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ESCOLA SUPERIOR DE BIOTECNOLOGIA
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SUSTAINABLE POSTBIOTICS FOR COSMETIC AND SKINCARE APPLICATIONS

by

Marco Filipe Pinto Duarte

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Biomedical Engineering

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Marco Filipe Pinto Duarte

Supervision: Dr. Raquel Madureira, PhD and Dr. Ana Amaro, PhD and Dr. Manuela Pintado, PhD

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Resumo

A pele é o maior órgão humano e desempenha funções vitais como proteção, preservação de água e eletrólitos, termorregulação e armazenamento de gordura. A manutenção da pele como limpeza, proteção e alteração é realizada com produtos cosméticos. No desenvolvimento de novos produtos cosméticos, tem sido sugerido o uso de pós-bióticos, uma nova classe de moléculas derivadas dos probióticos, definidos como uma "preparação de microrganismos inanimados e/ou seus componentes que conferem benefícios à saúde do hospedeiro". Podem ser produzidos por fermentação, utilizando meios de culturas com glucose como a fonte de carbono, e como microrganismos fermentativos, bactérias láticas do género *Lactobacillus*, e/ou leveduras, principalmente *Saccharomyces cerevisiae*. Os pós-bióticos compreendem diferentes metabolitos, e apresentam importantes propriedades biológicas (antioxidante, anti-inflamatória, entre outras), razão pela qual o seu uso em cosméticos deve ser considerado. Neste trabalho, a produção de pós-bióticos foi realizada por fermentação com a palha ou o bagaço de cana-de-açúcar, como fonte de carbono e compostos ativos e como processo mais sustentável e promissor na obtenção de extratos mais bioativos. Foi realizado o processo de sacarificação destas biomassas, tendo sido testado o uso de enzimas (Celluclast e Sherazyme Plus), o tipo de substrato (palha e bagaço) e a duração do processo (24h e 48h). A fermentação foi realizada de modo sequencial (SQSF) após a sacarificação, ou em simultâneo com a sacarificação (SSF). Os microrganismos fermentativos testados foram *L. plantarum, L. fermentum* e *S. cerevisiae*. Os extratos produzidos (sem células) foram caracterizados quanto à sua composição e principais propriedades biológicas como a antioxidante e antimicrobiana. O extrato mais promissor foi caracterizado quanto à sua citotoxicidade, atividade anti-inflamatória, produção de citoqueratina 14/colagénio I α1 e impacto na microbiota da pele *ex vivo*. Os melhores extratos foram obtidos realizando fermentações em modo sequencial (SQSF), com bagaço durante 48h e palha durante 72h, usando *L. plantarum* e *S. cerevisiae*, respetivamente. De entre estes, o extrato que apresentou os melhores resultados foi o SQSF *S. cerevisiae* de palha fermentado durante 72h (SQSF ScStr 72h). O seu uso demonstrou ser seguro em concentrações abaixo ou iguais a ~2% para queratinócitos e ~0,7% para fibroblastos. Apresentou atividade antioxidante, com Ec_{50} de 1,88 mg mL^{-1}, e inibiu as atividades de elastase e tirosinase em 83,4% e 42,3%, respetivamente, à concentração máxima testada (2%). Para além disso, promoveu a produção de citoqueratina, e demonstrou atividade anti-inflamatória à concentração de 1%. Por fim, na microbiota da pele de voluntários humanos, o extrato inibiu a bactéria *Cutibacterium acnes* e o género de fungos *Malassezia*. Em suma, foram produzidos pós-bióticos, com sucesso, com palha como substrato, fonte de carbono e de compostos fenólicos. Estes pós-bióticos apresentaram propriedades bioativas que potenciam o seu uso no desenvolvimento de produtos cosméticos e de *skincare* como, por exemplo, o tratamento da acne ou outras doenças da pele, devido ao seu efeito anti-inflamatório e inibitório da bactéria responsável pela acne, bem como de fungos potencialmente colonizadores.

Palavras-chave: Pele; cosméticos; tratamento de pele; pós-bióticos; fermentação.

Abstract

Skin is the largest human organ and performs vital functions such as protection, water and electrolytes preservation, thermoregulation and fat storage. Skin maintenance and preservation such as cleaning, protection and alteration is carried out with cosmetic products. In the development of new cosmetic products, the use of postbiotics has been suggested, a new class of molecules derived from probiotics, 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 yeasts, 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 and bagasse, 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, the saccharification process of these biomasses was carried out, testing the use of enzymes (Celluclast and Shearzyme Plus), the type of substrate (straw or bagasse) and the duration of the process (24h and 48h). Fermentation was carried out sequentially (SQSF) after saccharification, or simultaneously with saccharification (SSF). The fermentative microorganisms tested were *L. plantarum*, *L. fermentum* and *S. cerevisiae*. The extracts produced (cells-free) were characterized regarding their composition, and main biological properties such as antioxidant and antimicrobial. The most promising extract was also characterized for its cytotoxicity, anti-inflammatory activity, cytokeratin 14/collagen I α1 production, and impact on skin microbiota *ex vivo*. The best extracts were obtained by performing fermentations in sequential mode (SQSF), with bagasse for 48h and straw for 72h, using *L. plantarum* and *S. cerevisiae*, respectively. Among these, the extract that showed the best results was the SQSF *S. cerevisiae* with straw fermented for 72h (SQSF ScStr 72h). Its use has been shown to be safe at concentrations below or equal to ~2% for keratinocytes and ~0.7% for fibroblasts. It showed antioxidant activity, with Ec_{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 (2%). In addition, it promoted the production of cytokeratin 14, and demonstrated anti-inflammatory activity at a concentration of 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.

Keywords: Skin; cosmetics; skincare; postbiotics; fermentation.

Contents

Introduction

1. Skin

1.1. Anatomy and functions

Skin is the largest human organ with a 2 m^2 surface and approximately 3.6 kg of weight in adults **(Gilaberte *et al.*, 2016)**. This organ is composed by two distinct layers – epidermis and dermis **(Byrd *et al.*, 2018)**. There are also several associated appendages such as hair follicles, sebaceous and sweat ducts, apocrine glands, and nails **(Walters and Roberts, 2002)**, which constitute the integumentary system that develops from surface ectoderm and the underlying mesenchyme **(Gilaberte *et al.*, 2016)**. The skin provides a life-sustaining interface between the body and the surrounding environment, carrying out important functions such as protection, water and electrolytes preservation , thermoregulation and water and fat storage. Furthermore, skin also plays a major role in the endocrine and immunological systems **(Gilaberte *et al.*, 2016)**. It also produces infectious preventing antimicrobial peptides, hormones, neuropeptides, and cytokines that exert biological effects, not only on the skin but also systemically throughout the whole body.

1.2. Microbiota

The skin microbiota refers to the millions of bacteria, fungi and viruses that inhabit our skin **(Byrd *et al.*, 2018)** and includes two main groups: (i) resident microorganisms, which are a relatively fixed group of microorganisms (the core microbiota), commonly found in the skin and that re-establish themselves after some perturbation. The core skin microbiota is commensal, meaning that these microorganisms are usually harmless and beneficial to the host; and (ii) transient microorganisms which do not establish permanent placement, but rather arise from the environment and persist for hours to days before disappearing. Under normal conditions, both groups should be non-pathogenic **(Dréno *et al.*, 2016)**. Some of the most abundant microorganisms present on skin are *Cutibacterium acnes* (previously known as *Propionibacterium acnes*), *Staphylococcus epidermidis*, *Malassezia restricta*, and *Malassezia globosa* **(Byrd *et al.*, 2018)**.

There is a balanced interplay between the host and resident and/or transient bacterial populations, being this balance continuously affected by intrinsic (host) and extrinsic (environmental) factors which alter skin microorganism communities composition and the host skin barrier function. Changing this equilibrium results in microbiota dysbiosis **(Dréno *et al.*, 2016)**. Skin microbial communities composition was found to be primarily dependent on the skin site physiology, with changes in the relative abundance of bacterial taxa associated with moist, dry, and sebaceous microenvironments **(Byrd *et al.*, 2018)**.

1.2.1. Impact of skin microbiota on skin diseases

Interactions between microbiota members both shape resident microbial community and prevent colonization by pathogenic bacteria, in a process termed 'colonization resistance'. However, in certain contexts, bacteria ordinarily beneficial to their hosts, can become pathogenic and, therefore, many common skin diseases are associated with dysbiosis. This mechanism is often driven by common commensal species and is described in the literature for several skin diseases, such as *acne vulgaris*, *atopic dermatitis* and chronic wounds **(Byrd *et al.*, 2018) (Copper *et al.*, 2015)**.

1.3. Aging

The skin changes that occur over time are much more related to the interaction skin-environment than to genetic predisposition. Therefore, in humans, it is known that skin aging process is mainly related to personal lifestyle. For instance, smoking or having excessive exposure to solar radiation and low air humidity causes wrinkles appearance. In addition, poor diet, and excess alcohol intake, as well as some diseases such as diabetes mellitus, significantly accelerate the premature skin aging **(Silva *et al.*, 2017)**.

There are two independent processes that accelerate skin aging: chronological or intrinsic, and environmental or extrinsic **(Silva *et al.*, 2017)**, being their impact particularly concerning, especially when oxidative stress is involved **(Peres *et al.*, 2011)**. Chronological aging signs usually appear around 30 years of age when the cell renewal work becomes slower and hormone production undergoes changes that reflect directly on the skin. On the other hand, extrinsic agents are the result of exposure to external elements, especially to UV radiation **(Silva *et al.*, 2017)** (a process called photoaging **(Kammeyer and Luiten, 2015)**) without protection, that affects the rate of normal skin aging through the formation of free radicals, which attack the skin structures, destroying collagen and elastin fibers, impairing hydration, with consequences such as: dyschromias, changes in skin relief and wrinkles **(Silva *et al.*, 2017)**. The skin degeneration by UV radiation is a cumulative process and the rate of degeneration depends on the frequency, duration, and intensity of solar exposure and on the natural protection by skin pigmentation **(Kammeyer and Luiten, 2015)**. Both intrinsic and extrinsic mechanisms lead to reduced structural integrity and loss of physiological function **(Kammeyer and Luiten, 2015)**.

1.3.1. Reactive oxygen species (ROS) and skin cells oxidation

Reactive oxygen species (ROS) play an important role in skin aging. In the skin, about 1.5-5% of the consumed oxygen is converted into ROS, by intrinsic processes. ROS are continuously produced as side products of the skin cells aerobic metabolism and are considered the main cause of intrinsic aging. Keratinocytes and fibroblasts are the main producers of 'mitochondrial' ROS in the skin, with reactive superoxide anion radical ($\bullet O_2^-$) as the most predominantly formed one, by the addition of an electron to each oxygen (O_2) molecule. These ROS-particles abundant generation may harm the cellular function **(Kammeyer and Luiten, 2015)**. ROS have also been

associated with the process of UV-induced skin damage, including photoaging, immunomodulation, melanogenesis and, ultimately, photo-carcinogenesis. Besides direct photochemical reactions, UVB generates a high level of ROS in the skin resulting in photooxidative damage of the cells and extracellular matrix (ECM) **(Peres *et al.*, 2011)**.

Moreover, events such as cellular senescence (loss of cells replicative ability) and the increased expression of enzymes that degrade the dermis ECM (skin aging processes) may be triggered by ROS but can also be reduced by antioxidants **(Kammeyer and Luiten, 2015)**. Antioxidants are compounds that inhibit or block the process of formation of free radicals. They must exhibit a redox potential lower than that of the compound that they want to protect, i.e., they must be oxidized first than the protected agent.

2. Cosmetics

Why is the skin appearance important? Skin is the outermost tissue of the human body. As a result, people are aware of, and very sensitive to, the appearance of their skin. Consequently, skin appearance has been a subject of great interest in several fields of science and technology. The desire to have beautiful and healthy-looking skin has been a centuries-old quest for humans, as skin with brighter complexion and smoother surface tends to be perceived as healthier and more attractive **(Igarashi *et al.*, 2007)**. It is in this context that cosmetics begin to emerge as products whose primary function would be to "hide" imperfections, especially when related to the skin.

So, the term 'cosmetics' derives from the Greek "Kosm tikos" meaning 'having the power to arrange, skilled in decoration'. The Council of European Union Regulation defines cosmetic product as "any substance or mixture intended to be placed in contact with external parts of the body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity, with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition, or correcting body odours" **(Halla *et al.*, 2018)**.

Cosmetics can be classified according their use, fields of application, functions, form of preparation, consumer's age, or gender as: (1) cosmetics for personal cleansing (soaps, deodorants, shampoos); (2) cosmetics for the skin, hair, and integument care (toothpastes, products for external intimate care); (3) cosmetics for embellishment (perfumes, lip colors); (4) protective cosmetics (solar products, anti-wrinkle products); (5) corrective cosmetics (beauty masks, hair dyes); (6) maintenance cosmetics (shaving cream, moisturizing creams); and (7) active cosmetics (fluoridated toothpastes, antiseptics) **(Halla *et al.*, 2018)**.

2.1. Cosmetics preservation and microbiological safety

A preservative is a natural or synthetic ingredient that is added to products, to prevent them from spoiling. In the case of cosmetics and skincare products, preservatives ensure that these remain

safe during their period of use by consumers. Without these ingredients, microbial contamination of cosmetic products is more likely to happen, increasing irritation or infection risk, especially in products used around the eyes and on the skin **(European Commission, n.d.)**.

2.1.1. The potential role of antioxidants in cosmetic preservation

The use of antioxidant ingredients can also be considered important for maintaining the formulations stability. The fragrance and color of a cosmetic product are decisive factors in the product acceptance by consumers. Thus, the protection of lipid oxidation (most common type of oxidation, occurring in oils and fats) is important, since many products have limited shelf life due to oxidation of the excipients, resulting in fragrance and color alteration, for example. Interestingly, lipid oxidation cannot be avoided or stopped completely. Nonetheless, it can be reduced to achieve a significant increase in stability and useful life of the final product. Thus, some antioxidants are used in the formulation to protect the product, while others are used to protect the application site such as the skin **(Silva *et al.*, 2017)**.

2.1.2. Common microbial contaminants

In general, all products, including cosmetics, containing water and organic/inorganic compounds under appropriate physicochemical conditions, are exposed to microbial contamination. An ideal preservation system should protect the product from microbial degradation, both in its original closed packaging until use, and in an open container throughout its use. The main cosmetics contaminant microorganisms are *Pseudomonas aeruginosa*, *Klebsiella oxytoca*, *Burkholderia cepacian*, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Enterobacter gergoviae*, and *Serratia marcescens* **(Halla *et al.*, 2018)**.

Beyond product degradation, and although the skin and mucous membranes are protected against microorganisms, the presence of these in such products can increase the risk of microbial infection. Nonetheless, cosmetics are not supposed to be sterile, but they must not contain excessive quantities of specified microorganisms or microorganisms which may affect the quality of the product or the safety of the consumer **(Halla *et al.*, 2018)**. Microbiological specifications according to international regulations are described, by region (United States of America, Europe, and Japan), in **Halla *et al.* (2018)** review.

3. Postbiotics for cosmetic and skincare purposes

In recent years, the skin microbiota and its interactions with the skin itself and its environment have been the aim of the cosmetics and dermatology fields **(Souak *et al.*, 2021)**. Many research studies have been reporting the beneficial health effects of prebiotics and probiotics as both act by increasing the expression of some beneficial bacteria, and indirectly they influence the host's immune system, consequently influencing the respective microbiome. Derived from the probiotics, a new type of health-promoting molecules named postbiotics has claimed increasing

attention. These molecules can be metabolites from different origins and of several different types and although the mechanisms involved in their action are not clear yet, different biological properties, i.e., antimicrobial, antioxidant and immunomodulatory, have been assigned to them **(Rinaldi *et al*., 2020b)**. Technologically, these compounds are superior to probiotics since no viability in the topical formulation is required. Adding to that, postbiotics have longer shelf life (increased stability), greater safety (absence of living microorganisms), possess multiple health benefits **(Ciardiello *et al*., 2020; Majeed *et al*., 2020a)**, and they also can be administered in a safe way to immune-deficient or compromised patients for which live probiotics are not allowed. Nowadays postbiotics could represent an evolution in the way by which human health benefits derived from microorganisms can be obtained **(Rinaldi *et al*., 2020b)**.

3.1. How to define postbiotics

The World Health Organization (WHO) defines probiotics as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host" **(Mack, 2005**). This definition has been grammatically revised by an International Scientific Association of Probiotics and Prebiotics (ISAPP), a convened panel of experts, to "live microorganisms that, when administrated in adequate amounts, confer a health benefit on the host", and the panel supports the use of this wording going forward **(Hill *et al*., 2014)**. The ISAPP also proposes that a postbiotic is a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" **(Salminen *et al*., 2021)**. Comparing the two concepts, it seems that the distinction between both becomes clear as probiotics are evidently defined as "live microorganisms", and postbiotics as "inanimate microorganisms". Following this line of thought, the introduction of the 'postbiotic' term was an attempt to describe a product consisting of dead microorganisms, fractions and lysates or metabolites secreted by live microorganisms or released after cellular lysis. These include enzymes, peptides, teichoic acids, cell surface proteins, polysaccharides, and organic acids which promote health benefits to the host **(Aguilar-Toalá *et al*., 2018; Koleilat, 2019; Sabinsa, 2021; Żółkiewicz *et al*., 2020)**. Furthermore, postbiotics have several advantages over probiotics including biological activity in a non-viable state, a lower chance for microbial translocation and infection, improved inflammatory defence, favourable physicochemical properties (solubility), and advantageous pharmacokinetics properties (absorption, distribution, metabolism, and excretion) **(Sabinsa, 2021)**. Postbiotics have also been explored due to their safety dose parameters, long shelf life and the content of various signalling molecules possibly exhibiting anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, hypo-cholesterolemic, anti-proliferative, and antioxidant activities **(Aguilar-Toalá *et al*., 2018)**. Additionally, it has been reported that probiotic bacteria may not be safe at all times leading to an increasing interest in the application of cell components and metabolites derived from probiotics themselves **(Teame *et al*., 2020)**. In fact, some recent papers from prestigious medical journals, such as Lancet, New England Journal of Medicine, and Nature, have started to question the actual safety of probiotics **(Freedman *et al*., 2018; Kunk, 2019;**

Schnadower *et al*., 2018; Suez *et al*., 2019). Nevertheless, probiotics are "a *priori* nonpathogenic", which means that they are not meant to cause any adverse effects on the host, independently of the source of their intake **(Žuntar *et al*., 2020)**. For example, *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* are claimed to have GRAS (generally recognized as safe) status in fermented foods and beverages **(Koirala and Anal, 2021; Žuntar *et al*., 2020)**. Following this, the safety of probiotics should not be taken as a linear and totally predictable factor. It may depend on the type of microorganism used, on the application for which it is intended, or even on the condition of the individual in question (age, pregnancy, immunosuppression, etc.) **(Žuntar *et al*., 2020)**.

3.2. Postbiotics types and their identified biological effects

As mentioned before, the term postbiotics refers to many different types of metabolites exhibiting health benefits. In **Table 1** we can observe a short summary of some *in vitro* and *in vivo* studies of postbiotics and their bioactivity and/or effects. In most of the cases presented in **Table 1**, tested postbiotics are intracellular compounds, cell-wall components and/or cell-free supernatants without metabolite specification/individualization. Nonetheless, other studies refer to some specific metabolites such as exopolysaccharides, biosurfactants or lipoteichoic acid as "a major cell-wall factor of Gram-positive bacteria" **(Tominari *et al*., 2021)**. In most cases referred to on **Table 1**, the active compounds are produced by lactic acid bacteria and demonstrate many potential health benefits like immunomodulatory, antioxidant, anti-inflammatory, antimicrobial, and anti-aging/anti-senescence activities. Moreover, lipoteichoic acid shows an immunomodulatory effect in different types of cells (i.e., human monocyte THP-1-cells, human HT29 cells, and raw 264.7 macrophages) **(Matsuguchi *et al*., 2003; Sokol *et al*., 2008; Vidal *et al*., 2002)** which, even though that no study presented has been done on skin cells, may indicate a potential transfer of these benefits towards skin care cosmetic applications. Interestingly, lipoteichoic acid also demonstrates some capacity to induce different effects such as anti-aging **(Wang, S. *et al*., 2020)**.

3.3. Postbiotics in cosmetic products market

Cosmetic ingredients that may promote a healthy microbiome (like postbiotics) and that are mainly derived from fermented products have drawn much interest recently **(Ciardiello *et al*., 2020)**. The products derived from the fermentation of microorganisms, like antimicrobial peptides and fragments of dead cells - the so called postbiotics - have been highlighted due to their physiological effects. The concept has been growing since the majority of the health benefits from microorganisms come from their metabolites. Following this thought, when compared to probiotics, postbiotics are reported to induce similar beneficial effects but without the presence of live microorganisms **(Majeed *et al*., 2020b)**. For instance, the *Lactobacillus* species (probiotics) and their metabolites are reported to enhance skin hydration, elasticity, gloss, and reduce the extent of wrinkles in the skin **(Kimoto-Nira *et al*., 2012; Lee *et al*., 2015; Majeed *et al*., 2020b)**.

The principal benefits of the use of postbiotics over probiotics come from their higher specificity of action on the resident microbiota as of interaction with the cells of the host **(Ciardiello *et al*., 2020)**. For these reasons, postbiotics can help improve several cosmetic and dermatological conditions in which the influence of the microbiome has been hypothesized. Postbiotics are usually obtained through microbial fermentation and although the benefits of fermented products are mostly described for gut microbiome modulation **(Aguilar-Toalá *et al*., 2018; Tsilingiri and Rescigno, 2013; Yan *et al*., 2007; Yan *et al*., 2013)**, their effect on skin microbiome has also been hypothesized **(Callewaert *et al*., 2021; Ciardiello *et al*., 2020)**. In fact, the fermentation process is connected to the overproduction or ex-novo production of bioactive molecules exhibiting health benefits from antimicrobial to immunomodulatory, anti-inflammatory, anti-proliferative and antioxidant activity **(Ciardiello *et al*., 2020)**. Even though a few studies using probiotic metabolites have shown a positive effect on the skin **(Guéniche *et al*., 2010; Hoang *et al*., 2010; Lew and Liong, 2013)**, the use of postbiotics for skin applications is still in the beginning **(Majeed *et al*., 2020b)**. **Table 2** presents several postbiotics that are already produced and commercialized for cosmetic purposes by some companies. The key players involved in this market are companies that operate in several areas such as the chemical industry (e.g., Clariant), food innovation, pharmaceutical, and cosmetic industries (e.g., Sabinsa). These types of companies aim to contribute to the creation of innovative and sustainable solutions for customers **(Clariant, 2021)** and some of them focus on 'clean solutions' providing evidence-based natural products for human well-being **(Sabinsa, 2021)**, as the need for renewable resources is increasingly urgent. Companies aim to prioritise and address the most recent key trends as these also include energy efficiency, emission-free mobility, and finite resources conservation **(Clariant, 2021)**.

As postbiotics are an emerging trend, there are continuous research and ongoing development activities to find out what their potential applications may be. Following this, the governments of many countries are providing financial support for the research activities in the global postbiotics market to aid the expansion of this market. Some regions like North America, Europe, and the Middle East, amongst others, are key regions in the postbiotics market, North America being the dominant region. The main reasons supporting this expansion are the increased consumption of innovative cosmetic products and the predictable easy accessibility of cosmetic postbiotics in the near future which already happens with food products containing postbiotics through pharmacies and other distribution channels. Consequently, major enterprises are focusing on the development of innovative products that can fulfil the needs of the end user. To achieve this, there is a growing investment going on in the industry towards research and development and also a very intense focus on strengthening distribution channels. All of this promises a bright future for the cosmetic postbiotics market **(Transparency Research Market, n.d.)**.

