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Impact of a novel sugarcane straw extract-based ingredient on skin microbiota via a new preclinical *in vitro* model

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ABSTRACT

During cosmetic product development, the impact on the skin microbiota needs to be evaluated, as it plays an important role in skin health. Clinical studies are frequently used to assess cosmetic effects, but are expensive, time-consuming and require finished ingredients, which may be a limiting factor. Thus, this study had two main objectives, the development of a preclinical *in vitro* model for the evaluation of the effect of cosmetic ingredients on the skin microbiota, and its use to evaluate a novel sugarcane straw extract-based ingredient. In the development of the preclinical *in vitro* model, the microbiota incubation time and atmospheric conditions were optimized. The model was validated using a benchmark ingredient with reported *in vivo* effects on skin microbiota. Then, the new sugarcane straw extract-based ingredient was tested in the model, using skin microbiota samples from 12 healthy female volunteers. The impact on microbial groups was analyzed using qPCR and next-generation sequencing. The best conditions for the *in vitro* model were 24 h incubation under aerobic conditions. Furthermore, the results obtained with the benchmark ingredient agreed with those obtained *in vivo*, thus validating our model. Sugarcane straw extract-based ingredient was found to have no effect on community α-diversity, however it appears to affect *S. epidermidis*. In conclusion, the developed model can be used as a tool to assess the impact of novel cosmetic ingredients on skin microbiota. Also, the novel ingredient seems to have an impact on commensal *S. epidermidis*.

1. Introduction

The skin has an important barrier function that supports a complex microbial ecosystem (skin microbiota) that is in homeostasis with the host (Samaras and Hoptroff, 2020; Marchesi and Ravel, 2015). The skin microbiota is mainly composed of bacteria, including four main phyla, Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes, and the most abundant genera are *Cutibacterium*, *Staphylococcus* and *Corynebacterium* (Mukherjee et al., 2016). Fungal microbes are also present, being dominated by *Malassezia* species, representing 80% of fungus in the skin (Keum et al., 2020). An imbalance in the skin microbiota (dysbiosis) can lead to the development of skin diseases such as acne, psoriasis, and atopic dermatitis (Carmona-Cruz et al., 2022). Since the maintenance of skin microbiota equilibrium is the key to skin health, understanding this community has been the focus of novel therapies for skin diseases and microbiota friendly cosmetics (Kong and Segre, 2012; Wallen-Russell, 2019), which can include pre-, pro- and postbiotics that contribute to maintaining this community and should not introduce contaminants to the skin environment (Fournière et al., 2020). An example of these ingredients is Ecoskin®, a pre- and postbiotic ingredient composed of α-glucooligosaccharides (GOS), β-fructooligosaccharides (FOS) and inactivated *Lactobacillus* probiotic bacteria, namely *Lactobacillus casei* and *Lactobacillus acidophilus* (Solabia, 2019).

The addition of natural ingredients in the cosmetic industry has been observed over the years, and currently, around 55% of the total cosmetics placed on the market worldwide use natural ingredients (Mukherjee et al., 2011; Cosmetica Italia Association, 2022). These natural ingredients can be extracted from plants and fruits, but they can also be a byproduct of an industrial process, making this last option a more sustainable alternative (Barbulova et al., 2015; Rodrigues et al., 2015). Straw is a byproduct of sugarcane processing and it is estimated that 10–20 tons of straw are generated per hectare (Bilatto et al., 2020).

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This byproduct is a source of phenolic and bioactive compounds, such as phenolic acids and flavonoids, which are known to have anti-ageing properties such as antioxidant activity (Wittenauer et al., 2015; Ji et al., 2020). This activity contribute to the scavenging of reactive oxygen species (ROS) that cause oxidative damage to cellular components, provoking injury to the connective tissue of the skin (Kammeyer and Luiten, 2015). Although the impact of these compounds on the skin microbiota is unknown, their antimicrobial activity (Zhao et al., 2015) suggests that an imbalance in the skin microbiota might occur, thus testing its effect on the skin microbiota is essential.

A large variety of *in vivo* and *in vitro* models are available to study the effect of cosmetics on the skin microbiota (Ciardiello et al., 2020; Van Der Krieken et al., 2016; Cadau, 2017). *In vivo* models are the preferred approach for testing ingredients or products in the cosmetic industry. In these studies, after the application of the cosmetic product to the skin of human volunteers, a microbiota sample is collected and characterized for its taxonomic composition. For example, a previous work reported the testing of a cosmetic ingredient by swabbing the cheeks of volunteers in the beginning and after 1 month of using the product to determine the impact in the microbiota (Ciardiello et al., 2020). The disadvantages of this type of testing are time and cost consumption and ethical implications (Nigam, 2009). Alternatively, we can find *in vitro* models that are often combined with culture-dependent methods or only study a single microbial group at a time (Van Der Krieken et al., 2016; Cadau, 2017). One main constraint of these models is that the complexity of the skin microbiota-cosmetic ingredient interaction is not reproduced, which does not allow the determination of the full impact on the microbiota. Thus, alternatives for testing the impact of cosmetic ingredients on skin microbiota need to be studied.

Hence, this study aimed to develop a preclinical *in vitro* model by optimizing the conditions, such as incubation time and environmental conditions, and validation. After establishing this model, the impact of a novel sugarcane straw extract-based cosmetic ingredient on the skin microbiota was evaluated.

2. Materials and methods

2.1. Testing ingredients

Ecoskin® (Solabia, Pantin, France) is a pre- and postbiotic ingredient, with a reported effective dose of 0.5–3% (w/w) (Solabia, 2019), thus this ingredient was tested at 2.5% (w/v) and used as a benchmark to validate our preclinical *in vitro* model. The sugarcane straw extract-based ingredient was produced as described by Carvalho et al. (Carvalho et al., 2023). In short, straw was provided by by Raízen from São Paulo, Brazil. After a milling and drying process, it was performed a solid:liquid extraction using 50% ethanol (v/v) (Honeywell, Morris Plains, New Jersey, USA) followed by a purification process using amberlite XAD-2. At the end of this process, the straw extract was freeze-dried (Martin Christ, Osterode am Harz, Germany) obtaining a powder. The ingredient was obtained by mixing the straw extract with the cosmetic solvent 1,2 Hexanediol (Sigma-Aldrich, St. Louis, Missouri, USA) and it was tested at 0.5% (v/v) concentration.

