Skincare Potential of a Sustainable Postbiotic Extract Produced Through Sugarcane Straw Fermentation by Saccharomyces Cerevisiae

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Research Article

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Abstract

Postbiotics, a new class of molecules derived from microorganism's metabolism, are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host". They can be produced by fermentation, using culture media with glucose as the carbon source, and lactic acid bacteria of the genus *Lactobacillus*, and/or yeast, mainly *Saccharomyces cerevisiae* as fermentative microorganisms. Postbiotics comprise different metabolites, and have important biological properties (antioxidant, anti-inflammatory, etc), which is why their use in cosmetics should be considered. During this work, the production of postbiotics was carried out by fermentation with sugarcane straw, as a source of carbon and other active compounds, and as a more sustainable and promising process to obtain more bioactive extracts at the end. For this, its saccharification process was carried out with cellulase at 55 ºC for 24 h. Fermentation was performed sequentially after saccharification at 30 ºC, for 72h, using *S. cerevisiae*. The cells-free extract was characterized regarding its composition, antioxidant activity, and skincare potential. Its use was safe at concentrations below ~ 20 $mg.mL^{-1}$ for keratinocytes and ~ 7.5 $mg.mL^{-1}$ for fibroblasts. It showed antioxidant activity, with ABTS IC_{50} of 1.88 $mg.mL^{-1}$, and inhibited elastase and tyrosinase activities by 83.4% and 42.4%, respectively, at the maximum concentration tested (20 $mg.mL^{-1}$). In addition, it promoted the production of cytokeratin 14, and demonstrated anti-inflammatory activity at a concentration of 10 $mg.mL^{-1}$. Finally, in the skin microbiota of human volunteers, the extract inhibited the *Cutibacterium acnes* bacterium and the *Malassezia* fungal genus. In short, postbiotics were successfully produced using straw as substrate, and as source of carbon and phenolic compounds. These postbiotics showed bioactive properties that potentiate their use in the development of cosmetic and skincare products, such as the treatment of acne or other skin diseases, due to their anti-inflammatory and inhibitory effect on the bacteria responsible for acne, as well as on potentially colonizing fungi.

1. Introduction

Postbiotics (or fermented cosmetics) are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [1].These molecules can be several types of metabolites (enzymes, polysaccharides, teichoic acid, etc) and exert some relevant biological effects such as immunomodulatory, anti-inflammatory, antioxidant, antimicrobial, anti-proliferative and anti-aging activities [2–5], among others. They have been thought useful for cosmetic and skincare applications, owing to their benefits on skin such as antioxidant potential, anti-inflammatory effect, skin microbiota equilibrium, skin enzymes inhibition (over collagenase, elastase, and hyaluronidase). In addition, these compounds have shown antimicrobial effect over *Pseudomonas aeruginosa*, an opportunistic pathogen and cosmetic contaminant, and *Cutibacterium acnes*, the acne-causing bacteria and in the treatment of dermatological diseases (e.g. Alopecia areata) [6–15]. A recent review by our research group [16] describes what is currently known about these compounds, the benefits of using them, the main

postbiotics products available in the market and players, the production key trends and available production methods.

Postbiotics can be produced/obtained especially through fermentative processes, but most of companies' industrial processes are patented [17–20]. Furthermore, most of these compounds are usually derived from lactic acid bacteria, *Lactobacillus* genera and/or yeast, especially *Saccharomyces cerevisiae*, and as a substrate, lignocellulosic material can be used as a carbon/sugar and phenolic compounds (great antioxidant activity) source [16]. The main advantages identified for the use of postbiotics are related to their higher specificity of action on resident microbiota as of interaction with cells of the host compared to probiotics. Besides that, also relatively to probiotics, postbiotics have longer shelf life and greater safety and do not require viability in the topical formulation [9, 21], which turns them into an innovative approach within the cosmetic ingredients market. Moreover, they also can be safely administered to immune-deficient or compromised patients for which live probiotics are not allowed [14]. Adding to that, most of the postbiotics-based products present in the cosmetic market mention several claims such as anti-aging effect, skin defence/barrier/immunity boost, skin regeneration, skin elasticity improvement, anti-wrinkles effect, positive skin microbiota modulation and antioxidant defences improvement, among others [16]. The main players are companies that operate in several areas, such as food innovation, and chemical, pharmaceutical, and cosmetic industries, and the critical trends for production of these compounds include energy efficiency, emission-free mobility, conservation of finite resources and renewable raw material utilization [4, 22].

On this regard, sugarcane (*Saccharum officinarum* L.) processing by-products such as straw can be used as a source of sugar for the fermentation production of postbiotics but never was explored. Sugarcane is a perennial monocot plant, which belongs to the grass family (*Poaceae* or *Gramineae*) [23, 24]. The processing of sugarcane generates annually a great number of by-products such as bagasse and straw, which are the main resultant wastes [24–27]. Sugarcane straw is rich in polysaccharides and other compounds, being composed of 33–45% cellulose, 18–30% hemicellulose, 17–41% lignin, 1–12% ashes, and 5–7% extractives [24, 28]. Furthermore, sugarcane is also a source of phenolic compounds which exhibit several properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, and antioxidant activities [24, 29, 30].

Thus, the main objective of this work was to develop a new and sustainable postbiotic extract with high performance for skin cosmetics and skincare applications, using a fermentation process with *S. cerevisiae* and sugarcane straw as substrate.

2. Materials And Methods

2.1. Biomass

The lignocellulosic-biomass-based feedstock, sugarcane straw, was sourced in Bonfim and Paraíso, provided by Raízen (São Paulo, Brazil). The biomass composition was as follows: 38.77% cellulose,

26.01% hemicellulose, and 19.14% lignin. Samples were transported under controlled conditions, to CBQF-UCP laboratory (Porto, Portugal), and dried at 40 ºC using an oven (Nabertherm, Porto Salvo, Portugal). All sugarcane straw was primarily grinded using a knife mill SM100 (Retsch, Vila Nova de Gaia, Portugal) to a particle size < 4 mm, and then it was sifted, using a Retsch Vibratory Sieve Shaker AS 200 basic (Scansci, Vila Nova de Gaia, Portugal), before use.

2.2. Saccharification conditions to prepare the fermentation media

Sugarcane straw was suspended in 50 mM citrate buffer (pH 5), and supplemented with 10 $g.L^{-1}$ of peptone, 5 $g.L^{-1}$ of yeast extract (Sigma-Aldrich, Sintra, Portugal), 2 $g.L^{-1}$ of ammonium citrate (VWR International, Pennsylvania, USA), 2 $g.L^{-1}$ of potassium phosphate dibasic (Honeywell, North Carolina, USA), 5 $g.L^{-1}$ of sodium acetate, 0.1 $g.L^{-1}$ of magnesium sulfate, and 0.05 $g.L^{-1}$ of manganese sulfate (Merk KGaA, Darmstadt, Germany), using 250 mL Erlenmeyer flasks, in duplicate, in a 1:20 ratio (m/v) [31–33]. To promote water-soluble sugars release from the biomass, cellulase (Celluclast, Novozymes, Bagsværd, Denmark) was added at 20 FPU per gram of cellulose and the flasks were incubated at 55 ºC for 24 h, with the agitation of 150 rpm. Before adding the enzymes, every flask and its content were autoclaved, and the enzymes solutions filtered with 0.22 µm sterile filters. Samples were withdrawn from each flask at 0 and 24 hours, and then centrifuged (10 min., 5000 rpm, 25ºC) for biomass removal and quantification of total sugars by phenol-sulfuric acid.

2.3. Sugarcane straw fermentation process

2.3.1. Microorganisms

Fermentative microorganisms' inoculums were prepared by growing *S. cerevisiae* in Yeast Malt (YM; Biokar Diagnostics, Allonne, France) broth, overnight at 30 ºC. After confirmation of purity, plates and slants were prepared as stock cultures in Potato Dextrose Agar (PDA; Biokar Diagnostics). Inoculums were prepared from stocks using the same incubation conditions as used previously. After incubation, the growth media was centrifuged (5 min, 5000 rpm, 25 ºC). The supernatant was discarded, and cells were washed twice with sterile 50 mM citrate buffer (pH 5) and finally resuspended in 10 mL of sterile 50 mM citrate buffer (pH 5) [31]. These cells were then used to inoculate the fermentation media.

2.3.2. Sequential Saccharification and Fermentation (SQSF) conditions

After the saccharification process, the biomass was removed from the Erlenmeyer flasks, and the fermentation process was performed by inoculating the media with *S. cerevisiae*. For that, the flasks content was centrifuged (10 minutes, 5000 rpm, 25 ºC) in sterile falcon tubes and transferred to new sterile 250 mL Erlenmeyer flasks. After, the reactors were inoculated and incubated at the optimal temperature (previously described) along 72 hours, with agitation (150 rpm). The initial and final pH were recorded along the process with the Seven Compact pH meter (using an InLab Expert Pro-ISM pH

electrode (Mettler, Toledo; USA)). The samples collected along the experiments (0, 24, 48, and 72 h) were subject to different analysis. For the evaluation of microorganism's cellular concentrations, serial decimal dilutions were performed in peptone water and plated using the spread plating technique in PDA. Plates were incubated at 30 ºC during 24h. In addition, samples were withdrawn and centrifuged (10 min, 5000 rpm, 25 ºC) and the supernatant was analyzed for the total sugar content by the phenol-sulfuric acid method.

2.4. Preparation of cells-free extracts

Extracts were prepared as follows. The fermentation broths were subjected to ultrasonication (CY-500, Optic Ivymen System, Comecta, Barcelona, Spain) for disruption of cell membranes and release of the intracellular content, in an ice bath. The tested ultrasonication conditions set up were 10 minutes at 20 ºC, 25% of duty cycle, and 70% amplitude, according to the 'Q500 Protocol *E. coli* Cell Lysis' [34], with modifications. After cellular disruption, broths were centrifuged for 30 minutes at 800 x g and 25 ºC to remove only intact cells and leave components or part of lysed cell membranes [35]. To ensure that broths were not carrying on intact alive cells, these were filtered with 0.22 µm sterile filters, and a microbiological control was performed by plating them in nutrient agar incubated at 30 ºC during 48h. At the end, all extracts broths were freeze-dried (gamma 2–16 LSCplus, Martin Christ, Osterode am Harz, Germany), for further testing.

