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ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

VALORIZATION OF GLUCAN EXTRACTS INTO A HIGH-VALUE BIOACTIVE INGREDIENT

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

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Supervisor: **João Fernandes, PhD**
Co-supervisors: **Diana Valente, PhD**
Maria Amorim, PhD

November 2023

“Whether our efforts are, or not, favored by life, let us be able to say, when we come near to the great goal, I have done what I could.”

Louis Pasteur

This work was co-financed by European Regional Development Fund (ERDF) through the Operational Program for Competitiveness and Internationalization (POCI) supported by the Amyris Bio Products Portugal, Unipessoal Lda and Escola Superior de Biotecnologia – Universidade Católica Portuguesa through Alchemy project 'Capturing High Value from Industrial Fermentation Bio Products' (POCI-01-0247-FEDER-027578). Thanks, are also due to Universidade Católica Portuguesa and FCT/MCT for the financial support for the CBQF (project UIDB/50016/2020) research unit, through national funds and where applicable co-financed by the FEDER, within the PT2020 Partnership Agreement.

Resumo

As empresas de biotecnologia focadas no uso de leveduras para produção de compostos enfrentam um desafio crescente na criação de valor económico a partir dos subprodutos gerados no processo fermentativo. Além disso, fatores ambientais sem precedentes estão a forçar as empresas a adotar um sistema de economia circular mais sustentável, produzindo menos resíduos ou encontrando novas formas de os valorizar. As leveduras residuais obtidas após a fermentação, que normalmente não possuem valor, são uma fonte importante de compostos bioativos que podem ser aplicados a uma variedade de produtos, desde alimentos até às indústrias farmacêuticas.

Como tal, este doutoramento está focado na extração, purificação e modificação química de glucanas da levedura *Saccharomyces cerevisiae*, um polissacarídeo composto por uma mistura de ligações glicosídicas α/β, para explorar ainda mais as propriedades biológicas relacionadas à pele, através de ensaios celulares *in vitro* ou através de modelos *ex vivo* usando pele humana. Para isso, a primeira etapa desta tese concentrou-se na implementação de uma nova metodologia de extração e purificação para isolar glucanas de duas estirpes diferentes de *S. cerevisiae*: uma estirpe geneticamente desenhada fornecida pela Amyris e uma estirpe selvagem produzida à escala laboratorial. Dois extratos insolúveis foram obtidos com uma alta pureza de glucose e estrutura quimicamente semelhante. Devido à sua insolubilidade em água, foi realizada uma modificação química adicional (carboximetilação), resultando em dois extratos funcionalizados solúveis em água. Uma vez obtidos esses extratos, dois extratos insolúveis e dois carboximetilados, foi feita uma avaliação para garantir que as glucanas não representam um risco ao serem aplicadas na pele. Esta avaliação não demonstrou nenhum efeito genotóxico, nenhuma capacidade sensitizante e nenhum impacto na microbiota naturalmente presente em pele feminina.

Na segunda etapa deste estudo, os extratos insolúveis e carboximetilados foram analisados através de ensaios *in vitro* quanto às suas potenciais propriedades biológicas. Observou-se que as glucanas foram capazes de atenuar a produção de citocinas (IL-1α/IL-6/IL-8) num modelo de inflamação, utilizando lipopolissacarídeos bacterianos (LPS) e partículas de poluição (PM), induzido em células queratinócitos (HACAT) e células semelhantes a macrófagos (mTHP-1). Utilizando as células HACAT, também foi demonstrado que esses extratos, com as glucanas insolúveis a demonstrar um maior potencial bioativo, reduziram com sucesso a formação intracelular de espécies reativas

de oxigénio (ROS) induzida por PM como também promoveram a migração celular de queratinócitos num ensaio de cicatrização de feridas. Na fase final desta tese, todos os extratos testados foram incorporados individualmente numa formulação de creme para ser aplicada em modelos *ex vivo* de pele humana, desenvolvidos no sentido de avaliar potenciais propriedades biológicas relacionadas com a pele. Inicialmente, as formulações foram aplicadas em explantes de pele e foi avaliada a sua permeabilidade bem como o impacto na viabilidade da pele, sendo que todas as formulações não mostraram influência na sua viabilidade. Em seguida, foi analisado se os cremes formulados afetavam a presença de células imunes da pele (Langerhans e macrófagos M1/M2), onde foi observado que algumas das amostras possuíam capacidade de modular, aumentando ou reduzindo, o número destas células presentes na pele. Após analisar as componentes estruturais da pele, percebeu-se também que todas as formulações tinham a capacidade de aumentar a deposição de colagénio na camada dérmica, especificamente aumentava o colagénio do tipo-I. Numa vertente mais terapêutica, foi demonstrado que estes cremes possuíam capacidade de reduzir a produção de citocinas (IL-1α/IL-6) após a exposição da pele a SDS e PM, mostrando um grande potencial para usar as glucanas como agente anti-inflamatório para aplicação tópica. Adicionalmente, também foi demonstrado que a maior parte destes cremes detinha capacidade de induzir a migração epidérmica, demonstrando um potencial uso das glucanas como regenerador de feridas.

Em suma, as glucanas de levedura usadas neste estudo demonstraram ser uma molécula segura e com múltiplas propriedades bioativas para aplicação na pele. No entanto, é de realçar que a capacidade das glucanas de levedura é diretamente afetada pelas comunicações célula a célula. Adicionalmente, mais estudos devam ser feitos para analisar de que forma as glucanas podem ser aplicadas noutras áreas relacionadas com a pele, incluindo em doenças como a dermatite atópica ou no tratamento de infeções virais da pele, como o herpes simplex.

Palavras-chave: Valorização de levedura residual, Biotecnologia sustentável, Extração de glucanas, Carboximetilação, Propriedades biológicas, Saúde da pele, Aplicação em pele.

Abstract

Yeast-based biotechnology companies face an increasing challenge in deriving economic value from the byproducts generated through fermentative processes. Furthermore, growing environmental concerns are pushing companies toward adopting more sustainable and closed-loop economic systems, which aim to reduce waste production and find ways to valorize these byproducts. Spent yeasts obtained from fermentations represent an unused source of valuable bioactive compounds with potential applications across various industries, from food to pharmaceuticals. This PhD work focuses on the extraction, purification, and chemical modification of *Saccharomyces cerevisiae* glucans, complex polysaccharides composed of a mixture of α/β glycosidic linkages, to explore their biological properties related to skin health through *in vitro* assays and human skin *ex vivo* models.

For this, the first stage of this thesis concentrated on developing a novel extraction and purification methodology to isolate glucans from two different *S. cerevisiae* strains: an engineered strain designed for biotechnology fermentation provided by Amyris and a wild strain produced in a lab-scale fermentation process. Two water-insoluble extracts were obtained, which displayed a high glucose purity and a similar chemical structure. Due to their water-insolubility, an extra chemical modification (carboxymethylation) was performed, resulting in two water-soluble extracts with new functional groups added to the polysaccharide chain. Once these extracts were obtained, comprising two water-insoluble and two functionalized extracts, a safety assessment was conducted to ensure that glucans could be safely used as chemical ingredient for skin application. This assessment demonstrated that glucans had no genotoxic effect, no skin sensitization capacity, and no impact on the skin microbiota naturally present on healthy female skin.

In the second stage of this study, both water-insoluble and carboxymethylated extracts were analyzed through *in vitro* assays to assess their biological properties for potential skin application. It was observed that glucans were capable of attenuating cytokine release (IL-1α/IL-6/IL-8) in a cell-induced inflammation model, using bacterial lipopolysaccharides (LPS) and pollution particle matter (PM), involving keratinocytes (HACAT) and macrophage-like THP-1 cells (mTHP-1). Using HACAT cells, it was also demonstrated that these extracts, with water-insoluble glucans showing a higher bioactive potential, effectively reduced intracellular reactive oxygen species (ROS) generation induced by PM and promoted cell migration in a wound healing assay.

Abstract

In the final stage of this thesis, all the extracts tested until now were individually incorporated into a cream formulation and applied in newly developed human skin *ex vivo* models to evaluate potential skin-related biological properties. Initially, all the creams containing glucans were applied to skin explants, and its permeability and impact on skin viability were assessed, with all the formulations showing no adverse effects on the skin tissue. It was also analyzed whether these formulated creams affected the presence of skin immune cells (Langerhans and M1/M2 macrophage cells) in the explants, and it was observed that some of the samples had the ability to modulate, either by increasing or reducing, the number of these cells present in the skin. After analyzing the skin's structural components, it was also perceived that all cream formulations had the capacity to increase collagen deposition in the dermal layer, specifically collagen type-I. From a therapeutic perspective, it was demonstrated that the formulations tested had the capacity to reduce cytokine production (IL-1α/IL-6) when skin explants were exposed to SDS and PM, suggesting a potential application as an anti-inflammatory agent for topical use. Additionally, it was also shown that most of the cream formulations had the capacity to induce epidermal migration and wound closure, supporting the potential use of glucans as a wound repair agent.

Overall, spent yeast glucans were demonstrated to be a safe and bioactive choice with various skin-related properties for potential skin application, noting that yeast glucans capacity is directly influenced by cell-to-cell interactions. Moreover, more studies are needed to explore the ways in which glucans can be applied to other skin-related areas, such as managing skin diseases like atopic dermatitis or combating skin viral infections like herpes simplex.

Keywords: Spent yeast valorization, Sustainable biotechnology, Glucans Extraction, Carboxymethylation, Biological Properties, Skin health, Skin application.

Acknowledgments

As my PhD thesis comes to an end, I would like to use this section to thank everyone directly and indirectly helped me finalizing this journey.

I would like to thank my faculty *Escola Superior de Biotecnologia* from *Universidade Católica Portuguesa* for accepting me as PhD student and Amyris BioProducts Portugal Company, through the Alchemy Project, for fully provide all the necessary support throughout this PhD thesis.

To Professor Manuela Pintado, for leading a project that perfectly bounded industry and academia, which allowed the concretization of this thesis, as well as all the support and encouragement given.

I also would like to express my deeply gratitude to my supervisor João Fernandes for the guidance, assistance and support given during the whole journey. A big thank you and forever appreciation for all the incredible work and patient conferred by my co-supervisors, Diana Valente and Manuela Amorim, that tremendously helped me in everything I needed. An additional thanks to João Silva, Helena Moreira and Inês Ribeiro for the support, help and feedback brought to this work as well as the rest of "Quinoa" team for the companion and suggestions given. A special thanks to Doctor Inês Correia Sá and Doctor Margarida Mendes from *Centro Hospitalar de São João*, for providing the skin explants that significantly increased the novelty of this thesis work.

A forever thank you for the amazing group of friends that strongly supported me while doing my PhD, including Carlos Ferreira, Carla Pereira, Bruno Horta, Sofia Oliveira, Luís Carvalho, Pedro Castro, Nelson Carvalho and all the other great friends made along the last years. A very special thank you to my four gaming partners and lifetime friends, David, Jacinto, Luís and Marco for all the laughs and unforgettable hours spent in their companion.

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Table of contents

List of figures

List of tables

List of supplementary figures

List of supplementary tables

List of abbreviations

A

A.U – Arbitrary units

ABC – Avidin-Biotin complex

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

Alk – Alkaline extraction

ATCC – American Type Culture Collection

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

B

BCA – Bicinchoninic acid

BG – β-Glucans

BSA – Bovine serum albumin

C

C – Cysteine

CF – Cream formulation

CF CM-ES – Cream formulation containing CM ES-Glu

CF CM-WS – Cream formulation containing CM WS-Glu

CF-ES – Cream formulation containing ES-Glu

CF-WS – Cream formulation containing WS-Glu

CLS – Cell Lines Service

CM – Carboxymethyl

COX-2 – Cyclooxygenase-2

CR-3 – Complement receptors-3

D

D_2O – Deuterated water

DAB – 3,3-Diaminobenzidine

DC – Dermal cells

DCFDA – 2,7-Dichlorofluorescin diacetate

dH_2O – Deionized water

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DMSO-d6 – Deuterated DMSO

DP – Degree of polymerization

DPPH – 2,2-Diphenyl-1-Picrylhydrazyl

DPRA – Direct peptide reactivity assay

DS – Degree of substitution

E

EC – Epidermal cells

ECM – Extracellular matrix

EDTA – Ethylenediamine tetra acetic acid

ELISA – Enzyme-linked immunosorbent assay

ELSD – Evaporative light scattering detection

ES – Engineered strain

ES-Alk – Engineered strain alkaline extract

ES-Glu – Engineered strain acidified ethanol glucan extract

EWG – Electron-withdrawing group

F

FBS – Fetal Bovine Serum

FITC – Fluorescein isothiocyanate

FRAP – Ferric reducing antioxidant power

G

G-CF – Cream formulations containing glucans

Glu – Acidified ethanol extraction

H

H&E – Hematoxylin and Eosin Y

HACAT – Immortalized human keratinocytes

HPLC – High performance liquid chromatography

HPLC-SEC – HPLC size exclusion

HRP – Horseradish peroxidase

I

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

IL-1Ra – Interleukin-1 receptor antagonist

IONPs – Iron oxide nanoparticles

ITS - Internal transcribed spacers

K

KE – Key event

KRT – Keratin

L

L – Lysine

LC – Langerhans cells

LM – Laminin

LPS – Lipopolysaccharides

M

M1 – Pro-inflammatory macrophage phenotype

M2 – Anti-inflammatory macrophage phenotype

MC – Macrophage cells

Ms – Mouse

MT – Masson's Trichrome

mTHP-1 – Differentiated macrophage-like THP-1 cell line

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

N

NGS – Next-generation sequencing

nHDF – Normal human dermal fibroblasts

nHEK – Normal human epidermal keratinocytes

NLR – Nucleotide-binding leucine-rich repeat receptor

NLRP1 – NLR Family Pyrin Domain Containing 1

NMR – Nuclear Magnetic Resonance

NO – Nitric oxide

O

OECD – Organization for Economic Co-operation and Development

P

PAMPs – Pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PBMC – Peripheral blood mononuclear cells

PD-1 – Programmed cell death protein-1

PD-L1 – Programmed cell death-ligand 1

PI – Propidium Iodide

PM – Urban particulate matter

PMA – Phorbol-12-myristate-13-acetate

PRRs – Pattern recognition receptors

PSR – Picro Sirius Red

PVA – Polyvinyl alcohol

R

RFI – Relative Fluorescence Intensity

ROS – Reactive oxygen species

RPM – Rotations per minute

RPMI – Roswell Park Memorial Institute

Rb – Rabbit

S

SC – Stratum corneum

ssDNA – Single-stranded DNA

T

TAMs – Tumor-associated macrophages

TEWL – Transepidermal water loss

THP-1 – Human leukemia monocytic cell line

TK6 – Human lymphoblast cell line

TLRs – Toll-like receptors

TME – Tumor microenvironment

TNF – Tumor necrosis factor

TGF – Transforming growth factor

Trolox – 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

U

UV – Ultraviolet radiation

V

VEGF – Vascular endothelial growth factor

W

WS – Wild-type strain

WS-Alk – Wild-type strain alkaline extract

WS-Glu – Wild-type strain acidified ethanol glucan extract

Y

YPD – Yeast extract peptone dextrose medium

Thesis scope and objectives

This PhD comprises five main chapters that provide comprehensive descriptions and discussions of each stage of the work, as schematized in **Figure 1**. These chapters encompass all the work performed to achieve the following objectives:

(I) Development of a method for extracting a water-insoluble glucan powder obtained from engineered and wild type *Saccharomyces cerevisiae* strains, followed by successful functionalization through the addition of carboxymethyl groups.

(II) Validation of both water-insoluble and carboxymethylated extracts as safe chemical ingredients for application to the skin.

(III) Evaluation of relevant *in vitro* biological properties, considering the previously reviewed literature.

(IV) Incorporation of all the extracts into a cream formulation and validation of its safety, biological properties, and potential for use as a skincare ingredient through human skin *ex vivo* models.

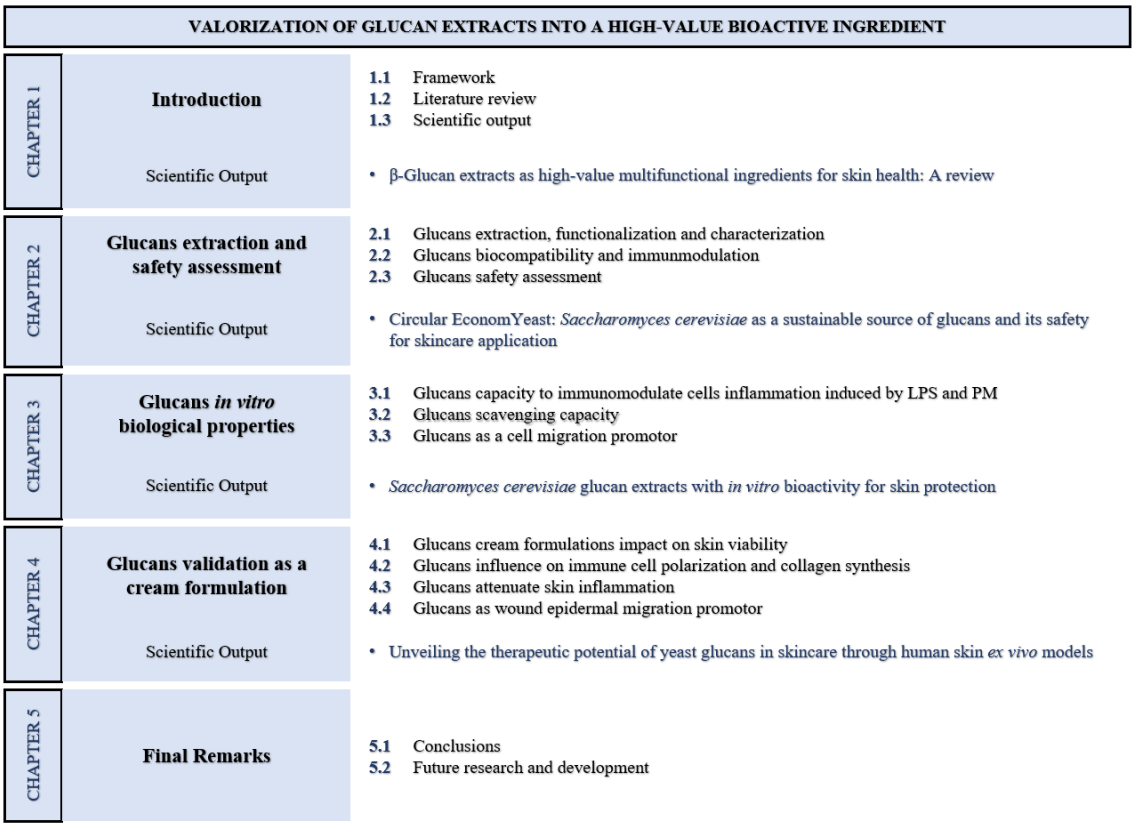

Figure 1 – Thesis structure.

Chapter 1 provides an overview of the framework, objectives, and scientific contributions of this doctoral work. It also includes a comprehensive review of existing knowledge concerning β-glucans from various natural sources and their applications in skincare. It emphasizes the existing gap in studies and information on this topic.

Chapter 2 describes the methodology for isolating and purifying glucan extracts. The focus is on their β-glucan content, which was obtained from two different yeasts: an engineered strain provided by Amyris from an industrial fermentation process and a wild type strain produced in a lab-scale fermentation. The resulting purified glucan extracts, both water-insoluble and carboxymethylated, from both yeasts were subjected to safety assessments through biological and chemical assays, including various tests based on OECD guidelines.

Chapter 3 evaluates the *in vitro* biological properties of our glucan extracts. Based on the properties of β-glucans reviewed previously, three relevant properties were tested: immunomodulatory capacity against an inflammatory stimulus, protective ability against intracellular oxygen radicals induced by airborne particulate matter, and their potential to promote keratinocyte migration for wound closure.

Chapter 4 investigates the transition from *in vitro* to *ex vivo* studies to enhance our understanding of how glucan extracts can be effectively used in skincare. A cream formulation was developed and applied to skin *ex vivo* explants to determine if the glucans could permeate through the skin layers and observe their impact on skin tissue viability. These formulations were then applied to different human skin *ex vivo* models to analyze their impact on immune cells, skin ECM components, and potential biological properties, including cytokine modulation and wound healing capacity.

Finally, **Chapter 5** presents the most significant conclusions of this PhD thesis and outlines future work and challenges. It aims to provide an explanation for some of the mechanisms observed in this study and contribute to a better understanding of how glucans can be a promising ingredient for skin application.

Chapter 1

This chapter provides an overview of the overall framework, the scope of this PhD thesis, and its anticipated scientific contributions. It also includes a state-of-the-art assessment of the availability of β-glucans from natural sources, along with a detailed analysis of their molecular structure, extraction methods, chemical modifications, and potential applications in skin.

João Fernandes hereby authorize the PhD student Pedro Sousa from the CBQF/ESBUCP to include the following publication in his doctoral Thesis entitled ***Valorization of glucan extracts into a high-value bioactive ingredient*** as a result of his activity on the review article cited bellow.

Information based on the following paper:

Sousa, P., Tavares-Valente, D., Amorim, M., Azevedo-Silva, J., Pintado, M., & Fernandes, J. (2023). β-Glucan extracts as high-value multifunctional ingredients for skin health: A review. Carbohydrate Polymers, 121329. doi.org/10.1016/J.CARBPOL.2023.121329.

1.1 – Framework

Saccharomyces cerevisiae is a microorganism widely used in various industries, including brewing and the production of specific molecules through biotechnological processes. Consequently, significant amounts of spent yeast are continuously generated, often with little or no value to the companies, leading to their disposal as waste or utilization as livestock feed. Given the growing importance of topics such as sustainability, the circular economy, and environmental responsibility, there is an increasing emphasis on reducing industrial waste. It is crucial for companies to design a production process that consider the generation of final waste products, create new value, and reduce their environmental footprint.

Amyris inc, a company primarily devoted to biotechnology and the sustainable production of molecules through yeast fermentation, collaborated with the *Escola Superior de Biotecnologia* at *Universidade Católica Portuguesa*, an academic institution specializing in leveraging byproducts generated by various industries. This collaboration explored innovative ways to create value from waste generated during Amyris' fermentation processes, including spent yeast. This collaborative research project was named Alchemy.

Against this backdrop, this PhD thesis primarily focused on *S. cerevisiae* spent yeast. This microorganism strain is extensively studied by the scientific community due to its potential as source of biologically active molecules. One of these compounds is yeast glucans, a complex polysaccharide found in the cell wall of this microorganism, which exhibits various biological properties, including immunomodulation and wound healing. Even though several commercial products already utilize this molecule as an active ingredient to promote skin health, there remains a lack of scientific understanding regarding the mechanisms by which glucans function in this context.

Consequently, this thesis aims to first design a method for extracting and chemically modifying this polysaccharide from the yeast cell wall using the spent yeast waste generated during the fermentation process. Subsequently, the thesis seeks to validate a final cream formulation, containing water-insoluble or carboxymethyl glucan extracts as the active ingredient, with respect to safety and suitability for promoting skin health.

1.2 – Literature review

β-glucan extracts as high-value multifunctional ingredients for skin health: A review

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Abstract

β-Glucans, which are naturally present in cereals, yeast, and mushrooms, have gained attention as a potential natural source for functional foods and pharmaceuticals. Due to the availability of β-glucans from several sources, different extraction methods can be employed to obtain high purity extracts that can be further modified to enhance their solubility or other biological properties. Apart from their known ability to interact with the immune system, β-glucans possess specific properties that could benefit overall skin health and prevent age-related signs, including soothing and antioxidant activities. As a result, the use of β-glucans to mitigate damage caused by environmental stressors or skin-related issues that accelerate skin aging or trigger chronic inflammation may represent a promising, natural, eco-friendly, and cost-effective approach to maintaining skin homeostasis balance. This review outlines β-glucan extraction methodologies, molecular structure, functionalization approaches, and explores skin-related benefits of β-glucans, along with an overview of related products in the market.

Keywords: Beta Glucans; Extraction; Chemical modification; Biological Properties; Immunomodulation; Skincare

1.2.1 – Introduction

Human skin serves as a natural physical barrier against various external factors, including ultraviolet radiation, urban air pollution, pathogens, and toxins, while also regulating body temperature and water loss (Abdo et al., 2020; Maarouf et al., 2019). Skin structure can be divided into three main layers: the epidermis, dermis, and hypodermis. Each of these layers supports a large number of different skin cells and more than 300 molecules present in the extracellular matrix (ECM). These molecules include fiber-forming molecules (e.g., collagen), non-fiber forming molecules (e.g., hyaluronan), and matricellular proteins (e.g., periostin) that contribute to the support, elasticity, strength, and homeostasis of the skin (Huang et al., 2022). The epidermis is the outermost layer of the skin and is mainly composed of keratinocytes (approximately 90%), as well as a smaller number of Langerhans cells, melanocytes, and Merkel cells (Mohamed & Hargest, 2022). Within this layer, keratinocytes can be found in various stages of differentiation, ranging from undifferentiated keratinocytes (innermost layer of the epidermis) to terminally differentiated non-nucleated dead keratinocytes (outermost layer of the epidermis) (Scieglinska et al., 2019). Langerhans cells are the only dendritic cells present in the epidermis. These are immune cells with antigen-presenting capacity, playing a crucial role in adaptive immune defense. Melanocytes are responsible by the production of melanin, which is transferred to keratinocytes and accumulates in the supranuclear region, providing protection against ultraviolet (UV) radiation (Correia et al., 2018). Finally, Merkel cells constitute a unique population of postmitotic cells scattered along the dermo-epidermal junction. It is believed that these serve as adapting mechanoreceptors.

Within the epidermis, various types of keratins (KRT) and laminins (LM) are present, contributing to its strength (Pfisterer et al., 2021). According to Dyring-Andersen et al., review (2020), this layer contains 47 different identified keratins, with KRT-1, 5, 10, 14, and 6A being the most abundant. Increased levels of certain keratins (KRT-6, 16, and 17) are also associated with cell proliferation and inflammation, which is a natural response to skin barrier damage or specific skin diseases like psoriasis. LM 332, 511, and 521 are the most prevalent laminins in the epidermis, particularly near the epidermal-dermal junction. These laminins are involved in wound repair processes, with LM 332 being synthesized by keratinocytes in the initial stages of wound healing (Rousselle et al., 2019).

The dermis is the region where skin fibroblasts reside. These are responsible for producing the molecules found in the ECM. The dermis also contains pericytes, which possess mesenchymal capacity and play an important role in tissue regeneration (Bodnar et al., 2018). Additionally, immune cells, endothelial cells, and cells that produce cytokines and chemokines are present in the dermis, contributing to immune responses (Rognoni & Watt, 2018; Sanchez et al., 2019). Collagen is the primary component of the ECM in the skin and is predominantly represented by fibrillary collagen. Collagen type-I is the most abundant type, followed by collagen type-III, and smaller amounts of collagen type-V. These collagens provide structural support and tensile strength to the skin. Elastic fibers (e.g., elastin), glycoproteins (e.g., fibronectin), and other molecules (e.g., hyaluronan) also contribute to the elasticity and stabilization of the skin (Pfisterer et al., 2021). The hypodermis, also known as the subcutaneous layer, serves as an insulator and protects the organs underneath (Supe & Takudage, 2021). It is primarily composed of blood vessels and adipocytes, which have various functions such as energy storage, synthesis of triglycerides, tissue regeneration by facilitating the recruitment of fibroblasts, and nutrient homeostasis (Gupta, 2014; Yucha et al., 2019). Given the importance of this organ, it is essential to minimize skin degradation by promoting the synthesis of vital skin components or mitigating the adverse effects of factors such as sun exposure or air pollution (Piquero-Casals et al., 2023; Zouboulis et al., 2019). In recent years, consumers' interest in innovative skincare products containing natural, bioactive compounds has surged (Ahsan, 2019a). These products, known as cosmeceuticals, may contain ingredients that occur naturally (e.g., minerals, plant extracts, products from microorganisms) or are chemically synthesized.

One of the most essential and widespread classes of bioactive compounds is polysaccharides, which play a crucial role in the structure of animal cell membranes, microbes, and plant cell walls, and possess well-known biological properties. Polysaccharides can be obtained from a wide variety of sources, including plants, algae, microorganisms, and animals (Li et al., 2018; Ullah et al., 2019; Yu et al., 2018). Several studies have indicated the use of polysaccharides extracted from natural sources as bioactive ingredients in skincare products (Du et al., 2014; Kanlayavattanakul & Lourith, 2015; Fernando et al., 2019). One such polysaccharide with bioactive properties is β-glucan (BG) (**Figure 2**), a structural polymer found in plants and fungi, which has documented biological activities suitable for skincare applications, such as wound healing, anti-wrinkle, and antioxidant activities (Du et al., 2014). BG can be obtained and

isolated from cereals, mushrooms, or microorganisms, in different conformations (Kaur et al., 2020).

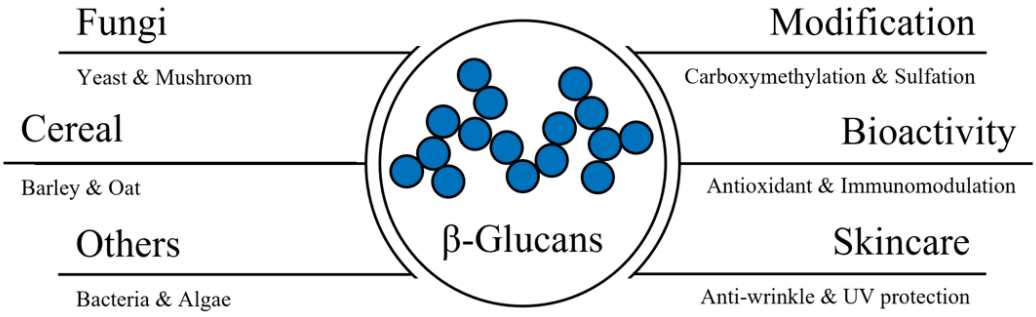

Figure 2 – β-Glucans as a high-value multifunctional ingredient for skin applications.

Products containing BG are commercially available and are present in the normal diet of many consumers due to their natural presence in oats and barley (Hughes & Grafenauer, 2021). Recently, some authors have pointed out BG as an excellent compound with applicability in skincare, not only due to its direct impact through antioxidant mechanisms or antimicrobial properties but also due to its indirect effect on the modulation and stimulation of the immune system and wound healing (Divya et al., 2020; Huang & Huang, 2021; Vetvicka et al., 2019). Numerous chemical functionalization strategies have been extensively employed to modify the inherent structure of specific types of glucans, with the aim of enhancing their physicochemical and biological properties. These modifications can considerably improve BG solubility and increase the potential use of these molecules as a bioactive ingredient (Liu et al., 2021; Yuan et al., 2019). In this case, certain properties demonstrated by functionalized BG, such as antioxidant (Calegari et al., 2017; Theis et al., 2019), antibacterial (Wan-Mohtar et al., 2016), wound healing (Yasuda et al., 2018), UV radiation protection (Zülli et al., 1998) and antiaging (Pillai et al., 2005), can be promptly used for skincare application.

This review comprehensively describes different BG extraction methodologies applied to various sources and the types of chemical functionalization typically employed to improve its solubility and biological properties. In the context of skincare, we will also review the relevant properties that have been reported for BG, which have potential applicability as a bioactive ingredient. The final chapter of this review will analyze the

potential application of this molecule and the current availability of skin products enhanced by the presence of BG in their formula or its use as an ingredient.

1.2.1.1 – β-Glucans extraction, structure and functionalization

1.2.1.1.1 – Extraction methods

The extraction of BG can be achieved through various methods, however, they can affect BG's primary physical properties, such as viscosity, molecular weight, and solubility. These methods include chemical extraction using acid, alkaline, and organic solvents, enzymatic treatment, autolysis, mechanical methods, or a combination of these to improve BG purity and remove impurities such as lipids (Kaur et al., 2020; Yuan et al., 2019).

Regarding yeast, several approaches have been reported, including enzymatic hydrolysis, autolysis, chemical treatments, or ultrasounds, mainly used to break down the cell wall (Avramia & Amariei, 2021; Liu et al., 2021). Purified BG from spent yeast from a brewing process with a final yield of 11% comparing with the initial mass of yeast, was obtained from the *Saccharomyces cerevisiae* yeast (Liu et al., 2008). The authors applied a methodology that consisted of autolysis for 24 hours (h) at 55 °C followed by a short heat treatment at 85 °C. The pellet was then subjected to a hot water treatment (autoclave) for 4h, homogenization by high pressure, lipid extraction, protein hydrolysis, and spray drying as a final process. The dry weight content of BG and total carbohydrates increased from 21% and 35% (spent yeast) to 93% and 96% (purified BG) at the end of the process. A more in-depth study was conducted to evaluate the best methods to disrupt this yeast cell wall, analyzing various methodologies such as hot water treatment (autoclaving), autolysis, bead mill, sonication, and more than one of these conditions simultaneously. After comparing and analyzing all the results, the authors reported that bead milling alone, autolysis followed by bead milling, and sonication were the most efficient methods to extract BG from yeast, obtaining extracts with a high quantity of saccharides and low protein content. All the extraction methods tested obtained a BG content ranging from 6% (autoclaving in deionized water/buffer) to 15% (autolysis + bead milling in deionized water) (Bzducha-Wróbel et al., 2014). Applying a “greener” approach, (Magnani et al., 2009) demonstrated that it is also possible to extract insoluble BG from *Saccharomyces cerevisiae* yeast without producing harsh residues. For this, an initial autolysis of 24 h at 55 °C followed by a high-temperature procedure in an autoclave for 4 h was performed. Afterward, three more processes took place: sonication to break the cell wall, lipid

extraction using isopropanol and petroleum ether, and a final protein hydrolysis with protamex (proteolytic enzyme) to cleave the residual protein remaining in the insoluble fraction. This extraction method had a yield of 11% and a BG recovery of 94%, compared with the amount of BG from the original yeast. In most cases, simple methods are not enough to destroy the cell wall structure, explaining the need to complement more than one method, such as heat treatment followed by acid-base extractions (Avramia & Amariei, 2021).

In order to obtain BG from cereals, various extraction conditions can be applied, such as alkaline extraction, acid extraction or enzymatic hydrolysis (Ahmad et al., 2010). Some authors observed that the most efficient condition to extract BG from oat was a simultaneous hot-water treatment and a thermostable α-amylase hydrolysis for 3 h at 100°C, following an initial reflux of oat brans in ethanol (82%) for 2 h to inactivate native enzymes. This method resulted in a yield of 76% (Ragaee et al., 2008). In a different study, two proteolytic enzymes, pancreatin and papain, were tested separately, resulting in two final BG concentrates with 57% BG content and a protein content of 18% and 6%, respectively (Immerstrand et al., 2009). Another study evaluated four types of extraction (alkaline, acidic, hot-water and enzymatic), following an initial reflux of barley flour in ethanol (80%) for 6 h. The authors reported that hot-water extraction was the best condition, recovering 83% of BG from the original source and an extraction yield of 5%, when compared with the other conditions studied (Ahmad et al., 2009). The same authors made a similar analysis for oat BG. In this case, the enzymatic procedure was the condition that obtained the highest yield, with a recovery of 87% and an extraction yield of 5%, when compared to alkaline and acid extractions, as hot-water extraction was not performed (Ahmad et al., 2010).

In another study, the impact of increasing concentrations of HCl [0.1-0.5 M] was evaluated for primary acid hydrolysis and an alkaline extraction with NaOH [1 M] to extract BG from barley. Then, the supernatant was hydrolyzed with α-amylase and later precipitated with ethanol (80%) overnight at 4°C. The authors concluded that as HCl concentration increased, the amount of total and soluble BG decreased, but the purity of both fractions increased. The molecular weight and viscosity of the soluble BG also decreased (Lee et al., 2015). BG was also extracted from *Eleusine coracana* seeds flour through an initial reflux with 80% ethanol and NaOH [1 M] for 8 h. Then, the sample was stirred at 45 °C for 90 min, and the insoluble fraction was incubated overnight with 80%

ethanol. The final purified BG from this plant seeds presented a value of 8% (Divya et al., 2020).

The drying process is equally important to the extraction method when it comes to achieving high purity. According to recent research by Zeko-Pivač et al., (2023), spray-drying has been identified as the most suitable method for preserving the structure of BG compared to lyophilization, which can cause agglomeration and result in a porous sheet-like surface, or air drying, which leads to the formation of larger, granular BG particles.

As summarized in **Table 1**, various approaches can be used to disrupt the matrix, although researchers typically begin with similar steps, which typically include: (I) Autolysis or autoclave treatment - to break down the cell wall and inactivate native enzymes; (II) Acid or alkaline extraction - primarily aimed at removing lipids and proteins; (III) Enzymatic hydrolysis - specifically targeting the breakdown of α/β glycosidic linkages or the removal of specific chemical components; (IV) Spray-drying method - used to obtain a fine and stable powder of BG. It is important to note that these methods, particularly the harsh chemical extractions, may gradually break down and alter the conformation of polysaccharides, potentially resulting in a BG extract with a lower molecular weight. Depending on the desired degree of purification, additional steps such as sonication, organic solvent treatments, or other complementary methods (e.g., acid-base extraction) may be included.