2.2. In vitro preclinical model development

2.2.1. Study population

A total of 30 female volunteers, aged between 25 and 35 years-old, without known skin diseases were selected. From these volunteers, six were recruited for the optimization of preclinical *in vitro* model, 12 volunteers were used to validate the *in vitro* model using Ecoskin® and the remaining 12 volunteers to the sugarcane straw extract-based ingredient testing. During the recruitment process one of the exclusion criteria was the use of prescribed antibiotics or other systemic medications in the three months before the study. The volunteers were instructed to not use any facial cosmetics in the 12 h period preceding the skin microbiota collection. All volunteers signed an informed consent after an explanation about the study and the procedure. Samples were delinked and unidentified from their donors. The study (project no. 83) was approved on 17th September 2020 by the Ethics Committee for Health of the Universidade Católica Portuguesa.

2.2.2. Skin microbiota sampling

Sampling from the face of the donors was performed using 4N6FLOQSwabs™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) moistened in a sterile solution of phosphate buffer solution (PBS at 0.1 M, pH 7.3 ± 0.2 at 25 °C) with 0.1% (v/v) Tween 80. This procedure was based on previous protocols for recovery of skin microbiota samples (Mukherjee et al., 2016; Ogai et al., 2018). The swabbing method was performed in a standardized pattern and pressure (20 times vertical swabbing and 20 times horizontal swabbing) to recover a representative sample of the facial skin microbiota (forehead, cheeks, nose, and chin). The control of the collection method was performed using the same procedure without the skin microbiota sample. All samples and controls were maintained on ice or at 4 ºC until their processing.

2.2.3. Development of the model assay

During the development of the preclinical *in vitro* model, two different incubation conditions (aerobic and anaerobic environments) and three incubation time periods (24, 48 and 72 h) were tested, since different microorganisms belonging to the skin microbiota could benefit from different incubation conditions. Thus, skin microbiota samples from six volunteers were collected. After sampling, both swabs were incubated in a tube with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), for 3 h at 34 °C with agitation (100 rpm) in an incubator shaker (Innova 40 New Brunswick, Eppendorf, Hamburg, Germany) to ensure that the microorganisms were transferred from the swabs to the medium. Next, swabs were removed, and each sample was divided in tubes containing RPMI medium and were incubated at the different testing conditions. At each time-point of incubation, the samples were centrifuged at 21,130 g for 10 min and the pellet was stored at −20 ºC until DNA extraction. Based on these results, the best time of incubation and the environmental conditions were chosen.

2.2.4. Validation of in vitro skin microbiota model

For the validation of the assay, skin microbiota samples were collected from 12 volunteers. After sampling, swabs were incubated with RPMI medium for 3 h at 34 °C with agitation (100 rpm) in an incubator shaker (Innova 40 New Brunswick, Eppendorf, Hamburg, Germany). Then, the swabs were removed, and each sample was split in two groups: RPMI (control) and test condition (RPMI + 2.5% (w/v) of Ecoskin®). All samples were incubated for 24 h at 34 ºC with agitation (100 rpm). After, samples were centrifuged at 21,130 g for 10 min and the pellet was stored at −20 ºC for future DNA extraction.

2.3. Assessment of the impact of the new sugarcane-based ingredient

The effect of a sugarcane straw extract-based ingredient on skin microbiota was assessed using our previously optimized and validated model. For that purpose, skin microbiota samples of 12 volunteers were processed similarly to the described above. Each of these samples was divided into two sterile tubes containing: RPMI (control), and RPMI + 0.5% (v/v) of sugarcane straw extract-based ingredient (test condition). As previously described, samples were incubated for 24 h at 34 ºC with agitation (100 rpm) and then they were centrifuged at 21,130 g for 10 min to recover the microbial (stored at −20 ºC).

2.4. Microbiota profiling techniques

2.4.1. DNA extraction and quantitative real-time PCR (qPCR)

DNA extraction of all pellets were performed with QIAamp DNA

Table 1
Primer used for qPCR.

Primer name	Foward primer (5′ ->3′)	Reverse primer (5′->3′)	Refs.
Universal Bacteria	TCCTACGGGAGGCAGCAGT	CGTATTACCGCGGCTGCTGGCAC	(Horz et al., 2005)
Universal Fungi	TCCGTAGGTGAACCTGCGG	GCTGCGTTCTTCATCGATGC	(Hoggard et al., 2018)
Firmicutes	ATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC	(Queipo-Ortuño et al., 2013)
Actinobacteria	TACGGCCGCAAGGCTA	TCRTCCCCACCTTCCTCCG	(Bacchetti De Gregoris et al., 2011)
Staphylococcus sp.	GGCCGTGTTGAACGTGGTCAAATCA	YATHACCATTTCWGTACCTTCTGGTAA	(Wampach et al., 2017)
Cutibacterium sp.	CGGATTTATTGGGCGTAAAGR	AGGGTATCTAAGCCTGTTCG	(Cazanave et al., 2013)
Streptococcus sp.	CCGGHCGTCACGGWAA	CCATACCAAGRTGAAGYTCCATA	(Cazanave et al., 2013)
Corynebacterium sp.	TCAGCGYGACTACGCCCTC	ACCTYGCCAGGGCTTCTC	(Xu et al., 2021)
Malassezia sp.	CGAAACGCGATAGGTAATGTG	CAAATGACGTATCATGCCATGC	(Vuran et al., 2014)
Staphylococcus aureus	AGGACAATCATGGCAAGCGTAC	AACGGACAACATCTAAACTGGC	(Van Der Krieken et al., 2016)
Staphylococcus epidermidis	GGCAAATTTGTGGGTCAAGA	TGGCTAATGGTTTGTCACCA	(Byrne et al., 2007)
Cutibacterium acnes	CGAGGAGCAATTTCTGGGAT	ATGGATGACTTCGACGATGA	(Van Der Krieken et al., 2016)

Table 2
Primers used for amplification of the V3- V4 regions (16S rRNA gene) and ITS 2 regions.