2.5. Phenol-sulphuric acid method for total sugar content determination

For phenol-sulfuric acid method, a 5% (m/v) phenol (Sigma-Aldrich) solution was prepared by solubilizing 5 g of phenol in 100 mL of deionized water (dH_2O). Tested samples were diluted in dH_2O until an appropriate absorbance value was obtained. Briefly, 80 µL of diluted sample were pipetted into glass tubes in duplicate followed by 150 µL of 5% phenol solution and 1 mL of 95% sulphuric acid (Sigma-Aldrich), as provided. The mixture was stirred using a vortex and incubated for 10 minutes at 100 ºC. After incubation, the mixture was left cooling for about 10 minutes and the absorbance was measured at 490 nm using a UV-1900 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Final values were calculated by interpolation with a glucose (Sigma-Aldrich) calibration curve (0.031–0.250 $mg.mL^{-1}$) and expressed as $mg.mL^{-1}$.

2.6. Monosaccharides and short-chain fatty acids identification by High Performance Liquid Chromatography (HPLC)

For the analysis of mono-, oligosaccharides and organic acids, an HPLC analysis was performed. The assayed samples were accurately weighed and dissolved in ultrapure water at a concentration of 25 $mg.mL^{-1}$. The samples were filtered into vials using 0.45 µm filters (Minisart, Sartorius stedim, Gottingen, Germany). For SCFAs identification and quantification, samples were analysed on an HPLC (Agilent 1260 Infinity II, Agilent Technologies, California, USA) attached to a Refractive Index Detector (RID) coupled to a

Aminex HPX 87H column (300 x 7.8 mm, BioRad, Hercules, CA). The composition of the mobile phase was as follows: 5 mM sulfuric acid solution in ultrapure water. The flow rate was set at 0.600 $mL.min^{-1}$ and an injection volume of 10 µL was used. The detector temperature was set at 35 ºC. For mono- and oligosaccharides identification and quantification, samples were analysed using a Shodex KS-802 column (300 x 8.0 mm). The utilized HPLC equipment and detector were the same. The column temperature was 80 ºC, and the utilized mobile phase was ultrapure water. The flow rate was set at 0.400 $mL.min^{-1}$ and an injection volume of 10 µL was defined. The detector temperature was set at 35 ºC. In both cases, for the determination of elution order (retention time) and obtention of the calibration curves, pure standards were injected. All samples were analysed at least in duplicate.

2.7. Folin-Ciocalteau method for total phenolics content determination

For total phenolic content quantification, the Folin-Ciocalteau's method was used according [36]. Briefly, tested samples were prepared in dH_2O at a 25 $mg.mL^{-1}$ concentration and then filtered using 0.45 µm filters. After, 50 µL of sample, or solvent (dH_2O) for blank were pipetted in triplicate into glass tubes, followed by 50 µL of Folin-Ciocalteau's reagent (Sigma-Aldrich) (1N) as provided, 1000 µL of 75 $mg.mL^{-1}$ sodium carbonate (Na_2CO_3) (Sigma-Aldrich) solution, and 1400 µL of dH_2O, by this exact order. The mixture was stirred using a vortex and incubated for 1h, in the dark, at room temperature. After incubation, the absorbance was measured at 750 nm, using a UV-1900 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Final values were calculated by interpolation with a gallic acid (Sigma-Aldrich) calibration curve (0.062–0.493 $mg.mL^{-1}$) and expressed as $mg.g^{-1}$ dry extract.

2.8. Individual polyphenols and organic acids identification by LC-ESI-UHR-QqTOF-MS

The identification and quantification of polyphenols and organic acids were attained by LC-ESI-UHR-QqTOF-MS, as described by Oliveira *et al*. [37]. Briefly, the assayed samples were accurately weighed and dissolved in ultrapure water, at a concentration of 50 $mg.mL^{-1}$. After that, samples were filtered into vials, using 0.45 µm filters. The separation of metabolites was performed in a Bruker Elute series liquid chromatograph, using an BRHSC18022100 intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 µm, Bruker). The composition of the mobile phase was as follows: (A) 0.1% aqueous formic acid (Sigma-Aldrich); and (B) acetonitrile (Sigma-Aldrich) with 0.1% formic acid. The separation was carried out for 24.5 min, under the following gradient conditions: 0 min, 0% B; 10 min, 21.0% B; 14 min, 27% B; 18.30 min, 58%; 20.0 min, 100%; 24.0 min, 100%; 24.10 min, 0%; 26.0 min, 0%. The flow rate was set at 0.250 $mL.min^{-1}$ and an injection volume of 5 µL was used. For MS analysis, an ultrahigh-resolution quadrupole – quadrupole time-of-flight (UHR – QqTOF) mass spectrometer with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany) was used. MS analysis parameters were set using negative ionization mode with spectra acquired over a range from *m/z* 20 to 1000 in an Auto MS scan mode. The selected parameters were as follows: End plate off set voltage, 500 V; capillary voltage, 3.0 kV; drying gas

temperature, 200°C; drying gas flow, 8.0 $L.min^{-1}$; nebulizing gas pressure, 2 bar; collision radio frequency (RF), from 250 to 1000 Vpp; transfer time, from 25 to 70 µs; collision cell energy, 5 eV; and pre-pulse storage, 6 µs. Post-acquisition internal mass calibration used sodium formate clusters, with sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

The elemental composition for the compound was confirmed according to accurate mass and isotope rate calculations designated mSigma (Bruker Daltonics). The accurate mass measurement was within the lowest elemental composition, and mSigma values provided confirmation. Compounds were identified based on its accurate mass $[M-H]^-$. For the determination of elution order (retention time), and obtention of the calibration curves, pure standards were injected. All samples were analysed in duplicate and results are expressed in $mg.g^{-1}$ dry extract.

2.9. Antioxidant activity

Antioxidant activity of the fermentation extracts was determined using three distinct methods: DPPH, ABTS and ORAC assays. For these assays, lyophilized samples were prepared in dH_2O at a 50 $mg.mL^{-1}$ concentration, filtered using 0.45 µm filters and then diluted in series at a 1:2 (v/v).

2.9.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Cation Decolorization Assay

The DPPH assay was used to measure the free radical scavenging capacity of the fermentation extract. Used as a reagent, DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants. For DPPH assay, the $DPPH^+$ concentrated solution was obtained by weighing 24 mg of DPPH (Alfa Aesar, Thermo Fisher Scientific, Massachusetts, USA) for 100 mL of methanol (600 µM). The solution was stirred and then stored in the dark, at -20 ºC. The $DPPH^+$ working solution was prepared by diluting the previous one using methanol until the absorbance was 0.600 ± 0.100 at 515 nm. Trolox (Sigma-Aldrich) stock solution was prepared by dissolving 15 mg of Trolox in 10 mL of methanol, in a volumetric flask. From the previous one, trolox working solution was prepared in a volumetric flask by transferring 1 mL to a final volume of 10 mL of methanol. Briefly, 25 µL of sample (each dilution), Trolox, or solvent (dH_2O) for the blank, were pipetted in duplicate into each well of a 96-well plate, followed by 175 µL of $DPPH^+$ working solution. The mixture was incubated for 30 min at room temperature, and the absorbance was measured at 515 nm, with a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal) [38].

The inhibition percentage (I) of the sample was calculated using the Eq. (1) and compared with trolox standard calibration curve (0.0075–0.075 $mg.mL^{-1}$). The results were expressed as IC_{50} ($mg.mL^{-1}$).

2.9.2. 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Cation Decolorization Assay

For ABTS assay, the $ABTS^+$ concentrated solution was prepared by separately solubilizing ABTS (Sigma-Aldrich) at 3.84 $mg.mL^{-1}$ and $K_2O_8S_2$ (potassium persulfate) (Sigma-Aldrich, Sintra, Portugal) at 0.66 $mg.mL^{-1}$ in dH_2O. Both solutions were then mixed using a magnetic stirrer and $ABTS^+$ was generated through a chemical oxidation reaction between both substances. The $ABTS^+$ working solution was prepared by diluting the previous one using dH_2O until the absorbance was 0.700 ± 0.020 at 734 nm. Trolox working solution was prepared similarly to DPPH assay. Briefly, 15 µL of sample (each dilution), Trolox, or solvent (dH_2O) for the blank, were pipetted in duplicate into each well of a 96-well plate followed by 200 µL of $ABTS^+$ working solution. The mixture was incubated for 5 min at 30 ºC, and the absorbance was measured at 734 nm, with a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal) [38]. The inhibition percentage (I) of the sample was calculated using the Eq. (1) and compared with trolox standard calibration curve (0.0075–0.075 $mg.mL^{-1}$). The results were expressed as IC_{50} ($mg.mL^{-1}$).

$$I\,(\%) = \left[\frac{(Abs_{A0} - Abs_{sample})}{Abs_{A0}}\right] \times 100\,(1)$$

where Abs_{A0} is the absorbance of blank and Abs_{sample} is the absorbance of the reaction between sample and the radicals.

2.9.3. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC method measures the antioxidant capacity of a specimen by its ability to prevent loss of fluorescence signal, by neutralizing peroxyl radicals. The decrease of fluorescence signal should be minimal if the specimen rich in antioxidant compounds [39]. For ORAC assay, a 75 mM PBS buffer was prepared by dissolving monosodium phosphate (NaH_2PO_4) (Sigma-Aldrich) in ultrapure water (9 $mg.mL^{-1}$) and adjusting the pH to 7.44, using a monovalent strong base. The fluorescein stock solution was prepared by solubilizing 0.01097 g of fluorescein di-sodium salt (Sigma-Aldrich) in 25 mL of previously made PBS buffer (1166.1 µM). This solution was stored at 4 ºC, for 1 month (maximum) and covered with aluminium foil. The fluorescein work solution was made from the previous one by sequentially diluting 100 µL of it in 10 mL with PBS and then 250 µL in 25 mL with PBS (116.66 nM). The Trolox stock solution was made by weighing 0.0125 g of Trolox and dissolving it in 1 mL of methanol (12.5 mg.ml-1), completing then the volume up to 50 mL with PBS. The Trolox working solution was prepared from the previous one by removing 1mL and making up the volume with PBS up to 10 mL (solution T0). Finally, AAPH (Acros Organics, Thermo Fisher Scientific, New Jersey, USA) solution was prepared by dissolving it in PBS (13.018 $mg.mL^{-1}$). Except for PBS buffer, all solutions were prepared in the dark, and in volumetric flasks covered with aluminium foil. Briefly, 20 µL of sample (each dilution), Trolox, or solvent (PBS buffer) for the blank were pipetted in duplicate into each well of a 96-well plate followed by 120 µL of fluorescein working solution. The mixture was incubated for 10 min at 37 ºC. After 10 min, 60 µL of AAPH solution was added rapidly with a multichannel pipette into each well of the plate. The mixture was

incubated for 70 min at 37 ºC, and the fluorescence signal was recorded every minute, using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal) [40].

