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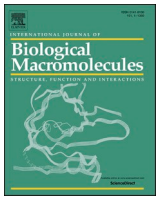

Circular economyeast: *Saccharomyces cerevisiae* as a sustainable source of glucans and its safety for skincare application

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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.

1. Introduction

An emerging integrated strategy to enhances industrial sustainability, promotes a 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) [1]. Glucan polymers, in particular, primarily consist of a β-(1,3)-glucans backbone with long β-(1,6)-glucans branches, which provide structural rigidity to the cell wall. Other linkages, such as α/β-(1,4), are also present [2–5]. 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 [6,7].

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 [8–10]. 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 [10–13]. 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 [14–16], function as antioxidants [13,17–19], and mitigate the effects of skin aging [20].

Glucans have the capacity to interact with specific receptors, such as

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dectin-1 or toll-like receptors (TLRs), on various skin cells, including keratinocytes in the epidermis layer and macrophages (M1 and M2) in the dermis layer [21,22]. 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 [23,24].

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. Materials and methods

2.1. Materials and chemicals

Yeast extract peptone dextrose medium (YPD) was purchased from Grisp. Titriplex III standard EDTA solution (0.1 M) was obtained from Merck. Enzymatic Yeast β-glucans Assay Kit (K-EBHLG) and β-glucans Assay Kit (Yeast and Mushroom) (K-YBGL) were sourced from Megazyme. 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, PureLink™ Microbiome DNA Purification Kit, Pierce BCA Protein Assay Kit and Bovine Serum Albumin (BSA) were obtained from Thermo Fisher Scientific. 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. Propidium Iodide (PI), Nickel(II) Sulfate, Phorbol-12-myristate 13-acetate (PMA), Mitomycin C from *Streptomyces caespitosus* and Deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Sigma. The AMES FT mutagenicity assay kit was obtained from Moltox, MicroFlow test kit was purchased from Litron Laboratories and Nutrient Broth Number 2 was bought from Oxoid. 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 2 L 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 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.

2.2. Glucan extraction

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 g of wet pellet to 1 mL of deionized water (1:1 ratio) 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.

2.2.2. 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. [25], 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.

2.2.3. 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 at 8000 rpm (Multifuge X1R, ThermoFisher Scientific) for 10 min 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.

2.3. Glucan chemical modification

2.3.1. Carboxymethylation

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.

2.3.2. Degree of substitution

The degree of substitution (DS) of this functionalization was performed according to Ding et al. [26], 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:

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

$$\text{DS value} = \frac{162 \times \%CH_2COONa}{8100 - 80 \times \%CH_2COONa}$$

C = EDTA standard solution concentration (mol/L).V = Blank volume (mL).v = Sample volume (mL).

2.4. Characterization

2.4.1. 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.

2.4.2. 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 [27] and Breil et al. [28] 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:

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

Total polysaccharides content was analyzed and derivatized according to Bastos et al. [29]. 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 1 M 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 [30]. 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.

2.4.3. 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}.

2.4.4. 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 to create the standard curve.

2.4.5. Nuclear magnetic resonance (NMR)

For the NMR experiment, glucans were solubilized at 20 mg/mL in DMSO-d_6 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.

2.5. In vitro assays

2.5.1. Cell culture

HACAT and nHDF cells were maintained in culture with DMEM supplemented with 10 % FBS (*v*/v) and 1 % (v/v) antibiotic-antimycotic. THP-1 cells were maintained in culture with RPMI medium supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), 1 % antibiotic-antimycotic (v/v), and 0.05 mM 2-Mercaptoethanol. TK6 cells were cultured in RPMI medium supplement with 10 % FBS (v/v) 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.

2.5.2. 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 1.0×10^5 cells/mL for HACAT and nHDF and incubated for 24 h. For THP-1, a cell suspension of 1.5×10^5 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 [31].

2.5.3. Cytokine profile

To analyze the impact of these 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 2.5×10^5 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.

2.6. Safety assessment

2.6.1. Mutagenicity test

The mutagenicity assessment was conducted using a high throughput fluctuation Ames test with an Ames FT test kit, based on the work published by Sui et al. [32] and followed OECD guideline No.471 [33]. 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 °C in nutrient broth N°2 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.

2.6.2. 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 [34]. 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 °C. 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).