Primer name	Primer Sequences	Region	Refs.
16S_V3V4_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	V3 – V4 regions of 16S rRNA gene	(Zheng et al., 2015)
16S_V3V4_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC		
ITS 2_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCRARTYTTTG	ITS 2 region	(Tedersoo and Lindahl, 2016; White et al., 1990)
ITS2_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTSCGCTTATTGATATGC		

Microbiome Kit (Qiagen, Hilden, Germany), according to manufacturing instructions. After extraction, DNA was quantified by Qubit dsDNA HS Assay Kit (Life Technologies, Foster City, CA, USA). qPCR was employed to determine the relative abundances of specific microbial genera and species. For this purpose, a universal assay, composed of universal primers targeting the 16 S rRNA conserve gene region (bacteria) and ITS2 region (fungi) were employed, as well a genus- or specie-specific assays, using specific primers (Table 1). The primer specificity was confirmed by Primer-Basic Local Alignment Search Tool (Primer-BLAST) from National Center for Biotechnology Information (NCBI). qPCR reactions were set to a final volume of 10 μL, using 1x of NZYSupreme qPCR Green Master Mix (NZYtech, Lisbon, Portugal), 2 μL of Microbial DNA-Free Water (Qiagen, Hilden, Germany), 1 μL of reverse and forward primers (Integrated DNA technologies, Heverlee, Belgium) and 1 μL of DNA sample (10 ng/μL). qPCR was performed using TOWER^3 G (Analytic-Jena, Jena, Germany) with the following conditions: 10 min at 95 ºC, followed by 40 cycles of denaturation at 95 ºC for 15 s and annealing/extension at 60 ºC for 1 min. To check for the generation of nonspecific products, a melt dissociation step was performed after the amplification steps. Additionally, 2% agarose gel electrophoresis was used to check the purity of the qPCR product. Microbial DNA-Free Water (Qiagen, Hilden, Germany) was used to create blanks rather than DNA templates to rule out any potential environmental contaminants in qPCR assays. It was also included a positive control for each assay, i.e., DNA of bacteria or fungi species of each group tested. Two replicates were performed for each sample and controls.

The individual microbial genera or species as well as the overall microbial load were measured using the relative standard curve methodology. First, standard curves were created using dilution series of known microbial CFU values opposed to the calculated threshold cycle (Ct) value. Then, the relative abundance of each microbial group was determined using the Log10 ratio between the CFU count from the genus- or specie-specific assay and the CFU count from the universal assay (16S rRNA or ITS2). For each volunteer, the variance between the benchmark or the sugarcane straw extract-based ingredient group and its control group was determined in an effort to diminish the inter-individuality variation (fold-variation).

2.4.2. 16S rRNA and ITS2 amplicon-based next-generation sequencing

The V3-V4 hypervariable regions of the 16S rRNA gene and the ITS2 region were amplified using universal primers fused with Illumina adapters sequences (Integrated DNA Technologies, Heverlee, Belgium) (Table 2). PCR reactions were performed in 25 μL containing 1x AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.2–0.4 μM of forward and reverse primers. Instead of DNA, microbial DNA-free water was supplied to the PCR negative control. Using the Axy Prep PCR Clean-Up Kit (Axygen, Union City, CA, USA), amplicons were purified with magnetic beads. Then amplicons were seen on 1.5% agarose gels and quantified using the Qubit dsDNA HS Assay Kit. Using the Illumina 16S Metagenomic Sequencing Library preparation methodology, an equal number of amplicons were employed to build the sequencing library. The final sequencing library was sequenced using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) and the Illumina MiSeq platform utilizing 300 bp paired-end sequencing reads, with an expected output of 100,000 reads per sample. QIIME2 v2021.4 was used to analyse the generated raw sequence data (Caporaso et al., 2010). The reads were denoised using the DADA2 plugin, which included the trimming and truncating low quality regions, dereplicating the reads and filtering chimeras (Callahan et al., 2016). The SILVA (version 138 QIIME) database was used to classify the filtered reads by taxon using operational taxonomic units (OTUs), with a clustering criterion of 97% similarity. OTUs containing at least ten sequence reads were considered as significant.

2.5. Statistical analyses

Statistical analysis was performed using GraphPad Prism software (version 7.04, GraphPad Software Inc., CA, USA) and IBM SPSS statistics (v. 21, 2012, IBM SPSS statistics, New York, USA). The Shapiro-Wilk normality test was used to determine whether the data had a normal distribution. As data did not follow a normal distribution, Kruskal-Wallis non-parametric test followed by Dunn's multiple comparisons test was performed when more than two groups were compared (IBM SPSS statistics), and the Mann-Whitney statistical test was performed when comparing two groups (GraphPad Prism software).

3. Results

3.1. Optimization of the preclinical in vitro model

To establish the preclinical *in vitro* model, the face skin microbiota samples of six volunteers were recovered and incubated in aerobe and