Each sample was analyzed at least in duplicate in the plate. Final ORAC-FL values were expressed as μmol of Trolox equivalent per gram of dry weight (μmol TE.g^{-1} dry weight). Values were calculated by interpolation with a Trolox calibration curve (10–80 μM).

2.10. In vitro chemical skin enzymes inhibition tests

Extracts were tested for their effect on two distinct skin enzymes inhibition: neutrophil elastase (NE), and tyrosinase. Samples were prepared in dH_2O according to the following final concentrations in the wells: 20, 15, 7.5, 3.75, and 1.875 mg.mL^{-1}.

2.10.1. Elastase

The assay was performed using a commercial kit of neutrophil elastase inhibitory screening (fluorometric) (ab118971, ABCAM, Cambridge, UK), according to manufacturer's instructions. Briefly, NE enzyme stock was first reconstituted in 220 μL of assay buffer and stored at -80 ºC. When testing, all reagents (assay buffer, substrate, NE solution, and inhibitor control (Succinyl-alanyl-alanyl-prolyl-valine chloromethyl ketone - SPCK) were equilibrated to room temperature. Then, NE enzyme stock solution, enzyme substrate, and inhibitor control were diluted 1/25, 2/25, and 1/25, respectively, in assay buffer, to required total volume. 50 μL of diluted NE solution was added to all wells. Then, 25 μL of sample, or assay buffer for blank (enzyme control), or inhibitor control were pipetted in duplicate, into each desired well of the microplate. The microplate was mixed and left incubating at 37 ºC, for 5 min. After incubation, 25 μL of diluted enzyme substrate were added to all wells and fluorescence was immediately measured at Ex/Em 400/505 nm at 37 ºC for 30 min, using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal) in kinetic mode. The RFU of fluorescence is $\Delta RFU = R_2 - R_1$, and the kinetic mode was used to choose the R_1 and R_2 at linear range. The percentage inhibition of this assay was calculated by Eq. (2):

$$\mathrm{Enzymeinhibitionactivity}\,(\%) = \frac{\Delta \mathrm{RFUsample}}{\Delta \mathrm{RFUenzymecontrol}} \times 100 \quad (2)$$

2.10.2. Tyrosinase

The assay was performed using a commercial kit of tyrosinase inhibitory screening (colorimetric) (ab204715, ABCAM), according to manufacturer's instructions. Briefly, tyrosinase substrate and lyophilized tyrosinase were dissolved in 220 μL of dH_2O and assay buffer, respectively, and stored at -20 ºC. Inhibitor control (kojic acid) was prepared in dH_2O to a 10 mM concentration and stored at -20 ºC. When testing, all reagents (assay buffer, tyrosinase substrate stock solution, tyrosinase stock solution, tyrosinase enhancer, and inhibitor control) were equilibrated to room temperature, prior to use. Then, tyrosinase enzyme was diluted 1/25 in assay buffer to required total volume. For diluted tyrosinase

substrate solution, tyrosinase substrate and tyrosinase enhancer were diluted 2/30, and 5/30, respectively, together in assay buffer to required total volume. 20 µL of sample, inhibitor control, assay buffer for enzyme control, or solvent for solvent control, were pipetted in duplicate into each desired well of the microplate. Prior to use, inhibitor control was set to a 0.75 mM concentration. Then, 50 µL of diluted tyrosinase enzyme were added to all wells and the plate was left incubating at 25 ºC, for 10 min. After incubation, 30 µL of diluted tyrosinase substrate solution was added to all wells and the absorbance (abs) was recorded at 510 nm, every 2–3 min for 30 to 60 min, using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal), in kinetic mode. Data was plotted as abs versus time for each sample. Two points (T_1 and T_2) were chosen in the linear range of the plot, and the corresponding values of absorbance were obtained (A_1 and A_2). The slope was calculated for all samples (S), inhibition control (IC) and enzyme control (EC) by dividing the net ΔA (A_2 - A_1) values with the time ΔT (T_2 - T_1). The percentage inhibition of this assay was calculated by Eq. (3):

$$\mathrm{Enzymeinhibitionactivity}\,(\%) = \frac{\mathrm{SlopeofEC} - \mathrm{SlopeofS}}{\mathrm{SlopeofEC}} \times 100 \quad (3)$$

2.11. Cell culture

Human dermal fibroblasts (HDF) and immortalized human keratinocytes (HaCaT) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fischer) and 1% antibiotic at 37 ºC, with 5% CO_2 in a humidified atmosphere.

Human monocytes THP-1 (ATCC TIB-202) were cultured in Roswell Park Memorial Institute (RPMI; Thermo Fischer) culture medium, supplemented with 10% FBS, 1% antibiotic, and 50 mM beta-mercaptoethanol, at the same conditions than HDF and HaCaT cells.

2.12. Cytotoxicity

Cytotoxicity of fermentation extracts was evaluated using PrestoBlue™ Cell Viability assay kit (Invitrogen), according to the manufacturer´s instructions. HDF and HaCaT cells were seeded at 1 × 10^4 cells/well in 96-well plates and incubated over-night to allow cells to adhere. Cells were then exposed to the fermentations extracts at desired concentrations (80, 40, 20, 10, 5, and 2.5 $mg.mL^{-1}$), in DMEM, for 24h, at 37 ºC, with 5% CO_2 in a humidified atmosphere. Each sample dilution was tested in quadruplicate in two independent experiments. Briefly, 100 µL of sample, culture medium for positive control, or culture medium with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich) for negative control, were pipetted into each appropriate well. After 24h incubation, 10 µL of PrestoBlue™ Cell Viability Reagent (Invitrogen, A13262) were added to each well and the plate was left incubating at 37 ºC, with 5% CO_2 in a humidified atmosphere, protected from the light, up to 3h. Finally, the fluorescence was recorded using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal). Results are expressed in percentage of metabolic

inhibition in comparison to positive control with an inhibition superior to 30% being considered cytotoxic in accordance with ISO 10993-5 standard.

2.13. Cytokeratin 14 quantification

HaCaT cells were seeded at 2.5×10^5 cells.mL^{-1} (1 mL per well) in 12-well plates. Cells were exposed to the fermentation extract at desired concentration (15 mg.mL^{-1} in DMEM), for 24h. Each sample was tested in duplicate in two independent experiments. Culture medium was used as negative control. After incubation, the growth medium was removed, and cells were washed twice with PBS. Cells were then harvested by the addition of ice-cold 1X cell extraction buffer PTR (Human Cytokeratin 14 SimpleStep ELISA® Kit, ABCAM, ab226895) directly to the plate and were mechanically scrapped into a microfuge tube. Cell lysates were incubated on ice for 15 minutes, centrifuged at 18000 x g for 20 minutes at 4 ºC and supernatants were collected. Total protein was quantified by the BCA method using the Pierce™ BCA Protein assay kit (Thermo Scientific), according to the manufacturer´s instructions. For cytokeratin 14 (CK14) quantification, 25 ng of total protein were used. CK14 abundance was determined by ELISA using the Human Cytokeratin 14 SimpleStep ELISA® Kit (ABCAM, ab226895), according to manufacturer's instructions.

2.14. Collagen I α1 quantification

HDF cells were seeded at 2.5×10^5 cells.mL^{-1} (1 mL per well) in 12-well plates. Cells were exposed to the fermentation extract at desired concentration (6 mg.mL^{-1} in unsupplemented DMEM), for 24h. Unsupplemented DMEM was used, as the presence of FBS is reported to inhibit collagen synthesis [41]. Each sample was tested in duplicate in two independent experiments. Cells with only media and with palmitoyl tetrapeptide-3 (GenScript, New Jersey, USA) were used as negative and positive controls, respectively. Total protein quantification was performed as described in the previous section. For collagen I α1 quantification, 100 ng of total protein were used. Collagen I α1 abundance was determined by ELISA, using the Human Pro-Collagen 1 alpha 1 CatchPoint® SimpleStep ELISA® Kit (ABCAM, ab229389), according to the manufacturer's instructions.

2.15. Production of IL-6 by macrophages

THP-1 cells were seeded at 3×10^5 cells/well in 24-well microplates and differentiated into macrophages by treatment with 50 nM of phorbol 12-myristate 13-acetate (Sigma-Aldrich), for 48 h. Cells were exposed to the fermentations extracts (10 and 1 mg.mL^{-1}) for 24h, in the presence or absence of lipopolysaccharides form *E. coli* O111:B4 (LPS, Sigma-Aldrich) to induce inflammation. For anti-inflammatory control, macrophages were treated with 20 nM of betamethasone (Sigma-Aldrich). After 24h, supernatants were collected and the level of proinflammatory cytokine IL-6 was determined by

ELISA, using the ELISA MAX™ Deluxe Set Human IL-6 kit (Biolegend), according to manufacturer's instructions. For total protein quantification, cells were lysed with water, and BCA method was performed, as previously described. The results were expressed in pg of cytokine.ug^{-1} of total protein.

2.16. Evaluation of the impact of extracts on skin microbiota

A study protocol for the evaluation of the effect of the developed extracts in skin microbiota modulation was established. This protocol was validated by the Commission of Ethics for Health of Universidade Católica Portuguesa before its execution. Additionally, the team members which proceed with the trials are certified with ICH good clinical practice E6 (R2) recognized by the Global Health Training Centre. Nine female volunteers without diagnosed dermatological diseases were included on the study. On the day of the collection, the selected volunteers could not perform any skincare routine. Facial skin microbiota was collected from those volunteers following the protocol of Carvalho *et al*. [42].

