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Multifunctional Biotechnological Lip Moisturizer for Lip Repair and Hydration: Development, *In Vivo* Efficacy Assessment and Sensory Analysis

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Abstract: The demand for sustainable cosmetics leads to the search for natural and biotechnological ingredients. The present study reports the development of a multifunctional lip moisturizer containing levan (LEV) from *Bacillus subtilis natto*, sophorolipids (SOPs) from *Starmerella bombicola* and *Citrus paradisi* (OCP) essential oil, using a simplex-centroid experimental design. The formulations were evaluated physicochemically, pharmacotechnically and by DPPH assay. The optimized formulation was selected through the Response Surface Method, and the evaluation of its efficiency in lip hydration was carried out using the bioimpedance method and sensory analysis. The formulations showed pH compatibility with lips and remained stable after a centrifuge test and thermal stress. Spreadability varied between 415.3 and 1217.1 mm^2, moisture retention was above 95% and antioxidant capacity was around 50% for all formulations. The optimized formulation, containing 0.4% LEV and 0.8% SOF, maintained the lip hydration already shown by the participants; 85% of them reported improvement in this aspect. For the first time, LEV and SOP were incorporated in lip moisturizers, which is an environmentally friendly product with marketing potential. Furthermore, the use of the Skin Analyzer Digital equipment, a low-cost and non-invasive technique, to evaluate the effectiveness of lip products is innovative; this methodology may help in the development of future cosmetology studies.

Keywords: lip moisturizer; levan; sophorolipid; *Citrus paradisi* essential oil; bioimpedance; sensory attributes; acceptance; buy intention; cosmetics

1. Introduction

Lip histology is well defined, with this region consisting of a tenuous stratum corneum (SC), formed by orthokeratotic cells that renew more quickly than those present in the

normal SC, and the epithelium characterized as a thin tissue, slightly keratinized and with less ceramide content [1]. Due to their prominent location in the facial region, the lips are constantly susceptible to influences from the external environment, such as solar radiation, wind, extreme temperatures and the use of cosmetics and dental treatments [2]. Of the different skin maintenance mechanisms, the hydration state of the SC is the most commonly altered on a daily basis, and, for an intact barrier to be maintained in the epidermis, an adequate amount of water needs to be present on this surface [3]. Due to the rapid cell renewal of the lip SC, immature corneocytes are exposed to the skin surface, allowing the water present in the lips to transpire more easily, resulting in a dry and rough region [4]. In order to prevent dryness and roughness of the lips, maintaining or increasing SC hydration levels, cosmetic products for lip treatment are an excellent alternative [3].

In recent years, there has been a growing demand for the development of more natural and sustainable cosmetics, mainly through the dissemination of these ideals through social media, which influence consumers' opinion and purchase of products [5]. In this way, research by the cosmetic industry into natural actives and inputs that are safe for human use has been highlighted [6]. These natural actives can be obtained from different sources, such as vegetal, which includes oils, butters, waxes, essential oils, among others; animal, which includes waxes, honey [7] and others; and microbial, which includes biopolymers and biomolecules and may provide additional benefits to the cosmetic formulation.

The global dermocosmetics market is expected to grow at a compound annual growth rate (CAGR) of 7.5% until 2030, due to investment and development of new skin and hair care solutions by the industry [8]. Cosmeceuticals, which are cosmetic products with medicinal and drug-like benefits, have been a growing demand in the last years, especially due to research into bioactive molecules, which present biological properties such as repairing, moisturizing and anti-aging [9] and renewable characteristics. This research has been carried out by the cosmetic industry in association with biotechnology, a science that allows the development of new inputs and products efficiently [10] through fermentative processes or genetic engineering using microorganisms and enzymes, which can help in the evaluation of these components in the skin [11].