Table 1 – Methods used for the extraction and purification of BG obtained from different sources.

Source	Method(s)	β-Glucans (%)	Reference
Bacteria	Basic extraction Acid precipitation Dialysis	-	(Shih et al., 2009)
	Ethanol precipitation Fractionation	>90	(Luo et al., 2019)
Algae	Acid/Water extraction Ethanol precipitation	4 - 5	(Kadam et al., 2015)
	Acid extraction Autoclave Filtration	-	(Garcia-Vaquero et al., 2019)
	Water extraction Ethanol precipitation	-	(Cuong & Xuan, 2020)
Fungi	Alkaline extraction Organic solvent extraction Autoclave	-	(Ishibashi et al., 2004)

Table 1 – Methods used for the extraction and purification of BG obtained from different sources (continued).

Fungi	Autolysis Autoclave High Pressure homogenization Organic solvent treatment Protease treatment	34 - 93	(Liu et al., 2008)
	Autolysis Autoclave Sonication Lipid extraction Enzymatic hydrolysis Dialysis	-	(Magnani et al., 2009)
	Autolysis Autoclave Bead mill Sonication	6 - 15	(Bzducha-Wróbel et al., 2014)
	Hot water extraction Ethanol precipitation Dialysis	9 - 17	(Khan et al., 2017)
	Autoclave Ethanol precipitation	3 - 48	(Vetvicka et al., 2019)

Table 1 – Methods used for the extraction and purification of BG obtained from different sources (continued).

Fungi	Alkaline extraction Acid extraction	-	(Amer et al., 2021)
	Mechanical cell lysis Alkaline extraction Acid extraction	79	(Avramia & Amariei, 2022)
Cereals	Ethanol reflux Alkaline extraction Acid extraction Enzymatic hydrolysis	-	(Ahmad et al., 2009)
	Ethanol reflux Hot water extraction Ultrasound Autoclave Enzymatic hydrolysis Dialysis Ethanol precipitation	57	(Immerstrand et al., 2009)

Table 1 – Methods used for the extraction and purification of BG obtained from different sources (continued).

Cereals	Ethanol reflux Alkaline extraction Acid extraction Hot water extraction Enzymatic hydrolysis	-	(Ahmad et al., 2010)
	Acid extraction Alkaline extraction Enzymatic hydrolysis Ethanol precipitation	1 - 7	(Lee et al., 2015)
	Ethanol reflux Alkaline extraction Ethanol precipitation	8	(Divya et al., 2020)
	Organic solvent extraction Enzymatic hydrolysis Ethanol precipitation Ammonium sulfate precipitation Dialysis	81	(Chen et al., 2021)

Table 1 – Methods used for the extraction and purification of BG obtained from different sources (continued).

Cereals	Ethanol reflux Hot water extraction Enzymatic hydrolysis Ethanol precipitation Dialysis	77 - 80	(Li et al., 2023)

1.2.1.1.2 – Molecular structure

Glucans consist of several glucose units linked by both alpha and beta glycosidic linkages, and the number of monomers directly affects its molecular weight (Ruiz-Herrera & Ortiz-Castellanos, 2019). Depending on its source, BG can have a linear or branched structure with different types of glycosidic linkages: β-(1→3), β-(1→4), and β-(1→6) (**Figure 3**). In the specific case of yeast, BG is composed of a backbone of β-(1→3)-glucan with long β-(1→6)-glucan branches. However, recent reports highlight the presence of a mix of α-(1→4) and β-(1→4) linked cell wall glucans (Bastos et al., 2022). In mushrooms, BG has a backbone of β-(1→3)-glucan with short β-(1→6)-glucan branches, while in cereals BG has a linear structure consisting of linked β-(1→4)-glucan and β-(1→3)-glucan (Du et al., 2019).

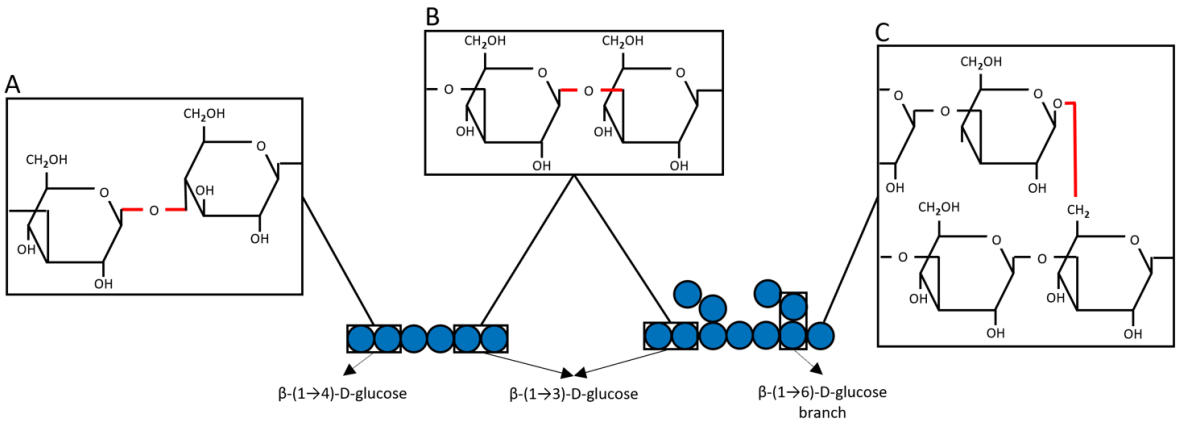

Figure 3 – Types of linkages that can be found in β-Glucans between glucose monomers: (A) β-(1→4)-D-glucose; (B) β-(1→3)-D-glucose; (C) β-(1→6)-D-glucose. These linkages are naturally found in cereals (A and B) or in yeast (B and C).

BG solubility is mainly affected by its degree of polymerization (DP), since a better solubility indicates a lower DP (Du et al., 2014; Yuan et al., 2019). Considering their linkages, BG are typically water-insoluble when their structure is mainly composed by β-(1→3)-glucans with few or no β-(1→6)-glucans, as seen in curdlan. Conversely, a more branched structure composed of the same linkages, such as lentinan, can be dissolved in water (Chen et al., 2022). Therefore, the solubility of BG is influenced by the frequency and distribution of side-chain branches within their molecular structure (Bacic et al., 2009).

1.2.1.1.3 – Chemical modification

Chemical modifications of BG can be achieved by altering its structure through processes such as carboxymethylation, sulfation, phosphorylation, or acetylation. These modifications can enhance the solubility and biological activity of BG. Less commonly, amination or gamma irradiation have been utilized to functionalize β-glucan (Kaur et al., 2020; Khan et al., 2016). Moreover, besides modifying the structure of BG itself, it is possible to functionalize it by conjugating it with other molecules. For example, for skincare applications, BG can be conjugated with chitin (Gautier et al., 2008), while for drug delivery purposes, it can be linked with silica (Hwang et al., 2018). These conjugations allow for the incorporation of additional properties or functionalities into BG, expanding its potential applications. These substitutions, as depicted in **Figure 4**, can have a significant impact on the properties of BG. The type and number of substituents linked to the structure will modify its biological capacity, molecular weight, and branching ratio (Huang & Huang, 2021).

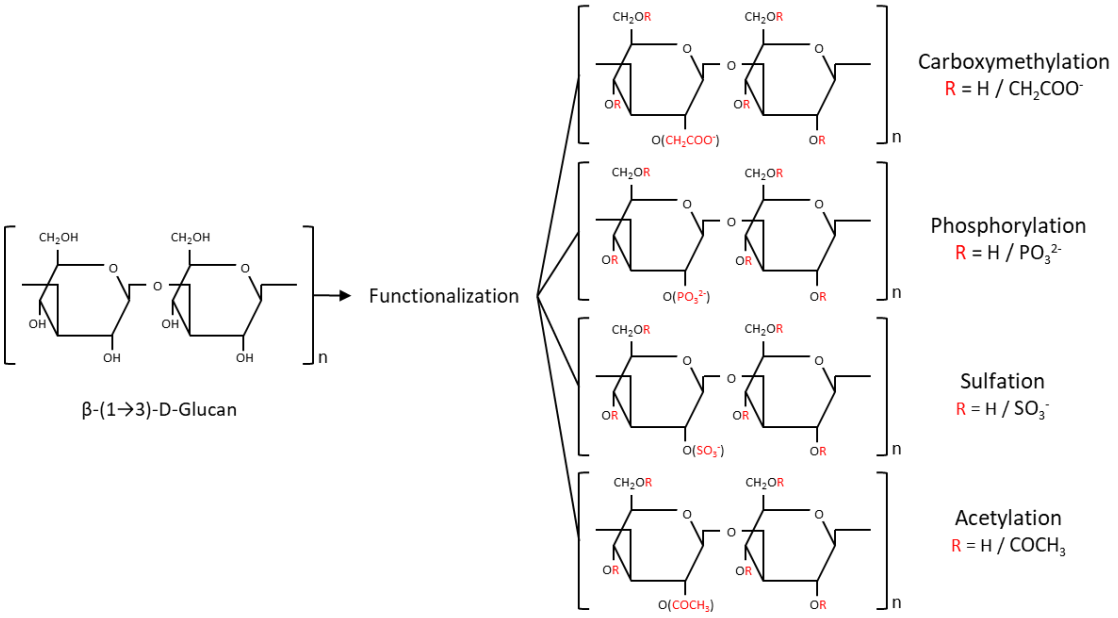

Figure 4 – Examples of most used chemical modifications of β-(1→3)-D-glucans through the addition of functional chemical groups.

Carboxymethylation and sulfation are the most commonly used methodologies for modifying BG, based on the available literature. Carboxymethylation provides an effective and low-cost way to functionalize BG while potentially enhancing its biological

activity (Chakka & Zhou, 2020). This process involves replacing hydroxyl groups on the polysaccharide chain with carboxymethyl groups. It typically involves three main steps: (I) formation of alkoxide groups through the reaction of the polysaccharide in a highly alkaline environment; (II) addition of a carboxymethylation agent, usually monochloroacetic acid, in a highly alkaline environment; (III) reaction of the polysaccharide with the carboxymethylation agent, resulting in the addition of carboxymethyl groups to the alkoxide groups.

Sulfation is another commonly used method, chosen for the resulting immunomodulatory properties observed in chemically modified polysaccharides (Huang et al., 2019). Sulfation involves the addition of sulfate groups by substituting hydroxyl groups in the polysaccharide. Various methods can be employed, but they generally follow a similar methodology (Kaur et al., 2020; Liu et al., 2014; Ray et al., 2013; Zhang et al., 2017): (I) dissolution of glucans in formamide or dimethyl formamide; (II) addition of a sulfation agent, such as sulfuric acid, sulfur trioxide-dimethylacetamide, or chlorosulfonic acid; (III) reaction of the polysaccharide with the sulfation agent, leading to the addition of sulfate groups into the polymer chain. The degree of substitution (DS) is a critical parameter used to evaluate the efficiency of these functionalization processes. It represents the average amount of functional groups inserted into the molecule.

Various DS values have been reported for carboxymethylated BG extracts obtained from different sources. For example, fungal exocellular Lasiodiplodan (*Lasiodiplodia theobromae*) showed a DS range of 0.32-0.68 (Theis et al., 2019) and Qingke barley (*Hordeum vulgare* L.) exhibited a range of 0.32-0.88 (Guo et al., 2020). BG extracted from yeast (*S. cerevisiae*) also showed varying DS values depending on the method specifications, such as 0.43 (Machová et al., 2014) or 0.80-1.03 (Ma et al., 2022). Many of these studies have indicated a potential correlation between a higher degree of substitution and improved biological activity, including enhanced antioxidant capacity, which can be further potentiated by reducing the molecular weight of BG (Ma et al., 2022).

Sulfated BG can also be obtained with high DS values, with variations depending on the modification methods and the sources of extraction. Examples include oat (*Avena sativa* L.) with a DS of 0.68 (Yun et al., 2006), Qingke barley with a range of 0.25-0.58 (Guo et al., 2019), and mushroom sclerotia (*Pleurotus tuber-regium*) with a range of 1.14-1.74 (Zhang et al., 2003). For yeast-derived BG (*S. cerevisiae*), different studies have reported DS values such as 0.16-0.27 (Lei et al., 2015), 0.75 (Zhang et al., 2017), 0.16 (Wang et

al., 2016, 2019). While a definitive relationship between the DS value and the biological activity of sulfated BG cannot be confirmed due to discordance in the results reported by various authors, there has been an observed relationship between lower molecular weight of BG and higher biological activity, independent of the DS value (Lei et al., 2015).

Other chemical methods, such as phosphorylation, acetylation, and amination, can also be employed to modify BG. These methods function similarly to carboxymethylation and sulfation by introducing functional groups into the polysaccharide chain. For fungal phosphorylated BG, two studies reported DS values of 0.06-0.15 for a *Poria cocos* extract (Chen et al., 2009) and 0.77-2.09 for a *S. cerevisiae* extract by Shi et al., (2014). Acetylated BG showed DS values of 0.03-0.12 for oat BG (Souza et al., 2015). BG was obtained by Shin et al., (2005) from oat with a DS value of 0.48, and more recently, *S. cerevisiae* BG was successfully modified with DS values of 0.55-1.15 (Qiu et al., 2022).

As observed, various methods can be utilized to chemically modify BG from different sources, leading to improved solubility and enhanced biological activity. The choice of method depends on the complexity of the functionalization process. Among all the methods, carboxymethylation appears to be the most commonly used due to its simplicity, requirement of fewer reagents, and lower overall cost of the resulting modified molecule.

1.2.1.2 – Biological properties for skin benefit

Native BG has been found to exhibit bioactive properties naturally, but the addition of functional groups can significantly enhance BG solubility and improve bioactive properties (Divya et al., 2020; Calegari et al., 2017). Therefore, some biological active properties, reported for both native and chemically modified BG, with potential use in skin care products, are summarized in **Table 2**.

Table 2 – Skin-applicable properties of various modified and non-modified β-glucans and their sources.

Source		Chemical modification	Properties	Reference
Bacteria	*Alcaligenes faecalis* (Curdlan)	Native	Wound healing	(Berg et al., 2014)
Fungi	*Agrocybe chaxingu*	Native	Anti-inflammatory	(Lee et al., 2009)
	Amanita muscaria	Native	Anti-cancer	(Zavadinack et al., 2021).
	Lasiodiplodia theobromae (Lasioplodan)	Native	Antioxidant	(Kagimura et al., 2015; Theis et al., 2017, 2019)
		Carboxymethylation		
		Sulfation	Antioxidant	(Calegari et al., 2017)
		Native		
	Schizophyllum commune	Native	Wound healing	(Seo et al., 2019)
	Cordyceps militaris	Native	Anti-inflammatory	(Smiderle et al., 2014)
	Saccharomyces cerevisiae	Sulfation	Antioxidant	(Lei et al., 2015; Zhang et al., 2017)
		Carboxymethylation	Antioxidant	(Ma et al., 2022; Machová et al., 2014)
			UV Protection	(Zülli et al., 1998)
		Native	Wound healing	(Medeiros et al., 2012)
			Antioxidant	(Khan et al., 2016)
	Schizophyllum commune	Native	Wound healing	(Seo et al., 2019)
			Anti-inflammatory	(Cao et al., 2021)
	Aureobasidum pullans	Native	Anti-inflammatory	(Choi et al., 2016)

Table 2 – Skin-applicable properties of various modified and non-modified β-glucans and their sources (continued).

Algae	*Euglena gracilis* (Paramylon)	Native	Anti-inflammatory Wound healing	(Yasuda et al., 2018)
	Laminaria digitate (Laminarin)	Native	Anti-inflammatory Antioxidant	(Ozanne et al., 2020)
Cereals	Barley	Sulfation	Antioxidant	(Guo et al., 2019)
		Native	Wound healing	(Fusté et al., 2019)
	Oat	Native	Anti-wrinkle	(Pillai et al., 2005)
			Anti-cancer	(Choromanska et al., 2015; Parzonko et al., 2015)
	Eleusine coracana	Native	Antioxidant	(Divya et al., 2020)

1.2.1.2.1 – Antioxidant

The use of ingredients with antioxidant capacity in skincare products is important for reducing skin damage caused by oxidative stress. Reactive oxygen species can be produced directly and indirectly in our body. Various mechanisms can generate radical species such as superoxide by the electron transport chain in the mitochondria or hydrogen peroxide by peroxisomes in the endoplasmic reticulum (Rinnerthaler et al., 2015). The human body can protect itself by enzymatic and non-enzymatic mechanisms against ROS generated by endogenous factors (e.g., genetics) and exogenous sources (e.g., environment). However, the inclusion of topical antioxidants like vitamin C and E in skincare products can provide additional protection against these free radicals. Nevertheless, there are certain drawbacks associated with these antioxidants, such as molecular instability. This has opened up new possibilities for utilizing polysaccharides for this purpose (Chen et al., 2012; Krutmann et al., 2021; Ngoc et al., 2023). Polysaccharides are known to possess antioxidant activity, although their efficacy is influenced by factors like molecular structure and chemical characteristics, including solubility and the presence of negatively and positively charged groups (Fernandes & Coimbra, 2023).

The antioxidant potential of BG extract obtained from a native Brazilian mushroom (*Geastrum saccatum*) was evaluated, and it was observed that this extract highly inhibited superoxide radicals and was efficient against hydroxyl radicals and lipid peroxidation in a dose-dependent manner (Dore et al., 2007). The antioxidant capacity was also analyzed for BG extracted from three mushroom species (*Agaricus bisporus*, *Coprinus atramentarius*, and *P. ostreatus*). According to the results obtained, *C. atramentarius* demonstrated the best antioxidant capacity against ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, as well as reducing power and chelating ability. On the other hand, BG from *P. ostreatus* demonstrated the best lipid peroxidation inhibition (Khan et al., 2017). Laminarin, a β-(1→3)-D-glucan obtained from the seaweed *Laminaria digitate*, was reported as a great protector against ROS formation in human dermal fibroblast cells (nHDF) cells and normal human epidermal keratinocyte cells (nHEK) upon exposure to hydrogen peroxide and UV-A radiation (Ozanne et al., 2020).

Antioxidative properties were also reported for γ-irradiated (5 to 50 kGγ) BG extracted from *S. cerevisiae*. According to the authors, irradiated BG demonstrated scavenging capacity against ABTS, DPPH, and hydroxyl radicals, as well as lipid peroxidation

inhibition and ferrous-ion chelating properties. The authors pointed out that this antioxidant capacity increased with the amount of radiation used (Khan et al., 2016). BG from barley (*Hordeum vulgare*), when γ-irradiated (2, 4, and 8 kGγ), also demonstrated increased antioxidant capacity (Shah et al., 2015).

Lasioplodan is a β-(1→6)-D-glucan obtained from the fungi *Lasiodiplodia theobromae*, which has been shown to be an interesting choice for developing antioxidant compounds through chemical modification. By adding carboxymethyl groups to the molecular structure of lasiodiplodan, its capacity to scavenge hydroxyl radicals was improved compared to its native form. The authors observed a positive correlation between the increase of the DS value of the derivatives and this scavenging capacity, although the native BG performed better for hydrogen peroxide scavenging and reducing power (Theis et al., 2019). These improvements were corroborated by the results obtained for carboxymethylated lasiodiplodan through the evaluation of ABTS and DPPH scavenging capacity and ferric reducing antioxidant potential (FRAP) (Kagimura et al., 2015). According to the results obtained, the modified derivative obtained great results for the FRAP assay when compared with the native form, although its scavenging capacity for the other assays was relatively similar.

In a study by Ma et al., (2022), the antioxidant capacity of carboxymethylated *S. cerevisiae* BG with three different molecular weights was investigated against hydroxyl radical and ABTS radical. The results indicated that the molecular weight had a notable impact on the antioxidant capacity. The fraction with the lowest molecular weight demonstrated the highest activity, and higher concentrations of BG (1-10 mg/mL) exhibited increased antioxidant activity. For instance, at a concentration of 1 mg/mL, the smallest molecule showed a scavenging percentage of 91% against the hydroxyl radical and 45% against the ABTS radical, whereas the largest fraction exhibited values of 7% and 8% for the same assays and concentration. Another study demonstrated that sulfation, as a way to modify lasiodiplodan, increased the overall antioxidant properties and reducing power when compared to its native form, by scavenging hydroxyl radicals in 74% and 44% and peroxide hydrogen radicals in 5% and 0%, at a concentration of around 0.9 mg/mL for the sulfated BG and the native BG, respectively (Calegari et al., 2017). Using the same modification method, hulless barley BG had its antioxidant capacity improved against DPPH and nitric oxide radicals and reducing power when compared with the native form, with this activity increasing with the DS value of the sulfated BG (Guo et al., 2019). Sulfated BG from *S. cerevisiae* also demonstrated strong antioxidant

activity against DPPH, superoxide, and hydroxyl radicals. Although the authors reported an inverse correlation between the DS value/molecular weight and antioxidant ability (Lei et al., 2015). Although no studies were done evaluating antioxidant activity by a direct topical application of BG, it was observed that sulfated BG from *S. cerevisiae* increased the activity of catalase and superoxide dismutase, enzymes responsible for the defence against free radicals and reactive species in the human body (Limón-Pacheco & Gonsebatt, 2009), when administered intragastrically to mice (Lei et al., 2015; Zhang et al., 2017).

In general, BG obtained from different sources demonstrate great *in vitro* antioxidant capacity, and in some cases, this capacity can be improved by chemical modification. Further *in vivo* studies should be done to understand the topical applicability of BG as an antioxidant ingredient for the skin in clinical trials.

1.2.1.2.2 – Immunomodulation

Inflammation is a complex biological response that involves both the innate and adaptive immune systems in response to stimuli such as infection or injury. Generally, an inflammatory process begins with the recognition of the stimulus, followed by the activation of inflammatory pathways, the release of inflammatory mediators, and immune cell proliferation. Numerous molecules are involved in the inflammation process, such as transcription factors (e.g., nuclear factor-κB), enzymes (e.g., cyclooxygenase-2), pro-inflammatory cytokines (such as interleukin IL-1β/6/8 and tumor necrosis factor [TNF]), anti-inflammatory cytokines (such as IL-10/11 and transforming growth factor [TGF]), and reactive oxygen species (ROS) (Chen et al., 2018). Typically, a compound demonstrates anti-inflammatory properties by suppressing pro-inflammatory cytokines, enzymes, and inflammation-related proteins (Xu et al., 2019). Immunomodulators such as BG are compounds capable of stimulating or suppressing the natural response of the immune system, such as inflammation, by modulating adaptive or innate immunity (Byrne et al., 2020).

BG is recognized by the innate immune system through pattern recognition receptors (PRRs) due to its conserved structures named pathogen-associated molecular patterns (PAMPs), which are naturally present in microorganisms. This causes a response from the innate system by interacting with BG and activating macrophages (Vetvicka et al., 2019). Macrophages, an important and ubiquitous type of cells in the innate immune system, when activated, exert their phagocytic activity, release cytokines (e.g., IL-1β),

generate ROS and nitric oxide (NO) (Schepetkin & Quinn, 2006). BG can bind to dectin-1, toll-like receptors (TLRs), complement receptors-3 (CR-3), lactosylceramide, and scavenger receptors present in various immune cells, such as macrophages or dendritic cells, triggering a response from the immune system (Chan et al., 2009; Kim et al., 2011). In topical applications, BG can bind to the receptors present in the skin resident cells, promoting an inflammatory response by immune cells, cytokine production by macrophages, and cell proliferation by non-immune cells such as keratinocytes and fibroblasts for skin repair (Majtan & Jesenak, 2018).

According to the literature, BG obtained from the mushroom *Agrocybe chaxingu* led to a reduction in the production of NO and the expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) *in vitro* in murine macrophages stimulated with lipopolysaccharide (LPS) (Lee et al., 2009). The same pattern in NO production was observed, with reduced NO levels as BG concentration increased, for BG obtained from black yeast (*Aureobasidum pullans*) when exposing macrophages to the same inflammatory stimulus (Choi et al., 2016). It was also found that BG extracted from mushroom (*Cordyceps militaris*) could reduce the impact of LPS on the expression of IL-1β, TNF-α, and COX-2 in macrophage cells (Smiderle et al., 2014). Laminarin, a BG obtained from seaweed, demonstrated the capacity to reduce the production of IL-6 by 27% and 54% in nHDF and nHEK, respectively, when exposed to LPS (Ozanne et al., 2020).

In an *in vivo* assay, BG was applied one hour after the application of 12-O-tetradecanoylphorbol 13-acetate (TPA) on the mice ear for two days, which caused a reduction in monocyte infiltration and ear weight and thickness compared to untreated mice with TPA-induced inflammation (Lee et al., 2009). To evaluate the potential use of BG for treating skin wounds in mice, dressing films containing BG were analyzed for their impact on the release of inflammatory cytokines, such as interferon gamma (IFN-γ), IL-6, and vascular endothelial growth factor (VEGF) in the mouse serum at 3 and 5 days after wounding. The results showed that BG maintained a modest cytokine production initially but tended to reduce and normalize in the following days, indicating their potential use for skin application (Yasuda et al., 2018). This may be due to the initial higher inflammatory response promoted by BG, as evidenced by the upregulation of pro-inflammatory cytokines, followed by a reduction and stabilization of inflammatory cytokine production (Russo et al., 2017).

1.2.1.2.3 – β-Glucans as therapy in skin cancer

Tumors are characterized as a mass of malignant transformed cells that communicate and induce recruitment of other cells, distorting their behavior (Balkwill et al., 2012). This extensive network of intracellular communications, such as cytokine release, occurs among various immune cells, both malignant and non-malignant, and is known as the tumor microenvironment (TME) (Arneth, 2019). In the skin, cancer is typically divided into two broad categories: melanoma and non-melanoma cancer (Khan et al., 2022). Melanoma is associated with mutations in melanocytes, while non-melanoma cancer involves other types of cancer, such as squamous cell and basal cell carcinomas (Dhanyamraju & Patel, 2022; Woo et al., 2022). The main reason for using BG for this type of disease is their capacity to modulate the immune system, as previously detailed, and interact with various immune cells, altering their behavior. In addition to immunomodulation, BG can also be utilized to transport specific molecules or anti-tumor drugs, such as single-stranded DNA (ssDNA) or doxorubicin, to target tumors (Geller et al., 2019).

Nine different glucans, including curdlan, zymosan, and yeast BG, were also analyzed for their anti-tumor capacity. The authors demonstrated that these BG were able to modulate tumor-associated macrophages (TAMs), immune suppressive cells, into a more pro-inflammatory phenotype (M1). They observed an increase in chemoattractant production, such as CCL3, by TAMs and TAMs derived from melanoma, which may help in understanding the role of BG in anti-tumor therapies (Graaff et al., 2021). Additionally, the ability of BG to polarize macrophages into an M1-like phenotype is significant, as M1 macrophages are associated with a more favorable prognosis in cancer patients and may result in tumor regression (Zhou et al., 2020).

Low molecular weight oat BG has also demonstrated *in vitro* anticancer properties by reducing the viability of human melanoma and human epidermoid carcinoma cells. It induces high levels of caspase-12 expression, leading to cell death due to oxidative stress in both cell types (Choromanska et al., 2015). Another study tested human melanoma cells to analyze the potential pro-apoptotic effects of *Avena sativa* BG. According to the authors' observations, these oat glucans induced cell cytotoxicity and demonstrated anti-proliferative and pro-apoptotic capacities (Parzonko et al., 2015).

BG obtained from the mushroom *Amanita muscaria* were reported to selectively reduce the proliferation of murine melanoma cells but not fibroblasts (normal cells around the

tumor). They also exhibited the ability to inhibit colony formation when cells were pre-treated with or in the presence of the BG extract (Zavadinack et al., 2021).

A nanocomplex of carboxymethylated BG from the mushroom *P. tuber-regium* and iron oxide nanoparticles (IONPs) was also reported to have a combined effect of stimulating the polarization of pro-inflammatory M1 macrophages, increasing cancer cell apoptosis, and suppressing melanoma growth. This study observed the capacity of BG to stimulate the immune system and serve as a vehicle for IONPs (Su et al., 2022, 2023). Another study investigated the potential tumor treatment using water-soluble BG (*Aureobasidium pullulan*) combined with a blocker of programmed death-ligand 1 (PD-L1), evaluating the synergistic effect of both compounds against melanoma (Hu et al., 2023). According to their findings, BG induced the production of cytokines, such as IL-6, by converting the phenotype of peritoneal exudate macrophages (CD11b+ cells). However, BG also enhanced the expression of programmed cell death protein 1 (PD-1) and PD-L1, two immune checkpoints known to negatively regulate the immune response (Zou & Yaguchi, 2023). To overcome this issue, the authors combined BG with a PD-L1 blocker for use in a mouse melanoma model. They observed that the anti-tumor capacity of BG was improved by initially changing the phenotype of CD11b+ cells to a pro-inflammatory state, blocking PD-1/PD-L1, and boosting T-cell function to inhibit tumor growth and metastasis.

Considering these studies, BG can potentially be used for melanoma skin cancer therapy as an active molecule or as a vehicle to transport specific anti-cancer drugs into tumors. However, it is important to consider and evaluate the different types of BG for both uses since their interactions with the immune system can vary based on their chemical characteristics. For example, soluble and insoluble BG from the same yeast may exhibit different interactions in cancer therapy (Qi et al., 2011).

1.2.1.2.4 – Wound healing and tissue repair

1.2.1.2.4.1 – Wound healing capacity

Wound healing is a regenerative process that occurs through four main events: coagulation, inflammation, proliferation, and remodeling. These events are accompanied by the release of a cascade of numerous signaling molecules, such as cytokines, chemokines, and growth factors, to promote tissue regeneration (Larouche et al., 2018).

In a study analyzing the effects of four BG obtained from different sources (black yeast, barley, *Schyzophilum commune*, and *Euglena gracilis*) on immortalized human

keratinocytes (HACAT) and nHDF, it was found that there was no cytotoxicity in these cells and an increase in HACAT migration was observed when exposed to all the BG studied at a concentration of 20 µg/mL. It was concluded that BG obtained from *S. commune* presented the best results, including promoting of cell migration and dermal contraction and *in vivo* properties, such as re-epithelialization capacity, wound closure, and scab loss observed in mice skin wounds (Seo et al., 2019). Similar results were obtained for *in vivo* studies, after monitoring a mouse wound for two weeks treated with aqueous BG (Barley origin) solution. A notable wound healing ability was observed after topical application of the BG solution, which may be related to the promotion of human fibroblast cell migration (Fusté et al., 2019). Another study evaluated if dectin-1 activation would induce cellular proliferation and migration. The authors observed that human keratinocytes had their proliferation, migration, and wound closure increased by Curdlan BG (*Alcaligenes faecalis*) exposure, *in vitro* and *ex vivo* (Berg et al., 2014). Yeast BG can also be an interesting wound healing agent, as it showed a capacity to promote proliferation and increased the number of inflammatory cells recruited to ulcer area (Medeiros et al., 2012). The same study also observed that BG could reduce the wound area in over 60% of a patient with a non-healing wound for over 15 years, which reinforces the potential use of this molecule for wound healing. These results are consistent with the ones obtained from the analysis, through a clinical study, of the impact of soluble BG from yeast (*S. cerevisiae*) applied as a topical wound healing promoter in diabetic patients with foot ulcers. It was concluded that BG were safe and successfully promoted skin healing, probably due to the interaction with various immune cells (e.g., macrophages), and it may accelerate the closure of leg ulcers (Zykova et al., 2014).

1.2.1.2.4.2 – β-Glucans in wound dressings

The capacity of BG to be used as immune modulators and wound healing agents has boosted research on these molecules for medical and pharmaceutical applications. Several reports have shown promising results using BG incorporated into composite scaffolds, such as collagen or chitin, for tissue repair (Reddy et al., 2021). Hydrogels, in particular, are highly useful for treating wounds due to their versatility, biodegradability, biocompatibility, and compatibility with a wide range of bioactive molecules (Firlar et al., 2022). However, the application of hydrogels may be limited in wounds where greater adsorption or adhesion is required (Latiyan et al., 2023). In such cases, BG can also be applied in other types of wound dressings, such as sponges (Zhou et al., 2023).

As a biopolymer, BG can also serve as a structural element rather than an active molecule in hydrogels and film dressings for wounds. For example, BG can be linked with collagen to explore the potential antioxidant and antibacterial properties of tannic acid (Michalska-Sionkowska et al., 2021a) or combined with glycerol to analyze the capacity of nanostructured zinc oxide as an antibacterial agent (Pino et al., 2023). Additionally, BG imparts physical properties to these hydrogels, improving material stability and rigidity (Choi et al., 2021; Michalska-Sionkowska et al., 2021b).

BG can also act as an active compound in hydrogels due to its biological potential. One study explored a sprayable hydrogel composed of yeast soluble BG, which demonstrated the capacity to promote wound closure in a diabetic mice model (Grip et al., 2021). Another study demonstrated the wound healing capacity of a BG hydrogel and its ability to inhibit scarring by modulating the production of skin extracellular matrix components such as collagen type-III and keratin-14/keratin-15 (Kang et al., 2022). A mixture of BG extracted from *Schizophyllum* spp. and polyvinyl alcohol (PVA) hydrogel was reported to promote human dermal fibroblast migration and wound closure in mice when treated for two weeks. It also exhibited a significant anti-scarring effect in the wound area (Muthuramalingam et al., 2019). Another hydrogel containing lasioplodan was found to be highly effective for wound application. This formulation demonstrated antioxidant capacity, promoted *in vivo* mice wound closure, initiated collagen type-III production, which later matured to collagen type-I, and stimulated keratinocyte proliferation (Nissola et al., 2021). Pullulans in gel form were studied for their wound healing capacity by applying them to excision wounds in mice. The results showed that the gel positively influenced all stages of the wound healing process, accelerating wound closure, promoting blood vessel formation, and stimulating collagen type-III biosynthesis (Thangavel et al., 2020).

In addition to hydrogels, BG films and sponges are also potential candidates for wound repair. Barley BG films were found to promote HACAT cells growth, contributing to wound closure (Michele et al., 2023). The wound healing ability induced by BG extracted from *E. gracilis*, using a film dressing with paramylon, was also demonstrated. These films exhibited significant wound healing capacity by reducing the initial wound area in mice skin by half after 5 days (Yasuda et al., 2018). A sponge incorporated with carboxymethyl yeast BG was evaluated for wound healing. According to the authors, this sponge accelerated blood coagulation, controlled hemorrhage through *in vivo* mice

wounds, and considerably improved wound repairing properties, such as *in vitro* cell migration, compared to native water-insoluble BG (Zhou et al., 2023).

The immunomodulatory capacity of BG plays a significant role in the process of wound healing, primarily through its interactions with skin and immune cells, thereby enhancing the cascade of events involved in skin regeneration. As discussed in a review by Majtan and Jesenak (2018), BG has a direct influence on keratinocytes, fibroblasts, and macrophages, leading to enhanced reepithelialization by stimulating cellular proliferation and migration through dectin-1 receptors. Additionally, the interaction between BG and fibroblasts stimulates collagen production, facilitating the restoration of the extracellular matrix and promoting wound closure and tissue repair (Pillai et al., 2005).

1.2.1.2.5 – Antiaging

Skin aging can be influenced by both intrinsic, natural aging processes that occur throughout life and extrinsic factors such as air pollution, smoking, or exposure to ultraviolet radiation. These factors contribute to the development of wrinkles, primarily caused by a thinner epidermis and the breakdown of ECM components like elastin (Graf, 2005). Furthermore, they promote the generation of ROS and trigger inflammation processes (Zhang & Duan, 2018). To reduce the impact of these factors, the antioxidant and immunomodulatory capacity of BG, paired with its anti-aging properties such as UV protection (Zülli et al., 1998) or wrinkle reduction (Pillai et al., 2005), demonstrate the potential of this molecule for anti-aging purposes. A representation of how BG interrelate with our skin is explained in **Figure 5**.

