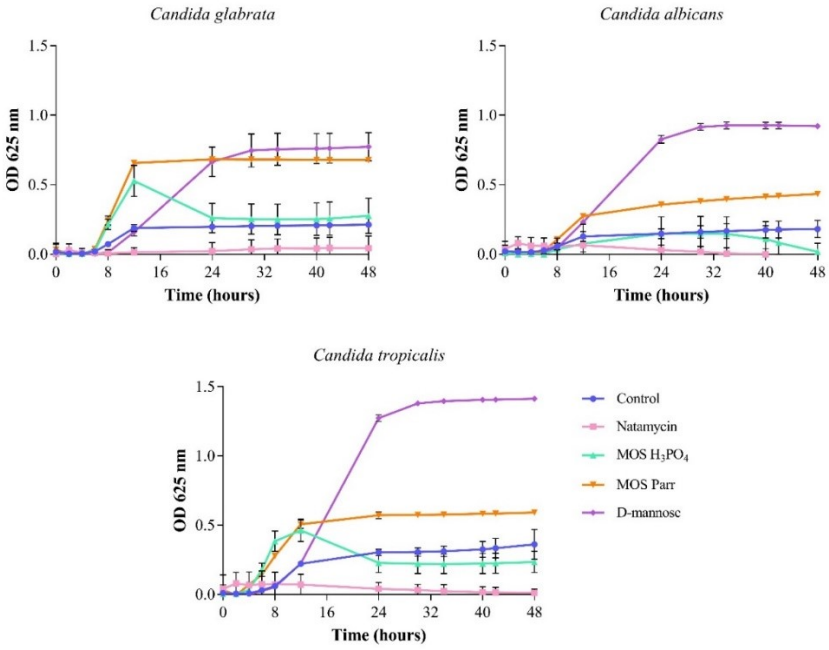


Figure 30. Screening of antimicrobial potential of several mannose-based extracts (MOS Parr, MOS H_3PO_4 and D-mannose) against *C. glabrata*, *C. albicans* and *C. tropicalis* in Muller Hinton medium.

Commercial D-mannose was used for comparison with MOS, as these are composed essentially of mannose monomers. *Candida* strains exhibited the highest growth rate when exposed to mannose, especially *C. tropicalis* and *C. albicans*. MOS Parr was the second-best growth promoting sample, able to promote growth of all strains of *Candida* sp. On contrary, MOS H_3PO_4 extract did not significantly promote the growth of the *Candida* strains, as evidenced by the similar OD values read when compared to the medium control, which indicates prevalence of the yeast but not the promotion of its growth. It is, thus, possible to conclude that, neither mannose nor MOS extracts present antifungal activity against the above tested *Candida* strains. Literature studies on the antifungal activity of MOS is scarce, however, other oligosaccharides such as chitosan and alginate oligosaccharides have been studied and demonstrated antifungal activity against *Candida* sp. (Ganan et al., 2019; Powell et al., 2023).

4.2.3.2. Assessing microbial growth in simulated vaginal fluid: competition and prophylaxis assays

Screening of growth curve for each microorganism in SVF

A healthy vaginal environment is frequently associated with microbiota dominated by *L. crispatus*, *L. gasseri*, and/or *L. jensenii* (Burton et al., 2003; Petrova et al., 2017). Nevertheless, some women present a microbiome with more facultative and anaerobe bacteria (Ravel et al., 2011), such as *Prevotella* or *Gardnerella* (Green et al., 2015; Hickey et al., 2012), which are also considered as healthy, typical vaginal flora in asymptomatic women, and their presence does not always indicate illness (Zangl et al., 2020). However, for the present study, the three above-mentioned lactobacilli were considered as the typical microbiota from healthy vaginal environment. Concerning *Candida* species, *C. albicans* was selected as the most representative pathogenic yeast (Turner & Butler, 2014).

Since SVF was used in this study as a good proxy for the vaginal environment (Borges et al., 2012, 2013; Owen & Katz, 1999), the behaviour of the microorganisms under study had to be, initially, evaluated on this fluid, i.e., growth curves needed to be performed in SVF to define infection times during prophylaxis assays. These curves were obtained for *C. albicans* and the above-mentioned *Lactobacillus* species, as depicted in **Figure 31**.

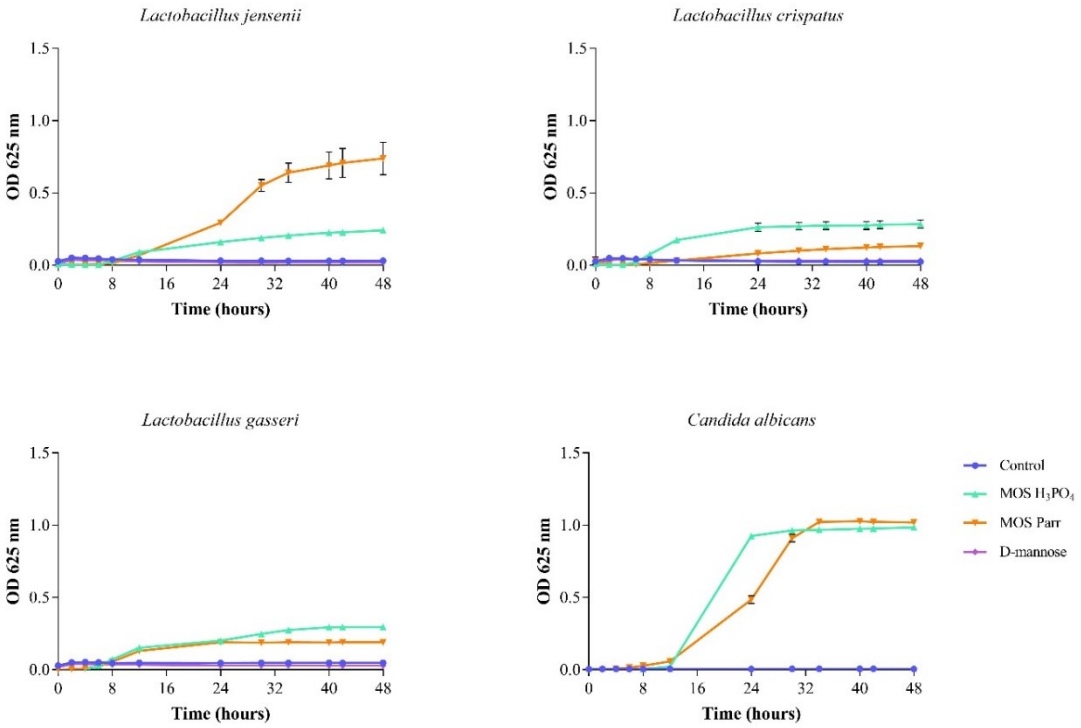


Figure 31. Growth curves of *L. jensenii, L. crispatus*, *L. gasseri* and *C. albicans* in SVF supplemented with several mannose-based extracts (MOS Parr, MOS H_3PO_4 and D-mannose).

When comparing the growth of the selected microorganisms in SVF with their growth in MH medium (**Figure 31**), most microorganisms present a lower growth, particularly in the control, where species only have access to SVF components. This is not surprising since SVF is not rich in nutrients, namely sugars. By analysing the maintenance curves in SVF of *L. gasseri* and *L. crispatus*, it can be observed that both vaginal lactobacilli maintain its viability in SVF supplemented with MOS extracts and D-mannose. Regarding the *L. jensenii* and *C. albicans*, the two MOS extracts showed a significant promotion of bacterial growth, while in D-mannose and in the control, only the maintenance of the tested microorganisms was revealed. Upon the conclusion of this study, valuable insights were gathered, confirming the viability of all microorganisms for a duration of 48 h in SVF. This fluid served as the designated vaginal environment to carry out the presented assays, namely competition and prophylaxis, within the simulated vaginal setting.

Moreover, this research was carried out to complement the results presented in **Supplementary material Figure S 4**. The focus of the results presented in data was to analyse the growth curves of vaginal lactobacilli in MRS broth medium, which is known to

be an optimal growth medium for these microorganisms. By analysing these growth curves, we were able to identify the different phases of microbial growth (lag, exponential, stationary and dead) and identify the exponential phase (approx. 16 h), which corresponds to a vaginal lactobacilli density similar to that found in the vaginal flora. This information played a crucial role in the subsequent experiments, as it allowed us to prepare the pre-infection vaginal environment in a consistent and relevant manner. By identifying the start of the exponential phase and understanding the growth curves of lactobacilli in MRS broth medium, we were able to establish the appropriate conditions to mimic the vaginal flora and to ensure the validity of the studies presented.

Prophylaxis assay (sequential exposure)

The protective effect of vaginal *Lactobacillus* strains is typically evaluated *in vitro* through its capacity to attach to the vaginal epithelium, as well as its antibacterial efficacy against *Gardnerella vaginalis* and *C. albicans*, probably through the production of hydrogen peroxide (H_2O_2) (Klebanoff et al., 1991; McGroarty et al., 1992). Single strains of vaginal lactobacilli inhibit *Candida* sp. development *in vitro* (Strus et al., 2005), and this has also been observed *in vivo* (Drutz, 1992). However, the mechanism underlying this growth suppression remains unknown. Thus, following the study of the individual behaviour of each microorganism in SVF supplemented with MOS extracts, a prophylaxis study was designed to simulate the potential preventive effect of MOS against *C. albicans* infection in a "healthy" vaginal environment, i.e., already containing a population of different *Lactobacillus* species. This study allowed to examine the cell viability of the *Lactobacillus* sp. pool (indicating a prebiotic effect of MOS) and the viability of the pathogenic yeast over a 40 h period. For this purpose, the *Lactobacillus* sp. pool was initially placed in SVF with 2% (w/v) of each extract for 16 h. The 16 h point was chosen to start mimicking the infection by *C. albicans* (**Supplementary material Figure S 4**), because in this set time the vaginal lactobacilli reach the desired concentration to resemble the vaginal microbiota, approximately 10^7-10^9 CFU/mL (Sobel & Chaim, 1996). Subsequently, 2% (v/v) of C. albicans was added to simulate the infection, and the study continued up to 40 h, with several sampling points, as previously detailed in **section 4.2.4.4**. Growth numbers of both lactobacilli and *C. albicans* along time can be seen in **Figure 32**.

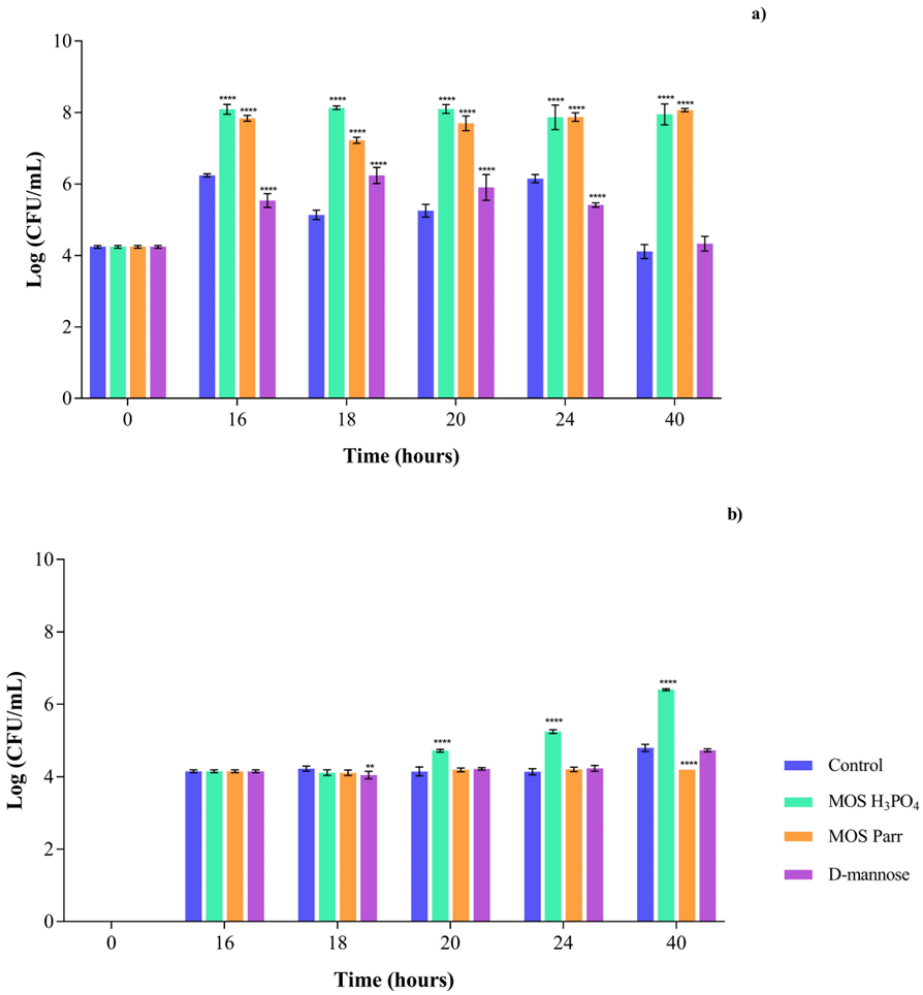


Figure 32. Cell viability by Log (CFU/mL) over 40 h of prophylactic effect, for a) *Lactobacillus* sp. pool and b) *C. albicans* with different extracts: D-mannose, MOS Parr, and MOS H_3PO_4 in SVF with 0.05% L-cysteine hydrochloride. ****$p < 0.0001$ and *** $p < 0.001$ indicate statistically significant differences between each sample and the control at each time point.