The microbial DNA was purified using the PureLink™ Microbiome DNA Purification Kit (Invitrogen) and it was quantified using the Qubit™ 1X dsDNA HS (High Sensitivity) Assay Kit (Invitrogen) according to the manufacturer's instructions. For quantitative real-time PCR (qPCR), it was used universal and specific primers to quantify the total load of bacteria or fungi, and the relative abundance of specific microbial genera, and species [43–50].

qPCR reactions were prepared to a final volume of 10 µL, containing 1x NZYSupreme qPCR Green Master Mix (NZYtech, Lisbon, Portugal), 0.5 to 1 µM of forward and reverse primers (Integrated DNA Technologies, IDT, Heverlee, Belgium), 2 µL of Microbial DNA-Free Water (Qiagen, Hilden, Germany) and 1 µL of DNA. The qPCR was performed in a qTOWER³ G (Analytik-Jena, Germany) with the following conditions: 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The amplification steps are followed by a melt dissociation step to check for nonspecific product formation. The samples were tested in triplicate.

The relative standard curve method was used to quantify the total microbial load and the specific microbial genus or species. To create standard curves, dilution series of known microbial CFU number were used to create a standard curve for each pair of primers, by plotting the $\log_{10}$ of each known CFU number in the dilution series against the determined threshold cycle (Ct) value. For each genus and species, the relative abundance was calculated by log10 ratio between the CFU number determined for the genus- or specie-specific assay and the CFU number determined for the universal assay. To reduce the inter-individuality, for each volunteer it was calculated a ratio between the condition test and its control condition (fold-difference or fold-change).

2.17. Statistical Analysis

For statistical analysis, it was used the IBM® SPSS® Statistics 26 software. Data was first analysed for normality distribution (Shapiro-Wilk test, n < 50, or Kolmogorov-Smirnov test, n > 50). Afterwards, a one-way ANOVA test (normal distribution) with Tukey's HSD post hoc test, or a Kruskal-Wallis test (non-normal distribution) were applied to determine differences between more than two groups. In case of a two-group comparison, a student's t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) were performed. In general, the significance level was set at 0.05.

3. Results And Discussion

3.1. Sugarcane straw saccharification and fermentation processes

The sugarcane straw was subject of a saccharification process, using a cellulase (Celluclast), to release monosaccharides from the cellulose and hemicellulose polysaccharide chains. This process was performed during 24 h and the total sugar concentration increased from 1.69 to 3.55 $mg.mL^{-1}$. The resultant sugar rich media was used for fermentation with *S. cerevisiae*. The broth was inoculated at an approximate initial cellular concentration of 6 $log\ CFU.mL^{-1}$, and the yeast grew to a concentration of 9.12 $log\ CFU.mL^{-1}$, at 72h, while consuming the available sugars, which decreased from 3.55 to 1.58 $mg.mL^{-1}$. All results are resumed in **Fig. 1**.

Within, the first 24 h, it was observed the highest cellular growth and, consequently, the highest sugar uptake. After, there was a stabilization of both parameters, suggesting that the microorganism reached the stationary phase of its growth after the 24 h. This may have been due to carbon and/or other nutrients starvation (lack of fermentable sugars or other nutrients), condition in which yeast cells are able to survive for long periods (from 24 to 72h, **Fig. 1**) as described by Werner-Washburne et al. [51]. Also, *S. cerevisiae* performs alcoholic fermentation. Several studies report that ethanol can disrupt the physical structure of cell membranes, and that this phenomenon can be observed in any cell membrane. Moreover, it is reported that ethanol increases the membrane permeability [52–56]. It is possible that cells who had been affected by ethanol, released monosaccharides, that may have already been taken up, back into the broth. This phenomenon is described in the literature, for example, for potassium, nucleotides, and amino acids. Adding to this, ethanol has been shown to inhibit glucose and maltose uptake by cells [55, 56]. Nevertheless, ethanol was not detected by HPLC as it possibly evaporated during the freeze-drying process.

3.2. Extracts compositional properties

3.2.1. Mono-, oligosaccharides and short-chain fatty acids profile

Both extracts were analyzed for mono-, and oligosaccharides (typically 2–10 monosaccharides) identification and quantification, to find the reasons why not all sugars were consumed during

fermentation. Recall that the sugars that are being analysed are free sugars present in the biomass, released along the saccharification, and not consumed during fermentation. Three different sugars were identified: glucose, sorbitol, and cellobiose. The results are summarized in Table 1. Glucose is a monosaccharide centrally involved in the processes of photosynthesis, respiration, and fermentation, serving as an energy source for metabolic activity in most organisms [57]. Sorbitol is a sugar alcohol, and it is synthesized from glucose 6-phosphate, via a NADP-dependent sorbitol 6-phosphate dehydrogenase and sorbitol 6-phosphatase [58, 59]. Cellobiose is a disaccharide consisting of two glucose molecules linked by a *β*-(1,4') glycosidic bond [60, 61]. In the case of glucose, this sugar composes the polysaccharide chain of cellulose (one of the main components of lignocellulose), so its presence was expected, resulting of cellulose degradation by heat and, posteriorly, enzymes. Also, cellobiose was expected since it is produced by the hydrolysis of cellulose [62]. Regarding sorbitol, it is the hydrogenation/reduction product of glucose [63].

Table 1
Mono-, oligosaccharides, and short-chain fatty acids identified in both non-fermented and fermented extracts (mg.g^{-1} dry extract, mean ± SD); * Significantly different from the control ($p < 0.05$).

Compound	**Non fermented straw extract**	S. cerevisiae **straw extract**
Mono-, oligosaccharides		
Cellobiose	12.60 ± 0.96	n.d.
Sorbitol	63.06 ± 1.87	95.58 ± 17.86*
Glucose	41.51 ± 2.63	n.d.
Short-chain fatty acids		
Citrate	294.55 ± 11.37	127.51 ± 19.16*
Lactate	n.d.	n.d.
Acetate	71.66 ± 9.70	51.14 ± 13.40*
Nd – not detected.		

Concerning the fermented sample, both cellobiose and glucose were fully metabolized. In case of glucose, this would be expected since it is the most used fermentable monosaccharide by microorganisms. In case of cellobiose, being a disaccharide its metabolization should not be easy. However, accordingly to the obtained results it was fully consumed or degraded. Regarding *S. cerevisiae*, some studies report that the metabolism of cellobiose is not easy, suggesting strategies that would allow a more efficient utilization of this disaccharide [64–68]. Alternatively, cellobiose may have been converted into glucose (fermentable sugar) by microbial *β*-glucosidases, which break the glycosidic bonds [69]. In contrast, *S. cerevisiae* did not metabolized sorbitol, and it seemed to produce it, which is accordingly to the absence of studies on sorbitol uptake by *S. cerevisiae*. In fact, some studies even report sorbitol

production by *S. cerevisiae* [70, 71]. Moreover, this sugar is reported to cause osmotic stress to this yeast, inducing trehalose and/or glycerol biosynthesis [72, 73]. However, no analysis was made for glycerol and/or trehalose quantification.

Both extracts were also analyzed by High Performance Liquid Chromatography (HPLC) for short-chain fatty acids (SCFAs) identification and quantification, and the results are resumed in Table 1. Regarding the non-fermented extract, only citrate and acetate were detected, with concentrations of 294.55 and 71.66 $mg.mL^{-1}$, for citrate and acetate, respectively. Citrate presence results from the citrate buffer used as buffer of the fermentation medium and the same with acetate which is derived from sodium acetate. As expected, no lactate was detected. Concerning the fermented sample, citrate concentration decreased suggesting that *S. cerevisiae* may have metabolized part of it. However, *S. cerevisiae* has been reported as incapable of metabolizing citrate [74, 75]. Nonetheless, some studies report that, when glucose is absent and in the presence of acetate, *S. cerevisiae* is capable of metabolizing isocitrate (an isomer of citrate) into glyoxylate [76, 77]. This finding suggests that the detected citrate was, in fact, a combination of citrate and isocitrate, and isocitrate was the isomer consumed. The mass spectrometry analysis allowed to confirm this hypothesis, as two distinct peaks with the same *m/z* and the same fragments and citrate characteristics were detected, but only one was attenuated by fermentation. Furthermore, a study by Shang *et al*. [78] showed that, with *S. cerevisiae*, and acetate supplementation, there was a slight reduction in acetate concentration. Also, and as expected, no lactic acid was produced by *S. cerevisiae*.

3.2.2. Total phenolics content and individual polyphenols and organic acids profile

Both non-fermented and fermented extracts were analysed by the Folin-Ciocalteau method, for total phenolic content determination, and by LC-ESI-UHR-QqTOF-MS, for individual polyphenols and organic acids identification and quantification. The respective results are summarized in Table 2.

Table 2
Total phenolic content and polyphenols and organic acids identification and quantification in both extracts (mg.g^{-1} dry extract, mean ± SD). * Significantly different from the control ($p < 0.05$).

Analyzed compound	Non fermented straw extract	Fermented straw extract
Total phenolics	12.328 ± 0.419	13.460 ± 0.488*
Organic acids		
Azelaic acid	0.809 ± 0.050	0.833 ± 0.014
Sebacic acid	0.104 ± 0.005	0.135 ± 0.005*
Hydroxybenzoic acids		
4-Hydroxybenzaldehyde	0.182 ± 0.006	0.006 ± 0.001*
3,4-Dihydroxybenzaldehyde	0.065 ± 0.005	0.232 ± 0.019*
Hydroxycinnamic acids		
p-Coumaric acid	1.056 ± 0.053	0.422 ± 0.013*
p-Coumaric acid derivate	0.057 ± 0.066	0.114 ± 0.001
Flavones		
Isochaftoside	0.003 ± 0.001	0.004 ± 0.000
Tricin 7-O-rhamnosyl-glucuronide	0.013 ± 0.000	0.014 ± 0.000*

The obtained values for total phenolics content were 12.328 and 13.460 mg.g^{-1} for non-fermented straw extract, and fermented straw extract, respectively. Regarding the fermented straw extract, it seems that during fermentation, a small quantity of phenolic compounds may have been released ($p < 0.05$), according to the Folin-Ciocalteau method. For instance, soybean meal fermentation by *S. cerevisiae* is reported to lead to a total phenolic content increase [79]. In the present work, the increase in total phenolics content can be due to the function of microbial *β*-glucosidase allowing to break the *β*-glycoside bonds that link some phenolics to proteins or polysaccharides in the cell walls, to release additional phenolics [69]. However, this does not seem to justify the higher antioxidant activity of the fermented extract when compared to the control. In this line, and as discussed in the next section, *S. cerevisiae* is reported to produce other metabolites which exert this type of activity.