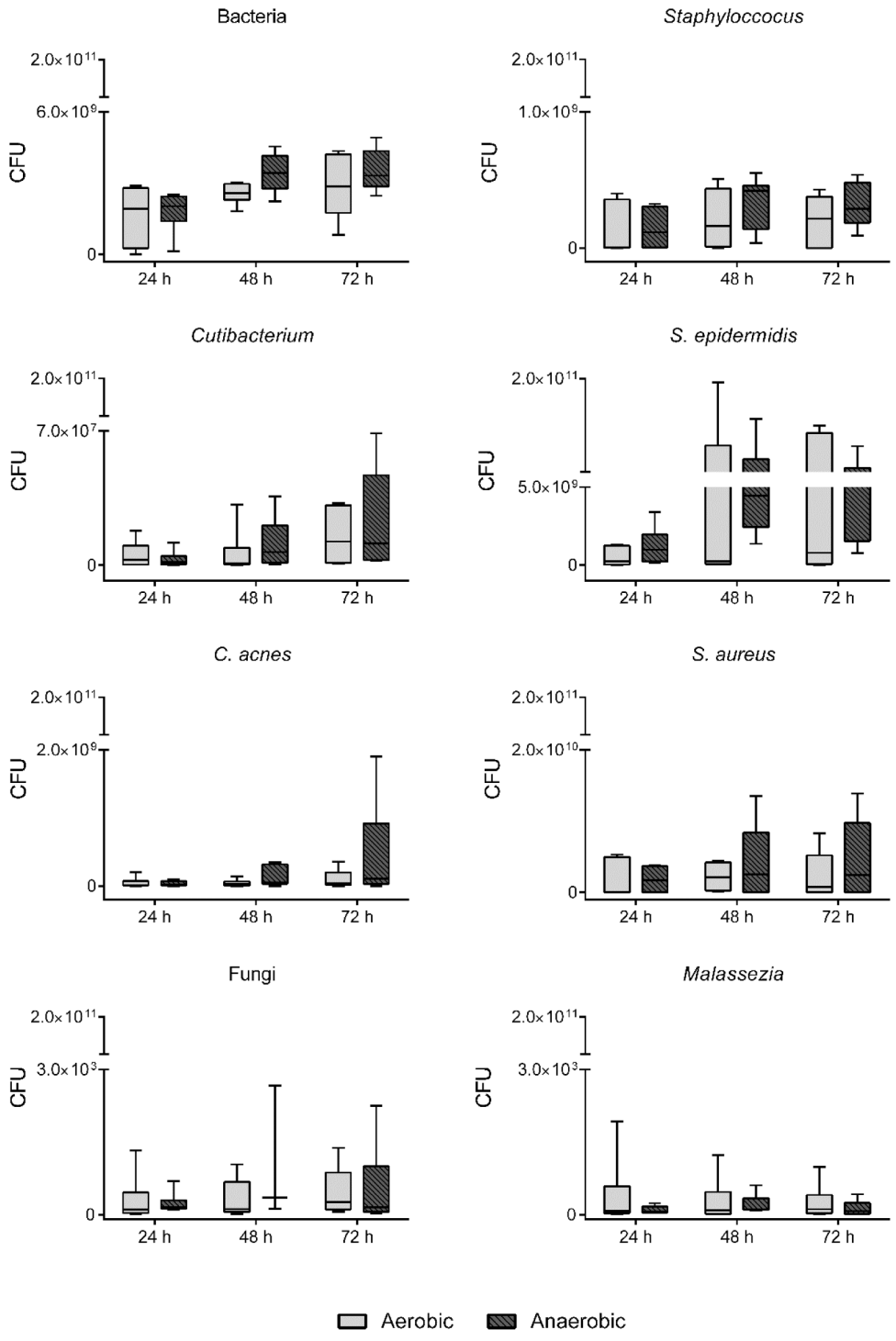

Fig. 1. Absolute abundances of specific microbial groups after the incubation of skin microbiota samples (n = 6) during 24, 48 and 72 h under aerobic (light grey) and anaerobic (dark grey) conditions evaluated by qPCR assays. Data was analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test. *Indicate significant differences of $p < 0.05$ (when comparing the 24 h incubation period with the 48 and 72 h period in anaerobic conditions).

anaerobe environments for 24, 48 and 72 h.

As shown in Fig. 1, most bacterial groups tested, including *Staphylococcus* sp., *Cutibacterium* sp., *Cutibacterium acnes* and *Staphylococcus aureus* did not display differences in absolute abundance ($p > 0.05$) between 24, 48 and 72 h of incubation and between aerobic and anaerobic environments. On the other hand, the total bacterial load and *Staphylococcus epidermidis* showed significant differences between the time point of 24 h and the remaining two time points (48 and 72 h) under anaerobic conditions. Under aerobic conditions, no differences were detected, which indicated that under anaerobic conditions these groups took longer periods of time to grow. Regarding fungi groups, total fungal load and *Malassezia* sp. did not display differences in absolute abundance ($p > 0.05$) between 24, 48 and 72 h of incubation and between aerobic and anaerobic conditions.

Therefore, 24 h of aerobic incubation were the optimal conditions for the preclinical *in vitro* model.

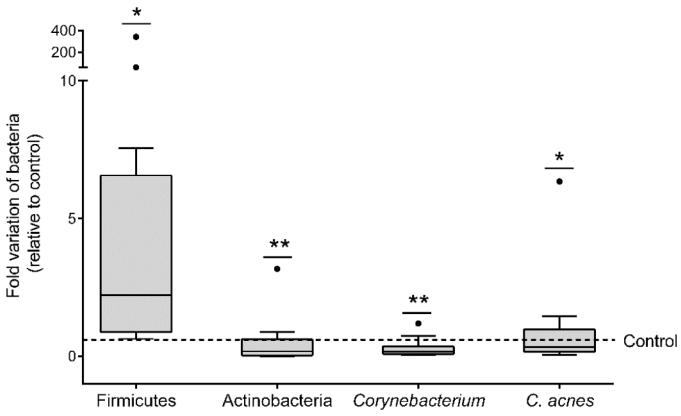

Fig. 2. Fold variation of specific bacterial groups after skin microbiota samples (n = 12) were incubated with Ecoskin®. The box-plot graphs include data obtained by qPCR. Data was submitted to Mann-Whitney statistical test in comparison with the control. * Indicate significant differences ($p < 0.05$) and ** indicate significant differences ($p < 0.0001$).

3.2. Validation of the preclinical in vitro model

To validate our preclinical *in vitro* model, a commercial prebiotic and postbiotic ingredient was used. The collected skin microbiota samples of 12 donors were incubated with and without Ecoskin®, at the optimized conditions described in the previous section (24 h, aerobic conditions, 34 °C). Then, the microbial groups described in the data sheet (Firmicutes, Actinobacteria, *Corynebacterium* sp. and *C. acnes*) were evaluated by qPCR. In Fig. 2, it is possible to see the results obtained by testing Ecoskin® with our preclinical *in vitro* model. The relative abundance of Firmicutes was significantly increased ($p < 0.05$) in the Ecoskin® group compared to that in the control group. In contrast, the relative abundance of Actinobacteria, *Corynebacterium* sp. and *C. acnes* significantly decreased in the Ecoskin® group compared to that in the control group ($p < 0.05$). The remaining groups listed in Table 1 were also tested and did not state any differences ($p > 0.05$) when comparing the Ecoskin® group with the control group (data not shown).