Figure 32a illustrates the cell viability of the *Lactobacillus* sp. pool after exposure to the different supplements (MOS extracts and D-mannose) since time zero, and exposure to the pathogen after 16 h. The cell viability remained stable throughout the 40 h assay (no significant variations in growth), displaying significant differences between the MOS extracts and the control (SVF). At 40 h incubation time, both MOS extracts exhibited a substantial increase of 4 log cycles in cell viability compared to the control ($p < 0.0001$). Contrarily, the control and D-mannose showed a lower increase in cell viability.

Figure 32b focuses on the cell viability of the selected pathogenic strain. It was observed that the cell viability remained consistent for approximately 20 h, except for the MOS H_3PO_4 extract, which displayed a 1.1 log cycle increase at 20 h (i.e., 4 h after infection). With MOS Parr extract, on the other hand, *C. albicans* demonstrated a smaller decrease of around 0.6 log cycles at the 40 h point, when compared to the control at the same time point. D-mannose exhibited similar behaviour to the control ($p > 0.05$) throughout the study, maintaining cell viability at approximately between 4 and 5 log cycles.

In summary, *Lactobacillus* sp. pool growth was promoted in the presence of both MOS extracts, reaching a cell viability of about 8 log cycles from 16 to 40 h of incubation. This shows the potential prebiotic effect of the MOS extracts, as they have clearly induced the growth of the vaginal lactobacilli pool, with no statistically significant distinction between the two types of extracts. On the other hand, the two MOS extracts have induced a different outcome in the *C. albicans* viability: MOS H_3PO_4 promoted the growth of pathogenic strain, whereas in the presence of MOS Parr no growth of the *C. albicans* was observed, maintaining a cell viability of around 4 to 5 log cycles until the end of the study (40 h), which was the same compared to the control at the same time point (**Figure 32b**).

As seen before (**Figure 31**), the MOS extracts have no differentiated effect on *C. albicans* growth, and thus we hypothesize that the changes observed in **Figure 32b** must be a result of the interference of MOS extracts in the relationship between vaginal lactobacilli and *Candida* sp. One theory is that, since it is known that vaginal lactobacilli may negatively impact *C. albicans* growth, as discussed above, it is possible that MOS H_3PO_4 protects *C. albicans* from these mechanisms in some way not currently understood. To the best of our knowledge, this is the first time this behaviour is observed, and further studies are needed to understand the underlaying mechanisms associated with it.

Competition assay (simultaneous exposure)

For VVC infections caused by *C. albicans*, the typical reported concentration is 10^7-10^9 CFU/mL for vaginal lactobacilli and 10^2- 10^4 CFU/mL for *Candida* sp. (Sobel & Chaim, 1996). To replicate this infection scenario, both *Lactobacillus* pool and *C. albicans* strains were simultaneously inoculated (at the above-mentioned concentrations) in SVF supplemented with the different extracts under study. The cell viability was then monitored for a duration of 48 h, as shown in **Figure 33**.

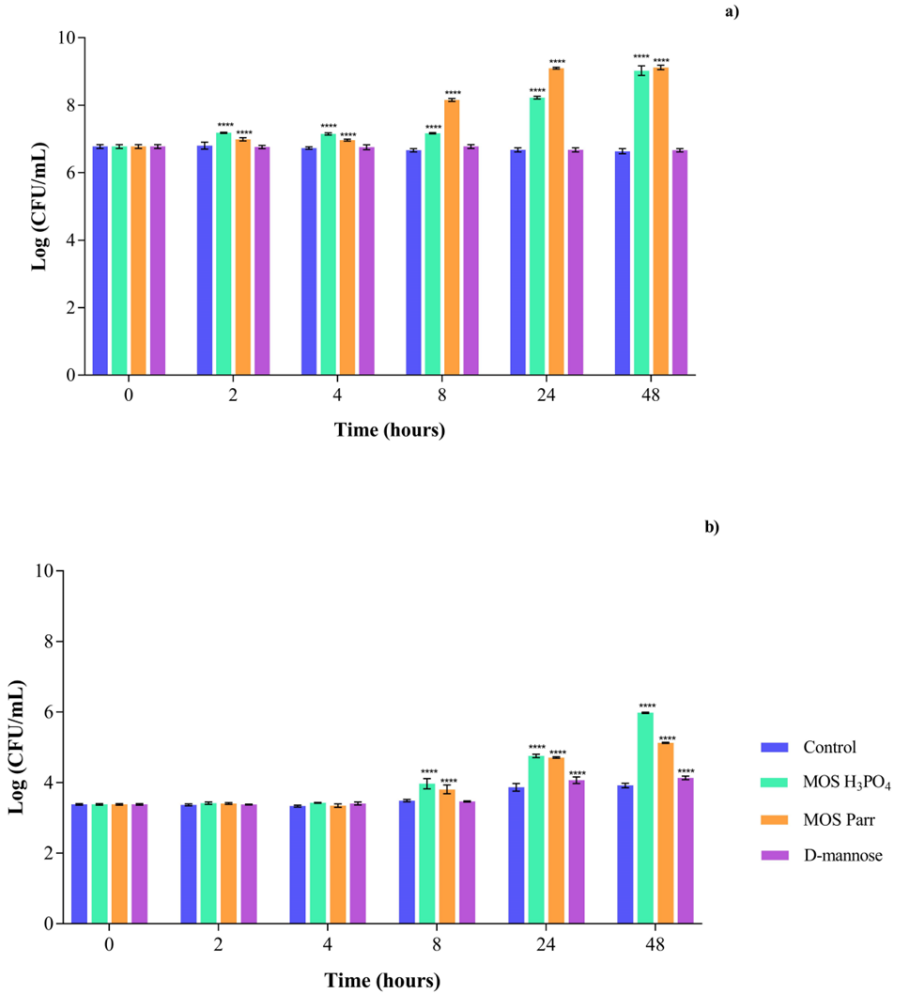


Figure 33. Cell viability in Log (CFU/mL) over 48 h of simultaneous exposure, for a) *Lactobacillus* sp. pool and b) *C.* albicans with different extracts: D-mannose, MOS Parr, and MOS H_3PO_4 in SVF with 0.05% L-cysteine hydrochloride. **** indicates statistically significant differences (****$p < 0.0001$), between the different samples and against the control at each time point.

In **Figure 33a**, the cell viability of vaginal lactobacilli, represented by the *Lactobacillus* sp. pool, remained stable over the 48 h period when exposed to control (SVF) and D-mannose. When considering the effect of the MOS Parr and MOS H_3PO_4 extracts on the vaginal lactobacilli pool, both extracts resulted in a 2.5 log cycle increase in cell viability. This, once more, demonstrates the prebiotic potential of these extracts. MOS Parr and MOS H_3PO_4 extracts also promoted the growth of *C. albicans*, resulting in a 1.5 log cycle increase for MOS Parr and a 2.5 log cycle increase for MOS H_3PO_4 at the 48 h (**Figure 33b**).

Ideally, the desired outcome of this assay would be the reduction or, at least, maintenance of *C. albicans* viability, while the growth of the *Lactobacillus* sp. pool would be promoted. In fact, the coexistence of *Lactobacillus* strains and *Candida* strains in the vaginal epithelium of healthy women has been reported (Falagas et al., 2006). *In vitro* and clinical trials have shown positive results regarding the efficacy of specific *Lactobacillus* strains against *C. albicans.* Nevertheless, it is important to note that different probiotic strains can have distinct qualities and effects on *C. albicans*, and thus, findings from research evaluating one *Lactobacillus* strain should not be generalized to others. However, although *C. albicans* growth has been observed in both MOS extracts, *C. albicans* grown in the presence of MOS H_3PO_4 still has shown less viability than that grown with MOS Parr, supporting our previous remarks about the protective effects that MOS H_3PO_4 may have.

4.2.3.3. Adhesion to cervical cells assays

Cytotoxicity

Cytotoxicity of samples against HeLa cells was assayed, by evaluating their impact upon cell metabolism using a cellular viability dye. According to ISO 10993–5 (2009), a sample is cytotoxic when a metabolic inhibition percentage above 30% is observed. MOS Parr (23.30 ± 3.40%) and MOS H_3PO_4 (-11.69 ± 6.04%) exhibited no cytotoxicity at the concentration of 2.5 mg/mL, with no metabolic inhibitions being observed. Commercial D-mannose exhibited no cytotoxicity up to 2.5 mg/mL (-9.51 ± 1.85%). In fact, the negative values illustrate an increase in cell metabolism observed in the presence of MOS H_3PO_4 and D-mannose. Differences observed in the cellular viability of HeLa cells when exposed to MOS Parr and MOS H_3PO_4 may have origin in the structure/composition of the extracts (**Chapter 3.1**).

Adhesion to vaginal epithelial cells

In order to increase the degree of complexity and, thus, obtain a more realistic approach of the biological human complexity, the extracts were also evaluated on their capacity to promote or inhibit the adhesion of *C. albicans* to HeLa cells. HeLa cells are a human cervical cancer cells (Rizzo et al., 2013) that have been used as a model system (Cautela et al., 2019; Facchinatto et al., 2021) of the vaginal environment and investigate the impact of vaginal lactobacilli on *C. albicans* adhesion capacity (Calonghi et al., 2017; Rizzo et al., 2013).

Procedure was performed according to the description in **section 4.2.2.6. Individual adhesion to vaginal epithelial cell line** and pathogen cell numbers were normalized by the number of HeLa cells in each well. Results are depicted in **Figure 34**.

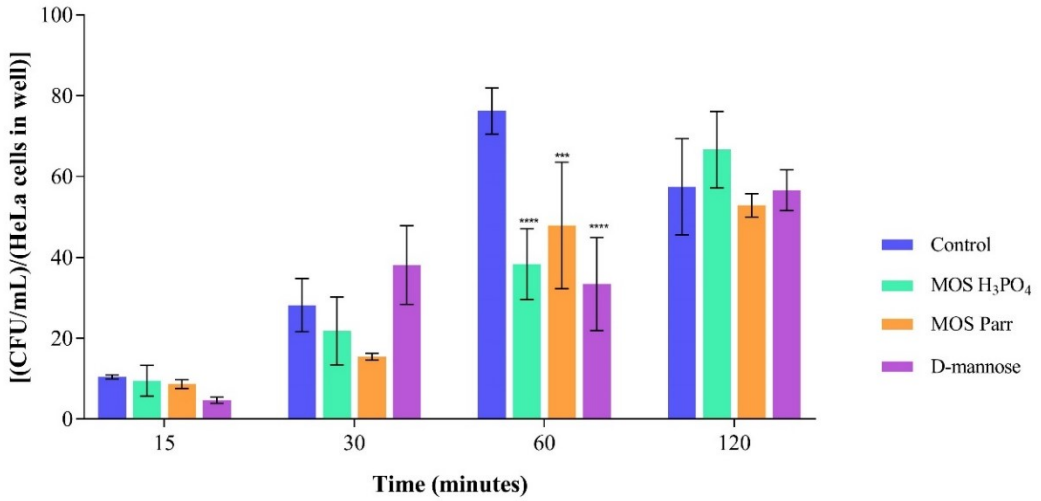


Figure 34. CFU/mL/ HeLa cells in well over 120 min of individual adhesion, for *C. albicans* with different extracts: D-mannose, MOS Parr, and MOS H_3PO_4. **** $p < 0.0001$ and *** $p < 0.001$ indicate statistically significant differences between the different samples and against the control at each time point.

Figure 34 illustrates the initial adaptation of *C. albicans* to the cellular system, and the fast-growing rate by which adhesion is observed. In the control group, *C. albicans* reached its peak at 60 min and declined by 120 min. In contrast, all other samples only peaked at 120 min. Despite these results, there were no statistically significant differences ($p > 0.05$) at 120 min, suggesting that our samples cannot delay the adhesion of *C. albicans* within the studied time period.

According to Mayer et al., (2013), *C. albicans*' capacity to infect and adhere to various host niches is supported by a diversified set of virulence and fitness variables. Virulence factors include the morphological transition between yeast and hyphal forms, the production of adhesins and invasins on the cell surface, thigmotropism (the capacity to detect and respond to surface contour changes), biofilm development, phenotypic switching, and the release of hydrolytic enzymes (Davies et al., 1999). Fitness characteristics include quick adaptability to changes in ambient pH, metabolic flexibility, potent food acquisition mechanisms, and robust stress response machinery. *C. albicans*' ability to form biofilms on abiotic or biotic surfaces is another major virulence factor (Tester et al., 2012).

As observed during the assays in SVF, the viability of both *Lactobacillus* sp. and *Candida* sp. species depended not only on the extract to which they were exposed, but also on potential interactions between microorganisms (e.g., growth of *C. albicans* in SVF with MOS extracts was similar for the two extracts when the yeast was cultivated alone, but different when lactobacilli were also present). Therefore, the subsequent step of the study focused on testing the ability of MOS extracts to inhibit the adhesion of *C. albicans* to HeLa cells in the presence of *L. crispatus*, again in a prophylaxis or competition mode assay.