As observed in **Table 2**, most of the postbiotics in the market are derived from bacteria, especially lactic acid bacteria and *Lactobacillus* genera. Few are from yeast, but when derived from these microorganisms most are from *Saccharomyces cerevisiae.* A great part are metabolites from lysates resulting from the microorganisms that are secreted/produced exogenously to the culture

media and further separated. Also, most of these lysates components are organic acids, teichoic acids, (exo)polysaccharides, cell-wall fragments, peptides, and cytoplasmic compounds, amongst others.

3.3.1. Production: processes and conditions

Postbiotics can be produced/obtained through processes that are extensively described in the literature. These processes have some essential steps such as (1) bacterial strains growth, (2) cell washing and resuspension, (3) cell disruption and (4) medium final centrifugation.

Bacterial strains are normally grown in modified media (e.g., MRS broth, etc) supplemented with determined substances (e.g., cysteine, etc), and under well-defined conditions (temperature, time, supplement quantity, etc). After cellular growth, and in case only intracellular content is wanted, cells are harvested by centrifugation and the cell pellets are washed usually at least three times with a buffer solution or deionized water, and then disrupted by methods like ultrasonication, high-pressure homogenization, using a French press, etc. To maintain the extracellular metabolites, cell washing is not performed if the aim is only supernatant (extracellular metabolites) or supernatant together with the intracellular content. If intracellular content is not the goal, cell disruption is not performed as well. Nevertheless, in all scenarios, the final step must be medium centrifugation to remove intact cells and/or cellular debris **(Abbas *et al*., 2016; Amaretti *et al*., 2013; Cicenia *et al*., 2016; Jensen *et al*., 2010; Kim *et al*., 2006; Kumar *et al*., 2020; Lee *et al*., 2002; Merghni *et al*., 2017; Ou *et al*., 2006; Sarikaya *et al*., 2017; Tejada-Simon and Pestka, 1999; Vidal *et al*., 2002; Wang, K. *et al*., 2020; Wang, S. *et al*., 2020; Zhang *et al*., 2011)**.

On the other hand, there are some works performed using different substrates as a sugar source. For example, in a research study, **Amiri *et al*. (2021b)** tested several variables (temperature, incubation time, and yeast extract concentration) to optimize a fermentative process for the co-production of postbiotics (conjugated linoleic acid, exopolysaccharides, and bacteriocins) by *Bifidobacterium lactis* BB12 in cheese whey. The fermentation bioprocess was performed in Erlenmeyer flasks with medium, inoculated with the mentioned bacterial strain. The fermentation medium consisted of a cheese whey broth (without precipitates) with adjusted pH 4.5 supplemented with linoleic acid in 2% Tween 80 and yeast extract. In conclusion, the researchers found the optimal conditions (incubation temperature (38 ºC), time (28 h), and supplement concentration (2.5 % yeast extract)) for the performed process. Nonetheless, it´s expected that these optimal conditions may vary from process to process. Also, there is a huge opportunity to find new substrates sources on by-products to produce postbiotics taking advantage of other compounds present in these substrates that could enhance the bioactivities of postbiotic extracts, even though there aren´t many reported studies using this type of substrate for postbiotics production whether for food or cosmetic purposes. For instance, besides cheese whey, milk permeate, and lignocellulosic biomass have also been reported as used substrates in the production of postbiotics **(Amiri *et al*., 2021a; Trif *et al*., 2021)**. This considered, the use of substrates derived from agricultural and industrial activities should be seriously considered as

they are abundant, cheap and renewable. Their use has the potential to significantly reduce the production costs of raw material as well as to minimize waste accumulation **(Vandenberghe *et al*., 2018)**. Following this, corn steep liquor, malt wastes, soybean meal, cotton seed, wheat and rice bran and fish waste have been reported as possible sources of nutrients **(Dhillon *et al*., 2013; Wee and Ryu, 2010)**, but on the other hand, starchy and algal biomasses, and molasses and soybean vinasses have been considered as carbon sources **(Karp *et al*., 2011; Rodrigues *et al*., 2017)**.

Regarding industrial production of postbiotics, many companies usually have their own patented fermentation procedure. According to the Bio Component Research company, Dermaspring IQ, Dermaforce IQ, Elastiflex IQ, Royal Tea IQ, and Gentleguard IQ (all postbiotics) are produced by a proprietary fermentation solution (Bio-Hacked BCR™) that combines biome-friendly probiotics, *Lactobacillus plantarum* and *Saccharomyces boulardi* with prebiotic superfoods. The process yields a postbiotic broth brimming with micronutrients that are supportive of the environment of the skin microbiome. It is a clean and sustainable methodology that extracts a full spectrum of plant-derived actives with enhanced bioavailability to boost efficacy and accelerate the onset of benefits to the skin **(Bio Component Research, n.d.)**. In Chemisches Laboratorium Dr. Kurt Richter GmbH company, Repair Complex CLR™ PF, a postbiotic product is obtained by fermentation of *Bifidobacterium longum*. After the growth is complete, the bacteria are disintegrated using ultrasound, releasing the cytoplasm fractions and cell wall constituents. After cell disintegration, no cell fractions are isolated thus ensuring the presence of all constituents in their natural distribution in the product **(Chemisches Laboratorium Dr. Kurt Richter GmbH, n.d.)**. In another example, EPS Seapur®, produced by Codif, is obtained by the fermentation of marine planktonic microorganisms in which the released exopolysaccharides are extracted and purified, and the hydro thermolysis process accelerated with supercritical CO_2 **(Codif, n.d.)**. Finally, the company Kalichem produces Kalibiome postbiotics by a patented fermentation process carried in minimal media and controlled conditions. The obtained product is living bacteria and bacterial fragments/toxins free, and it only contains ferment actives, i.e., active metabolites produced by fermentation **(Kalichem, n.d.)**.

Industrially, there is also a trend in the use of differentiated substrates of microorganisms, sugars, and metabolites, other than just the regular use of culture media and pure cultures. An example is TR-PRP plus-Celsi gel in which the main active ingredients include various postbiotics like plantaricin A (Pln A) and *Lactobacillus kunkei* bee bread (a fermented product and postbiotic). As another example, postbiotic Lactobiotyl® by Silab, a cyclic polyols pinitol type produced from jojoba fermentation by *Lactobacillus arizonensis* **(Fournière *et al*., 2020)**. These two scenarios show that there is an opportunity to use different substrates to produce postbiotics such as fermented products or even the use of other sources of sugars to feed the fermentation purposes.

3.3.2. Scientific evidence of postbiotics benefits on skin

A considerable amount of research and information shows that postbiotics demonstrate tremendous potential for skin care cosmetic applications. These molecules have shown the ability

to improve certain aspects related to skin health in different ways. Regarding the skin microbiota and function, some postbiotics have been shown to respect microbiota equilibrium and to restore/improve the skin barrier integrity. Other postbiotics have shown antioxidant activity and improved UV protection (UV radiation is the main cause of skin cells oxidative stress and aging), delaying the aging process of skin cells. Some of these postbiotics have also shown to inhibit certain enzymes associated with extracellular matrix disintegration. Moreover, certain postbiotics up/downregulate some genes to potentially reduce inflammatory response. Ultimately, some of these molecules also demonstrate antimicrobial activity with the potential to fight some skin conditions where the microbiota influence has been hypothesized (e.g., acne vulgaris, scalp infectious diseases).

3.3.2.1. Antioxidant potential and UV radiation effects block

The claims that are made for these products are diversified. Some formulations are claimed to possess antioxidant activity and block the UV radiation effects and protect or restore the balance of the cutaneous microbiota due to their antioxidant and/or anti-inflammatory activities **(Ngoc *et al*., 2019; Souak *et al*., 2021)**. Some products (i.e., LactoSporin®, by Sabinsa) have been shown to have antioxidant activity and found to protect skin cells from UV-induced apoptosis and cell death **(Majeed *et al*., 2020b)**. These effects may happen because of the indirect photoprotective properties of some compounds with origin in the cutaneous microbiota. Furthermore, some of the bacteria already have direct UV radiation blocking or absorbing effects as well as anti-inflammatory and anti-oxidative activities **(Souak *et al*., 2021)**.

3.3.2.2. Anti-inflammatory effect

LactoSporin® is also claimed to improve skin inflammation on both open and closed comedones (small bumps frequently found on the forehead and chin of those with acne) and to improve other signs of inflammation like redness and skin texture. For example, LactoSporin® treatment was as fast as three days suggesting rapid efficacy in relieving symptoms **(Majeed *et al*., 2020a)**. Moreover, bacteriocins produced by *Enterococcus faecalis* SL-5 in patients with acne lesions caused by *C. acnes* have shown the capacity to significantly reduce the inflammatory lesions and pustules **(Kang *et al*., 2009)**. Following these thoughts on anti-acne treatments, other products (Active EPS Seapur®, by Codif) are based in exopolysaccharide solutions from the fermentation of marine planktonic microorganisms and are suggested to re-equilibrate the *S. epidermidis* and *C. acnes* (skin commensal bacteria) ratio and decrease the inflammation induced by bacterial stress **(Fournière *et al*., 2020)**.

3.3.2.3. Skin microbiota equilibrium and skin barrier/immunity boost

Other products (i.e., Ecobiotys®, by Silab) based on yeast extract enriched with biopeptides from *Metschnikowia reukaufii* are identified as to specifically re-equilibrate the microbiota of mature skin and so restore the function of the immune skin barrier. Bacterial extracts from *Lactobacillus pentosus* (the active ingredient of Phytobioactive Biotilys®, by Greentech) also showed to have

significant effects on skin immunity after 6 days of treatment at 2% on an ex vivo skin mode which significantly increased (by 229%) hBD2 expression and decreased (by 57%) TLR2 expression **(Fournière *et al*., 2020)**. Some postbiotics (i.e., Lactobiotyl®, by Silab) were also shown to respect microbiota equilibrium, improve skin barrier integrity by cohesion and structure of the protein and lipidic markers and accelerate epidermal renewal. This postbiotic is based on a cyclic polyols pinitol type produced from jojoba fermentation by *Lactobacillus arizonensis* **(Fournière *et al*., 2020)**.

3.3.2.4. Skin enzymes inhibition

Skin enzymes inhibition has also been reported when using LactoSporin®, which inhibited collagenase, elastase, and hyaluronidase activities. Moreover, it upregulated hyaluronan synthase expression transforming growth factor and epidermal growth factor being these factors related to extracellular matrix integrity. In short, this suggests that LactoSporin® may function as a skin protector with wide potential applications in cosmetic formulations (**Majeed *et al*., 2020b)**.

3.3.2.5. Antimicrobial effect

Regarding antimicrobial activity against pathogens, LactoSporin® was shown to inhibit various skin pathogen bacteria such as *Pseudomonas aeruginosa*, an opportunistic pathogen, and *S. aureus* and *S. epidermidis*, the two most relevant skin pathogens. Nonetheless, its most relevant inhibitory effect was shown against *C. acnes*, the acne-causing bacteria, showing a minimum inhibitory concentration (MIC) of 4% (**Majeed *et al*., 2020a)**.

3.3.2.6. Treatment of dermatological diseases

Other postbiotics such as TR-PRP plus Celsi gel are shown to be a solution to treat dermatological diseases such as Alopecia areata (AA), where a significant improvement in hair regrowth is observed in the subjects treated with the product. Furthermore, both Plantaricin A (Pln A), a bacteriocin produced by *L. plantarum* with antimicrobial and pheromone activities **(Rinaldi *et al*., 2020b)**, and *Lactobacillus kunkei* bee bread (fermented product) (two of the TR-PRP plus Celsi gel components) have been shown to have a strong antioxidant activity **(Di Cagno *et al*., 2019; Marzani *et al*., 2012; Rinaldi *et al*., 2020a)**. This is a very interesting area considering the potential for hair growth, scalp skincare-related products and the importance of skin microbiome in the protection of the scalp and development of diseases.

Table 1 - Postbiotics (types of metabolites) and their potential biological effect

Microorganism	Components	Type of study	Biological effect	Reference source
Bifidobacterium, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus gasseri, Lactobacillus helveticus, Latobacillus reuteri & *Streptococcus thermophilus*	Cell wall components and cytoplasmic extract	RAW 264.7 macrophage cell line	Immunomodulation	Tejada-Simon and Pestka, 1999
Faecalibacterium prausnitzii A2–165 (DSM 17677)	Cytosolic fraction	Caco-2 cells		Sokol *et al*., 2008
Lactobacillus plantarum K8 (KCTC10887BP)	Lipoteichoic acid	Human monocyte THP-1 cells		Kim *et al*., 2011
Bifidobacterium bifidum BGN4	Cell-free extract, purified cell wall and supernatant	RAW 264.7 cells		Lee *et al*., 2002
Lactobacillus johnsonii La1 & *L. acidophilus* La10	Lipoteichoic acid	Human HT29 cell line		Vidal *et al*., 2002
L. casei YIT 9029 & *Lactobacillus fermentum* YIT 0159	Lipoteichoic acid	RAW 264.7 macrophages		Matsuguchi *et al*., 2003
Bacillus coagulans	Cell-wall components	Human polymorphonuclear cells	Immunomodulatory and anti-inflammatory	Jensen *et al*., 2010
Steptococcus salivarius ssp. *thermophilus* ATCC 19258 & *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842	Intracellular content	*In vitro*	Antioxidant	Ou *et al*., 2006
L. acidophilus KCTC 3111, *Lactobacillus johnsonii* KCTC 3141, *L. acidophilus* KCTC 3151 & *Lactobacillus brevis* KCTC 3498	Intracellular content	*In vitro*		Kim *et al*., 2006
L. casei subsp. *casei* SY13 and *L. delbrueckii* subsp. *bulgaricus* LJJ	Intracellular content	*In vitro*		Zhang *et al*., 2011
Bifidobacterium animalis subsp. *lactis* DSMZ 23032, *L. acidophilus* DSMZ 23033 & *L. brevis* DSMZ 23034	Intracellular content	*In vitro*		Amaretti *et al*., 2013

Lactobacillus paracasei B21060	Cell-free supernatants	Human mucosa explant of colon	Anti-inflammatory	Tsilingiri *et al*., 2012
Lactobacillus rhamnosus GG	Cell-free supernatants	Human colonic smooth muscle cells		Cicenia *et al*., 2016
Lactobacillus spp.	Cell-free supernatants	Vaginal epithelial cells	Anti-adhesion effect against *Escherichia coli*	Abbas *et al*., 2016
Bifidobacterium breve BASO-1 & *Lactobacillus* spp.	Exopolysaccharides	*In vitro*	Anti-biofilm effect against *Staphylococcus aureus*, *E. coli* 0157:H7, *E. coli* ATCC 35218 & *Pseudomonas aeruginosa*	Sarikaya *et al*., 2017
L. fermentum S1	Exopolysaccharides	*In vitro*	Anti-oxidative and anti-biofilm effect against *E. coli* and *S. aureus*	Wang, K. *et al*., 2020
L. casei B1	Biosurfactants	In vitro human epithelial cell line (HEp-2)	Anti-oxidative, anti-proliferative & anti-adhesion activity against *S. aureus*	Merghni *et al*., 2017
L. fermentum	Cell-free supernatants	3T3-L1 pre-adipocytes	Anti-senescence potential	Kumar *et al*., 2020
L. paracasei D3-5	Lipoteichoic acid	*In vivo*	Anti-aging	Wang, S. *et al*., 2020

Table 2 - Postbiotics present in the cosmetic products market

Product name	Company/Brand	Producer microorganism	Main compounds in product	Claims / Benefits	Reference source
LactoSporin®	Sabinsa	*Bacillus coagulans* MTCC 5856	Proteins	Antimicrobial, Anti-inflammatory, Anti-aging, Anti-collagenase, Antioxidant, UV protection, Hair growth promotion, Oral hygiene	Sabinsa, 2021
BifiDa	Clariant	*Bifidobacterium longum*	Vitamins, proteins, lactic acid, and minerals	Skin conditioning, Skin smoothing	Clariant, 2021
Lactobiotyl®	Silab	*Lactobacillus arizonensis*	Maltodextrin, *Lactobacillus* ferment (pinitol type cyclic polyols)	Skin barrier integrity, Epidermal renewal, Cutaneous microbiota equilibrium	Silab, n.d.
Kalibiome (AGE, SENSITIVE, ECOGENETIC)	Kalichem	*Lactobacillus paracasei*	*Lactobacillus* ferment	Immunomodulation, Skin regeneration, Skin wellness, Microbial control, Anti-aging, Skin irritation reduction, Skin barrier integrity	Kalichem, n.d.
Biotilys®	GreenTech	*Lactobacillus pentosus*	*Lactobacillus* Extract (Metabolites)	Skin defense booster, Skin moisturizing effect, Microbiota-friendly skin repair	GreenTech, n.d.
EPS SEAPUR	Codif	Marine planktonic microorganism	Exopolysaccharide	Decrease of the number of lesions, Rebalancing of the skin, Skin texture unification	Codif, n.d.
BeBebiome	LABIO	*L. paracasei, Weissella confusa, Bacillus flexus*	Cis-vaccenic acid, Sarcosine, Gamma-aminobutyric acid, Ectoine	De-stress, Skin conditioning, Anti-wrinkle, Anti-inflammatory	LABIO, n.d.
WORESANA®Rye	Woresan	*Lactobacillus*	*Lactobacillus*, Rye flour, ferment	Anti-inflammatory, immunomodulatory, antiproliferative, antioxidant	Woresan, 2021
DermaSpring IQ	Bio Component Research (BCR)	*Lactobacillus plantarum, Saccharomyces boulardi*	*Lactobacillus* Ferment Lysate, *Saccharomyces* Lysate	Anti-aging	Bio Component Research, n.d.
DermaForce IQ				Skin energy and radiance boost	

Elastiflex IQ				Skin elasticity primer	
Royal Tea IQ				UV and Blue-light protection, 24hr antioxidant protection	
GentleGuard IQ				Skin barrier protection enhancement	
Repair Complex CLR™ PF	Chemisches Laboratorium Dr. Kurt Richter GmbH (CLR)	*Bifidobacterium*	*Bifidobacterium* lysate (products of metabolism, cytoplasm fractions, cell wall constituents and polysaccharide complexes)	Skin protection, Anti-aging	Chemisches Laboratorium Dr. Kurt Richter GmbH, n.d.
ProRenew Complex CLR™ NP		*Lactococcus Lactis*	*Lactococcus* Ferment Lysate (DNA, metabolites, cytoplasmic compounds, and cell wall materials)	Skin regeneration, Skin barrier maintenance and repair, Skin protection, Desquamation promotion	
Sooth-BA	Onlystar Bio-Technology	*Bifidobacterium*	*Bifidobacterium* Ferment Lysate	Skin allergies and sensitivity reduction, Repair and anti-aging effects, Brightening, Skin-smoothing, Anti-wrinkles	Onlystar Bio-Technology, n.d.
LactoLift		*Lactobacillus casei*	*Lactobacillus* Ferment Lysate (peptidoglycan, teichoic acid, protein, phospholipid, sterol, fatty acid, enzymes, peptide, amino acid, nucleotide, extracellular polysaccharides)	Anti-wrinkles, Skin elasticity improvement, Skin moisturizing effect, Skin complexion improvement	
Lava-DermaBiotics	SK-Bioland	*Lactobacillus*	*Lactobacillus* Ferment Lysate	Skin barrier function, Skin moisturizing effect	SK-Bioland, n.d.
DermaBiotics				Anti-wrinkles	
Bioptimized™ Guava	Innovacos	*Saccharomyces*	*Saccharomyces* Lysate Extract	Anti-aging, Anti-senescence, Anti-wrinkles, Sun care, After sun care	Innovacos, 2017
PREBIOME™	Radiant	*L. plantarum*	*L. plantarum* ferment lysate	Skin microbiome balance improvement, Skin hydration, Skin Moisturizing effect, Anti-aging, Skin barrier function	Radiant, n.d.

Inspira[SEA]	Contipro	*Thalassospira xiamenensis*	Deep sea bacteria ferment	Anti-aging, Anti-wrinkles, Skin elasticity improvement, Anti-inflammatory and anti-spots effect,	Contipro, 2019
OXY 229 PF	DSM Nutritional Products Europe Ltd	*Saccharomyces cerevisiae*	*Saccharomyces* Lysate (cytoplasmic and mitochondrial constituents), amino acids	Skin redness control, Skin-smoothing, Dull-looking skin revival, Youthful skin look improvement	DSM, 2021
ALOFERM HF	Phenbiox	*S. cerevisiae*	*Saccharomyces* ferment lysate filtrate	Keratinocyte metabolism boost, Skin Moisturizing (long-term, short-term and deep) effect, Skin scaliness reduction, Skin firmness increase,	Phenbiox, n.d.
LENTIMYRT HF		*Saccharomyces*		Skin antioxidant defences improvement, Positive skin microbiota modulation, Skin hydration, Hydrolipidic film improvement	
ACQUA DI RISO FERMENTATA		*Saccharomyces*		Hair hydration, Hair dryness prevention	

4. Fermentation as a production process of postbiotics

Fermentation is a chemical process/pathway by which molecules such as glucose are broken down anaerobically (in the absence of oxygen) **(Encyclopedia Britannica, 2020)**, and it is performed by many types of organisms and cells. In this process, the only energy extraction pathway is glycolysis **(Khan Academy, 2021)**. Glycolysis is the process by which glucose is broken down within the cytoplasm of a cell to form pyruvate. Under aerobic conditions, pyruvate can diffuse into the mitochondria, where it enters the citric acid cycle and generates reducing equivalents in the form of NADH and $FADH_2$. These reducing equivalents then enter the electron transport chain, leading to the production of 32 ATP molecules per molecule of glucose (oxidative phosphorylation). In the absence of oxygen, pyruvate has a different fate. Instead of entering the mitochondria, pyruvate is converted to lactate (lactic acid fermentation) or ethanol (alcohol fermentation), allowing the regeneration of NAD+ from NADH. NAD+ is an oxidizing cofactor necessary to maintain the flow of glucose throughout glycolysis, which only produces 2 ATP molecules per glucose molecule, providing a direct mean of producing energy under anaerobic conditions **(Melkonian and Schury, 2020)**. In **Table 9** (Appendix 1) are resumed several fermentation processes using different kinds of microorganisms, substrates, and conditions.

4.1. Commonly used microorganisms

4.1.1. Lactobacillus

The genus *Lactobacillus* is the largest genus among lactic acid bacteria (LAB), consisting of more than 237 species, with continuous new species discoveries, such as *Lactobacillus metriopterae* and *Lactobacillus timonensis* **(Teame *et al*., 2020)**. *Lactobacillus* are rod-shaped, gram-positive, non-spore-forming bacteria of the family *Lactobacillaceae*. Like other genera in the family, *Lactobacillus* are characterized by their ability to produce lactic acid as a by-product of glucose metabolism **(Encyclopedia Britannica, 2021)** and some of the species are among the most widely used probiotics **(Teame *et al*., 2020)**. These bacteria are generally nonmotile and can survive in both aerobic and anaerobic environments **(Encyclopedia Britannica, 2021)**.

The amount of lactic acid produced by different *Lactobacillus* organisms varies. In several species, including *L. acidophilus*, *L. casei*, and *L. plantarum*, glucose metabolism is described as homofermentative, since lactic acid is the primary by-product, representing at least 85% of final metabolic products. However, in other species, such as *L. brevis* and *L. fermentum*, glucose metabolism is heterofermentative, with lactic acid representing about 50% of metabolic by-products and ethanol, acetic acid, and carbon dioxide making up most of the other half **(Encyclopedia Britannica, 2021)**.