2.6.3. Direct peptide reaction assay (DPRA)

The DPRA assay was performed according to OECD guideline test No. 442C [35]. 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.

2.6.4. Human cell line activation test (h-CLAT)

The h-CLAT assay was done according to OECD guideline test No. 442E [36]. In summary, THP-1 monocyte cells were exposed to various concentrations of glucan extracts, and cell viability was analyzed using PI 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 °C. 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, 5 μL 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 °C in the dark. Then, cells were 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.

2.6.5. Impact upon skin microbiota

The effect of the extracted and functionalized molecules on the skin microbiota was evaluated according to Carvalho et al. [37]. 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 °C 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. [38] 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.

2.7. 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.

3. Results and discussion

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 [39]. The high rigidity of this structural component requires the use of multiple methods to isolate a higher quantity of polysaccharides [10,40].

According to the extraction process applied (Fig. 1), it was utilized two types of yeast: a wild strain and an engineered strain. These strains underwent an alkaline extraction followed by a subsequent purification step to isolate yeast polysaccharides. Prior to the extraction, a thermal

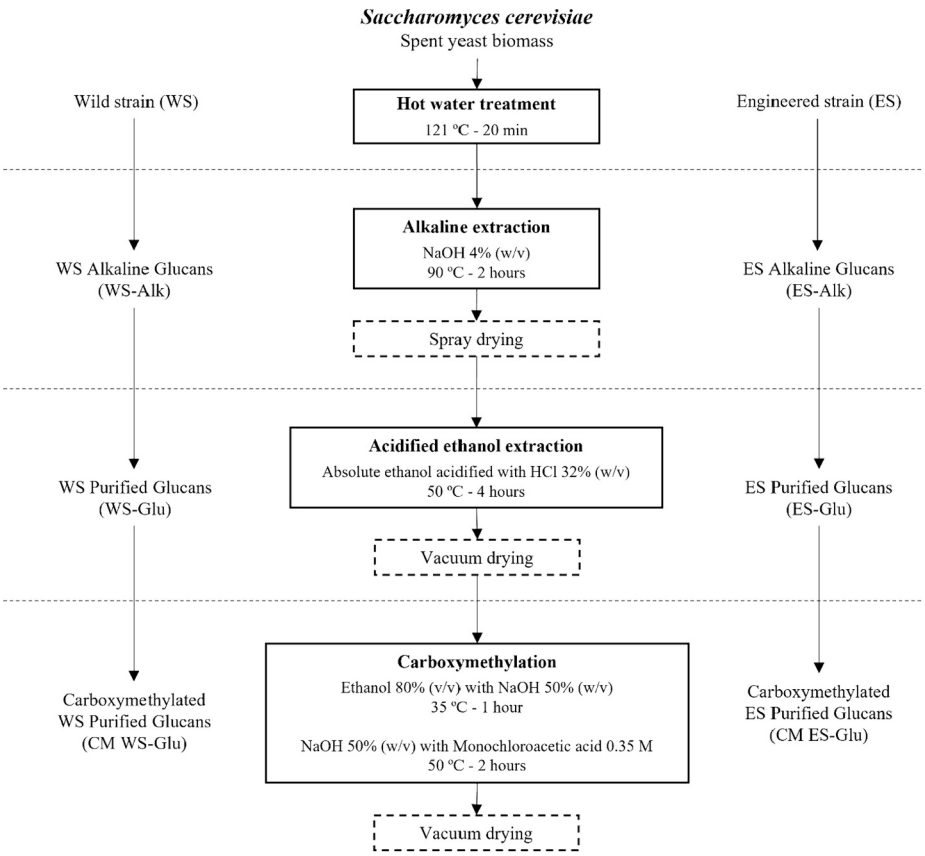

Fig. 1. Extraction, purification and chemical modification scheme of glucans from wild strain (WS) and engineered strain (ES): Alkaline extraction (Alk), Acidified ethanol extraction (Glu) and Carboxymethylation (CM).

process was employed to rupture the yeast cell wall and remove intracellular compounds [41,42]. Subsequently, an alkaline extraction was performed to eliminate impurities, primarily targeting the removal of proteins [43], alkali-soluble glucans, mannans, and lipids [44]. To further enhance the purity of the extracts, a final extraction step using an acidified ethanol solution was employed primarily to eliminate lipids [41] 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.