Regarding individual identification, six distinct polyphenols and two organic acids were identified among the assayed extracts: azelaic acid, sebacic acid (organic acids), 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde (hydroxybenzoic acids), *p*-coumaric acid, *p*-coumaric acid derivate (hydroxycinnamic acids), isochaftoside, and tricin 7-O-rhamnosyl-glucuronide (flavones). Among these, it is important to highlight the ones found in higher quantities, like azelaic acid, 4-hydroxybenzaldehyde,

and *p*-coumaric acid. The first one did not seem to be affected by the fermentation process. In contrast, the results regarding the other two suggest that they may have been metabolized (degraded) during fermentation as reported by Carvalho et al. [80]. It should also be noted that *p*-coumaric acid derivate was detected in greater amounts in the fermented extracts. Instead, *p*-coumaric acid was found in smaller amounts, suggesting that the fermentation process can lead to the degradation of this type of compound. Phenolic compounds like the ones here found have biological importance especially by their known antioxidant activity. However, these compounds are also reported to have skin care applications, for example, *p*-coumaric acid and derivatives were suggested to have potential use as a skin-lightening active ingredient [81] and to be an inhibitor of tyrosinase activity [82], and to exert anti-inflammatory effect [83]. 4-Hydroxybenzaldehyde and derivatives were reported to promote wound healing and reepithelization in an in vivo animal model [84], and tyrosinase activity inhibition [85]. Another example is azelaic acid, which is reported as an anti-acne ingredient [86–89]. For instance, some compounds presence, such as 3,4-dihydroxybenzaldehyde and sebacic acid, was enhanced by the fermentation process. In fact, 3,4-dihydroxybenzaldehyde is reported to lower reactive oxygen species generation, and to inhibit oxidative DNA damage and apoptosis due to its antioxidant activity [90, 91]. Also, a derivate of it (5-bromo-3,4-dihydroxybenzaldehyde) has been reported to promote hair growth in dermal papilla cells [92]. Possibly, not all the present polyphenols have been identified. However, some common ones derived from sugarcane such as chlorogenic acid, caffeic acid, ferulic acid, and vanillic acid [93, 94] were evaluated but were not detected. Perhaps the fact that the samples were prepared in dH_2O caused this, since in most of the studies reporting the presence of such molecules, a percentage of an organic solvent is used (e.g. ethanol, methanol).

3.3. Extracts biological properties

3.3.1. Antioxidant activity

The obtained results regarding the antioxidant performance of both extracts by the ABTS, DPPH, and ORAC assays are represented in Table 3. In all cases, the fermented extract presented higher antioxidant potential (lower IC_{50} and higher ORAC value). The obtained values for non-fermented and fermented extracts were 5.46 $mg.mL^{-1}$, 9.62 $mg.mL^{-1}$, 171.21 µmol $TE.g^{-1}$, and 1.88 $mg.mL^{-1}$, 8.94 $mg.mL^{-1}$, 302.23 µmol $TE.g^{-1}$ for ABTS, DPPH and ORAC assays, respectively. Some studies report autolysates of *β*-glucans and protein fractions (free thiols from denatured proteins) derived from *S. cerevisiae* with potential to be explored as natural antioxidants [95–97]. Furthermore, cell-wall polysaccharides from *S. cerevisiae* have also been associated with this kind of activity [98]. Moreover, as mentioned before, there are polyphenols that may be entrapped in the biomass and that, during fermentation, may be released. However, the DPPH assay results were quite different from the ones of ABTS assay. The IC_{50} values of ABTS were much lower suggesting that most of antioxidant compounds present were water soluble. In fact, the main identified polyphenols (previous section), such as 4-hydroxybenzaldehyde, and *p*-coumaric acid are described as being water soluble at the detected concentrations [99, 100]. Nevertheless, these compounds are reported to be more soluble with ethanol-like solvents. However, the samples were prepared in water, and, in an aqueous reaction medium these can present radical scavenging activity

[101]. In this line, it is expectable that the ABTS assay presented the best results once it was performed in aqueous conditions. Instead, DPPH assay was carried out in methanol.

Table 3
Antioxidant activity values (mean ± SD) determined by ABTS, DPPH, and ORAC assays of both extracts and two antioxidant benchmarks (ascorbic acid and BHT). * Significantly different from the control ($p < 0.05$).

Analysis	**Non fermented straw extract**	S. cerevisiae **straw extract**	**Ascorbic acid**	**BHT**
ABTS IC_{50} ($mg.mL^{-1}$)	5.46 ± 0.35	1.88 ± 0.12*	0.05	0.13
DPPH IC_{50} ($mg.mL^{-1}$)	9.62 ± 0.10	8.94 ± 0.46	0.04	0.28
ORAC (µmol $TE.g^{-1}$)	171.21 ± 11.27	302.23 ± 19.49*	2652.93 ± 274.39	—

Also, two antioxidant benchmarks (ascorbic acid and BHT) were tested. The values obtained for ABTS, DPPH and ORAC assays were 0.05 $mg.mL^{-1}$, 0.04 $mg.mL^{-1}$, 2652.93 µmol $TE.g^{-1}$, and 0.13 $mg.mL^{-1}$, 0.28 $mg.mL^{-1}$, for ascorbic acid and BHT, respectively. As expected, these substances presented higher antioxidant activity, comparing to the fermented extract, since they are pure substances. On the other hand, the fermented extract is a result of a microbiological fermentation, being this a mix of various kinds of components, also exerting biological activities other than antioxidant.

3.3.2. Cytotoxicity

The obtained results regarding the cytotoxicity of the fermented extract, by the PrestoBlue assay, are presented in Fig. 2. The safety of the extract was evaluated only on HaCaT and HDF cells, demonstrating to be safe at concentrations approximately below 20 $mg.mL^{-1}$ (2%) and 7.5 $mg.mL^{-1}$ (0.75%), respectively. In this line, fibroblasts proved to be more sensitive to the fermented extract. Nonetheless, in a biological tissue context (*in vivo*), cells tend to be more resilient to the exposure with foreign substances. In all cases, the negative results of metabolic inhibition are suggested to indicate an increase in cellular proliferation. Even so, additional studies should be performed to evaluate such a hypothesis [102].

In short, due to the “compatibility” between the safe concentrations range and the obtained ABTS IC_{50} value, the fermented extract was further explored for several other biological properties, such as CK14 and collagen I α1 production, skin enzymes inhibition, anti-inflammatory activity, and influence on skin microbiota. The non-fermented extract was not evaluated for cytotoxicity and skincare properties since its antioxidant activity was significantly lower comparing to the fermented extract.

3.3.3. Cytokeratin 14 and collagen I α1 production

The obtained results regarding collagen I α1 and CK14 production by fibroblasts and keratinocytes, respectively, under exposure to the fermented extract are represented in Fig. 3. The tested concentrations were defined accordingly to the cytotoxicity assay results.

Regarding collagen I α1, the assayed sample (6 $mg.mL^{-1}$ concentration) did not significantly affect its production by fibroblasts ($p > 0.05$). The obtained values of fold change relative to control for the positive control and assayed samples were 1.45 and 0.90, respectively. The objective was to evaluate if the assayed extract could induce collagen I α1 production *in vitro*. In fact, a study by Schlotmann *et al*. [103] demonstrated that skincare products can stimulate natural processes in the skin, such as the synthesis of collagen. Interestingly, collagen and its hydrolysates benefits are highly reported in literature, but also as cosmetic nutraceutical products [104–108], which represents a different approach. However, the obtained results with the assayed extract were not as promising as initially intended, which was not expected as sugarcane-derived polyphenols (e.g. caffeic acid, ellagic acid, gallic acid) are reported to induce collagen synthesis [24, 109, 110]. Perhaps, the tested concentration was not enough to positively induce the production of collagen. Nevertheless, no studies reporting *S. cerevisiae*-derived molecules effects on collagen synthesis were found. Still, it is important to understand the importance of collagen on skin integrity, function, and appearance. Collagen is the major structural protein of the skin, being the most prevalent component of the extracellular matrix (ECM) [108, 111]. It is responsible for structure, stability, and strength especially within the dermal layers [107] and plays a key role in preventing skin aging. In fact, decreased collagen density is reported to be associated with the progression of skin aging, causing it to lose its integrity and flexibility [106]. The decrease of collagen density has been associated with the passage of time, and particularly with exposure to the sun (photo-aging) [107]. In this line, Asserin *et al*. [105] described the accelerated fragmentation of the collagen network as an “hallmark of skin aging”. Furthermore, collagen is suggested to maintain skin firmness and elasticity, and its hydrolysates to keep the skin hydrated [112, 113].

Regarding CK14, the assayed sample (15 $mg.mL^{-1}$ concentration) showed a significantly positive effect (increase) on its production ($p/2 < 0.05$). The obtained value of fold change relative to control for the assayed sample was 2.40. Similarly, to collagen I α1, the objective was to evaluate if the assayed extract could induce CK14 production *in vitro*. CK14 is an intermediate filament protein and a precursor of keratin 14 (K14) protein, and it is a component of the epithelial cells cytoskeleton [114]. Normally, it functions with a pair keratin, which is keratin 5 (K5) [115–117]. These two keratins have long been biochemical markers of the stratified squamous epithelia, including epidermis [115]. In this line, the obtained results will only serve as an indication of the possible effect of the assayed extract on the skin as only CK14 was evaluated. Moreover, regarding these results, there was no prediction since no studies were found that related products fermented by *S. cerevisiae* and/or molecules derived from sugarcane with the biosynthesis of CK14. Nonetheless, microbial benefits have been studied before as a work by Boni *et al*. [118] reported the overproduction of CK14 for a wound site re-epithelization when exposed to a bacterial cellulose from *Acetobacter xylinum*. So, this molecule is reported to help in maintaining the epidermal cell shape, to provide resistance to mechanical stress, and to act as negative regulator of terminal

differentiation of keratinocytes [119]. Moreover, the K5-K14 pair is reported to provide mechanical support in basal keratinocytes [116]. A study by Mendoza-Garcia *et al*. [120] reports that CK14 is known to be closely related with skin tensegrity. In the same study, increased re-epithelization, and extracellular matrix reconstruction and remodelling coincided with an increase of CK14 in the epidermis. Furthermore, the presence of CK14 is reported as potentially important in epidermal replacement by Kurokawa *et al*. [121] and Zhang *et al*. [122]. Some studies also report the K5-K14 apparatus as a regulator of melanin distribution, with an impact on skin pigmentation and tone. For example, a loss-of-function mutation in K5 gene was found in individuals with Downling-Degos disease (progressive and disfiguring reticulate hyperpigmentation of the flexures). Furthermore, it is reported that epidermolysis bullosa simplex (skin disease characterized by blistering, due to mechanical stress-induced degeneration of basal epidermal cells) with mottled pigmentation has also been reported as a disorder of these keratins [123–125].