4.1.2. Saccharomyces cerevisiae

S. cerevisiae is a unicellular fungus **(Parapouli *et al*., 2020)**, of the *Saccharomyces* genus of yeasts, belonging to the family *Saccharomycetaceae* **(Encyclopaedia Britannica, 2013)**. It is the

principal yeast utilized in biotechnology worldwide, and it is also the best studied eukaryote and a valuable tool for most aspects of basic research on eukaryotic organisms **(Johnson and Echavarri-Erasun, 2011; Karathia *et al*., 2011; Parapouli *et al*., 2020)**. It has been used as a model to study aging, regulation of gene expression, signal transduction, cell cycle, metabolism, apoptosis, neurodegenerative disorders, and many other biological processes **(Karathia *et al*., 2011)**. This is due to its unicellular nature, offering the combination of nearly all biological functions found in eukaryotes within a single cell **(Parapouli *et al*., 2020)**. Among the most prominent applications of *S. cerevisiae* are the manufacture of baked goods, beverages (beers, wines, distilled spirits), industrial alcohol, and biofuel by fermenting sugars **(Encyclopaedia Britannica, 2013; Parapouli *et al*., 2020)**. Interestingly, in 1864, it was reported that alcoholic fermentation was performed by *S. cerevisiae* cell-free extracts, leading to the discovery of enzymes, literally meaning "in yeast" **(Johnson and Echavarri-Erasun)**. In this line, an outstanding characteristic of this yeast is its ability to convert sugar into carbon dioxide and alcohol, by means of enzymes **(Encyclopaedia Britannica, 2013)**.

4.2. Potential of lignocellulosic material as fermentative substrate

Lignocellulosic biomass comprising forestry, agricultural and agro-industrial wastes are abundant, renewable, and inexpensive energy sources. Such wastes include a variety of materials such as sawdust, poplar tree, sugarcane bagasse and straw, wastepaper, brewer's spent grains, switchgrass, stems, stalks, leaves, husks, shells, and peels from cereals like rice, wheat, corn, sorghum, and barley, among others. Since these materials are mainly composed of sugars, they could be used for producing several valuable products, such as ethanol, food additives, organic acids, enzymes, and others. Every year, lignocellulosic wastes are accumulated in large quantities, causing not only environmental problems but also leading to the loss of potentially valuable energy sources in case of non-use **(Mussatto and Teixeira, 2010)**.

Sugarcane (*Saccharum officinarum* L.) is a perennial monocot plant, which belongs to the grass family (*Poaceae* or *Gramineae*). It has been widely cultivated around the world, being India (306 million tons) the second largest producer after Brazil (758 million tons). This plant has been considered one of the most relevant crops with high economic value due to the high value of its stalks as a source of sucrose used to produce sugar and ethanol **(Alokika *et al*., 2021; Carvalho *et al*., 2021)**. The processing of sugarcane for this production generates annually a great number of byproducts such as bagasse and straw, which are the main resultant wastes **(Carvalho *et al*., 2021; Dotaniya *et al*., 2016; Santos *et al*., 2020; Sarker *et al*., 2016)**. Bagasse consists of 39-45% cellulose, 23-27% hemicellulose, 19-32% lignin, 1-3% ashes, and 5-7% extractives, while straw is composed of 33-45% cellulose, 18-30% hemicellulose, 17-41% lignin, 2-12% ashes, and 5-17% extractives **(Carvalho *et al*., 2015; Carvalho *et al*., 2021)**.

4.2.1. Composition

Lignocellulosic biomass is mainly characterized by the presence of two carbohydrate polymers: cellulose and hemicellulose, and an aromatic polymer: lignin **(Koupaie *et al.*, 2019) (Ravindran and Jaiswal, 2016)**. Specifically, sugarcane is composed, in average, of 40% cellulose, 27% hemicellulose and 10% lignin **(Kucharska *et al.*, 2018)**. Beyond these components, there are others such as ash, pectin, proteins, and phenolics which are found in smaller amounts. Lignocellulose is the main component of plant cell wall **(Koupaie *et al.*, 2019)**, and therefore lignocellulosic materials are considered as the most abundant renewable carbon resources in the world **(Ravindran and Jaiswal, 2016)**.

Cellulose is a polysaccharide polymer of D-glucose disaccharide units, cellobiose **(Koupaie *et al.*, 2019)**, linked by β(1→4) glycoside bonds, forming linear chains **(Ravindran and Jaiswal, 2016)**. Cellulose is also an insoluble compound, being this a result of the hydrogen bonds holding the crystalline structure **(Koupaie *et al.*, 2019)**. However, it also exists in amorphous form, which is soluble and can be easily digested by enzymes **(Ravindran and Jaiswal, 2016)**. Hemicellulose is a random and branched heterogeneous polymer of different short chains of polysaccharides **(Ravindran and Jaiswal, 2016)** composed by five different sugar monomers: pentoses (D-xylose and L-arabinose), hexoses (D-glucose, D-galactose, and D-mannose) and sugar acids. Lignin is a complex and large compound made from phenylpropane units **(Koupaie *et al.*, 2019)**, that differ only in the substitution of methoxyl groups on the aromatic rings **(Ravindran and Jaiswal, 2016)**, linked in a three-dimensional structure. The covalent bonding with cellulose prevents the carbohydrates exposure to enzymatic hydrolysis. Furthermore, lignin has the capacity to irreversibly adsorb enzymes **(Ravindran and Jaiswal, 2016)**, inactivating them, and making it difficult for lignocellulosic biomass to be converted into fermentable sugars **(Kim *et al.*, 2016)**. Phenolic compounds are secondary metabolites that derive from several pathways in plants. Structurally, they have an aromatic ring with one or more hydroxyl groups, ranging from simple molecules to highly polymerized compounds. They are mostly found in fruits, vegetables, nuts, seeds and cereals, and, amongst these sources, agricultural and industrial wastes resulting from processing (sugarcane bagasse and straw) still contain high amounts of phenolics. In certain cases, the wastes may present a superior phenolic content than the raw source material. Also, sugarcane phenolic compounds exhibit several properties, such as anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, and antioxidant activity **(Balasundram *et al.*, 2006; Carvalho *et al.*, 2021; Ignat *et al.*, 2011)**.

The main objectives of this master thesis were:

1. To develop a new postbiotic extract produced with straw and bagasse, as a substrate with high performance for skin cosmetics and skincare applications;
2. Propose a new sustainable process for production of these extracts.

Materials and Methods

1. Materials / Biomass

The lignocellulosic-biomass-based feedstocks, sugarcane bagasse and straw, were sourced in Bonfim and Paraíso, provided by Raízen (São Paulo, Brazil). The biomass composition of the bagasse and straw was as follows: 41.62% cellulose, 24.72% hemicellulose, 20.04% lignin, and 38.77% cellulose, 26.01% hemicellulose, 19.14% lignin, respectively. Samples were transported to CBQF-UCP laboratory (Porto, Portugal), and dried at 40 ºC using an oven (Nabertherm, Porto Salvo, Portugal). In case of sugarcane straw, it was primarily grinded using a knife mill SM100 (Retsch, Vila Nova de Gaia, Portugal) to a particle size < 4 mm. All biomass was sifted, using a Retsch Vibratory Sieve Shaker AS 200 basic (Scansci, Vila Nova de Gaia, Portugal), before use, and for the milling process, both substrates were reduced to powder using a Bimby® (Vorwerk, Portugal). In case of unmilled biomass, only the sifting process was performed.

2. Production of postbiotics

2.1. Screening of the best saccharification conditions to prepare the fermentation media

Sugarcane bagasse or sugarcane straw were suspended in 50 mM citrate buffer (pH 5), and supplemented with 10 $g.L^{-1}$ of peptone, 5 $g.L^{-1}$ of yeast extract, 2 $g.L^{-1}$ of ammonium citrate, 2 $g.L^{-1}$ of potassium phosphate dibasic, 5 $g.L^{-1}$ of sodium acetate, 0.1 $g.L^{-1}$ of magnesium sulfate, and 0.05 $g.L^{-1}$ of manganese sulfate, using 250 mL Erlenmeyer flasks, in duplicate, in a 1:20 ratio (m/v) **(Nagarajan *et al.*, 2020; Zhang and Vadlani, 2015; Zhang *et al.*, 2016)**. To evaluate the best saccharification conditions with or without enzymes, a control without enzymes (named No enzymes), with cellulase (named Celluclast) and with both cellulase and xylanase (named Shearzyme Plus) were prepared. Celluclast (Novozymes, Bagsværd, Denmark) was added at 20 FPU per gram of cellulose, and Shearzyme Plus (Novozymes, Bagsværd, Denmark) was added at the dosage of 100 U. The process of saccharification of samples without enzymes and with Celluclast was performed at 55 ºC, with agitation of 150 rpm, while with both Celluclast and Shearzyme Plus, it was performed at 45 ºC and 150 rpm of agitation. Before adding the enzymatic cocktails, 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, 24, and 48 hours, and then centrifuged (10 min., 5000 rpm, 25ºC) for biomass removal and quantification of total sugars by phenol-sulfuric acid method.

2.2. Fermentation process

2.2.1. Microorganisms

Fermentative microorganisms inoculums were prepared by growing *L. plantarum* and *L. fermentum* in Man, Rogosa and Sharpe (MRS) broth at 37 ºC, and *S. cerevisiae* in Yeast Malt (YM) broth, overnight at 30 ºC. After confirmation of purity, plates and slants were prepared as stock cultures in MRS agar in case of *Lactobacilli* bacteria, and in Potato Dextrose Agar (PDA) for *S. cerevisiae*. 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) **(Nagarajan *et al.*, 2020)**. These cells were then used to inoculate the fermentation media.

2.2.2. Simultaneous and Sequential Saccharification and Fermentation (SSF and SQSF)

For SSF experiments, after the saccharification process, the biomass was kept in the Erlenmeyer flasks, and the fermentation process was performed by inoculating the media with a 10^5–10^7 CFU .mL^{-1} cellular concentration range. The reactors were incubated at the optimal temperatures for each microorganism (previously described) along 24, 48, and 72 hours, with agitation (150 rpm). The initial and final pH were recorded along the process with a potentiometer.

For SQSF experiments, the biomass was removed before inoculation. For that, the flasks content following saccharification was centrifuged (10 minutes, 5000 rpm, 25 ºC) in sterile falcon tubes and transferred to new sterile 250 mL Erlenmeyer flasks. All remaining conditions (temperature, agitation, and initial pH) were the same as for SSF experiments.

The samples collected along SSF and SQSF were subject to different analysis. For the evaluation of microorganism's cellular concentrations, decimal dilutions were performed in peptone water plated with the drop technique **(Miles and Misra, 1938)** in MRS agar for quantification of Lactobacillus species and *S. cerevisiae* using the spread plating technique in PDA. Plates were incubated at 37 and 30 ºC, respectively during 24h. In addition, samples were withdrawn and centrifuged (10 min, 5000 rpm, 25 ºC) and the supernatant was analysed for the total sugar content by the phenol-sulfuric acid method.

2.3. Preparation of cells-free extracts

Extracts were prepared as follows. The fermentation broths from SSF and SQSF experiments, were subjected to ultrasonication 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' by **Qsonica (n.d.)**, with modifications. After cellular disruption, broths were centrifuged for 10 minutes at 5000 rpm and 25 ºC to remove intact cells, cell debris and other insoluble materials

(Nagarajan *et al*., 2020). In case of SQSF the centrifugation process was performed in different conditions, for 30 minutes at 800 x g and 25 ºC to remove only intact cells and leave components or part of lysed cell membranes **(Tejada-Simon and Pestka, 1999)**. To ensure that broths were not carrying on intact alive cells, these were filtered with 0.22 um 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 lyophilized, for further testing.

3. Analytical methods

3.1. Phenol-sulfuric acid method for total sugar content determination

For phenol-sulfuric acid method, a 5% (m/v) phenol 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% sulfuric acid, 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 calibration curve (0.031 – 0.250 $mg.mL^{-1}$) and expressed as $mg.mL^{-1}$.

3.2. Monosaccharides and short-chain fatty acids (SCFAs) 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.

For SCFAs identification and quantification, samples were analysed on an HPLC (Agilent 1260 Infinity II) attached to a Refractive Index Detector (RID) coupled to a Aminex HPX 87H column (300 x 7.8 mm, Biorad). 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.

3.3. Methods for determination of 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 weighed, solubilized in dH_2O and diluted in series at a 1:2 (v/v) proportion.

3.3.1. 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 extracts. 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 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 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) **(Gonçalves *et al.*, 2009)**.

The inhibition percentage (I) of the sample was calculated using the Equation (1) and compared with trolox standard calibration curve (0.0075 – 0.075 $mg.mL^{-1}$). The results were expressed as EC_{50} milligrams per gram of dry extract ($mg.g^{-1}$ dry extract). Each of the duplicate extracts, obtained for each different methodology, were analysed in duplicate in the plate.

3.3.2. 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 0.03841 g of ABTS and 0.0066 g de $K_2O_8S_2$ (potassium persulfate) in 10 mL of dH_2O. Both solutions were then mixed using a magnetic stirrer and $ABTS^+$ was generated through a chemical oxidation reaction between both substances. The resultant solution was left stirring overnight, and then stored at 4 ºC, for 1 month maximum. 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) **(Gonçalves *et al.*, 2009)**.

The inhibition percentage (I) of the sample was calculated using the Equation (1) and compared with trolox standard calibration curve (0.0075 – 0.075 mg.mL^{-1}). The results were expressed as EC_{50} milligrams per gram of dry extract (mg.g^{-1} dry extract). Each of the duplicate extracts, obtained for each different methodology, were analysed in duplicate in the plate.

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

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

3.3.3. 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 is rich in antioxidant compounds **(Dasgupta & Klein, 2014)**. For ORAC assay, a 75 mM PBS buffer was prepared by dissolving 9 g of NaH_2PO_4 in 1 L of ultrapure water 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 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, 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 solution was prepared by dissolving 0.13018 g in 10 mL of PBS. 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) **(Dávalos *et al.*, 2004)**.
Each sample was analyzed at least in duplicate in the plate. Final ORAC-FL values were expressed as mg of Trolox equivalent.mL^{-1}. Values were calculated by interpolation with a Trolox calibration curve (10 – 80 µM).

3.4. Antimicrobial activity evaluation

Antimicrobial activity of the fermentation extracts was assessed as follows. Microbial cosmetic contaminant strains (*S. aureus* DSM799) were activated in TSB (Tryptic Soy broth) at 37 ºC overnight. After growth, the cultures were plated in TSA (Tryptic Soy agar), left incubating for

~24h, and then stored unrenewed at 4 ºC for 3 months maximum (contaminant strains stock). To perform the method, colonies of the contaminant strains were transferred using a metal loop and grown overnight in MH (Mueller Hinton) broth at 37 ºC. After growth, the absorbance of the inoculant was adjusted to 0.08 – 0.1 at 660 nm using a UV-1900 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan), representing a cellular concentration of 10^8 CFU.mL^{-1}. The inoculants were then used to inoculate the several dilutions of the tested extracts, in a 2% ratio (v/v), representing an initial cellular concentration of 10^6 CFU.mL^{-1}. Fermentation extracts were previously weighed, solubilized in dH_2O, and then filtered, using 0.22 µm sterile filters. After that, a series of dilutions were performed using MH broth, to obtain different extracts concentrations. These steps were performed so that each dilution would have a minimum volume of 600 µL after being inoculated. The tested extract concentrations were the following: 5, 4, 3, 2, and 1%. Briefly, 200 µL of each inoculated dilution, MH broth for media control, MH broth inoculated at 2% for inoculum control, or each dilution for sample control were pipetted in triplicate into each well of a sterile 96-well plate. The mixture was incubated for 24h at 37 ºC, and the absorbance was measured hourly at 625 nm, with an Epoch 2 microplate reader (BioTek, Vila Nova de Gaia, Portugal). After the 24h, any well with no visible growth was plated using the spread plating technique in MH agar and then incubated for ~24h at 37 ºC, for MIC (minimum inhibitory concentration) - visible growth - and MBC (minimum bactericidal concentration) - no growth - determination.

3.5. Folin-Ciocalteau method for total phenolics content determination

For Folin-Ciocalteau method, a 7.5% (m/v) Na_2CO_3 solution was prepared in a glass flask by dissolving 15 g of in 200 mL of dH_2O. Tested samples were prepared in dH_2O at a 25 mg.mL^{-1} concentration. Briefly, 50 µL of sample, or solvent (dH_2O) for blank were pipetted in triplicate into glass tubes, followed by 50 µL of Folin-Ciocalteau reagent as provided, 1000 µL of 7.5% Na_2CO_3 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 calibration curve (0.062 – 0.493 mg.mL^{-1}) and expressed as mg.g^{-1}.

3.6. DUMAS method for total protein content determination

The Dumas method relies on the quick combustion of a sample in pure oxygen atmosphere, followed by an analysis of the resulting combustion gases. These gases cause a change in the thermal conductivity, which is registered by a thermal conductivity detector (TCD), resulting in a signal that corresponds to the amount of nitrogen (N_2) in the combusted sample **(FOSS, n.d.)**. Samples were analysed according to ISO 16634 by using a Dumatec™ 8000 from FOSS Analytical A/S (Hilleroed, Denmark). Briefly, 100 mg of dry sample were carefully weighted,

wrapped and tightly pelleted in aluminium foil, and then inserted in the analysis device for protein quantification. EDTA (EthyleneDiamineTetraacetic Acid) was used as the nitrogen calibration standard at a (10 – 150 mg) range. Five to eight different standards are recommended for an adequate calibration. Oxygen flow and factor for samples analysis were 300 $mL.min^{-1}$, and 1.4 $L.mg^{-1}$, respectively (method B1.4). Combustion reactor, reduction reactor, and degassing oven temperatures were, respectively, 980 ºC, 650 ºC, and 300 ºC. The protein factor used for the calculation of the sample's protein content from its total nitrogen content was 6.25. For calibration, oxygen flow was 400 $mL.min^{-1}$, and oxygen factor was 1 $mL.mg^{-1}$ (method A1).

4. Final postbiotics extracts safety, physicochemical and biological properties evaluation

The extracts that showed best biological potential were selected for an evaluation of physicochemical and biological analysis.

4.1. Physicochemical properties

4.1.1. Individual polyphenols identification by LC-ESI-UHR-QqTOF-MS

The identification and quantification of polyphenols were attained by LC-ESI-UHR-QqTOF-MS, as described by **Oliveira *et al*. (2015)**. Briefly, the assayed samples were accurately weighed and dissolved in ultrapure water, at a concentration of 50 $mg.mL^{-1}$. After, the 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; and (B) acetonitrile 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 at least in duplicate.

4.1.2. Individual aminoacids identification by High Performance Liquid Chromatography (HPLC)

The assayed samples were prepared as follows, 10 mg of sample were weighed accurately to a 5 mL vial. Then, 3 mL of HCl (6M) were added and the vials were closed with the septum and vortexed. In case of tryptophan, 3 mL of NaOH (5M) were added instead of HCl. After that, vials were flushed with nitrogen gas, for 1 min (placed 2 needles in the septum, one for nitrogen inlet and the other for gas outlet). Then, the outlet needle was removed, and the vials were bubbled with nitrogen, for 3 minutes. The inlet needle was then removed, and the vials were carefully sealed with paper tape. After that, the vials were incubated at 115 ºC, for 20h. After incubation, the vials were left to cool and 4 mL of dH_2O were added. The samples pH was adjusted to 3.5 with NaOH 10 M, and then they were transferred to 10 mL volumetric flasks. The volume was completed with HCl 0.1 M. In case of tryptophan, the samples pH was adjusted to 7, with HCL 6M. Then, the content was transferred to 25 mL volumetric flasks and the volume was completed with sodium borate buffer (1 M, pH 9). All samples were, finally, filtered using 45 µm filters, 5x diluted, and then 20 µL were pipetted into the inserts for HPLC analysis.

For individual aminoacids identification, samples were analysed on an HPLC (Agilent 1260 Infinity II) attached to a High-Resolution Fluorescence Detector (HRFD) coupled to a Chromolith® Performance RP 18 column (4.6 x 100 mm, Merck REF:1.02129.0001). The column temperature was set at 25 ºC, and the compositions of the mobile phases were as follows: (A) 2.8 g of Na_2HPO_4, 7.6 g of $Na_2B_4O_7$, 0.064 g NaN_3 in 2 L of ultrapure water, with pH adjusted to 8.2 with HCl; (B) methanol/acetonitrile/ultrapure water (45:45:10, v/v). The flow rate was set at 0.700 $mL.min^{-1}$ and an injection volume of 10 µL was defined. The detector was set to read fluorescence at Ex/Em 356/445 nm. For the determination of elution order (retention time) and obtention of calibration curves, pure standards were injected. All samples were analysed at least in duplicate. The method was performed according to the 'Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column' protocol by **Long (2017)**, with modifications.

4.1.3. In vitro chemical skin enzymes inhibition tests

Extracts were tested for their effect on skin enzymes inhibition on three distinct enzymes: neutrophil elastase (NE), matrix metalloproteinase-1 (MMP1) collagenase, and tyrosinase. Samples were prepared in dH_2O according to the following final concentrations in the wells: 2, 1.5, 0.75, 0.375, and 0.1875%.

4.1.3.1. Elastase

The assay was performed using a commercial kit of neutrophil elastase inhibitory screening (fluorometric) (ab118971, ABCAM), 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 (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 equation (2):

$$\text{Enzyme inhibition activity } (\%) = \frac{\Delta\text{RFU sample}}{\Delta\text{RFU enzyme control}} \times 100 \quad (2)$$

4.1.3.2. MMP1 collagenase

The assay was performed using a commercial kit of MMP1 inhibitory screening (colorimetric) (ab139443, ABCAM), according to manufacturer's instructions. Briefly, MMP substrate and MMP inhibitor (NNGH) were warmed up to room temperature. Then, MMP inhibitor, MMP substrate, and MMP1 enzyme were diluted 1/200, 1/25, and 1/40, respectively, in assay buffer to required total volume, and warmed to reaction temperature (37 ºC). 90, 70, or 50 µL of assay buffer were pipetted in duplicate into each desired well of the ½ volume microplate for blank (no MMP1), enzyme control (no inhibitor), and MMP inhibitor, respectively. Regarding tested samples, assay buffer was added depending on wanted concentration. 20 µL of diluted MMP1 were added to the enzyme control, MMP inhibitor, and sample wells, and 20 µL of MMP inhibitor were added to the MMP inhibitor wells only. Then, the desired volume of samples was added to the correspondent wells, depending on wanted concentration. The plate was incubated for 30-60 min at 37 ºC to allow inhibitor/enzyme interaction and the reaction was started by adding 10 µL of diluted MMP1 substrate. Absorbance (abs) was recorded at 412 nm every minute for 10 to 20 min using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal). Abs values were plotted versus time for each sample, and the points of the linear phase of the reaction were selected. The reaction velocity (V) in abs.min^{-1} was determined by the slope of a line fit to the linear portion of the data plot, and an average of the slopes was calculated for duplicate samples. The percentage inhibition of this assay was calculated by equation (3):

$$\text{Enzyme inhibition activity } (\%) = 1 - \left(\frac{\text{V inhibitor}}{\text{V enzyme control}} \times 100\right) \quad (3)$$

4.1.3.3. *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 uL of dH_2O and assay buffer, respectively, and stored at -20 ºC. Inhibitor control (kojic acid) was prepared in dH_2O to a 10mM 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 equation (4):

$$\text{Enzyme inhibition activity } (\%) = \frac{\text{Slope of EC} - \text{Slope of S}}{\text{Slope of EC}} \times 100 \quad (4)$$

4.2. Safety and biological properties

4.2.1. Cytotoxicity evaluation

Cytotoxicity of fermentation extracts was evaluated using a PrestoBlue Cell Viability assay (Invitrogen), according to the manufacturer´s instructions. Human dermal fibroblasts and immortalized human keratinocytes (HaCaT) were cultured in DMEM (Dulbecco's Modified Eagle Medium) culture medium, supplemented with 10% FBS (Fetal Bovine Serum) and 1% antibiotic at 37 ºC, with 5% CO_2 in a humidified atmosphere. For the experiments, the cells were seeded at 1×10^4 cells/well in 96-well plates. Cells were exposed to the fermentations extracts at desired concentrations (8, 4, 2, 1, 0.5, and 0.25%), 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% DMSO 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, covered

with aluminium foil, for 3h maximum. Finally, the fluorescence was recorded four times using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal) and a mean was calculated.