Examples of biotechnological molecules used in cosmetic formulations correspond to polysaccharides and derived lipids, such as levan (LEV) and sophorolipids (SOPs) [12]. LEV corresponds to a fructose exopolysaccharide (EPS), formed through glycosidic bonds of the β(2→6) type [13], which can be obtained by fermentative processes from a variety of microorganisms, such as *Bacillus subtilis natto*. This EPS has several bioactive properties of cosmetic interest, such as moisturizing, antioxidant and filler effects [14]. Kim et al. [15] evaluated the properties presented by LEV from *Zymomonas mobilis*, and they verified that this molecule has moisturizing properties similar to hyaluronic acid, as well as similar proliferation of human fibroblasts and keratinocyte cells, which demonstrates that LEV can be used as a cosmeceutical agent. Choi et al. [16] evaluated the potential of LEV as a dermal filler. They verified that levan-based hydrogel enhanced cell proliferation, showed better collagen synthesis than hyaluronic acid and also demonstrated anti-wrinkle efficacy. Pei et al. [17], Domżał-Kędzia et al. [18] and Bouallegue et al. [19] reported that LEV presents great antioxidant activity due to its capacity to donate electrons to the free radicals. Da Silva et al. [20] developed a facial cosmetic formulation containing LEV (0.75 g) and almond oil and evaluated its spreadability, antioxidant and moisture-retention capacity. They verified that the incorporation of these actives helped to improve all the parameters evaluated (805 mm^2, 72% and 100.3%, respectively). Helenas et al. [21] developed a new biocosmetic gel-anionic type containing LEV, and, using the Simple Lattice Design, they evaluated the optimized formulation, which was composed of LEV (1%), avocado oil (0.9 mL) and aloe vera extract (0.1 mL). According to statistical analyses, this formulation would have good spreadability (767.30 mm^2), antioxidant capacity (76%) and moisturizing capacity (98.37%). All studies have shown that LEV has useful properties as active ingredients in cosmetics.

SOPs correspond to biosurfactants, which consist of disaccharide sophoroses (2′-O-β-D-glucopyranosyl-1-β-D-glucopyranose) linked by glycosidic bonds to a fatty acid chain [22], which are obtained from non-pathogenic fungal strains such as *Starmerella bombicola*. These have moisturizing, antioxidant and antimicrobial properties, which have high applicability in the cosmetic industry [22–26]. Maeng et al. [27] produced SOP from *Candida bombicola* using horse oil, and they verified its properties. This biomolecule presented the capacity of expressing collagen I and helped in fibroblast migration in human skin. Gaur et al. [23] evaluated the antimicrobial capacity of SOP and showed that this biosurfactant presented activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* by destabilizing the permeability of bacterial cytoplasmic membranes. Filipe et al. [26] developed a self-preserving cosmetic formulation containing SOP (0.4 g) and palmarosa essential oil (0.04 g) that showed action against acne-causing microorganisms. Costa et al. [25] developed a multifunctional lipstick, containing SOP (1 g) and palmarosa essential oil (0.2 g), due to the antioxidant activity presented by SOP (59.4%). They also verified that the lipstick with the actives presented better spreadability (201.5 mm^2), moisture-retention capacity (91.57%), occlusive factor (85.6) and fusion (63 °C) and breaking (89 g) points when compared to the formulation without the actives (179.1 mm^2, 90.83%, 80.47, 59 °C and 85 g, respectively). Many studies have shown the potential of SOPs as cosmetic ingredients.

The use of essential oils (EO) by the cosmetic industry has stood out [28], both due to their aroma and the various bioactive and pharmacological properties they present [29]. *Citrus paradisi* (grapefruit) essential oil (OCP), which is recognized as GRAS (Generally Recognized as Safe by the FDA—Food and Drug Administration), has several bioactive properties, such as antimicrobial, which can be applied in the development of cosmetic products [30].

The quality of a cosmetic product involves its effectiveness and safety of use, the stability of the formula and the sensorial aspect. In order to evaluate formulation effects, the biophysical study of the skin has been widely used, as it allows the application of methods that evaluate skin characteristics, such hydration and oiliness, *in vivo* through non-invasive, fast and safe techniques through impedance or capacitance methods [31]. The bioimpedance method consists of passing an electrical current of low intensity (500 to 800 μÄ) and high frequency (50 kHz) through the labial region (which appears as conductive). With the resistance of this conductor to the passage of electrical current, the results of the analysis will be obtained through percentages for skin hydration and oiliness. The Skin Analyzer Digital equipment is based on this method [32]. The Corneometer®equipment is based on the capacitance method, with an acoustic signal triggered due to electromagnetic contact, and it is cited in the literature as a sensitive instrument for water content measurements [33]. In addition to the methods presented, other techniques can be used for lip evaluation, such as clinical and histological analyses to investigate lip healing after treatment [34]. Apart from proving the clinical efficacy presented by cosmetic products, it is essential that the formulation presents a good sensorial, which implies the well-being of the consumer, the acceptance of the product and its long-term use [35], with sensorial analysis a useful and relevant tool, which guarantees the quality of products developed considering consumer expectations and the benefits highlighted [36].