Thus, it was possible to conclude that the model was hereby validated, since the results obtained by the preclinical *in vitro* data were the same as the ones reported by *in vivo* testing.

3.3. Impact of a sugarcane straw extract-based ingredient on the skin microbiota

After the preclinical *in vitro* model was established, the impact of the sugarcane straw-based ingredient on the skin microbiota was evaluated. For this purpose, microbial samples from 12 donors were collected from the face and incubated with and without the sugarcane straw extract-based ingredient. Next, NGS and qPCR were performed to evaluate the impact of the sugarcane straw-based ingredient on the microbial groups.

Next-generation sequencing results demonstrated that the sugarcane straw-based ingredient did not alter the α-diversity of skin microbiota when comparing the ingredient and the control groups (Fig. 3). Additionally, it is possible to conclude that the ingredient did not affect the main phylum, including Firmicutes, Actinobacteria and Proteobacteria (Bacteria) and Ascomycota and Basidiomycota (Fungi) as no significant differences were detected ($p > 0.05$) when comparing the ingredient with the control groups (Figs. 4 and 6A). The same was visible when analyzing the main genera, such as *Staphylococcus*, *Cutibacterium*,

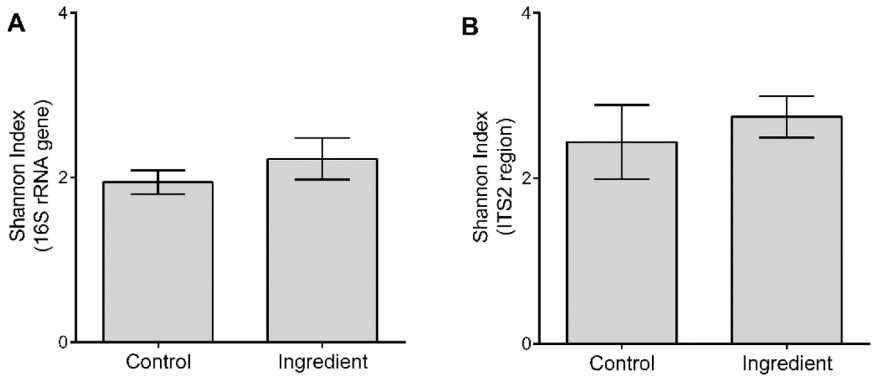

Fig. 3. Alpha-diversity of skin microbiota was calculated by the Shannon index. The average of bacterial (A) and fungal (B) alpha-diversity of samples of ingredient group (straw extract-based ingredient) demonstrated to be similar to those of control group (without straw extract-based ingredient). Bars represent the average ± SEM and the Mann-Whitney test was performed.

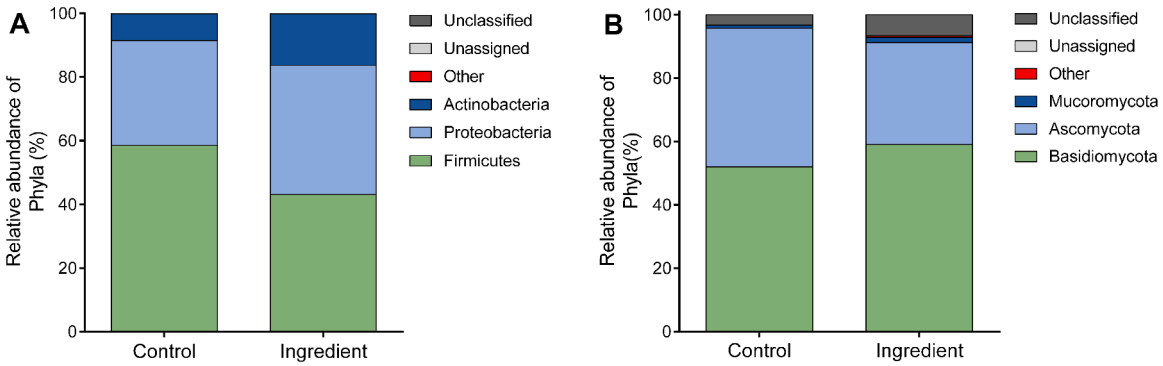

Fig. 4. Profile of skin microbial community in ingredient (straw extract-based ingredient) and the control (without straw extract-based ingredient) groups obtained by 16S rRNA gene- and ITS2 amplicon-based NGS. The graphs show the relative abundance of bacterial (A) and fungal (B) phyla with an average higher than 0.5%.