4.2.2. Cytokeratin 14 quantification

Immortalized human keratinocytes (HaCaT) were cultured in DMEM (Dulbecco's Modified Eagle Medium) culture medium, supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic at 37 ºC and 5% CO_2 in a humidified atmosphere. For the experiments, 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 extracts at desired concentration (1.5%), for 24h. Extracts were prepared using DMEM culture medium. Each sample was tested in duplicate in two independent experiments. Briefly, 1000 µL of sample, were pipetted into each appropriate well. 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, and then centrifuged at 18000 x g for 20 minutes at 4 ºC. Supernatants were transferred to clean tubes, and the corresponding pellets were discarded. Total protein content was quantified by the BCA method using the Pierce™ BCA Protein assay kit (Thermo Scientific), according to the manufacturer´s instructions. Briefly, the BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 25 µL of each standard or sample (supernatant), followed by 200 µL of WR were added to each microplate well. Then, the plate was mixed for 30 seconds, covered in aluminium foil and incubated at 37 ºC, for 30 minutes. After incubation, the absorbance was measured at 562 nm using a Synergy H1 microplate reader (Agilent Technologies, Inc., Santa Clara, CA, USA). A calibration curve was obtained using bovine serum albumin (BSA) standard solutions at a concentration range of 3.91 - 2000 µg.mL^{-1}. Total protein content in samples was determined by interpolation of absorbance values in the linear fit equation.

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. Briefly, 50 µL of sample or standard followed by 50 µL of the respective antibody cocktail were added to each well of the stripped microplate, and left incubating for 1h, at room temperature, with agitation at 400 rpm. After incubation, each well was washed 3 times using 1X wash buffer PT, and the plate was gently tapped against clean paper to remove excess liquid. After, 100 µL of TMB Development solution were added to each well and the plate was incubated for 10 minutes in the dark with agitation at 400 rpm. 100 µL of Stop Solution were then added to each well, the plate was mixed for 1 minute and the OD was recorded at 450 nm using a Synergy H1 microplate reader (Agilent Technologies, Inc., Santa Clara, CA, USA).

4.2.3. Collagen I α1 quantification

Human dermal fibroblasts (HDF) were cultured in DMEM culture medium, supplemented with 10% FBS, and 1% antibiotic at 37 ºC, with 5% CO_2 in a humidified atmosphere. For the experiments, 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 extracts at desired concentration (0.6%), for 24h. Extracts were prepared using plain DMEM culture medium, as the presence of FBS is reported to inhibit collagen synthesis **(Bilgen *et al.*, 2007)**. Each sample was tested in duplicate in two independent experiments. Briefly, 1000 µL of sample were pipetted to each well. Plain DMEM and palmitoyl tetrapeptide-3 (GenScript) were used as negative and positive controls, respectively. Total protein quantification was performed as described in **section 4.2.2. (Materials and Methods)**.

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. Briefly, 50 µL of sample or standard followed by 50 µL of the respective antibody cocktail were added to each well of the stripped microplate, and left incubating for 1h, at room temperature, on a plate shaker set to 400 rpm. After incubation, each well was washed 3 times using 1X wash buffer PT, and the plate was gently tapped against clean paper to remove excess liquid. After, 100 µL of catchpoint HRP Development Solution were added to each well of the microplate and incubated, in the dark, for 10 minutes on a plate shaker set to 400 rpm. After incubation, fluorescence was recorded at Ex/Cutoff/Em 530/570/590 nm using a Synergy H1 microplate reader (Agilent Technologies, Inc., Santa Clara, CA, USA).

4.2.4. Evaluation of immunostimulatory and anti-inflammatory activities

Human monocytes THP-1 (ATCC TIB-202) were cultured in RPMI (Roswell Park Memorial Institute) culture medium, supplemented with 10% FBS, 1% antibiotic, and 50 Mm beta-mercaptoethanol, at 37 ºC, with 5% CO_2 in a humidified atmosphere. For the experiments, 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, for 48 h. Cells were exposed to the fermentations extracts at desired concentrations (1% and 0.1%) for 24h, in the presence or absence of lipopolysaccharides form *E. coli* O111:B4 (LPS, Sigma) to induce inflammation. Samples were prepared using RPMI culture medium. For anti-inflammatory control, macrophages were treated with 20 nM of betamethasone. 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. Briefly, all wells were coated with 100 µL of diluted Capture Antibody the day prior to running the ELISA, and the plate was left incubating overnight, between 2 ºC and 8 ºC. Then, the plate was washed 4 times with 300 µL of Wash Buffer. After that, 200 µL of 1X Assay Diluent A were added, and the plate was left incubating on a plate shaker (500 rpm) for 1 hour, at room temperature. After incubation, the plate was washed again 4 times, and 100 µL of diluted standards and samples were added.

The plate was incubated under the same conditions as before, but for 2 hours. Then the plate was washed 4 times, and 100 µL of diluted Detection Antibody were added. After 1h incubation and wash (4 times), 100 µL of Avidin-HRP were added, and the plate was left incubating, under the same conditions, for 30 minutes. After that, the plate was washed 5 times, and 100 µL of TMB Substrate Solution were added. The plate was then left incubating for 15 minutes in the dark, at room temperature, without agitation. After incubation, 100 µL of Stop Solution were added and the absorbance was measured at 450 nm and 570 nm, using a Synergy H1 microplate reader (BioTek, Vila Nova de Gaia, Portugal). 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.

4.2.5. Evaluation of the influence on skin microbiota

4.2.5.1. Skin microbiota collection

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. The skin microbiota was collected from the face of volunteers using a swab previously moistened in a sterile solution of PBS with 0.1% Tween 80. In parallel, the control method was performed consisting in a swab moistened in a sterile solution of PBS with 0.1% Tween 80 without skin microbiota. After the collection, the swabs containing microbiota and controls were incubated in RPMI, for 3 hours, at 34 ºC, with agitation. Afterwards, the swabs were aseptically removed, and the content was divided into two new sterile tubes. Then, to each of these tubes was added the ingredient (test condition) or the culture medium (control condition) in a 1:1 proportion. Due to that, the sample was prepared with a concentration 2 times superior to the one to be tested. All tubes were incubated at 34 ºC with agitation, for approximately 24h. After incubation, the tubes were centrifuged at room temperature and 14000 x g for 10 minutes, and the supernatants were transferred into new sterile tubes. The tubes containing pellets and supernatants were stored at -20 ºC until further testing.

4.2.5.2. DNA purification, quantification, and amplification by q-PCR

The microbial DNA was purified using the PureLink™ Microbiome DNA Purification Kit (Invitrogen) according to the manufacturer's instructions. For lysate preparation, the microbial pellets were resuspended in 800 mL of Lysis buffer and transferred to the bead tube, where and 100 mL of the Lysis Enhancer were added. The mixture was incubated at 65 ºC, for 10 minutes and homogenized by bead beating for 10 minutes, at maximum speed. After that, the samples were centrifuged at 14000 x g, for 2 minutes, and 500 mL of the supernatant were transferred to a clean microcentrifuge tube, avoiding the bead pellet and any debris.

For DNA biding to the column, 900 mL of binding buffer were added and the mixture was briefly vortexed. Then, 700 mL of sample mixture were loaded onto a spin column-tube assembly and

centrifuged at 14000 x g, for 1 minute. The flow-through was discarded and the previous step was repeated with the remaining sample mixture.

For DNA washing and elution, a spin column was placed in a clean collection tube and 500 mL of wash buffer was added, and the spin column-tube assembly was centrifuged at 14000 x g, for 1 minute. The flow-through was discarded, and the spin column-tube assembly was centrifuged at 14000 x g for 30 seconds (for optimal removal of wash buffer). Then, the spin column was placed in a clean tube, 100 mL of elution buffer was added, and the mixture was incubated at room temperature, for 1 minute. Finally, the spin column-tube assembly was centrifuged at 14000 x g, for 1 minute, and the column was discarded. The purified DNA was quantified using the Qubit™ 1X dsDNA HS (High Sensitivity) Assay Kit (Invitrogen) according to the manufacturer's instructions, and after it was stored at -20 ºC, until further use. Briefly, 10 µL of each Qubit™ standard (two standards) for calibration, or 1 µL (1-20 uL are recommended) of each sample were added to the appropriate tubes, followed by Qubit™ 1X dsDNA working solution to a final volume of 200 µL. After, each tube was mixed by vortexing for 3-5 seconds, and incubated for 2 minutes, at room temperature. Finally, the standard tubes, followed by the sample tubes, were directly introduced in a Qubit™ 4 Fluorometer, for DNA quantification.

For quantitative PCR (qPCR), it was used universal and specific primers to quantify the total load of bacteria or fungi, specific microbial genera, and species. Some primers were designed using Eurofins software based on the gene sequences retrieved form the NCBI gene. After construction, the primers were checked concerning their specificity in Blast program of PubMed. All other cases were obtained from scientific papers, where similar processes are described. The sequence of used primers for qPCR is shown in **Table 3**.

Table 3 - Primer sequences used for qPCR.

Primer	Reference	ºC	[µM]f	[Work solution] (µM)
Universal bacteria	Horz et al., 2005	60	0.1	1
Universal fungi	Hoggard et al., 2018	60	0.5	5
Staphylococcus	Wampach et al., 2017	60	0.5	5
Corynebacterium	Xu et al., 2021	60	1	10
Propionibacterium	Cazanave et al., 2013	60	1	10
Malassezia	Vuran et al., 2014	60	1	10
S. aureus	VAN DER KRIEKEN et al., 2016	60	1	10
S. epidermidis	Byrne et al., 2007	60	0.5	5
C. acnes	VAN DER KRIEKEN et al., 2016	60	0.5	5
Propioniferax innocua	Custom designed	60	1	10

The qPCR mastermix components (**Table 4**) were added into nuclease-free microtubes, and the qPCR thermocycler (qTOWER³ G, Analytic-Jena) was set with the following program: 95 ºC for 10 minutes (polymerase activation); denaturation at 95 ºC for 10 minutes; annealing at 60 ºC for 1 minute (40 cycles); melt curve from 60 ºC to 95 ºC (1ºC increase for 5 seconds). The samples were tested in triplicate for each sample.

Table 4 - qPCR Mastermix components.

Component	Volume	Final concentration
NZYSpeedy qPCR Green Master Mix (2X)	5 µL	1x
1 to 10 µM forward primer	1 µL	0.1 to 1 µM
1 to10 µM reverse primer	1 µL	0.1 to 1 µM
Template (10 ng/uL)	1 µL	---
Microbial DNA-Free water (Qiagen)	Up to 10 µL	---

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.

5. 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.

Results and Discussion

1. Saccharification

1.1. Effect of time, enzymes and milling on the total sugar content

For lignocellulosic biomass enzymatic hydrolysis, four variables were tested: type of biomass (straw and bagasse), biomass grinding degree (milled and unmilled), saccharification time (24 and 48h) and used enzymatic cocktail (no enzymes, Celluclast, and Celluclast + Shearzyme Plus). The obtained results were as follows (**Figures 1 & 2)**.

1.1.1. Sugarcane Bagasse

Regarding sugarcane bagasse enzymatic hydrolysis (**Figure 1**), the absence of enzymes had no positive effect on what was intended, i.e., release of fermentable sugars (monosaccharides) directly from both milled and unmilled biomass ($p>0.05$). On the other hand, both enzymatic cocktails allowed a significant increase in total sugar content along saccharification ($p<0.05$). Comparing both, Celluclast + Shearzyme Plus resulted in higher sugar concentrations in average, at 48h of saccharification, although no significant differences were verified with milled bagasse ($p>0.05$). On opposite, with unmilled bagasse there were significant differences between both enzymes, at 48h ($p<0.05$). This outcome was as expected since Shearzyme Plus should assist in the sugar release from hemicellulose molecules. Interestingly, at 24h of saccharification, there were no evident differences between both cocktails ($p>0.05$), suggesting that hemicellulose hydrolysis was more intense in the last 24h. The fact that Celluclast alone did not seem to represent a significant advantage in 48h processes ($p>0.05$) suggests that this enzyme may lose most of its activity in the first 24h. The possibility of irreversible adsorption of enzymes by lignin may be the main cause for this. However, and contrary to expectations, the milling process did not seem to represent any advantage. Depending on the milling degree of the biomass, a “facilitated” enzymatic hydrolysis would be expected with smaller particles, due to the larger available surface area for enzymes to perform **(Koupaie *et al.*, 2019; Ravindran & Jaiswal, 2016)**. In this case, the best saccharification conditions found for sugarcane bagasse were 48h, with unmilled bagasse, using Celluclast and Shearzyme Plus. Technologically, it is very important to understand that this process does not require any milling procedure, in the mentioned conditions.

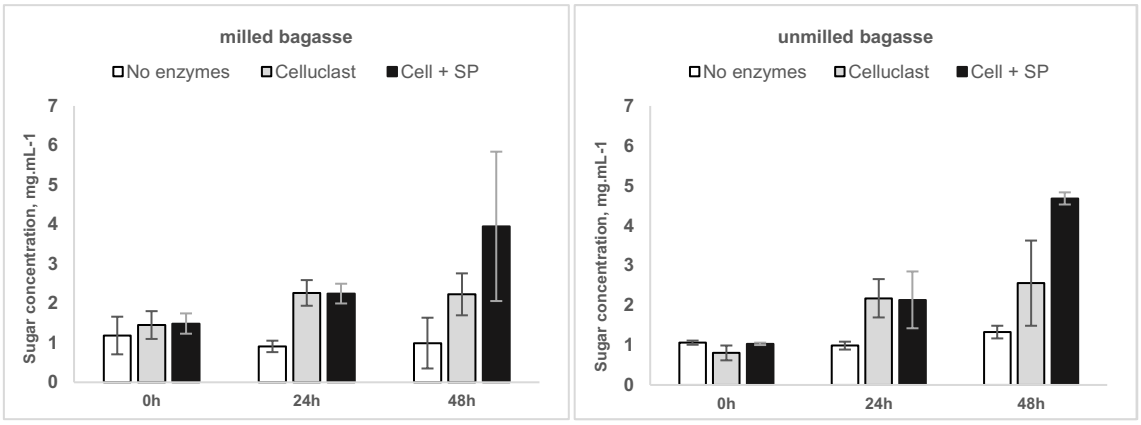

Figure 1 - Total sugar content along saccharification process of sugarcane bagasse without enzymes, added with cellulase (Celluclast) and with both cellulase and xylanase (Cell+SP). Mean values (solid bars) are expressed as mg.mL^{-1}, and standard deviation is represented by bars.

1.1.2. Sugarcane Straw

For sugarcane straw enzymatic hydrolysis (**Figure 2**), the results were, in general, like those observed with bagasse. Briefly, with no enzymes, no significant saccharification was observed with milled biomass ($p>0.05$). The exception was in the last 24h using unmilled straw ($p<0.05$), where the raise in sugar content may have been due to agitation. Regarding both enzymatic cocktails, the saccharification tendencies were the same, i.e., both promoted sugar release during saccharification, with significant differences between measured times ($p<0.05$). With 48h processes, Celluclast + Shearzyme Plus appears to be the most advantageous option in average, but no significant differences were verified for both milled and unmilled straw ($p>0.05$) when comparing with Celluclast. However, with 24h experiments that advantage was not observed. Moreover, in case of unmilled straw, celluclast alone was more effective at that point of saccharification, with a significantly higher sugar content ($p<0.05$). Furthermore, the milling process did not seem to represent any advantage to the process.

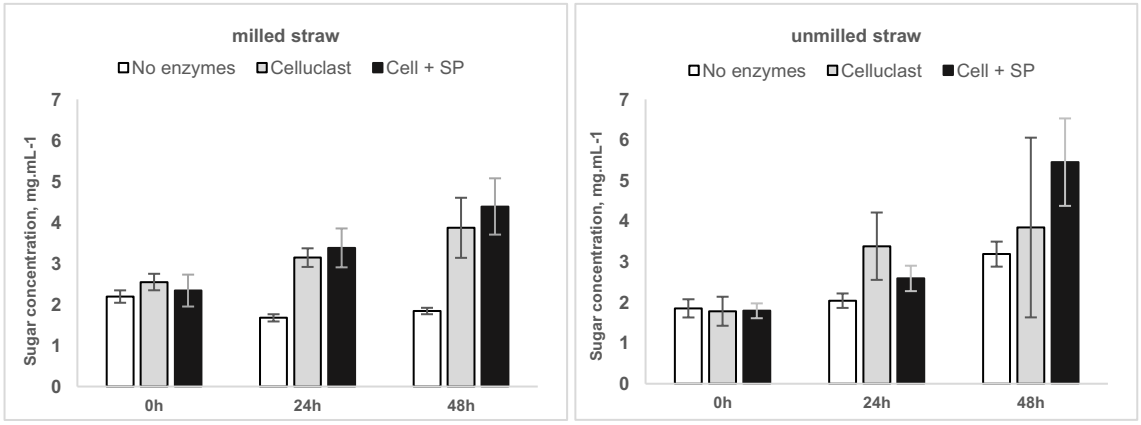

Figure 2 - Total sugar content along saccharification process of sugarcane straw without enzymes, added with cellulase (Celluclast) and with both cellulase and xylanase (Cell+SP). Mean values (solid bars) are expressed as mg.mL^{-1}, and standard deviation is represented by bars.

However, the type of biomass used affected the sugar concentration along saccharification. Although the tendencies between both are similar, with straw, the total sugars concentration is significantly higher, in most of the tested conditions ($p/2<0.05$). The exceptional conditions were Celluclast + Shearzyme Plus, at 48h, with milled biomass, and Celluclast at 48h, Celluclast + Shearzyme Plus, at 24h and 48h, with unmilled biomass, where no significant differences were verified between bagasse and straw ($p/2>0.05$). If we analyse the sugar release with each substrate (**Figure 3**), it is possible to observe that there are not significant differences between them, in any tested condition that would explain such a discrepancy ($p>0.05$). Nonetheless, available sugarcane bagasse had already been industrially processed (with water and higher temperatures) for molasse extraction and then recovered for further exploration. On the other hand, sugarcane straw was collected directly from nature. This considered, during biomass sterilization by autoclaving, the sugar release with straw was significantly higher when compared to bagasse ($p/2<0.05$) (**Figures 1 & 2**). In this line, milled biomass also allows a superior initial sugar concentration prior to saccharification, for both substrates ($p/2<0.05$).

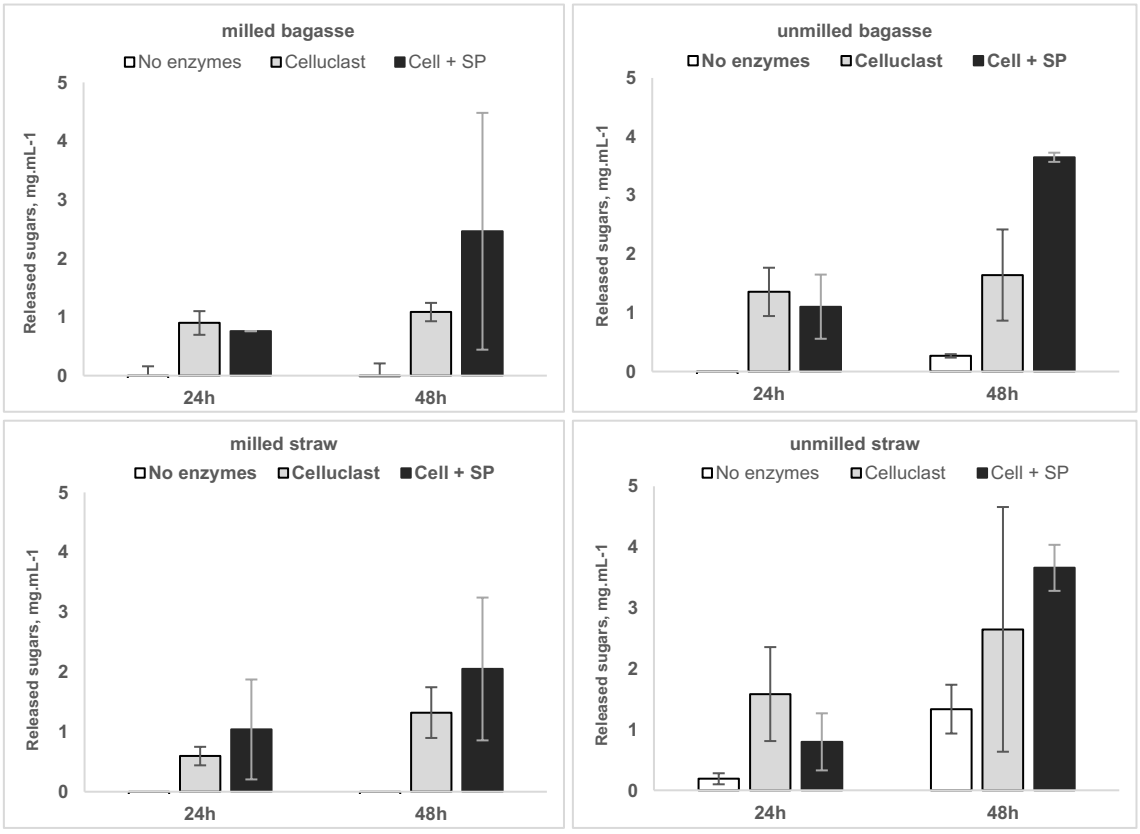

Figure 3 - Total sugar production along saccharification process without enzymes, added with cellulase (Celluclast) and with both cellulase and xylanase (Cell+SP). Mean values (solid bars) are expressed as $mg.mL^{-1}$, and standard deviation is represented by bars.

2. Simultaneous Saccharification and Fermentation (SSF)

2.1. Effect of time and biomass in the sugar uptake

For fermentation experiments, the tested variables were the following: time of fermentation and type of biomass. Since there were no statistically significant differences between the enzymes used ($p>0.05$) in most of the tested conditions, and opting for a lower production cost process, all fermentation procedures were preceded by a saccharification process using only Celluclast for enzymatic hydrolysis purposes.

Three distinct microorganisms were tested due to their different metabolic pathways. *L. plantarum* (homofermentative lactic acid bacteria **(Xu *et al.*, 2019)**), *L. fermentum* (heterofermentative LAB **(Bassi *et al.*, 2018)**), and *S. cerevisiae* (yeast **(Malik *et al.*, 2020)**). Fermentations along 24h were preceded by 48h saccharification processes. All the other fermentations were preceded by a 24h saccharification process. This was decided because in the 24h experiments, the last 24h of saccharification didn´t represent any advantage. Considering the saccharification results, this was not expected. On the other hand, the goal was not only to improve the final extract but also to attenuate the final sugar concentration. Both lactic acid bacteria were inoculated at an initial cellular concentration range of 10^5 - 10^7 $CFU.mL^{-1}$, and the yeast at a range of 10^5 - 10^6 $CFU.mL^{-1}$. The results for sugar uptake and cellular concentration are resumed in **Figures 4, 5 and 6** for *L. plantarum*, *L. fermentum*, and *S. cerevisiae*, respectively.

2.1.1. Lactobacillus plantarum

Regarding *L. plantarum*, at 24h, there was already a significant sugar uptake with both substrates, what seems to be consistent with the significant raise in cellular concentration during that period ($p<0.05$). Some variation between samples, resulting in high standard deviations of the average, may come as result of the biomass itself, which being biological material retrieved directly from nature, may present distinct responses under similar conditions. Moreover, the use of unmilled biomass did not allow to guarantee its homogeneity between independent fermentation experiments.