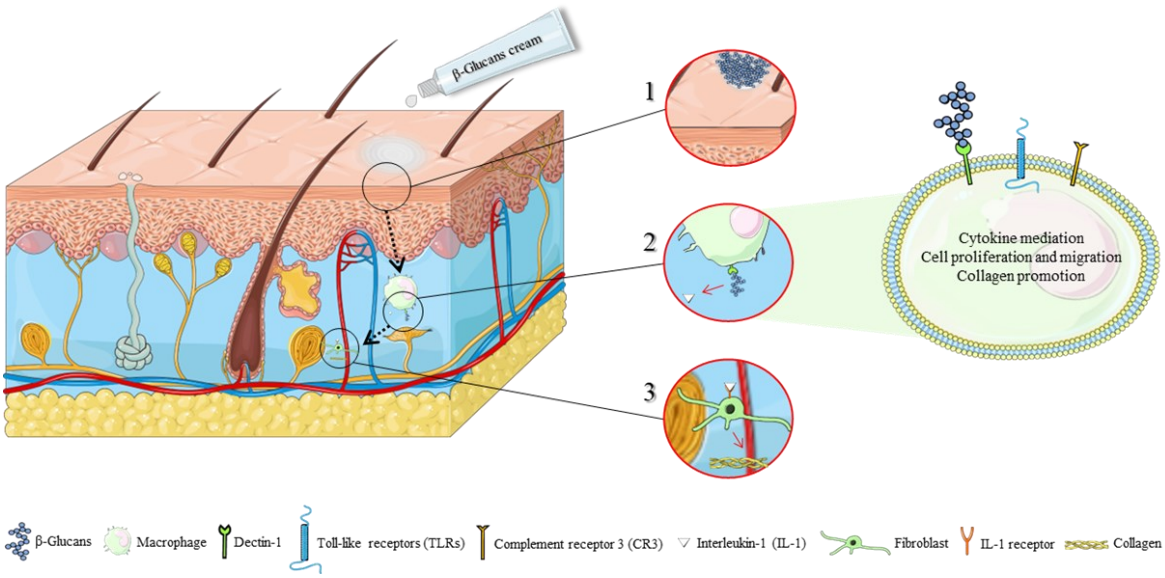

Figure 5 – Overview of β-glucans cream impact upon skin application and potential cellular mechanisms regarding its use as a skincare product (Cordier-Dirikoc et al., 2022; Majtan & Jesenak, 2018; Pillai et al., 2005) (1) A topical formulation containing β-glucans could boost skin permeation and allow the passage of this molecule through epidermis. (2) Once β-glucans reach the dermis, they are recognized by macrophages receptors (e.g., dectin-1) and initiate an immune response, releasing interleukins (e.g., IL-1) and starting an inflammatory cascade. (3) This immunomodulatory capacity could lead to activation of several other skin cells, such as dermal fibroblasts inducing the production of ECM components (e.g., collagen deposition) or epidermal keratinocytes prompting migration and proliferation in a wound closure scenario. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. Some of the original images used to assemble this figure were edited, modified or their color was altered.

The protective capacity of carboxymethylated BG obtained from *S. cerevisiae* against UV radiation was analyzed *in vitro* and *in vivo*. According to the results obtained, pre-treatment with the modified BG effectively protected human keratinocyte cells from UV-A radiation. For the *in vivo* test, the authors observed a concentration-dependent reduction in squalene hydroperoxides when using an oil-in-water emulsion with BG at 0.04% and 0.2%, after pre-treating the volunteer's skin for 5 days before exposing it to the UV-A stimulus (Zülli et al., 1998). Oat β-glucan has demonstrated potential anti-wrinkle capacity by reducing wrinkle height and depth, as well as skin roughness, in 27 subjects over 8 weeks (Pillai et al., 2005). Additionally, the same study concluded that β-glucan could penetrate deeply into the skin layers, explaining its ability to reach the dermis and epidermis and interact with skin cells in these layers, thereby reducing wrinkles and improving skin elasticity.

Zhu et al., (2023) evaluated the potential of a water-soluble BG produced by the bacteria *Agrobacterium* sp. and Panthenol (pro-vitamin B5) spray formulation to alleviate itchy and inflamed skin using an *in vivo* model. They induced chronic pruritus in mice with an acetone-ether solution and observed several positive effects. The authors noted a reduction in transepidermal water loss (TEWL), decreased inflammation indicated by lower levels of cytokines such as IL-1β and TNF-α, and a decrease in scratching bouts, indicating relief from itching sensation. This suggests that incorporating BG into a spray formulation with common skin moisturizers can provide a feasible solution for skin itching and irritation. Corroborating these findings, Cao et al., (2021) also found that a cream containing BG from *Schizophyllum Commune* is effective in reducing TEWL and improving skin erythema in patients undergoing fractional laser therapy, a procedure that naturally induces skin inflammation and irritation.

Potential to be used as a moisturizer is also a great property to maintain our skin healthy. Skin outer layer can be affected the same factors causing aging (e.g., UV exposure) or low humidity, which can impact normal enzymatic processes, causing flaky and dry skin (Rittié, 2016). The first study evaluated the potential use of a chitin-glucan formulation and concluded that it caused no harmful effects in volunteers with sensitive skin, and improved skin firmness, tonicity, SC hydration, and roughness compared to the placebo (Gautier et al., 2008). In the second study, a formulation containing a plant extract, plant oil, BG, and sodium hyaluronate was effective in improving skin hydration and texture in subjects with sensitive skin (Wang et al., 2018).

Overall, several BG from different sources, extracted by diverse methodologies and with unequal chemical properties, water-soluble, water-insoluble, or chemically modified, have demonstrated throughout the years a very interesting potential to be used in skin (**Figure 6**).

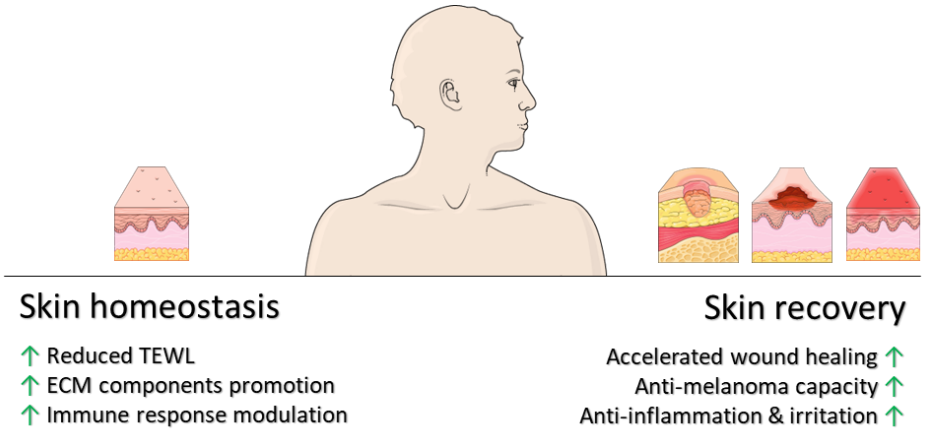

Figure 6 – Beneficial properties of BG molecules in maintaining skin homeostasis, such as preventing TEWL or by applying it in skin disorders, such as wounds, melanoma, and inflammation. This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. Some of the original images used to assemble this figure were edited, modified or their color was altered.

BG not only significantly contributes to skin homeostasis by forming a protective layer that reduces water loss and promotes collagen deposition but also has potential applications as a natural compound to accelerate wound closure and in tumor therapies. Its strong immunomodulatory capacity and easy recognition by various cellular receptors expressed in skin and immune cells make it highly versatile. Consequently, the broad applicability of BG positions this polysaccharide as a valuable ingredient skincare, biomedical or cosmetic industry.

1.2.1.3 – β-Glucans as a skin health ingredient

Due to increasing consumers' interest in how skin products impact their health, their choice is changing towards the use of these products with natural bioactive ingredients, which have a beneficial effect when compared with synthesized ones (Ahsan, 2019b; Bilal & Iqbal, 2019). Additionally, was observed a preference for "green" products obtained from natural sources without preservatives (Rizzi et al., 2021). Interestingly, yeast and oat BG seem to be heavily used in skin products, as shown in **Table 3**, with a broad diversity of biological properties described.

Table 3 – Examples of commercial BG used for skincare products as a multipurpose bioactive ingredient.

Product name	BG source	Manufacturer	Described properties available in their website	Reference
PromOat Oat BG	Oat	Lantmännen Oats	• Thickener and emulsion stabilizer	(Lantmännen Oats, 2023)
SWEOAT Flours	Oat	Swedish Oat Fiber	• Skin protectant and moisturizer • Repairing, rejuvenating and anti-aging capacity • Anti-inflammatory, anti-itchy and soothing properties • Skin pH stabilizer	(Swedish Oat Fiber, 2023)
SymGlucan	Oat	Symrise	• Cellular renewal (*in vitro*) • Moisture, firmness and elasticity enhancer (*in vivo*) • Fades wrinkles, skin smoother and improve skin recovery (*in vivo*)	(Symrise, 2023)
IMoist BG	Oat	Bioalkemia	• Anti-aging, anti-irritant and non-greasy • Wrinkles improvement and accelerate wound healing • Attenuate sunburns and protects against UV light redness • Skin moisturizer	(Bioalkemia, 2023)

Table 3 – Examples of commercial BG used for skincare products as a multipurpose bioactive ingredient (continued).

Yeast essence glucare C90	Yeast	Angel Yeast	• Enhances natural skin self-protective capacity • Improves skin recovery and wound healing • Restores skin moisture barrier • Repair damage induced by sun light • Anti-aging and anti-radiation	(Angel Yeast, 2023)
Herbex Yeast BG	Yeast	Biospectrum	• Immune cells mobilization • Phagocytic capacity of immune cells to destroy pathogens • UV protection and sunburn recovery	(Biospectrum, 2023)
CM-Glucan forte	Yeast	Mibelle AG Biochemistry	• Reduces the release/expression of inflammatory cytokines • Inhibits the adhesion of *S. aureus* to skin • Reinforce the skin barrier • Alleviate 6 symptoms of skin eczema	(Mibelle Biochemistry, 2023a)
CM-Glucan granulate			• Soothes capacity • Protection against skin dehydration after stress • Accelerates skin regeneration • Protects against UV damage, preventing loss of firmness and skin lipids degradation • Reduces wrinkle depth	(Mibelle Biochemistry, 2023b)

Considering consumers opinion in cosmetic products, BG seems to be a perfect candidate for this purpose, since it is a molecule possible to be extracted from a wide variety of natural sources with exploitable bioactive properties for skincare. In some cases, by-products from already stablished industrial processes, such as spent yeast generated in beer production, can be used as sustainable source. As Thomas et al., (2022) emphasize, industrial waste should be the main choice for the extraction of these type of bioactive compounds, prioritizing sustainability, and a circular economy, protecting the environment. Therefore, spent yeast seems to be the obvious choice to extract BG, since it's produced in relatively large quantities, can be obtained in a pure form, creating value in a by-product that is mostly discarded (Caruso et al., 2022).

As mentioned previously, BG can be obtained from a wide variety of sources, in some instances, subjected to carboxymethylation to enhance solubility and modify specific bioactive properties, as discussed earlier. Due to the diverse sources from which BG can be obtained, including by-products like spent yeast, the extraction of this polysaccharide can remain relatively cost-effective and may be optimized to reduce expenses and increase purity. However, it is important to note that the extraction methods for BG cannot be universally applied to all sources, which poses challenges in reproducing large-scale extractions of this compound when multiple sources are utilized (Zhu et al., 2016). This diversity of extraction methods also complicates the comparison of the chemical structure of BG in its native and functionalized forms, as well as its potential for skin care applications across the numerous available studies. To mitigate this heterogeneity, it would be beneficial to employ more standardized extraction methods specific to each source, resulting in less variability in the obtained results. Further research is required to comprehensively understand the true impact of BG on more complex systems, such as 3D cell models or *ex vivo* skin models, and how BG interacts with different layers of the skin to maintain or enhance skin health. Additionally, there is a paucity of information regarding the utilization of BG for skin diseases like atopic dermatitis or psoriasis and how it can positively modulate the inflammatory response in such cases. These areas warrant additional investigation to fully explore the potential benefits of BG in managing and treating various skin conditions.

1.2.1.4 – Conclusion

The development of new skin products enriched with novel ingredients that promote skin health may represent a significant step forward for the cosmetic, biomedical, and pharmaceutical industries. β-glucans, due to their biological properties (e.g., wound healing) and immunological relevance related to skincare, have great potential to be used in such products. This polysaccharide can be obtained from a wide range of natural products (e.g., cereals) or by-products (e.g., brewer's spent yeast), and in some cases, it can be chemically modified to adjust or improve its innate characteristics and biological capacity. Already, some products containing BG as a natural, bioactive, and innovative ingredient are commercially available in a growing market. However, practical formulations and *in vivo* studies must be conducted to ensure that their *in vitro* properties are transposable and comparable with what would occur in human skin without losing their biological capacity. Nonetheless, BG are a promising skin health enhancer with a strong scientific background supporting their applicability in the skincare industry.

1.3 – Scientific output

Throughout this PhD thesis, various outputs were generated, such as a poster communication, four scientific papers, published or in submission process, and two patents.

Sousa, P., Moreira, H., Conde, A., Azevedo-Silva, J., Amorim, M., Tavares-Valente, D., Pintado, M., & Fernandes, J. Glucans: from spent yeast to an ingredient with skin repair properties. Poster presentation at EuChemS. August 2022, Lisbon, Portugal.

Sousa, P., Tavares-Valente, D., Amorim, M., Azevedo-Silva, J., Pintado, M., & Fernandes, J. (2023). β-Glucan extracts as high-value multifunctional ingredients for skin health: A review. Carbohydrate Polymers, 121329. https://doi.org/10.1016/J.CARBPOL.2023.121329.

Sousa, P., Tavares-Valente, D., Pereira, C., Pinto-Ribeiro, I., Azevedo-Silva, J., Madureira, R., Ramos, Ó., Pintado, M., Fernandes, J., & Amorim, M. (2023). Circular EconomYeast: *Saccharomyces cerevisiae* as a sustainable source of glucans and its safety for skincare. – Submitted to International Journal of Biological Macromolecules (Elsevier).

Sousa, P., Amorim, M., Mendes, A., Azevedo-Silva, J., Pintado, M., Fernandes, J., Tavares-Valente, D., (2023). *Saccharomyces cerevisiae* glucan extracts with *in vitro* bioactivity for skin protection – In submission process.

Sousa, P., Moreira, H., Tavares-Valente, D., Amorim, M., Correia-Sá, I., Mendes, M., Azevedo-Silva, J., Pintado, M., & Fernandes, J. (2023). Unveiling the therapeutic potential of yeast glucans in skincare through human skin *ex vivo* models. – In submission process.

Fernandes, J. C., Amorim, M., **Sousa, P**., Valente, D., Silva, J. A. & Pintado, M. E., 11 Feb 2022, (Submitted) IPC No. A61K47/36, C08B37/00, C12P19/04, World Intellectual Property Organization, Patent No. WO2022172087A1, 11 Feb 2022, Priority date 14 Feb 2021, Priority No. PT11706221A – *Yeast glucans, methods and uses thereof.*

Fernandes, J. C., Amorim, M., **Sousa, P**., Valente, D., Silva, J. A. & Pintado, M. E., 18 May 2023, (Submitted) European Patent Office, Patent No. 18/253,484, 18 May 2023, Priority date 21 Dec 2021, Priority No. PT117662 - *Yeast β-glucan emulsion, methods and uses thereof.*

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Chapter 2

In this chapter, a comprehensive explanation of the various methods used to obtain purified glucans extracts from the two different yeast strains, both in their water-insoluble and carboxymethyl forms, will be provided. Additionally, an extensive safety assessment through biological and chemical assays was conducted to ensure the safety of the extracts for use in skincare applications.

Information based on the following paper:

Sousa, P., Tavares-Valente, D., Pereira, C., Pinto-Ribeiro, I., Azevedo-Silva, J., Madureira, R., Ramos, Ó., Pintado, M., Fernandes, J., & Amorim, M. (2023). Circular EconomYeast: *Saccharomyces cerevisiae* as a sustainable source of glucans and its safety for skincare. – Submitted to International Journal of Biological Macromolecules (Elsevier).

2.1 – Glucans extraction, modification and safety

Circular EconomYeast: *Saccharomyces cerevisiae* as a sustainable source of glucans and its safety for skincare application

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Submitted at International Journal of Biological Macromolecules

Abstract

Glucans, a polysaccharide naturally present in the yeast cell wall that can be obtained from side streams generated during the fermentation process, have gained increasing attention for their potential as a skin ingredient. Therefore, this study focused on the extraction method to isolate and purify water-insoluble glucans from two different *Saccharomyces cerevisiae* strains: an engineered strain obtained from spent yeast in an industrial fermentation process and a wild strain produced through lab-scale fermentation. Two water-insoluble extracts with a high glucose content (> 90%) were achieved and further subjected to a chemical modification using carboxymethylation to improve their water solubility. All the glucans' extracts, water-insoluble and carboxymethylated, were structurally and chemically characterized, showing almost no differences between both yeast-type strains. To ensure their safety for skin application, a broad safety assessment was undertaken, and no cytotoxic effect, immunomodulatory capacity (IL-6 and IL-8 regulation), genotoxicity, skin sensitization, and impact on the skin microbiota were observed. These findings highlight the potential of glucans derived from spent yeast as a sustainable and safe ingredient for cosmetic and skincare formulations, contributing to the sustainability and circular economy.

Keywords: Spent yeast; Glucans extraction; Skin safety.

2.1.1 – Introduction

An emerging integrated strategy to enhances industrial sustainability, promotes circular economy, and creates additional commercial value on industrial byproducts. Among the various byproducts generated by *Saccharomyces cerevisiae* fermentation processes, spent yeast biomass stands out as a key target for valorization. The yeast cell wall, from a chemical perspective, consists of three main groups: polymers of glucose (α/β-glucans, accounting for approximately 60% of the cell wall dry mass), polymers of mannose (mannoproteins, approximately 40% of the cell dry mass), and polymers of N-acetylglucosamine (chitin, around 2% of the cell wall dry mass) (Aguilar-Uscanga & François, 2003). Glucan polymers primarily consist of a β-(1→3)-glucans backbone withs long β-(1→6)-glucans, which provide structural rigidity to the cell wall. Other linkages, such as α/β-(1→4), are also present (Bastos et al., 2022; Kapteyn et al., 1999; Lesage & Bussey, 2006; Teparić et al., 2020). The specific types of linkages in the cell wall can vary depending on the strain, fermentation process, or growth medium used, as yeast cells can adapt to their environment, leading to structural changes in the cell wall (Boutros et al., 2022; Latgé, 2007).

Given the substantial quantities of spent yeast generated during industrial fermentation processes and the industrial applications of glucans present in their cell structure, several studies have focused on obtaining and purifying this biomolecule (Avramia & Amariei, 2021; Caseiro et al., 2022; Zhu et al., 2016). However, due to the water-insoluble nature of this complex polysaccharide, various methods, such as carboxymethylation, can be employed to significantly enhance its solubility and potentially explore new biological properties (Avramia & Amariei, 2021; Liu et al., 2021; Machová et al., 2014; Yuan et al., 2019). Until now, several studies have demonstrated the remarkable biological properties of yeast glucans, which make them suitable for skincare applications. These properties include their potential to reduce skin damage, aid in skin regeneration, act as wound healing agents (Medeiros et al., 2012; Seo et al., 2019; Zykova et al., 2014), function as antioxidants (Khan et al., 2016; Kogan et al., 2005; Lei et al., 2015; Machová et al., 2014), and mitigate the effects of skin aging (Zülli & Suter, 1995).

Glucans have the capacity to interact with specific receptors, such as dectin-1 or Toll-like receptors (TLRs), on various skin cells, including keratinocytes in the epidermis layer and macrophages in the dermis layer (Majtan & Jesenak, 2018; Quaresma, 2019). This interaction and the ability to modulate inflammatory responses are particularly interesting

for skin applications, as most skin cells can release specific cytokines and chemokines to initiate an immune response.

Even though the molecule by itself demonstrates potential for use in skincare, it is essential to analyze the safety of any chemical ingredient before it reaches the market. This is done through several test guidelines made available by the Organization for Economic Co-operation and Development (OECD). Appropriate *in vitro* assays should be performed to ensure the safety of new molecules. For instance, tests for genotoxicity are conducted to confirm that these innovative compounds do not induce mutagenicity or DNA damage. Additionally, skin sensitization potential is assessed through a critical group of tests to ensure that the chemical ingredient does not cause allergic reactions (Almeida et al., 2017; Barthe et al., 2021)

Despite numerous studies conducted on the capacity and biological potential of glucans, there is a lack of information regarding their effects on skin cells, such as keratinocytes, and how they can modulate cytokine production and inflammatory pathways within these cells. Furthermore, only a few studies have addressed the safety of glucans for skin application, in accordance with OECD guidelines. Therefore, this study aimed to develop a methodology for extracting highly pure glucans, enhance their solubility through functionalization, and assess how these two forms of the biomolecule: (1) influence the viability of various cell types; (2) act as immunomodulators in keratinocytes and macrophage-like cells; and (3) can be safely used as chemical ingredients for skincare applications.

2.1.2 – Materials and Methods

2.1.2.1 – Materials and chemicals

Yeast extract peptone dextrose medium (YPD) was purchased from Grisp (Porto, Portugal). Titriplex III standard EDTA solution (0.1 M) was obtained from Merck (Darmstadt, Germany). Enzymatic Yeast β-glucans Assay Kit (K-EBHLG) and β-glucans Assay Kit (Yeast and Mushroom) (K-YBGL) were sourced from Megazyme (Wicklow, Ireland). Immortalized human keratinocyte cells (HACAT) were obtained from Cell Lines Service (CLS). Normal human dermal fibroblasts (nHDF) were acquired from Lonza. Human leukemia monocytic cell line (THP-1) and lymphoblast cell line (TK6) were obtained from the American Type Culture Collection (ATCC). TrypLEX, Dulbecco's Modified Eagle Medium (DMEM, GlutaMAX™), RPMI 1640, Fetal Bovine Serum (FBS), antibiotic-antimycotic (penicillin–streptomycin–amphotericin B), Dimethyl Sulfoxide (DMSO), PrestoBlue Cell Viability Reagent and PureLink™ Microbiome DNA Purification Kit, Pierce BCA Protein Assay Kit and Bovine Serum Albumin (BSA) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). ELISA assay kits (IL-6/IL-8), FITC anti-human CD54 (353108), FITC anti-human CD86 (374204), Human TruStain FcX (422302), and FITC Mouse IgG1 k Isotype Ctrl (981802) were sourced from Biolegends (San Diego, CA, USA). Propidium Iodide (PI), Nickel(II) Sulfate, Phorbol-12-myristate 13-acetate (PMA), Mitomycin C from *Streptomyces caespitosus* and Deuterated dimethyl sulfoxide (DMSO-d6) were purchased from Sigma (St. Louis, MO, USA). The AMES FT mutagenicity assay kit was obtained from Moltox (Boone, NC, USA), MicroFlow test kit was purchased from Litron Laboratories (Rochester, NY, USA) and Nutrient Broth Number 2 was bought from Oxoid (Basingstoke, England). All other reagents used were of analytical grade.

Two different types of yeast were used in this work, a wild-type strain (WS) and an engineered strain (ES). The WS yeast (CEN.PK *S. cerevisiae* strain) was grown in YPD using bioreactors (Eppendorf) with 2L as working (max. bioreactor volume: 2.7 L. The conditions of pH (5.0), temperature (30 °C), agitation and oxygen levels (30%) were carefully controlled and maintained throughout the process. A pre-culture was grown over-night, in the same culture conditions, and used for media inoculation at 10% (v/v) of the total volume. The biomass was harvested once the oxygen levels began to rise due to complete glucose consumption, approximately 36 h of incubation. After collection, the biomass was centrifuged at 8000 rpm (Sorval LYNX 4000, ThermoFisher Scientific) for 10 minutes (min) at 4 °C, and the supernatant was discarded. The resulting pellet was

then stored at -20 °C for subsequent extraction. The ES yeast (engineered *S. cerevisiae* strain) was provided by Amyris Inc (Emeryville, CA, USA) and was obtained after the completion of an industrial fermentation process. The pellet from the ES strain was isolated and stored using the same method as described above.

2.1.2.2 – Glucan extraction

2.1.2.2.1 – Hot water treatment

Both types of yeast biomass were used to extract glucans from the cell wall. Briefly, the whole spent yeast recovered from the reactor was initially centrifuged at 8000 rpm (Sorval LYNX 4000, ThermoFisher Scientific) for 10 min to separate the supernatant from the biomass. The biomass was then diluted in deionized water, 1 gram of wet pellet to 1 mL of deionized water, and autoclaved at 121 °C for 20 min. After thermal extraction, a wash step was performed by adding deionized water, followed by centrifugation at 8000 rpm (Sorval LYNX 4000, ThermoFisher Scientific) for 10 min. The supernatant was discarded, and the insoluble fraction was preserved for further purification.

2.1.2.2.2 – Alkaline extraction

After autoclaving and washing the insoluble fraction, a basic extraction using sodium hydroxide and high temperature was performed based on the method used by Shokri et al., (2008), with some modification. Initially, a 20% (w/v) solution was prepared by diluting the insoluble fraction (wet) in 4% (w/v) NaOH. The solution was then placed in a water bath at 90 °C for 2 h and centrifuged to isolate the insoluble fraction at 8000 rpm (Sorval LYNX 4000, ThermoFisher Scientific) for 10 min. The insoluble fraction was washed twice with deionized water. Subsequently, the insoluble fraction was re-suspended in deionized water and neutralized with hydrochloric acid. After neutralizing the pH, a centrifugation step was performed to remove the supernatant, followed by a final wash. The resulting pellet, containing insoluble glucans, was homogenized in deionized water and dried in a spray dryer, resulting in a fine white powder.

2.1.2.2.3 – Acidified ethanol extraction

To further purify the polysaccharides content in the extracted insoluble fraction, acid-organic purification was applied to remove impurities. For this, spray dried glucans obtained from the alkali extraction were diluted ethanol, at a ratio of 1:80 (w/v), and then it was added 1.6 mL of HCl 32% (w/v) for each gram of glucan used. This solution reacted

at 50 °C during 4 h in an orbital shaker, centrifuged and the insoluble fraction was washed twice with absolute ethanol and a fourth time with acetone, in a ratio of 1:20 (w/v) for the initial mass of sample used. Purified glucans were dried in a vacuum oven at 50 °C overnight.

2.1.2.3 – Chemical modification

For the carboxymethylation of both yeast extracts, 2 g of purified glucans were diluted in 50 mL of 80% (v/v) ethanol and 5 mL of 50% (w/v) NaOH. The mixture was reacted for 1 h at 35 °C with slight agitation. After initial dissolution, 5 mL of NaOH and 70 mL of 0.35 M monochloroacetic acid (diluted in absolute ethanol) were slowly added to the solution. The final solution was incubated in a water bath at 50 °C for 2 h with agitation. Once the reaction was complete, the solution was neutralized with concentrated acetic acid and centrifuged at 8000 rpm (Multifuge X1R, ThermoFisher Scientific) for 10 min. The pellet was washed four times with 40 mL of 80% (v/v) ethanol and then dried at 40 °C in a vacuum oven overnight.

2.1.2.4 – Degree of substitution

The degree of substitution (DS) of this functionalization was performed according to Ding et al., (2013), with modifications. Briefly, 150 mg of purified glucans were weighed and diluted in 1 mL of ethanol. Then, 50 mL of deionized water and 20 mL of ammonium chloride buffer (pH 10) were added to the solution and left stirring for a few minutes until total dissolution. After that, the solution was neutralized to a pH of 7.5-8.0. Next, 50 mL of copper sulfate (0.05 M) was added, and the solution was allowed to react for 15 min with intermittent stirring. The volume was then completed to 250 mL and filtered to retain the copper precipitate generated. An aliquot (100 mL) of this filtered sample was titrated with a standard solution of EDTA (0.05 M – 0.1 M) using murexide as an indicator. A blank was prepared under the same conditions but without the addition of the sample at the beginning of the experiment. The DS value was calculated using the following equations 1 and 2:

$$\% \, CH_2COONa = \left(\frac{C(V - v) \times 2 \times 2.5 \times 0.081}{\text{sample weight (g)}}\right) \times 100 \quad (1)$$

$$\text{DS value} = \frac{162 \times \% \, CH_2COONa}{8100 - 80 \times \% \, CH_2COONa} \quad (2)$$

C = EDTA concentration (mol/L)

V = Blank volume (mL)

v = Sample volume (mL)

2.1.2.5 – Characterization

2.1.2.5.1 – α/β-Glucans content

To quantify the percentage of α/β-glucans present in the samples throughout the extraction process, α-glucans were quantified through K-YBGL assay kit while β-glucans were quantified through K-EBHLG kit, both enzymatic kits were done by following the manufacturer's protocol.

2.1.2.5.2 – Chemical characterization

The protein content was determined using the DUMAS combustion method with the Dumatec 8000 instrument (FOSS, Denmark). The quantification involved an O2 factor of 1.4 mL/mg of sample and a flow rate of 300 mL/min. EDTA ($C_{10}H_{16}N_2O_8$) was used for the calibration curve. The results obtained from the samples were analyzed using the integrated Dumatec software, and the general protein-to-nitrogen conversion factor of 6.25 was used for the calculations.

Total lipid content was determined based on the protocols by Bligh & Dyer (1959) and Breil et al., (2017) with modifications. Approximately 0.5 g of sample was accurately weighed and mixed with 3 mL of ethyl acetate and ethanol solution (2:1), along with 0.1 mL of deionized water. The mixture was homogenized for 1 min, followed by the addition of 2.25 mL of ethyl acetate, 0.5 mL of absolute ethanol, and 4.15 mL of deionized water. The solution was homogenized again and centrifuged at 3000 rpm (Multifuge X1R, ThermoFisher Scientific) for 1 min. After phase separation, the volume of the organic phase (ethyl acetate) was measured, and an aliquot was taken and evaporated in a speed vacuum system at 50 °C. The ratio of total lipids was calculated using the equation below:

$$\text{Total lipids (\%)} = \left(\frac{\text{Dried aliquot (g)} \times \text{Organic phase (ml)}}{\text{Aliquot (mL)} \times \text{Sample (g)}}\right) \times 100 \quad (3)$$

Total polysaccharides content was analyzed and derivatized according to Bastos et al., (2015). Briefly, 2 mg of sample was weighed and mixed with 200 µL of 72% H_2SO_4

(w/w) and stirred for 3 h. Afterward, the reaction was diluted with deionized water to obtain a 1M H_2SO_4 solution, and the samples were further hydrolyzed at 100 °C for 1 h. The results were analyzed using gas chromatography with flame ionization detection (GC-FID).

Dry weight was determined by drying a known weight of the sample for 24 h at 105 °C to remove moisture. The samples were then left in a desiccator until room temperature for a new weight measurement to obtain the moisture content (Association of Official Analytical Chemists, 2005). For ash content determination, a known weight of the sample was incinerated at 550 °C for 36 h in a muffle furnace. The remaining weight of the sample after incineration was considered the amount of ash present.

2.1.2.5.3 – ATR-FT-IR profile

All the purified dry glucan extracts, both native and carboxymethylated forms, were analyzed using Attenuated Total Reflection Fourier Transform Infrared (ATR-FT-IR). Each solid sample was scanned in the range of 550 to 4000 cm^{-1} using a mid-IR and far-IR spectrophotometer (MIR/FIR) from PerkinElmer. A total of 16 scans were performed with a spectral resolution of 4 cm^{-1}.

2.1.2.5.4 – Molecular weight

Molecular weight estimation was carried out using high-performance size exclusion chromatography (HPLC-SEC) with evaporative light scattering detection (ELSD) using an Agilent instrument. Carboxymethyl glucans were dissolved in ultra-pure water at a concentration of 1 mg/mL. Separations were performed on Agilent PL aquagel-OH MIXED-H column. The ELSD detector settings and flow rates were adjusted accordingly. Pullulans were used for the standard curve.

2.1.2.5.5 – Nuclear Magnetic Resonance

For the Nuclear Magnetic Resonance (NMR) experiment, glucans were solubilized at 20 mg/mL in DMSO-d6 and deuterated water (D_2O) for native and carboxymethylated forms, respectively. ^{13}C NMR spectra were acquired using a Bruker Avance III 600 HD spectrometer at CEMUP (Centro de Materiais da Universidade do Porto, Porto – Portugal) and processed with Bruker TopSpin 4.1.3 software. Chemical shifts are reported in ppm (δ units) using internal tetramethylsilane (TMS) as the reference.

2.1.2.6 – *In vitro* assays

2.1.2.6.1 – Cell culture

HACAT and nHDF cells were maintained in culture with DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. THP-1 cells were maintained in culture with RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) antibiotic-antimycotic, and 0.05 mM 2-Mercaptoethanol. TK6 cells were cultured in RPMI 1640 medium supplement with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic. Cell lines were cultured in a CO_2 incubator with an humified atmosphere containing 5% CO_2 at 37 °C.

2.1.2.6.2 – Cell viability

Cell viability was evaluated using the PrestoBlue cell viability reagent. Briefly, 96-well microplates were seeded with 100 µL of a cell suspension at a concentration of 1.0 x 10^5 cells/mL for HACAT and nHDF and incubated for 24 h. For THP-1, a cell suspension of 1.5x10^5 cells/mL was prepared, and PMA (50 nM) was added before seeding to differentiate THP-1 into macrophage-like THP-1 (mTHP-1). The cells were then incubated for 48 h. After incubation, the media was removed, and 90 µL of each glucan sample at 2-fold serial dilutions (10.0-0.3 mg/mL), previously diluted in phosphate-buffered saline (pH 7.4), was added to each well. DMSO (10% v/v) was used as a metabolic inhibitor. After incubating for 24 h, 10 µL of PrestoBlue was added to each well and allowed to react for 1 h. The cell viability was then measured by fluorescence spectrometry, with an emission and excitation wavelength of 560 nm and 590 nm, respectively. The dashed line at 30% metabolic inhibition represents the cytotoxicity limit according to ISO 10993-5 (2009).

2.1.2.6.3 – Cytokine profile

To analyze the impact of our extracts on cellular cytokine release, HACAT and mTHP-1 cells were exposed to native and carboxymethyl glucans. Briefly, both forms of glucans were cultured at a concentration of 2 mg/mL for 24 h in a 12-well plate with a volume of 1 mL per well. The wells were previously seeded with 2.5 x 10^5 cells/mL. As a negative control, the anti-inflammatory corticosteroid betamethasone (20 µM) was used. After the exposure, all the supernatants were collected, centrifuged, and stored at -80 °C for further analysis. The cells in the 12-well plate were scraped, and the protein content was quantified using the Pierce BCA assay kit. The production of IL-1α, IL-6 and IL-8 were

analyzed and quantified according to the manufacturer's assay procedure using ELISA MAX kits with a sensitivity of 0.6 pg/mL, 2 pg/mL and 8 pg/mL, respectively. All the results were normalized to pg of interleukin per µg of total protein to account for cell variations within wells, and the fold change variation was calculated.

2.1.2.7 – Safety assessment

2.1.2.7.1 – Mutagenicity test

The mutagenicity assessment was conducted using a high throughput fluctuation Ames test with an Amest FT test kit, based on the work published by Sui et al., (2009) and followed OECD guideline No.471 (OECD Guidelines for the Testing of Chemicals, 2020). The test was performed according to the manufacturer's manual. Briefly, glucan extracts (5, 2.5, 1.3, and 0.6 mg/mL) were exposed to two mutated *Salmonella typhimurium* strains (TA98/TA100) with and without the presence of a metabolic system obtained from rat liver (S9). The bacteria were grown overnight at 37 ºC in nutrient broth Nº2 with ampicillin (25 µg/mL) until reaching an optical density of 1.0-1.4 (600 nm). Then, the bacteria were inoculated in an exposure media containing small quantities of histidine, along with the test samples and controls, for 90 min to allow sufficient bacterial growth. This mix was subsequently diluted in histidine-free reverse media and aliquoted into a 384-well microplate, which was incubated for two days at 37 °C. Any wells showing turbidity or color change were counted as reverted. A mutagenic agent would allow these bacteria, which are unable to synthesize histidine, to revert this mutation and grow. An increase ≥ 2-fold in this reversion compared to the counts obtained in the untreated or negative control wells would indicate mutagenicity. For the assay without S9, 2-Nitrofluorene (50 µg/mL) and 4-Nitroquinoline-N-oxide (50 µg/mL) dissolved in DMSO were used as mutagenic controls for TA98 and TA100, respectively. In the presence of S9, 2-Aminoanthracene (100 µg/mL) dissolved in DMSO was used as a positive mutagenicity control for both strains.

2.1.2.7.2 – Micronucleus quantification

Micronucleus induction and quantification were performed using the MicroFlow test kit, following the manufacturer's instructions, and based on the OECD guideline test No. 487 (OECD Guidelines for the Testing of Chemicals, 2014a). For this assay, 1 mL (7.5×10^5 cells) of a TK6 cell suspension were seeded in a 12-well plate and exposed to 3 different concentrations (1.0, 0.5 and 0.1 mg/mL) of each glucan's extracts, insoluble and

carboxymethylated, for 24 h at 37 ºC. Mitomycin C was used as a positive control (micronucleus inducer) at 25 ng/mL. After exposure, cells were centrifuged, stained, and lysed according to the procedure described for the Basic Protocol. Subsequently, the cells were kept in the dark and later analyzed by flow cytometry (Accuri C6 Plus).