3.3.4. Skin enzymes inhibition

The obtained results regarding the skin enzymes inhibitory capacity of the fermented extract are represented in Fig. 4.

Regarding neutrophil elastase inhibition, the assayed sample showed an inhibitory effect. The values obtained for relative inhibition were 83.4, 80.7, 62.9, 51.1, and 31.3% at the concentrations of 20, 15, 7.5, 3.75, and 1.875 $mg.mL^{-1}$, respectively. In fact, the inhibitory effect of postbiotics (LactoSporin®) over this enzyme has already been reported [16]. Nonetheless, the amount of information on this subject is still very few. Also, a polyphenol rich sugarcane concentrate (Officinol™) has been shown to inhibit tyrosinase and elastase activities [126]. The same effect caused by sugarcane polyphenols is reported by Carvalho *et al*. [24]. In this work, it is possible to observe that the inhibitory effect was concentration dependent, as it was higher at higher concentrations. However, the two highest concentrations exert a very similar inhibitory effect, suggesting that, at these concentrations, the highest possible inhibitory effect produced by this sample could have almost been reached. Comparing to the inhibition control (SPCK), that presented a relative inhibition of 99.5%, it is reasonable to consider the obtained results as being very promising. The inhibition of neutrophil elastase can represent several advantages for the skin. Human neutrophil elastase, a major product of neutrophils [127], is a protease capable of degrading most connective tissue components, and it has been suggested to participate in the tissue injury of emphysema, rheumatoid arthritis, adult respiratory distress syndrome, and septic shock [128]. This enzyme is suggested to be induced by solar exposure, and it is reported in the literature that neutrophil elastase is strongly associated with solar elastosis, being this the “hallmark” of photoaged skin [127]. Photoaged skin is characterized by dryness, rough texture, irregular pigmentation, and fine and deep wrinkles, among other undesirable features [127, 129, 130]. Starcher and Conrad [131] described neutrophil elastase as an important mediator in the development of solar elastosis resulting from continued exposure to UVB radiation. In this line, a study performed on a hairless mouse model as shown that neutrophil infiltration and neutrophil elastase activity were elevated in photoaging. Furthermore, activated MMP-2 and MMP-1 levels were increased by neutrophil elastase treatment, suggesting that neutrophil elastase indirectly plays a role in skin photoaging through MMP activation [130]. Moreover, a

study by Li *et al*. [132] identified neutrophil elastase as a potential mediator for sun exposure-induced collagen degradation in human skin, by inducing decorin degradation (predominant proteoglycan in human dermis), which binds and protects type I collagen fibrils from proteolytic degradation by enzymes such as MMP-1. Nonetheless, solar elastosis is also described a product of elastic fibers degradation [127], and may result from a cycle of elastase mediated elastin fiber injury, followed by elastin synthesis and repair. The net result over time could be an accumulation of irregular, and thickened elastin fibers [131]. On a different study performed on hairless mice, it was suggested that neutrophil elastase can be an important factor in squamous cell tumour development, suggesting that the inhibition of this enzyme may supress the development of skin tumours [133].

Regarding tyrosinase inhibition, the assayed sample also exerted a considerable inhibitory effect. The values obtained for relative inhibition were 42.3, 40.5, 32.7, 24.7, and 21.3% at the concentrations of 20, 15, 7.5, 3.75, and 1.875 $mg.mL^{-1}$, respectively. The tendencies for this enzyme inhibition were like what happened with neutrophil elastase. Comparing to the inhibition control (NNGH), that presented a relative inhibition of 83.9%, it is reasonable to consider the obtained results as being interesting. Considering the origin of the assayed samples (fermentation of sugarcane straw), some inhibition of tyrosinase should be expected as plant polyphenols are reported as natural tyrosinase inhibitors [134, 135]. In a study, Lee *et al*. [136] demonstrated that, for example, *p*-coumaric acid and caffeic acid were highly effective as tyrosinase inhibitors. Tyrosinase is a copper-containing enzyme which catalyses two rate-limiting reactions in melanogenesis (process by which melanin is synthesized) [134, 137–140]. It is widely distributed in microorganisms, animals and plants and it engages in determining the color of mammalian skin and hair [138]. Therefore, inhibition of tyrosinase has been the prime target for researchers to regulate melanin production. Hyperpigmentation can occur through inflammation of the skin, chronic heat exposure, hormonal imbalance, mechanical stimulation, and medication applications, but under normal physiological conditions, pigmentation is beneficial on the photoprotection of human skin against UV injury [135]. Tyrosinase inhibitors are claimed to have preventive effects on pigmentation disorders (melasma, solar lentigo (age spots), and lentigo simplex (freckles) [135]) as well as skin-whitening effect, and those with high efficacy and less adverse side effects have huge demand in cosmetic and medicinal industries [137]. In fact, the downregulation of tyrosinase has been the most prominent approach for the development of melanogenesis inhibitors [139]. For example, a study by Boissy *et al*. [141] showed that DeoxyArbutin, a reversible tyrosinase inhibitor, had potential tyrosinase inhibitory activity resulting in skin lightening and that it might be used to improve hyper-pigmentary lesions.

3.3.5. Immunostimulatory and anti-inflammatory activities

The obtained results regarding the anti-inflammatory and immunostimulatory activities of the fermented extract are presented in Fig. 5.

Regarding anti-inflammation, the assayed sample showed considerable activity, even though in a concentration-dependent manner. The anti-inflammatory effect is mediated through the regulation of various inflammatory cytokines, such as interleukins (ILs) [142]. In this case, IL-6 was used as biomarker.

The obtained values for the assayed samples were 14.07, and 73.03 pg IL6.ug cell protein^{-1}, for 10 and 1 mg.mL^{-1} concentrations, respectively. When compared to the result of the LPS treatment (70.50 pg IL6.ug cell protein^{-1}), at 10 mg.mL^{-1} concentration was observed a significant decrease ($p/2 < 0.05$) in IL-6 level, indicating a possible anti-inflammatory effect. Furthermore, no immunostimulatory effect was caused by any of the tested sample concentrations. Interleukin 6 (IL-6) is a 184 amino acid proinflammatory cytokine produced by many types of cells and is expressed during several states of cellular stress, such as inflammation, infection, wound sites, and cancer [143]. This is a relevant result since inflammatory states are reported to be related with several dermatological conditions, such as *acne vulgaris* which is characterized by inflammatory papules, pustules, and nodules [144]. Another example is atopic dermatitis, seen as an exaggerated cutaneous immune response to environmental antigens (allergens), and it is a widespread inflammatory skin condition marked by flares and remissions [144, 145]. Moreover, psoriasis is a chronic inflammatory skin disease, and considered to be immune-mediated and organ-specific, and it is characterized by scaly, red cutaneous plaques that contain inflammatory infiltrates and epidermal hyperproliferation [144, 145]. Thus, products such as fermented extracts like the assayed sample, that present anti-inflammatory activity may be explored for the prevention and/or treatment of this kind of conditions. In a study by Ai *et al*. [146], the use of microorganisms, such as *S. cerevisiae*, is seen as a "*a more effective and economical way to convert and synthetize natural compounds with more biological activities*". In the same study, it was shown that fermented ginseng polysaccharides by *S. cerevisiae* exhibited superior antioxidant and anti-inflammatory activities than nonfermented ginseng polysaccharides. Furthermore, *β*-glucans are also reported in the literature as exerting anti-inflammatory properties [142]. Glucans from *S. cerevisiae* are highly reported to present this type of biological activity [142, 147–151]. Nonetheless, in potential future developments it would be important to evaluate the presence of this type of molecule in the tested extract. Also, *S. cerevisiae*-based probiotics are reported to exert anti-inflammatory activity. It was demonstrated that a *S. cerevisiae*-based probiotic markedly reduces the inflammatory response, which is a key player in vaginal candidiasis, and anti-fungal activity against *C. albicans* (one of the main cosmetic contaminants) [152]. In another study, it was demonstrated that synthetic wines, obtained from different *S. cerevisiae* strains exhibited antioxidant and anti-inflammatory properties [153, 154]. Nonetheless, it is also reported that these properties are strain specific. Regarding the used feedstock, sugarcane straw-derived polyphenols are also reported to exert anti-inflammatory activity and specifically reduce IL-6 cytokine expression [24, 155].

3.3.6. Modulation of skin microbiota and metabolism

To evaluate the effect of the fermented extract (at the concentration of 10 mg.mL^{-1}) on the skin microbiota, we determined the relative abundance of specific microbial components in the collected samples from 9 female volunteers. The bacterial load was not altered in skin microbiota with fermented extract (Fig. 6a). Concerning the bacterial genera, the relative abundance of *Staphylococcus*, *Cutibacterium* and *Corynebacterium* genera presented no statistical significant differences between fermented extracts and control groups (Fig. 6b). Additionally, *S. aureus*, *S. epidermidis*, *C. acnes* and *P. innocua* were evaluated in the skin microbiota samples. Our fermented extract statistically significant decreased the relative abundance of *C. acnes* comparing to control (Fig. 6c). This bacterium is highly

described in the literature as having a major role in acne vulgaris development in human skin [156, 157], reason why the obtained results may be an indicator of the potential of the evaluated extract as an anti-acne ingredient. Although large-scale studies on the microbiome of acneic follicles have not yet been performed, published data suggests a dominance of *Cutibacterium*, *Staphylococcus* and *Malassezia* genera (previously known as *Pityrosporum* genus) [158–161]. Interestingly, as described before, the azelaic acid, which applications and effectiveness in *acne vulgaris* treatment were highly reported in the literature [86–89], was found to be present in our extract. In fact, the azelaic acid was also found in the controls, therefore the fermentation process was not responsible for its production. Nonetheless, other phenolic compounds extracts tested by our lab did not show this kind of activity against *C. acnes* (Carvalho, 2023 (unpublished)). In line with this, it seems reasonable to assume that some compound or compounds derived from fermentation might contribute for the anti-acne potential. Despite not presenting significant differences, it matters to understand the impact that some bacterial genera and/or species may have on skin health [162]. For example, the imbalance of skin microbiota, known as dysbiosis, is strongly associated with the progression of psoriasis [162–164] and with chronic inflammatory skin diseases [166]. The relative abundance of *Cutibacterium*, *Corynebacterium* and *Staphylococcus* genera in the skin of individuals with those diseases is altered when compared with healthy individuals [167]. Furthermore, it has been suggested that the presence of *S. aureus* exacerbates the *atopic dermatitis*, since individuals with those diseases presented deficiency in *S. aureus* inhibitors produced by skin commensal bacteria [165, 168].