With 24h experiments not all monosaccharides were consumed, and so it was decided to advance to 48h experiments. In these fermentations, and as expected, the sugar uptake at 48h was in average higher when compared to 24h, although no significant differences were verified for both substrates ($p>0.05$), suggesting metabolic activity from the microorganism and consequent cellular growth during this period. However, at 48h, a decrease in cellular concentration was observed, corresponding to the detection limit of cellular concentration of the quantification method used. Interestingly, what this data suggests, is that the fermentation broth became fully inhibitory for *L. plantarum* at some point throughout the last 24 hours of fermentation. This could be explained, in part, by considering the metabolic pathways of the bacteria. *L. plantarum* is a homofermentative LAB, which means that at least 85% of the extracellular content produced will be lactic acid, whose antimicrobial properties have been reported in the literature **(Ibrahim *et al.*,**

2008; In *et al.*, 2013). Furthermore, *L. plantarum* strains are reported to produce a bacteriocin named 'plantaricin' which also exerts antimicrobial activity **(Kareem & Razavi, 2019)**. Also, polyphenols, which are expected to be present from sugarcane, are reported to exert inhibitory effect over *L. plantarum* **(Landete *et al.*, 2008; Ruiz-Barba *et al.*, 1993)**. However, with this data, it is impossible to define the moment when the full inhibition happens.

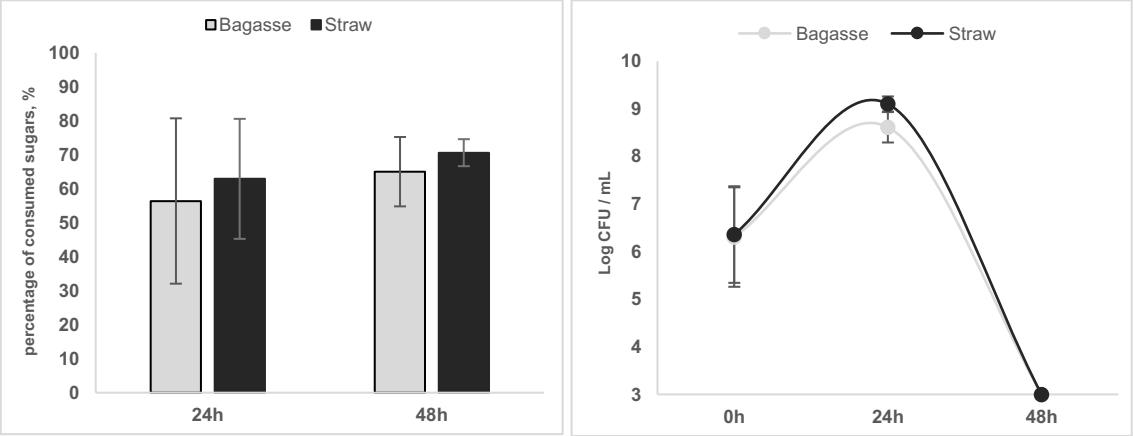

Figure 4 – Percentual sugar uptake (left) by *Lactobacillus plantarum* and its viability in Log CFU.mL^{-1} (right) during SSF experiments performed along 48h. Mean values are represented as solid bars/circles, and standard deviation is represented by bars.

2.1.2. Lactobacillus fermentum

In case of *L. fermentum*, in what concerns sugar uptake, the obtained results were the expected, occurring a significant higher sugar uptake at 48h ($p/2<0.05$), ca. 70-85% for both biomasses. However, and quite similarly to what happened with *L. plantarum*, there was a non-significant reduction (with bagasse, $p/2>0.05$) and a significant increase (with straw, $p/2<0.05$) in cellular concentration, within the last 24h hours of fermentation, what is inconsistent with the significant raise in sugar uptake. In this line, being this the case of two bacteria of the *Lactobacillus* genus, this reduction may suggest some degree of inhibition. This could be explained, in part, by considering the metabolic pathways of *L. fermentum*. It is a heterofermentative LAB, which means that only approximately 50% of extracellular content produced will be lactic acid, whose antimicrobial potential is known. However, this tendency in cellular growth may simply be 'synonymous' with the stationary phase of microbial growth. In fact, the expressive raise in sugar uptake during this period seems to point towards this explanation. The differences between substrates in cellular concentration at 48h, seems to point in a different direction. Comparing both substrates, at 48h of fermentation, there is a higher sugar uptake, in average, with straw. At 24h, the opposite is observed, although no significant differences were verified ($p/2>0.05$) on both cases.

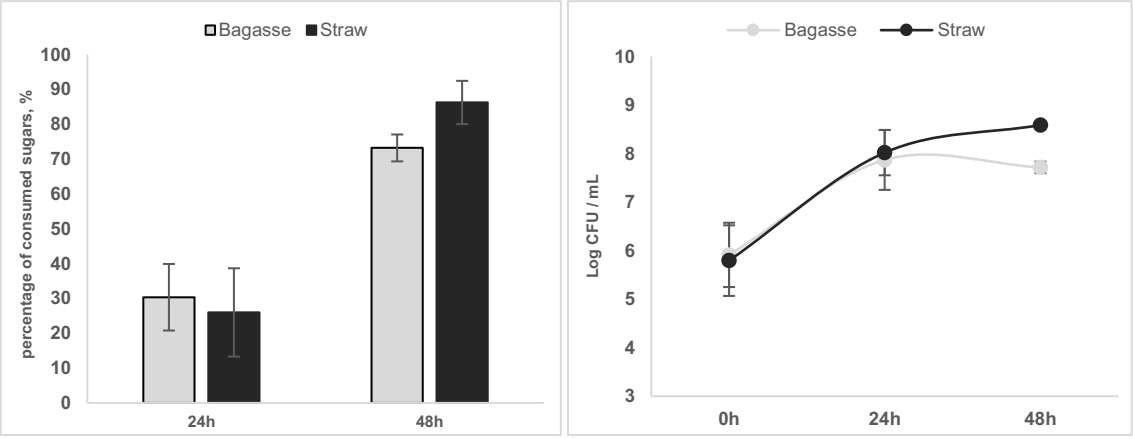

Figure 5 - Percentual sugar uptake (left) by *Lactobacillus fermentum* and its viability in Log CFU.mL^{-1} (right) during SSF experiments performed along 48h. Mean values are represented as solid bars/circles, and standard deviation is represented by bars.

2.1.3. Saccharomyces cerevisiae

In case of *S. cerevisiae*, the sugar uptake was in average superior at 48h, comparing to 24h, for both substrates, although only in case of bagasse there were significant differences ($p/2<0.05$). Comparing both substrates, at 24h, there is a higher sugar uptake, in average, with straw. At 48h, the opposite is observed, although no significant differences between substrates were confirmed, in both cases ($p>0.05$). Regarding cellular concentration, the results seem to be consistent with sugar uptake, as the cellular concentration grows continually during the first 48h of fermentation, indicating metabolic activity. Comparing both substrates, with straw, cellular concentration was significantly superior at 24h and 48h ($p/2<0.05$).

Since not all monosaccharides were consumed with 24 or 48h fermentations and at 48h, the cellular growth was still at stationary phase, it was decided to move on to 72h experiments. In these experiments, within the last 24h, no sugar consumption was observed, and in the case of bagasse fermentation there was even a significant increase in its concentration ($p/2<0.05$). Being this a fermentative process, the obtained data seems to be illogical, but the observed cellular growth attenuated, whereby sugar consumption could be inferior. Regarding the attenuation of cellular growth, this may have been due to an increase in the cellular death rate (stationary phase), and not to antimicrobial inhibition, which would not be expected considering the microorganism. Yet, it is important to emphasize that in the transition between saccharification and fermentation processes, the enzymes remained in the broth, whereby sugar release by enzymatic hydrolysis was possible to happen during fermentation, resulting in the stabilization or even the raise of sugar concentration, in cases where attenuated sugar consumption and cellular growth were observed. Interestingly, the raise in sugar concentration within the last 24h is much higher with bagasse, suggesting that this substrate could have a lower enzymatic adsorption capacity when compared to straw. Adding to this, *S. cerevisiae* performs alcoholic fermentation. Many 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 **(Goldstein, 1986; Jones & Greenfield, 1987; Komatsu**

& Okada, 1997; Mizoguchi & Hara, 1998; Piper, 1995). This considered, it is possible that cells who had been affected by ethanol, released monosaccharides, that may had already been uptook, back into the broth. This effect is referred 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 **(Mizoguchi & Hara, 1998; Piper, 1995)**. A study showed that lysosomes isolated from *S. cerevisiae* can cause its own cellular death, although at concentrations much higher than those expected under these conditions (90% mortality at a lysosome concentration of 40%) **(Yoon *et al*., 2009)**. Ethanol has also been associated with loss of cellular viability **(Jones & Greenfield, 1987; Mizoguchi & Hara, 1998; Piper, 1995)**. However, ethanol inhibitory effect over *S. cerevisiae* growth is strongly influenced by medium composition and is remarkably strain-dependent **(Piper, 1995)**.

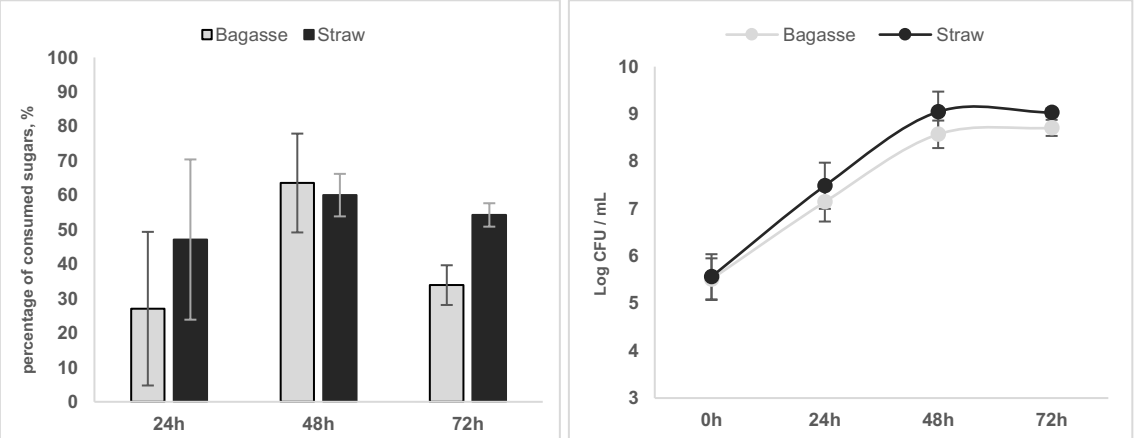

Figure 6 - Percentual sugar uptake (left) by *Saccharomyces cerevisiae* and its viability in Log CFU.mL^{-1} (right) during SSF experiments performed along 72h. Mean values are represented as solid bars/circles, and standard deviation is represented by bars.

2.2. SSF extracts chemical and biological potential screening

2.2.1. Antioxidant activity

The obtained results regarding the antioxidant performance of SSF samples by the ABTS, DPPH, and ORAC assays are represented in **Figure 7**.

The values obtained for the ABTS assay showed that *S. cerevisiae* extracts presented higher antioxidant activity than *L. fermentum* and *L. plantarum* ones, since these last two had higher EC_{50} values with 24 and 48h extracts. Regarding *L. plantarum* and *L. fermentum* extracts there wasn't an expressive increase in antioxidant activity with longer fermentation processes, suggesting that these microorganisms did not produce many antioxidant metabolites. In fact, in case of *L. plantarum*, it appeared to show a loss of antioxidant activity in case of longer fermentation processes with bagasse. In this line, *L. plantarum* has been reported to be able to metabolize phenolic compounds, whose antioxidant activity is described in literature **(Landete *et al*., 2008; Rodríguez *et al*., 2008)**. Moreover, the main component produced by this microorganism should be lactic acid, that as discussed before, is known by its antimicrobial activity. Regarding *S. cerevisiae* extracts, which had the best results, there was a general

decrease of the Ec_{50} values with longer fermentations. However, with 72h, the advantages over 48h processes did not seem to be significant ($p>0.05$), for both biomasses. In fact, the results obtained for sugar uptake and cellular concentration, discussed in **section 2.1.3. (Results and Discussion)**, also led to that conclusion. Nonetheless, there was an approximate 50% reduction of the Ec_{50} values between the 24h and 72h experiments (6.15 $mg.mL^{-1}$ to 2.86 $mg.mL^{-1}$ and 5.75 to 2.80 $mg.mL^{-1}$, for bagasse and straw samples, respectively).

The DPPH assay results were quite different from the ones of ABTS assay. On one hand, the Ec_{50} values were much higher for all samples, suggesting that most of antioxidant compounds present were water soluble. In fact, the main identified polyphenols (**section 4.1.4., Results and Discussion**), such as azelaic acid, 4-hydroxybenzaldehyde, and p-coumaric acid., are described as being water soluble at the detected concentrations **(Azepur99®, 2021; FooDB, 2020; Human Metabolome Database, 2021)**. However, they are reported to be even more soluble with ethanol-like solvents. Nonetheless, the samples were prepared in water, and, therefore, only in an aqueous reaction medium the present metabolites could present radical scavenging activity **(Tournour, 2016)**. In this line, it would be expectable that the ABTS assay presented the best results once it was performed in aqueous conditions. Instead, DPPH assay was carried out in methanol. Moreover, the conditions where it was observed a significant decrease of DPPH Ec_{50} values, with longer fermentations, suggest that in that period there was metabolites production which would dissolve in methanol, and, consequently, present higher antioxidant activity in DPPH assay. On the other hand, in contrast with ABTS, *S. cerevisiae* extracts had the worst results, mainly with 24h extracts. In fact, the differences between the three microorganisms, at 24h, with ABTS were not much expressive either. What this suggests is that the ratio between water insoluble antioxidant compounds and water-soluble ones is lowest in *S. cerevisiae* extracts. Nonetheless, with 48h extracts, the results already were very similar between the three microorganisms, mainly with straw (9.95 $mg.mL^{-1}$, 9.13 $mg.mL^{-1}$, and 9.89 $mg.mL^{-1}$ for *L. plantarum*, *L. fermentum*, and *S. cerevisiae*, respectively).

Considering the ORAC values of all samples, these were concordant with ABTS assay. *S. cerevisiae* extracts had the best results with a raise from 139.80 to 246.93 µmol $TE.g^{-1}$ and from 147.55 to 283.84 µmol $TE.g^{-1}$, between 24h and 72h extracts for bagasse and straw, respectively. In case of both *Lactobacillus* bacteria, there were not great differences between them. In all assays, straw presented higher antioxidant activity for all fermentation conditions performed, suggesting a higher phenolic compounds content, derived from sugarcane straw.

In short, the sample that presented the highest antioxidant activity, in average was the 72h fermentation extract from *S. cerevisiae*, using straw as substrate (ScStr 72h). In this line, some studies report that autolysates, β-glucans and protein fractions (free thiols from denatured proteins) derived from *S. cerevisiae* have the potential to be explored as natural antioxidants **(Hassan, 2011; Jaehrig *et al.*, 2007; Song *et al.*, 2006)**. Furthermore, cell-wall polysaccharides from *S. cerevisiae* have also been associated with this kind of activity **(Kogan *et al.*, 2008)**. Moreover, there are polyphenols that may be stranded to the biomass and that, during fermentation, may be released also by the presence of the microorganisms.

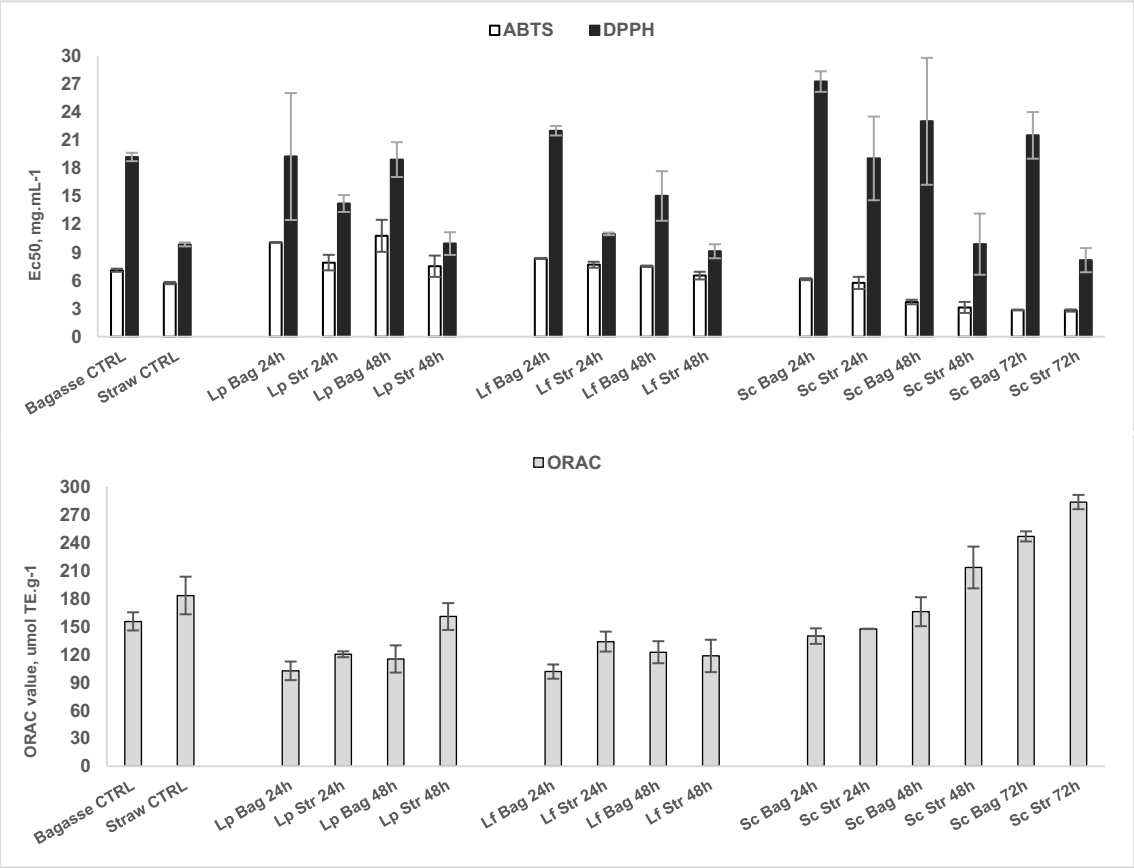

Figure 7 - Antioxidant values determined by ABTS, DPPH, and ORAC assays for all SSF extracts. Mean values (solid bars) are expressed as mg.mL^{-1} (ABTS, DPPH) or µmol TE.g^{-1} (ORAC), and standard deviation is represented by bars.

2.2.2. Antimicrobial activity

Regarding antimicrobial activity (**Table 5**), within the range of assayed concentrations, only *L. plantarum* 48h (LpBag 48h and LpStr 48h) extracts exhibited some degree of inhibition. The extract using bagasse presented higher antimicrobial potential than using straw, suggesting that some compounds derived from bagasse also contribute to this activity. Interestingly, the opposite tendency was observed for antioxidant activity. This extract presented a minimum inhibitory concentration (MIC) of 4 % and a minimum bactericidal concentration (MBC) of 5 %, against *S. aureus*. As discussed in **section 2.1.1. (Results and Discussion)**, several studies report the antimicrobial activity of *L. plantarum*-derived metabolites such as lactic acid and plantaricin. This considered, such results would be expectable. Furthermore, some available postbiotics on the market, such as LactoSporin®, claim to be antimicrobial and present a similar MIC (4%), even for a different microorganism **(Majeed et al., 2020a)**. In this line, the obtained results were interpreted as being very promising.

Table 5 - Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the bagasse and straw control (CTRL) and fermentation extracts from Simultaneous Saccharification and

Fermentation (SSF) process with *L. plantarum* (Lp), *L. fermentum* (Lf), and *S. cerevisiae* (Sc) in bagasse (Bag) and Staw (Str).

Sample	Biomass	MIC	MBC
CTRL	Bagasse	>5%	>5%
	Straw	>5%	>5%
Lactobacillus plantarum SSF extracts			
Lp 24h	Bagasse	>5%	>5%
	Straw	>5%	>5%
Lp 48h	Bagasse	4%	5%
	Straw	5%	>5%
Lactobacillus fermentum SSF extracts			
Lf 24h	Bagasse	>5%	>5%
	Straw	>5%	>5%
Lf 48h	Bagasse	>5%	>5%
	Straw	>5%	>5%
Saccharomyces cerevisiae SSF extracts			
Sc 24h	Bagasse	>5%	>5%
	Straw	>5%	>5%
Sc 48h	Bagasse	>5%	>5%
	Straw	>5%	>5%
Sc 72h	Bagasse	>5%	>5%
	Straw	>5%	>5%

3. Sequential Saccharification and Fermentation (SQSF)

3.1. Effect of biomass removal in sugar uptake of the most promising SSF extracts

3.1.1. Lactobacillus plantarum

The effect of removing biomass after saccharification, i.e. Sequential Saccharification and Fermentation (SQSF), was compared with the Simultaneous Saccharification Fermentation (SSF). These experiments were performed using sugarcane bagasse as substrate since it was the condition that showed the most promising results with *L. plantarum*. Comparing SSF and SQSF 48h experiments (**Figure 8**), some differences were identified. In this line, at 24h there were no differences between experiments ($p>0.05$) and at 48h, SSF experiments allowed a higher sugar uptake in average, despite no significant differences were confirmed as well ($p>0.05$). Moreover, between the 24 and 48h of SQSF, the sugar uptake remained practically unchanged ($p>0.05$), suggesting that during this period the metabolic activity may have been extremely reduced. Regarding cellular concentration, significant differences were obtained at 0h and 24h ($p<0.05$). At 48h, the opposite was observed ($p>0.05$). Also, it matters to remember that at 48h, similarly to what happened in SSF experiments, the cellular concentration decayed to 3 log $CFU.mL^{-1}$ (quantification method detection limit of cellular concentration) since no growth was obtained. In short, regarding the sugar uptake no obvious advantages were obtained by removing

the biomass in between the saccharification and fermentation processes. Nonetheless, the presence of biomass, especially unmilled, may introduce some unwanted variability to the process by releasing sugar more slowly along the fermentation.

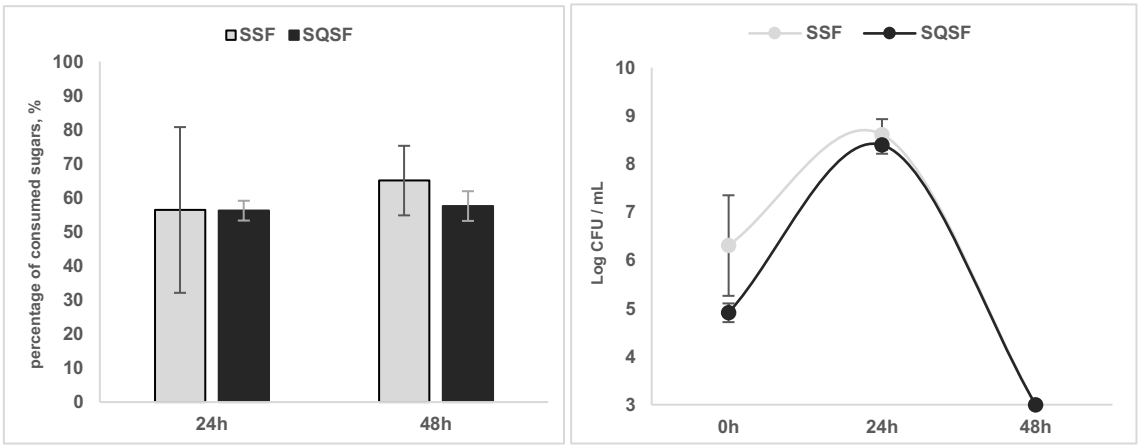

Figure 8 - Percentual sugar uptake (left) by *Lactobacillus plantarum* and its viability in Log CFU.mL^{-1} (right) during SQSF experiments performed along 48h. Mean values are represented as solid bars/circles, and standard deviation is represented by bars.