According to the chemical characterization (Table 1), 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, 4–5 % protein content, and 1–2 % lipid content. However, differences were observed in the β-glucans and α-glucans 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.

Table 1
Overall chemical characterization of glucan extracts after the alkaline extraction (ES-Alk/WS-Alk), purification process (ES-Glu/WS-Glu) and chemical modification through carboxymethylation (CM ES-Glu/CM WS-Glu) for the engineered strain (A and C) and the wild strain (B and C) yeasts. 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

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 *Sacchromyces pastorianus* during the brewing process, to enhance cell wall strength. Additionally, more linkages may be present but were not quantified, such as β-(1,4) [29,45] or non-hydrolyzed enzymatically. Further conformational modifications can also occur during the extraction process, such as the disruption of hydrogen bonds [46] and partially open of the triple-helix conformation [47]. 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-glucans 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) [48], 72 % (60 % of β-glucans) [48], and 96 % polysaccharides (93 % of β-glucans) [42].

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 [49,50]. This type of reaction involves replacing hydroxyl groups present within the polysaccharide chain with carboxymethyl groups [51]. 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 the extracts chemical characterization (see Table 1), 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 $\times$ 10^5 Da and 4.89 $\times$ 10^5 Da for CM ES-Glu and CM WS-Glu, respectively. Comparing the DS values with those reported by Ding et al. [26], the values observed in this study (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. [13] and Ma et al. [52], 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, an ATR-FT-IR analysis was conducted. As shown in Fig. 2, 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 [53].

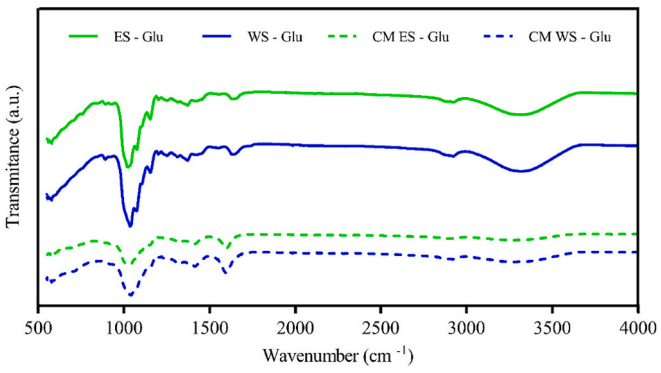

Fig. 2. – ATR-FT-IR of water-insoluble glucan extracts from WS and ES type yeast and its carboxymethylated form.

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 [54]. Two other peaks at 1150 and 1075 cm^{-1} exhibited reduced intensity after carboxymethylation, suggesting a possible degradation of a specific type of β-glucans, as these peaks are associated with linear β-(1,3) [55,56]. 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 [57,58]. Another strong peak at 1035 cm^{-1} can be attributed to β-glucans linkages [59].

In addition to ATR-FT-IR analysis, it was performed a ^{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 (Fig. 3A) 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 [60]. 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. [61]. 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.

Concerning the carboxymethyl form (Fig. 3B), 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 [54], 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 to 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.

3.2. Biocompatibility and immunomodulation

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

According to the cytotoxicity results (Fig. 4), 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) (Fig. 4C).

Comparing both carboxymethylated samples, only the WS showed a cytotoxic effect on HACAT (Fig. 4B) and nHDF cells (Fig. 4D) 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 [62], 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, their immunogenic potential was tested using keratinocytes (Fig. 5A) and THP-1-derived macrophages (Fig. 5B), respectively. Specifically, it was