Regarding fungi, our results demonstrated that fungi community was statistically significant increased after the incubation with fermented extracts in comparison to the control group (Fig. 6a). Additionally, the fermented extract induced a significant decrease of *Malassezia* genus when comparing the control with test groups (Fig. 6b). Yeasts of *Malassezia* genus have pathogenic potential being related with skin diseases such as head and neck dermatitis, seborrheic dermatitis, pityriasis versicolor, and Malassezia folliculitis [169, 170]. In line with this, our extract showed that might have an anti-acne potential and the capability to be useful in the treatment of other skin diseases. For instance, the interactions between *C. acnes* and fungi, in particular *Malassezia* species, appear to be important in the development of dandruff [159, 171]. Biofilms of *C. acnes* and *M. restricta* were observed in a pre-clinical cell-culture-based dandruff model [159, 172]. Currently, the molecular basis for the interactions between *C. acnes* and fungi in these polymicrobial communities is unknown.

4. Conclusions

The main purpose of this work was the sustainable production of postbiotics using a sugarcane by-product, through its sequential saccharification with cellulase and fermentation with *S. cerevisiae*, and its evaluation to be used for the development of a postbiotic ingredient for skincare applications.

The extract had in its composition a sugar alcohol, sorbitol, two SCFAs, acetate and citrate, and two organic acids, azelaic and sebacic acid. In addition, there were several polyphenols from three main groups, being these hydroxybenzoic acids, hydroxycinnamic acids, and flavones. In short, the fermented

extract exhibited potential for cosmetic and skincare applications as it inhibited the activity of skin degrading enzymes (elastase and tyrosinase) and potential inflammatory states. Also, it worked as a stimulus for CK14 production, and as a down-regulator of some skin diseases-associated microorganisms.

Regarding the sugarcane straw, it left the possibility of being a potential promising source of bioactive compounds as a fermentation substrate, with applications in skincare industry, considering a sustainable approach within a circular economy context.

Declarations

Authors contribution

Conceptualization: [Marco Duarte, Ana Amaro, Manuela Pintado, Ana Raquel Madureira]; Methodology: [Marco Duarte, Maria João Carvalho, Nelson Mota de Carvalho, Adélia Mendes, João Azevedo Silva, Inês Pinto Ribeiro, Ana L. S. Oliveira]; Investigation: [Marco Duarte, Adélia Mendes, João Azevedo Silva, Inês Pinto Ribeiro]; Formal Analysis: [Marco Duarte, João Azevedo Silva, Inês Pinto Ribeiro]; Writing – original draft: [Marco Duarte]; Writing – review and editing [João Azevedo Silva, Inês Pinto Ribeiro, Ana L. S. Oliveira, Carla Oliveira, Ana Amaro, Ana Raquel Madureira]; Supervision: [Ana Amaro, Manuela Pintado, Ana Raquel Madureira].

Compliance with ethical standards

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Conflict of interest: The authors have no conflict of interest to declare.

Informed consent: Informed consent was obtained from all individual participants included in the study. Additionally, the participants have consented to the submission of the case report to the journal.

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Figures

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Figure 1

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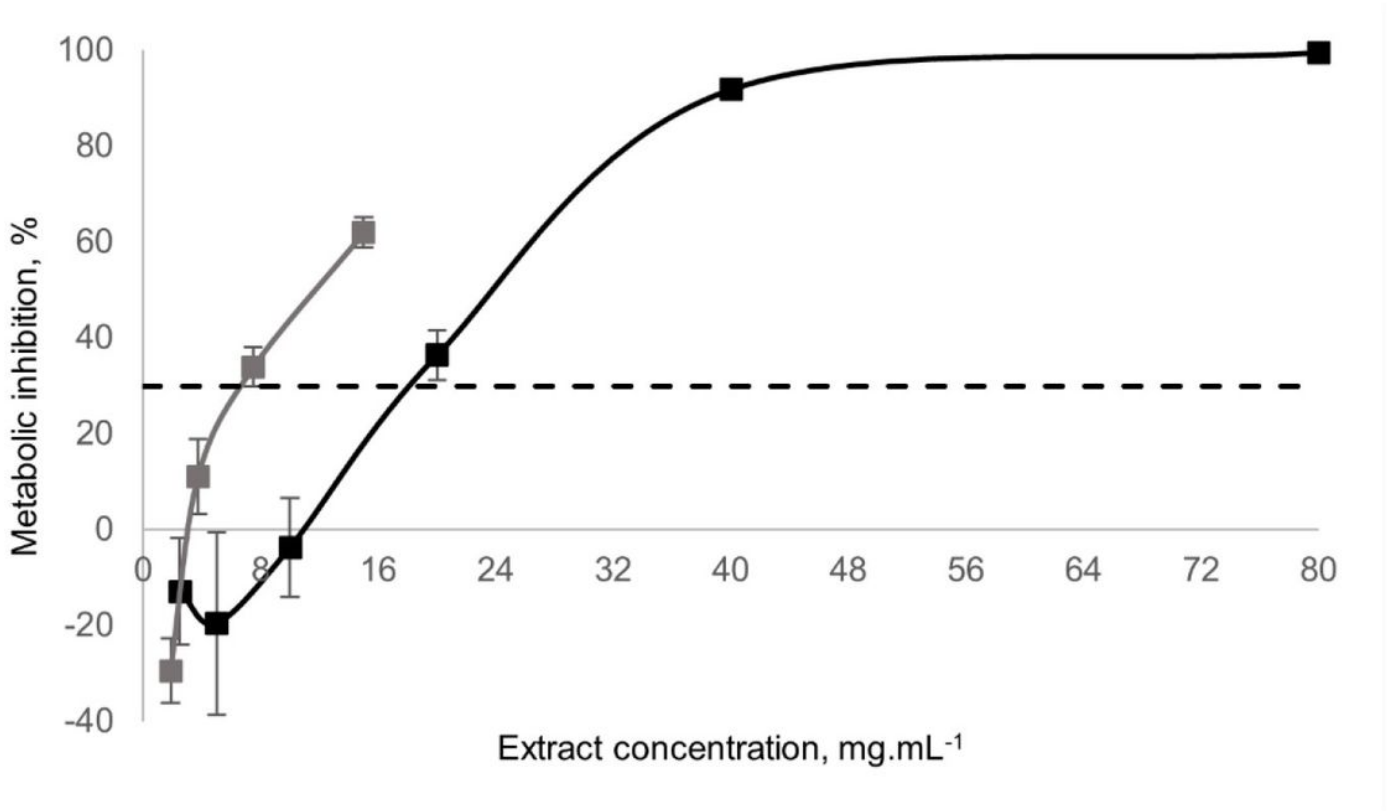

Figure 2

Metabolic inhibition (%, mean ± SD) of the postbiotic extract when in contact with keratinocytes (■) and fibroblasts (■). The dotted line (- - - -) represents the 30 % cytotoxicity limit.

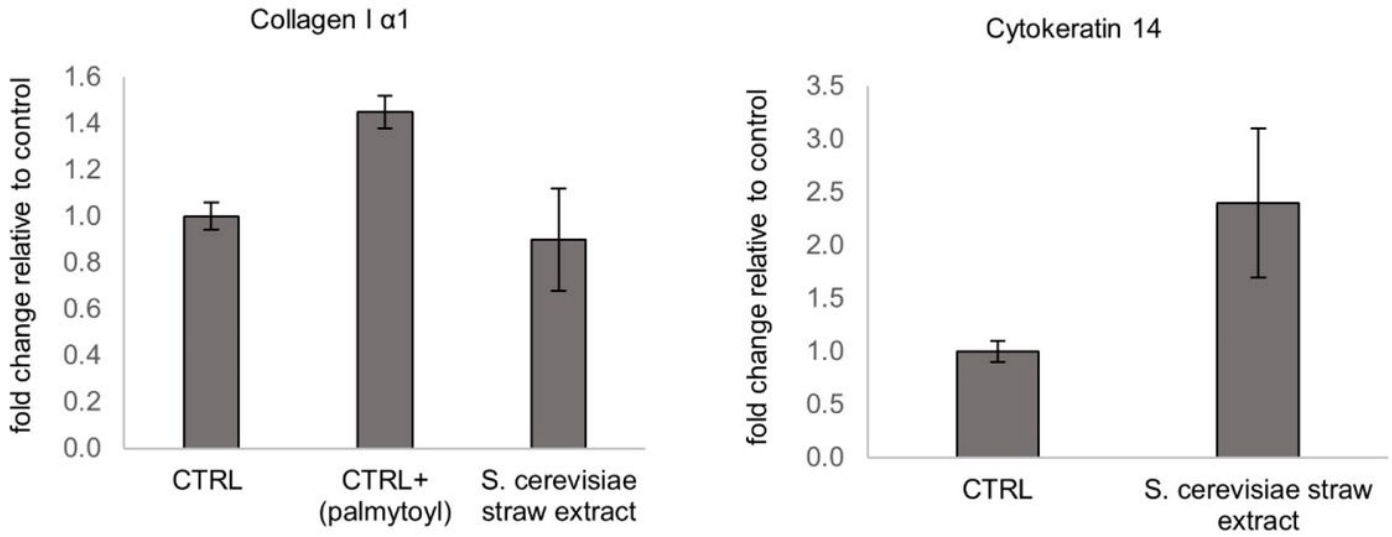

Figure 3

Effect of the fermented extract in collagen I α1 and cytokeratin 14 production by fibroblasts and keratinocytes, respectively. Mean values (solid bars) are expressed as fold change relative to control, and standard deviation is represented by bars.