3.1.2. Saccharomyces cerevisiae

For SQSF comparing with SSF experiments (**Figure 9**), within the first 24h of fermentation, cellular growth was more accentuated (curve slope), suggesting that the biomass removal may have facilitated the cellular growth of *S. cerevisiae*. Perhaps a higher sugar uptake should also be expected in that period, since the monossacharides would be more available for cells without the biomass presence, but this was not clearly confirmed, as no significant differences were verified ($p>0.05$). After the 24h of fermentation in SQSF experiments, the cellular concentrations seemed to stabilize, suggesting that the conditions discussed in **section 2.1.3. (Results and Discussion)** may have been reached sooner. In this line, sugar uptake in SQSF extracts slightly improved, between 24h and 48h, yet with significant differences ($p<0.05$), and remained constant within the last 24h of fermentation ($p>0.05$), what seems to agree with the stabilization of cellular concentration. On the other hand, the changes in cellular membrane permeability, discussed in **section 2.1.3. (Results and Discussion)** may also have influence in this stabilization. Comparing both approaches, no significant differences were verified at any measured time ($p>0.05$). Finally, and similarly to the observed with *L. plantarum*, with biomass removal, the obtained standard deviations were overall substantially smaller, especially at 24h, suggesting that the presence of biomass may be a source of variability to the process. These experiments were performed only with sugarcane straw as substrate once it showed the most promising SSF results with *S. cerevisiae*, as discussed in **section 2.2.1 (Results and Discussion)**.

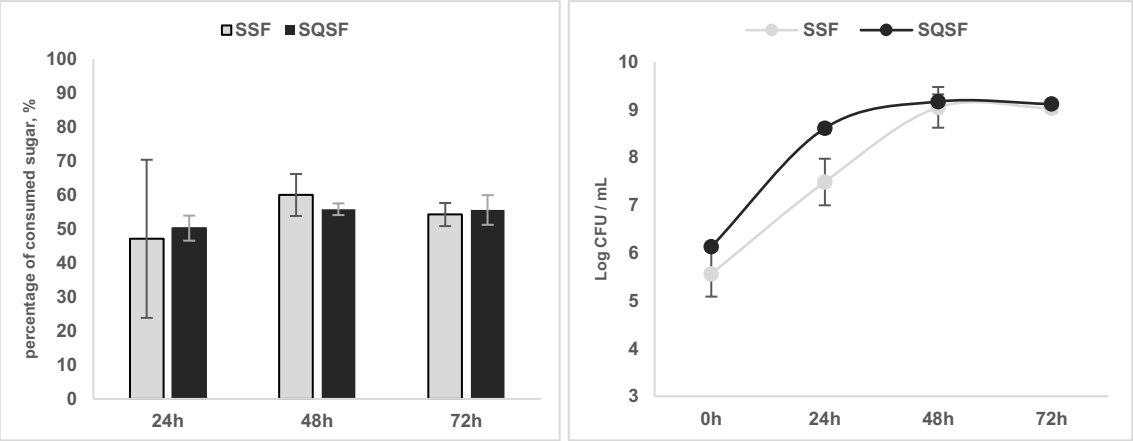

Figure 9 - Percentual sugar uptake (left) by *Saccharomyces cerevisiae* and its viability in Log CFU.mL^{-1} (right) during SQSF experiments performed along 72h. Mean values are represented as solid bars/circles, and standard deviation is represented by bars.

Both SQSF extracts were analyzed for mono-, and oligosaccharides (typically 2-10 monosaccharides) identification and quantification, in order to try to understand the reasons why not all sugars were consumed during fermentation. Remember that the sugars that are being analysed are free sugars present in the biomass, released along the saccharification, and not consumed along fermentation. Three different sugars were identified: glucose, sorbitol, and cellobiose. The results are resumed in **Figure 10**. 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 **(Galant *et al*., 2015)**. 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 **(Dutta *et al*., 2018; Sharma *et al*., 2014)**. Cellobiose is a disaccharide consisting of two glucose molecules linked by a β-(1,4') glycosidic bond **(Ouellette and Rawn, 2015; Ouellette and Rawn, 2018)**. In case of glucose, being this the constituent monosaccharide of cellulose (one of the main components of lignocellulose), its presence was in accordance with the expected, resulting of cellulose degradation by heat and, posteriorly, enzymes. Regarding sorbitol, it is the hydrogenation/reduction product of glucose **(Singh *et al*., 2018)**, reason why its presence seems justifiable. Also, the presence of cellobiose was expected since it is produced by the hydrolysis of cellulose **(Carbosynth, n.d.)**.

Regarding control analysis, cellobiose was found in higher quantities when using sugarcane straw. In opposite, sorbitol was more present when using sugarcane bagasse. In case of glucose, there were not significant differences between both substrates, although in average it was slightly higher with straw. Generally, by this analysis, it seemed that sugarcane bagasse allows a higher content of free sugars. However, the phenol-sulfuric acid analysis pointed in the opposite direction, suggesting that some mono-, and/or oligosaccharide, which could be more present in straw, may not have been detected by HPLC analysis. Interestingly, some initially expected sugars such as xylose and arabinose were not, in fact, detected. Concerning the fermented samples, both cellobiose and glucose were fully consumed by both microorganisms. In case of glucose, this would be expectable since it is the most used fermentable monosaccharide exactly

by its easiness in being metabolized by microorganisms. In case of cellobiose, being this a disaccharide, microorganisms should not easily metabolize it. However, accordingly to the obtained results both microorganisms consumed it. The low quantities that were detected also contributed to the total uptake of cellobiose. In higher quantities, it was most likely that this sugar would not be fully consumed. In fact, regarding *S. cerevisiae*, some studies report its difficulty in metabolizing cellobiose and the need to resort to other strategies that would allow a more efficient utilization of this disaccharide. For example, the engineering of multiple proteins and/or genes involved in cellobiose uptake and metabolization have been explored. Also, the introduction of exogenous DNA for enhanced cellobiose catabolism has been reported **(Eriksen *et al*., 2013; Ha *et al*., 2011a; Ha *et al*., 2011b; Sadie *et al*., 2011; Yuan and Zhao, 2013)**. In case of *L. plantarum*, a study by **Ucar et al. (2020)** showed that several LAB, including *L. plantarum*, were able to ferment disaccharides (cellobiose and gentibiose). Nonetheless, these sugars degradation by these bacteria was pH dependent. Interestingly, at pH 4.7 $\pm$ 0.1 (fairly like what was used in this work), *L. plantarum* was able to metabolize cellobiose. Despite this, cellobiose, being a disaccharide, would not be the primary carbon source for *L. plantarum* metabolism, in the presence of glucose. In case of sorbitol the results were different. *L. plantarum* was able to consume this sugar alcohol, although not in its entirety. In fact, some papers in the literature report that this microorganism can grow in the presence of sorbitol as the sole energy source **(Glaasker *et al*., 1998; Yang, X. *et al*., 2019)**. Nonetheless, when in the presence of other sugars, the addition of sorbitol is reported to not increase the growth rate of *L. plantarum*, because the microorganism fails to accumulate this sugar against its concentration gradient **(Glaasker *et al*., 1998)**. In opposite, *S. cerevisiae* did not metabolize sorbitol. Instead, it seemed to have produced it. In this line, no studies were found reporting sorbitol uptake by *S. cerevisiae*. In fact, this sugar is reported to cause osmotic stress to this yeast, inducing trehalose and/or glycerol biosynthesis **(Garcia *et al*., 1997; Kaino and Takagi, 2008)**. However, no analysis was made for glycerol and/or trehalose quantification. Furthermore, sorbitol (1.5 M) has been reported to inhibit *S. cerevisiae* growth in about 70% **(García *et al*., 1997)**.

Figure 10 – Concentration of mono-, and oligosaccharides identified in SQSF extracts from *L. plantarum* and *S. cerevisiae* fermentations. Mean values (solid bars) are expressed as $mg.g^{-1}$, and standard deviation is represented by bars.

Although the study of enzymatic hydrolysis and fermentation processes was not the main purpose of this work, the analysis of the obtained data may be of crucial importance for obtaining the most optimized fermentation extracts possible. For example, being the antimicrobial activity one of the assessed properties, it is important that the final sugars concentration in the extracts are as low as possible. For that, saccharification time, fermentation time, and inoculation ratio represent variables that may have a huge influence on this parameter. Furthermore, data from cellular concentration, for example, in case of *L. plantarum* fermentation, indicates that somewhere between 24 and 48h of fermentation, the broth becomes self-inhibitory for *L. plantarum* itself, preventing a possible evolution towards longer fermentation processes, that, in this case, would not represent any obvious advantage.

4. Final (SQSF) extracts properties

4.1. Physicochemical properties

4.1.1. Antioxidant activity

The obtained results regarding the antioxidant performance of SQSF samples, compared to the correspondent SSF samples, by the ABTS, DPPH, and ORAC assays are represented in **Figure 11**.

The values obtained for the ABTS assay showed that both SQSF extracts presented higher antioxidant activity, once they presented lower EC_{50} values compared to SSF extracts (from 10.78 to 6.86 $mg.mL^{-1}$, and from 2.80 to 1.88 $mg.mL^{-1}$, for *L. plantarum* and *S. cerevisiae*, respectively), suggesting the presence of higher levels of water-soluble antioxidant compounds. In case of *L. plantarum*, the initial cell concentration was, in average, lower, although within range, and so this outcome should be due to some water-soluble components of its cellular membrane. For DPPH assay, *L. plantarum* SQSF extract also showed higher antioxidant activity (from 18.92 ± 1.86 to 9.00 ± 2.37 $mg.mL^{-1}$). The conclusions are similar as for ABTS assay. However, in this case, the components contributing for this outcome should mainly be water insoluble. For instance, lipoteichoic acid, present in gram-positive bacteria such as *L. plantarum*, exerts several biological activities, as resumed in **Table 1**. In this line, it is possible that other components may also present some antioxidant activity. In case of *S. cerevisiae*, the initial cellular concentration was similar (slightly higher) in SQSF experiments, suggesting that the improvement of antioxidant activity may also have been due to its cellular membrane components. However, for DPPH assay, there were no significant differences between SSF and SQSF extracts ($p>0.05$), suggesting that most of the antioxidant compounds produced by this microorganism are water soluble. Considering the ORAC values, these agree with ABTS, for both microorganisms, and with DPPH assay for *L. plantarum*, as the SQSF extracts presented higher antioxidant activity, e.g., higher ORAC values (from 115.24 to 155.27 µmol $TE.g^{-1}$ and from 283.84 to 302.23 µmol $TE.g^{-1}$ for *L. plantarum* and *S. cerevisiae*, respectively).

Two antioxidant benchmarks (ascorbic acid and BHT) were tested. The values obtained for ABTS, DPPH and ORAC assays were 0.051, 0.036, 2652.93, and 0.131, 0.284 µmol TE.g^{-1}, for ascorbic acid and BHT, respectively. BHT results in ORAC assay were inconclusive, and therefore are not presented. As expected, these substances presented higher antioxidant activity, comparing to the samples, since they are pure substances. On the other hand, the assayed extracts are a result of microbiological fermentations, being these a mix of various kinds of components, also exerting biological activities other than antioxidant.

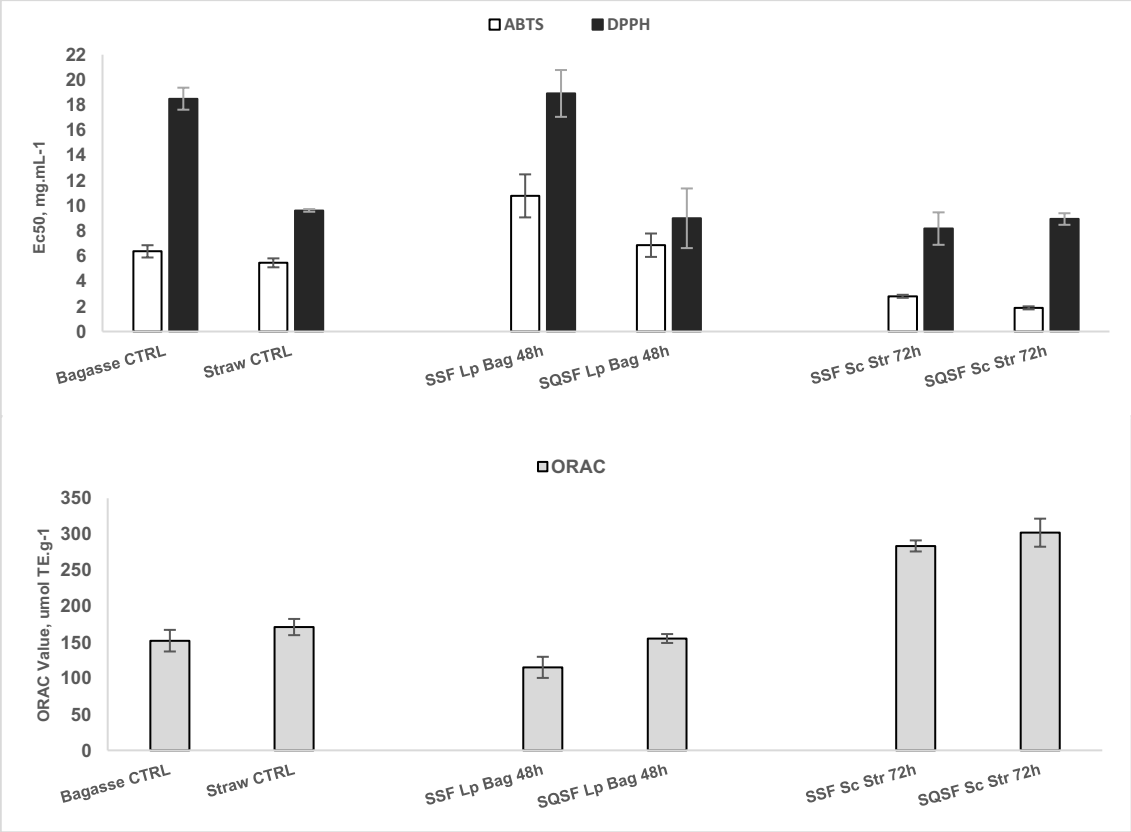

Figure 11 - Antioxidant activity values determined by ABTS, DPPH, and ORAC assays of the SSF extracts and SQSF extracts with higher potential. Mean values (solid bars) are expressed as mg.mL^{-1} (ABTS, DPPH) or µmol TE.g^{-1} (ORAC), and standard deviation is represented by bars.

4.1.2. Antimicrobial activity

Regarding antimicrobial activity (**Table 6**), no improvements in the inhibition over *S. aureus* were observed. In case of *S. cerevisiae*, what this suggests is that its cellular membrane components do not present antimicrobial properties. In fact, these results would be expectable since no studies were found reporting the antimicrobial activity of this microorganism. In case of *L. plantarum*, the results would also be expectable. The initial cellular concentration was maintained and, therefore, the production of the reported antimicrobial metabolites (lactic acid and plantaricin) should be similar. Nonetheless, it was possible to assess that the cellular membrane components did not present any antimicrobial activity. Also, removing the biomass did not represent any improvement in the antimicrobial activity of both extracts, as it could be a helpful solution to reduce the final sugar concentration of the extracts.

Table 6 - Comparison of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) between extracts from Simultaneous Saccharifications and fermentation (SSF) and extracts from Sequential Saccharification and Fermentation (SQSF) processes with *L. plantarum* (Lp) in bagasse, and with *S. cerevisiae* (Sc) in straw.

Sample	Biomass	MIC	MBC
CTRL	Bagasse	>5%	>5%
	Straw	>5%	>5%
Lactobacillus plantarum extracts			
SQSF Lp 48h	Bagasse	4%	5%
SSF Lp 48h		4%	5%
Saccharomyces cerevisiae extracts			
SQSF Sc 72h	Straw	>5%	>5%
SSF Sc 72h		>5%	>5%

4.1.3. Short-chain fatty acids

Both SQSF extracts were analysed by High Performance Liquid Chromatography (HPLC) for short-chain fatty acids (SCFAs) identification and quantification. Three SCFAs were identified: citrate, acetate, and lactate. The results are resumed in **Figure 12**.

Regarding control analysis, only citrate and acetate were detected, and in a fairly similar concentration between bagasse and straw, as it would be expected. Citrate came from the 50 mM citrate buffer which served as buffer of the fermentation medium. On its side, acetate was derived from one of the used supplements: 5 $g.L^{-1}$ sodium acetate. Also, and as expected, no lactate was detected in the controls. The obtained values using bagasse and straw were 309.91 and 71.57, and 294.55 and 71.66 $mg.mL^{-1}$, for citrate and acetate, respectively.

Concerning the fermented samples, citrate concentration decreased on both extracts suggesting that both microorganisms may have metabolized part of it. In case of *L. plantarum*, some studies reported that this bacterium, if present in large enough numbers, can metabolize citrate in the absence of an energy source such as glucose **(Hugenholtz, 1993; Kennes *et al*., 1991; Palles *et al*., 1998)**. Considering the SQSF *L. plantarum* extract with bagasse fermented for 48h (SQSF LpBag 48h), the acetate concentration increased, and lactate was detected. The production of lactate was initially expected since *L. plantarum* is classified as a homofermentative lactic acid bacteria, as described in **section 4.1.1. (Introduction)**. On the other hand, this increase in acetate would not be so predictable. However, of the remaining 15% of the extracellular metabolites produced by *L. plantarum*, it is possible that acetate was present. Furthermore, *L. plantarum* has also been described as a facultative heterofermentative lactic acid bacterium **(Kleerebezem *et al*., 2003)**, reason why acetate could, in fact, be a more representative substance of *L. plantarum* metabolism. Moreover, it is reported by **Palles *et al*. (1998)** that one of the main products of citrate metabolization by *L. plantarum* is, in fact, acetate. Before that, also **Hugenholtz (1993)** and **Kennes *et al*. (1991a)** had already described this. Regarding the SQSF *S. cerevisiae* extract with straw fermented for 72h (SQSF ScStr 72h), this microorganism metabolized more of the available citrate in the culture medium, comparing to *L. plantarum*. However, *S. cerevisiae* has been reported as incapable of metabolizing citrate **(Barnett and**

Kornberg, 1960; Kennes *et al.*, 1991b). 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 **(Polakis and Bartley, 1965; Takada and Noguchi, 1985)**. What this seems to suggest is that the detected citrate was, in fact, a combination of citrate and isocitrate, being the isocitrate the one consumed. In this line, the mass spectrometry analysis allowed to confirm this hypothesis, as two distinct peaks with the same *m/z* and the same fragments, citrate characteristics, were detected, but only one was attenuated by fermentation. Acetate also was slightly consumed. In fact, and adding to what was discussed before, a study by **Shang *et al.* (2016)** showed that, during fermentations using *S. cerevisiae*, and preceded by acetate supplementation, there was a slight reduction in acetate concentration. Nonetheless, as the controls and the samples were independent experiences it is normal that some small variations, like this one, may be verified. Moreover, and as expected, no lactic acid was produced by *S. cerevisiae*.

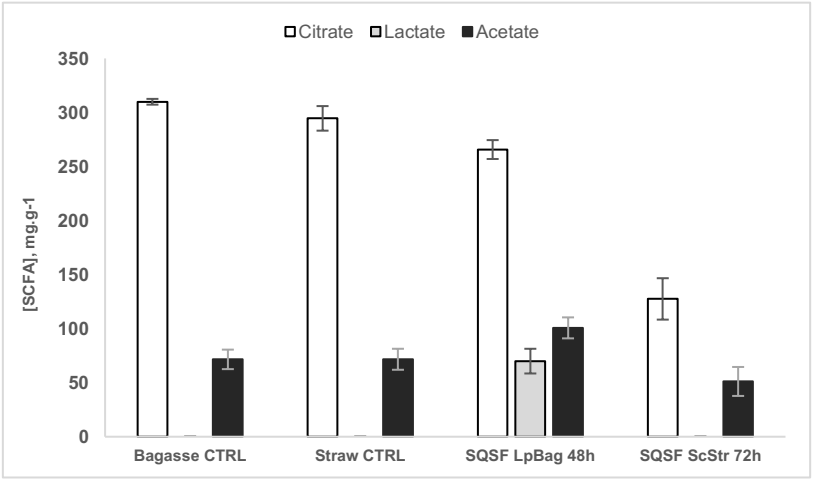

Figure 12 - Short-chain fatty acids identified in both SQSF extracts. Mean values (solid bars) are expressed as $mg.g^{-1}$, and standard deviation is represented by bars.

4.1.4. Total phenolics content and individual polyphenols

Both SQSF extracts were analysed by the Folin-Ciocalteau method, for total phenolic content determination, and by LC-ESI-UHR-QqTOF-MS, for individual polyphenols identification and quantification. The respective results are resumed in **Table 7**.

The obtained values for total phenolics content were 8.620, 6.810, 12.328 and 13.460 $mg.g^{-1}$ for bagasse control, SQSF LpBag 48h extract, straw control, and SQSF ScStr 72h extract, respectively. As hypothesized earlier in **section 2.2.1. (Results and Discussion)**, the notion that *L. plantarum* is capable of metabolizing phenolic compounds seems to be corroborated by the obtained results, since there is a decrease in their concentration comparing the bagasse control with the SQSF LpBag 48h extract. In this line, the decrease in antioxidant activity seems to be justified, as *L. plantarum* is not known to produce metabolites exerting that kind of activity. Regarding SQSF ScStr 72h extract, it seems that during fermentation, a small quantity of phenolic compounds may have been produced. However, this discrepancy does not seem to justify the

much higher antioxidant activity of the SQSF ScStr 72h extract when compared to the straw control. In this line, and as discussed before, *S. cerevisiae* is reported to produce other metabolites which exert this kind of activity. Comparing bagasse with straw, it is possible to observe that straw makes available a significantly higher concentration of phenolic compounds, reason why the higher antioxidant activity of straw is justified.

Regarding individual identification, eight distinct polyphenols were identified among all the assayed samples: azelaic acid, sebacic acid (organic acids), 4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde (protocatechuic aldehyde, hydroxybenzoic acids), p-coumaric acid, p-coumaric acid derivate (hydroxycinnamic acids), iso-chaftoside, 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 was found to be more present with straw and its content did not seem to be affected by the fermentation process on both cases. Contrary, the other two were found in higher amounts with bagasse, and the obtained results suggest that they may have been metabolized by both microorganisms during fermentation. Concerning *L. plantarum* this was expectable, as discussed above. Nonetheless, no studies were found reporting this phenomenon with *S. cerevisiae*. In fact, this metabolization of phenolic compounds seemed to be more expressive when using the bacteria. It should also be noted that p-coumaric acid derivate was detected in greater amounts in the fermented extracts, suggesting that the fermentation process can lead to the degradation of this type of compound. Phenolic compounds like the ones here explored have biological importance, by their known antioxidant activity. However, these compounds are also reported to have some usefulness in skin care applications. For example, p-coumaric acid is suggested to have potential to be used as a skin-lightening active ingredient **(Boo, 2019)** and to be an inhibitor of tyrosinase activity **(Varela *et al*., 2020)**. Furthermore, p-coumaric acid and its derivates are also reported to exert some anti-inflammatory effect **(Lee *et al*., 2019)**.4-Hydroxybenzaldehyde is reported to promote wound healing and reepithelization in an in vivo animal model **(Kang *et al*., 2017)**. Moreover, this compound and its derivates are reported to also present some tyrosinase activity inhibition **(Yi *et al*., 2010)**. Another example is azelaic acid, which is reported as an anti-acne ingredient **(Iraji *et al*., 2007; Nazzaro-Porro *et al*., 1983; Webster, 2000; Worret and Fluhr, 2006)**. For instance, some polyphenols, such as 3,4-dihydroxybenzaldehyde and sebacic acid, also seemed to be enhanced by the fermentation process. In fact, 3,4-dihydroxybenzaldehyde is reported to lower ROS generation, and to inhibit oxidative DNA damage and apoptosis due to its antioxidant activity **(Jeong *et al*., 2009; Maheshwari *et al*., 2018)**. Also, a derivate of it (5-bromo-3,4-dihydroxybenzaldehyde) has been reported to promote hair growth in dermal papilla cells **(Kang *et al*., 2022)**. Naturally, not all the present polyphenols must have been identified. However, some common ones derived from sugarcane such as chlorogenic acid, caffeic acid, ferulic acid, and vanillic acid **(Deseo *et al*., 2020; Freitas *et al*., 2018)** were evaluated but were not detected.