2.1.2.7.3 – Direct peptide reaction assay (DPRA)

The DPRA assay was performed according to OECD guideline test No. 442C (OECD Guidelines for the Testing of Chemicals, 2023a). Briefly, native and carboxymethyl glucans were incubated at a concentration of 2 mg/mL with cysteine- and lysine-containing peptides for 24 h. Cinnamic aldehyde (100 mM) was used as positive control. The relative peptide concentration was measured by high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm. The depletion percentage values of cysteine and lysine peptides were calculated against the negative control and used in a prediction model referred to in the OECD guideline, which allows assigning the glucans to one of four reactivity classes used to discriminate between sensitizers and non-sensitizers.

2.1.2.7.4 – Human cell line activation test (h-CLAT)

The h-CLAT assay was done according to OECD guideline test No. 442E (OECD Guidelines for the Testing of Chemicals, 2023b). In summary, THP-1 monocyte cells were exposed to various concentrations of glucan extracts, and cell viability was analyzed using propidium iodide staining to determine the CV75, which is the concentration of the test chemical that allows 75% cell survival. Based on this, dilutions starting at 1.2 mg/mL were applied to THP-1 cells (1.0×10^6 cells/mL) in a 24-well plate with a final volume of 1 mL/well and incubated for 24 h at 37 ºC. The cells were then centrifuged and washed twice with a solution of PBS containing 0.1% (w/v) BSA. The cells were resuspended in 150 µL of the same solution and transferred to a 96-well microplate. Next, 5µL of Trustain was added to each well and incubated for 20 min. The volume was then divided into three wells (50 µL/well), and 5 µL of CD54 antibody, CD86 antibody, and Isotype-control FITC were added to the respective wells. The plate was incubated for 30 min at 4 ºC in the dark. Then, cells centrifuged and washed five times with PBS-BSA. Cells were resuspended in 200 µL of the same solution and 10 µL of PI solution (12.5 µg/mL) was added. All the results were analyzed by flow cytometry (Accuri C6 Plus). If the relative fluorescence intensity (RFI) levels of CD86 and CD54 were above the stipulated values

present in the OECD guideline (RFI CD86>150 and RFI CD54>200), the test samples were considered sensitizers. Nickel(II) sulfate (100 µg/mL) was used as a positive sensitizer control.

2.1.2.7.5 – Impact upon skin microbiota

The effect of the extracted and functionalized molecules on the skin microbiota was evaluated according to Carvalho et al., (2022). Briefly, skin microbiota samples were collected from 20 healthy female volunteers without skin disease. The samples were exposed to native and carboxymethyl glucans at a concentration of 2 mg/mL in RPMI (test groups) for 24 h at 34 ºC with agitation at 100 rpm. For each volunteer, a control condition was included in which the skin microbiota was incubated without any ingredients. After the incubation period, the pellet was recovered by centrifugation, and DNA was extracted using the PureLink™ Microbiome DNA Purification Kit. Subsequent DNA analysis was performed according to the work of Luz-Veiga et al., (2023) using amplicon-based next-generation sequencing (NGS) to evaluate the impact of native and carboxymethyl glucans on the relative abundance of microbial populations naturally present in the volunteers' skin.

2.1.2.8 – Statistical analysis

All the analysis and graph representation were done using GraphPad Prism software and Statistica software. All the data normality was ensured and then analyzed through Student's t-test to compare the values obtained from control test and each glucan extract, insoluble and carboxymethylated. In exception to evaluating the impact of the compounds on the skin microbiota, where initially were evaluated by the D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests to assess data normality. Based on the results, the Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test was performed.

2.1.3 – Results and Discussion

2.1.3.1 – Glucan extraction and functionalization

Disrupting the yeast cell wall is a crucial step in obtaining a purer product, particularly rich in glucans and other complex polysaccharides (Bzducha-Wróbel et al., 2014). The high rigidity of this structural component requires the use of multiple methods to isolate a higher quantity of polysaccharides (Avramia & Amariei, 2021; Ruiz-Herrera & Ortiz-Castellanos, 2019).

In our extraction process (**Figure 7**), we utilized two types of yeast: a wild strain and an engineered strain with the capability to produce a steviol glycoside. These strains underwent an alkaline extraction followed by a subsequent purification step to isolate yeast polysaccharides. Prior to the extraction, an autolysis process was employed to rupture the yeast cell wall and remove intracellular compounds (Freimund et al., 2003; Liu et al., 2008). Subsequently, an alkaline extraction was performed to eliminate impurities, primarily targeting the removal of proteins (Kushnirov, 2000), alkali-soluble glucans, mannans, and lipids (Magnani et al., 2009). To further enhance the purity of the extracts, a final extraction step using an acidified ethanol solution was employed primarily to eliminate lipids (Freimund et al., 2003) and other impurities. This resulted in the production of a final insoluble extract rich in polysaccharides from the yeast cell wall, only composed by glucose monomers.

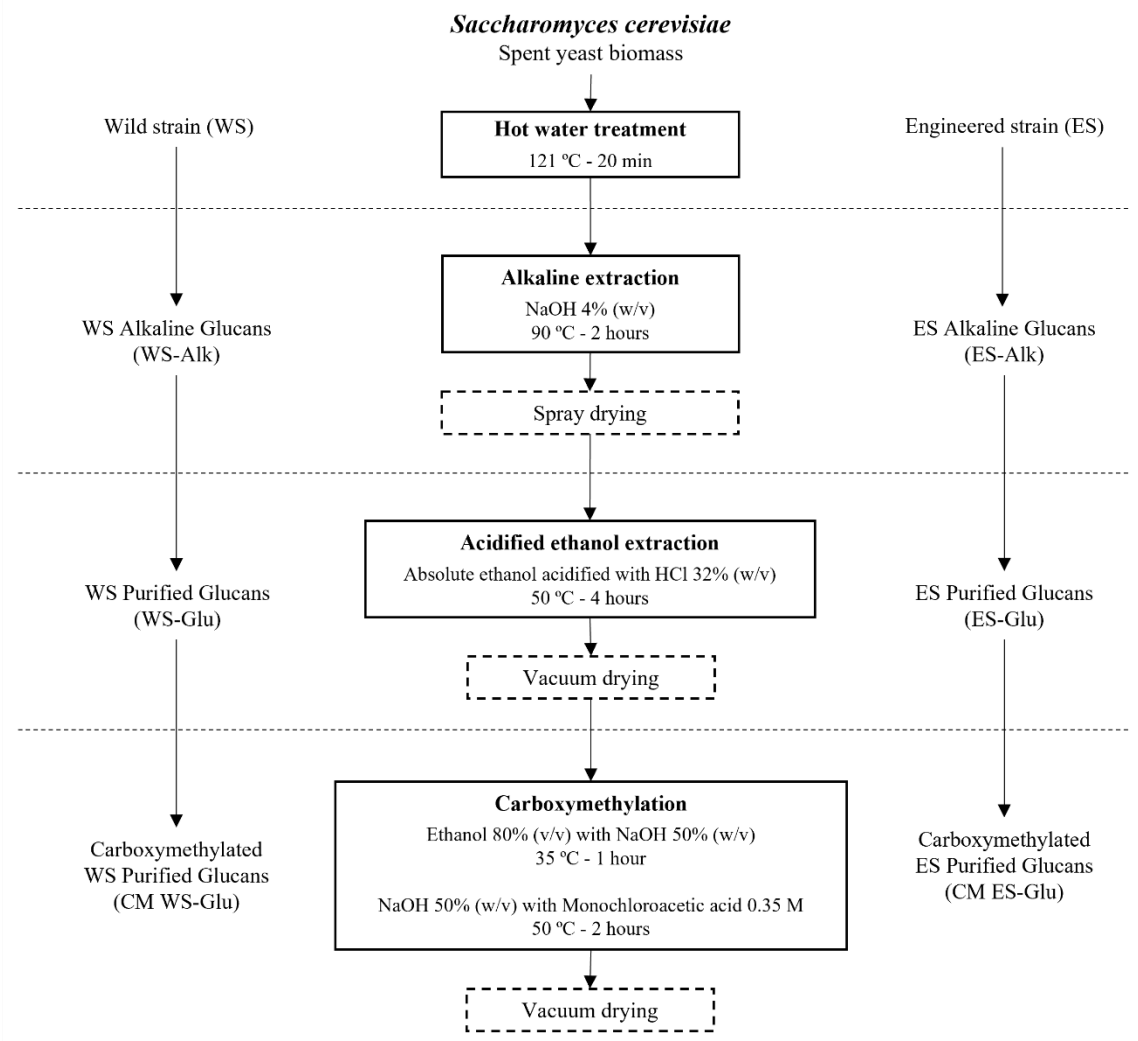

Figure 7 – Extraction, purification and chemical modification scheme of glucans from wild strain (WS) and engineered strain (ES): Alkaline extraction (Alk), Acidified ethanol extraction (Glu) and Carboxymethylation (CM).

According to the chemical characterization (**Figure 8**), the purified extracts from both yeast sources exhibited very similar chemical profiles, with approximately 90% total glucose content, with no traces of other sugar monomers, 3-5% protein content, and 1-2% lipid content. However, differences were observed in the β-glucan and α-glucan content of the two extracts. WS-Glu exhibited a higher content of β-glucans (56%), but a smaller content of α-glucans (10%) compared to ES-Glu, which showed values of 46% and 26%, respectively, for these linkages.

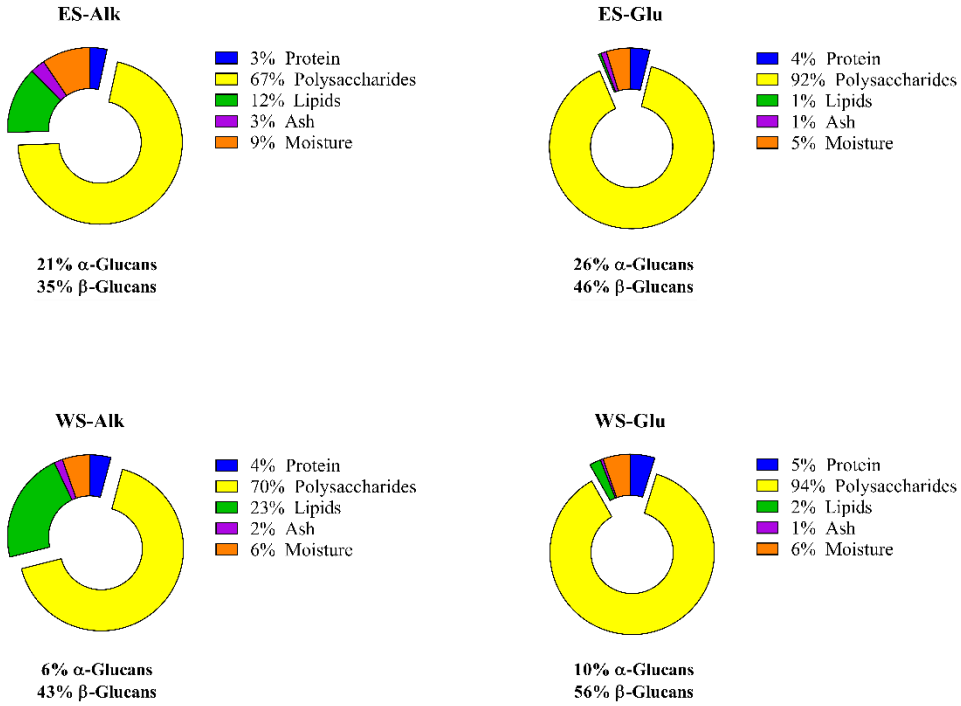

Figure 8 – Chemical characterization of glucan extracts after the alkaline extraction (Alk) and purification (Glu), for both yeasts.

These differences can be attributed to the origin of the yeasts, since WS-Glu is a wild strain produced at lab-scale, while ES-Glu is a spent yeast originated from an engineered strain produced through industrial fermentation. This industrial fermentation process may lead to a change in glucans content, as observed in *Saccharomyces pastorianus* during the brewing process, to enhance cell wall strength (Bastos et al., 2015). Additionally, more linkages may be present but were not quantified, such as β-(1→4) (Bastos et al., 2015; Pinto et al., 2015) or non-hydrolyzed enzymatically. Further conformational modifications can also occur during the extraction process, such as the disruption of hydrogen bonds (Bzducha-Wróbel et al., 2013) and partially open of the triple-helix conformation (Young et al., 2000). Comparing the extracts obtained after the alkaline extraction and the purification process, the final purification step effectively removed around 90% of the total lipids, increased the total polysaccharides to 92-94%, and resulted in a 10% increase in the β-glucan content. According to the literature, water-insoluble glucans with similar chemical profiles can be obtained through various methods, with a high percentage of polysaccharides, such as 65% (55% β-glucans) (Thammakiti et al., 2004), 72% (60% of β-glucans) (Thammakiti et al., 2004), and 96% polysaccharides (93% of β-glucans) (Liu et al., 2008).

Due to the water-insoluble nature of the extracts, their application could pose a challenge. One option to address this issue is to functionalize the molecules to increase their solubility. Carboxymethylation is one of the most commonly used methods to chemically improve glucan solubilization and enhance biological properties (Kagimura et al., 2014; Kaur et al., 2020). This type of reaction involves replacing hydroxyl groups present within the polysaccharide chain with carboxymethyl groups (Theis et al., 2019). The degree of substitution (DS) achieved in this modification can vary depending on factors such as the polysaccharide used, reaction time, and solvents employed. Therefore, the efficiency of this modification is often measured by the DS value attained.

According to our results (see **Table S1**), both glucan extracts were successfully functionalized through carboxymethylation with high efficiency, yielding 91-96%. The DS value obtained was 0.33, and the molecular weight was determined to be 4.91 x 10^5 Da and 4.89 x 10^5 Da for CM ES-Glu and CM WS-Glu, respectively. Comparing the DS values with those reported by Ding et al., (2013), our values (0.329-0.333) fell within the range reported by the authors for the optimization process of carboxymethyl glucans from *S. cerevisiae* (0.292-0.507). A lower value was obtained compared to the DS values reported by Machová et al., (2014) and Ma et al., (2022), which were 0.43 and 0.8, respectively, for *S. cerevisiae* carboxymethyl glucans. However, a direct comparison is not possible since differences in yeast cell wall structure or carboxymethylation methods used in the studies directly impact the DS values obtained. In order to analyze the structural differences between the native glucans from both strains and their carboxymethyl counterparts, we conducted ATR-FT-IR analysis. Our results (**Figure 9**) showed that both native samples exhibited a broad peak at 3650-3300 cm^{-1}, indicating the presence of OH groups, and a peak at 2950-2850 cm^{-1}, corresponding to C-H groups commonly found in polysaccharides (Barrientos et al., 2016).

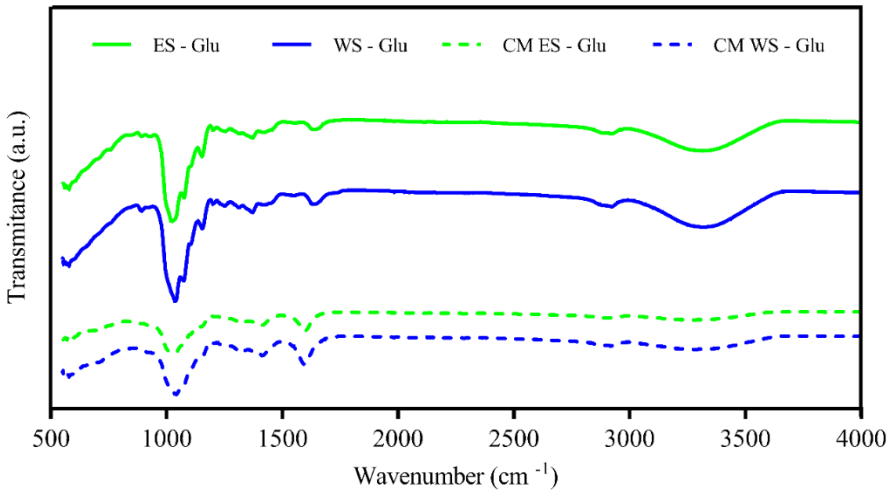

Figure 9 – ATR-FT-IR of water-insoluble glucan extracts from WS and ES type yeast and its carboxymethylated form.

A notable difference was observed between carboxymethyl and native glucans, with two peaks at 1650-1550 cm^{-1} and 1450-1400 cm^{-1}, indicating the presence of COO^- groups and the addition of carboxymethyl groups to the structure of native glucans (Kagimura et al., 2015). Two other peaks at 1150 and 1075 cm^{-1} exhibited reduced intensity after carboxymethylation, suggesting a possible degradation of a specific type of β-Glucan, as these peaks are associated with linear β-(1→3) (Fusté et al., 2019; Wang et al., 2005). Additionally, a strong peak was observed at 1230-950 cm^{-1}, indicating the presence of C-O-C, C-O, and C-C groups, which are characteristic of polysaccharides (Amer et al., 2021; Šandula et al., 1999). Another strong peak at 1035 cm^{-1} can be attributed to β-glucan linkages (Bikmurzin et al., 2022).

In addition to ATR-FT-IR analysis, we performed ^{13}C NMR analysis to further investigate the types of sugar units, type of linkages, and positions of substituent groups in the structure of native and carboxymethyl glucans. The ^{13}C NMR spectrum of native glucans (**Figure 10A**) displayed well-defined carbon signal peaks with high resolution, corresponding to β-(1→3)-linked backbone D-glucosyl units. The chemical shifts were as follows: 103.49 ppm (C-1), 73.29 ppm (C-2), 86.57 ppm (C-3), 69.03 ppm (C-4), 76.85 ppm (C-5), and 61.34 ppm (C-6), consistent with previous reports on water-insoluble yeast glucans (Naruemon et al., 2013). Additional signals at 73.39 and 69.85 ppm were assigned to C-5 and C-6, respectively, of the branch-point units, as described by Šoltés et al., (1993). The signal at 75.51 ppm corresponded to C-5 of the β-(1→6)-linked side-chain D-glucosyl units, while other carbon signals of the side-chain units were

indistinguishable due to overlap with more intense signals from the backbone. The spectra of WS and ES overlapped, and no differences in chemical shifts were observed.

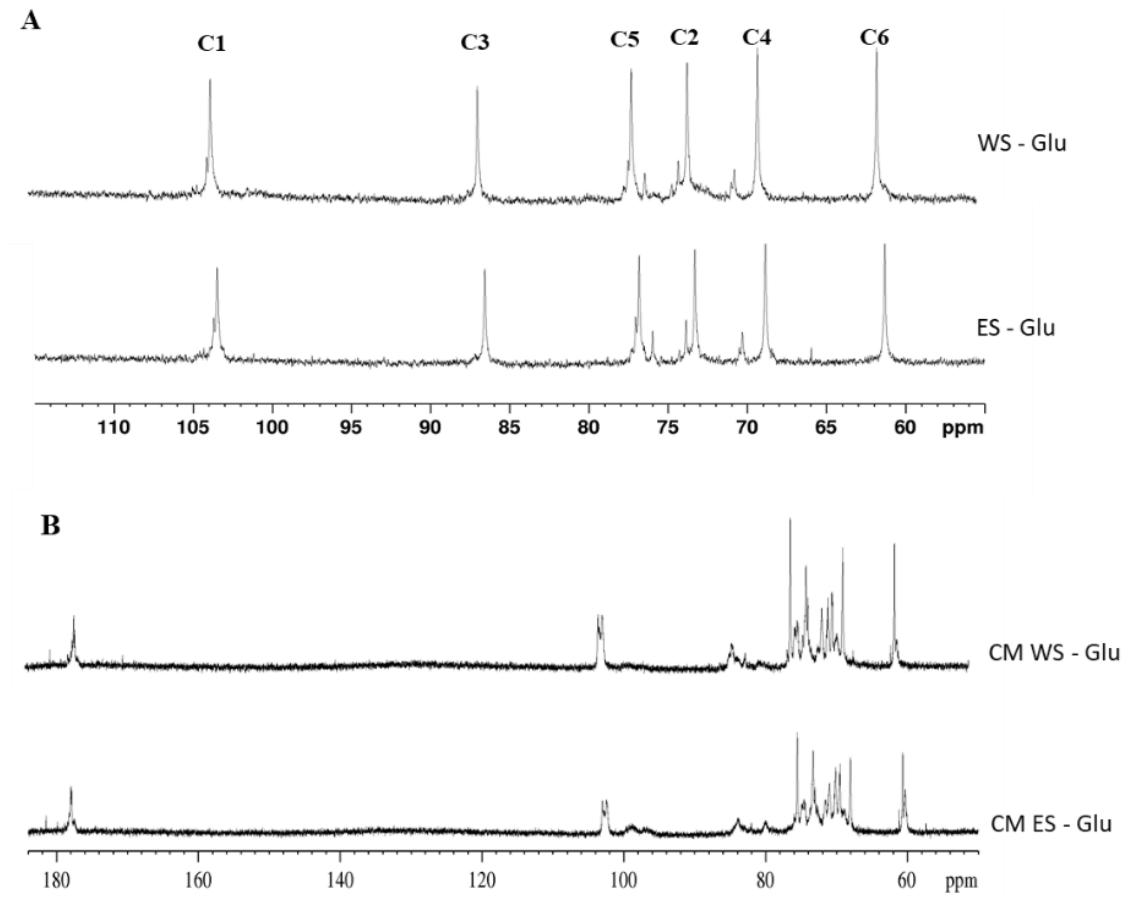

Figure 10 – ^{13}C-NMR spectra of (A) native and (B) carboxymethylated glucans.

Concerning the carboxymethyl form (**Figure 10B**), the main chain structure of the glucan polysaccharides remained unaltered, and the signal peaks corresponding to carbon resonance (C-1 to C-6) were similar to those observed in the native form, concentrated in the range of 60-110 ppm. Additionally, a carbonyl group appeared at 179 ppm and a methylene carbon signal at 70.3 ppm, attributed to the carboxymethyl group (Kagimura et al., 2015), confirming the carboxymethylation process as indicated by ATR-FT-IR analysis. Notably, the C-3 and C-5 signals were split into several signals (ranging from 86.8-76 to 70-75 ppm), indicating a change in conformation and chemical shift, possibly due to the introduction of carboxymethyl substituents into the polymer structure. No differences were found between the carboxymethylation spectra of ES and WS.

2.1.3.2 – Biocompatibility and immunomodulation

To ensure the safety of our extracts on the skin, we evaluated their cytotoxic effects on three types of cells: keratinocytes (HACAT), dermal fibroblasts (nHDF), and macrophage-like THP-1 cells (mTHP-1).

According to our results (**Figure 11**), the native glucans showed very similar behavior, with no cytotoxic effects on HACAT and mTHP-1 cells at concentrations ranging from 0.3 to 10 mg/mL. However, a reduction in nHDF cells viability was observed when exposed to concentrations above 5 mg/mL (WS-Glu) and 10 mg/mL (ES-Glu) (**Figure 11C**).

Comparing both carboxymethylated samples, only the WS showed a cytotoxic effect on HACAT (**Figure 11B**) and nHDF cells (**Figure 11D**) at concentrations of 5 mg/mL and 1.25 mg/mL, respectively. Although dermal fibroblasts are generally more sensitive to carboxymethyl glucans than HACAT cells (Costa et al., 2022), the addition of carboxymethyl groups appears to have a negative impact on WS-Glu extract for both cell types. The increased solubility of WS-Glu seems to directly affect cell viability in both skin cell types, with a greater impact in the fibroblast cells.

To further understand the modulatory effect of the native glucans and their functionalized form on the inflammatory response, we evaluated their immunogenic potential in keratinocytes (**Figure 12A**) and THP-1-derived macrophages (**Figure 12B**), respectively. Specifically, we assessed their capacity to stimulate the production of two pro-inflammatory cytokines, IL-6 and IL-8 (Liu et al., 2021).

Based on the results obtained by exposing HACAT cells to the glucans extracts produced in this project (**Figure 12A**), only the carboxymethyl form of ES-Glu did not lead to significant changes in IL-6 levels compared to the non-exposed control ($p > 0.05$). However, all other glucans resulted in a significant increase in IL-6 levels. As for IL-8, only CM WS-Glu showed a significant increase in this cytokine compared to the non-exposed control ($p \leq 0.0001$), suggesting a potentiated immunomodulatory effect after carboxymethylation. None of the other conditions appeared to affect the basal levels of IL-8 release by HACAT cells. Among all the glucans tested, CM WS-Glu was the only extract capable of stimulating the production of IL-6 and IL-8 by keratinocytes, with a fold change variation of 1.8 and 2.2, respectively. To our knowledge, no other studies have demonstrated such an immunomodulatory effect on keratinocytes by glucans.

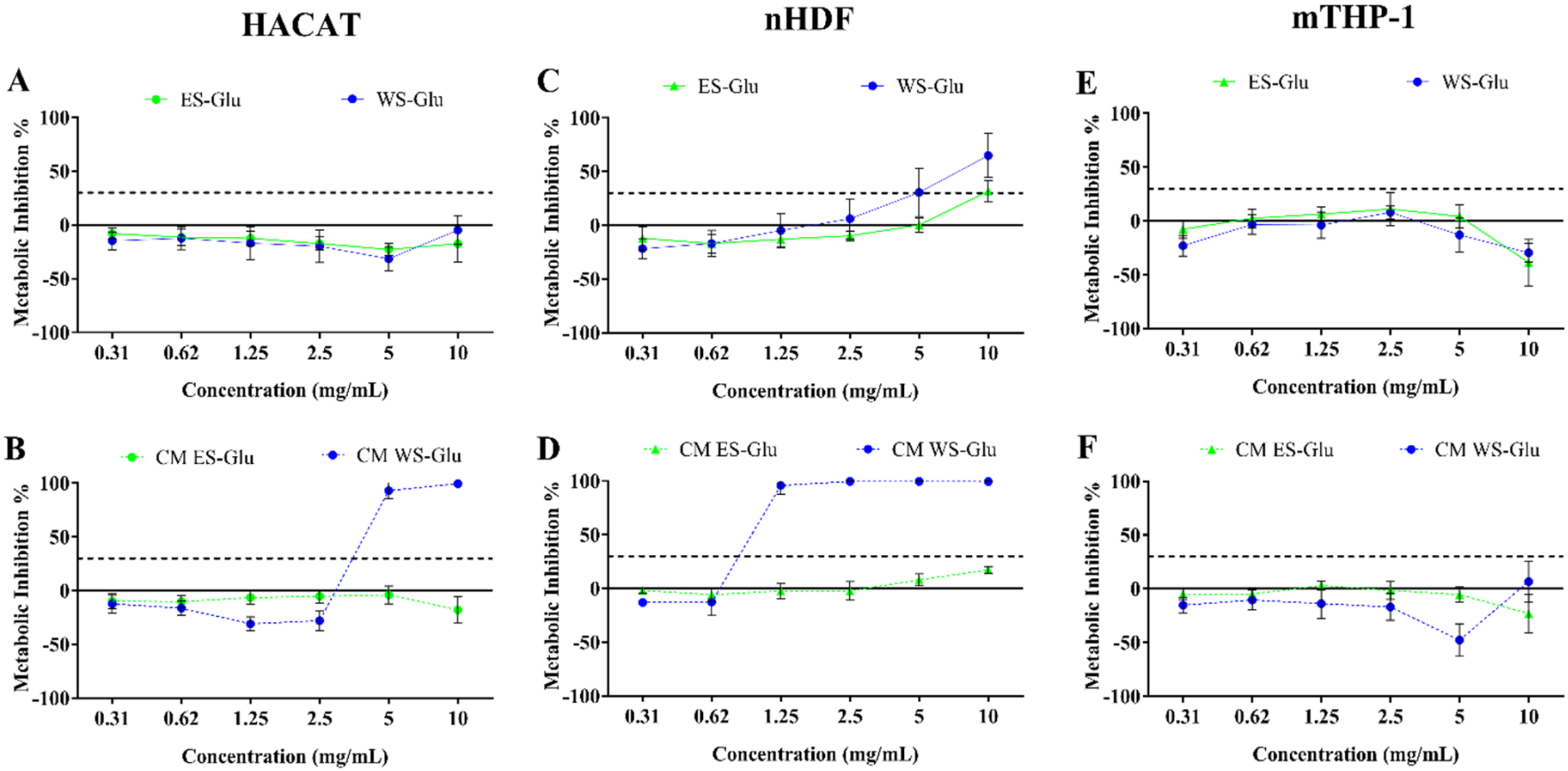

Figure 11 – Metabolic inhibition of HACAT cells (A and B), nHDF cells (C and D), and mTHP-1 cells (E and F) treated with insoluble glucans (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu). Data are represented as mean ± SD from three replicates for each sample.

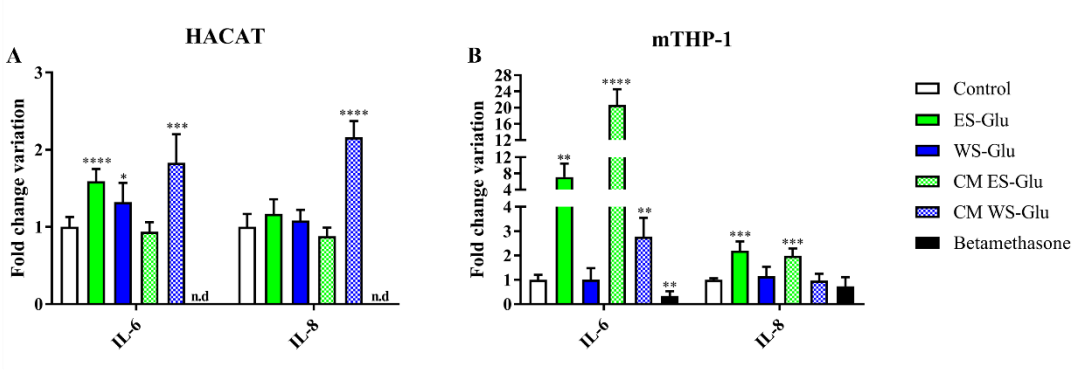

Figure 12 – Cytokine production of (A) HACAT cells and (B) mTHP-1 cells upon exposure to native glucans (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu). Data are represented as mean ± SD from three replicates for each sample. Significance differences between samples and control are indicated as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. No detected values (n.d) were assigned for concentrations below the detection limit of the kit. This figure is based on the concentrations shown in **Figure S1**.

Regarding mTHP-1 (**Figure 12B**), ES-Glu and its carboxymethyl form seem to considerably stimulate the production of IL-6, with a fold change increase of around 7.1 and 20.7, and in smaller scale IL-8, with a fold change variation of around 2.2 and 2.0, respectively. However, WS-Glu and CM WS-Glu had no significant differences ($p > 0.05$) in comparison to control for IL-8, and only the carboxymethylated format was significantly different when compared to the control ($p \leq 0.01$) for the IL-6, showing a very different behavior between the extracts obtained from the wild strain and an engineered strain.

This type of interaction is well-known to occur due to the recognition of glucans by macrophage cells through dectin-1, TLR2/TLR6, and CR3 receptors (Brown et al., 2002; Chan et al., 2009; Herre et al., 2004). It has been observed that baker's yeast glucans (*S. cerevisiae*) strongly stimulate cytokine production, particularly IL-6 and IL-8, when exposed to human whole blood cultures (Noss et al., 2013). Similarly, a particulate β-(1→3)-Glucan from *S. cerevisiae* (Zymosan) was found to upregulate IL-6 and IL-8 in mTHP-1 cells (Kanjan et al., 2017). β-Glucans from *S. cerevisiae* were also shown to enhance the synthesis and secretion of IL-8 in differentiated THP-1 cells, especially when combined with Vitamin D (Bergandi et al., 2021). Blocking the Dectin-1 receptor with an antibody appeared to downregulate cytokine production in the presence of these glucans,

highlighting the significance of this receptor in glucan recognition by immune cells (Kanjan et al., 2017; Sahasrabudhe et al., 2016).

Comparing the results obtained from both cell types, it is evident that immune cells exhibit a higher upregulation compared to skin cells, which is expected due to the potent ability of immune cells to induce cytokine production and the presence of essential glucan receptors in these cells (Duque & Descoteaux, 2014; Lacy & Stow, 2011; Mishra et al., 2023). IL-6 showed the greatest upregulation in both cell types, and this cytokine is known to play a role in the wound healing process (Johnson et al., 2020), which may be directly related to the well-established wound healing properties of glucans *in vivo* (Abedini et al., 2022; Cerci et al., 2008; Wu et al., 2016). Additionally, the slight increase in interleukin production observed in HACAT cells may provide insights into the wound healing capacity of yeast glucans using this cell line (Seo et al., 2019; Vetvicka & Vetvickova, 2011; Xin et al., 2022).

2.1.3.3 – Safety assessment

New cosmetic ingredients require proven efficacy combined with a comprehensive safety assessment to ensure no risk to human health. The OECD provides guidelines as an alternative to animal studies to assess the safety of new cosmetic products and ingredients. *In vitro* assays, such as genotoxicity, skin sensitization, skin irritation, dermal absorption, and others, are utilized for this purpose (Barthe et al., 2021). In our study, we followed some of the OECD-approved assays to evaluate the safety of our glucan extracts.

To assess the genotoxic potential, we conducted the bacterial mutagenicity assay – AMES fluctuation test and the *in vitro* mammalian cell micronucleus test, following OECD guidelines (OECD Guidelines for the Testing of Chemicals, 2014a, 2020). In the first assay, we used two *S. typhimurium* strains, TA100 and TA98, to detect point mutations by base substitutions and frameshifts, respectively. Our glucans did not induce revertants, indicating no mutagenic effects compared to the negative control (**Figure S2**). The same result was observed in the presence of the S9 activation enzymes. Regarding the micronucleus assay (**Figure S3**), which is used to observe micronucleus formation resulting from chromosome damage or anomalies during cell division, all our compounds did not promote the development of micronuclei in lymphoblast TK6 cells compared to the negative control. The number of micronucleated cells was considerably lower than the mutagenic agent mitomycin C, which was used as the positive control for this assay.

Based on the results of the AMES test and the micronucleus assay, our native and carboxymethyl glucans can be considered non-mutagenic according to the guidelines. In both assays, both forms of glucans showed behavior very similar to the negative control, and no concentration-related increased response was observed, which is an important criterion described for both assays.

Moving on to the evaluation of potential skin sensitization, we analyzed four key events (KE) established by the OECD: covalent binding with cysteine/lysine (KE-1), keratinocyte inflammatory/antioxidant response (KE-2), dendritic cells activation (KE-3), and T-cell proliferation (KE-4). For this assessment, we conducted two different tests: the DPRA assay for KE-1 and the h-CLAT assay for KE-3 (OECD Guidelines for the Testing of Chemicals, 2014b). Regarding the DPRA test results (**Table S2**), our extracted glucans did not induce a haptenation reaction, which is a complexation reaction between low molecular weight substances and proteins (Chipinda et al., 2011), against peptides containing cysteine or lysine. This information is useful in understanding the reactivity of glucans with these two amino acids, and it is expected that our molecules will not become antigenic and prompt an immune response. However, evaluating the results obtained through the h-CLAT test, we observed that all the glucans tested increased the biomarkers CD86 and CD54 expressed on the cell surface of monocytic THP-1 cells. According to OECD guidelines, this suggests a sensitizing potential. This result was expected since THP-1 cells possess several receptors capable of recognizing glucans, such as toll-like receptors or dectin-1 receptor and initiate an immune response (Vargas-Hernández et al., 2020; Zhang et al., 2022). This finding is consistent with the cytokine profile observed in macrophage-like cells (**Figure 12B**), showing an upregulation of cytokines, particularly IL-6, due to the high affinity of glucans for receptors present in immune cells. To mitigate potential hazards related to skin sensitization, OECD employs a "2 out of 3" approach, where at least two tests must be negative. Therefore, a third assay should be performed. Focusing on KE-2 for keratinocyte inflammatory response, the OECD has two approved methods for this analysis, KeratinoSens and LuSens. However, a simpler method named HaCaSens has been established in the literature, which uses HACAT cells to test chemical products and quantify the resulting cytokines produced, specifically IL-6 and IL-1α (Kim, et al., 2018; Quan, et al., 2018; Jung et al., 2016; Kalka et al., 2022). An increase in these cytokines is used as an indicator of sensitization if it is observed, with a fold change variation equal to or higher than 3.0 compared to the basal control (Kim, et al., 2018). In this study, we adapted HaCaSens to analyze the production

of IL-1α, IL-6, and IL-8 upon exposure to insoluble and carboxymethyl glucans. As shown in **Figure 12A**, none of the samples surpassed this threshold for IL-6 and IL-8. However, it was not possible to quantify the production of IL-1α (data not shown), which may limit the conclusions obtained. IL-1α was probably present in such low concentration that it could not be quantified due to the kit's sensitivity. Of particular importance, the levels of IL-8, which we included in this analysis as it represents an important interleukin that mediates immune cell recruitment in the skin, were below a fold change variation of 3 for both HACAT and mTHP-1 cells (**Figure 12**). Based on the results of the three assays conducted for skin sensitization, our extracted glucans may not pose a risk when used as an ingredient in cosmetic products. However, it is important to note that a higher reactivity is expected when in contact with immune cells, such as monocytes or macrophages.