Competition (Simultaneous exposure)

This study evaluates if MOS promotes the inhibition of *C. albicans* adhesion on HeLa cells in synergy with *L. crispatus*. This specie was selected as the most representative from the vaginal lactobacilli since it would be very complicate to work with the previous pool of lactobacillus in the cell line. **Figure 35** demonstrates the inhibitory effect of the extracts in synergy with *L. crispatus* on the adhesion of *C. albicans* to HeLa cells.

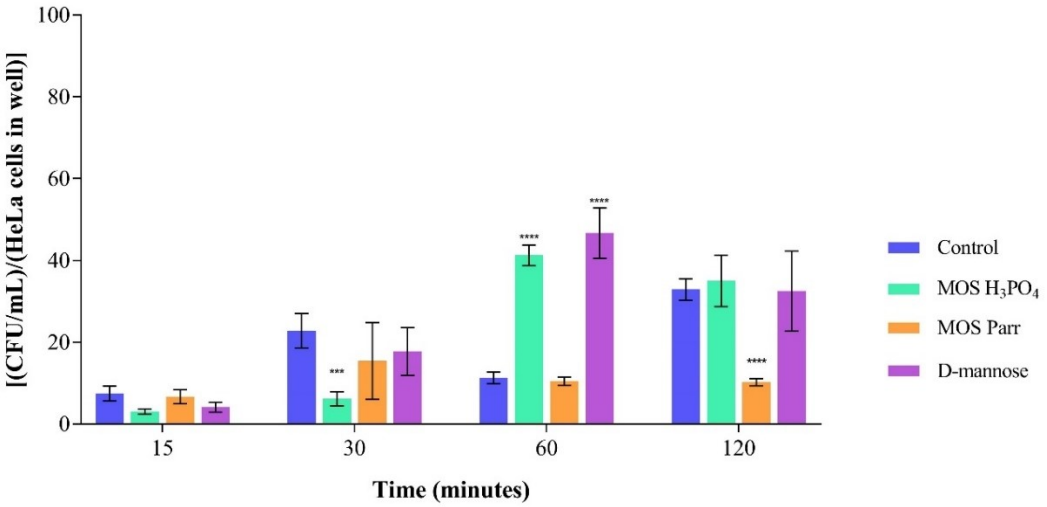


Figure 35. CFU/mL/ HeLa cells in well over 120 min of simultaneous exposure, for *C. albicans* with different extracts: D-mannose, MOS Parr, and MOS H_3PO_4. ****$p < 0.0001$ and *** $p < 0.001$ indicates statistically significant differences between the different samples and against the control at each time point.

With respect to MOS Parr, results show a significant reduction in *C. albicans* adhesion when inoculated simultaneously with vaginal lactobacilli, with an adhesion result of 10.21 ± 0.88 CFU/mL/HeLa cells in the well. This indicates that the combination of MOS Parr

extract and *L. crispatus* has an inhibitory effect on the adhesion of *C. albicans* to the HeLa cells. The control group, which consists of *L. crispatus* alone, demonstrated an adhesion result of 32.92 ± 2.60 CFU/mL/HeLa cells in the well. Results observed for MOS Parr are in line with those previously obtained in the microbiological prophylaxis assay, showing that MOS Parr has a negative impact on *C. albicans* growth and adhesion on HeLa vaginal cells, while MOS H_3PO_4 has no effect on the virulence inhibition of *C. albicans*.

According to Niu et al., (2017) *L. crispatus*, is one of the most predominant microorganisms on the vaginal microbiota, and has been shown to decrease the virulence of *C. albicans* and increase the local immune response of the vaginal epithelium by modulating the immune cytokine and chemokine profile. Another study by Sun et al., (2023) has shown that vaginal lactobacilli can inhibit the adhesion of *C. albicans* to vaginal epithelium. Lactobacilli produce lactic acid, hydrogen peroxide, and other antimicrobial substances that can create an acidic environment in the vagina and prevent the overgrowth of pathogenic microorganisms such as *C. albicans*. Additionally, lactobacilli can compete with *C. albicans* for adhesion sites on the vaginal epithelium, further inhibiting the growth and colonization of *Candida* strains. This is why maintaining a healthy vaginal microbiota that is dominated by lactobacilli is important for preventing urogenital infections, including vaginal candidiasis.

There is limited information available regarding the specific use of MOS in inhibiting the adhesion of *C. albicans* on vaginal cells. Nevertheless, according to Al-Ghazzewi et al., (2007), hydrolysed glucomannan increases the growth, metabolism, and antibacterial capabilities of probiotic microorganisms, including vaginal healthy lactobacilli strains (Sutherland et al., 2009), implying that hydrolysed glucomannan might be used for vaginal treatment. A study conducted by Tester et al., (2012) showed hydrolysed glucomannan's symbiotic potential to restore the healthy microbiota of vagina treated with antifungal drugs. Tester and colleagues also found that inserting pessary capsules containing hydrolysed glucomannan inserted into the vagina aided in the recovery and optimization of healthy vaginal microbiology, hence preventing future infection. Therefore, a possible reason for the obtained results is that MOS H_3PO_4 either protects *Candida* sp. from these antifungal mechanisms, or *L. crispatus* is not as promoted by MOS H_3PO_4 as they are by MOS Parr, resulting in the observed patterns in **Figure 35**. However, further studies are needed to address this issue and draw definitive conclusions.

Prophylaxis assay

From another perspective, adhesion of the lactobacilli to the epithelium is the first step in the barrier formation able to prevent undesirable microbial colonisation (Borchert et al., 2008). Probiotic microorganisms have been recommended as an alternate preventative and therapeutic form of therapy for human *Candida* sp. infections (Matsubara et al., 2016; Meurman, 2005) and have been widely examined for their ability to diminish infections by *C. albicans* on mucosal surfaces, not only in urogenital and gastrointestinal infections (Hu et al., 2013; Kovachev & Vatcheva-Dobrevska, 2015; Roy et al., 2014), but also in oral infections (Ishikawa et al., 2015; Kraft-Bodi et al., 2015; Li et al., 2014; Matsubara et al., 2012). According to the literature, *Lactobacillus* sp. species in the female urogenital system act as a barrier to infection and contribute to vaginal microbiota control by competing with other microorganisms for epithelial cell adherence, displacing pathogen biofilm (Boris et al., 1998; Saunders et al., 2007), and/or inhibiting the growth of potential pathogens (Atassi et al., 2006; Spurbeck & Arvidson, 2008; Strus et al., 2005). Furthermore, supernatants from *L. gasseri* and *L. crispatus* reduce the *C. albicans* ability to adhere to HeLa cells (Matsuda et al., 2018). Thus, the prophylaxis assay aims to simulate the probiotic/prophylactic effect of vaginal lactobacilli (*L. crispatus*) in synergy with MOS extracts against the adhesion of *C. albicans*. The results are represented in **Figure 36**.

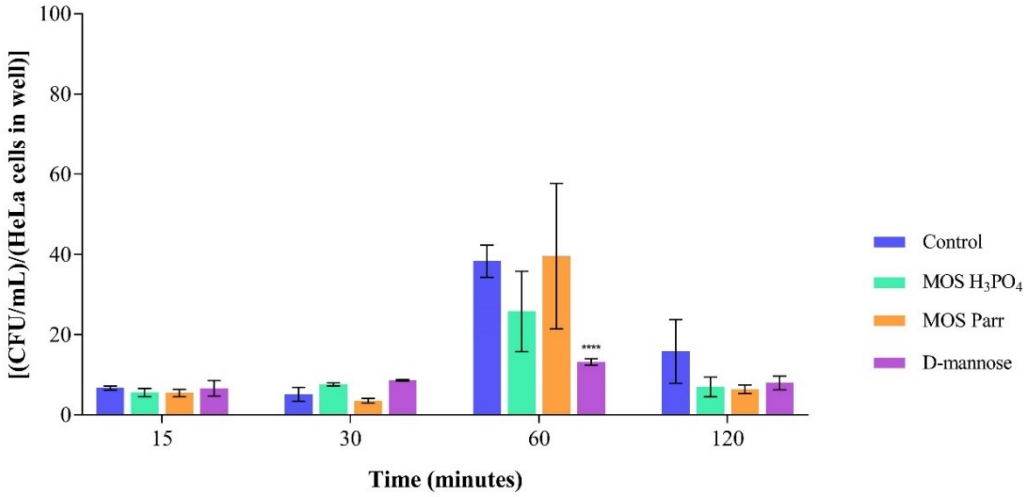


Figure 36. CFU/mL/ HeLa cells in well over 120 min of prophylaxis assay, for *C. albicans* with different extracts: D-mannose, MOS Parr, and MOS H_3PO_4. **** Indicates statistically significant differences (**** $p < 0.0001$) between the different samples and against the control at each time point.

In this assay, a robust population of lactobacilli was established in the cells for 120 min, after which the pathogen was inoculated (5.08 x 10^5 CFU/ml). At the end of 120 min, the production of compounds (biosurfactants (De Gregorio et al., 2020) for example, which prevent the adhesion) by *L. crispatus* may occur, in synergy with MOS extracts which affects the adhesion of *C. albicans* and the subsequent proliferation and infection of the cells by this pathogen. Based on the analysis of **Figure 36**, it can be inferred that the presence of an established population of vaginal lactobacilli has a detrimental effect on the adhesion of *C. albicans*, particularly when in synergy with MOS extracts (no statistically significant differences were observed at 60 min and 120 min between the MOS extracts and the control). This synergistic effect is evident when comparing **Figure 34**, where *C. albicans* alone does not show inhibition of adhesion in the presence of the extracts, with **Figure 36**, where the presence of vaginal lactobacilli and the extracts leads to a decrease in *C. albicans* adhesion after 120 min. This demonstrates a synergistic effect that promotes the inhibition of adhesion.

The adaptation of *C. albicans* can be observed between the initial 15 and 30 min, as it adjusts to the presence of the lactobacilli population. However, at the end of 120 min, the adhesion is inhibited, and this inhibition is positively affected by the MOS extracts and D-mannose (MOS H_3PO_4 = 7.00 ± 2.43, MOS Parr = 6.42 ± 1.06, D-mannose = 7.96 ± 1.69 CFU/mL/HeLa cells in the well) in synergy with *L. crispatus* establish population, mainly, even though there is no statistically significant evidence between the tested samples and the control (15.83 ± 7.94 CFU/mL/HeLa cells in the well).

In literature it was reported that lactobacilli generate a wide range of antibacterial active chemicals (primary and secondary metabolites). A few of these metabolites are likewise anti-*C. albicans*. Bacteriocins (Pascual et al., 2008) and bacteriocin-like peptides (Okkers et al., 1999), cyclic dipeptides (Ström et al., 2002), proteinaceous compounds (Lakhtin et al., 2010), enzymes (Charlet et al., 2020), fatty acids (Lipinska-Zubrycka et al., 2020), biosurfactants (Ceresa et al., 2015; Gomaa, 2013), and other organic molecules such as reuterin (Schaefer et al., 2010) and 3-Phenyllactatic acid (Ström et al., 2002) are the most investigated metabolites. Metabolites change fungi physiology by causing oxidative stress or depleting ATP, resulting in cytotoxicity or growth suppression. Other metabolites weaken the fungal cell's structural integrity, causing changes in cell shape, membrane permeability, and death, whereas biosurfactants prevent adhesion to mucosal surfaces (Vazquez-Munoz & Dongari-Bagtzoglou, 2021). Lactobacilli may produce inorganic chemicals such hydrogen

peroxide (Strus et al., 2005), which cause oxidative stress and genotoxicity in larger concentrations. These chemicals have antibacterial action against a wide range of bacteria and fungi (Crowley et al., 2013; Lipinska-Zubrycka et al., 2020; Ribeiro et al., 2020; Siedler et al., 2019). *L. gasseri* and *L. crispatus* vaginal strains have been shown to prevent *C. albicans* adhesion via the following mechanisms: i) alteration of polar lipid structure and physical characteristics, and ii) alteration of α5β1 integrin exposure (Matsubara et al., 2016). Lactobacilli adhesion to the epithelium is the first stage in the creation of a barrier to prevent undesirable microbial colonisation (Ocaña & Nader-Macías, 2001).

More research is needed to fully understand the mechanisms by which MOS may affect the adhesion of *C. albicans* to host cells and whether it has potential as a therapeutic agent for preventing vaginal infections. It is well described that *C. albicans* activates the host immune response leading to the production of several cytokines by epithelial cells (Zangl et al., 2020). Mannans were described to be capable of stimulating the immune system and promoting the production of antibodies against *C. albicans*, which can also help to prevent the adhesion/colonization and infection (Nelson et al., 1991; M. Shukla et al., 2021).