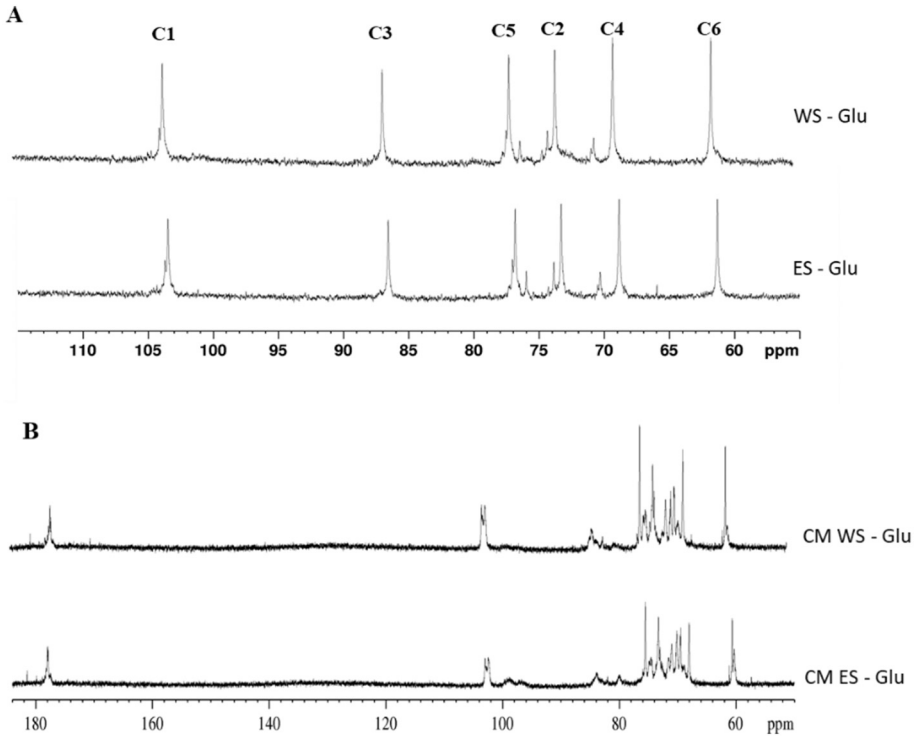

Fig. 3. ^{13}C NMR spectra of native (A) and carboxymethylated (B) glucans.

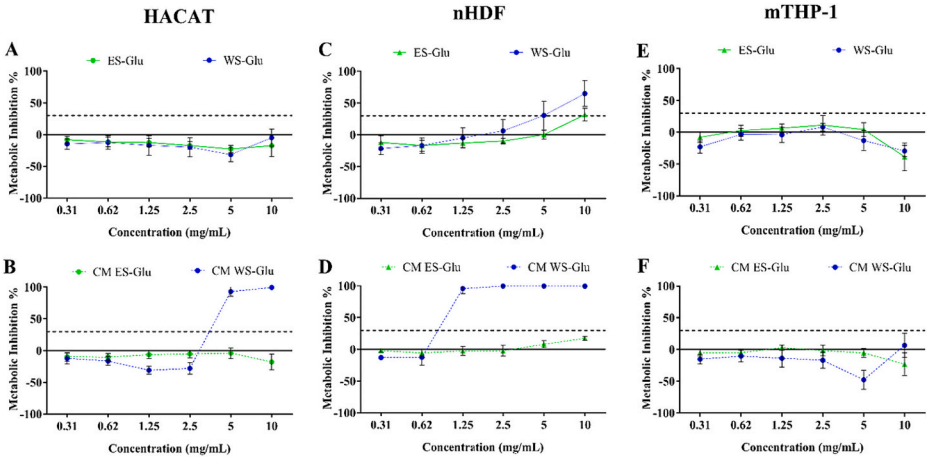

Fig. 4. Metabolic inhibition of HACAT cells (A and B), nHDF cells (C and D), and mTHP-1 cells (E and F) treated with insoluble glucans (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu). Data are represented as mean ± SD from three replicates for each sample.

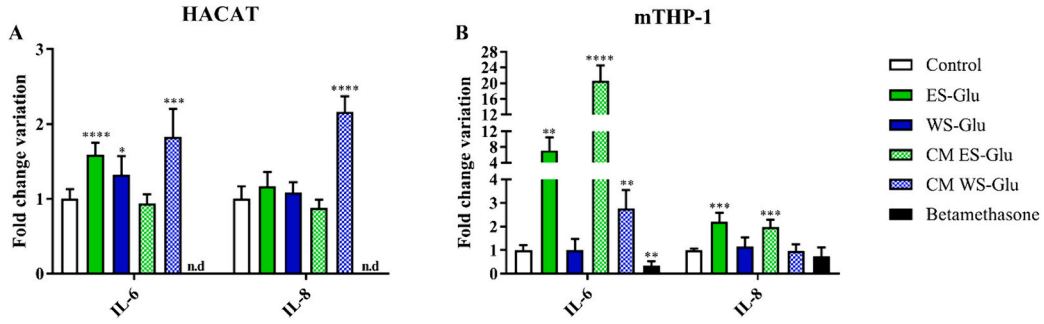

Fig. 5. Cytokine production of HACAT cells (A) and mTHP-1 cells (B) upon exposure to native glucans (ES-Glu and WS-Glu) and carboxymethyl glucans (CM ES-Glu and CM WS-Glu). Data are represented as mean ± SD from three replicates for each sample. Significance differences between samples and control are 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.

assessed their capacity to stimulate the production of two pro-inflammatory cytokines, IL-6 and IL-8 [63].