To further enhance the safety of the insoluble and carboxymethyl extracts, we evaluated the potential effects of our extracts on the naturally occurring microbial community in facial skin using a preclinical *in vitro* model followed by amplicon-based NGS (Carvalho et al., 2022). This methodology is essential for characterizing the taxonomic profiles of the skin microbiota by targeting conservative genes such as the 16S rRNA gene for bacterial communities and internal transcribed spacers (ITS) for fungal communities (Carvalho, et al., 2022). Ideally, the skin microbiome remains stable throughout adulthood but varies depending on the skin characteristics of each body site. For example, areas with oily or moist conditions may exhibit differences in the abundance of microbial components (Byrd et al., 2018).

As shown in **Figure 13A** and **Figure 13C**, Proteobacteria (56-62%) and Ascomycota (62-68%) were the most prevalent phyla in our volunteers' facial skin samples. Among bacteria, *Staphylococcus* sp. (26-32%) was the most representative genus, while *Candida* sp. (42-49%) was the most abundant genus among fungi (**Figure 13B** and **Figure 13D**). Importantly, no significant differences ($p > 0.05$) were observed in the relative abundance of both genera between the control and test groups (**Figure S4**). The skin is naturally colonized by microorganisms belonging to these dominant phyla, such as *Acinetobacter* sp. (Proteobacteria) and *Candida* sp. (Ascomycota) (Cosseau et al., 2016; Limon et al., 2017), as well as genera such as *Staphylococcus epidermidis* (*Staphylococcus* sp.) and *Candida parapsilosis* (*Candida* sp.) (Proctor et al., 2021; Severn & Horswill, 2023). Li et al., (2020) reported similar findings when analyzing the microbiome of facial skin (cheek) from 80 individuals, where they observed that Firmicutes and Ascomycota were

the most abundant phyla, with a dominance of *Staphylococcus* sp. and *Malassezia* sp. genera. Furthermore, Lee et al., (2021), in their study focusing on the bacterial community in human facial skin of male volunteers, reported that Firmicutes and the genus *Staphylococcus* were the most dominant, which corroborates our findings regarding the most abundant genus. The difference in the abundance of phyla between studies may be explained by a higher presence of Proteobacteria than Firmicutes in female skin (Robert et al., 2022).

Compounds that disrupt the balance of microbial communities can have detrimental effects on the skin microbiota, potentially leading to skin conditions. For example, an increase in the relative abundance of *Candida albicans* or *Staphylococcus aureus* can result in skin infections, particularly in elderly and immunocompromised individuals (Kashem & Kaplan, 2016; Parlet et al., 2019). Therefore, the results obtained in these findings indicate that native and carboxymethyl glucans do not significantly affect the relative abundance of the dominant genera found in our volunteers' skin.

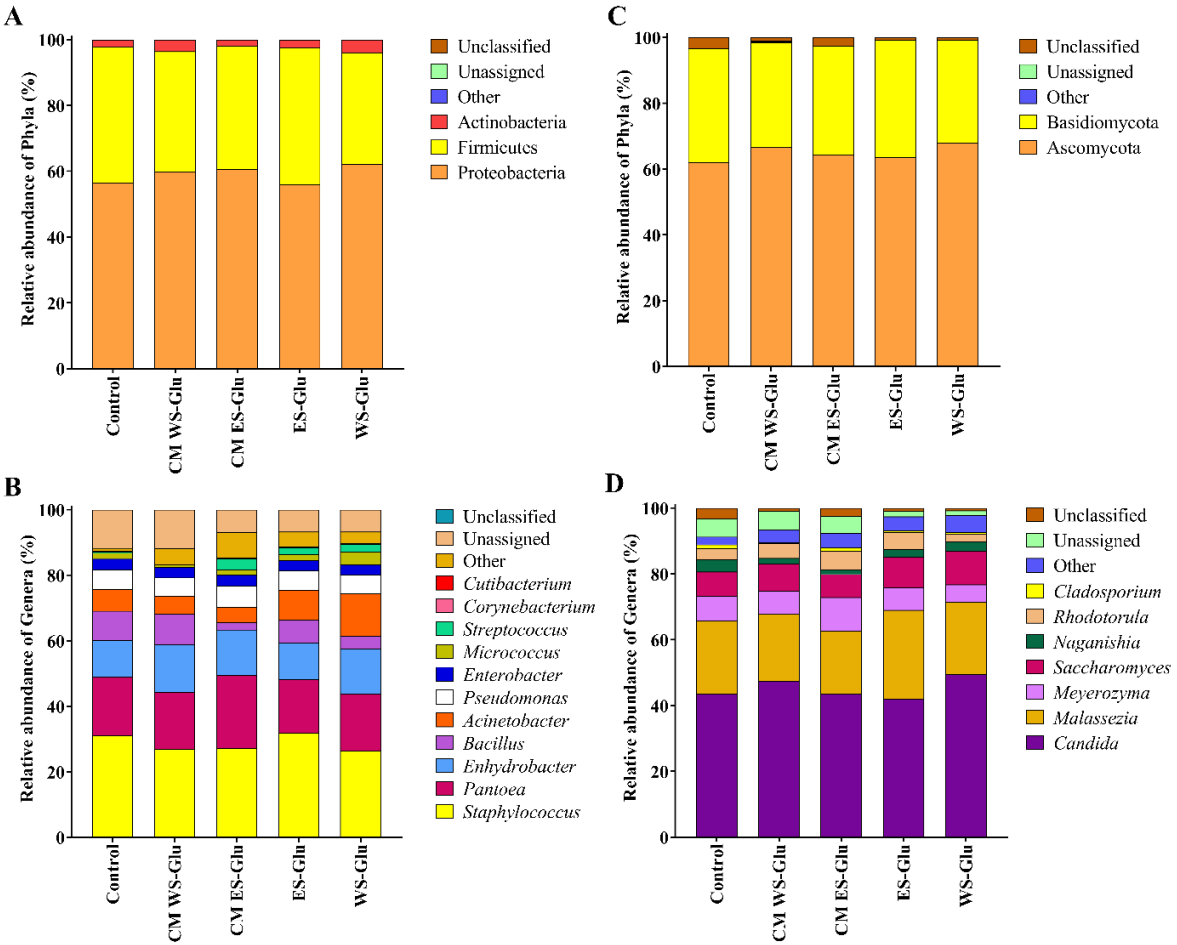

Figure 13 – Impact of glucan extracts, native and carboxymethylated, on the relative abundance of bacterial phyla and genera (A and B) and fungal phyla and genera (C and D) in the naturally occurring skin microbiota of female volunteers.

2.1.4 – Conclusion

We successfully established an extraction process to obtain high-purity glucan extracts from two different *S. cerevisiae* strains. These extracts were further functionalized to enhance their water-solubility, opening up new possibilities for their utilization as cosmetic ingredients. To assess the safety of the glucan extracts for cosmetic applications, we conducted comprehensive evaluations, which indicated that these extracts do not possess sensitization and genotoxic potential, capacity to induce acute skin irritation/corrosion, or the ability to disrupt the diversity of the natural skin microbiota. Furthermore, we tested the effects of these biomolecules on various cell lines and found that, at different concentrations, most of the extracts did not negatively impact the metabolism of keratinocytes, dermal fibroblasts, and macrophage-like cells. Additionally, we conducted further analyses to explore how native and carboxymethyl glucans can modulate the immune response in keratinocytes and macrophage-like cells. Our findings revealed that under certain conditions, these glucans had the capacity to upregulate IL-6 and IL-8 cytokines in both cell lines, suggesting an immunomodulatory capacity, however further studies are warranted to comprehensively understand the underlying mechanisms. Concerning the safety of glucans as a chemical ingredient for skincare, we conducted a comprehensive evaluation of their genotoxic and skin sensitization potential. As demonstrated, both forms of glucans did not show the capacity to induce genetic alterations through the AMES or Micronucleus test. For skin sensitization, our results indicate the safety of these molecules based on the DPRA and an adapted form of HaCaSens, despite a positive result observed for h-CLAT. Additionally, our molecules did not show an influence on the microbiota naturally present in female human skin. Overall, glucans have the potential to be safely used for skin application. However, further studies are necessary to assess their influence on more complex cellular structures, such as organotypic models or human skin *ex vivo* tissue. This will provide a more comprehensive understanding of their suitability for skincare products.

Supplementary material

Table S1 – Chemical characterization of glucan extracts throughout the extraction for the Engineered Strain type (A), Wild Strain type (B) and subsequent functionalization through carboxymethylation (C). Data are represented as mean ± SD from two replicates for each sample.

A **Engineered Strain Yeast**

Sample	Protein (%)	Polysaccharides (%)	Lipids (%)	Ash (%)	Moisture (%)	β-Glucan (%)	α-Glucan (%)
ES-Alk	3.23 ± 0.70	67.36 ± 5.94	12.47 ± 3.53	2.78 ± 0.30	8.99 ± 1.64	35.38 ± 0.80	20.80 ± 2.61
ES-Glu	3.93 ± 0.22	92.09 ± 1.96	0.52 ± 0.09	1.14 ± 0.14	4.85 ± 0.38	46.13 ± 1.48	25.82 ± 0.41

B **Wild Strain Yeast**

Sample	Protein (%)	Polysaccharides (%)	Lipids (%)	Ash (%)	Moisture (%)	β-Glucan (%)	α-Glucan (%)
WS-Alk	4.36 ± 0.57	70.24 ± 4.51	22.92 ± 4.03	1.78 ± 0.09	5.66 ± 1.71	43.13 ± 1.69	6.47 ± 0.27
WS-Glu	5.23 ± 0.49	94.48 ± 1.21	2.43 ± 1.10	0.59 ± 0.10	5.88 ± 2.69	55.58 ± 5.27	9.60 ± 0.27

C **Carboxymethyl Glucans**

Sample	Molecular Weight (Da)	Process Yield (%)	DS Value
CM ES-Glu	4.91×10^5	95.77 ± 0.30	0.333 ± 0.028
CM WS-Glu	4.89×10^5	90.76 ± 0.40	0.329 ± 0.019

Table S2 – DPRA prediction model considering the peptide depletion percentage of Cysteine (C) and Lysine (L) for insoluble and carboxymethyl glucans at 2 mg/mL. Data are represented as mean ± SD from two replicates for each sample. Cinnamic aldehyde (100 mM) was used as positive control.

	Mean peptide depletion (%)			Prediction model				
Sample	C	L	C and L	Reactivity (C)	Reactivity (C and L)	Based on mean of C	Based on mean of C and L	Potential sensitizer?
ES-Glu	1.7 ± 0.0	5.0 ± 0.3	3.4 ± 2.3	Minimal	Minimal	Negative	Negative	No
WS-Glu	0.4 ± 0.3	3.2 ± 3.0	1.8 ± 2.0	Minimal	Minimal	Negative	Negative	No
CM ES-Glu	2.9 ± 0.3	5.0 ± 0.2	3.4 ± 0.2	Minimal	Minimal	Negative	Negative	No
CM WS-Glu	1.7 ± 0.4	0.0 ± 0.1	1.8 ± 0.8	Minimal	Minimal	Negative	Negative	No
Control (+)	72.3 ± 0.5	64.6 ± 1.0	68.3 ± 5.0	Moderate	High	Positive	Positive	Yes

Table S3 – h-CLAT sensitization test based on the RFI obtained for the cell markers CD54 and CD86 after exposing native and carboxymethyl glucans to THP-1 cells. Nickel(II) sulfate (100 µg/mL) was used as a positive control.

		Assay 1			Assay 2		
Sample	**Concentration (mg/mL)**	**RFI CD54**	**RFI CD86**	**Outcome**	**RFI CD54**	**RFI CD86**	**Outcome**
Control (-)	-	100	100	-	100	100	-
Control (+)	**0.1**	268	317	Sensitizer	1126	232	Sensitizer
ES-Glu	**1.2**	4032	126	Sensitizer	858	177	Sensitizer
	1.0	3620	110	Sensitizer	701	121	Sensitizer
	0.8	3675	115	Sensitizer	695	131	Sensitizer
	0.7	3807	118	Sensitizer	731	63	Sensitizer
	0.6	4132	98	Sensitizer	1487	81	Sensitizer
	0.5	2827	82	Sensitizer	1338	66	Sensitizer
	0.4	2774	102	Sensitizer	1593	41	Sensitizer
WS-Glu	**1.2**	2590	106	Sensitizer	698	30	Sensitizer
	1.0	1792	75	Sensitizer	594	54	Sensitizer
	0.8	1765	96	Sensitizer	437	17	Sensitizer
	0.7	3896	128	Sensitizer	911	45	Sensitizer
	0.6	3309	128	Sensitizer	340	18	Sensitizer
	0.5	3398	108	Sensitizer	594	39	Sensitizer
	0.4	2791	121	Sensitizer	818	44	Sensitizer
CM ES-Glu	**1.2**	649	36	Sensitizer	476	37	Sensitizer
	1.0	668	34	Sensitizer	682	12	Sensitizer
	0.8	678	52	Sensitizer	744	20	Sensitizer
	0.7	776	44	Sensitizer	933	114	Sensitizer
	0.6	971	45	Sensitizer	737	25	Sensitizer
	0.5	1073	50	Sensitizer	828	13	Sensitizer
	0.4	784	74	Sensitizer	928	39	Sensitizer
CM WS-Glu	**1.2**	1061	65	Sensitizer	661	39	Sensitizer
	1.0	861	79	Sensitizer	626	54	Sensitizer
	0.8	891	96	Sensitizer	812	65	Sensitizer
	0.7	849	66	Sensitizer	701	75	Sensitizer
	0.6	778	114	Sensitizer	847	93	Sensitizer
	0.5	849	90	Sensitizer	857	90	Sensitizer
	0.4	717	90	Sensitizer	688	120	Sensitizer

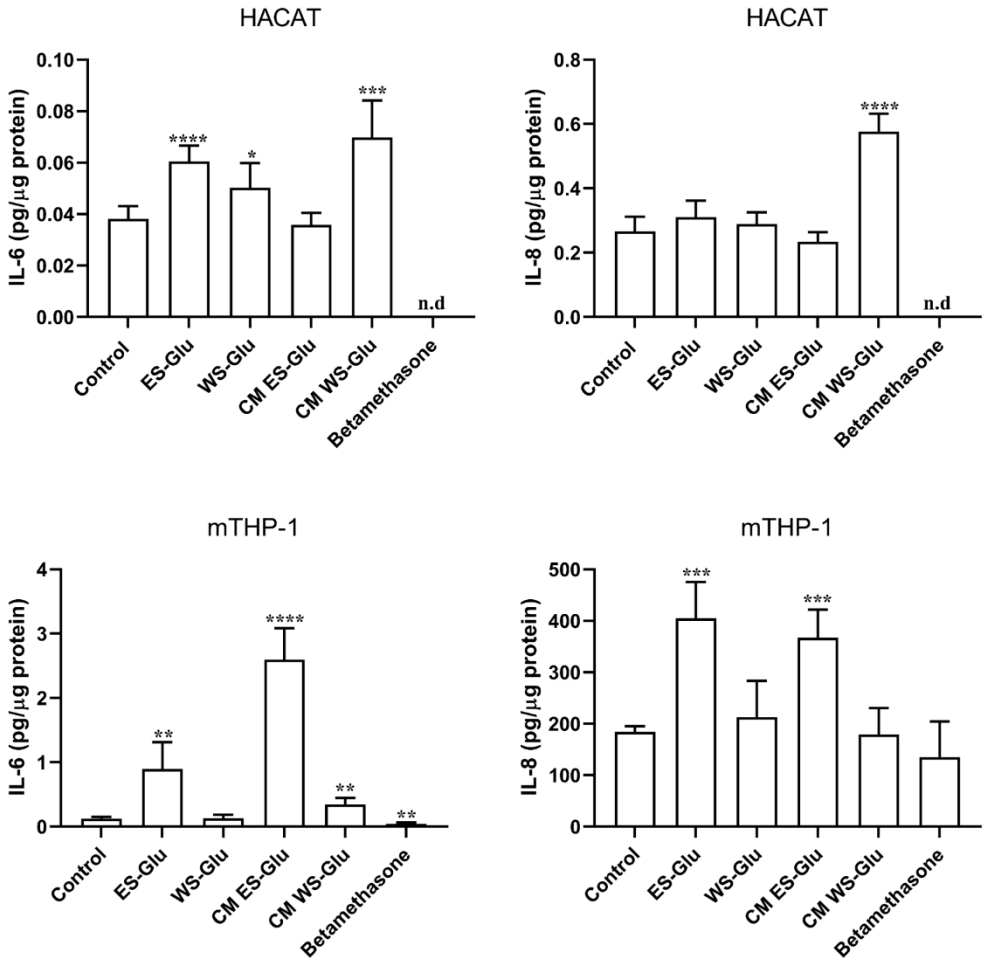

Figure S1 – Levels of (A) IL-6 and (B) IL-8 on HACAT and (C) IL-6 and (D) IL-8 on mTHP-1 cells upon exposure to native (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu). The data are presented as mean ± SD from three replicates for each sample. Significant differences between the samples and the basal control are denoted by *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$. Betamethasone (20 µM) was used as anti-inflammatory control.

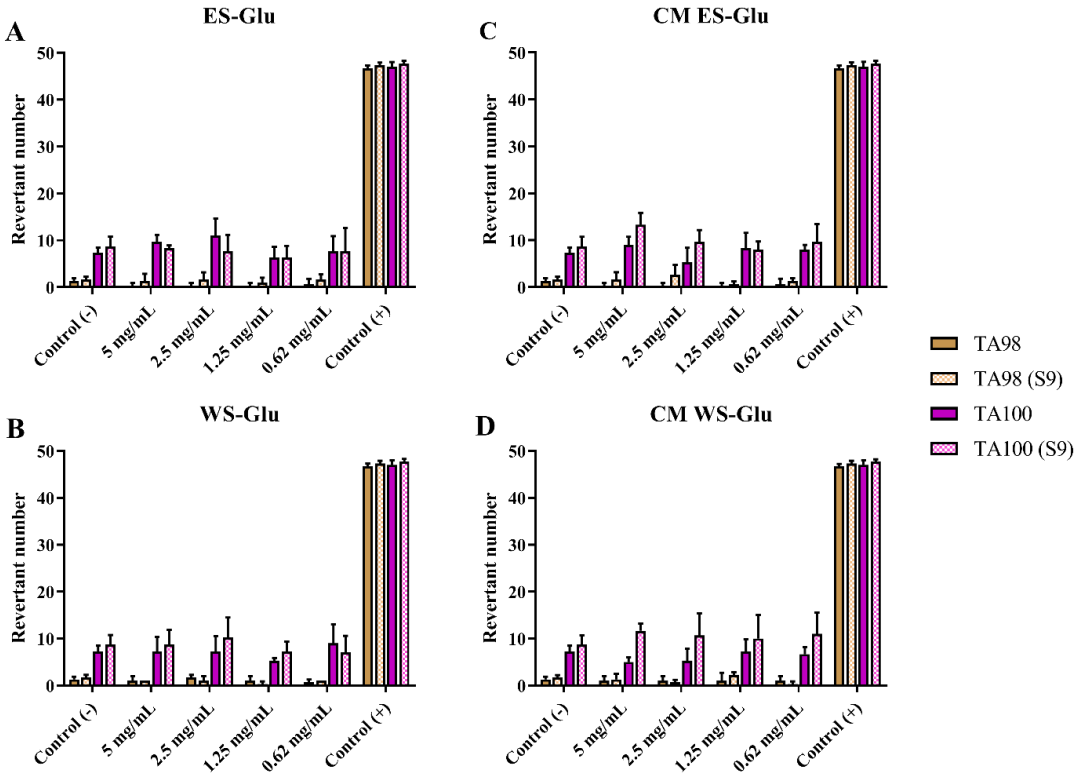

Figure S2 – Number of revertant wells observed in mutagenic assay test (AMES) upon exposure to insoluble glucans extracts (A and C) and its carboxymethylated form (B and D), to TA98 and TA100 strains with and without presence of metabolic activation (S9). Data are represented as mean ± SD from three replicates for each sample. 2-Nitrofluorene (50 µg/mL) and 4-Nitroquinoline-N-oxide (50 µg/mL) were used as positive controls for TA98 and TA100 strains, respectively.

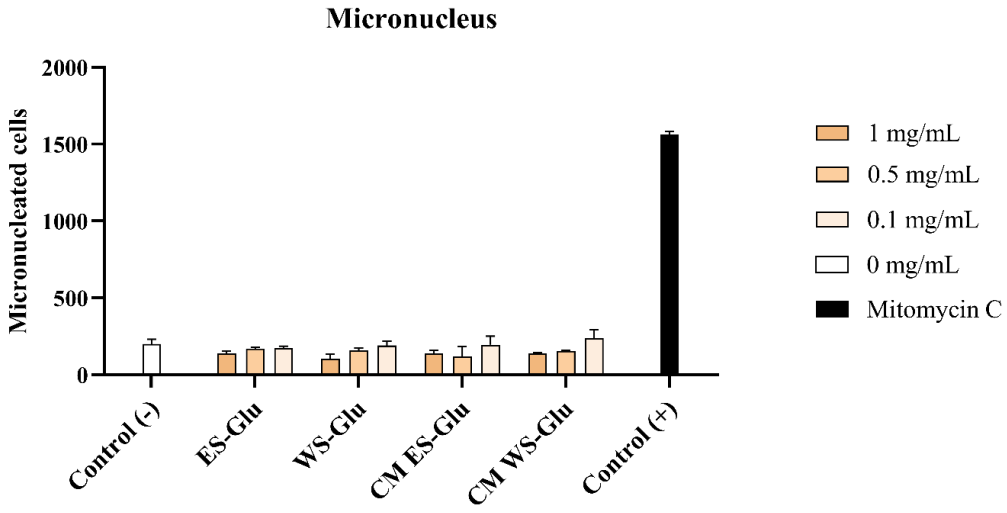

Figure S3 – Micronucleated cells quantification through flow cytometry in human lymphoblastoid TK6 cells exposed to insoluble and carboxymethyl glucans. Data are represented as mean ± SD from two replicates for each sample. Mitomycin C (25 ng/mL) was used as positive control.

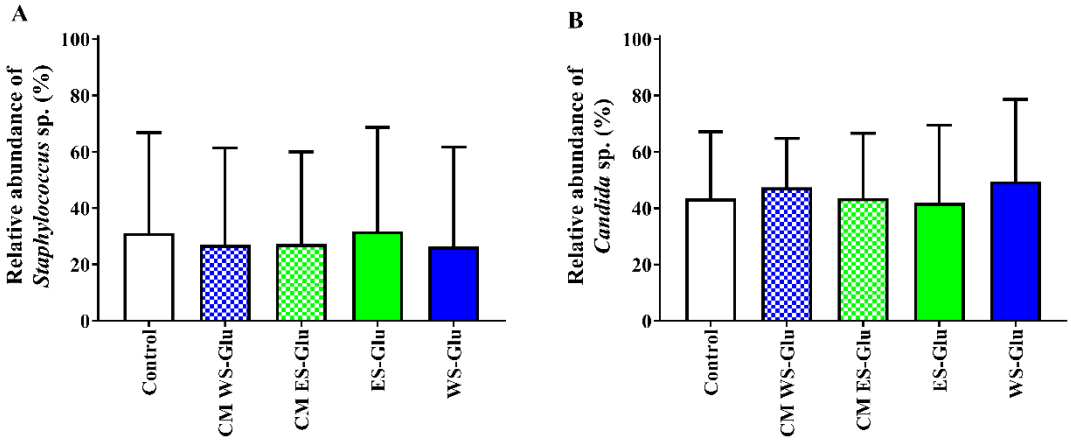

Figure S4 – Impact of the test groups in the relative abundance of *Staphylococcus* sp. (A) and *Candida* sp. (B). Data are represented as mean ± SD obtained from 20 healthy female volunteers. No significant differences were observed between the control and test groups by Dunn's multiple comparisons test.

Chapter 3

This chapter will analyze the *in vitro* biological properties of water-insoluble and carboxymethyl glucan extracts. These properties include their anti-inflammatory capacity when cells are exposed to LPS and PM, their ability to neutralize ROS production induced by PM, and their capacity to promote keratinocyte migration for wound closure.

Information based on the following paper:

Sousa, P., Amorim, M., Mendes, A., Azevedo-Silva, J., Pintado, M., Fernandes, J., & Tavares-Valente, D. (2023). *Saccharomyces cerevisiae* glucan extracts with *in vitro* bioactivity for skin protection – In submission process.

3.1 – Glucans *in vitro* biological properties

Saccharomyces cerevisiae glucan extracts with *in vitro* bioactivity for skin protection

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Abstract

There is a growing interest in discovering new healthy compounds derived from industrial by-products in line with the principles of the circular economy. Glucans extracted from *S. cerevisiae*, specifically from cell walls, have garnered significant attention in both biomedical and skincare fields due to their inherent immunomodulatory capabilities and other bioactive properties, such as wound healing and anti-aging capacity. This study explores the *in vitro* potential of water-insoluble and water-soluble glucan extracts obtained from spent yeast, evaluating their anti-inflammatory capacity against two inflammatory stimuli (bacterial lipopolysaccharide and particle pollution), antioxidant potential by reducing intracellular ROS formation, and wound healing abilities by assessing HACAT migration until wound closure. The results demonstrate that yeast glucan extracts can effectively modulate inflammation, reduce the release of IL-1α, IL-6, and IL-8 when cells are exposed to inflammatory stimuli, lower intracellular ROS formation in the presence of pollution particle matter, and enhance HACAT migration for wound repair. This study highlights the potential application of water-insoluble and water-soluble glucans as a bioactive ingredient in skincare products.

Keywords: Yeast Glucans, Bioactivity, Skincare

3.1.1 – Introduction

The skin acts as a protective barrier against environmental damage and plays a vital role in temperature and water regulation. It consists of three main layers: epidermis, dermis, and hypodermis, with various cell types such as keratinocytes and fibroblasts contributing to essential extracellular matrix components like keratin and collagen (Hofmann et al., 2023). However, intrinsic, and extrinsic factors can accelerate aging, disrupt the skin barrier, and interfere with inflammatory pathways, ROS production, and wound healing (Gushiken et al., 2021; Martic et al., 2022). Among extrinsic factors, human-generated airborne pollution particles containing harmful chemicals are a significant contributor to skin aging, triggering an immune response upon contact (Dijkhoff et al., 2020; Fitoussi et al., 2022). Skin cells that naturally produce cytokines such as interleukin-1 (IL-1α/β), IL-4, IL-6, IL-8, or tumor necrosis factor (TNF-α), crucial for communication and cell differentiation (Hänel et al., 2013; Noske, 2018). When exposed to air pollution particles, their production can be altered, resulting in an abnormal increase in pro-inflammatory cytokines (Abolhasani et al., 2021). Particle pollution, and various other extrinsic and intrinsic factors contributing to skin aging also have a direct impact on the skin's ability to repair itself. This includes altering skin cell communication, response mechanisms, and the capacity for cell proliferation. As a result, it becomes essential to preserve the skin's natural ability to restore itself through processes like immune response, re-epithelization (the regeneration of the epidermal layer), and ECM remodulation (the restructuring of the extracellular matrix). By supporting these reparative mechanisms, the skin can maintain its balance and overall health, ensuring homeostasis is upheld (Vu et al., 2022). Bioactive molecules with antioxidant and cell regulatory properties have emerged as potential solutions to combat such skin aging inducers. These compounds offer protective effects against oxidative stress and inflammation among other stressors that play significant roles in the aging process. By incorporating such bioactive molecules into skincare products, it is possible to enhance the skin's ability to counteract the damaging effects of aging inducers and promote healthier and more youthful-looking skin.

In general, consumers now prefer greener and more sustainable skincare products, aligning with the increasing awareness of environmental concerns. Embracing natural and eco-friendly ingredients has become a significant trend in the skincare industry, as people seek effective and environmentally conscious solutions for their skin health (Fernandes et al., 2023; Martic et al., 2022; Rizzi et al., 2021). Some microorganisms, such as *Saccharomyces cerevisiae*, are naturally rich in compounds with potential applications to

alleviate the effects of aging, including polysaccharides or peptides. Among these compounds are glucans, which make up more than half of the yeast cell wall and have been extensively studied in both their native form (insoluble) and chemically modified forms, demonstrating appealing biological properties for skin applications, such as wound healing, anti-wrinkles, antioxidant capacity, and more (Avramia & Amariei, 2021; Majtan & Jesenak, 2018; Sousa et al., 2023). The immunomodulatory capacity of glucans is particularly noteworthy, as they can interact with specific receptors, such as dectin-1 or toll-like receptors, expressed in various skin cells, including keratinocytes in the epidermis and macrophages in the dermis (Lima et al., 2021; Seo et al., 2019). Despite numerous studies demonstrating the bioactive properties of glucans, such as antioxidant and anti-inflammatory effects, from various sources (Gou et al., 2023; Ozanne et al., 2020; Zhou et al., 2023), there is still a lack of information regarding how skin cell mechanisms can be beneficially modulated by insoluble yeast glucans and their chemically modified form using carboxymethylation. Additionally, the properties of glucans obtained from spent yeast from industrial fermentation processes remain poorly understood. Further research is needed to fully explore and understand the potential of these glucans in skincare applications.

In this study, we explored the impact of native and carboxymethyl water-soluble glucan extracts obtained from two different sources of *S. cerevisiae* upon skin cells using different methodologies. We evaluated the inflammatory effects of two external stimuli, bacterial lipopolysaccharide (LPS) and particle pollution (PM), on cytokine production in differentiated leukemia monocytic cells (mTHP-1) and immortalized human epidermal keratinocytes (HACAT). Our aim was to understand the potential of these extracts in regulating cellular inflammatory pathways caused by external factors. Additionally, we analyzed the ability of the glucan extracts to reduce reactive oxygen species (ROS) generation induced by PM in HACAT cells, as ROS can contribute to skin damage and aging. Furthermore, we investigated the potential of glucan extracts to promote HACAT cell migration, a critical process for wound closure and skin repair.

3.1.2 – Materials and Methods

3.1.2.1 – Materials

Two water-insoluble glucan extracts obtained from the cell wall of *Saccharomyces cerevisiae* along with their respective carboxymethylated forms (CM ES-Glu and CM WS-Glu), were used in this study: one extracted from an engineered spent yeast strain collected from Amyris industrial fermentation wastes (ES-Glu) and a second one extracted from a wild strain produced via lab-scale fermentation (WS-Glu). The details of obtention and characterization of these extracts are described on **Chapter 2.**

Human immortalized epidermal keratinocyte cells (HACAT) and human leukemia monocytic cells (THP-1) were purchased from the Cell Lines Service (CLS) and American Type Culture Collection (ATCC), respectively. Dulbecco's Modified Eagle Medium (DMEM) with and without phenol red, Glutamax 100X, RPMI 1640, Fetal Bovine Serum (FBS), antibiotic-antimycotic (penicillin–streptomycin–amphotericin B) and Pierce BCA assay kit were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Culture insert 2-well silicone 24 plates were obtained from Ibdi (Munich, Germany). Phorbol-12-myristate-13-acetate (PMA), lipopolysaccharides (LPS) from *Escherichia coli* (O111:B4), urban particulate matter (PM) SRM 1648a, fluorescent probe 2′,7′-Dichlorofluorescin diacetate (DCFDA), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Betamethasone and Mitomycin C from *Streptomyces caespitosus* were acquired from Sigma-Aldrich (St. Louis, MO, USA). Black clear-bottom 96-well plates were purchased from Corning (Corning, NY, USA). ELISA assay kits (IL-1α/IL-6/IL-8) were purchased from Biolegends (San Diego, CA, USA). All other reagents used were of analytical grade.

3.1.2.2 – Cell culture

HACAT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS) (10% v/v) and antibiotic-antimycotic (1% v/v). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) antibiotic-antimycotic and 2-Mercaptoethanol at 0.05 mM. All cultures were maintained in a humified atmosphere at 37 °C with 5% CO_2. THP-1 cells were used in its differentiated macrophage-like form (mTHP-1). For this, PMA at a final concentration of 50 nM was added immediately before seeding and let incubate for 48 h at 37 °C with 5% CO_2, before exposing the cells to the compounds.

3.1.2.3 – Cytokine profile

The analysis of pro- or anti-inflammatory activity was performed following the method described in **Chapter 2**. Briefly, HACAT and mTHP-1 cells were exposed to insoluble and carboxymethylated glucans (2 mg/mL) simultaneously with the inflammatory stimuli, LPS at 1 µg/mL (mTHP-1) or 20 µg/mL (HACAT), and PM (500 µg/mL). As an anti-inflammatory control, betamethasone at 20 µM was used. Cytokine quantification was conducted using enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions. The protein cell content was calculated using the Pierce BCA assay kit. All results were normalized to pg of IL-1α, IL-6, and IL-8 per µg of protein.

3.1.2.4 – Intracellular ROS induced by PM

Intracellular ROS was generated by adding PM to HACAT cells, and its levels were monitored over time using the fluorescent probe DCFDA. Briefly, cells were seeded in a black clear-bottom 96-well plate at a density of 1 x 10^5 cells/mL in DMEM without phenol red (DMEM-) supplemented with Glutamax 1X. A ROS medium solution containing DMEM- and PM (500 µg/mL) was prepared and incubated overnight with agitation, and the soluble fraction of PM was isolated. After 24 h of cell incubation, 100 µL of ROS medium, previously centrifuged and filtered (0.22 µm), was added to each well in the presence of glucan extracts, insoluble, and carboxymethylated, at a final concentration of 2 mg/mL. The water-soluble antioxidant Trolox (vitamin E analogue) was used as an antioxidant control at 15 µM. Cells were exposed to all samples for an additional 24 h. Then, DCFDA at a final concentration of 25 µM was added to the wells, considered as time 0, and fluorescence was immediately read at 495/529 (Excitation/Emission). ROS generation was measured again after 5 h. The 96-well plate was kept in the dark during incubation and fluorescence measurements. Results are expressed as fold change variation compared to the control (untreated cells exposed to PM) at 0 h.

3.1.2.5 – Cell migration assay

To assess the wound healing capacity of the glucan extracts, HACAT cells were seeded in a 24-well plate with a 2-well silicone insert, creating two separate zones with a normalized wound size of approximately 500 µm. Briefly, a cellular suspension containing 5 x 10^4 cells was added (70 µL per well) to each side of the silicone insert and incubated for 24 h. Before adding the compounds, both silicone inserts were removed, and 500 µL of Mitomycin C diluted in media (25 µg/mL) was added to inhibit HACAT

proliferation for 2 h. The media was then aspirated, cells were washed with PBS, and 500 µL (2 mg/mL) of each glucan sample diluted in media was added to the cells. Images were captured at different time points (24 and 48 h) using a Zeiss inverted microscope with an Axiocam 208 color camera. CellProfiler software was used to analyze all the images (Stirling et al., 2021). The results are presented as the reduction of the initial wound area (0 h) after 24 and 48 h of treatment with the glucan extracts.

3.1.2.6 – Statistical analysis

All the graphics were created using GraphPad Prism software, and the statistical analysis was conducted using Statistica software. The data were analyzed using Student's t-test to determine if the values obtained for native and carboxymethyl glucans were significantly different from the control in all the assays tested. Significance was considered for *p* values below 0.05, 0.01, 0.001, and 0.0001.

3.1.3 – Results and Discussion

3.1.3.1 – Glucans' immunomodulation against environmental inflammatory stimuli

To assess the immunomodulatory capacity of native and carboxymethyl glucans, HACAT and mTHP-1 cells were exposed to two different inflammatory stimuli: LPS from *Escherichia coli* and PM derived from urban air dust pollution.

3.1.3.1.1 – Glucans' capacity to modulate cytokine production in LPS-induced inflammation

LPS, a cell membrane component of gram-negative bacteria, triggers inflammation in skin by activating pattern-recognition receptors (PRRs) in various cells, including keratinocytes and macrophages (Cohen et al., 2023; Crompton et al., 2016; Gvirtz et al., 2020). In the case of microorganism invasion, human skin cells recognize the invaders, leading to the activation of various pro-inflammatory pathways, including the nuclear factor κB (NF-κB) pathway. This activation results in a significant increase in the production of cytokines such as IL-1α, IL-6, IL-8, and others. These cytokines play crucial roles in promoting an immune response and inflammation to combat the invading microorganisms (Bang et al., 2021; Guo et al., 2022; Juráňová et al., 2018; Seo et al., 2020).