4.2.4. Conclusions

Lactobacilli, which are the most common vaginal microbes in healthy premenopausal women, protect against bacterial vaginosis and urinary tract infections. It has been stated that some lactobacilli are also anti-*Candida* vaginitis. The processes through which these lactobacilli suppress *Candida* sp. vaginal populations are described, but scarce information was found about the influence of MOS extract against *C. albicans* and the promotion of vaginal lactobacilli. The present study has shown that SVF was an adequate medium to mimic the vaginal fluid to grow/maintain *Lactobacillus* sp. pool and *Candida* strains. Both MOS extracts appear to have a positive prebiotic effect in *Lactobacillu*s strains in SVF medium. Additionally, *C. albicans* growth is negatively impacted in the presence of MOS Parr, while unaffected in the presence of MOS H_3PO_4, which make us believe that MOS H_3PO_4 may exert some sort of protective umbrella over *Candida* sp. and protect it from lactobacilli antifungal mechanisms.

Furthermore, in cell line assays, MOS Parr extract in synergy with *L. crispatus* has also demonstrated an inhibitory effect on the adhesion of the *C. albicans*. This inhibition is likely due to a potentiation effect of lactobacilli antifungal properties by MOS Parr, since situation does not occur when adhesion of *C. albicans* occurs in HeLa cells alone, where none of the

MOS extracts promote *C. albicans* adhesion inhibition. However, with the simultaneous exposure of both the microorganisms, we observed decreased adhesion of *C. albicans* in synergy with the *L. crispatus* cultured with MOS Parr. In the prophylactic assay a lactobacilli vaginal population was previously stablished when the infection by pathogenic yeast was tested. In all the tested conditions the MOS extracts in synergy with the population of *L. crispatus* inhibited the adhesion of *C. albicans.* Based on these results, the two tested MOS extracts exhibit promising potential for the treatment of VVC. These extracts have a heterogeneous composition, with different oligomer populations characterized by differences in molecular weights (**Chapter 3.1**). These variations may contribute to distinct activities. It would be interesting to identify the specific compounds that exert the inhibitory effect on pathogens. The ability of MOS extracts to inhibit the adhesion of *C. albicans* to vaginal cells and their potential to modulate the vaginal microbiota make them a promising option for managing VVC.

Supplementary material

Table S 1. Strains were used in this study.

Microorganisms		Catalogue #	URL
Bacteria	*Lactobacillus jensenii*	20557	https://www.dsmz.de/collection/catalogue/details/culture/DSM-20557
	Lactobacillus crispatus	20356	https://www.dsmz.de/collection/catalogue/details/culture/DSM-20356
	Lactobacillus gasseri	20243	https://www.dsmz.de/collection/catalogue/details/culture/DSM-20243
Yeast	*Candida albicans*	3454	https://www.dsmz.de/collection/catalogue/details/culture/DSM-3454
	Candida glabrata	28718	https://www.dsmz.de/collection/catalogue/details/culture/DSM-28718
	Candida tropicalis	11952	https://www.dsmz.de/collection/catalogue/details/culture/DSM-11952

Table S 2. Simulated vaginal fluid at 4.2 pH.

Reagent	Concentration g/L	Purchased from
Sodium Chloride	3.5	Honeywell Fluka, Seelze, Germany
Potassium Hydroxide	1.4	Merck, Darmstadt, Germany
Calcium Hydroxide	0.2	Sigma, St. Louis, MO, USA
Bovine serum albumin	0.02	Sigma, St. Louis, MO, USA
Lactic Acid	2.0	Sigma, St. Louis, MO, USA
Acetic Acid	1.0	Sigma, St. Louis, MO, USA
Glycerol	0.2	Fisher Scientific, Loughborough, UK
Urea	0.4	Sigma, St. Louis, MO, USA
D-Glucose	5.0	Merck, Darmstadt, Germany
L-cysteine hydrochloride	0.05	Sigma, St. Louis, MO, USA

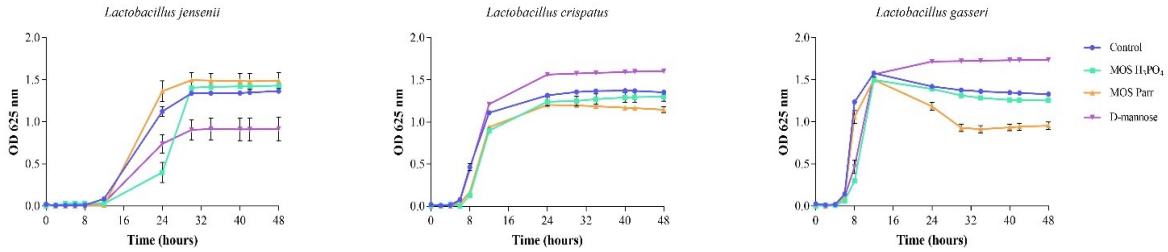


Figure S 4. Growth curves of *Lactobacillus jensenii*, *L. crispatus* and *L. gasseri* and in MRS broth supplemented with several mannose-based extracts.

Chapter 5

Chemical modification of mannans extract

This chapter aims to evaluate the chemical modification method for mannans and characterize structural, physicochemical, and biological properties of the carboxymethylated extracts.

Chapter 5.1 *Carboxymethylation of mannans extract*

Adapted from the following submitted scientific publication:

Faustino, Margarida; Pereira, Carla F.; Durão, Joana; Pereira, Ana Margarida; Oliveira, Ana Sofia; Pereira, Joana Odila; Ferreira, Carlos; Pintado, Manuela E.; Carvalho, Ana P.; 2023. Carboxymethylation of *Saccharomyces cerevisiae* mannans: preparation and properties evaluation. Manuscript in preparation.

5.1. Carboxymethylation of mannans extract

Carboxymethylation of Saccharomyces cerevisiae mannans: preparation and properties evaluation

Abstract: Mannans are polysaccharides derived from various sources, and their physicochemical and biochemical characteristics allow them to be commercialised in a wide range of products. This study aims to prepare carboxymethylated yeast mannans (CMTH), using mannans from yeast (*Saccharomyces cerevisiae*) extracted by thermal hydrolysis (TH) as starting material, and to stress their impact on the physicochemical and biological properties. The structural characterization of the carboxymethylated product, and the corresponding starting material was performed using a myriad of solid-state techniques: ATR-FT-IR, PXRD and SEM, being also determined the degree of substitution. The physicochemical properties were evaluated based on sugars, protein, ash and water contents, solubility, and molecular weight distribution, and the thermal behaviour was analysed by DSC. The biological properties were assessed by studying the antioxidant capacity (DPPH, ABTS and ORAC assays) and the antimicrobial potential against *Candida albicans* and *Escherichia coli*. The parameters which revealed major differences, for both extracts, in terms of structural and physicochemical properties regarded morphology (SEM), physical appearance (colour), moisture and solubility. The mannans chemical modification showed an improved capacity for the inhibition of pathogenic microorganisms, and similar antioxidant capacity regarding to the starting material.

Keywords: Mannans, Chemical modification, Carboxymethylation, Structural characterization, Physicochemical and Biological properties

5.1.1. Introduction

Saccharomyces cerevisiae has undergone extensive research and is considered a valuable model for exploring the cell walls of other yeast species (Lee et al., 2001). The cell wall of *S. cerevisiae* primarily consists of three major components: glucans, mannoproteins (mannans bound to proteins), and chitin (Kath & Kulicke, 1999; Kwiatkowski et al., 2009; Orlean, 2012; Pinto et al., 2015).

Mannans, which are the focus of this study, are mainly composed of mannose units with α-(1-6) linked backbones and additional α-(1-2) and α-(1-3) linkages (Ballou, 1974; Peat, Whelan, et al., 1961). These polysaccharides are connected to asparagine through N-acetyl-glucosamine, while oligosaccharides are attached to serine or threonine residues. Moreover, mannans exhibit various biological properties such as inhibiting pathogens, modulating bacterial growth (Smith et al., 2020), enhancing immune responses (Lee & Dugoua, 2011; Onitake et al., 2015), and are commonly utilized as antibiotic alternatives in animal feed (Smith et al., 2020; Spring et al., 2015). Mannans possess techno-functional properties that make them appealing for various applications. Physicochemical characteristics like water solubility, viscosity, and stability contribute to their suitability in food applications, including uses as hardening ingredients and emulsion stabilizers (Singh et al., 2018). These advantageous properties extend beyond the realm of food (Butylina et al., 2007; Singh et al., 2018) and feed (Shurson, 2018; Singh et al., 2018) and find application in diverse fields such as cosmetics (Gaspar et al., 2008; Yoon et al., 2019) and drug administration (Yu et al., 2010).

In the realm of chemical modification of polysaccharides, which is often pursued to enhance a range of properties, including various biological activities, there is a noticeable discrepancy between the knowledge available for mannans and that for other yeast cell wall polysaccharides, such as glucans (Ding et al., 2013; Song et al., 2020; Theis et al., 2019). Information specifically related to mannans remains scarce (Faustino et al., 2021; Machová, Bystrický, et al., 2014; Machová, Čížová, et al., 2014).

Chemical modification methods involve introducing additional active groups into the polysaccharide chain through chemical reactions. This process leads to the emergence of new or enhanced bioactivities and, in some cases, reduces the molecular weight of the native polysaccharides (Li et al., 2016). Common chemical modification methods include carboxymethylation, sulfation, phosphorylation, acetylation, alkylation, and selenization

(Xu et al., 2019). Chemical modification is widely considered the most effective approach for improving the water solubility and bioactivities of natural polysaccharides. The development of novel polysaccharide derivatives through chemical modification is an area of active research. Among the various methods, carboxymethylation is the most employed technique to enhance water solubility and bioactivities of polysaccharides, being commonly used the Williamson etherification (Ding et al., 2013). This method offers advantages such as ease of processing, affordability of chemical reagents, and non-toxicity of the resulting products (Chakka & Zhou, 2020).

The chemical modification of mannans from yeast strains is currently in its beginning. Carboxymethylation has been the primary focus, especially in pathogenic *Candida* sp. strains, primarily *Candida albicans* (Korcová, Machová, Filip, Bystrick, et al., 2015; Machová, Bystrický, et al., 2014; Machová, Čížová, et al., 2014; Oka et al., 1972). Research on the chemical modification of mannans from *S. cerevisiae* began in 2014, optimizing the synthesis pathway for carboxymethyl derivatives and characterizing their structure and properties (Machová, Bystrický, et al., 2014). While carboxymethylation has been extensively studied for other polysaccharides, such as cellulose and starch (Dos Santos et al., 2015; Heinze & Koschella, 2005; Rahman et al., 2022), and even glucans (Ding et al., 2013; Song et al., 2020; Theis et al., 2019) there is still a need for further understanding of its application to mannans.

Carboxymethylated mannans from *S. cerevisiae* and *Candida* sp. strains have shown improved performance compared to unmodified mannans, exhibiting enhanced antitumor activity by reducing toxicity while maintaining efficacy (Oka et al., 1972), and also antioxidant properties, effectively scavenging radicals and offering potential antioxidant effects (Korcová, Machová, Filip, Bystrick, et al., 2015; Liu & Huang, 2018; Machová, Čížová, et al., 2014). Additionally, carboxymethylated mannans also demonstrated thrombolytic activities (Korcová, Machová, Filip, Bystrick, et al., 2015). Further research in this area is warranted to explore the full potential of mannans' chemical modification.

The first report in 2014 focused on the optimization of the synthetic pathway to obtain carboxymethyl derivatives of *S. cerevisiae* mannans (Machová, Bystrický, et al., 2014). Various characterization techniques, including ATR-FT-IR spectroscopy, UV measurements, optical rotation, and potentiometric titration, were employed to elucidate the structure and properties of the modified mannans. This study addressed the need for more information on the functionalization of mannans, as carboxymethylation is a well-studied

method for modifying other polysaccharides. Furthermore, it is worth highlighting that while carboxymethylation has been extensively studied for the functionalization of insoluble polysaccharides like cellulose and starch, as well as soluble polysaccharides like dextran and hyaluronan, there is a clear lack of information regarding the functionalization of mannans. The previously mentioned study represents one of the few investigations into the carboxymethylation of mannans, employing three different procedures/media: a water/alcohol system, a non-aqueous dimethyl sulfoxide system, and a water-only system. In the same year, the carboxymethyl derivatives of *S. cerevisiae* mannans obtained using the optimized conditions mentioned above (Machová, Bystrický, et al., 2014) were evaluated as potential antioxidants. This evaluation included a comparison with carboxymethyl derivatives of β-glucan and dextran (Machová, Čížová, et al., 2014).

Overall, this study aims to evaluate the impact of the carboxymethylation (CMTH) of yeast mannans extracted by *S. cerevisiae* from thermal hydrolysis (TH) in the structural (ATR-FT-IR, PXRD, and SEM); physicochemical (colour, total protein, neutral sugars, molecular weight distribution, solubility, dry weight, ashes, and thermal stability) and biological properties (antioxidant activity and antimicrobial potential against pathogenic *C. albicans* and UPEC). With this study is expected to lay the scientific foundation for the recovery of well-characterized carboxymethylated mannans from *S. cerevisiae* yeast, which will pave the way for their application in various fields.