Based on the information presented, the main objective of this work was to develop a multifunctional lip moisturizer as a new biotechnological cosmetic containing LEV from *Bacillus subtilis natto*, SOP from *Starmerella bombicola* and OCP as active ingredients, in addition to evaluating the clinical efficacy of the formulation against the parameters of hydration and lip oiliness through a non-invasive technique, as well as evaluating sensory aspects of this, such as intensity of attributes, acceptance and purchase intention. This is an innovative work, which adds knowledge in the field of cosmetology.

2. Materials and Methods

2.1. Microorganisms and Essential Oil

The bacteria *Bacillus subtilis natto* (CCT 7712), obtained from the Tropical Cultures Collection, was provided by the Department of Biochemistry and Biotechnology (State University of Londrina, Londrina, Brazil) and used for the production of levan (LEV). *Starmerella bombicola* yeast (ATCC® 22214™), obtained from the American Type Culture Collection (Manassas, VA, USA), was supplied by the Department of Biochemistry and Biotechnology (State University of Londrina, Londrina, Brazil) and used for the production of sophorolipids (SOPs). The microorganisms *Staphylococcus aureus* (ATCC® 25923™), *Staphylococcus epidermidis* (ATCC® 12228™) and *Streptococcus mutans* (UA 159) were provided by the Basic and Applied Microbiology Laboratory (State University of Londrina, Londrina, Brazil). *Citrus paradisi* essential oil (OCP) was obtained commercially (ViaAroma, Porto Alegre, Brazil).

2.2. Obtaining Biomolecules

2.2.1. Levan (LEV)

LEV was produced by the enzyme levansucrase, obtained through the bacteria *B. subtilis natto* (CCT 7712), according to the methodology described by Helenas et al. [21]. LEV extraction occurred through precipitation with absolute ethanol in a ratio of 1:3 v/v (supernatant: ethanol), with the system kept at rest for 12 h at 4 °C. Then, centrifugation was used at 6500 G for 20 min and at 4 °C. After complete evaporation of the ethanol, the LEV was dialyzed against distilled water for 48 h, with three daily water changes, and was subsequently frozen and lyophilized. Gravimetry was used for quantification.

2.2.2. Sophorolipids (SOPs)

The production of SOPs occurred from the yeast *S. bombicola* (ATCC® 22214™) in a benchtop bioreactor using glucose and oleic acid as substrates, according to the methodology described by Silveira et al. [37]. The yeast inoculum was standardized at 0.5 $g{\cdot}L^{-1}$. The SOPs were extracted using organic solvents, which were subsequently dried in an oven and lyophilized. Gravimetry was used for quantification.

2.3. Assessment of the Antibacterial Activity of Actives

The tests were carried out against *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Streptococcus mutans* UA 159.

2.3.1. Minimum Inhibitory Concentration (MIC)

The MIC value of SOPs and OCP was determined by the broth microdilution method (Clinical and Laboratory Standards Institute Guidelines, CLSI) [38]. The tested concentration ranged from 0.003 $mg{\cdot}mL^{-1}$ to 0.184 $mg{\cdot}mL^{-1}$ for SOP and from 0.66 $mg{\cdot}mL^{-1}$ to 41.75 $mg{\cdot}mL^{-1}$ for OCP. SOP and OCP were solubilized in DMSO. The assay was carried out in microtubes, in triplicate and on three different occasions.

Colonies isolated from bacterial culture grown on Muller–Hinton agar medium (MHA, Difco, Sparks, MD, USA) (*S. aureus* and *S. epidermidis*) and Brain Heart Infusion Broth agar medium (BHI, Himedia, Maharashtra, India) (*S. mutans*) were suspended in saline solution (0.85% sodium chloride, m/v, Merck) and adjusted to the 0.5 McFarland scale to obtain 1.5×10^8 colony-forming units per milliliter ($CFU{\cdot}mL^{-1}$). After diluting the bacterial suspension 1:100 in Muller-Hinton Broth (MHB, Difco, Sparks, MD, USA) or BHI Broth (Himedia, Maharashtra, India), 50 µL were inoculated into a culture medium supplemented with antimicrobials at the concentrations described previously. The culture medium, antimicrobial actives and DMSO in the absence of inoculum were tested as sterility controls. Positive control for bacterial growth was performed with inoculum in the culture medium without antimicrobials.