According to our results, HACAT cells exposed to glucans in the presence of LPS showed significant differences (**Figure 14A** and **Figure 14B**). Both native extracts induced an increase in IL-6 values, indicating a pro-inflammatory effect. As for carboxymethyl glucans, only CM ES-Glu significantly reduced the inflammatory induction of LPS for IL-6 and IL-8. While no studies directly compare the modulatory effects of insoluble and carboxymethyl glucans from yeast in HACAT cells, Ozanne et al., (2020) reported that laminarin (1-100 µg/mL) reduced IL-6 but had no effect on IL-8 production by cells exposed to LPS, similar to our findings with CM ES-Glu, which significantly reduced IL-6 while showing no differences in IL-8 compared to the control.

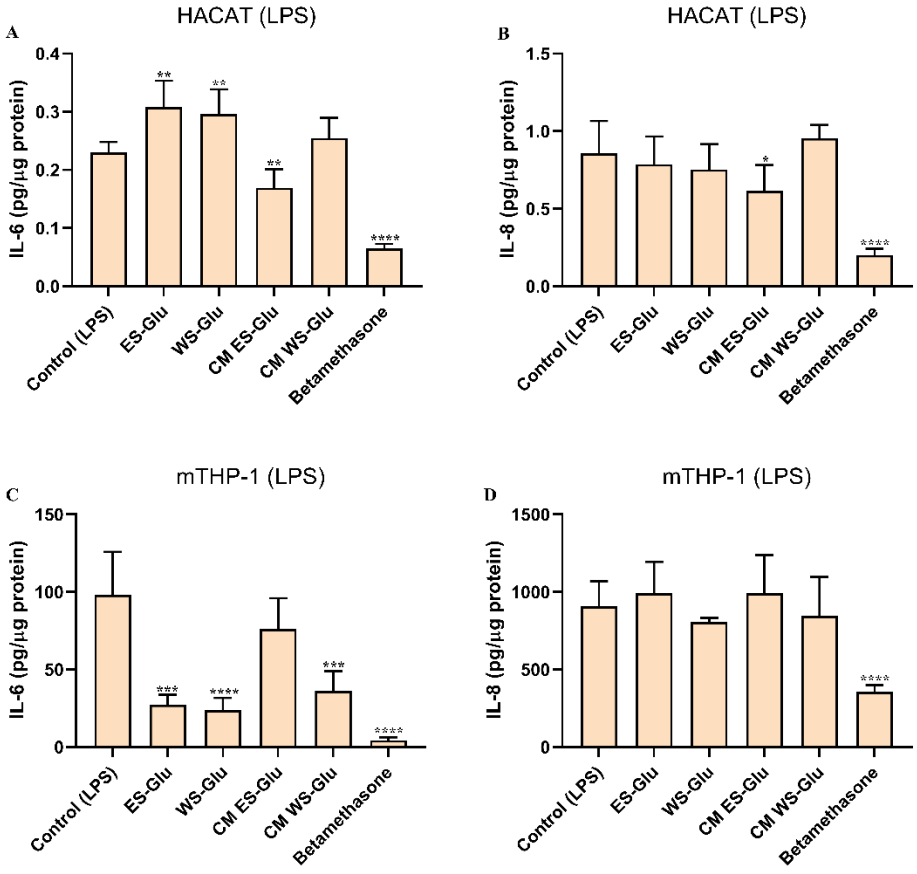

Figure 14 – Levels of (A) IL-6 and (B) IL-8 on HACAT and (C) IL-6 and (D) IL-8 on mTHP-1 cells upon exposure to native (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu) in the presence of bacterial LPS. The data are presented as mean ± SD from three replicates for each sample. Significant differences between the samples and the control exposed to LPS are denoted by *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ and ****$p \leq 0.0001$. Betamethasone (20 µM) was used as anti-inflammatory control.

Regarding mTHP-1 cells (**Figure 14C** and **Figure 14D**), insoluble glucans exhibited an opposite behavior compared to HACAT cells, significantly reducing the production of IL-6, indicating a potential anti-inflammatory capacity. Additionally, carboxymethyl glucans from the wild strain (CM WS-Glu) also showed the ability to significantly reduce IL-6 production, indicating its potential to reduce inflammation. However, none of the samples were able to modulate the immune response of mTHP-1 cells for IL-8 production, with values similar to the positive control. Similar findings were reported by Xin et al., (2022), where they observed an anti-inflammatory effect of water-soluble glucans from yeast at different concentrations (0.01-0.1 mg/mL) in LPS-induced macrophage-like THP-1 cells. It's worth noting that the concentration of glucans used in our study (2 mg/mL) may have played a role in the observed differences.

The notable differences in cytokine production between HACAT and mTHP-1 cells can be explained by the nature of both cell lines. Human keratinocytes primarily use TLRs to recognize various microbial markers, such as LPS or β-glucans, and initiate an immune response (Piipponen et al., 2020). On the other hand, macrophages, including mTHP-1 cells, express similar receptors but dectin-1 is widely reported as the "prime" receptor for glucans, responsible for mediating cytokine responses triggered by this polysaccharide (Mata-Martínez et al., 2022; Zhang et al., 2022).

3.1.3.1.2 – Glucans' capacity to modulate cytokine production in PM-induced inflammation

In the case of PM-induced inflammation in HACAT cells (**Figure 15A** and **Figure 15B**), no significant anti-inflammatory effect was observed, except for CM WS-Glu, which significantly increased the production of IL-6. However, all the samples showed the ability to reduce IL-1α content compared to the induced control, indicating their anti-inflammatory potential. No values for IL-8 were obtained in this study.

Air pollution, particularly particulate matter, is a well-known inducer of skin inflammatory diseases and is commonly used as a reference material to assess inflammation, oxidative stress, and visible skin alterations, such as wrinkles (Fitoussi et al., 2022; Hieda et al., 2020; Yang et al., 2022). The exact mechanisms underlying this inflammatory stimulus are not yet fully understood, although one study has pointed out that IL-6 production can be directly related to TLR5 recognition (Ryu et al., 2019). Additionally, this inflammatory stimulus is responsible for the increased production of IL-1α and IL-8 cytokines within the epidermal layer (Verdin et al., 2019).

For mTHP-1 cells (**Figure 15C** and **Figure 15D**), similar to the results observed for LPS-stimulated mTHP-1 cells, native glucans significantly reduce the production of IL-6. In addition, all the glucans showed a significant reduction in IL-8 production, demonstrating a potent anti-inflammatory capacity of glucans when macrophages are exposed to pollution particles. However, no values for IL-1α could be quantified in this case.

Overall, while most glucans did not significantly influence IL-6 production in HACAT cells, all of them downregulated IL-1α production. This observation is important due to the significance of controlling the production of this cytokine, which is associated with various skin diseases, in the epidermal layer (Macleod et al., 2021; Martin et al., 2021).

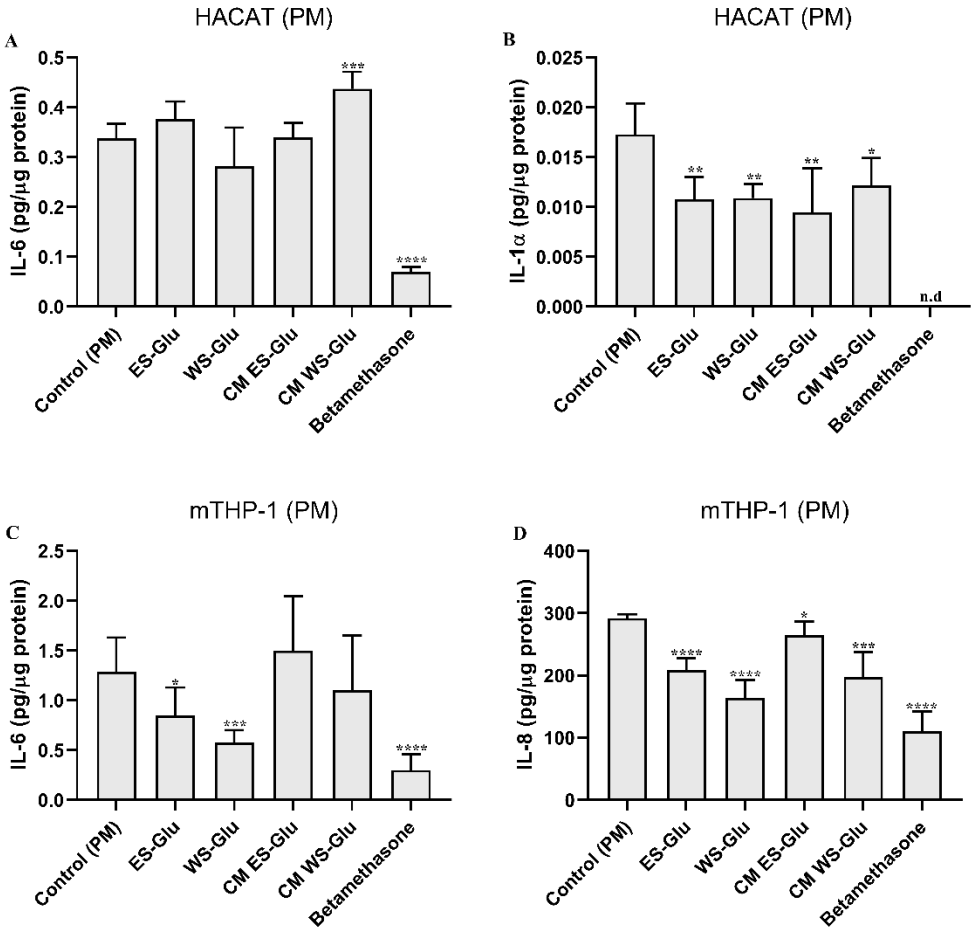

Figure 15 – Levels of (A) IL-6 and (B) IL-1α on HACAT and (C) IL-6 and (D) IL-8 on mTHP-1 cells upon exposure to native (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu) in the presence of PM. The data are presented as mean ± SD from three replicates for each sample. Significant differences between the samples and the control exposed to PM are denoted by $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $****p \leq 0.0001$. Betamethasone (20 µM) was used as anti-inflammatory control.

To the best of our knowledge, no previous studies have been conducted on the anti-inflammatory capacity of glucans from any source using particulate matter as an inflammation inducer. Therefore, this study demonstrates a new potential anti-inflammatory property of both insoluble and water-soluble (i.e., carboxymethyl) glucans when exposed to airborne pollution particles.

3.1.3.2 – Glucans scavenging capacity against intracellular ROS generated by PM in HACAT cells

Beyond its capacity to act as an inflammatory inducer, PM has been reported as a potent inducer of intracellular ROS (Kim et al., 2019; Neo et al., 2022). ROS are highly reactive

species derived from oxygen metabolism, such as hydroxyl radical (•OH) or superoxide radical ($O2^{\bullet-}$), which are normally neutralized by skin membrane mechanisms, including enzymatic and nonenzymatic protectants (e.g., superoxide dismutase and Vitamin E) (Godic et al., 2014; Yadav et al., 2019). However, when the skin is continuously exposed to damaging agents like pollution particles and its homeostasis is disrupted, ROS production surpasses the skin's neutralizing capacity, leading to oxidative damage and skin diseases. Given that yeast glucans are reported as natural antioxidant agents (Khan et al., 2016; Ma et al., 2022; Machová et al., 2014; Tang et al., 2017), their antioxidant ability makes them valuable ingredients for skincare products. Therefore, in this study, native and carboxymethyl glucan extracts were applied to HACAT cells, both in the absence and presence of PM (**Figure 16**), to evaluate their potential as antioxidant molecules.

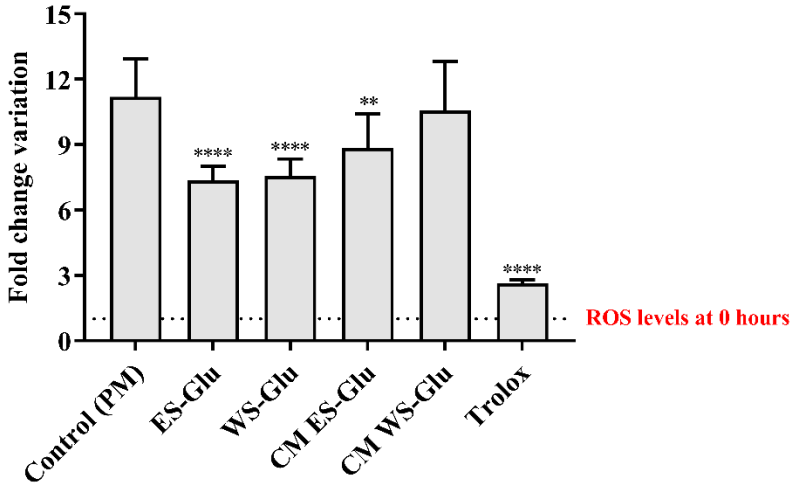

Figure 16 – Intracellular generation of ROS in HACAT cells after exposure to native and carboxymethyl glucans in the presence of PM for 24 h. This assay starts upon addition of the fluorescent probe DCFDA, followed by a fluorescence measurement at 0 h (Fold change variation = 1.0) and after 5 h. Data are represented as mean ± SD from three replicates for each sample. Were considered significative differences between samples and control exposed to PM after 5 h for a $^{**}p \leq 0.01$ and $^{****}p \leq 0.0001$. Trolox (15 µM) was used as an antioxidant control.

According to our results, both native extracts (ES-Glu and WS-Glu) and carboxymethyl glucans from the engineered strain (CM ES-Glu) significantly reduced ROS production, effectively protecting HACAT cells from the development of a strong pro-oxidant environment induced by PM. The results suggest that these glucan extracts have the potential to counteract the harmful effects of pollution-induced ROS in the skin, making

them effective in protecting the skin from oxidative damage and related skin issues. However, no significant differences ($p > 0.05$) were observed between the HACAT control cells exposed to PM and cells treated with CM WS-Glu. This suggests that CM WS-Glu may not have a significant antioxidant effect under the conditions of the experiment, or its antioxidant capacity may be weaker compared to the other glucan extracts tested. Another possibility could be related to the activation of the NLRP1 inflammasome by PM through TLR activation, which has been previously reported in HACAT cells, resulting in an increase in both IL-8 and IL-1α cytokines, as well as in ROS levels (Dong et al., 2020; Fenini et al., 2022). This last possibility suggests that the absence of interaction between CM WS-Glu and the TLRs present on the surface of HACAT cells could be responsible for the observed lack of antioxidant and anti-inflammatory activities within the methodologies employed in this study. This contrasts with all the other three glucan extracts, which led to a reduction in pro-inflammatory cytokines and ROS production. Further investigation is warranted to gain a better understanding of the interaction between different glucans and the TLRs present on the surface of HACAT cells, as well as their potential intervention on NLRP1 inflammasome activation. Elucidating these mechanisms in other cell types beyond the extensively studied innate immune system cells (such as phagocytes, dendritic cells, mast cells, basophils, eosinophils, or innate lymphoid cells), particularly in cell types like keratinocytes and dermal fibroblasts, could provide valuable insights into the unique anti-inflammatory and antioxidant properties of each glucan extract. This broader investigation could enhance our understanding of the potential applications of these extracts in skincare products. Nevertheless, there are various studies published reporting the direct radical scavenging capacity by water-soluble and insoluble glucans, determined through chemical methodologies, such as ABTS radical cation decolorization assay, DPPH free radical spectrophotometric assay, or ferric reducing antioxidant power assessment (Boddapati et al., 2020; Kagimura et al., 2015; Machová et al., 2014; Theis et al., 2019). This scavenging activity was not the primary focus of this study. However, its potential to serve as a protective shield against free radicals, which are responsible for causing premature signs of aging, is noteworthy.

In general, insoluble glucans can effectively be used as a ROS neutralizer in skin cells, while its carboxymethyl form can have a divergent behavior. In our case, insoluble glucan's structure subjected to functionalization through carboxymethylation seem to have a reduced antioxidant capacity against the ROS produced by HACAT cells. The

impact of carboxymethyl groups in the antioxidant capacity of modified polysaccharide is widely studied with a few studies pointed a reduction on the antioxidant capacity of the modified polysaccharides (Chen et al., 2014; Liu et al., 2019) and since the carboxymethyl group is an electron-withdrawing group (EWG), it can indeed negatively impact the resulting scavenging capacity (Lee et al., 2020), thus explaining the results observed.

3.1.3.3 – Glucans promote wound closure by stimulating HACAT cells migration

Wound closure is a complex process involving various cells and molecules that coordinate the four main stages of the healing process: coagulation, inflammation, proliferation, and remodeling. Keratinocytes play a crucial role in the proliferation phase due to their ability to proliferate and migrate, as well as their production of specific factors such as keratinocyte growth factor (KGF), which promotes re-epithelization (Fang & Lan, 2023). Glucans are widely known for their wound healing capabilities (Du et al., 2014; Majtan & Jesenak, 2018) and previous studies have evaluated the potential of yeast glucan extracts to promote HACAT migration (Vetvicka & Vetvickova, 2011; Xin et al., 2022). Therefore, in this study, the researchers analyzed the capacity of two types of molecules, insoluble and carboxymethylated glucans, to promote HACAT migration and wound closure after 24 and 48 h at two different concentrations (2 and 0.5 mg/mL).

According to our results (**Figure 17**), no significant differences were observed when comparing the control and the test conditions during the first 24 h of exposure. However, after 48 h, both native glucans at 2 and 0.5 mg/mL and the carboxymethyl glucan from the wild strain (CM WS-Glu) at 0.5 mg/mL significantly impacted the migration of HACAT cells by promoting wound closure, in comparison to the control. CM ES-Glu was observed to significantly ($p \leq 0.05$) delay HACAT migration at 2 mg/mL but not at 0.5 mg/mL, which seems to indicate that reducing the concentration of this condition may improve its capacity to increase HACAT migration. On the other hand, CM WS-Glu significantly ($p \leq 0.05$) promoted cell migration at 0.5 mg/mL.

These findings suggest that both insoluble and carboxymethylated glucans have the potential to enhance wound closure and promote HACAT cell migration, which is valuable information for the development of skincare products aimed at supporting the wound healing process and overall skin health. Further studies may explore the underlying mechanisms and optimize the concentrations of these glucan extracts for enhanced wound healing benefits.

The observed differences between the two concentrations of glucan extracts may be attributed to aggregation, a natural occurrence observed in insoluble glucans (Kaur et al., 2020) that could also affect chemically modified glucans, reducing their biological capacity at higher concentrations. Comparing our results with the literature, Vetvicka and Vetvickova (2011) demonstrated wound healing capacity for insoluble yeast (*S. cerevisiae*) glucans using HACAT cells. The authors reported wound recovery after subjecting keratinocytes to 0.001 mg/mL of the compound for 24 h. In our study, we observed a similar behavior but at a different time point (48 h) and using a higher concentration (0.05 mg/mL). Additionally, water-soluble glucans have also been reported to promote HACAT migration at 0.05 and 0.1 mg/mL (Xin et al., 2022), which is consistent with our results for CM WS-Glu at 0.5 mg/mL.

Considering the basal interleukin production in HACAT cells (**Chapter 2**), insoluble glucans can significantly upregulate IL-6 production, and CM WS-Glu doubled the amount of IL-6 and IL-8 produced, in comparison to the control. However, CM ES-Glu didn't affect the production of both interleukins. Since CM ES-Glu was the only sample that did not show the capacity to promote wound closure and upregulate IL-6 or IL-8 in HACAT cells, this lack of immunomodulation may directly explain the absence of promotion in cell migration. Both interleukins play important roles in the skin repair process, where IL-6 has a straightforward effect on keratinocyte motility and proliferation (Hänel et al., 2013; Piipponen et al., 2020), while IL-8 seems to influence various stages of wound healing, including keratinocyte migration (Jiang et al., 2012; Takada et al., 2017).

Overall, in line with interleukin modulation, the extract concentration also seems to influence the way carboxymethyl glucans impact HACAT migration, and a better capacity may be achievable at lower concentrations, while insoluble glucans retain their capacity even at reduced concentrations. These findings highlight the importance of considering the concentration and type of glucan extract in wound healing applications, as they can have varying effects on cell migration and interleukin production, ultimately affecting the wound closure process. Further investigations are needed to optimize the concentration.

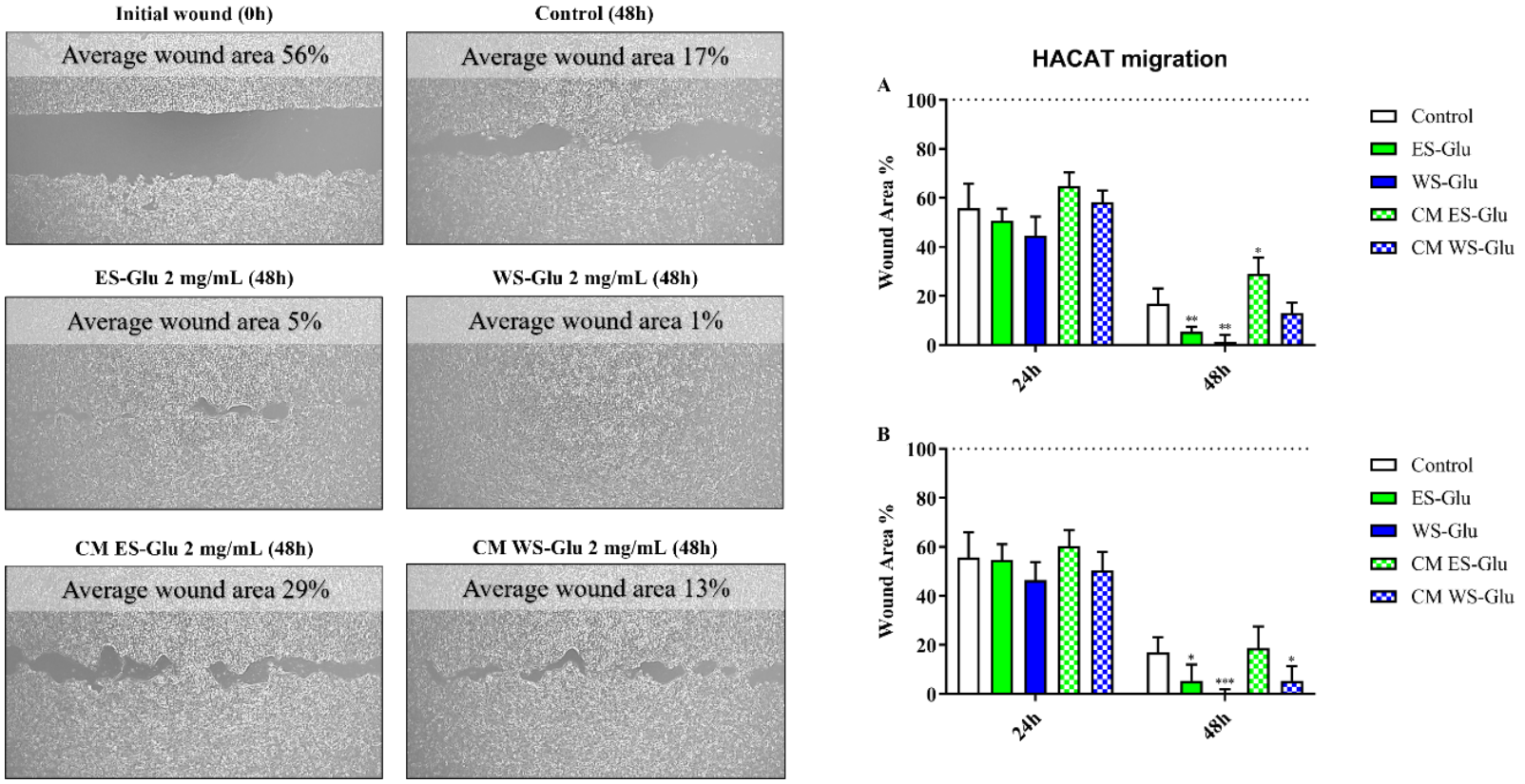

Figure 17 – Representative images of wound closure percentage after 48 h in HACAT cells, not exposed (control) or exposed to insoluble (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu) at two different concentrations (A) 2 mg/mL and (B) 0.5 mg/mL. Data are represented as mean ± SD from three replicates for each sample. Significative differences between samples and control were considered for $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$. Wound area at 0 h is considered as 100%.

3.1.4 – Conclusion

The glucan extracts obtained from two different strains demonstrated the capacity to reduce the inflammatory response induced by LPS and PM, with a stronger effect observed in macrophage-like THP-1 cells. Additionally, these extracts were found to significantly reduce ROS production in keratinocytes exposed to air pollutants. Furthermore, the water-insoluble glucan extracts showed a remarkable ability to promote keratinocyte motility and facilitate wound closure. Taken together, these findings suggest that yeast insoluble and carboxymethylated glucans hold significant potential for use in skincare products due to their beneficial biological properties, overall safety, and wide availability from industrial fermentation processes that generate residual yeast. Utilizing these glucans as ingredients can add value by repurposing waste materials and contributing to sustainable practices. Although this study has shed light on the positive effects of glucans, further investigations should be conducted to evaluate their performance against other skin inflammatory inducers, such as UV radiation, and expand the panel of cytokine analysis to gain a more comprehensive understanding of their mechanisms of action. Despite this, the extensive existing literature on glucans' biological capacity underscores their potential usefulness in cosmetic and skin therapeutic applications, making them a valuable consideration for future skincare formulations.

Chapter 4

This chapter validates glucan extracts as a suitable ingredient for a cream formulation using human skin *ex vivo* models. Initially, the permeability and the impact of these formulations on skin tissue viability were tested. Subsequently, the influence of these formulations on immune cell polarization and collagen deposition was analyzed. Furthermore, the chapter explores whether these creams have the capacity to attenuate induced skin inflammation, using SDS and PM as inflammatory inducers, or whether they can be used as epidermal migration promoters for wound repair purposes.

Information based on the following paper:

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4.1 – Glucans validation as a cream formulation

Unveiling the therapeutic potential of yeast glucans in skincare through human skin *ex vivo* models

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Abstract

Glucans, a structural component naturally present in the yeast cell wall, are well known for their immunomodulatory capacity and biological significance. Nowadays, various skin products containing this polysaccharide are commercially available and use it as an active ingredient within their formulations. However, there is a significant lack of scientific evidence and studies analyzing the mechanisms and the real influence of this molecule on skin cells or skin extracellular matrix components. Thus, this work aims to understand how cream formulations containing glucans, obtained from spent yeast, can effectively be beneficial for skin application using human skin *ex vivo* models. For this, we incorporated water-insoluble and water-insoluble carboxymethyl glucans extracts into cream formulations and applied them to skin explants, observing the formulations' permeability and their impact on skin viability, influence on skin immune cells (Langerhans and macrophage cells), effect on collagen production, protective capacity against an inflammatory stimulus, and their potential wound healing capability. Overall, it was unveiled that glucans can, in fact, upregulate collagen deposition, specifically collagen type-I, can attenuate skin inflammation upon exposure to an irritant and pollutant stimuli (IL-6/IL-1α), and even induce wound closure and epidermal layer migration. These results are essential to further understand and scientifically support the cosmetic and therapeutic potential of glucans when applied to the skin.

Keywords: Yeast Glucans, Skincare, Human skin, *Ex vivo*.

4.1.1 – Introduction

Skin, one of the largest organs in the human body, is the ultimate barrier against environmental factors that can disrupt its health. It also plays a crucial role in maintaining homeostasis by regulating water retention and body temperature. This skin consists of three interconnected main layers: the epidermis, the outermost layer primarily composed of keratinocytes; the dermis, which houses fibroblasts and several immune cells; and the deeper adipose tissue layer, mostly containing adipocytes. Supporting this network of cells are various extracellular matrix (ECM) components, including keratin in the epidermis and collagen in the dermis, which serve as essential structural elements of the skin (Lynch et al., 2022). Over the years, the skin's structure is affected by intrinsic and extrinsic aging processes, leading to natural changes that negatively affect dermal thickness, ECM deposition, the number of immune cells in the dermis, and an increased concentration of cytokines (Costello et al., 2022). This intrinsic aging is further accelerated by exposure to various environmental factors, such as air pollution or UV radiation, which expedite structural modifications in the skin. These alterations can include the breakdown and reduced biosynthesis of ECM components, as well as other microstructural alterations such as collagen cross-linking (Fernandes et al., 2023; Lynch et al., 2022). Besides aging, these environmental factors can also trigger an inflammatory response initiated by various cells, including keratinocytes and dendritic cells. This response involves the release of a wide range of cytokines, such as IL-1, IL-6, IL-8, and others (Makowska et al., 2023), which can exacerbate or even incite inflammatory skin diseases like rosacea or atopic dermatitis (Lee et al., 2021).

Currently, a comprehensive number of molecules, including vitamins and polyunsaturated fatty acids, have been used to mitigate various aging and inflammatory mechanisms(Bjørklund et al., 2022). However, there is a growing demand for new, natural, and sustainable sources in the (dermo)-cosmetic industry driven by economic, ethical, and environmental considerations. This has led to the exploration of methodologies and extraction processes using waste products as raw materials, less environmentally harmful chemicals, and more environmentally friendly product development (Alves et al., 2020; Dini & Laneri, 2021). Glucans are one such molecule, a complex polysaccharide found in a wide variety of natural sources, including yeast, mushrooms, and oats. This molecule plays an important role as a structural component in their cell walls. Interestingly, glucans possess numerous skin-related biological properties, including anti-aging (Sousa et al., 2023), yet there is a lack of robust scientific

studies, such as *ex vivo* or *in vivo* research, to demonstrate their skin-applicable properties for topic application. For instance, it is believed that glucans can directly interact with fibroblasts or indirectly with macrophages, inducing an immune response which, in turn, modulates fibroblast activity toward procollagen biosynthesis (Pillai et al., 2005). Moreover, the interaction between glucans and skin cells goes beyond anti-aging properties, as they have the capacity to modulate immune responses, benefiting areas such as wound healing and even skin cancer therapy. Several studies have directly linked these properties to glucans' ability to interact with skin cells through pathogen recognition receptors (PRRs), toll-like receptors (TLRs), dectin-1, and others (Murphy et al., 2023), activating specific cellular pathways. Distinctively, in the case of human chronic wounds, glucans activate the dectin-1 and TLR2 signaling pathways in wound-site macrophages, leading to increased TNF-α transcription and protein expression (Roy et al., 2011). Thus, the use of glucans may benefit health conditions where an inefficient inflammatory response is one of the underlying causes of impaired healing.

With this in mind, our hypothesis revolved around the potential of yeast glucans as (dermo)-cosmetic ingredients. Hence, we set out to assess the potential of yeast glucans extracted from both industrial and lab-scale production sources, in both its water-insoluble and water soluble (i.e., carboxymethylated) forms. To accomplish this, these molecules were incorporated into cream formulations and applied to skin explants to primarily assess their safety for skin applications. Additionally, we investigated their capacity to promote ECM components and activate various skin immune cells. Moreover, we delved into the anti-inflammatory properties of glucans using different inflammatory *ex vivo* skin models and explored their potential to promote wound healing.

4.1.2 – Materials and Methods

4.1.2.1 – Materials

Two water-insoluble and carboxymethyl glucan extracts, from an engineered (ES-Glu/CM ES-Glu) and a wild strain (WS-Glu/CM WS-Glu) yeast, were used for this study and its characterization/obtention methods are further detailed on **Chapter 2** of this thesis. Montanov 68 and Soy oil were purchased from Acofarma (Madrid, Spain). Squalene was obtained from Aprinnova (Emeryville, CA, USA). Euxyl PE 9010 and Matrixyl® 3000 were acquired on Lotioncrafter (Eastsound, WA, USA). Proteinase K was obtained from Analytik Jena (Jena, Germany). Triton X-100, α-Tocopherol, Tween-20, urban particulate matter (PM) SRM 1648a, Entellan, Fluoromount, Dimethyl sulfoxide (DMSO) and Litium chloride (LiCl) were gotten from Sigma Aldrich Sigma-Aldrich (St. Louis, MO, USA). VECTASTAIN Elite Avidin/Biotin complex and horseradish peroxidase (ABC-HRP) Kit and 3,3-diaminobenzidine (DAB) were obtained from Vector Laboratories (Newark, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Sodium Dodecyl Sulfate (SDS), Penicillin-Streptomycin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 4',6'-diamino-2-fenil-indol (DAPI) and 2-Hematoxylin were bought from Thermo Fisher Scientific (Waltham, MA, USA). Masson's Trichrome (MT) and Picro Sirius Red (PSR) staining kits were bought from Bio-optica (Milan, Italy). Eosin Y Alcoholic was obtained from Epredia (Kalamazzo, MI, USA).

4.1.2.2 – Cream formulations

For this study, a cosmetic cream formulation (CF) was developed with the test samples, insoluble (CF-ES/CF-WS) and carboxymethyl glucan (CF CM-ES/CF CM-WS) extracts. All ingredients were weighed according to **Table 4**. Initially, phase A and phase B or C were heated on to 70 °C. Phase B or C was added into phase A and homogenized through a with high-speed mixing Turrax for 1 min. The mixture was cooled to 35 °C and phase D (preservative) was added by mixing. Note that phase C which contains insoluble glucans, was previously prepared, where all the ingredients were subjected to ultrasonic irradiation at 80% amplitude for 30 s with an ultrasonic homogenizer (JP Selecta CY-500 Barcelona, Spain). All formulations were left to stabilize overnight. Formulations containing commercial active compounds were also produced and used as positive controls in respective assays. α-Tocopherol was prepared according to formulation 2 and Matrixyl® 3000 to formulation 3.

Table 4 – Cream formulation used to incorporate water-insoluble, carboxymethyl and positive controls throughout this study. Depending on the solubility on the compounds, formulation 2 (water-insoluble compounds) or formulation 3 (water-soluble compounds) was used.

Phase	Ingredient	Vehicle Control (Formulation 1)	Insoluble Glucans (Formulation 2)	Soluble Glucans (Formulation 3)
A	Deionized Water	76%	25%	75%
	Soluble Glucans or Matrixyl® 3000	-	-	1%
B	Montanov 68	3%	3%	3%
	Soy oil	10%	10%	10%
	Squalane	10%	5%	10%
C	Insoluble Glucans or α-Tocopherol	-	1%	-
	Squalane	-	5%	-
	Water	-	50%	-
D	Euxyl PE 9010	1%	1%	1%

4.1.2.3 – Human skin tissue

For all the *ex vivo* assays, healthy skin was obtained from abdominoplasty surgeries from *Centro Hospitalar Universitário de São João in Porto*, in the department of *Cirurgia Plástica, Reconstrutiva e Estética* under a stablished protocol with the *Universidade Católica Portuguesa.* The ethics committee of both institutions approved this study (project number 283) and a written informed consent from all donors was obtained. The samples used were limited to female donors with less than 45 years and an IMC lower than 25. Skin was collected to sterile containers with PBS and Penicillin-Streptomycin at 10% (v/v) and used within hours after surgery. To prepare the *ex vivo* models, the skin was placed dermis side up and the adipose layer was carefully removed with scissors, remaining only the epidermal and dermal skin layers. A 12 mm biopsy punch (3 cm^2) was pierced through the skin explants, which were then placed inside transwell inserts (with a diameter of 12 mm and porosity of 1 µm), epidermis side up in air-liquid interface, with 600 µL of supplemented DMEM with FBS (10% v/v) and Penicillin-Streptomycin (1% v/v) on the bottom of the 12-well plate. Skin models were left to stabilize overnight at 37 °C in 5% CO_2. These conditions were used to maintain the skin tissue for all the further assays.

4.1.2.4 – Cream formulations safety assessment

Insoluble and carboxymethyl glucans included into the cream formulation were evaluated for its irritation potential in *ex vivo* tissue, based on the OECD guideline No. 439 (OECD Guidelines for the Testing of Chemicals, 2013). Briefly, 100 µL of PBS 1x (negative control), formulations or SDS 5% (v/v) (positive control) were added to the epidermis of each biopsy punch and incubated for 1 h. Then, all the skin explants were washed vigorously with PBS 1x, dried with a sterile cotton tip swab, and incubated for two days, with fresh media changed daily. On the third day, skin explants were moved to a 24-well plate with the dermis side up, and 1 mL of MTT solution (1 mg/mL in supplemented DMEM) was added into each well, and incubated for 3 h at 37 °C. Afterwards, the solution was completely discarded, 2 mL of MTT extraction solution (HCl diluted in Isopropanol until 0.1M) was added and the extraction process occurred under a gently shake (~300 rpm) for 4.5 h. Finally, aliquots of 200 µL were transferred to a 96-well microplate and the absorbance was read at 570 nm.

4.1.2.5 – Glucans skin permeation

After the safety assessment, it was necessary to understand if all the formulations containing glucans were permeated through the skin using the Franz diffusion cell system. Briefly, 20 mm-diameter skin slices were thawed at room temperature and placed in each cell. At the beginning of the permeation experiment, 0.1 mL of glucans formulations were applied in duplicate, onto the stratum corneum (SC) of each skin sample in the donor compartment of each cell. Receptor compartments were filled with PBS (phosphate buffer) pH 7.4. The system was kept at 32 °C by a circulating water bath and the membrane surface temperature was maintained at this temperature throughout 24 h of experiment. At the end, the skin was removed from the Franz diffusion cell and each side was gently treated with 1 mL of DMSO with LiCl (0.25 M) to wash out the unabsorbed water insoluble glucans, or 1 mL of PBS for carboxymethyl glucans. A standard curve was done with various concentrations of glucans and its corresponding fluorescence with calcofluor stain, to quantify the non-absorbed fraction. Fluorescent intensity was determined using a microplate reader (Synergy H1, BioTek) with excitation at λex 360 nm and emission at λem 430 nm. Results are expressed as percentage of non-absorbed formulation.