5.1.2. Materials and methods

5.1.2.1. Materials

Mannans extract was produced from spent yeast (*S. cerevisiae*) kindly provided by Amyris Company's. The extraction methodology was previously described by Faustino et al., (2022). *C. albicans* and UPEC were purchased from DSMZ (Braunschweig, Germany). For reagents: Ethanol (≥ 99%) was purchased from CHEM-LAB (Zedelgem, Belgium); Ether (≥ 99%) and Methanol (≥ 99%) were purchased from VWR (Pennsylvania, USA), NaOH was purchased from LabChem (Johannesburg, South Africa); Monochloroacetic acid was purchased from VWR (USA), Ethylene diamine tetra acetic acid (EDTA) at 0.1 M, Murexide indicator and Acetic acid (≥ 99%) was purchased from Sigma-Aldrich (USA). Ammonium chloride and Copper sulphate was purchased from Merck (Darmstadt, Germany). Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS

(2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt, AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride), Fluorescein was also purchased from Sigma-Aldrich. DPPH (2,2-Diphenyl-1-picrylhydrazyl) was purchased from Thermo-Fisher Scientific (USA). All reagents were used as received, without further purification.

5.1.2.2. Chemical modification of mannans

Reaction conditions optimization

The carboxymethylation of the *S. cerevisiae* mannans extract obtained by pH neutral hydrolysis (TH) – details **in section 2.1.2.2. of Chapter 2.1** - implied the study of the impact several parameters: reagents ratio; different solvents; washing steps and the evaluation of different drying processes. Herein, we report the reaction conditions to afford the carboxymethylated products CMTH1-CMTH7.

CMTH1: The CMTH1 product was obtained as previously described by (Machová, Bystrický, et al., 2014). Briefly, 100 mg of the mannans extract was dissolved in 1 mL of 6 M NaOH and the reaction mixture was stirred at room temperature for 30 min (250 rpm). Afterwards, of monochloroacetic acid (300 mg) was slowly added (250 rpm), and the reaction proceeded at 70 °C for 5 h (120 rpm). The reaction mixture was neutralized by acetic acid to pH ~ 7.0 and dialyzed against distilled water (Visking Dialysis Membranes, MWCO 12–14 kDa) (250 rpm). The solid was collected by centrifugation and dried by freeze drying for approximately 72 h (Alpha 2–4 LSCplus, Germany).

CMTH2: The CMTH2 product was obtained using the conditions previously described for CMTH1, but using a higher quantity of starting material, and the corresponding reagents. Mannans (500 mg) were dissolved in 5 mL of 6 M NaOH, and the reaction mixture was stirred at room temperature for 30 min (250 rpm). Afterwards, 1.5 g of monochloroacetic acid were slowly added (250 rpm) and the reaction proceeded at 70 °C for 5 h (120 rpm). The reaction mixture was neutralized and dialyzed was following the procedure described in CMTH1. Subsequently, the freeze-drying process was carried out.

CMTH3: The CMTH3 product was obtained using the reaction conditions described for the CMTH2 product but using several washings with ethanol (6 washings with ethanol

80% v/v (2739 x *g*; 5 min), followed by absolute ethanol and drying in a vacuum oven at 40 °C for 16 h.

CMTH4: The CMTH4 was obtained as previously described by (Oka et al., 1972), but using a lower quantity of the starting material due to availability issues. Briefly, 0.22 g of monochloroacetic acid and 0.20 g of NaOH were dissolved in 1 mL of distilled water and mixed with 4 mL of isopropanol. Then, 500 mg of mannans were added to the mixture and allowed to react at 70 ºC for 4 h with stirring. The reaction product was collected, centrifuged (2739 x *g*; 5 min), successively washed thoroughly with 80% aqueous methanol (6 times), absolute ethanol and petroleum ether (twice), followed by drying in a vacuum oven at 40 ºC for 16 h.

CMTH5: The CMTH5 product was obtained in a similar way previously described for CMTH4 but using a higher ratio of starting material: monochloroacetic acid (1:3).

CMTH6: The CMTH6 was obtained also using a water-miscible organic system, as previously described for CMTH4, but using a higher quantity of isopropanol. Briefly, 0.22 g of monochloroacetic acid and 0.20 g of NaOH were dissolved in 0.4 mL of distilled water and mixed with 4.6 mL of isopropanol. Then, 500 mg of mannans were added to the mixture and allowed to react at 70 ºC for 4 h with stirring. The reaction product was collected, centrifuged (2739 x *g*; 5 min), successively washed thoroughly with 80% aqueous methanol (6 times), absolute ethanol and petroleum ether (twice), followed by drying in a vacuum oven at 40 ºC for 16 h.

CMTH7: The carboxymethylated product CMTH7 was obtained using the conditions previously described for CMTH6 but using a higher ratio of starting material: chloroacetic acid (1:3).

5.1.2.3. Structural characterization

ATR-FT-IR

The FT-IR spectra of Attenuated Total Reflection were performed according to **section 2.1.2.3 of Chapter 2.1**.

PXRD

PXRD was carried out according to previously described in **section 2.1.2.3 of Chapter 2.1**.

SEM

The morphology of mannans and carboxymethylated mannans was evaluated by Scanning Electron Microscopy (SEM) on Thermo Scientific™ Pro Scanning Electron Microscope as described in **section 2.1.2.3 of Chapter 2.1**.

5.1.2.4. Determination of degree of substitution

The degree of substitution (DS) was determined using a complexometric titration technique devised by Stojanović et al., (2000) described in Ding et al., (2013). Briefly, 1 mL of 95% ethanol was added to the carboxymethyl derivative (0.1 g). The mixture was then stirred for 2 min with 50 mL of water and 20 mL of ammonium chloride buffer solution (pH 10). After the pH was adjusted to 7.5-8.0 with acetic acid (1 M) or NaOH (1 M), 50 mL of 0.05 M copper sulphate was added, stirred for 5 min, placed for 15 min, diluted to 250 mL with distilled water, and filtered through Whatman filter paper Nº 1. Then, 100 mL of the filtrate was taken out, and the solution was titrated with ethylene diamine tetra acetic acid (EDTA) at 0.05M standard solution using murexide as an indicator. Titrated copper sulphate under the same condition was used as a blank control group. The DS was calculated by the equation (8 and 9) as follows Ding et al., (2013):

$$\text{W (Content of sodium acetate)} = \left[\frac{(0.05\ (V-v)\times 2\times 2.5\times 0.081)}{m}\right] \quad (8)$$

$$\text{Degree of substitution (DS)} = \frac{162\times W}{(8100-80)\times W} \quad (9)$$

W = content of sodium acetate based, C = molarity of EDTA standard solution, V = EDTA used to titrate blank (mL), v = EDTA used to titrate sample (mL) and m = sample mass (g).

5.1.2.5. Physicochemical properties

Colour

A portable CR-410 Chroma meter was used to find the colour point according to the methodology described in **section 2.1.2.4 of Chapter 2.1**.

Total protein

Total protein concentration was determined in microplates using the BCA Protein Assay Kit as previously described in **section 2.1.2.4 of Chapter 2.1**.

Neutral sugars

According was described Faustino et al., (2022), the neutral sugars were derivatized to their alditol acetates derivatives and analysed by GC-FID (Agilent Technologies, Inc., CA, USA) using a 7890B GC System with a DB-225 capillary column (30 m length, 0.25 mm diameter, 0.15 µm thickness).

Molecular weight distribution

The molecular weight distribution was analysed using High-performance liquid chromatography (Agilent 1260 Infinity II HPLC) according to what was previously done in **section 2.1.2.4 of Chapter 2.1**.

Dry weight and ashes

Mannans samples were put in a convection oven at 105 °C for 24 h to determine moisture content according to described in **section 2.1.2.4 of Chapter 2.1**.

Solubility test

The tests were carried out in accordance with the European Pharmacopoeia (Pharmacopoeia, 2017), was previously described in **section 2.1.2.4 of Chapter 2.1**.

DSC

DSC observations were carried out in a nitrogen environment using Netzsch DSC 204 F1 Phoenix equipment as performed according to **section 2.1.2.4 of Chapter 2.1**.

5.1.2.6. Antioxidant capacity

ABTS scavenging assay

The ABTS scavenging assay was carried out in 96-well microplates, as modified by Gonçalves et al., (2009). This method was performed as described in **section 2.2.2.5 of Chapter 2.2**.

DPPH scavenging assay

The free-radical scavenging activity of DPPH was measured using the methodology reported by Schaich et al., (2015), with adjustments for a 96-well microplate scale as described previously in **section 2.2.2.5 of Chapter 2.2**. For the analysis, 175 µL of DPPH daily solution was combined with 25 µL of extracts at 10 - 5 mg/mL for TH and CMTH samples.

Oxygen radical absorbance capacity (ORAC assay)

The oxygen radical absorbance capacity (ORAC assay) assay was performed according to the methodology used by Contreras et al., (2011), previously described by Hernández - Ledesma et al., (2005). The reaction occurred at 37 °C in 75 mM of PBS (pH 7.4). This assay was performed in 96-well U bottom, polypropylene, black microplates (Thermo Scientific™, Nunc, Denmark), with 20 µL of the samples (TH extract at 1-0.5 mg/ mL, CMTH extract at 5 – 1.25 mg/mL, blank and Trolox at 10 – 80 µM) being added to 120 µL of fluorescein (116.66 nM), and equilibrated for 10 min at 37 °C in a microplate reader (Synergy H1, Biotek Instruments, VT, USA). Afterwards, the reaction was initiated through the addition of 60 µL of AAPH (48 mM), and immediately placed in a microplate reader with fluorescence being read (485 nm excitation and 580 nm emission), throughout 120 min in 1 min intervals. All reactions were performed in triplicate and three independent runs were done. Final ORAC values were expressed as µmol TE (Trolox equivalent)/100 g of sample.

5.1.2.7. Antimicrobial activity – growth inhibition curves

The potential antimicrobial activity of TH and CMTH samples was evaluated using a time-growth inhibition curve. The samples were dissolved in PBS solution to a final concentration 2-fold higher than the desired and sterilized using the autoclave at 100 °C for 20 min. After sterilization, the solutions were twofold diluted in Mueller Hinton (MH) (Biokar Diagnostic, Beauvais, France) to achieve the final a concentration of 10 mg/mL. The *Candida albicans* and uropathogenic *Escherichia coli* (UPEC) strain was used as a monoculture and, before the assay was grown in Yeast & Mould and Tryptic Soy Agar (YM and TSA; Biokar Diagnostic, Beauvais, France) with 3 g/L of yeast extract (Sigma-Aldrich, Munich, Germany) at 30 °C and 37 °C for 24 h under aerobic conditions, respectively. Ampicillin at 10 mg/mL (Sigma, St. Louis, USA) was used as dead control for UPEC and Fluconazole at 0.29 mg/mL (≤ 98%, Merck, Sigma, St. Louis, USA) was used as death control for *C. albicans*. The protocol was performed according as described in **section 4.1.2.8. of Chapter 4.1**, with the respective modifications for the microorganisms tested.

5.1.2.8. Statistical analysis

Data are expressed as the mean values ± SD (standard deviation) of replicates. To analyse the differences between the TH mannans extracted from *S. cerevisiae* and the corresponding carboxymethylated product (CMTH), when a normal distribution was observed (Shapiro-Wilke normality tests) the T-student test were carried out in association with Tukey's Multiple Comparison Test with a 95% confidence level was carried out. Tests were carried out using GraphPad Prism 7.04 software (Dotmatics, Boston, MA, USA). All tests were realized with a $p < 0.05$ for considered statistically significant.

5.1.3. Results and discussion

Carboxymethylation of polysaccharides is one of the most studied chemical modifications, rendering improved biological properties. While being highly reported in the literature for cellulose, starch, pullulan, dextran, hyaluronan or even for β-glucans, it is clear that the field of mannans chemical modifications is only in its infancy (Ding et al., 2013; Faustino et al., 2021).

The Williamson etherification is a well-documented synthetic pathway to afford carboxymethyl derivatives, encompassing a main reaction (two-consecutive steps) where the sodium hydroxide reacts with the hydroxyl groups of the polysaccharide starting material affording an alkoxide group (equilibrium reaction), followed by a SN2 reaction between the alkoxide specie and the chloroacetic acid, where the carboxymethyl group is formed. A side reaction also occurred between the chloroacetic acid and sodium hydroxide, leading to sodium glycolate and sodium chloride (Ding et al., 2013). This synthetic pathway was then selected to perform the carboxymethylation of yeast mannans from *S. cerevisiae* extracted by the pH-neutral Thermal Hydrolysis (TH), using as a starting point the conditions previously described by Machová et al., (2014) for the chemical modification of *S. cerevisiae* (Entry 1, **Table 15**), and a water-miscible organic solvent system (Entry 4, **Table 15**) described by Oka et al., (1972) for the carboxymethylation of yeasts of *Candida utilis*, both occurring at 70 °C.

The optimization performed to obtain carboxymethyl mannans extracted from *S. cerevisiae* is summarized in Table 1, keeping in mind the high yields, the sustainability, and economic viability of the production process. All products (CMTH1-CMTH7) were characterized by ATR-FT-IR to attest the success of the functionalization, and by PXRD analysis, which prompts to infer about the purity of the carboxymethylated derivatives (PXRD patterns with the sharp characteristic reflections of sodium glycolate and sodium chloride allowed to conclude that the sample contains these secondary products).