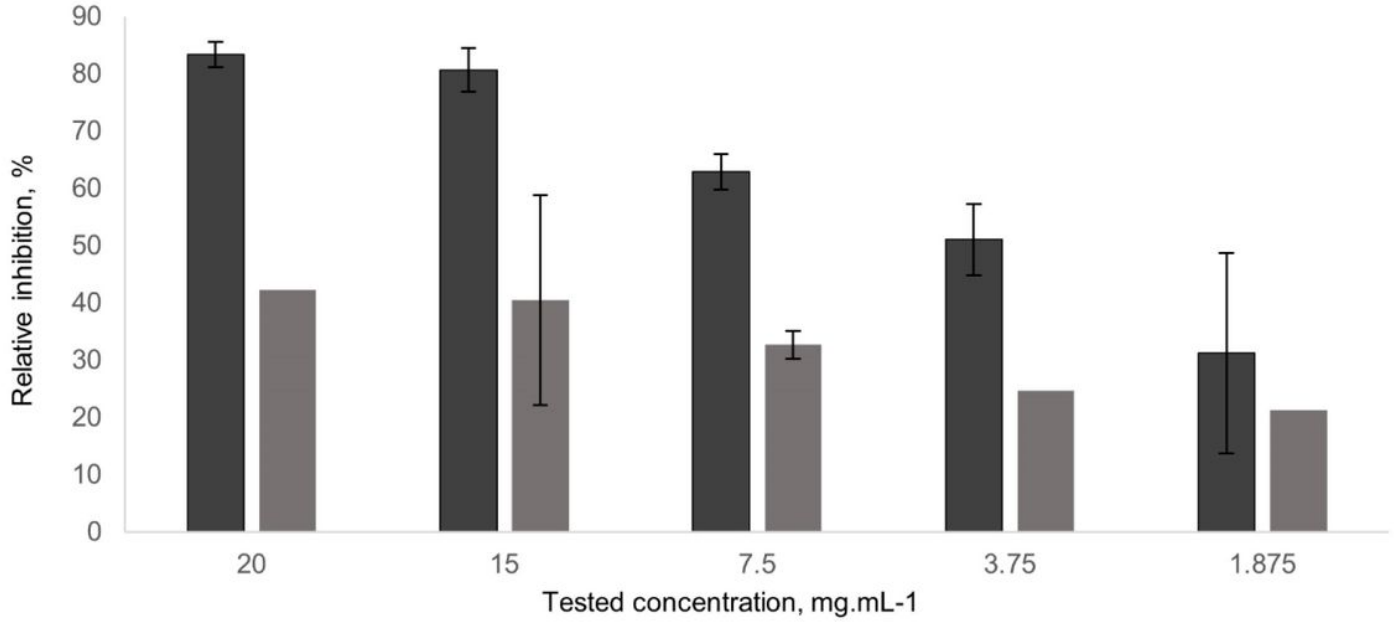

Fig. 4 Relative inhibition of skin enzymes (elastase (■), tyrosinase (■)) by the fermented extract. Mean values (solid bars) are expressed as percentual, and standard deviation is represented by bars.

Figure 4

See image above for figure legend.

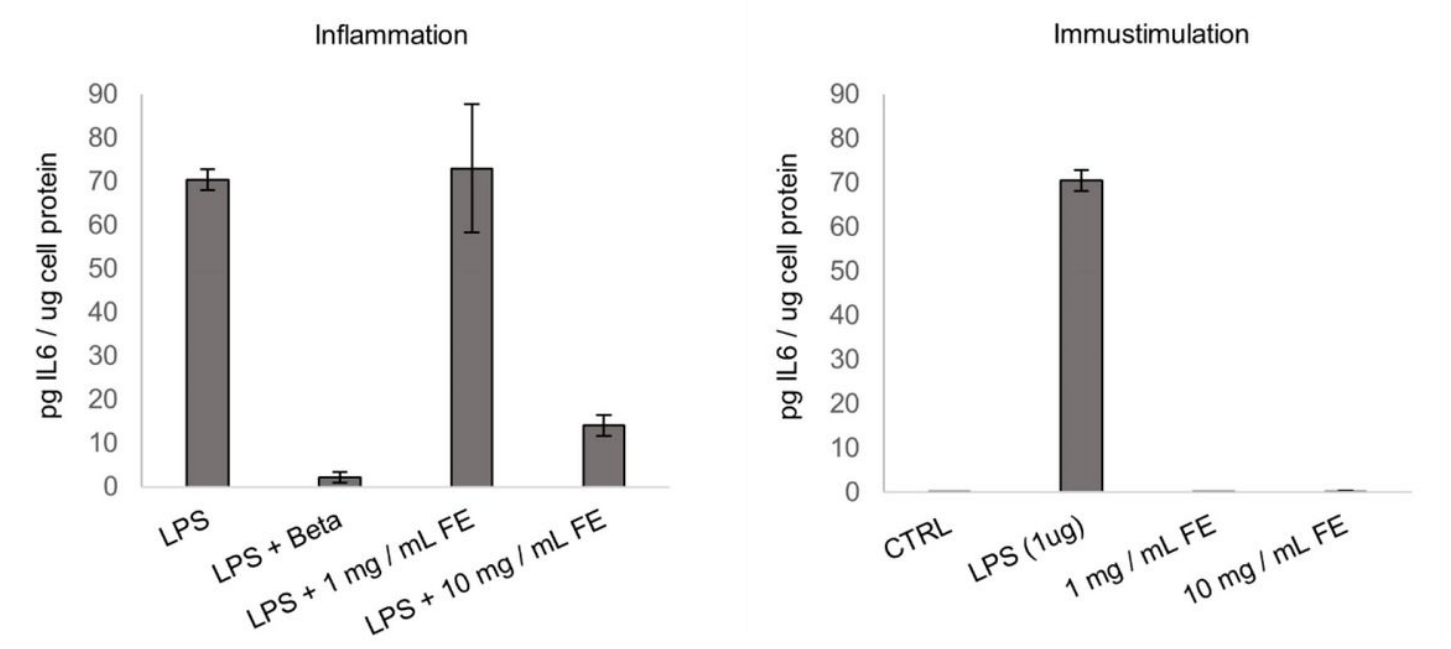

Figure 5

Effect of the fermented extract (FE), at the concentrations of 1 and 10 mg.mL^{-1}, on macrophages by evaluation of IL-6 levels under an inflammatory stimulus (LPS). Mean values (solid bars) are expressed as pg IL-6.µg^{-1} cell protein, and standard deviation is represented by bars. Betamethasone (Beta) was used as an anti-inflammatory control.

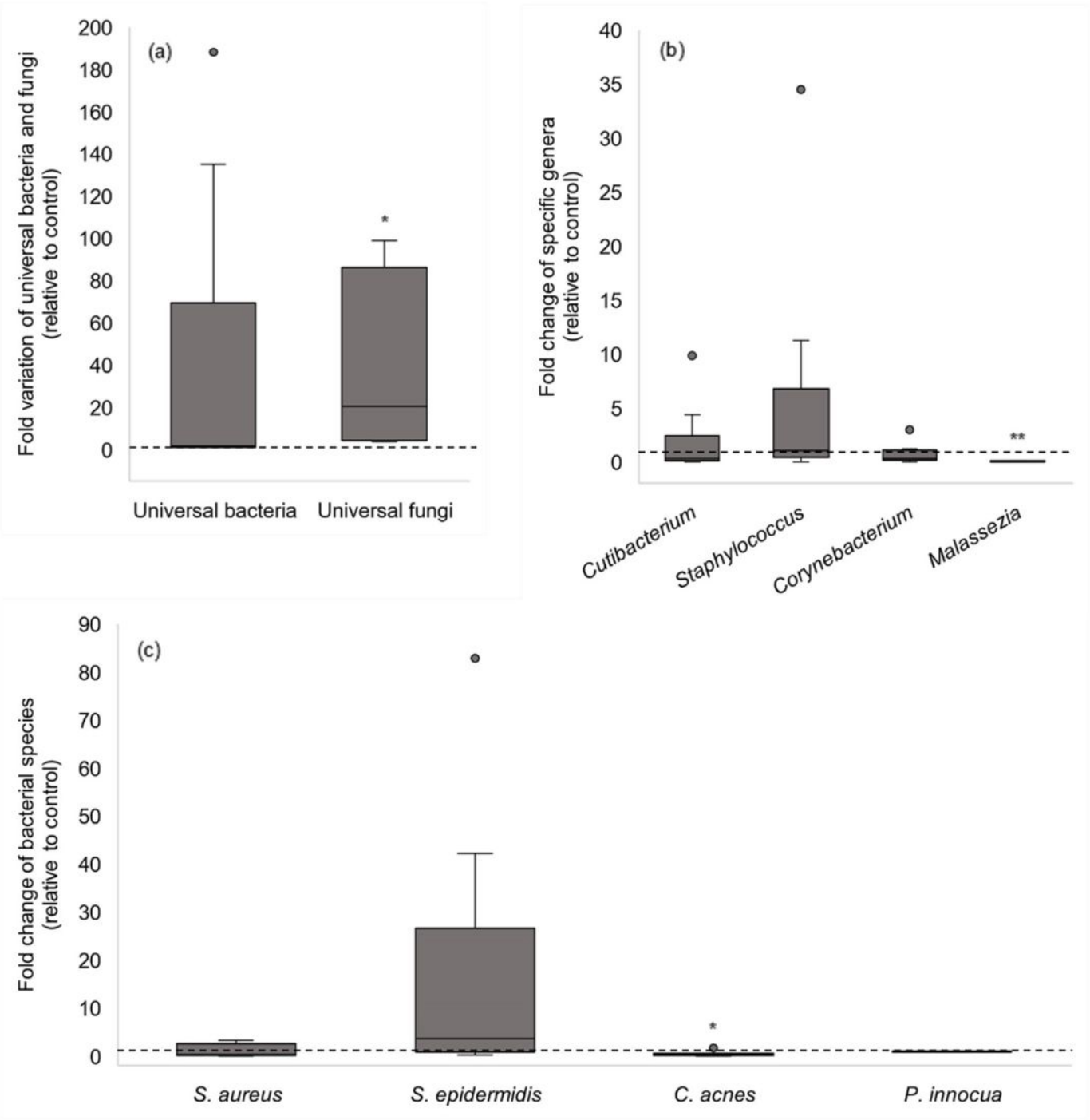

Figure 6

Effect of the fermented extract on the skin microbial (a) universal, (b) genera, and (c) species communities. The results are presented as fold-change (mean ± SEM) relative to control (dotted lines) group (skin microbiota sample without fermented extract). * $p < 0.05$; ** $p < 0.0001$.