Based on the results obtained by exposing HACAT cells to the glucans extracts produced in this project (Fig. 5A), 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.

Regarding mTHP-1 (Fig. 5B), 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 [64–66]. 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 [67]. Similarly, a particulate β-(1,3)-glucan from *S. cerevisiae* (Zymosan) was found to upregulate IL-6 and IL-8 in mTHP-1 cells [68]. 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 [69]. 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 [68,70].

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 [71–73]. IL-6 showed the greatest upregulation in both cell types, and this cytokine is known to play a role in the wound healing process [74], which may be directly related to the well-established wound healing properties of glucans in vivo [75–77]. 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 [16,78,79].

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 [23]. For this study, some of the OECD-approved assays were followed to evaluate the safety of the extracted glucans.

To assess the genotoxic potential, it was conducted the bacterial mutagenicity assay - AMES fluctuation test and the in vitro mammalian cell micronucleus test, following OECD guidelines [33,34]. In the first assay, two *S. typhimurium* strains were used, TA100 and TA98, to detect point mutations by base substitutions and frameshifts, respectively. As was possible to observe, glucans did not induce revertants, indicating no mutagenic effects compared to the negative control (Fig. S1). The same result was observed in the presence of the S9 activation enzymes. Regarding the micronucleus assay (Fig. S2), which is used to observe micronucleus formation resulting from chromosome damage or anomalies during cell division, all the compounds tested 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, 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, four key events (KE) established by the OECD were analyzed: 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, two different tests were conducted: the DPRA assay for KE-1 and the h-CLAT assay for KE-3 [80]. Regarding the DPRA test results (Table S1), glucans did not induce a haptenation reaction, which is a complexation reaction between low molecular weight substances and proteins [81], 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 molecules used on this study will not become antigenic and prompt an immune response. However, evaluating the results obtained through the h-CLAT test, it was 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 [82,83]. This finding is consistent with the cytokine profile observed in macrophage-like cells (Fig. 5B), 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 [84–87]. 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 [87]. In this study, an adapted HaCaSens was performed to analyze the production of IL-1α, IL-6, and IL-8 upon exposure to insoluble and carboxymethyl glucans. As shown in Fig. 5A, 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 were 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 (Fig. 6). Based on the results of the three assays conducted for skin sensitization, glucan extracts obtained on this study 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, it was evaluated the potential effects of the extracts on the naturally occurring microbial community in facial skin using a pre-clinical in vitro model followed by amplicon-based NGS [37]. This