4.1.2.6 – Skin's structural components analysis

The developed cosmetic formulation containing glucan extracts, insoluble and carboxymethylated, were used to evaluate its impact on the ECM components, as well on skin immune cells. For this, 25 µL of CF was spread on the top of the *ex vivo* skin explants. Sterile ultrapure water was used as negative control and Matrixyl® 3000 was used as positive control. Vehicle control was also included to remove any interferences caused by the formulation ingredients. After a 24 h incubation with the formulations at 37 °C in 5% CO_2, the cream formulations were re-applied, after removing the excess of the day before with a sterile cotton tip swab. Culture media was changed daily throughout the assay. At the third day, all the tissues were collected, fixed, and embedded in paraffin until further use.

4.1.2.7 – *Ex vivo* inflammatory-like models

4.1.2.7.1 – Erythema-like skin model

The erythema-like *ex vivo* model was used to evaluate the capacity of glucans CF to protect skin exposed to SDS at 2% (v/v). Briefly, skin explants were initially subjected to 100 µL of SDS 2% (v/v) for 10 min to cause an inflammatory response. Then, skin was washed with PBS 1x and dried with a sterile cotton tip swab. Afterwards, 50 µL of CF were added to each explant and 600 µL of fresh media was added per well and incubated overnight at 37 ºC. On the second day, formulations were removed with a sterile cotton tip swab and re-applied. At the third day, all the tissues were collected, fixed, and embedded in paraffin until further use. Culture media was changed daily throughout the assay.

4.1.2.7.2 – Air pollution inflammatory-like skin model

To assess the capability of glucans CF on the protection against PM, skin explants were firstly treated with all the test formulations and daily exposed to the inflammatory stimuli. Briefly, after skin stabilization, 25 µL of each CF was added and incubated overnight at 37 ºC. On the morning of the following day, excess CF were removed with a sterile cotton tip swab and new aliquots were added. On the afternoon, excess CF were again cleaned from the skin and 25 µL of a PM solution (2.3 mg/mL) diluted in ethanol 10% (v/v) was added. This process was repeated the following two days, totalizing 3 exposures to PM solution. On the fifth day, all the tissues were collected, fixed, and embedded in paraffin until further use. Culture media was changed daily throughout the assay.

4.1.2.8 – Wound healing assay

The capacity of glucans CF to promote cell migration and proliferation in an *ex vivo* wound healing assay was evaluated. Briefly, skin explants were wounded using a 3 mm biopsy punch, removing the epidermal and dermal layer, resulting in a circular deep open wound. After 24 h, 25 µL of CF were added around the wound to avoid interferences between the cream and the formation of the new epidermal layer and incubated overnight at 37 ºC. In the following day, additional 25 µL of CF were added, and repeated for a total of 3 times (one per day). α-Tocopherol (a form of vitamin E) in CF was used as positive control. Culture media was changed daily. At the fourth day, all the tissues were collected, fixed, and embedded in paraffin until further use.

4.1.2.9 – Histological analysis

Skin explants embedded in paraffin were cut with a thickness of 5 µm. Then, tissue sections were deparaffinized, rehydrated and stained with (I) Hematoxylin and Eosin Y (H&E) to observe the overall skin structure; (II) Masson's Trichrome (MT) to identify collagen deposition; and (III) Picro Sirius Red (PSR) to identify and distinguish thin (green) and thick (yellow) collagen fibers under polarized light. All kits were done according to the manufacturer's instructions. After staining, slides were cleared with xylene and mounted using non-aqueous Entellan media.

For immunohistochemistry, paraffin tissue sections were deparaffinized in xylene, re-hydrated and submitted to different types of antigen retrieval. According to the antibody specification, heat-induced antigen retrieval with Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA and Tween-20 at 0.05% (v/v), pH 9, microwaved for 10 min) or Sodium citrate buffer (10 mM Sodium citrate and Tween 20 at 0.05% (v/v), pH 6, microwaved for 10 min), or enzyme-induced antigen retrieval with proteinase K (20 µg/mL, 15 min at 37 °C in a humidified chamber) was performed. Sections were then washed twice for 2 min with dH_2O. Endogenous peroxidases were blocked with 3% (v/v) H_2O_2 for 15 min. For intracellular antigens (e.g., macrophages M1/M2), skin sections were permeabilized with a triton X-100 solution in PBS at 0.2% (v/v) for 15 min at room temperature. Then, non-specific binding was blocked by incubation with universal horse serum (HS) at 2.5% (v/v) for 45 min at room temperature. Samples were incubated with the primary antibodies diluted in HS 1% (v/v), at a pre-determined concentration, overnight at 4 °C (**Table 5**).

Table 5 – Immunolabelling antibodies, from mouse (Ms) or rabbit (Rb), used for the various assays applied to the immunohistochemistry and immunofluorescence methods.

Antibody		Brand	Reference	Dilution	Host	Assay
Primary antibodies	Keratin 10	Abcam (UK)	Ab9026	1:100	Ms	- Structural analysis - Wound healing
	Keratin 14	Covance (USA)	PRB-160P	1:800	Rb	- Structural analysis - Wound healing
	Collagen Type-I	Abcam (UK)	Ab34710	1:100	Rb	- Structural analysis
	IL-1α	Abcam (UK)	Ab227482	1:100	Rb	- Inflammatory-like assays
	IL-6	Abcam (UK)	Ab9324	1:250	Ms	- Inflammatory-like assays
	Langerhans cells (Langerin)	Abcam (UK)	Ab192027	1:1000	Rb	- Immune cells polarization
	Macrophage M1 ($CD86^+$)	Cell Signaling (USA)	19589S	1:100	Rb	- Immune cells polarization
	Macrophage M2 ($CD163^+$)	Abcam (UK)	Ab182422	1:500	Rb	- Immune cells polarization
Secondary antibodies	Alexa Fluor 488	Invitrogen (USA)	A21206	1:500	Rb	
	Alexa Fluor 594	Invitrogen (USA)	A21203	1:500	Ms	

For fluorescent detection, slides were washed with PBS-T (PBS and Tween-20 at 0.05% (v/v)) and incubated with the respective secondary antibody diluted in HS 1% (v/v) for 1 h at room temperature. DAPI at 0.02 mg/mL was added to each slide and incubated for 15 min for nuclear staining. Samples were mounted with aqueous Fluoromount mounting media.

For chromogenic detection, the Vectastain Elite ABC-HRP kit and the respective peroxidase substrate DAB were used according to the manufacturers' protocol. Briefly, sections were incubated with universal biotinylated antibody that bind to the previously added primary antibody, and then with the ABC (Avidin/Biotin Complex) reagent to form a conjugate between a biotinylated target and a detection peroxidase enzyme. Afterwards, DAB was added and its oxidization by the peroxidase forms a brown precipitate that allows to detect the binding of the primary antibody to the samples. Depending on the antibody used, the DAB exposure time is different and therefore a previous optimization step was performed. DAB reaction was stopped by washing the slides with dH_2O. Then, slides were counterstained by incubation for 2 min with a diluted Gill's hematoxylin solution (1:1) in dH_2O. A further wash step was done two times with dH_2O for 2 min and slides were mounted with aqueous Fluoromount media.

All the sections were analyzed through an upright Zeiss Imager.M2 microscope (Zeiss, Germany), using the Zen Blue 3.2 software (Zeiss, Germany).

4.1.2.10 – Image analysis

Images of three different random fields were acquired for each condition and experiment and used for image analysis. Cellprofiler software (Stirling et al., 2021) was used to analyze and quantify the collagen amount from the histological samples. The images were automatically quantified using an algorithm for the identification of collagen mean intensity with both MT staining and immunohistochemistry for collagen type I. The same software was used to identify the different color pixels from the PSR stained sections – green pixels were considered thin fibers and yellow pixels were considered thick collagen fibers. The Fiji software (Schindelin et al., 2012) was used to count the number of $Langerin^+$, $CD86^+$ and $CD163^+$ cells, in relation to the total number of cells of each layer (epidermis or dermis). Moreover, this software was also used to measure keratinocytes migration, using the image scale as reference (100 μm), on the wound healing assay.

4.1.2.11 – Statistical analysis

Data was illustrated using GraphPad Prism software, and the statistical analysis was conducted using Statistica software. Data normality was analyzed, and the suitable statistical analysis was done using either Student's t-test or Wilcoxon matched pairs test, to compare G-CF in relation to VC. In the case of inflammatory assays, G-CF were compared to positive control. Significance was considered for *p* values below 0.05, 0.01, 0.001, and 0.0001.

4.1.3 – Results and Discussion

4.1.3.1 – Glucan cream formulations impact on skin viability

The use of novel molecules for skin application is often limited due to a lack of studies addressing their functionality, safety, stability, and performance. Typically, such molecules are evaluated primarily based on their in *vitro* chemical properties, and only a few and only a few studies involve skin *ex vivo* models with a scientific foundation. The use of such models is not only a more reliable way to study the effects of novel molecules for skin application, particularly in anti-aging research, but also helps in replacing the need for evaluating experimental compounds in animals, which has been restricted since 2009 and entirely banned in 2013 within Europe (Vinardell & Mitjans, 2017). As the development of new skincare ingredients continues, several safety tests can be performed to determine if such molecules pose any risk to consumers. The ideal validation for safety should involve *ex vivo* models.

As discussed in **Chapter 2** and previously evaluated, all glucan extracts underwent tests for cytotoxicity, genotoxicity, and skin sensitization potential using *in vitro* models. Overall, both water-insoluble and carboxymethyl yeast glucan extracts were considered safe. However, to further validate the safety of these polysaccharides and to understand if they induce any alteration in the skin's structure, they were included in a carrier CF and applied to human skin *ex vivo* models. Considering the potential topical use of glucans, a permeation test was conducted to assesses the ability of these polysaccharides to permeate the skin. According to the results presented in **Table 6**, all glucans formulated into a cream (G-CF) exhibited a notable capability to permeate the skin, with an absorbed dose ranging from approximately 40% to 50% after 24 h. This aligns with previous findings that glucans are known to reach the epidermis and dermis by being absorbed through the intercellular space when applied in a CF or specific vehicle (e.g., hyaluronic acid) (Du et al., 2014; Pillai et al., 2005). These results support the notion that our formulations

effectively pass through the SC and reach deeper layers of the epidermis, increasing their probability to exert a biological effect.

Table 6 – Skin permeation quantification after exposing skin explants to insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucans.

Cream formulation	Within the skin (%)	Non-absorbed (%)
CF-ES	40.0 ± 3.0	60.0 ± 3.0
CF CM-ES	41.6 ± 3.9	58.4 ± 3.9
CF-WS	50.5 ± 5.2	49.5 ± 5.2
CF CM-WS	51.8 ± 7.2	48.0 ± 7.2

Following this, the safety of the cream formulations was assessed based on OECD guidelines to evaluate their irritative potential on the skin. Therefore, the G-CF and one irritant inducer control (SDS) were applied to viable skin explants, allowing a direct comparison between the skin tissue viability of the creams and an actual irritating agent (**Figure 18**).

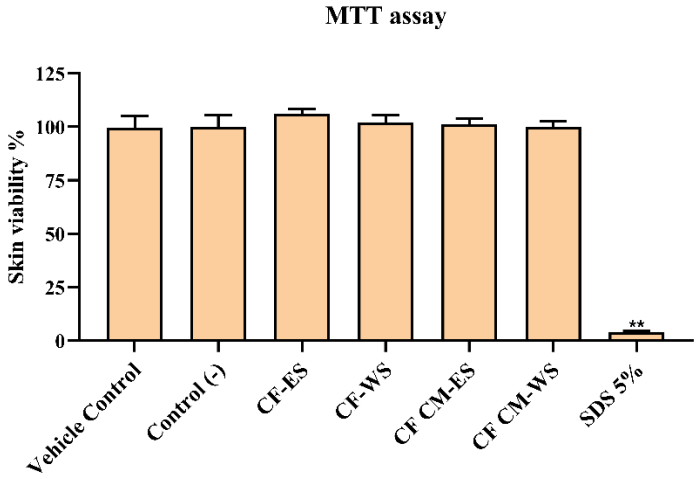

Figure 18 – Safety assessment of cream formulations containing insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucans extracts, performed through an MTT viability assay. The negative control (-) corresponds to skin treated with PBS. SDS at 5% (v/v) was used as an irritation inducer. The data is represented as mean ± SD from three replicates for each sample. Significant differences between the vehicle control and the other conditions are denoted by $^{**}p \leq 0.01$.

Based on the results, SDS was the only compound capable of significantly reducing skin tissue viability (viability < 5%, $p \leq 0.01$), as expected according to the OECD guideline

for *in vitro* irritation tests (OECD Guidelines for the Testing of Chemicals, 2013). Exposure to all CFs did not negatively affect skin viability, as viability exceeded 95% for all formulations. Furthermore, after applying G-CF to the skin for several days, there were no alterations in the protein expression of keratin (KER)-10 and KER-14 (**Figure 19**). Keratins are filament-like proteins naturally produced by keratinocytes and are directly linked to certain cell mechanisms, such as keratinocyte proliferation. Dysregulation in the expression of KER-10 and KER-14, expressed in the *stratum granulosum* and *stratum basale*, respectively, is directly correlated with changes in the skin's barrier properties. Hyperregulation of keratins can increase epidermal thickness and alter the skin's barrier function (Yang et al., 2022; Zhang et al., 2019). These results suggest that the CFs prepared for this study can be considered safe for topical applications, as they do not affect skin viability or the structure of the epidermis.

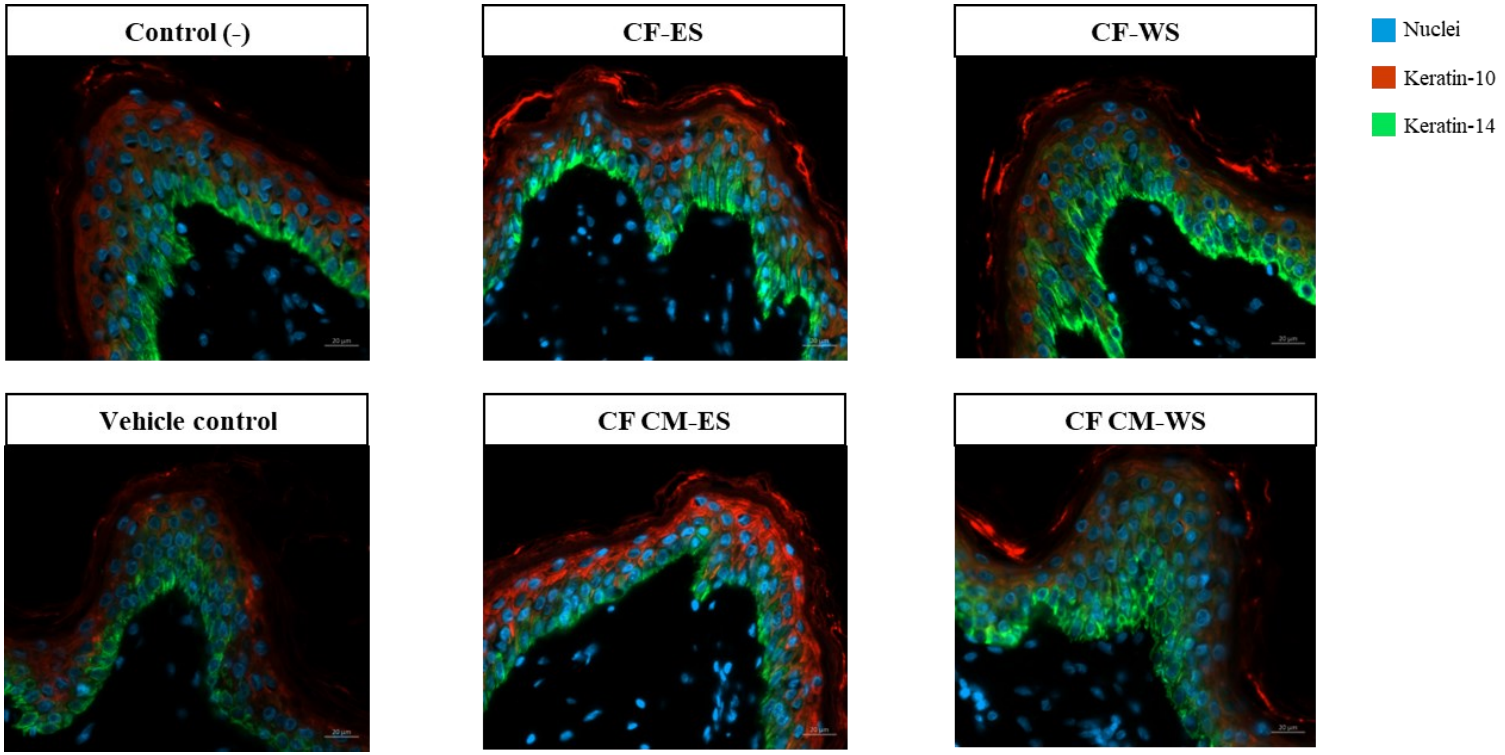

Figure 19 – Immunofluorescence staining of keratin-10 (red, KER-10) and keratin-14 (green, KER-14) after exposure to cream formulations containing insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucan extracts. The scale bar is set at 20 µm. Nuclei (blue) were counterstained with DAPI.

Overall, we have successfully formulated creams containing the different glucan extracts that are able to permeate the skin without affecting both skin viability and epidermal structure.

4.1.3.2 – Glucans influence immune cells polarization and collagen synthesis

As previously mentioned, glucans have several skin-related biological properties related to their ability to modulate the behavior of immune cells (Sousa et al., 2023). Therefore, it is essential to investigate the impact of glucans on skin immune cells, namely Langerhans (LC) and macrophages (MC), to determine if these polysaccharides alter the normal immune cell population. Both LC and MC play roles in modulating inflammatory responses (Bhattacharjee et al., 2019) and can change in number under certain conditions. In response to an inflammatory stimulus, LC change their localization within the epidermis layer or migrate from it through the dermal-epidermal junction due to factors like IL-1β production and release by keratinocytes (Neagu et al., 2022; Villablanca & Mora, 2008). MC, on the other hand, include M2-polarized cells with anti-inflammatory characteristics that reside in the dermis and help maintain tissue homeostasis while patrolling the local environment. In response to an inflammatory signal, they can recruit more MC and polarize towards a pro-inflammatory M1-phenotype (Bhattacharjee et al., 2019; Horiba et al., 2022). Considering these dynamics, we evaluated whether our glucan extracts influenced the number of LC and M1- and M2-polarized MC in the skin models after exposure to G-CF (**Figure 20**). To assess this, we calculated a ratio between the number immune cells observed and the number of the respective epidermal or dermal cells in which they were located.

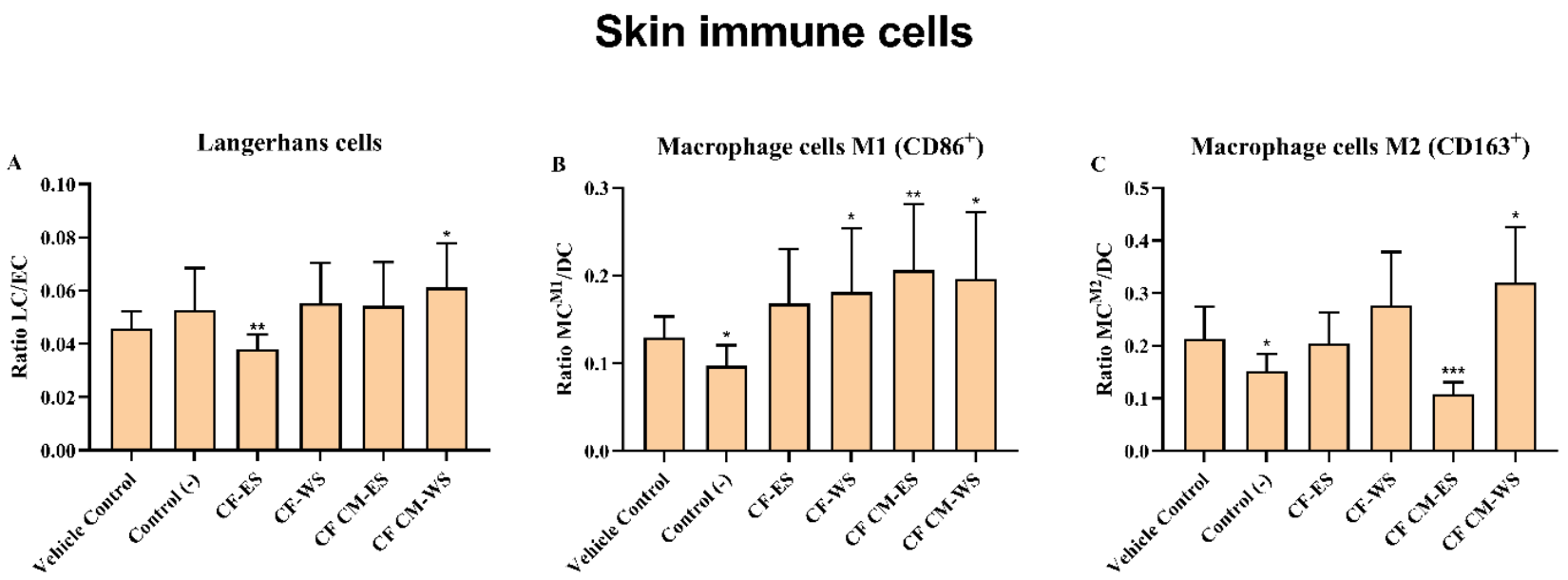

Figure 20 – Impact of cream formulations containing insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucan extracts on immune cells ratios for glucans CF. A) The ratio of Langerhans cells to the total number of epidermal cells (LC/EC). B) The ratio of M1 macrophages to the total number of cells present in the dermis (MC^{M1}/DC). C) The ratio of M2 macrophages to the total number of cells present in the dermis (MC^{M2}/DC). The data is represented as mean ± SD from three replicates for each sample. Significant differences between the vehicle control and the other conditions are denoted by $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Based on the results for the epidermal LC (**Figure 20A**), only two extracts showed significant differences compared to the vehicle control condition. The water-insoluble form of the engineered strain (CF-ES) significantly ($p \leq 0.01$) decreased the LC/EC ratio, resulting in a decrease in LC presence in the epidermis. In contrast, CF CM-WS significantly ($p \leq 0.05$) increased the LC presence. Looking into the MC, both M1 and M2 phenotypes had a significant ($p \leq 0.05$) increase in presence when compared with the negative control, indicating that the vehicle control cream formulation enhanced immune cell presence. Regarding M1 macrophages (**Figure 20B**), the G-CF seemed to increase the overall presence of this type of macrophage in the dermal layer when compared to the vehicle control. However, with the exception for CF-ES, where no significant differences were observed ($p > 0.05$). In the case of M2 macrophages (**Figure 20C**), no differences were observed for both insoluble formulations, but significant differences were noted in both carboxymethyl CF. CF CM-ES significantly decreased M2 presence ($p \leq 0.001$), while CF CM-WS appeared to significantly increase it ($p \leq 0.05$).

Taking these results into account, CF CM-WS seems to increase the number of all the immune cells tested in this assay. This aligns with the cytokine profile observed *in vitro*, as this condition had the highest IL-6 and IL-8 upregulation when keratinocytes were exposed to it. This cytokine increase may be triggering an immune chain response, leading to a higher recruitment of immune cells, which is a known capacity of keratinocytes (Jiang et al., 2020). Intriguingly, CF CM-ES considerably reduced M2 macrophages in the dermis while increasing the presence of M1 macrophages, indicating that this condition induces a pro-inflammatory immune response. This aligns with previous *in vitro* observations, where an increase IL-6 and IL-8 cytokine production by mTHP-1 cells was observed (**Chapter 2**).

Hypothesized to modulate collagen production by directly interact with fibroblasts or indirectly through immune cells, glucans are important for skin health (Pillai et al., 2005). We wanted to understand how the number of macrophages correlated with collagen deposition. After applying the G-CF on skin for several days, we stained our skin samples with Masson's Trichrome (MT) to observe the total collagen deposition within the dermis (**Figure 21**).

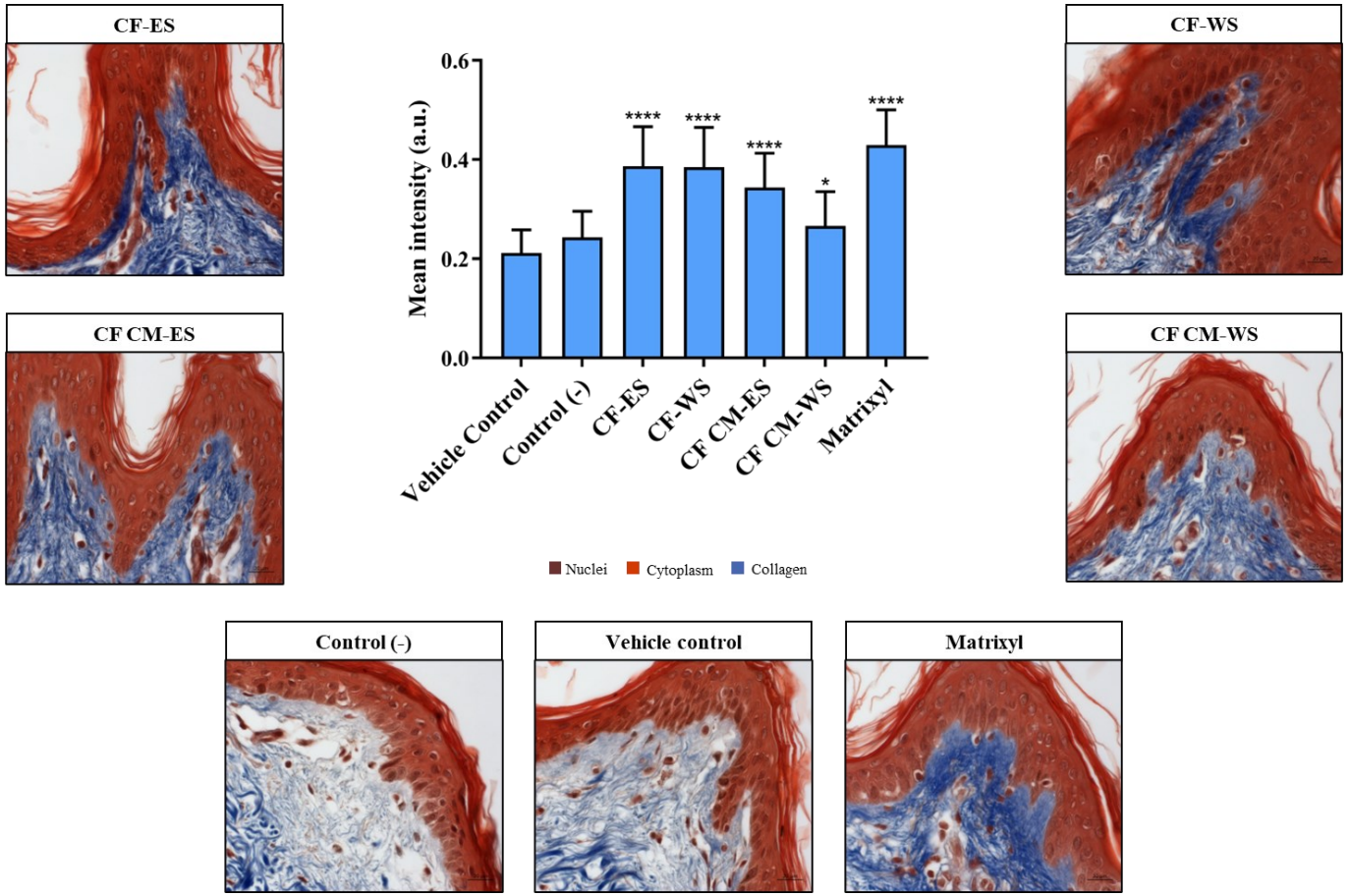

Figure 21 – Collagen deposition upon exposure to cream formulations containing insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucan extracts, using Masson's Trichrome staining. Matrixyl® 3000 was used as a positive control for collagen deposition. Nuclei are stained in brown, cytoplasm in red and collagen in blue. The scale bar is set at 20 µm. The data is represented as mean ± SD from three replicates for each sample. Significant differences between the vehicle control and the other conditions are denoted by *$p \leq 0.05$ and ****$p \leq 0.0001$.

Our results demonstrate that all the G-CF significantly ($p \leq 0.05$) increased collagen deposition when compared to the vehicle control. However, there seems to be a tendency for lower collagen deposition with the carboxymethyl glucans when compared with the insoluble ones. Interestingly, insoluble glucans had a very similar results in comparison to the commercial control Matrixyl® 3000, a well-known pentapeptide used as an anti-aging ingredient in cosmetic formulations due to its known activity in boosting collagen production (Badenhorst et al., 2016). Considering this information, we wanted to infer about what type of collagen fibers has been promoted (**Figure 22**). In line with the MT staining results, in the G-CF that were able to boost collagen deposition, higher amount of thick collagen fibers was attained when looking to the collagen distribution. Moreover, in these conditions, significantly ($p \leq 0.0001$) lower amount of thin collagen fibers was observed. With the PSR staining, usually thinner fibers are related to collagen type-III or newly formed immature collagen, while thicker fibers are related with collagen type-I, mature collagen and fiber crosslinking (Zerbinati & Calligaro, 2018). Ideally, G-CF application would lead to deposition of collagen type-I, which has a higher structural capacity and strength represents around 85% of the total dermis ECM (Davison-Kotler et al., 2019; Singh et al., 2023). To confirm, that indeed, collagen type-I was the one being deposited in the dermal layer, we performed an immunofluorescence analysis (**Figure 23**). Again, all the G-CF significantly ($p \leq 0.0001$) increased the deposition of collagen type-I in the dermis. Although the positive control (Matrixyl® 3000) had a more pronounced effect, both insoluble glucans lead to a substantial increase in the presence of collagen type-I. These results undoubtedly confirm that the glucans present in the CF are the ingredient responsible for the collagen deposition as the vehicle control (blank cream formulation) seems to negatively affect the presence of this specific collagen in comparison to negative control (skin treated with sterile water).

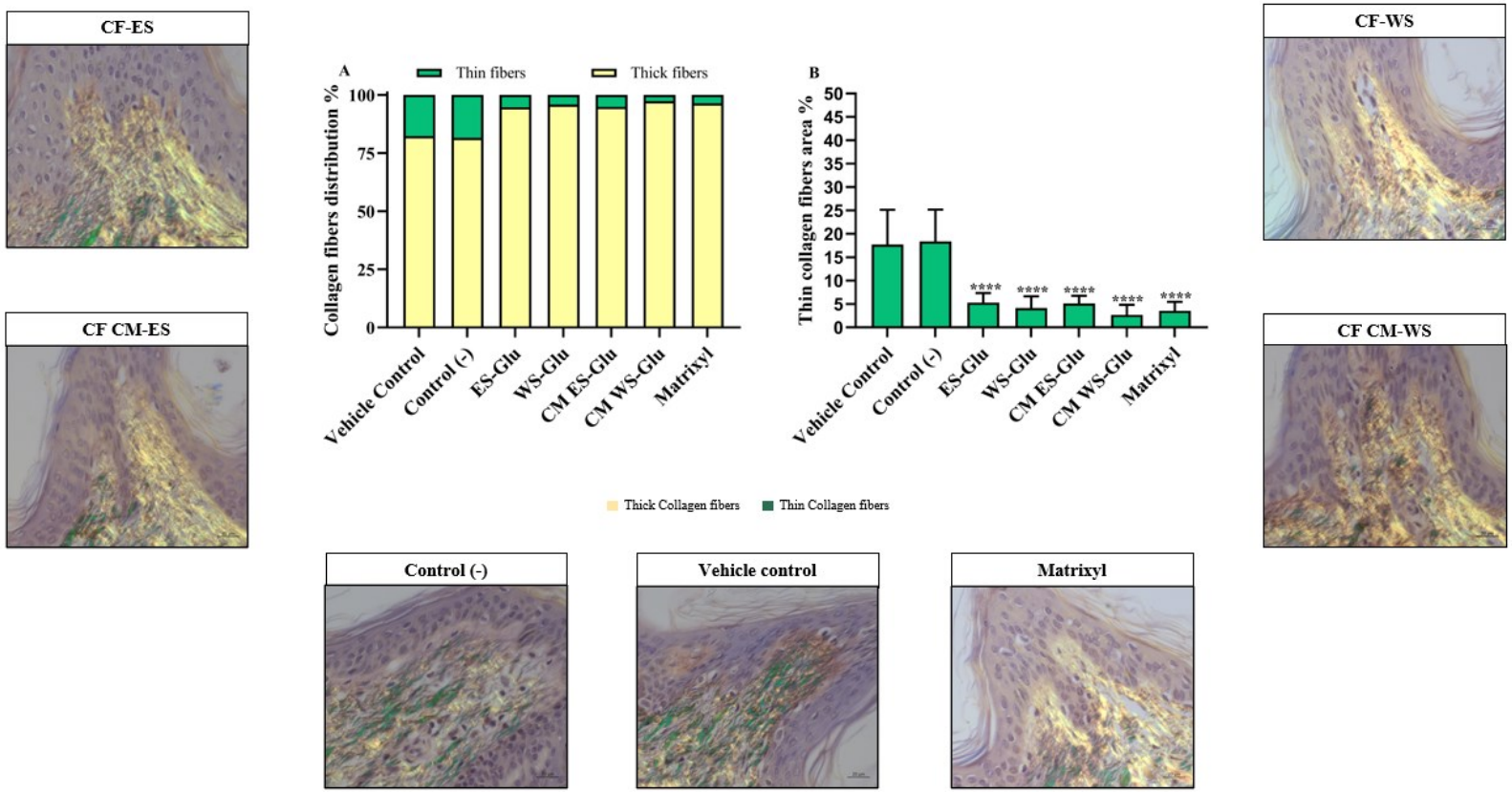

Figure 22 – Analysis of collagen fibers analysis upon exposure to cream formulations containing glucan extracts, using Picro Sirius Red staining. Yellow staining corresponds to thick fibers, while green represents thin collagen fibers. The scale bar is set at 20 µm. A) The distribution of thick and thin collagen fibers within skin explants. B) The quantification of the area covered by thin collagen fibers on the skin explants. Matrixyl® 3000 was used as a positive control for collagen promotion. Data is presented as mean ± SD from three replicates for each sample. Significant differences between the vehicle control and the other conditions are denoted by ****$p \leq 0.0001$.