By the analysis of **Table 15** is possible to infer that the reagent concentrations and ratios have been adjusted to ensure optimal efficiency while minimizing waste. This optimization helps to reduce the reagent consumption, making the process more sustainable and cost-effective. Second, the choice of solvents was also conducted with a focus on selecting more environmentally friendly alternatives. By replacing conventional solvents with greener options, the overall environmental impact of the process is reduced. In addition to these

optimizations, thorough investigations were performed to explore purification methods that effectively remove the secondary products while minimizing resource usage. By implementing efficient purification techniques, the overall sustainability of the production process is improved. Lastly, the drying methods used were also evaluated to minimize energy consumption. By addressing these various aspects: reagent concentrations, green solvents, purification methods and drying techniques, the aim is to minimize waste, reduce environmental impact and increase the overall efficiency and appeal of the process. These prompted us to select the reaction conditions to afford the carboxymethylated product CMTH3 in 122% yield (Entry 4, **Table 15**) as the greenest and more economically viable approach to afford the carboxymethyl yeast mannans. In this approach, the reaction solvent is water, and the washing step is fast and implies a green solvent.

Table 15. Summary of the reaction conditions studied in the optimization of mannans carboxymethylation.

Entry	Product	Reaction medium	Temperature (°C)	Time (h)	TH Starting Material (mg)	TH:Chloroacetic acid or the corresponding sodium salt	Product Washing	Drying Process	Yield (%)
1	CMTH1	Water	70	5	100	1:3	Dialysis	Freeze drying	53
2	CMTH2				500				36
3	CMTH3						Ethanol (80%; v/v)	Vacuum oven at 40 °C	122
4	CMTH4	Water/Isopropanol (1:4)		4		1:0.44	Methanol (80%; v/v), followed by absolute Ethanol and Petroleum Ether		86
5	CMTH5					1:1.3			103
6	CMTH6	Water/Isopropanol (1:11.5)				1:0.44			129
7	CMTH7					1:1.3			103

Figure 37a depicts an example of the ATR-FT-IR spectra obtained for the carboxymethylated products, where is possible to observe three new vibrations at *ca.* 1320, 1420 and 1590 cm^{-1} corresponding to the O-H group, $–CH_2–$ connected to the carboxyl, and COO^- stretching (data analysed in detail in the section of structural analysis of this chapter), attesting the successful carboxymethylation – a successful carboxymethylation was afforded for all conditions studied in **Table 15**. The efficacy of the washing steps demanded for the secondary products' removal was assessed by PXRD analysis. **Figure 37b** highlights the PXRD of a sample with secondary products, demanding the need of further purification processes. This scenario was observed for CMTH6-7.

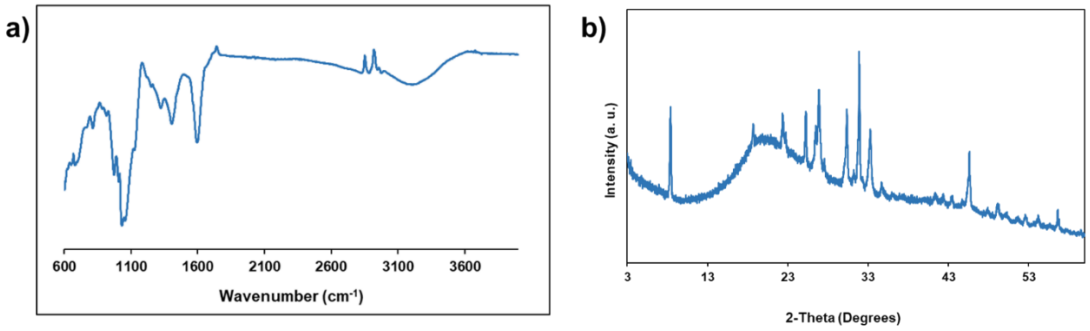


Figure 37. ATR-FT-IR spectrum obtained for carboxymethylated yeast mannans (a), and an example of a PXRD pattern exhibiting the contamination of the carboxymethylated product with the secondary reaction products.

After choosing the best approach to afford the carboxymethylated product (**Table 15**, CMTH3) from the yeast mannans extracted by the pH-neutral Thermal Hydrolysis (TH), the robustness and reproducibility of this methodology were investigated. This methodology revealed to be highly reproducible, since the carboxymethylated products resultant from five different reactions were obtained in similar yield (124-126%) and showed the same structural features, investigated by ATR-FT-IR and PXRD. These resultant carboxymethylated products were then mixed (CMTH), a detailed solid-state structural characterization was performed (ATR-FT-IR, PXRD, and SEM), and the substitution degree was determined. A comprehensive study was also performed regarding to the effect of the carboxymethylation of yeast mannans on the physicochemical (colour, total protein content, neutral sugar composition, molecular weight distribution, solubility, dry weight, ash content

and thermal behaviour) and biological properties (antimicrobial activity against pathogenic *C. albicans* and UPEC and antioxidant capacity).

5.1.3.1. Structural characterization

The structural features of the starting material (TH) and the corresponding carboxymethylated yeast mannans (CMTH) were evaluated using the following set of solid-state techniques: ATR-FT-IR, PXRD, and SEM (**Figure 38**).

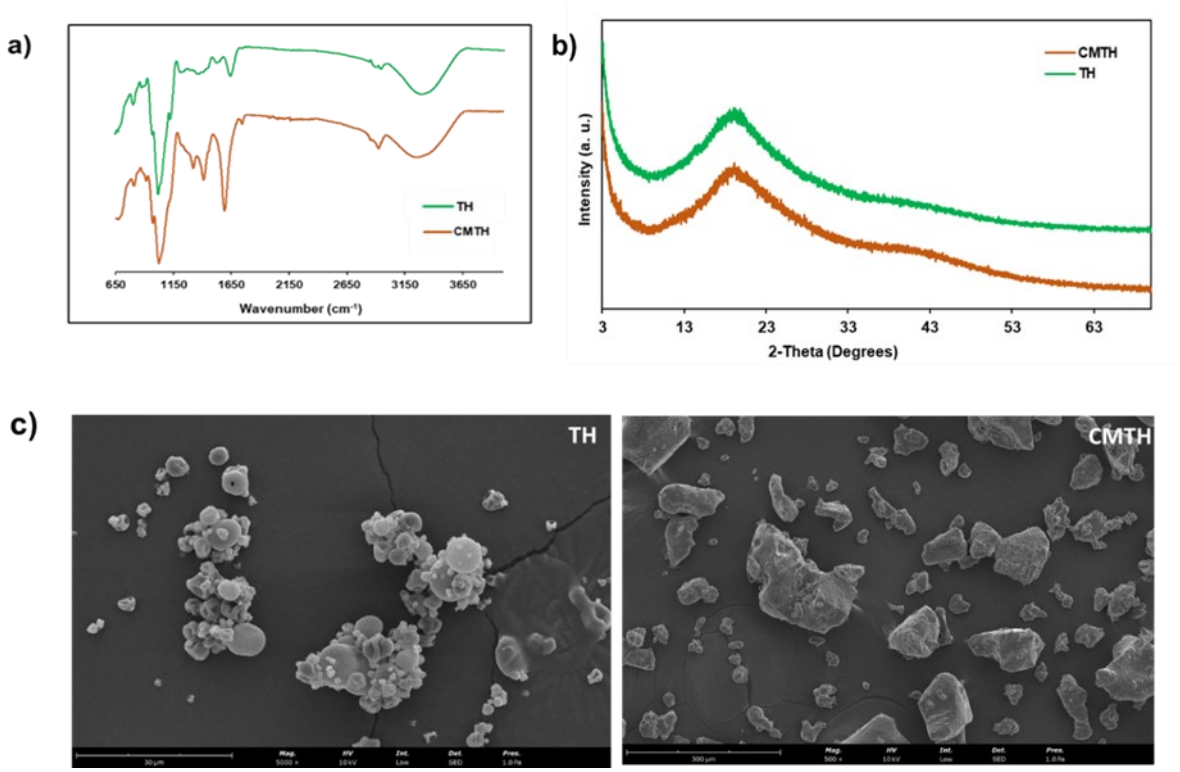


Figure 38. Structural characterization of the yeast mannans extracted by the pH-neutral Thermal Hydrolysis (TH) and the corresponding Carboxymethyl mannans (CMTH) by a) Attenuated Total Reflection Fourier- Transform Infrared Spectrometry; b) Powder X-Ray Diffraction analysis and c) Scanning electron microscopy.

The normalized ATR-FT-IR spectra of the TH and CMTH samples are depicted in **Figure 38a**. Both spectra exhibit a broad vibration band at 3000-3688 cm^{-1} corresponding to the stretching of the hydroxyl groups (Liu & Huang, 2018; Zhao et al., 2022); a vibration at *ca*. 2940 cm^{-1} which can be attributed to the C–H stretching vibration; a vibration band at *ca.* 1025 cm^{-1} that can be assigned to the O–H variable angle vibrations and the characteristic absorption of the mannan α-chain at *ca.* 820 cm^{-1} (Liu & Huang, 2018; Zhao et al., 2022).

The ATR-FT-IR spectra of the initial TH material exhibit a distinct peak at 1646 cm^{-1}, indicating the presence of the C–O asymmetric stretching vibration. (Liu et al., 2015), and the spectra corresponding to the carboxymethylated product (CMTH) exhibits the appearance of three new absorption bands at 1318, 1417 and 1598 cm^{-1} that can be assigned to the O-H group, $–CH_2–$ connected to the carboxyl, and COO^- stretching, respectively, attesting the successful carboxymethylation of the yeast mannans (An et al., 2011; Machová, Bystrický, et al., 2014).

The PXRD analysis of the TH starting material and the carboxymethylated mannans (CMTH) was performed not only to evaluate the crystallinity, but also to infer about the CMTH purity, since the presence of the secondary reaction products (sodium glycolate and sodium chloride) can be detected in the PXRD pattern by sharp and characteristic diffraction peaks corresponding to the salts resulting from the side reaction.

The PXRD patterns depicted in **Figure 38b** exhibits a single broad diffraction at 2θ = 19.3°, consistent with the predominant amorphous character of both samples. In the specific case of the CMTH product it can be also conclude that the washing steps were efficient in the secondary products removal.

The morphology of the TH spray dried sample used as starting material for the carboxymethylation and the carboxymethylated product was investigated by SEM (**Figure 38c**). The TH sample exhibit rough spherical particles, while the CMTH product oven-dried is mainly characterized by large particles that seem to be perforated.

5.1.3.2. Physicochemical properties

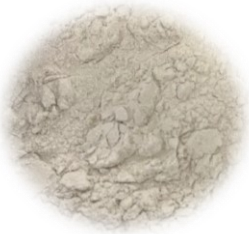
The integrated and comparative analysis of the physical appearance of the TH yeast mannans extract and the corresponding carboxymethyl derivatives (CMTH) along with their physicochemical properties and thermal analysis, is presented in this section.

Physical appearance

The physical appearance of both samples (TH and CMTH) is noticeably different, as seen in **Table 16**. Whereas TH mannans extract appears as a homogenous beige fine powder (spray dried extract), the products resulting from the carboxymethylation (CMTH) demonstrated to be a golden extract, which after the milling process exhibited a fine powder with small plates. The colour point of TH and CMTH determined according to Ordóñez-

Santos et al., (2017) using the CIELAB system (L*, a*, b*) is depicted in **Table 16**. The L* value (lightness) for the TH mannans extract is 84.32 ± 0.00, while the CMTH product exhibited a L* value of 75.46 ± 0.42. The total colour difference (E*) was calculated to quantify the colour difference between the TH mannans extract and the carboxymethylated yeast mannans. The ΔE* (1.50 < ΔE* > 3.0) between the TH and CMTH samples corresponds to 15.72 ± 0.78. As the overall colour difference measured exceeds 3.0, these findings support the distinct visual perception between both samples (Bellary et al., 2016).

Table 16. Results of physical appearance and colour characteristics of the yeast mannans extracted by the pH-neutral Thermal hydrolysis (TH) and the corresponding Carboxymethyl mannans (CMTH).

	Mannans extract (TH)	**Carboxymethyl mannans (CMTH)**
Physical appearance		
	Colour Characteristics	
L*	84.32 ± 0.00 [a]	75.46 ± 0.42 [b]
a*	2.30 ± 0.01 [a]	-0.63 ± 0.25 [b]
b*	8.04 ± 0.01 [a]	20.69 ± 0.74 [b]

[a,b,] means within the same line, marked with the same letter, do not differ from each other ($p > 0.05$).

Physicochemical properties

The physicochemical analyses performed for the TH mannans extract from *S. cerevisiae* and for CMTH are summarized in **Table 17**. Both samples were evaluated regarding to their composition – protein and total sugars content (mannose and glucose); molecular weight by HP-SEC; moisture; ash content and solubility.

Table 17. Results of physicochemical characterization of the yeast mannans extracted by the pH-neutral Thermal Hydrolysis (TH) and the corresponding Carboxymethyl mannans (CMTH).