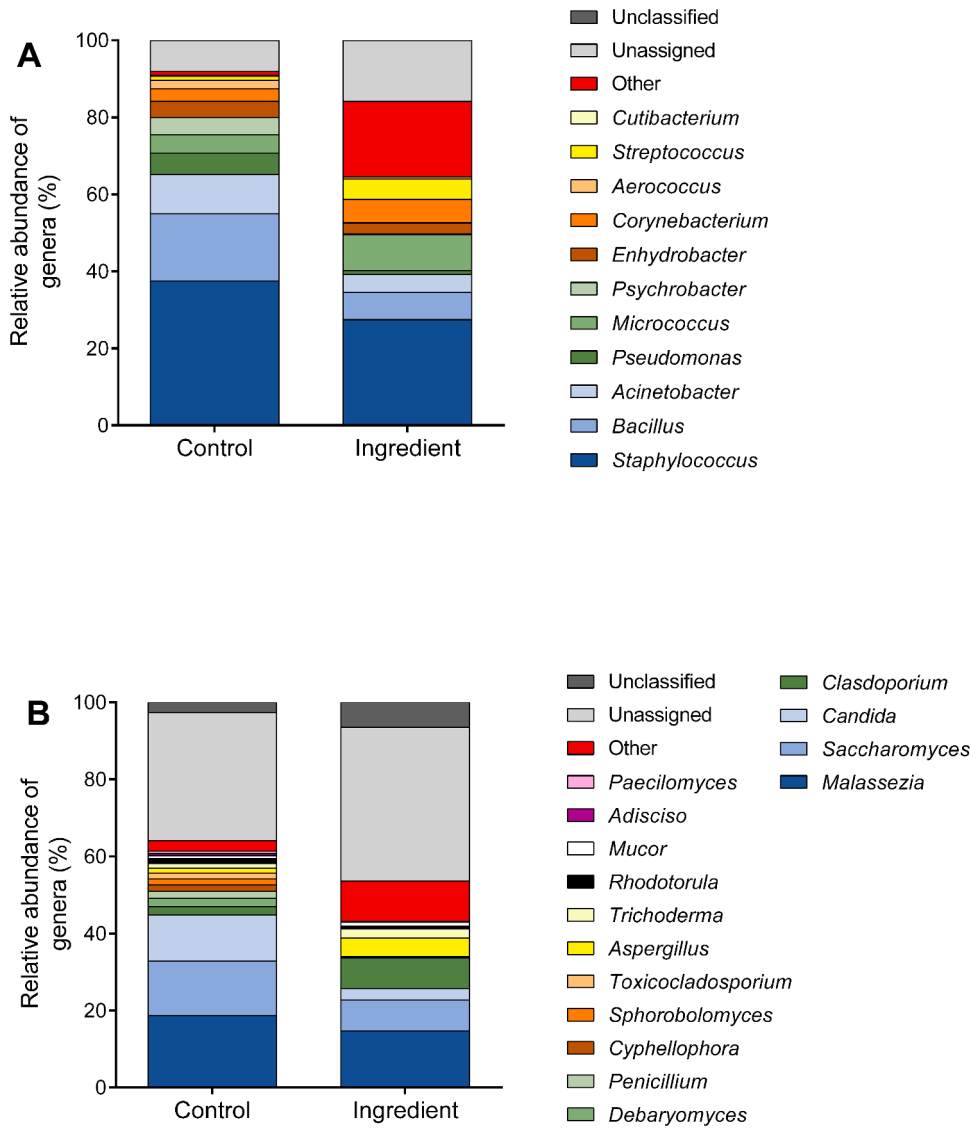

Fig. 5. Profile of skin microbial community in ingredient (straw extract-based ingredient) and the control (without straw extract-based ingredient) groups obtained by 16S rRNA gene- and ITS2 amplicon-based NGS. The graphs show the relative abundance of bacterial (A) and fungal (B) genera with an average higher than 0.5%.

Streptococcus and *Malassezia* (Figs. 5 and 6B).

When resorting to qPCR, the genera studied, *Staphylococcus*, *Cutibacterium, Streptococcus* and *Malassezia* did not show significant differences ($p > 0.05$) in the sugarcane straw extract-based ingredient group when compared to the control group (Fig. 7A). These results indicate that the sugarcane straw extract-based ingredient did not affect some of the main genera of the skin microbiota. When examining the impact of the ingredient on *S. epidermidis*, *C. acnes* and *S. aureus* (Fig. 7B), an imbalance seemed to occur in the ingredient group because *S. epidermidis* decreased and *S. aureus* increased in abundance ($p < 0.05$).

In addition to the specific microbial groups, the ratios *Cutibacterium* sp./*Staphylococcus* sp. and *C. acnes/ S. epidermidis* were calculated (Fig. 8). The *Cutibacterium* sp./ *Staphylococcus* sp. ratio did not differ significantly ($p > 0.05$) when comparing the control and ingredient groups, whereas the *C. acnes/ S. epidermidis* ratio displayed significant differences ($p < 0.05$) when comparing both groups.

It was also noted that NGS and qPCR techniques exhibit the same results when comparing both groups in *Staphylococcus*, *Cutibacterium, Streptococcus* and *Malassezia* species.

Thus, it was possible to conclude that the sugarcane straw extract-based ingredient did not impact skin microbiota α-diversity, the main phylum, and genera. However, the ingredient seems to inhibit *S. epidermidis* and promote *S. aureus*.

4. Discussion

Cosmetics have in their formulation ingredients that remain on the skin surface after several washes, which have an impact on the skin microbiota (Bouslimani et al., 2019). Therefore, the cosmetic industry has been engaged in the development of products that do not disturb or benefit the skin microbiota (Fournière et al., 2020). To assess the impact of cosmetics on the skin microbiota, *in vivo* studies are often employed, although they are time and cost consuming (Nigam, 2009). Hence, in this study, a new preclinical *in vitro* model was developed for testing cosmetic ingredients.

First, the preclinical *in vitro* model was optimized. The parameters chosen to develop the model were based on real skin conditions during the use of a cosmetic product. Thus, a temperature of 34 °C was chosen because it is the average temperature of the exterior layers of the skin (Yang et al., 2017), and RPMI medium was used as a basal medium to mimic the skin nutrients. In addition, the incubation time and