Table 7 - Polyphenols identified and respective concentrations (Mean ± SD) in the SQSF extracts.

n.d. - not detected; * significantly different from the respective control ($p < 0.05$).

	Extracts			
Polyphenols	Bagasse CTRL	SQSF LpBag 48h	Straw CTRL	SQSF ScStr 72h
	Concentration (Mean ± SD), $mg.g^{-1}$ of sample			
Total phenolics	8.620 ± 0.406	6.810 ± 0.227	12.328 ± 0.419	13.460 ± 0.488
Organic acids				
Azelaic acid	0.204 ± 0.009	0.238 ± 0.005*	0.809 ± 0.050	0.833 ± 0.014
Sebacic acid	0.013 ± 0.002	0.017 ± 0.001*	0.104 ± 0.005	0.135 ± 0.005*
Hydroxybenzoic acids				
4-hydroxybenzaldehyde	0.562 ± 0.034	0.350 ± 0.003*	0.182 ± 0.006	0.006 ± 0.001*
3,4-dihydroxybenzaldehyde	0.005 ± 0.009	0.073 ± 0.004*	0.065 ± 0.005	0.232 ± 0.019*
Hydroxycinnamic acids				
p-coumaric acid	2.285 ± 0.105	0.002 ± 0.001*	1.056 ± 0.053	0.422 ± 0.013*
p-coumaric acid derivate	n.d.	0.007 ± 0.000*	0.057 ± 0.066	0.114 ± 0.001
Flavones				
Iso-chaftoside	n.d.	n.d.	0.003 ± 0.001	0.004 ± 0.000
Tricin 7-O-rhamnosyl-glucuronide	0.007 ± 0.001	0.006 ± 0.000	0.013 ± 0.000	0.014 ± 0.000*

4.1.5. Total protein content and individual aminoacids

The SQSF fermented extracts were also analysed for total protein content. The obtained results are resumed in **Table 8**. The values for total protein content were 36.82, 33.54, 37.64 and 33.64 $g.100\ g^{-1}$ for bagasse control, straw control, SQSF LpBag 48h sample, and SQSF ScStr 72h sample, respectively.

Comparing the controls with the respective extracts, there seems to be no significant differences. However, and since we are dealing with fermentation, it was expectable that the fermented extracts would present a higher concentration of protein comparing to the controls, due to the predictable production of peptides by both microorganisms. Still, the utilized method for protein quantification was DUMAS method, which directly quantifies nitrogen, and not protein **(Mihaljev *et al.*, 2015)**. In this line and considering that for protein production microorganisms would have to have available amino acids, the nitrogen content of aminoacids/proteins supplementation should be similar to the final amino acids/proteins content. For this reason, fermentation did not represent an augment in total protein content. Furthermore, the addiction of enzymes to the medium must be considered. In fact, this is the reason samples with bagasse present a higher protein content. With this substrate, obtaining the same enzymatic activity (20 $FPU.g\ cellulose^{-1}$) required a higher volume of Celluclast cocktail. Moreover, it would be expectable that the biomass adsorbed the enzymes (by lignin action), resulting in their removal when removing the biomass, reason why the protein content would probably be higher, if not for this.

Table 8 - Total protein content (Mean ± SD) of the assayed samples, determined by the DUMAS method.

Sample	Bagasse CTRL	Straw CTRL	SQSF LpBag 48h	SQSF ScStr 72h
Total protein content ($mg.g^{-1}$)	36.82 ± 1.36	33.54 ± 0.56	37.64 ± 1.21	33.64 ± 0.57

For amino acids profiling, 17 different amino acids were tested: aspartic acid, glutamic acid, cysteine, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, and tryptophan. The obtained results are resumed in **Figure 13**.

Regarding both assayed extracts, cysteine, histidine, and arginine were not detected in either of them, and tryptophan was detected in both, in residual quantities. The cases where SQSF LpBag 48h extract presented a slight superiority in amino acids concentration, such as serine, threonine, alanine, valine, and lysine, were due to the difference on the added volume of enzymatic cocktail, depending on the substrate. In the cases of phenylalanine, isoleucine, and leucine, where those differences are more significant, it seems reasonable to assume that the fermentation process may also have contributed to the observed discrepancies. In the cases of aspartic acid, glutamic acid, glycine, and methionine (detected only on SQSF ScStr 72h extract), where the aminoacids concentration was superior in the SQSF ScStr 72h extracts, it is possible to conclude that the fermentation process by this microorganism promoted the synthesis of these amino acids, at least at a higher scale than *L. plantarum*. For both assayed extracts, the most present amino acid was glutamic acid, followed by glycine, aspartic acid, and alanine, by that order. All the other amino acids were detected in much smaller concentrations. It matters to mention that these conclusions are only possible since the supplementation to the fermentation medium was equal for all tested conditions.

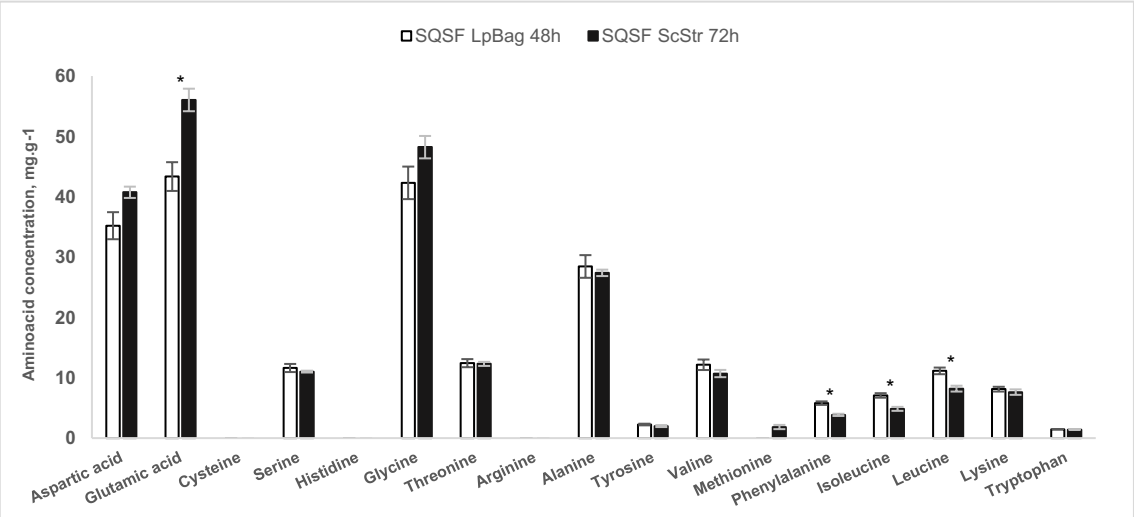

Figure 13 - Individual amino acid concentration in both SQSF assayed extracts. Mean values (solid bars) are expressed as $mg.g^{-1}$, and standard deviation is represented by bars. *Significant differences between samples ($p < 0.05$)

4.2. Skin biological properties of the most promising extract

The most promising extracts (SQSF) were tested for their skin biological activities. In this line, the safety of the extracts was first evaluated to test the non-cytotoxic concentrations.

4.2.1. Cytotoxicity

The obtained results regarding the cytotoxicity of the final (SQSF) samples, by the PrestoBlue assay, are represented in **Figure 14**. At an early stage, the safety of the SQSF extracts was evaluated only in keratinocytes, and the results would be extrapolated to the fibroblasts. However, these two types of cells may present distinct tolerances to the same substance. Having said that, when testing for collagen I α1 production, the assayed concentration ended up stressing the fibroblasts, and so the cytotoxicity of the sample had to be tested also in these cells. Only SQSF ScStr 72h extract was tested for fibroblasts cytotoxicity since it would be the only assayed extract for CK14 and collagen I α1 production.

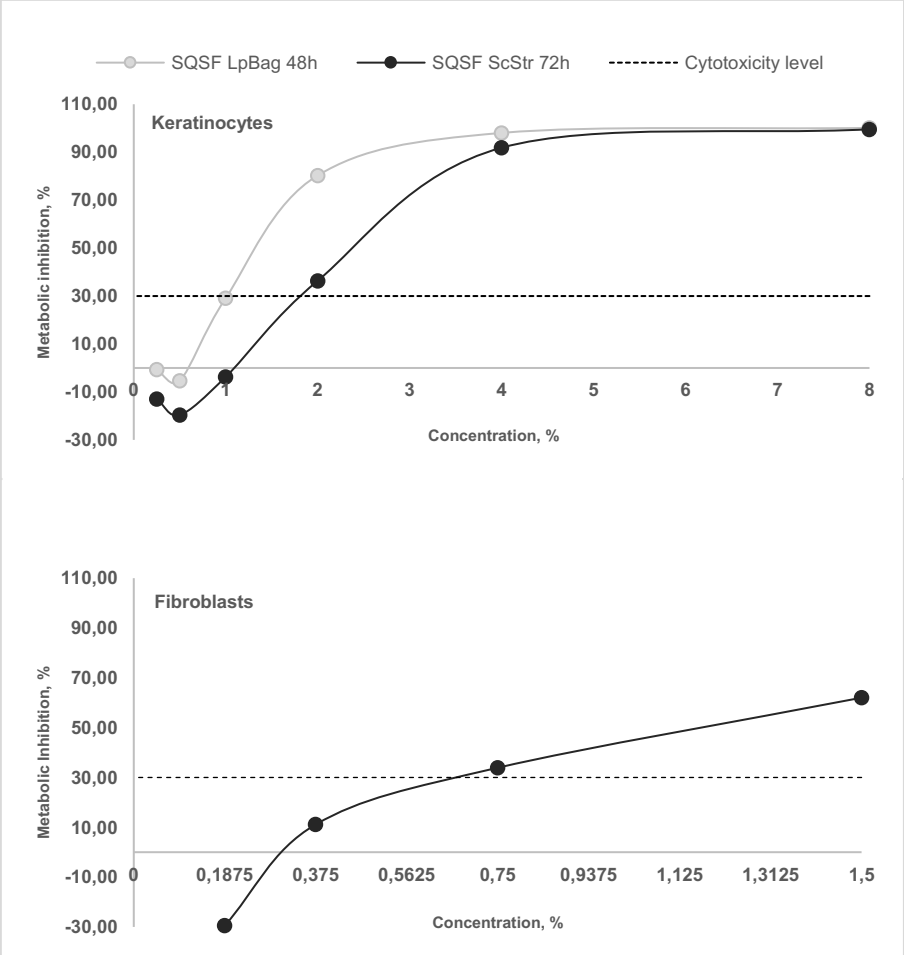

Figure 14 - Cytotoxic effect of the SQSF extracts in skin cells (keratinocytes and fibroblasts). Mean values (solid circles) are expressed as percentual.

Regarding keratinocytes metabolic inhibition, the SQSF LpBag 48h and SQSF ScStr 72h extracts presented cytotoxicity at concentrations higher than 1% and 2%, respectively. In case of the SQSF LpBag 48h extract, the cytotoxicity presented by the 1% concentration went to the limit

(29.13 ± 15.11%). On the other hand, the 2% concentration of the SQSF ScStr 72h extract already passed the cytotoxicity limit (36.38 ± 5.10%). Nonetheless, the standard deviation obtained, in SQSF LpBag 48h extract case, seems to suggest that the cells may respond differently to the same stimulus. Moreover, in a biological tissue context (*in vivo*), cells tend to be more resilient to the exposure with foreign substances. Considering what was discussed in **section 4.1.2. (Results and Discussion)**, the MIC obtained for the SQSF LpBag 48h extract was far superior to the maximum safe concentration. In this line, and under these conditions, this extract could not be successfully used for microbiological preservation and/or safety of a cosmetic formulation. So, only the SQSF ScStr 72h extract, explored by its highest antioxidant activity, was evaluated for other biological properties, reason why it was the only extract whose safety was tested in fibroblasts. Regarding fibroblasts metabolic inhibition, these proved to be more sensitive to the SQSF ScStr 72h extract than keratinocytes, reaching a metabolic inhibition of 30% (limit) at a concentration slightly lower than 0.75%. At this concentration, the obtained metabolic inhibition value was 33.90 ± 4.08%. In all cases, the negative results of metabolic inhibition are suggested to indicate an increase in cellular proliferation. Nonetheless, additional studies should be performed to evaluate such an hypothesis **(Teixeira *et al.*, 2021)**.

In short, due to the "compatibility" between the safe concentrations range and the obtained Ec_{50} value, only the SQSF ScStr 72h 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.

4.2.2. 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 assayed extract (SQSF ScStr 72h) are represented in **Figure 15**. The tested concentrations were defined accordingly to the cytotoxicity assay results, and only one concentration was tested for each quantification. It was opted to test a close to maximum safe concentration for each case.

Regarding collagen I α1, the assayed sample (0.6% concentration) showed no significant effect on 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 ± 0.07, and 0.90 ± 0.22, 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.* (2001)** demonstrated that skin care 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 **(Aguirre-Cruz *et al.*, 2020; Asserin *et al.*, 2015; Jhawar *et al.*, 2019; Kwatra, 2020; Sibilla *et al.*, 2015)**, which represents a different approach. However, the obtained results with the assayed extract were not as promising as initially intended, but it is still important to understand the importance of collagen on skin integrity, function, and appearance. Collagen is the major structural protein of connective tissues such as skin, tendons, ligaments, and bones, being the

most prevalent component of the extracellular matrix (ECM), together with elastin and hyaluronic acid **(Alves *et al*., 2017; Sibilla *et al*., 2015)**. It is responsible for structure, stability, and strength especially within the dermal layers **(Kwatra, 2020)**. In addition to the mentioned functions, collagen also 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, which manifest as lax and wrinkled skin **(Jhawar *et al*., 2019)**. The decrease of collagen density has been associated with the passage of time, and particularly with exposure to the sun (photo-aging) **(Kwatra, 2020)**. The consequences of photo-aged skin are widely described in **section 4.2.3. (Results and Discussion)** In this line, **Asserin *et al*. (2015)** described the accelerated fragmentation of the collagen network as an "hallmark of skin aging". Moreover, collagen XII is reported to be a key component for re-establishing skin homeostasis after injury **(Schonborn *et al*., 2020)**. Furthermore, collagen is reported to maintain skin firmness and elasticity, and its hydrolysates to keep the skin hydrated **(Sandhu *et al*., 2012)**. In a study by **Marini *et al*. (2012)**, the improvement of skin elasticity and hydration induced by Pycnogenol® (a commercialized product) coincided with increased gene expressions of collagen type I and hyaluronic acid synthase, suggesting the involvement of these two molecules on skin advantageous features. Nonetheless, not everything about collagen is good for skin. In fact, collagen overproduction has been reported to hinder wound healing, and to cause abnormal scar formation **(Sandhu *et al*., 2012)**.

Regarding CK14, the assayed sample (1.5% 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 ± 0.70. 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 **(abcam, 2022)**. Normally, it functions with a pair keratin, which is keratin 5 (K5) **(Coulombe *et al*., 1989; Guo *et al*., 2020; Zhang, 2018)**. These two keratins have long been biochemical markers of the stratified squamous epithelia, including epidermis **(Coulombe *et al*., 1989)**. 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. CK14 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 **(Revi *et al*., 2013)**. Moreover, the K5-K14 pair is reported to provide mechanical support in basal keratinocytes **(Guo *et al*., 2020)**. Furthermore, an increase in expression of K14 was found to accompany the differentiation of embryonic basal cells into basal epidermal cells **(Kopan and Fuchs, 1989)**, and a reduction in K14 expression seemed to be associated with tumorigenesis leading to basal cells carcinomas **(Roop *et al*., 1988; Stoler *et al*., 1988)**. A study by **Mendoza-Garcia *et al*. (2015)** reports that CK14 is known to lead to proliferation *in vivo*, and to be closely related with skin tensegrity. In the same study, the healing effects of photodynamic therapy (increased reepithelization, 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*. (2006) and Zhang *et al*. (2021)**. 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 **(Betz *et al*., 2006; Gu and Coulombe, 2007; Uttam *et al*., 1996)**. Also, the reduced expression of K5 and K14 in mice basal keratinocytes is reported to lead to a severe blistering phenotype that resembles the clinical features of *epidermolysis bullosa simplex* **(Zhang, 2018)**.

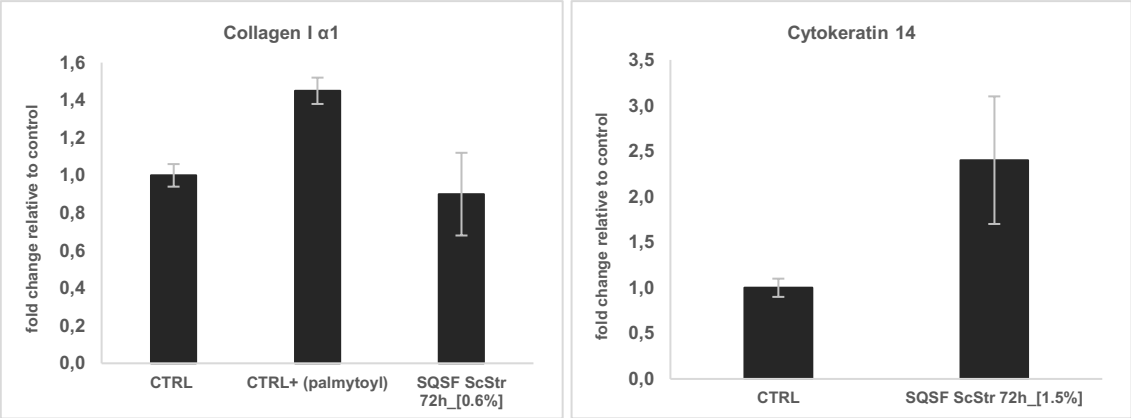

Figure 15 - Effect of the assayed extract in collagen I α1 and cytokeratin 14 production by fibroblasts and keratinocytes, respectively. Mean values (solid bars) are expressed as fold change relative to control, and standard deviation are represented by bars.

4.2.3. Skin enzymes inhibition

The obtained results regarding the skin enzymes inhibitory capacity of the assayed extract (SQSF ScStr 72h) are represented in **Figure 16**.

Regarding neutrophil elastase inhibition, the assayed sample exerted a considerable inhibitory effect. The values obtained for relative inhibition were 83.4 $\pm$ 2.2, 80.7 $\pm$ 3.8, 62.9 $\pm$ 3.1, 51.1 $\pm$ 6.2, and 31.3 $\pm$ 17.5% at the concentrations of 2, 1.5, 0.75, 0.375, and 0.1875%, respectively. 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 **(Rijken and Bruijnzeel, 2009)**, 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 **(Kawabata *et al*., 1991)**. 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 **(Rijken and Bruijnzeel, 2009)**. Photoaged skin is characterized by dryness, rough texture, irregular pigmentation, and fine and deep wrinkles, among other undesirable features **(Gilchrest, 1989; Rijken and Bruijnzeel, 2009; Takeuchi *et al*., 2010**). **Starcher and Conrad (1995)** 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 **(Takeuchi *et al*., 2010)**. Moreover, a study by **Li *et al*. (2013)** 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 **(Rijken and Bruijnzeel, 2009)**, 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 **(Starcher and Conrad, 1995)**. 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 **(Starcher *et al*., 1996)**.

Regarding tyrosinase inhibition, the assayed sample also exerted a considerable inhibitory effect. The values obtained for relative inhibition were 42.3, 40.5 $\pm$ 18.3, 32.7 $\pm$ 2.5, 24.7, and 21.3% at the concentrations of 2, 1.5, 0.75, 0.375, and 0.1875%, 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 **(Lee *et al*., 2015; Panzella and Napolitano, 2019)**. In a study, **Lee *et al*. (2012)** 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) **(Ferrer *et al*., 1995; Lee *et al*., 2015; Masum *et al*., 2019; Parvez *et al*., 2007; Pillaiyar *et al*., 2017)**. It is widely distributed in microorganisms, animals and plants and it engages in determining the color of mammalian skin and hair **(Parvez *et al*., 2007)**. 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 **(Panzella and Napolitano, 2019)**. Tyrosinase inhibitors are claimed to have preventive effects on pigmentation disorders (melasma, solar lentigo (age spots), and lentigo simplex (freckles) **(Panzella and Napolitano,**

2019)) as well as skin-whitening effect, and those with high efficacy and less adverse side effects have huge demand in cosmetic and medicinal industries **(Masum *et al*., 2019)**. In fact, the downregulation of tyrosinase has been the most prominent approach for the development of melanogenesis inhibitors **(Pillaiyar *et al*., 2017)**. For example, a study by **Boissy *et al*. (2005)** showed that DeoxyArbutin, a reversible tyrosinase inhibitor, had potential tyrosinase inhibitory activity resulting in skin lightening and that it might be used to improve hyperpigmentary lesions.
Regarding MMP-1 inhibition, there were some unexplainable variations in the results, making it difficult to take definitive conclusions. These variations were due to color interferences inherent to the assayed extract. The values obtained for relative inhibition were 6.86 ± 18.58, 30.78 ± 23.57, 13.73 ± 0.55, 14.71 ± 2.5, and 25.88 ± 13.31 % at the concentrations of 2, 1.5, 0.75, 0.375, and 0.1875%, respectively. The inhibition control (kojic acid) presented a relative inhibition of 88.5 %. Despite the irregular results, it matters to discuss the benefits of potential MMP-1 inhibition in human skin. MMPs are a family of zinc-dependent endoproteinases that play crucial roles in the regeneration of the extracellular matrix (ECM). MMP-1 is the most abundant MMP and is produced by fibroblasts. Like elastase, MMPs synthesis is induced by UV radiation, and they lead to the inhibition of collagen (at least 70% of the dry weight of dermis **(Lahmann *et al*., 2016)**) production **(Choi *et al*., 2016)**. MMP-1, also known as interstitial collagenase, is mainly responsible for the degradation of dermal type I collagen. Shortly, and completing what has already been described, exposure to UV radiation causes skin aging, which is characterized by skin fragility, laxity, roughness, dryness, hyper-pigmentation, blister formation, leathery appearance, and formation of wrinkles **(Mohamed *et al*., 2014)**. In this line, MMP-1 potential inhibitors have been explored as skin aging counter agents. For example, in a study by **Mohamed *et al*. (2014)** it was shown that DDE (*Disporum sessile* D.Don herbal extract) restored collagen production by inhibiting MMP-1 expression, and therefore, that it can be utilized as a potential agent for prevention and treatment of skin photoaging. In another research work, **Agren and colleagues (2014)** showed that the augmented collagenolytic activity of MMP-1, possibly through up-regulation of MMP-3 induced by TNF-α, led to gradual loss of type I collagen in human skin. Furthermore, and as mentioned in **section 1.3. (Introduction)**, smoking is a major factor for skin aging. Interestingly, it is suggested, in the literature, that smoking-induced MMP-1 might be important in the skin-aging effects of tobacco as significantly more MMP-1 mRNA in the skin of smokers than non-smokers has been reported **(Lahmann *et al*., 2016)**.

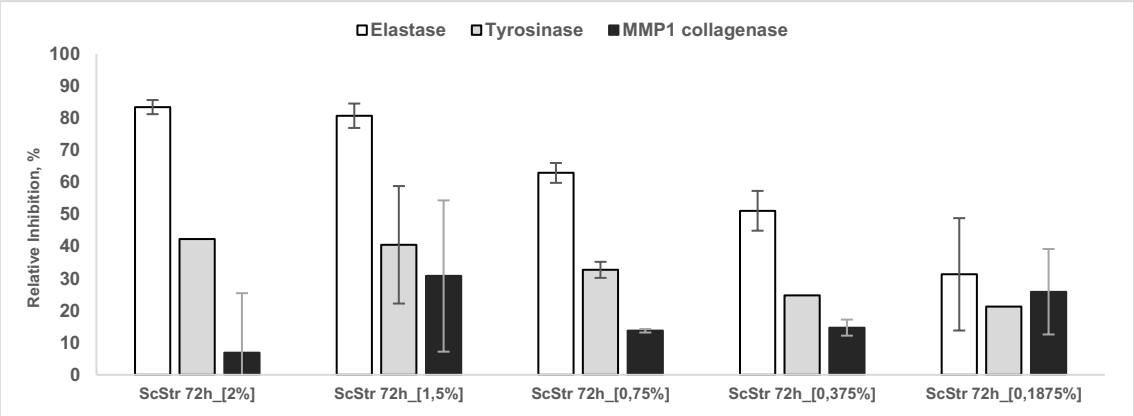

Figure 16 - Relative inhibition of skin enzymes by the SQSF 72h extract *of S. cerevisiae*. Mean values (solid bars) are expressed as percentual, and standard deviation is represented by bars.