	Mannans extract (TH)	Carboxymethyl mannans (CMTH)
Protein Content (% w/w)	10.46 ± 2.64 [a]	1.21 ± 0.19 [b]
Total Sugars (% w/w) *	73.88 ± 14.71 [a]	33.06 ± 1.90 [b]
Mannose (% w/w)	60.85 ± 11.84 [a]	26.71 ±1.92 [b]
Glucose (% w/w)	12.13 ± 2.47 [a]	5.24 ±0.32 [b]
Most Significant Population (Molecular Weight (kDa))	223 ± 13 [a]	256 ± 0 [a]
Area %	85 ± 2	79 ± 14
Moisture (% w/w)	9.55 ± 0.17[a]	9.75 ± 0.01[a]
Ash (% w/w)	5.26 ± 0.00 [a]	17.89 ± 0.17 [b]
Solubility	Very slightly soluble (approx. 1 mg/ml)	Sparingly soluble (approx. 5 mg/ml)
Degree of Substitution	n.a.	0.326 ±0.04

*Total sugars are the sum of the glucose content (% w/w) plus the mannose content (% w/w). [a,b,] means within the same line, marked with the same letter, do not differ from each other *($p > 0.05$)*. n.a. – not applicable.

The carboxymethylation of the TH mannans extract revealed a significant impact on the mannose content, which decreases from 60.85 ± 11.84% in the starting material to 26.71 ± 1.92%. The carboxymethyl mannans also revealed the lowest protein content (1.21 ±0.19%) and less-glucose residue (5.24 ± 0.32%) The CMTH ashes content increased in comparison with the ash content observed for the starting material (TH), which was expected since the CMTH product has COO^-Na^+ groups on the mannans chain, which contributes to the increase of the mineral content present in the sample (Machová, Bystrický, et al., 2014). Regarding to the moisture content, no significant difference was observed between the two samples.

The molecular weight (MW) of TH and CMTH samples was evaluated by HP-SEC, being reported a relationship between the MW and the bioactivity properties (Liu et al.,

2021). The carboxymethylation prompt an increase in the molecular weight of the mannans extract (Machová, Bystrický, et al., 2014). From the results presented in **Table 17**, it is possible to conclude that there is a difference regarding the MW of the population (256 kDa for CMTH against 223 kDa for the TH starting material). Carboxymethylated yeast mannans, on the other hand, reveals a roughly population of 470 kDa, but with limited representation (data not shown), that is not present in the mannans extract. The molecular weight is primarily related to the extraction or production of the initial extract and dictates its composition. It is reported in Machová et al., (2014) that the molecular weight of mannans of *S. cerevisiae* carboxymethylated increased from 67 kDa to 183 kDa.

The polysaccharides solubility has a major role in their applications, since most of their activities, such as emulsion capacity, drug delivery, among many others, are mainly achieved in an aqueous media. The TH carboxymethylation revealed to prompt a solubility increase from 1 mg/mL to 5 mg/mL. The solubility increase was also observed in the carboxymethylated products using other polysaccharides as starting materials, namely β-glucans and chitin (Taubner et al., 2020)., which may play a vital role in the study of biological properties.

In general, regarding the TH and CMTH composition highlighted in **Table 17**, it is possible to conclude that the TH carboxymethylation prompted a reduction in the protein, mannose, and glucose contents. Concerning the other physicochemical parameters evaluated, it was observed that carboxymethyl mannans exhibited, as expected, a higher molecular weight and ashes content and improved solubility regarding to the TH starting material. The CMTH product exhibits a degree of substitution of 0.326 ± 0.04, which is slight lower, when compared to the value obtained by Machová et al., (2014), using the reaction conditions previously described in this chapter to afford the CMTH1 product – 0.43, being important to emphasize that the CMTH product resulted from a scale-up and a more economically process.

Thermal analysis

The thermal properties of the TH mannans extract and the corresponding carboxymethylated product CMTH were studied by differential scanning calorimetry (DSC), as highlighted in **Figure 39**.

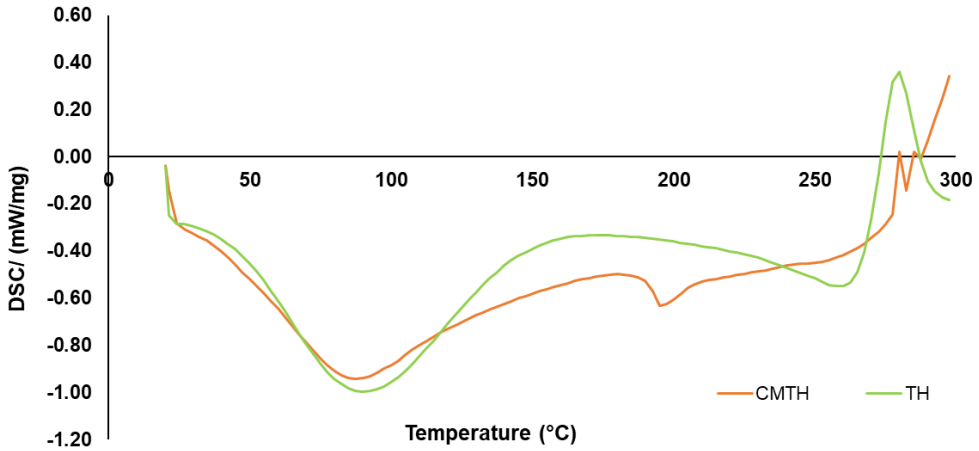


Figure 39. DSC thermograms of the yeast mannans extracted by the pH-neutral Thermal Hydrolysis (TH) and the corresponding Carboxymethyl mannans (CMTH). Mannans extract and Carboxymethylated mannans obtained at a heating rate of 10 °C/min under nitrogen atmosphere.

The DSC thermograms of the TH and CMTH samples exhibit a broad endothermic peak centered at 87.52 and 92.45 °C, respectively, which can be attributed to the evaporation of the residual water (Harish Prashanth, 2002).

The CMTH product exhibits an endothermic event (peak centered at 195.07 °C), which for other carboxymethylated, using β-glucans or cellulose as starting materials, can be attributed to the melting temperature or crystallization transitions, respectively (El-Sayed et al., 2011; Li et al., 2019). In this case, this event may be attributed to a melting temperature.

In both samples is observed an exothermic event, peaks centered at 280.06 and 277.55 °C for TH and CMTH, respectively. According to the literature (Ospina Álvarez et al., 2014), this event may be related to the thermal decomposition of polysaccharides, since during the degradation of the saccharide structure, it is common to observe events occurring near or above to 300 °C.

5.1.3.3. Antioxidant capacity

Several studies have shown that polysaccharides derived from many sources (plants, yeast, fungus, bacteria, and algae) have substantial antioxidant capabilities and might be investigated as potentially efficient antioxidants for the protection of oxidative damage in the human body (Machová & Bystrický, 2013). Antioxidants are a class of essential compounds capable of deactivate reactive species by transferring a hydrogen atom or an

electron to those species. Nevertheless, chemical modifications, namely carboxymethylation, can interfere and even enhance the bioactivities. The studies to obtain carboxymethyl at mannans from *S. cerevisiae* are scarce. However, it is possible to find, even very limited, studies that report the antioxidant activity of functionalized mannans. According to Schaich et al., (2015), radicals are typically quenched by either a hydrogen atom or an electron transfer to convert the radical to a stable species: HAT - hydrogen atom transfer (hydrogen atom transferred to target radical, possible secondary quenching by radical recombinants) and ET - electron transfer (one or more electrons transferred to reduce target compounds). The ABTS (also known as the TEAC assay), DPPH, and ORAC studies were developed to study the effect of mannans' carboxymethylation in the antioxidant capacity (**Table 18**). The ABTS and DPPH assays according to Munteanu & Apetrei, (2021) are mixed-mode assays that use both HAT and ET processes (with H atom donors and reducing agents, respectively) depending on the reaction circumstances (such as pH and solvent). The ORAC assay is the most well-known antioxidant test. According to Schaich et al., (2015), ORAC has several benefits over the ABTS and DPPH assays. This assay employs peroxyl radicals, which are better models of antioxidant interactions with oxidising lipids and reactive oxygen species (ROS) in foods and *in vivo*, and it generates radicals on a realistic time scale (more like actual reactions *in situ*). This method can be customized to detect hydrophilic and hydrophobic antioxidants in general or specifically by changing the radical source, solvent, and target molecule. The ORAC assay measures an antioxidant's capacity to quench radicals by hydrogen atom transfer rather than electron transfer.

From **Table 18**, it can be observed that the use of chemical modification processes promotes a significant variation in the antioxidant capacity. In all antioxidant assays, ascorbic acid presented a significantly higher values of μmol of TE/100 g (more Trolox units, the greater the antioxidant capacity are) than TH and CMTH samples, with the carboxymethylated product revealing the lowest value.

In the literature, the antioxidant activity of carboxymethyl mannans extracted from *S. cerevisiae* was only study by using the DPPH Machová et al., (2014) and Machová & Bystrický, (2013), being reported values slightly lower than the original extract or molecule. According to Sun et al., (2008), in carboxymethyl chitosan, a similar behaviour was observed, being observed a decrease in the scavenging effect of DPPH with the increasing of the substitution degree.

Table 18. ABTS radical, DPPH radical, and ORAC radical expressed in concentration µmol of Trolox (TE)/100 g in the yeast mannans extracted by the pH-neutral Thermal Hydrolysis (TH) and the corresponding Carboxymethyl mannans (CMTH).

	Mannans extract (TH)	Carboxymethyl mannans (CMTH)	Ascorbic acid
ORAC (µmol of TE/100 g of sample)	645 ± 155 ^{b}	31 ± 17 ^{c}	7654 ± 5 ^{a}
DPPH (µmol of TE/100 g of sample)	714 ± 110^{b}	698 ± 171^{b}	424019 ± 17148 ^{a}
ABTS (µmol of TE/100 g of sample)	4013 ± 328 ^{b}	1105 ± 131 ^{c}	463444 ± 25265 ^{a}

^{a,b} means within the same column, labelled different subscripts, differ significantly ($p < 0.05$)

To the best of our knowledge, this is the first report where the antioxidant capacity of the carboxymethyl yeast mannans is evaluated using the ABTS and ORAC assays. In these assays, the carboxymethylation did not reveal to improve the antioxidant capacity, which can be attributed to the protein content. In fact, protein and peptides present in yeast *S. cerevisiae* are reported due to their antioxidant properties (Costa et al., 2023), and the CMTH product exhibited approximately 1%, while the TH mannans extract exhibited 10% of protein. This protein content may be related to the observed antioxidant activity as there are no significant differences in terms of significant populations in molecular weight. However, it is reported that smaller populations (below for 3 kDa) show greater antioxidant capacity (Costa et al., 2023). In the TH mannans extract exists only two populations with 223 kDa and 127 kDa (data not shown), which contrasts with what is observed for the CMTH product -more than two populations, one of them with MW of 470 kDa.

5.1.3.4. Antimicrobial potential - screening

Several natural polysaccharides have been shown to have antibacterial action. According to the literature, carboxymethylation of polysaccharides improves antibacterial activity, which might have uses in the food industry to prevent food spoilage and food poisoning, as well as the pharmaceutical sector, with positive impacts on human health (Chakka & Zhou, 2020). Results presented in **Figure 40** show the impact of the TH mannans extract and the carboxymethylated product (CMTH) on the microorganism's growth. Furthermore, it is possible to observed that, both samples reveal similar growth curves with

the respective death controls (Fluconazole and Ampicillin). The CMTH sample showed a better ability to inhibit the growth of the *C. albicans* and UPEC when compare with Mannans extract and with the death controls.

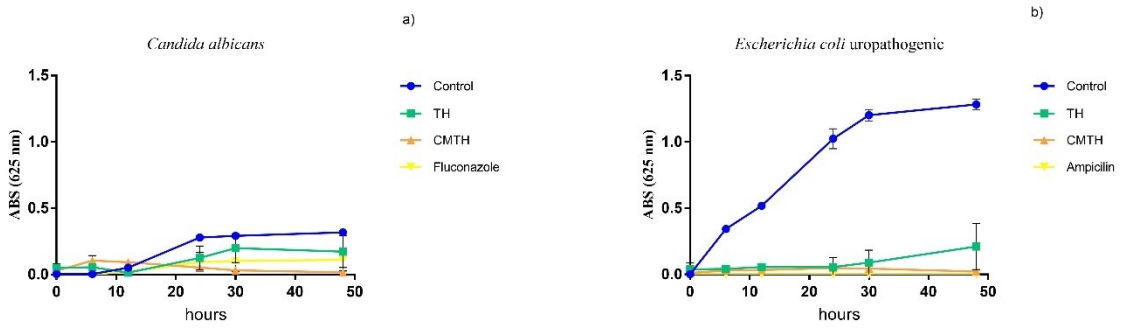


Figure 40. Antimicrobial growth curves of *Candida albicans* (a) and *Escherichia coli* uropathogenic UPEC (b) in MH broth supplemented with Mannans extract (TH), carboxymethyl mannans (CMTH) at 10 mg/mL and dead control (Fluconazole and Ampicillin), over 48 h incubation at 37 °C.

It is possible to conclude that carboxymethyl mannans (CMTH) reveal an antimicrobial potential against *C. albicans* and UPEC, the modification in the structure of the mannans potentiated its growth inhibition activity of the tested pathogens. In this study, the antibacterial activity of mannans may have been influenced by impurities, molecular weight, and chemical modification.