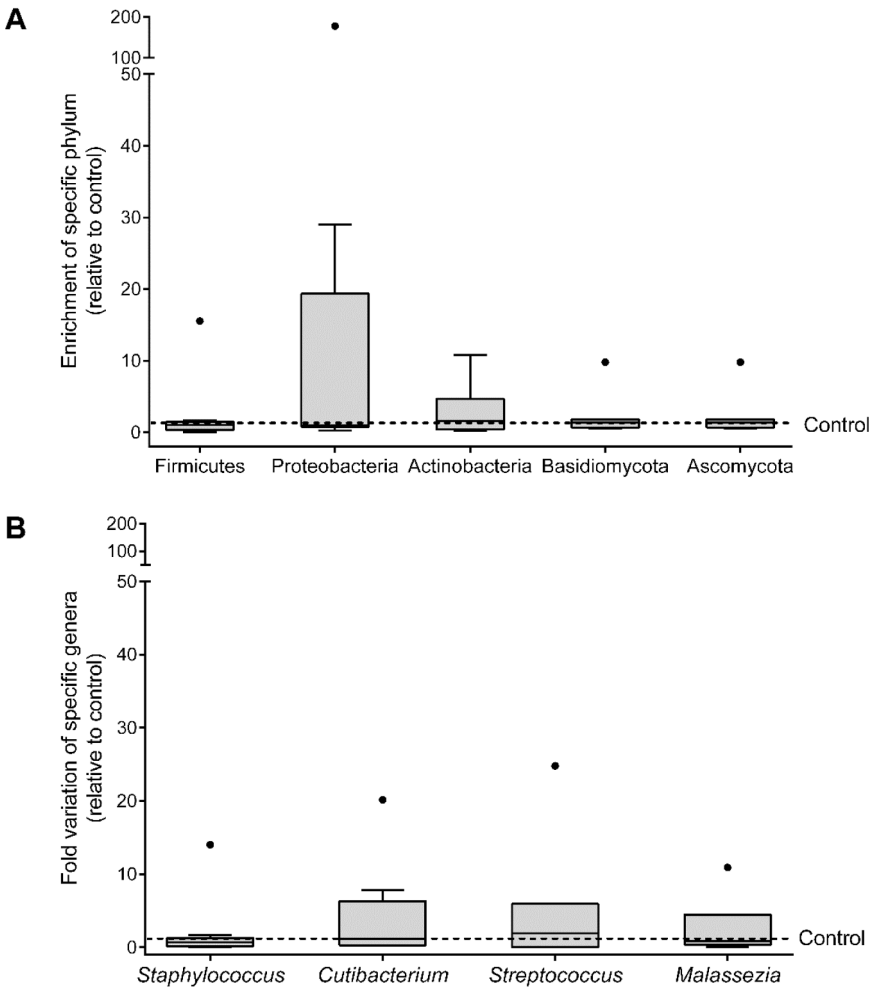

Fig. 6. Fold variation of the most abundant phyla (A) and the main genera (B) obtained after skin microbiota profiling (n = 12) by 16S rRNA gene and ITS2 amplicon-based NGS. Data was analyzed using the Mann-Whitney test in comparison to control.

environment conditions were optimized. These two variables (incubation time and environment) can affect the growth of some fastidious groups of microorganisms belonging to the skin microbiota. For instance, *Malassezia* species are usually incubated for longer periods of time in culture methods, 48–72 h (Hamdino et al., 2022), while *C. acnes* is usually grown under anaerobic conditions because it is a facultative anaerobic bacteria (Nakase et al., 2021). Besides total bacterial and fungal load, *Staphylococcus* sp., *Cutibacterium* sp., and *Malassezia* sp., were evaluated since these are described to be the main microbial genera of the skin microbiota (Mukherjee et al., 2016; Keum et al., 2020). *S. epidermidis* and *C. acnes* were analyzed since both are considered sentinels of the skin microbiota (Fournière et al., 2020), whereas *S. aureus* was tested since this bacteria is connected to several skin diseases and could be an indicator of dysbiosis (Guzik et al., 2005). Because no differences in absolute abundance were observed when samples were incubated in different environments, the aerobe environment was chosen. Regarding the incubation time, since no differences were detected under aerobic ($p > 0.05$), the 24 h period was chosen. Furthermore, a 24 h period allows us to better mimic the usage of daily cosmetic products, since some of the cosmetics are applied and removed daily. Therefore, it can be concluded that 24 h of incubation in an aerobic environment represents the best incubation conditions.

The model was validated by testing Ecoskin®, a benchmark cosmetic ingredient, with published *in vivo* data (Solabia, 2019). The findings of this investigation are consistent with the *in vivo* information provided in the Ecoskin® datasheet (Solabia, 2019), concluding that the developed model can achieve the same results as *in vivo* testing. Thus, this model may be a good alternative to *in vivo* testing, with several advantages, such as less invasiveness, time and cost savings, and may allow the simultaneous screening of more than one product at the same time.

After establishing the model, a novel sugarcane straw extract-based ingredient was tested. It was possible to see that the ingredient did not affect the relative abundance of the main phylum and genera belonging to the skin microbiota (Keum et al., 2020; Khayyira et al., 2020; Kim et al., 2021). Furthermore, the α-diversity (community diversity within-sample) of skin microbiota samples measured by the Shannon index (Fig. 3) (Johnson and Burnet, 2016) was not affected by the tested ingredient. At the species level, the results indicated that the sugarcane straw extract-based ingredient significantly decreased the relative abundance of *S. epidermidis,* while *S. aureus* abundance increased in the test condition in comparison to the control condition. *S. epidermidis* have been reported to produce antimicrobial peptides (AMP) that eliminates *S. aureus*; can inhibit *S. aureus*–induced neutrophil recruitment and pro-inflammatory cytokine production, which could potentially be protective against more severe skin infection; and produces a serine protease (Esp) which inhibits and degrades *S. aureus* biofilms (Iwase et al., 2010; Vandecandelaere et al., 2014; Brown and Horswill, 2020). Therefore, the sugarcane straw extract-based ingredient significantly decreased the relative abundance of S. epidermidis which might lead to an overrepresentation of S. aureus in the skin microbiota samples. The main issue of *S. aureus* colonization is that this bacterium is a pathogen commonly associated with some skin disorders, including atopic

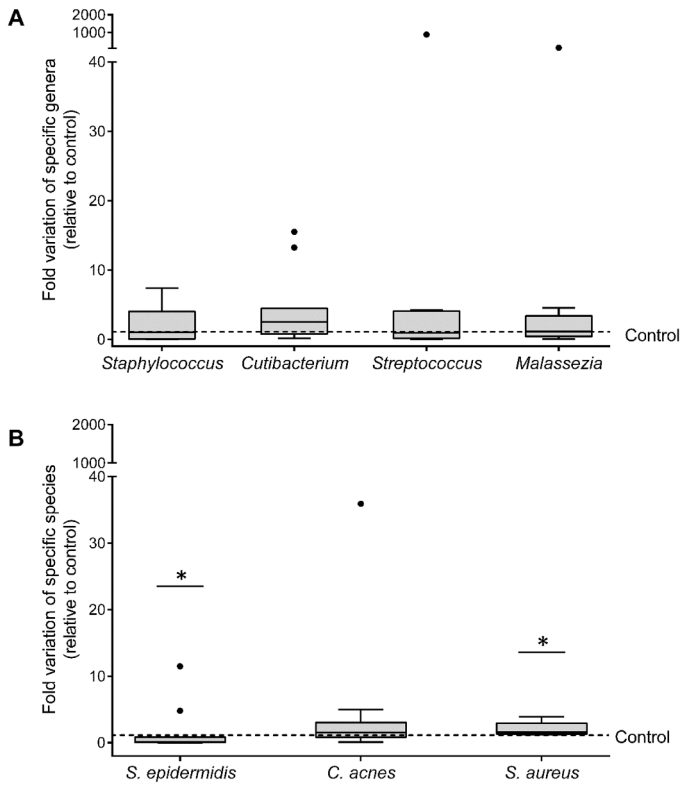

Fig. 7. Fold change of specific genera (A) and species (B) of skin microbiota of 12 female volunteers after the incubation with the sugarcane-based ingredient obtained by qPCR. The results are represented in fold-variation, which were analyzed by Mann-Whitney in comparison to control. * Indicate significant differences ($p < 0.05$).