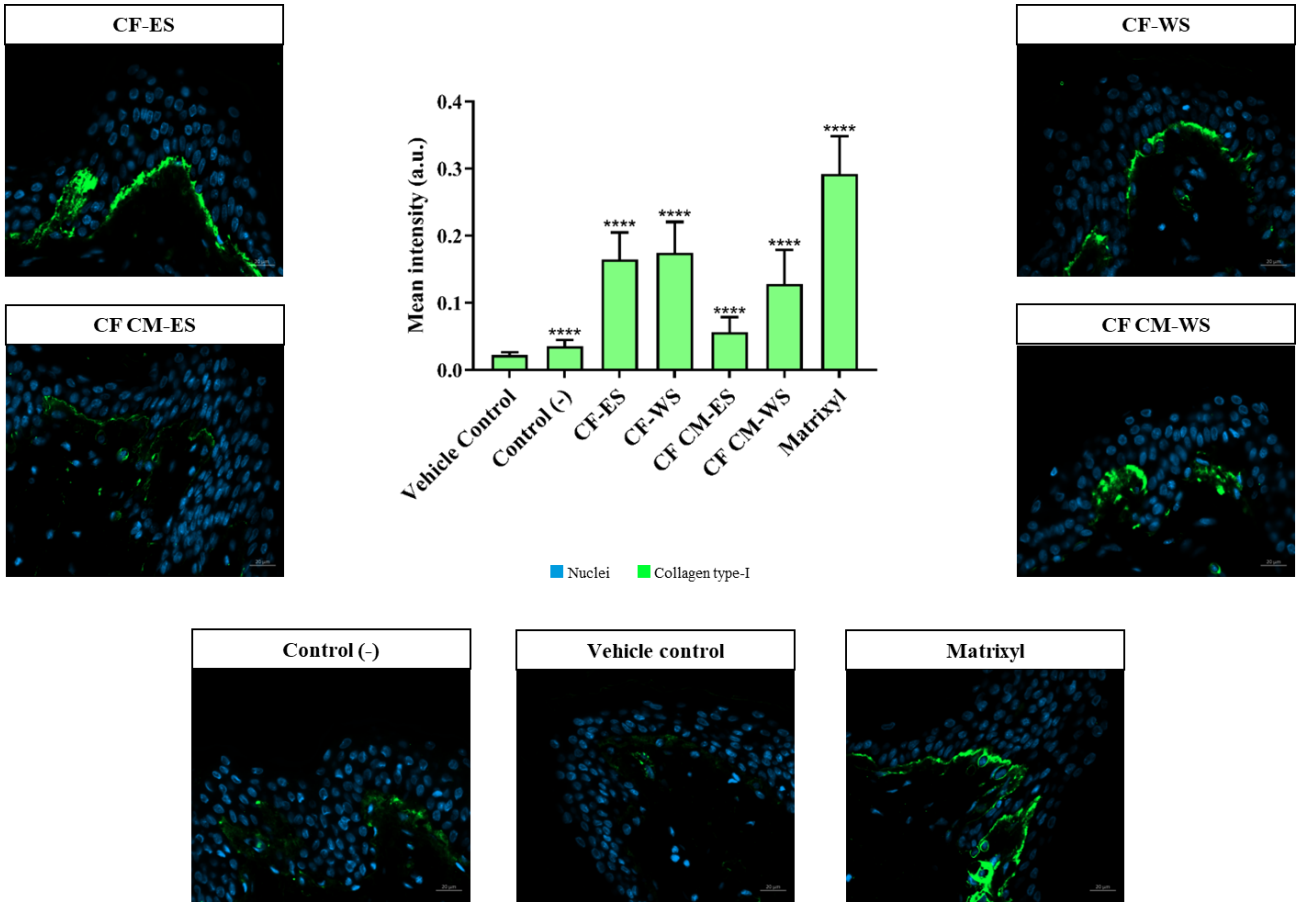

Figure 23 – Immunofluorescence images of collagen type-I (green) in skin explants that were exposed to cream formulations containing insoluble (CF-ES/WS) and carboxymethyl (CF CM-ES/WS) glucan extracts. Matrixyl® 3000 was used as a positive control. Nuclei (blue) are stained with DAPI. The scale bar is set at 20 μm. Data is presented as mean ± SD from three replicates for each sample. Significant differences between the vehicle control and the other conditions are indicated by ****$p \leq 0.0001$.

Aging leads to the breakdown of various ECM components in the skin, resulting in visible alterations such as wrinkles and reduced elasticity. Therefore, the capacity to positively modulate ECM components production is a key characteristic of an anti-aging ingredient. This effect was observed in all the glucan extracts used in this study, which significantly increased collagen deposition, with a particular focus on collagen type-I. Nonetheless, insoluble glucans proved to be more effective in modulating collagen synthesis. It appears that carboxymethylation, a chemical process used to increase glucans solubility, may affect the bioactivity of the molecules. As previously explored in **Chapter 3**, carboxymethylation negatively affected glucans' scavenging capacity and their ability to promote HACAT cell migration. Although the exact reasons for this reduction in bioactivity with carboxymethylation are not well understood, it is known to negatively affect the antioxidant capacity of polysaccharides, as observed in previous studies(Chen et al., 2014; Lee et al., 2020; Liu et al., 2019).

Considering the capacity of glucans to modulate immune skin cells (**Figure 20**), it was observed that CF CM-ES reduced the number of M2 macrophages in the skin and had a lower capacity to promote collagen type-I. M2 macrophages have an important role on collagen production, as they promote collagen fibril assembly (Horiba et al., 2023). In pair with glucans direct interaction with fibroblasts for collagen deposition, our results demonstrate the role of MC on fibroblasts modulation, establishing a correlation between them and collagen synthesis.

Ex vivo models are known to be more reliable and mimetic for studying the effects of novel molecules on skin application, including anti-aging effects. Indeed, when comparing the *ex vivo* assays with the *in vitro* results for ECM components modulation (data not shown), it was observed that the effects of glucans may be dependent on cell-to-cell communication between various skin cells, such as immune cells and fibroblasts. This effect is not a direct action on a specific cell. For instance, *in vitro* assays with only fibroblasts did not significantly lead to an increase of procollagen. On the other hand, *ex vivo* models demonstrated a clear upregulation of collagen, as shown in this chapter. These results allow us to better understand that glucans can modulate skin cells and promote collagen production through a network of cytokines and interactions among various cell types, which is not observed in a cell monolayer.

In summary, G-CF not only had the capacity to significantly modulate skin immune cells but also increased collagen deposition in the dermal layer, specifically collagen type-I, which is highly sought after in the skincare industry for anti-aging products.

4.1.3.3 – Glucans attenuate skin inflammatory responses after exposure to an inflammatory stimulus

As demonstrated in previous *in vitro* studies in **Chapter 3**, glucan extracts have the capacity to modulate the inflammatory response in immune cells, such as MC, after an inflammatory stimulus. This anti-inflammatory ability has been previously explored in *in vivo* models, where the inflammatory response was significantly reduced after topical and dietary application of glucans in an induced atopic dermatitis mouse model or after topical application of glucans on skin subjected to laser treatments (Cao et al., 2021; Kim et al., 2021). However, there is limited information on the effects of glucans in inflammatory skin *ex vivo* models. Given the capacity of glucans to influence the ratio of immune cells in the epidermal and dermal compartments, we sought to investigate if their anti-inflammatory effects could be extended to *ex vivo* models. For this, we used two different irritants, SDS (a molecule that causes a contact dermatitis-like reaction in the skin and PM (an air pollutant), to induce skin irritation.

To evaluate glucans' capacity to mitigate SDS-induced irritation, skin explants were exposed to SDS and then treated with G-CF for 2 days. We quantified the levels of two important cytokines, IL-1α (**Figure 24**) and IL-6 (**Figure 25**), which play an important role in skin immune responses and are known to increase with skin barrier disruption and inflammation (Diaz et al., 2022; Uehara et al., 2019). Our results showed that SDS successfully induced irritation by significantly increasing both IL-1α and IL-6 cytokines compared to the negative control. However, all G-CF significantly ($p \leq 0.01$) reduced IL-1α levels, nearly returning them to normal levels seen in the negative control. In contrast, only CF CM-ES couldn't significantly reduce IL-6 secretion to negative control levels, likely due to its unique effect on macrophage polarization, reducing M2 macrophages and increasing M1 macrophages (**Figure 20**). It is important to note that keratinocytes play an important role in the production of both cytokines in an induced inflammatory context, as these cells represent the first line of defense in the outer layer of the epidermis (Calabrese et al., 2022; Jiang et al., 2020; Wang et al., 2004). These cells can interact with and be modulated by glucans to reduce cytokine production. Therefore, the fluctuation in cytokine production is likely due to multiple factors and not just the direct correlation with macrophages.

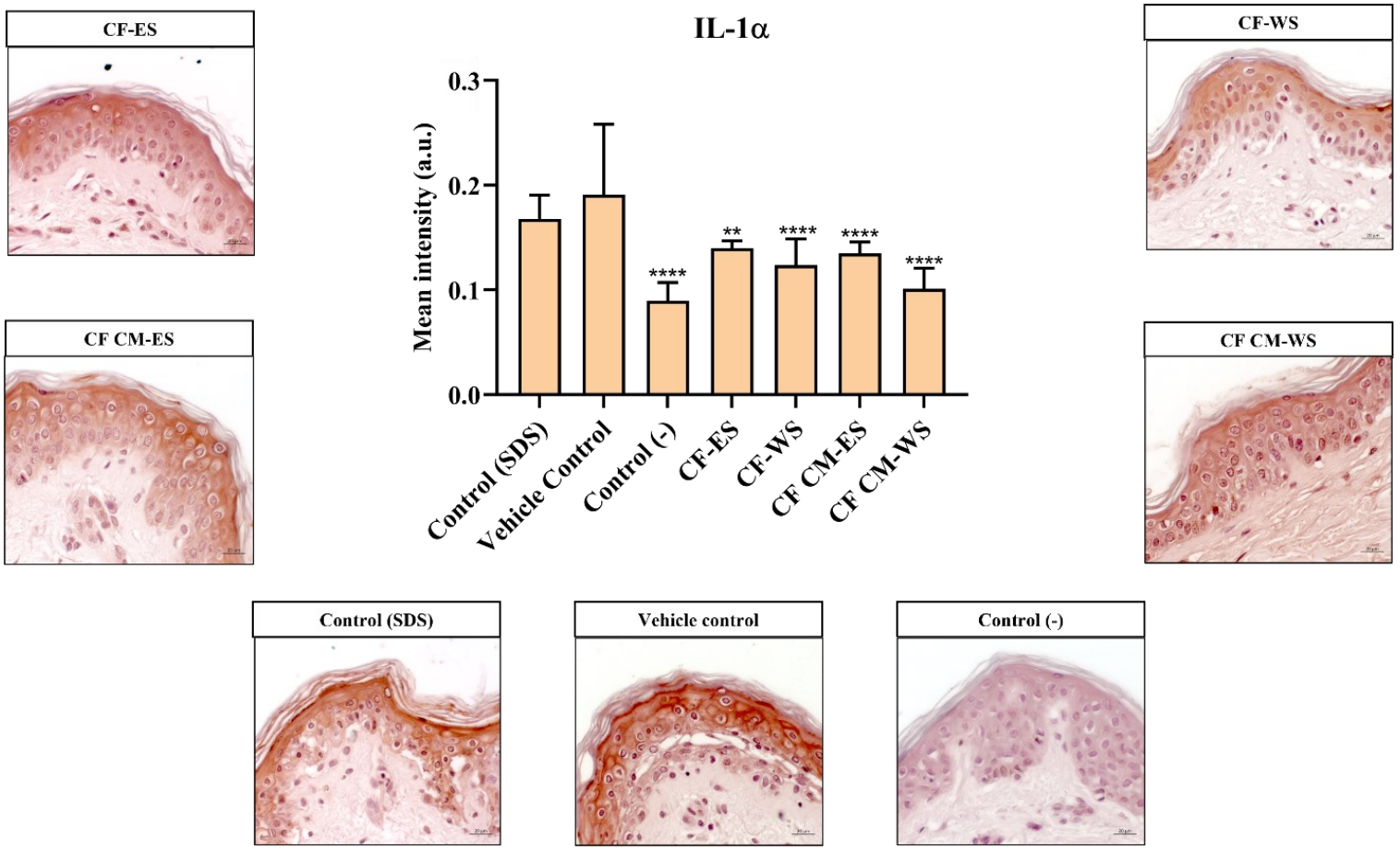

Figure 24 – IL-1α staining after exposing skin explants to SDS 2% (v/v), followed by the application of a cream formulation containing glucan extracts. Data is presented as mean ± SD from three replicates for each sample. The scale bar is set at 20 μm. Significant differences between the positive control (skin exposed to SDS) and the other conditions are denoted by $^{**}p \leq 0.01$ and $^{****}p \leq 0.0001$.

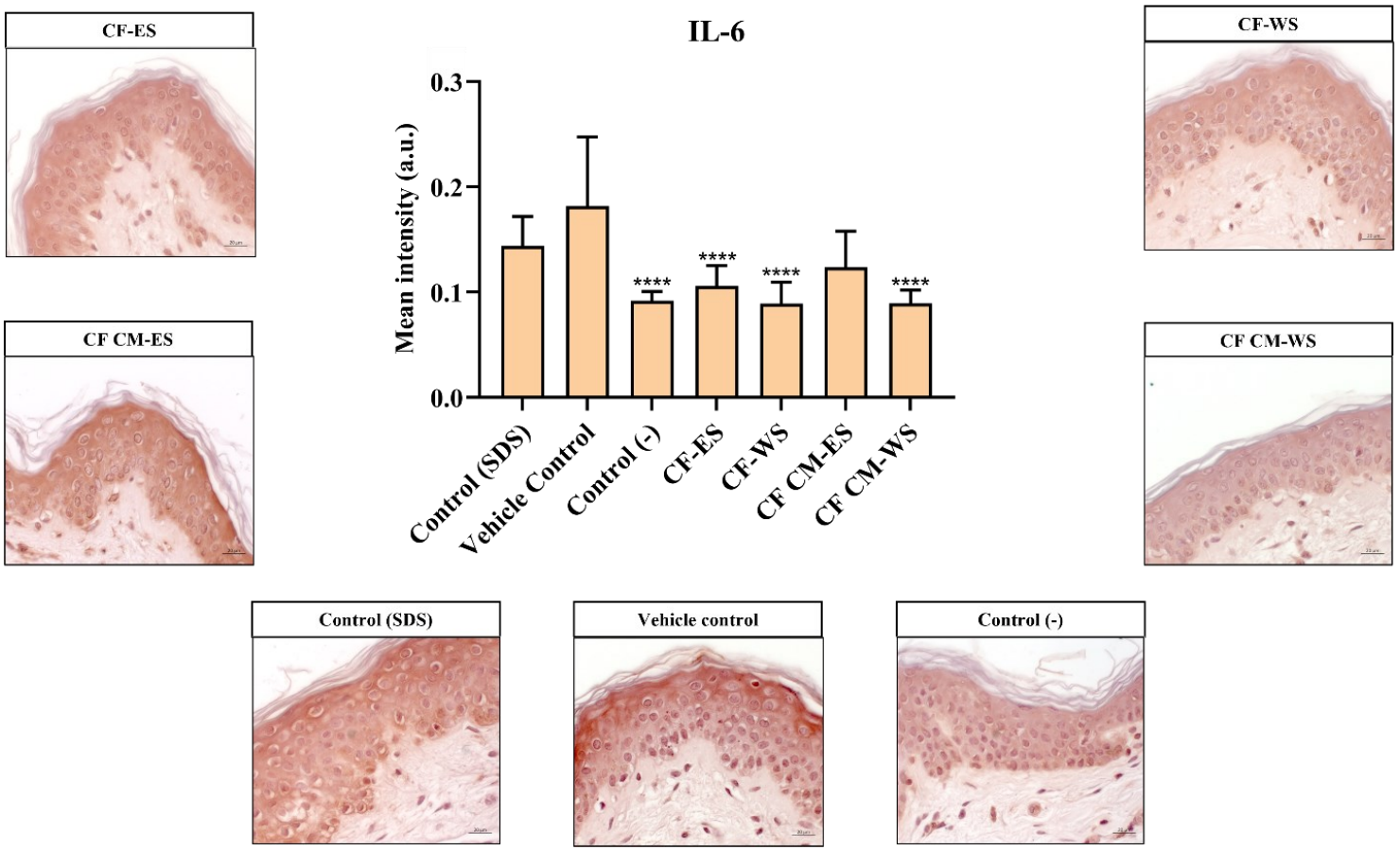

Figure 25 – IL-6 staining after exposing skin explants to SDS 2% (v/v), followed by the application of a cream formulation containing glucan extracts. Data is presented as mean ± SD from three replicates for each sample. The scale bar is set at 20 μm. Significant differences between the positive control (skin exposed to SDS) and the other conditions are denoted by ****$p \leq 0.0001$.

Environmental pollution, particularly airborne PM, is well documented to exacerbate and expedite skin aging (Martic et al., 2022). PM, depending on its size, can penetrate the epidermis and modulate several cytokines, including IL-1, IL-6, IL-8, and increase oxidative stress (Fitoussi et al., 2022; Jin et al., 2018), leading to inflammation. Since PM, in various sizes, predominantly affects the skin's surface barrier, keratinocytes are highly stimulated, resulting in rapid IL-1 release (Jiang et al., 2020). Consequently, we aimed to investigate the G-CF's anti-inflammatory potential following exposure to air pollution-like stimuli, exposing the skin to PM (**Figure 26**). Our findings demonstrate that exposure to PM significantly ($p \leq 0.001$) increased IL-1α secretion in our *ex vivo* model simulating air pollution-induced inflammation. Both the insoluble and carboxymethyl formulations significantly ($p \leq 0.05$) reduced inflammation caused by PM by decreasing IL-1α production. This supports previous data obtained for the anti-inflammatory capacity of glucans on HACAT cells exposed to PM, where all the extracts significantly reduced *in vitro* IL-1α production.

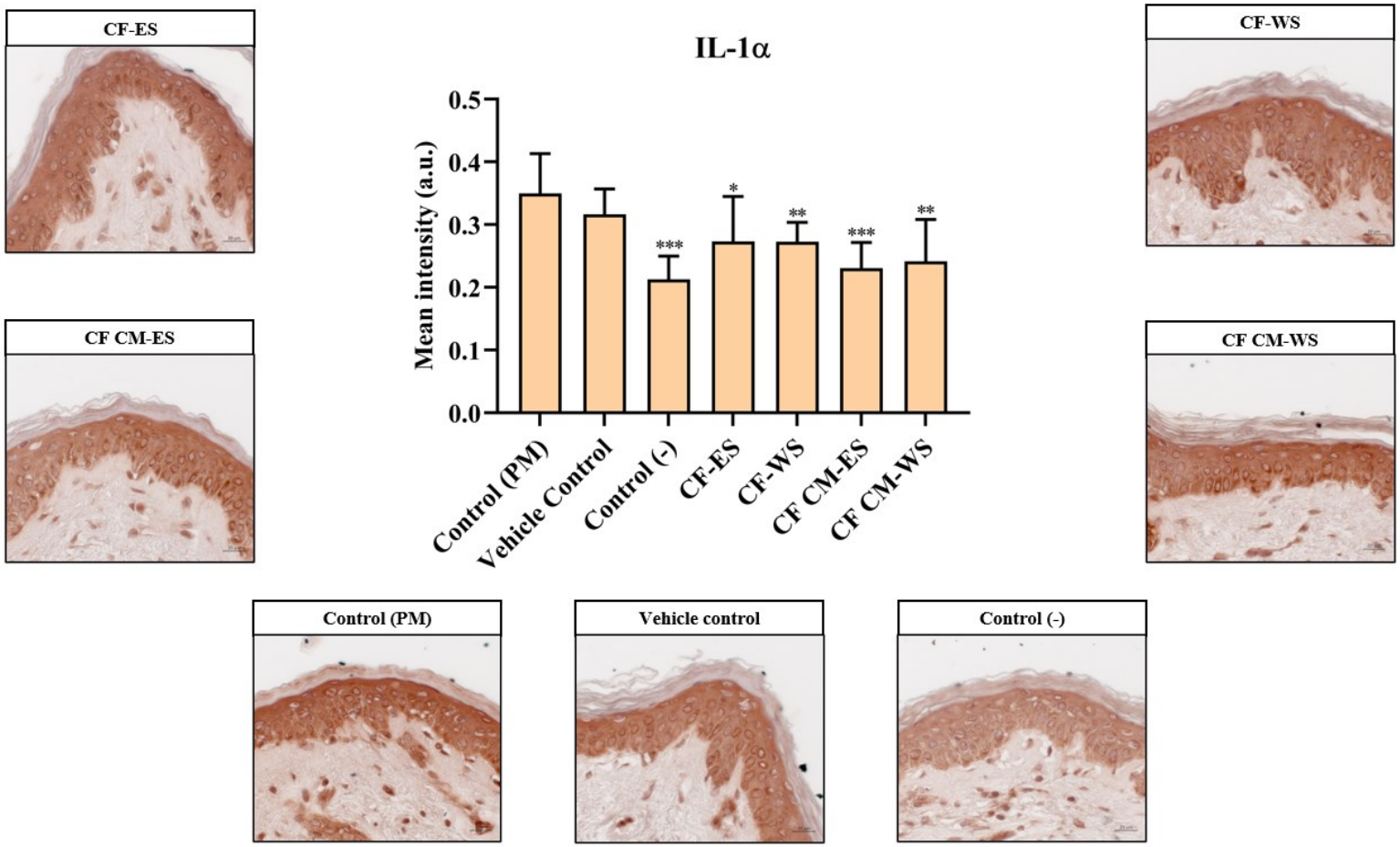

Figure 26 – IL-1α production following exposure to pollution particulate matter, followed by treatment with a cream containing insoluble and carboxymethyl glucans. Data is presented as mean ± SD from three replicates for each sample. The scale bar is set at 20 µm. Significant differences between the positive control (skin exposed to PM) and the other conditions are indicated by *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

When considering the overall effect of glucans on cells and skin exposed to an inflammatory stimulus, their anti-inflammatory capacity likely stems from their ability to bind to immune cell receptors, such as TLRs or dectin-1, which can reduce the inflammatory response. However, previously studies have highlighted the complexity and lack of complete understanding regarding the mechanisms or receptors responsible for the anti-inflammatory capacity of glucans from various sources (Du et al., 2015; Murphy et al., 2020). A study by Smeekens et al., (2015) observed that glucans from *Candida albicans* strongly induced IL-1 receptor antagonist (IL-1Ra) on peripheral blood mononuclear cells (PBMC), by binding to a novel receptor and activating an unknown pathway, which in turn competitively bind to the same receptor as IL-1 and reduce their effectiveness. This could validate the observed anti-inflammatory capacity of our glucans, potentially leading to the production of anti-inflammatory cytokines or attenuating the production of pro-inflammatory cytokines. An interesting observation from our *in vitro* studies is that while glucans increased the levels of pro-inflammatory cytokines in HACAT and mTHP-1, in the presence of an inflammatory stimulus (LPS and PM), they had an anti-inflammatory effect. This phenomenon might be related to the fact that yeast glucans not only competitively bind to the same receptors as the external stimulus but may also bind to alternative receptors, potentially mitigating the inflammatory cascade generated during the immune response.

4.1.3.4 – Glucans promote epidermal migration in wound healing

The process of skin wound healing involves several key stages including haemostasis, inflammation, proliferation, and remodelling, as recently revised by Wang et al., (2023). During the initial phases of wound healing, several immune cells are recruited to the wound site. M1 macrophages and neutrophils play essential roles in controlling microbial infections and releasing a variety of mediators and cytokines, such as IL-6, which promote angiogenesis and re-epithelialization of the wound. In the proliferation and remodelling stage, M1 macrophages polarize into M2 macrophages, secreting important factors that recruit fibroblast to deposit new ECM. This ECM serves as a scaffold for keratinocytes differentiation and epidermal stratification. Glucans are well known for they strong wound healing capacity, mainly by interacting with skin cells through dectin-1, TLRs, and CR-3 cellular receptors, which induce skin cell migration and proliferation (Majtan & Jesenak, 2018). As previously observed after exposing HACAT cells to our glucan extracts, we found that they directly promote cytokine release, particularly IL-6, and cell

migration (**Chapter 2** and **Chapter 3**). Therefore, it is essential to understand if this behaviour is also replicated in an *ex vivo* study.

Our results (**Figure 27**) indicate that CF-ES and both carboxymethylated forms significantly promoted wound closure. α-Tocopherol, known for its skin healing properties, also induced wound recovery. As expected, the vehicle control did not influence wound migration compared to the negative control. Unexpectedly, CF-WS did not induce wound migration, which contradicted the results from *in vitro* studies using a HACAT wound model (**Chapter 3**). However, when analysing the *in vitro* basal cytokine production when exposing mTHP-1 to glucan extracts (**Chapter 2**), CF-WS was the only condition that did not upregulate IL-6 and IL-8 production on mTHP-1, which may explain its limited potential to promote skin restoration. Therefore, while HACAT migration can be induced directly by glucans, this behaviour may not be transposable to a more complex wound model as its repair primarily depends on macrophages activation. In this line, despite the fact that CF CM-ES did not promote HACAT migration in the same study, it proved to be beneficial in *ex vivo* wound closure in this model (**Figure 27**). When analysing the influence of the glucans extracts on the immune cells previously studied, carboxymethyl glucans naturally significantly increased M1 macrophages, which explains why they demonstrate wound healing capacity in the *ex vivo* model. This was not observed in the *in vitro* study, as the *ex vivo* model is based on an orchestrated response by various immune cells and others skin elements, while the *in vitro* model only relies on the effect of glucans on keratinocytes.

Overall, both native and chemically modified glucans from various sources have well documented immunomodulatory capacity and wound healing potential (Abedini et al., 2022; Michele et al., 2023; Majtan & Jesenak, 2018; Nissola et al., 2021; Qiu et al., 2022). Our study demonstrates that yeast glucans have an inherent capacity not only to modulate immune cells and cytokine production but also to promote collagen formation, specifically collagen type-I. These characteristics are key factors in a potential wound healing agent, as we have shown.

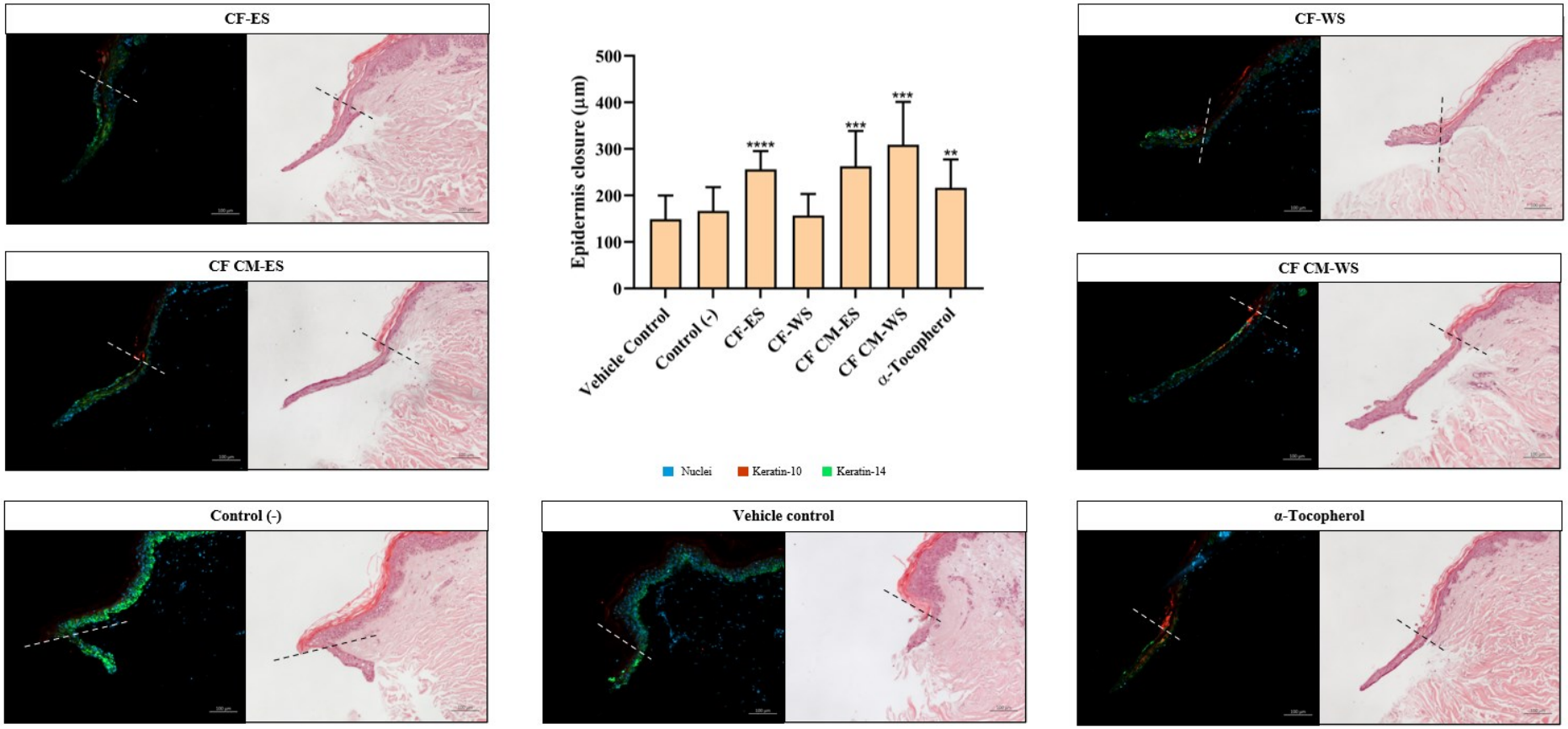

Figure 27 – Epidermal migration after exposure to all glucan extracts, water-insoluble and carboxymethyl forms, following a deep wound that extends through the skin's epidermis and dermis. Brightfield (H&E) and fluorescence (KER-10, KER-14, Nuclei) stained images illustrate epidermal migration. Data is presented as mean ± SD from three replicates for each sample. The scale bar is set at 100 µm. Significant differences between the vehicle control and the other conditions are denoted by $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$. α-Tocopherol was used as wound repairing agent.

4.1.4 – Conclusion

In conclusion, the cream formulations containing yeast water-insoluble and carboxymethyl glucans exhibit the unique capacity to regulate the number of immune cells present in the epidermis and dermis while promoting collagen deposition in the dermal layer, enhancing skin strength and elasticity. Additionally, these formulations mitigate the inflammatory effects of SDS and PM, demonstrating potential as natural healing agents for wound repair. These properties shed new light on how yeast glucans, whether newly developed or already commercially available, can enhance our skin health, reduce signs of aging, and be used for skin barrier conservation. However, further research is needed to explore the underlying mechanisms of glucans´ interactions with a multitude of skin cells, including cellular receptors and cell-to-cell communications, to define new directions for future research on glucans development and potential applications in cosmetics and therapeutics.

Chapter 5

This chapter will summarize the main conclusions derived from this research work as highlight areas for future research that need further exploration to understand the cellular and skin mechanisms induced by yeast glucans.

5.1 – Conclusions

The primary focus of this PhD thesis was to explore the potential of *Saccharomyces cerevisiae* yeast, an industrial fermentation byproduct, as a safe and bioactive skincare ingredient for diverse applications. Among its biological active constituents, glucans have shown potential for use in the cosmetics industry as a collagen enhancer and in therapeutic fields as an immunomodulator and wound healing agent. This research involved the development of an extraction and chemical modification method for glucans, as well as an exploration of their bioactive properties through *in vitro* and *ex vivo* models (**Figure 28**).

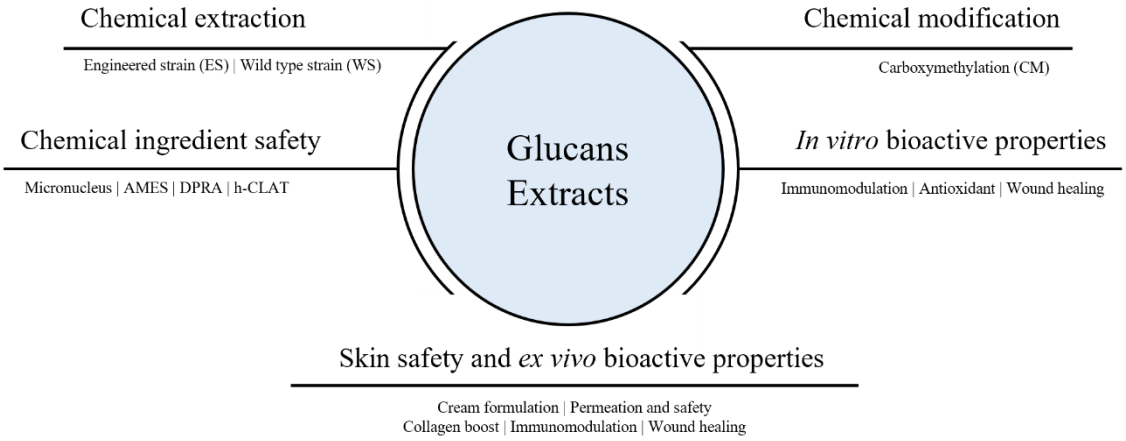

Figure 28 – Summary of main achievements and conclusions attained throughout this PhD thesis.

In **Chapter 2,** an efficient extraction methodology was developed to obtain a glucan extract with a high glucose content, initially water-insoluble, which were successfully converted into water-soluble forms through carboxymethylation. While there were slight differences between the industrial and lab-scale fermentation strains, both extracts exhibited substantial similarity in terms of structure, molecular weight, and reaction yield after chemical modification. Within the same Chapter, the safety assessments indicated that glucans did not induce skin damage. The extracts showed no inflammatory effect on epidermal cells, as fold change variations in cytokine profiles were mostly lower than 2. Additionally, various safety tests based on OECD guidelines, including AMES, micronucleus, and DPRA tests, demonstrated no harmful effects. Glucans did not impact the skin microbiota naturally present in healthy female donors. However, some glucans exhibited a skin sensitizing potential result in the h-CLAT assay and, in some cases,

induced cytokine upregulation in mTHP-1 cells. This immunomodulatory response was expected, as mTHP-1 cells are known to be strongly activated by glucans.

Chapter 3 explored the *in vitro* biological properties of water-insoluble and carboxymethyl glucan extracts. Glucans exhibited anti-inflammatory properties by reducing cytokine release in response to inflammatory stimuli such as bacterial LPS and airborne pollution PM. They also demonstrated antioxidant effects by downregulating ROS formation induced by PM. In a scratch wound assay, glucans promoted keratinocyte migration, suggesting their potential as wound healing agents. These findings revealed that glucans could modulate cell immune responses depending on the stimulus provided.

In **Chapter 4**, a proof-of-concept phase incorporated glucan extracts into cream formulations for application on human skin *ex vivo* models. These creams exhibited good permeability and did not affect skin tissue viability. Immune cell modulation and collagen boosting were observed, as most glucans influenced the number of immune cells and promoted collagen deposition, including collagen type-I and mature fibers. This discovery holds promise for reducing wrinkles and addressing aging effects on dermal collagen. The glucan extracts also successfully attenuated inflammation induce wound closure in deep wound *ex vivo* models. These results demonstrate the versatility and potential of glucans for various skin applications.

In summary, this PhD work developed a new method for the purification and chemical modification of spent yeast glucans. It revealed innovative biological properties through *in vitro* and human skin *ex vivo* models, contributing to an increased understanding of glucans' immunomodulation, wound healing capacity, and their potential as collagen boosters and anti-aging agents.

5.2 – Future research and development

This PhD thesis was fundamental in developing an extraction method for obtaining glucans from the yeast cell wall, a functionalization method through carboxymethylation, and ensuring the safety of all the extracts. Furthermore, various biological properties were observed through *in vitro* and *ex vivo* assays, demonstrating their efficacy. However, more work needs to be performed to truly understand how glucans can exhibit such a diverse and well documented portfolio of biologic activities. This involves defining the which mechanisms and pathways through which this polysaccharide intervenes at the cellular or tissue level. To address this, several areas for future work and research in glucans development for skincare have been identified.

Regarding the glucan extraction methodology (**Chapter 2**), this method should be applied to other sources to determine if the achieved purity can also be replicated with different yeast types or other glucan sources, such as mushrooms or oats. It is also important to investigate whether the molecular weight of glucans can affect their biological properties. This can be explored by using a fractionation method to obtain various glucan extracts with different weights and sizes. While carboxymethylation is the most commonly described chemical modification for glucans, other types of functionalization (e.g., sulfation) mentioned in the literature should be considered and tested to observe potential differences in the behavior of insoluble glucans after the addition of other functional groups. As previously described, glucan modification can enhance existing biological properties, such as antioxidant activity, which should be studied further for its relevance to this subject.

For the biological properties (**Chapter 3**), several other properties should be tested to investigate additional skin-related effects. These may include other damage inducers such as UV radiation, beyond air pollution. Additionally, it is important to explore anti-enzymatic activities such as anti-collagenase or the inhibition of matrix metalloproteinases, to observe if glucans can inhibit ECM degradation. This degradation can lead to the formation of wrinkles, due to the action of these enzymes in stressful environments. Furthermore, it is important to test the capacity of glucans to induce cellular proliferation and migration in a wound healing context or determine if they possess an anti-senescence effect, reducing systemic inflammation caused by senescent cells. Alongside the bioactive properties, is crucial to understand the mechanisms underlying glucan interactions with skin cells, particularly identifying the type of

receptors involved. Apart from the well-known receptors (e.g., dectin-1 or TLRs), various studies suggest that glucans may interact with unknown receptors to modulate certain cellular responses, including inflammation. This knowledge can be complemented by *in vitro* co-culture assays, which are ideal for exploring interactions between this molecule and various skin cells. For example, it can help better understand immunomodulation in HACAT and mTHP-1 cells or determine if ECM promotion (e.g., collagen promotion) is dependent on background pathways activated through other skin cells.

Translating *in vitro* knowledge to *ex vivo* skin models is a critical step in mimicking how glucans would behave *in vivo* (**Chapter 4**). Several new skin models can be developed using human viable skin explants to test new inflammatory stimuli, such as UV radiation or even more complex models involving a combination of UV radiation and PM. Additionally, it is essential to evaluate the impact of glucans on other ECM components, such as elastin or laminin, and their effect on well-known skin conditions, such as acne or psoriasis. Since some studies have already used glucans from other sources to observe some of these properties through *in vitro* and *in vivo* assays, it would be interesting to determine if yeast glucans demonstrate the same potential in an *ex vivo* model. Other cream formulation mixtures should also be developed to assess whether glucans' permeability into the skin layers can yield different bioactive outcomes based on their penetration depth. Furthermore, the inclusion of other compounds with glucans can be examined to identify potential synergies between this molecule and other bioactive compounds (e.g., vitamins or antioxidant agents).

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