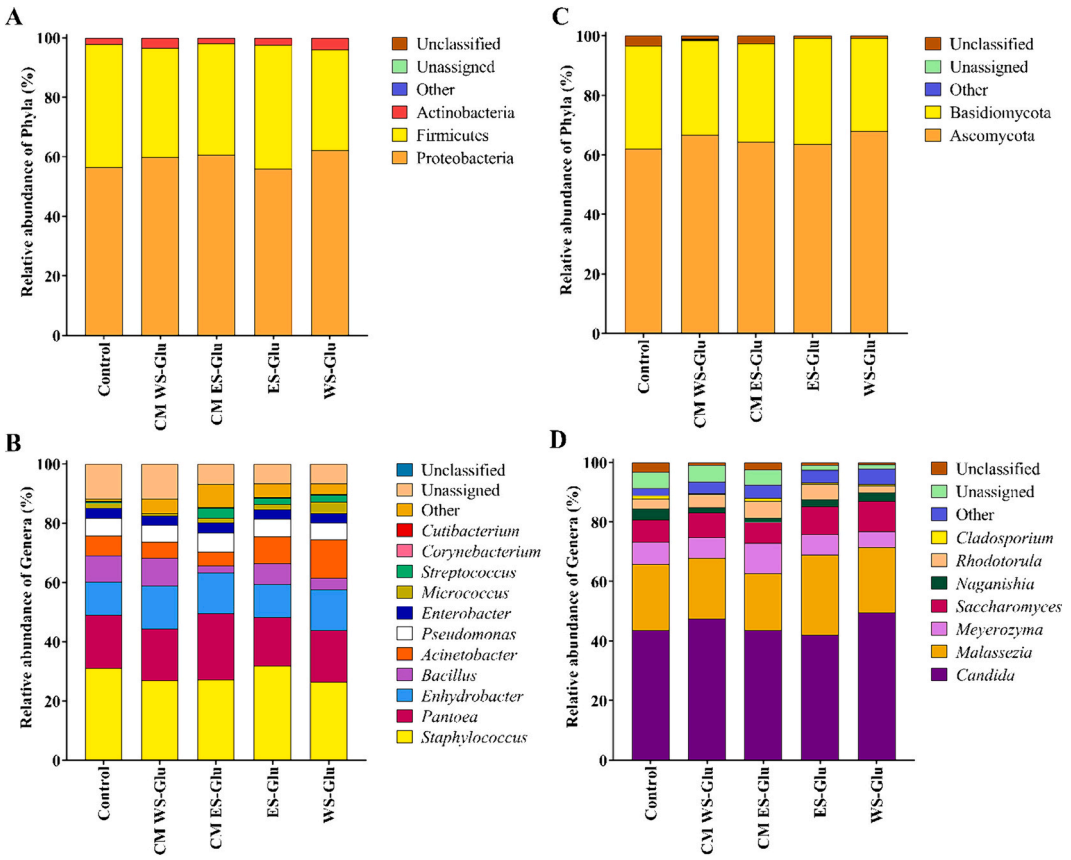

Fig. 6. Impact of glucan extracts, native and carboxymethylated, on the relative abundance of bacterial phyla and genera (A and B) and fungal phyla and genera (C and D) in the naturally occurring skin microbiota of female volunteers.

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 [88]. 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 [89].

As shown in Fig. 6A and C, Proteobacteria (56–62 %) and Ascomycota (62–68 %) were the most prevalent phyla in 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 (Fig. 6B and D). Importantly, no significant differences ($p > 0.05$) were observed in the relative abundance of both genera between the control and test groups (Fig. S3). The skin is naturally colonized by microorganisms belonging to these dominant phyla, such as *Acinetobacter* sp. (Proteobacteria) and *Candida* sp. (Ascomycota) [90,91], as well as genera such as *Staphylococcus epidermidis* (*Staphylococcus* sp.) and *Candida parapsilosis* (*Candida* sp.) [92,93]. Li et al. [94] 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. [95], 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 [96].

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 [97,98]. 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 volunteers' skin.

4. Conclusion

The study successfully developed a process to extract and chemically modify high-purity glucan extracts from two strains of *S. cerevisiae* to potentially be used as a natural ingredient for skincare formulations, in its native and functionalized form. Safety evaluations showed minimal to no impact on the metabolism of the tested cell lines as well as no genotoxic or skin sensitization capacity. Furthermore, these extracts had no impact on the microbiota diversity naturally present on volunteers' faces. Expectedly and under certain conditions, some of these extracts exhibited immunomodulatory capacity by upregulating cellular cytokine (IL-6/IL-8) production. Considering this and while the overall safety assessment yielded reassuring results, further studies are needed to understand the mechanisms and assess the influence of glucans on more complex cellular structures, such as organotypic models or human skin ex vivo, which could provide a more comprehensive understanding of their suitability for skin application. To conclude, spent yeast glucans is shown to be a promising biomolecule that can be safely included into novel skincare products.

Author statement

All authors have read and agreed with the final submission. The authors hereby declare that the submitted work is original and has not been submitted elsewhere.

CRediT authorship contribution statement

Pedro Sousa: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Diana Tavares-Valente:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Carla F. Pereira:** Methodology, Investigation. **Inês Pinto-Ribeiro:** Methodology, Investigation, Formal analysis. **João Azevedo-Silva:** Methodology, Investigation. **Raquel Madureira:** Methodology. **Óscar L. Ramos:** Methodology. **João Fernandes:** Writing – review & editing, Supervision. **Manuela Amorim:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.130933.

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