dermatitis and atopic eczema (Kong et al., 2012; Clausen et al., 2017; Nakatsuji et al., 2016), which are often accompanied by skin microbiota dysbiosis that contribute to increase the inflammation. For instance, in atopic dermatitis, a previous report demonstrated a significantly increase in the relative abundance of *S. aureus*, and a decrease in the relative abundances of *Staphylococcus*, *Cutibacterium* and *Streptococcus* genera (Kong et al., 2012). Our results demonstrated statistically significant differences in the relative abundances of *S. aureus* and *S. epidermidis* between the test and the control conditions without suggesting a greater unbalance of the skin microbiota. In fact, the relative abundance of *S. aureus* increased in the test condition lower than two-fold in relation to the control condition. Although, our preliminary data suggests that the sugarcane straw extract-based ingredient seems to negatively affect the skin microbiota balance, we need to confirm this in a study with more volunteers, in which should be included individuals with healthy skin and with skin diseases.

The sugarcane straw extract-based ingredient is mostly composed by phenolic compounds, namely hydroxybenzoic and hydroxycinnamic acids, and flavones (Carvalho et al., 2023). These phenolic compounds have been reported to exhibit antimicrobial activities (Zhao et al., 2015; Oliveira et al., 2022), which might explain the decrease in the relative abundance of *S. epidermidis* detected in this study. Regarding the impact of phenolic compounds on the skin microbiota, a *Rhodomyrtus tomentosa* fruit extract containing phytochemical compounds, including flavonols and phenolic acids, was reported to decrease the abundance of some *C. acnes* phylotypes in patients with acne (Gervason et al., 2020). Additionally, previous studies have evaluated the impact of novel cosmetic ingredients on skin microbiota (Ciardiello et al., 2020; Hong et al., 2020). In fact, a previous study that evaluated the effect of fermented oils on cheek skin microbiota showed a decrease in Proteobacteria and an increase in *Staphylococcus* sp. abundances (Ciardiello et al., 2020). In a different study, a cosmetic serum containing prebiotics (GOS) was applied to the face in a randomized controlled trial, and it was possible to observe a decrease in *S. aureus* and *Corynebacterium* (Hong et al., 2020).

Overall the described *in vitro* model demonstrated that the sugarcane straw extract-based ingredient had a negative effect on the skin microbiota, specifically on the relative abundance of *S. epidermidis* leading to an increase in *S. aureus*. Although, it is important to take it into account that the assay performed was a preliminary study, and further testing should be achieved with a high sample number in order to reduce variability and assure the significancy of the results. Therefore, further studies should be performed to evaluate the impact on these *Staphylococcus* species.

5. Conclusions

In summary, we successfully developed a preclinical *in vitro* model that allowed to mimic the impact of cosmetic ingredients on skin microbiota, indicating that might be used as an alternative or complementary method to *in vivo* models. Furthermore, our preclinical *in vitro* model showed that the sugarcane straw extract-based ingredient has a negative effect on the skin microbiota, specifically on the relative abundance of *S. epidermidis,* leading to an increase in *S. aureus*. Additionally, since the ingredient was tested directly in the skin samples, the results obtained may also be used to advise formulators to develop a new ingredient and product formulation that improve this potential effect on skin microbiota, e.g., using prebiotic ingredients in the recipe. However, despite the results obtained in this work, more research should be performed regarding the impact of the sugarcane straw extract-based ingredient on the skin microbiota composition.

Ethics approval

Ethical review and approval were waived for this study, as it was conducted according to the internal rules legally established, based on the research ethics recommendation and approved on 17th September 2020 by the Ethics Committee for Health of the Universidade Católica Portuguesa (project no. 83).

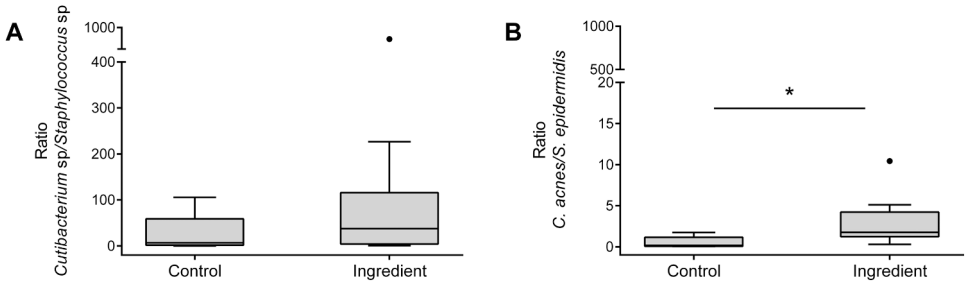

Fig. 8. Ratios of relative abundances of Cutibacterium sp./ Staphylococcus sp. (A) and C. acnes/ S. epidermidis (B). The skin microbiota samples collected from 12 female volunteers were analyzed by qPCR. Data was analyzed by Mann-Whitney statistical test. * Indicate significant differences ($p < 0.05$).

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CRediT authorship contribution statement

Maria João Carvalho: Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Inês Pinto-Ribeiro**: Investigation, Methodology, Data curation, Formal analysis, Conceptualization, Writing – review & editing. **Cláudia Castro**: Investigation, Methodology, Data curation, Formal analysis, Writing – review & editing. **Sílvia Santos Pedrosa**: Supervision, Writing – review & editing. **Ana L. S. Oliveira**: Supervision, Writing – review & editing. **Manuela Pintado**: Project administration, Funding acquisition. **Ana Raquel Madureira**: Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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