4.2.4. Immunostimulatory and anti-inflammatory activities

The obtained results regarding the anti-inflammatory and immunostimulatory activities of the assayed extract (SQSF ScStr 72h) are represented in **Figure 17**.

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) **(Du *et al*., 2015)**. In this case, IL-6 was used. The obtained values for the assayed samples were 14.07 $\pm$ 2.36 , and 73.03 $\pm$ 14.76 pg IL6.ug cell protein^{-1}, for 1% and 0.1% concentrations, respectively. In this line, at 0.1% concentration there was no activity observed compared to the treatment with LPS (70.50 $\pm$ 2.39 pg IL6.ug cell protein^{-1}), and at 1% concentration there was a significant decrease ($p/2<0.05$) in cytokine IL-6 level, comparing to the LPS treatment. Despite this, the exerted activity was still lower than the one presented by betamethasone (2.18 $\pm$ 1.25 pg IL6.ug cell protein^{-1}). 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 **(Choy and Rose-John, 2017)**. Inflammatory states are reported to be related with several dermatological conditions. For instance, *acne vulgaris* is characterized by inflammatory papules, pustules, and nodules **(Lai and Gallo, 2008)**. Another example is atopic dermatitis, which can be viewed as an exaggerated cutaneous immune response to environmental antigens (allergens), and it is a widespread inflammatory skin condition marked by flares and remissions **(Epstein, 1999; Lai and Gallo, 2008)**. 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 **(Epstein, 1999; Lai and Gallo, 2008)**. In this line, products, such as fermented extracts like the assayed sample, that present anti-inflammatory activity may be explored for the treatment of this kind of conditions. In a study by **Ai *et al*. (2020)**, the use of microorganisms, in this case *S. cerevisiae*, as a valuable tool of biological method is described as "*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 **(Du *et al*., 2015)**. Glucans from *S. cerevisiae* are highly reported to present this type of biological activity **(Du *et al*., 2015; Jawhara *et al*., 2012; Vetvicka and Vetvickova, 2011; Wang, S. *et al*., 2014; Xu *et al*., 2012; Zhu *et al*., 2013)**. Interestingly, *S. cerevisiae*-based probiotics are also reported to exert anti-inflammatory activity. In a **Gabrielli *et al*. (2018)** research work, it is demonstrated that a *S.cerevisiae*-based probiotic markedly reduces the inflammatory response, which is a key player in vaginal candidiasis. Also, anti-fungal activity against *C. albicans* (one of the main cosmetic contaminants) was reported. In another study, **Guerrini *et al*. (2018)** demonstrated that synthetic wines, obtained from different S. cerevisiae strains exhibited antioxidant and anti-inflammatory properties. Nonetheless, it is also reported that these properties are strain specific. This type of biological properties in wines obtained from *S. cerevisiae* is also reported by **Grieco *et al*. (2019)**.

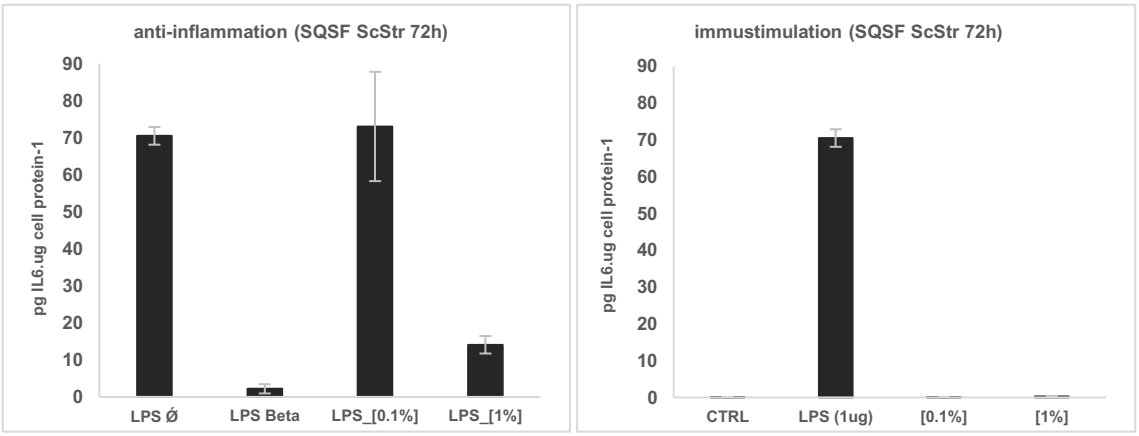

Figure 17 - Effect of the assayed sample on macrophages by evaluation of IL-6 levels under an inflammatory stimulus (LPS). Mean values (solid bars) are expressed as pg of IL-6.ug cell protein^{-1}, and standard deviation is represented by bars. Betamethasone (Beta) was used as an anti-inflammatory control.

4.2.5. Influence on skin microbiota

The effect of the assayed extract (SQSF ScStr 72h at the concentration of 1%) on the skin microbiota was tested using the samples collected from 9 volunteers. The results are resumed in **Figures 18** and **19**. The obtained values of fold change relative to control, expressed as mean ± SEM (standard error of the mean), were 37.16 ± 23.92, 5.84 ± 3.76, 1.73 ± 1.11, 0.77 ± 0.31, 1.01 ± 0.76, 16.59 ± 9.39, 0.44 ± 0.19, 0.92 ± 0.10, 38.43 ± 14.91, and 0.04 ± 0.02 for universal bacteria, *Staphylococcus* genus, *Propionibacterium* genus, *Corynebacterium* genus, *S. aureus*, *S. epidermidis*, *C. acnes*, *Propioniferax innocua*, universal fungi, and *Malassezia* genus, respectively. Contrary to all the other results presented during this work, the values from microbiota were expressed using standard error of the mean (SEM) over standard deviation (SD), because it is normal that in this type of test there is a lot of variability between subjects, as the same ingredient may alter the skin microbiota expression in different ways, depending on the subject. In this line, SEM was used because it considers those differences better than SD. Also,

the presented results of bacteria species were normalized to the universal bacteria and not to the correspondent genus.

Regarding bacteria, only *C. acnes* presented significant differences ($p < 0.05$) comparing to control. In this case, a decrease of this microorganism was observed. This bacterium is highly described in the literature as having a major role in *acne vulgaris* development in human skin **(Dréno *et al*., 2018; Platsidaki and Dessinioti, 2018)**, reason why the obtained results may serve as an indicator of the potential of the assayed extract as an anti-acne ingredient. Actually, even though large-scale studies on the microbiome of acneic follicles have not yet been performed, the initial data point to a dominance of *Cutibacterium* spp., *Staphylococcus* spp. and *Malassezia* spp. (previously known as *Pityrosporum* spp.) **(Akaza *et al*., 2015; Coenye *et al*., 2022; Findley and Grice, 2014; Xu and Li, 2019)**. Interestingly, as described in **section 4.1.4. (Results and Discussion)**, azelaic acid, whose applications and effectiveness in *acne vulgaris* treatment are highly reported in the literature **(Iraji *et al*., 2007; Nazzaro-Porro *et al*., 1983; Webster, 2000; Worret and Fluhr, 2006)**, was found to be present in the assayed extract. However, azelaic acid was also found in the controls, and therefore the fermentation process was not responsible for its production. Nonetheless, other phenolic compounds extracts, tested by the internal investigation group which encompasses this thesis, did not show this kind of activity against *C. acnes* **(Carvalho, 2022 (unpublished))**. In this line, it seems reasonable to assume that some compound or compounds derived from fermentation are contributing for the anti-acne potential of the assayed sample. In case of universal bacteria, *Staphylococcus* genus, *Propionibacterium* genus, *S. aureus*, and *S. epidermidis*, an increase, in average, of their abundance over control was observed, although without significant differences. In case of *Corynebacterium* genus and *P. innocua*, the opposite was observed, also without significant differences ($p>0.05$). Despite not presenting significant differences, it matters to understand the impact that some of these microorganisms genera and/or species may have on skin. For example, the imbalance (dysbiosis) of the *Cutibacterium*, *Corynebacterium*, and *Staphylococcus* genera has been strongly associated with the progression of psoriasis **(Chang *et al*., 2018; Quan *et al*., 2019; Rozas *et al*., 2021)**, a chronic inflammatory skin disease. Furthermore, *S. aureus* is suggested to exacerbate *atopic dermatitis* and some of its inhibitors produced by skin commensal

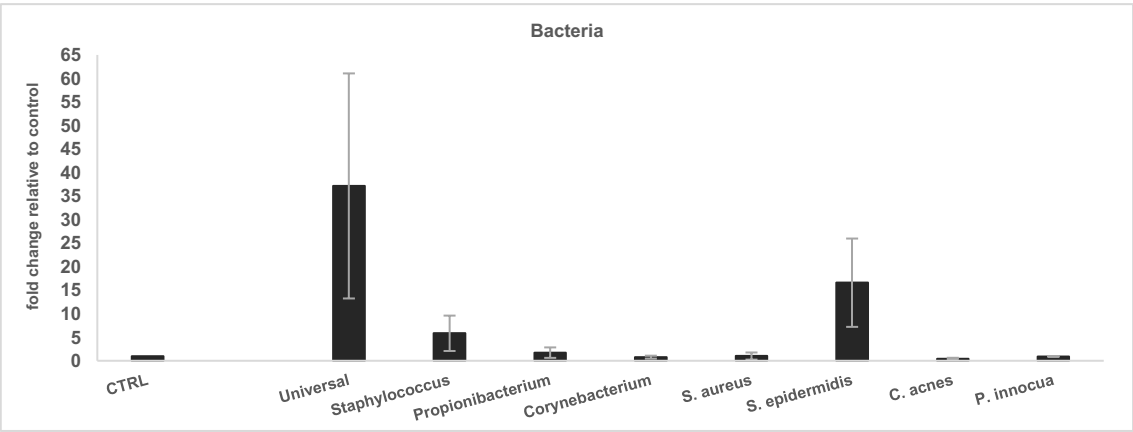

Figure 18 - Effect of the assayed extract on the skin bacteria expression. Mean values (solid bars) are expressed as fold change relative to control (CTRL), and standard error of the mean is represented by bars.

bacteria were found to be deficient in *atopic dermatitis* patients **(Nakatsuji *et al*., 2017; Rozas *et al*., 2021)**.

Regarding fungi, both universal fungi load (increase, $p<0.05$) and *Malassezia* (decrease, $p<0.0001$) expressions were significantly different from control. The *Malassezia* yeasts have pathogenic potential and have been related with skin diseases such as *head* and *neck dermatitis*, *seborrheic dermatitis*, *pityriasis versicolor*, and *Malassezia folliculitis* **(Gupta *et al*., 2004; Saunte *et al*., 2020)**. In this line, the assayed extract showed, not only anti-acne potential, but also the capability of possibly fighting other skin diseases. In the context of development of dandruff, interactions between *C. acnes* and fungi (in particular species belonging to the genus *Malassezia*) also appear to be important **(Clavaud *et al*., 2013; Coenye *et al*., 2022)** and mixed-species biofilms of *C. acnes* and *M. restricta* were observed in a pre-clinical cell-culture based dandruff model **(Coenye *et al*., 2022; Meloni *et al*., 2021)**. Currently the molecular basis for the interactions between *C. acnes* and fungi in these polymicrobial communities is unknown.

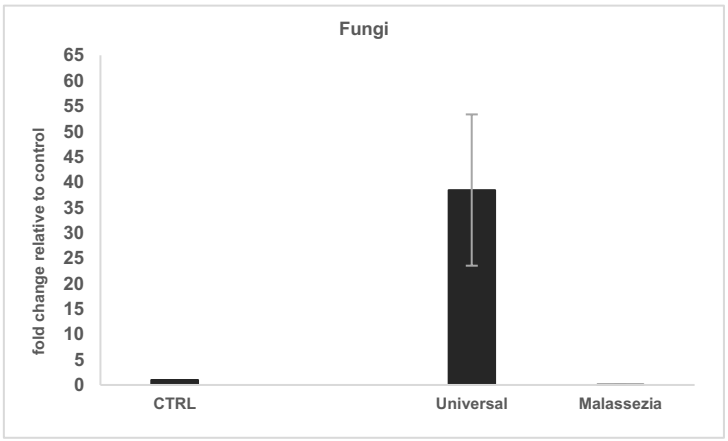

Figure 19 - Effect of the assayed extract on the skin fungi expression. Mean values (solid bars) are expressed as fold change relative to control (CTRL), and standard error of the mean is represented by bars.

Conclusions

The main purpose of this work was the sustainable production of postbiotics, through fermentation, with potential to be further utilized in skincare applications. Some fermentation and saccharification parameters were studied in the attempt of obtaining the best possible conditions for an efficient postbiotics production. In this line, regarding saccharification, four variables were evaluated: biomass type, biomass milling degree, saccharification time and used enzymatic cocktail (no enzymes, Celluclast, and Celluclast + Shearzyme Plus). As expected, without enzymes, no significant saccharification occurred, in contrast with the use of both enzymes. In general, sugarcane straw allowed higher sugar concentrations. Celluclast was considered as being the most advantageous enzyme over the mixture with Sherazyme Plus, as no differences were observed in most of the tested conditions, and as a low-cost option. Regarding fermentation, the sugar uptake was not affected by the type of process (SSF and SQSF) nor type of biomass used. Concerning SSF, in the case of *L. plantarum*, it was possible to conclude that within the last 24h of fermentation a fully inhibitory state was reached, since no significant differences in sugar uptake were registered between 24h and 48h of fermentation and no cellular growth was observed at 48h. The metabolic pathways of the bacteria were proposed as the main causes for this. Regarding *L. fermentum* and *S. cerevisiae*, the 48 h of fermentation was considered the most advantageous condition. The increment of time to 72 h in case of *S. cerevisiae* was not an advantage to the process, owing to the accumulation of metabolites such as ethanol and changes in cellular membrane permeability.

One of the hypotheses raised was the release of metabolites along fermentation, product of metabolic activity of microorganisms, release of phenolics from biomass, or even from cellular membrane and intracellular content.

The SQSF runs were only performed using *L. plantarum* (48h with bagasse, LpBag 48h) and *S. cerevisiae* (72h with straw, ScStr 72h) since these presented the best antimicrobial and antioxidant potentials, respectively. The change of process to SQSF allowed to improve the biological activities such as antioxidant, maybe due to the cellular fragments. Concerning antimicrobial metabolites, only *L. plantarum* 48h extracts presented an inhibitory effect over *S. aureus*.

In the case of organic acids, it was possible to observe that both microorganisms consumed citrate (or derivates) and presented acetate in both extracts. *L. plantarum* extracts showed the presence of lactic acid, product of its metabolism. Straw derived extracts showed a higher phenolics content than bagasse, and *L. plantarum* seemed to metabolize some phenolics. Both extracts exhibited the presence of aminoacids such as aspartic acid, glutamic acid, and glycine, which were the most present, being more expressively produced by *S. cerevisiae* when compared to *L. plantarum*. The SQSF ScStr 72h extract also showed considerable inhibition of skin enzymes such as neutrophil elastase and tyrosinase.

In terms of safety, SQSF LpBag 48h and SQSF ScStr 72h extracts were found to be toxic at concentrations superior to 1% and 2% for keratinocytes, respectively. For fibroblasts, the SQSF ScStr 72h extract was found to be safe at concentrations below 0.7%. It was possible to conclude

that SQSF LpBag 48h extract, could not be used as a cosmetic preservative ingredient since the obtained MIC and MBC were far superior to the limit toxic concentration. On the other hand, SQSF ScStr 72h extract antioxidant activity Ec_{50} (ABTS) was far inferior to the limit toxic concentration. Since the SQSF ScStr 72h extract showed to be promising when compared with the remaining, a deep analysis of the biological activity was performed. Regarding collagen I α1, no significant effect on its production was observed. In case of CK14, a significant increase was achieved. Regarding anti-inflammatory activity, it was concluded that the extract allowed a significant decrease in IL-6 expression (exacerbated during states of cellular stress, such as inflammation), although in a concentration dependent manner. In addition, no immunostimulatory effect was promoted by the extract. Finally, regarding the influence on skin microbiota, the significant reduction in *C. acnes* expression, made it possible to conclude that the extract may, in fact, present some anti-acne potential. Also, the significant decrease in *Malassezia* expression showed the extract's potential capability of fighting other skin conditions (head and neck dermatitis, seborrheic dermatitis, etc), besides acne.

In short, SQSF ScStr 72h extract showed to be the most promising as it presented the best antioxidant activity, being this compatible with the obtained cytotoxicity results. It revealed itself as a potential inhibitor of some skin 'degrading' enzymes, and of potential inflammatory states. Also, it worked as a stimulus for CK14 production, and as a down regulator of some skin diseases-associated microorganisms. This considered, it was possible to conclude that the final extract exhibits potential for cosmetic and skincare applications.

Future work

Since the production process related experimental work was exploratory, targeting a potential scale up of the process, the study of other parameters should be performed. It would be important to study the influence of initial cellular concentration in the exhibited properties. Even in cases where it has no significant influence on sugar uptake, it would be important to realize what potential consequences the initial cell concentration may have on the physicochemical composition and biological activities of the extracts. Also, the possibility of combining the benefits from different microorganisms, using co-fermentation processes, could be an interesting approach. However, from this perspective, studies would be needed to overcome some problems such as competition between microorganisms, to ensure that both could be metabolically active during fermentation. Also, the utilization of bacteria other than lactic acid ones, such as skin commensal bacteria, could be a very interesting path to follow. After all, these are the bacteria which inhabit our skin and that, when in balance, have no negative effects on it.

Regarding the extracts obtained, the presence of colour could be a problem for future commercialization in skin cosmetics market, so an intermediate step of colour removal should be considered.

Also, after the process development stage, an economical evaluation of the cost of production should be performed, to evaluate if the final ingredient is worth of being produced comparing with other benchmark costs. The performance should also be compared with other benchmarks to weight in the decision of scale up. Hence, in the product development stage and characterization, some benchmark products should be used to compare the performance as postbiotics.

Appendix

Appendix 1. Fermentation studies using several different substrates and microorganisms (table)

Table 9 - Fermentation studies using several different substrates and microorganisms. na: not available.

Microorganism	Substrate	Supplements	Conditions	Products	Reference source
Skin microbiota					
Staphylococcus epidermidis EFRL 12	Sugarcane bagasse, rice husk, molasses, and date syrup	Peptone, casein soluble or hydrolysed, tryptone, corn steep liquor, yeast extract, urea, sodium nitrate, ammonium nitrate, potassium nitrate, ammonium chloride, ammonium sulfate	37 ºC, pH 7.5	Proteases	Qureshi and Dahot, 2009
S. epidermidis	Glycerol (20 g/L)	na	Annaerobiose, 30 ºC, rich medium	4 SCFAS (succinic acid, acetic acid, butyric acid, lactic acid)	Wang, Y. *et al.*, 2014
	Sucrose (20 g/L	na	Annaerobiose, 37 ºC, rich medium, 200 rpm	4 SCFAS (succinic acid, acetic acid, butyric acid, lactic acid)	Wang *et al*., 2016
	Glycerol (20 g/L)	na	Annaerobiose, 37 ºC, rich medium	Butyric acid	Keshari *et al*., 2019
	Sodium L-lactate	na	TSB medium, 37 ºC	malodors (diacetyl, acetaldehyde, acetic acid)	Kumar *et al*., 2019
	Glycerol (2%)	na	Rich medium	4 SCFAS (succinic acid, acetic acid, butyric acid, lactic acid)	Yang, A. J. *et al.*, 2019
	PEG-8 Laurate (20 g/L)	na	Annaerobiose or aerobiose, 37 ºC, rich medium, 200 rpm	4 SCFAS (succinic acid, acetic acid, butyric acid, lactic acid)	Marito *et al*., 2020

Cutibacterium acnes	Glucose (2%)	na	Anaerobiosis, 37 ºC, rich medium	5 SCFAs (acetic acid, butyric acid, propionic acid, isobutyric acid, isovaleric acid)	Keshari *et al*., 2020
Lactic acid bacteria					
Lactococcus lactis 53 & *Streptococcus thermophilus* A	Cheese whey	Peptone, yeast extract	na	Biosurfactant	Rodrigues *et al*., 2006
Lactococcus garvieae	Fruit peels	na	na	na	Patel *et al*., 2020
Kefir (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*)	Mango peel	na	30 ºC, static conditions	Lactic acid, bioactive peptides, extracellular polysaccharides	Vicenssuto and de Castro, 2020
Lactobacillus plantarum & *Lactobacillus brevis*	Poplar hydrolysate, Corn stover	Peptone, yeast extract, ammonium citrate, sodium acetate, K_2HPO_4, magnesium sulphate hepta-hydrated, manganese sulphate tetra-hydrated, Tween 80	37 ºC, 150 rpm, pH 6.5	Lactic acid (both), acetic acid, ethanol (*L. brevis*)	Zhang and Vadlani, 2015
L. plantarum	Corn stover & sorghum stalks	Peptone, yeast extract, ammonium citrate, sodium acetate, K_2HPO_4, magnesium sulphate hepta-hydrated, manganese sulphate tetra-hydrated	37 ºC, 150 rpm, pH 6.5	Lactic acid, acetic acid	Zhang *et al*., 2016
	Rice straw	na	37 ºC, 150 rpm, pH 5	Lactic acid, acetic acid (residual)	Tu *et al*., 2019
	Sugarcane bagasse, Cheese whey, microalga *Chlorella vulgaris* ESP-31 & green macroalgae *Ulva sp*. (seaweeds)	Peptone, beef extract, tween 80, sodium acetate, K_2HPO_4, ammonium citrate, magnesium sulphate hepta-hydrated, manganese sulphate tetra-hydrated	Anaerobiosis, 30 ºC, pH 5.5, 200 rpm	Lactic acid, acetic acid (residual) & ethanol (residual)	Nagarajan *et al*., 2020
L. brevis	Rice straw	MRS medium (bacto-peptone, yeast extract, ammonium citrate sodium acetate, dipotassium phosphate)	37 ºC, pH 6	Lactic acid, acetic acid, ethanol	Kim *et al*., 2010

L. brevis & L. plantarum	Corn stover, corncobs	Peptone, yeast extract, beef extract, magnesium sulphate, manganese sulphate, sodium chloride, iron sulphate, $NaCH_3CO_2$, diammonium citrate, KH_2PO_4, Tween 80	30 ºC, 160 rpm	Lactic acid, acetic acid	Guo *et al*., 2010
L. brevis	Glucose / xylose + deoiled cottonseed cake	KH_2PO_4, $NaCH_3CO_2$, magnesium sulphate, calcium carbonate	37 ºC, pH 6	Lactic acid, acetic acid	Grewal and Khare, 2018
	Deoiled cottonseed cake and wheat straw	Calcium carbonate	37 ºC, pH 6, 65% relative humidity		
	Wheat straw, deoiled cottonseed cake, sugarcane bagasse	Yeast extract	37 ºC, pH 5.5, 120 rpm		
Yeasts					
Saccharomyces cerevisiae	Wheat straw, corn stover	Urea, potassium phosphate, magnesium sulphate, D-biotin	30 ºC, 150 rpm	Ethanol	van Dijk *et al*., 2020
	Cotton stalk	No supplements	35 ºC, pH 5, anaerobiosis	Ethanol	Malik *et al*., 2020

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