Studies on the antimicrobial capacity of functionalized mannans are limited or non-existent, which limits us in comparing and discussing data. However, this activity is reported for carboxymethylated β-glucans against *Staphylococcus aureus*, revealing significant antibacterial activity (Song et al., 2020).

5.1.4. Conclusions

The present study reports the effect of carboxymethylation on the structural, physicochemical, and biological characteristics of mannans obtained from spent yeast *S. cerevisiae*. From the different methodologies evaluated, it was possible to establish a more sustainable and economically viable approach to afford carboxymethyl *S. cerevisiae* mannans.

This chemical modification affected the composition (in terms of protein, mannose, glucose, and ashes content) and the physical appearance in terms of colour when compared

with the initial extract of mannans. Furthermore, differences in antioxidant capacity were also observed, where the carboxymethylated product reveals a lowest activity when compared with mannans extract. However, the carboxymethyl yeast mannans showed a high capacity of inhibited pathogenic such as UPEC and *C. albicans*. It is important to emphasize that these findings were from a screening study and further research will be conducted to determine the potential health impact of the observed effects.

Chapter 6

Final Remarks

6.1. General conclusions

The experimental work presented in this PhD thesis is an effort to find a scientific basis for the valorization of one by-product of the Amyris company: yeast. Overall, this doctoral dissertation contributes to the development of sustainable and economically viable biotechnological processes for the valorization of spent yeast to obtain mannans and MOS extracts well characterized in terms of structural, physicochemical, and biological properties (e.g., screening for prebiotic potential, immunological response, antioxidant capacity) for food, feed, and nutraceutical industries. Thereupon, in this thesis, the nutraceutical effect for human consumption of the MOS extracts was explored, an area with limited reports studies. The two potential applications studied focused on the extracts' promising putative ability to inhibit the adhesion of pathogens; firstly, as a supplement intended to prevent urinary infections against UPEC, and secondly as a topic agent to improve vaginal microbiota by preventing and helping vaginal lactobacilli against candidiasis. In addition, and in a parallel line of investigation, mannan carboxymethylation was explored to potentially improve mannans' extracts regarding their previously assessed biological properties.

More specifically, results from **Chapter 2** made possible to select an extraction process to obtain mannans from residual yeast (**Chapter 2.1**). The use of sustainable methodologies was considered a relevant point, as well as yields and purity. Thus, six different extraction methods were tested (thermal hydrolysis with water, thermal hydrolysis with sodium hydroxide at 0.25 M and 1.5 M, autolysis, autolysis followed by thermal hydrolysis with water and enzymatic hydrolysis), and the extract obtained by each of these methodologies was structurally, physiochemically, and biologically characterized. The performance of the different processes and their cost were also evaluated. It was concluded that each production process resulted in extracts with different characteristics, yields and costs. The enzymatic hydrolysis produced the maximum solid yield, around 23.88 ± 1.16%, however it was excessively expensive, having an estimated cost of 31 €/g of extract. The thermal hydrolysis with NaOH at a concentration of 0.25 M produced a higher mannose yield, 58.82 ± 1.57%, but the extract was contaminated with sodium chloride, thus requiring additional (and costly) purification procedures. The thermal hydrolysis, which used water as a solvent, provided an extract with a high mannose concentration, 53.46 ± 0.44%, without incurring in considerable expenditures (15 €/g of extract). As a result, this was the methodology selected for mannans extraction from spent yeast *S. cerevisiae*. In order to preserve mannans extracts along time,

in **Chapter 2.2** a comparison of two different drying processes was performed: spray drying over freeze drying. Physical appearance and physicochemical properties of the mannans extracts dried using the two methodologies were assessed, in order to evaluate the impact of the drying processes on the extract characteristics. Although some variations were found with respect to their physical appearance, no significant differences were observed regarding their physicochemical properties, except for the solubility of the mannans extract that were ascertained as “very slightly soluble” when dried by freeze drying and shifted to "sparingly soluble" when dried in a spray-dryer. Differences in the solubility of the mannans extract resulting from the drying process are of outmost importance as this parameter can substantially influence the results from several bioassays that rely on sample homogeneity. Henceforth, all subsequent studies were performed with mannans extracts spray dried as starting material.

In **Chapter 3** the effects of the two different MOS production methodologies (hydrothermal reaction - MOS Parr, and acidic hydrolysis reaction - MOS H_3PO_4) on the properties of the resulting extracts (including structural, physicochemical, compositional, and biological properties) were evaluated. The results showed no significant differences in the structural parameters between the two extracts produced. However, significant differences in yields, and molecular weight distribution were found. MOS Parr extract showed higher percentages of solid and mannose yield, as well as higher molecular weight populations, when compared to MOS H_3PO_4 extract. Considering the potential use of MOS extracts for nutraceutical applications, a simulation of their gastrointestinal tract digestion was performed to observe digestion effects on the bio accessibility of MOS. After cytotoxicity evaluation, immunomodulatory assays (with and without an inflammatory stimulus) were also performed, with both non-digested and digested extracts, using an *in vitro* model of the human intestinal epithelial barrier (CaCo-2 cells) and by measuring the expression of levels of two pro-inflammatory cytokines (IL-6 and IL-8). Results indicated that the digested MOS extracts induce an immune response in the presence of the inflammatory stimulus. The potential intestinal absorption of mannose present in the extracts was also evaluated using a co-culture model of an enterocyte-like cell line (CaCo-2) and mucus-producing cell line (HT-29-MTX), revealing that only small amounts of mannose were absorbed. The results described in this chapter were difficult to discuss as they were not found in the literature, so it is our understanding that they greatly contribute to deepen

our understanding and study of MOS *in vitro* for future applications in humans, given their already proven bioactive potential in animals.

Chapter 4 presents two case-study nutraceutical applications of MOS extracts: i) the inhibition of UPEC adhesion to bladder cells (to prevent UTI) and ii) the promotion of the vaginal flora and inhibition of vaginal pathogens (to prevent vulvovaginal candidiasis). The effectiveness of MOS extracts against UPEC adhesion was evaluated in **Chapter 4.1**. Results showed a significant reduction in UPEC adhesion when the extracts were used in a competition scenario, with MOS H_3PO_4 being the most effective one. The prophylaxis assay revealed that, although reaching inhibition values similar to those of D-mannose after 3 h, both extracts (MOS H_3PO_4 and MOS Parr) lost their effectiveness in inhibiting UPEC adhesion over time. Furthermore, the extracts did not present a significant immunomodulatory effect nor induced a cytotoxic response up to an equivalent mannose concentration of 2.5 mg/mL. Overall results suggest that both MOS may represent a promising alternative for managing UTIs, through a putative competitive inhibition of FimH adhesins by their mannose content, validated by assessing the extracts' impact on bacterial growth. Nevertheless, more studies are needed.

In **Chapter 4.2** it was investigated whether MOS extracts could promote the growth of vaginal lactobacilli and inhibit *C. albicans*. Results showed that in simulated vaginal fluid (SVF), considered as a suitable replica of the chemical environment present in vagina, the MOS H_3PO_4 extract was considered the most effective one in promoting the growth of *Lactobacillus* sp. pool, while the MOS Parr extract inhibited the growth of *C. albicans* and promoted the growth of *Lactobacillus* sp. pool. *In vitro* assays in HeLa cells demonstrated that MOS Parr extract, in combination *with L. crispatus*, inhibited *C. albicans* adhesion. Prophylactic assays also showed that all tested samples assisted the *L. crispatus* population in inhibiting *C. albicans* adhesion.

Chapter 5 was focused on the chemical modification of mannans by carboxymethylation with the aim of improving their biological potential. Overall, this chemical modification affected the composition (in terms of protein, mannose, glucose, and ashes content) and the physical appearance, in terms of colour, when compared with the initial extract of mannans (TH). The carboxymethylated product (CMTH) showed a high capacity for inhibiting pathogenic microorganisms such as UPEC and *C. albicans*. It is important to note that these findings are preliminary, and further research will be conducted to fully understand the potential health implications of these observed effects.

6.2. *Future work*

This PhD thesis proposes a novel approach to the valorization of spent yeast by applying sustainable extraction and production methods. These new methodologies and concepts support sustainability and connect with the circular economy idea, opening new study opportunities in the future. However, to fully realise this approach's potential and develop new high-value-added compounds that can be used in the food chain or as nutraceutical agents in human nutrition and health, several scientific, technological, and regulatory issues must be addressed comprehensively. As a result of the results of this PhD thesis, more research is required to continue investigating the potential of mannans and MOS as significant resources for various applications.

Concerning **Chapter 2**, the extraction of mannans extracts showed a promising purity and sustainability methods, despite the purity percentage could be increased. Accordingly, purification methods are needed, albeit bearing in mind that extraction methods continue to follow sustainability rules, namely in its economic and environmental areas. Regarding the second potential research line, MOS production in **Chapter 3**, it would be interesting to understand the effect of digested MOS extracts on *ex vivo* fermentation studies using donor faecal samples, that would also be important for prebiotic potential validation. Further monitorization of organic acids profiles in these fermentation studies and the effect on different groups of the microbiota using the polymerase chain reaction (PCR) technique are also in line for the future. The methodology usually followed needs to be optimized for this particular situation, because the prebiotic effect of MOS is related with the inhibition of the adhesion by the pathogens to host cells, and not with the promotion of specific groups such as *Lactobacillus* sp. and *Bifidobacterium* sp. As described in literature (Wang et al., 2018) MOS has the potential to be employed as a novel prebiotic to help regulate the gut flora (by decreasing the ratio of filo Firmicutes/Bacteroidetes and to alleviate metabolic problems (e.g., high-fat diet induced metabolic syndrome). Another important study to do is the improvement of the purification of the samples after the gastrointestinal tract (GIT) for a more precise characterization. The GIT protocol performed resorts to a considerable complex matrix, and the products generated after this process considerably affected the complexity of the extracts, as well as the interpretation of results. It is important to improve this step by removing the interferences that hinder the process of both characterization and evaluation of biological potentials. Another interesting approach will be the study of the

permeability of mannose oligomer standards and compare them with MOS extracts. Furthermore, collecting the samples after passing through the Transwells® to conduct immunological studies would provide valuable information. This strategy allows for a comprehensive assessment of the permeability and immunological effects of standardized mannose oligomers and MOS extracts. By comparing the results already obtained with those from these experiments, a better understanding of the potential benefits and mechanisms of action of MOS extracts can be obtained.

For the potential applications of MOS (**Chapter 4**), it would be interesting to better understand the effect of the digested MOS extracts on the inhibition of the adhesion of UPEC (**Chapter 4.1**). But first, it would be necessary to remove the interferences of the digested extracts associated to the GIT matrix. It would also be relevant to select the right vehicle for the final formulation of MOS. This step would be critical since it may affect the bio accessibility, bioavailability and the potential effect of the extracts. This topic is also pivotal in the research line (**Chapter 4.2**) since the application of our product could be in gel capsule, eggs with probiotics such as vaginal lactobacilli or incorporated into a topical gel. These formulations can affect the synergy of the MOS extracts with vaginal lactobacilli to inhibit the promotion and adhesion of *C. albicans*. In this context, it would also be interesting to increase the complexity of the adhesion in HeLa cells methodology. For example, using SVF instead of a culture medium, using not just a specie of *Lactobacillus* but a consortium of *Lactobacillus* to get closer to the actual vaginal flora of women. Other studies using vaginal exudates from healthy women more susceptible to candidiasis would be very interesting and enriching. An evaluation of the IL-6, IL-8 or tumour necrosis factor alpha (TNF-α) cytokines could also be an enriching result, since these cytokines are related to bacterial and/or fungal infections and are major mediators of the host's inflammatory response during an infection. As an example, IL-6 is produced by cells of the immune system in response to pathogens and plays a role in activating specific immune cells, such as T and B lymphocytes, which are important for defence against bacterial and fungal infections. The assessment of different *C. albicans* to vaginal lactobacilli ratios that would either be able to better mimic vaginal microflora, should also be assessed. These ratios should also be considered in other scenarios such as stablished infections or vaginal flora dysregulations. In addition, other vaginal lactobacilli populations could also be tested in different ratios and compositions to further assess their effects on non-*Candida* species.

Finally, with respect to the study of the chemical modification of mannans from the yeast *S. cerevisiae* (**Chapter 5**), it is our belief that the results presented in this dissertation establish the basis for future research in this field. Future research might focus on the comparison of the optimized methodologies presented in this thesis with additional carboxymethylation mechanisms to be developed for mannans, paying attention to the "greenness" of the process and to the economic availability. Other chemical modifications, such as phosphorylation, acetylation, crosslinking, glycosylation, and sulfonation, might be investigated to prompt the establishment of a structure-properties relationship. Furthermore, functionalized mannans could be used to produce MOS, and the resulting products could be evaluated to investigate the improvements in their properties. Additionally, chemical modifications of MOS extracts can also be performed, and the potential applications discussed in the previous chapter (**Chapter 4**) must be re-evaluated in order to assess the impact of the chemical modifications on these properties.

These findings might greatly contribute to the development of new and better mannans and MOS products to be used, with enormous potential advantages, in several applications.

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