The microtubes were incubated for 24 h at 37 °C (for *S. mutans*, incubation occurred in an oven with 4.5% CO_2 circulation), and the MIC was determined as the lowest concentration of antimicrobial agents capable of inhibiting visible bacteria growth.

2.3.2. Antimicrobial Effect of SOPs and CP in Combination

The MIC values for each active in combination was determined by the broth microdilution in a double concentration gradient, based on turbidity in a similar way to that described in Section 2.3.1. The type of antibacterial interaction between the actives was determined by the fractional inhibitory concentration index (FICI), as described by García-García et al. [39], with some modifications. The tested concentrations ranges were 0.003–0.092 mg·mL^{-1} for SOPs and 5.22–41.75 mg·mL^{-1} for OCP.

The bacterial inoculum was prepared as described in Section 2.3.1 using the McFarland scale and subsequent dilution of 1:100 in the appropriate culture media for each bacterial species, as well as sterility and bacterial viability controls. The microtubes were incubated for 24 h at 37 °C (for *S. mutans*, incubation took place in an oven with 4.5% CO_2 circulation). The interaction of the actives was analyzed using the Fractional Inhibitory Concentration Index (FICI), obtained in Equation (1) by adding the Fractional Inhibitory Concentration (FIC, Equation (2)) of the SOPs and the OCP. The FICI is interpreted as synergistic ($\leq$0.5), additive (>0.5 and $\leq$1), indifferent (>1 and <4) or antagonistic ($\geq$4) (Odds, 2003), according to the equation

$$FICI = FIC_{SOF} + FIC_{OCP} \quad (1)$$

where:

$$FIC = \frac{MIC_{combination}}{MIC_{individual}} \quad (2)$$

2.4. Assessment of the Antioxidant Activity of Actives

The analysis of the antioxidant capacity of the active ingredients was carried out according to the methodology described by Srikanth et al. [40], with adaptations. Initially, DPPH (2,2-diphenyl-1-picrylhydrazyl) was solubilized in ethanol to obtain a concentration of 250 µM and kept away from light. Test samples were prepared at different concentrations in the form of a curve: LEV was dissolved in water at concentrations of 1 mg·mL^{-1} to 100 mg·mL^{-1}; SOP was dissolved in ethanol at concentrations of 5 mg·mL^{-1} to 50 mg·mL^{-1}; and OCP was diluted in ethanol at concentrations of 1 µL·mL^{-1} to 50 µL·mL^{-1}. For the reaction mixture, 1 mL of the test solutions plus 0.3 mL of the DPPH solution were added to test tubes wrapped in craft paper, a procedure carried out in triplicate, and these were kept at room temperature and protected from light for 30 min. For the blank, 1 mL of test solutions and 0.3 mL of ethanol were used. The control was composed of 1 mL of ethanol and 0.3 mL of DPPH solution. The reading was carried out on a UV-Vis spectrophotometer at a wavelength of 517 nm. The inhibition rate (%) of the free radical was calculated according to Equation (3):

$$Inhibition\ (\%) = \frac{(controle\ absorbance - sample\ absorbance)}{control\ absorbance} \times 100 \quad (3)$$

2.5. Development of Cosmetic Formulations

The simplex-centroid experimental design was used to optimize active concentrations. Seven formulations were developed, varying the components x1 (LEV), x2 (SOPs) and x3 (OCP), based on a control formulation (CF) of the O/W emulsion type. The maximum concentration of active ingredients used was 2% (LEV), 1% (SOP) and 0.3% (OCP). The formulations were prepared by mechanical agitation: phase A composed of distilled water, disodium EDTA, sodium saccharin, glycerin and copolymer of sodium acrylate and lecithin; phase B composed of castor oil, capric and caprylic acid triglycerides, shea butter, BHT, menthol and polysorbate 80; phase C composed of phenoxyethanol and methylisothiazolinone; and phase D composed of 20% sodium hydroxide solution.

Initially, phase A was heated to 75 °C on a heating plate for addition of the sodium acrylate and lecithin copolymer under mechanical stirring at 1500 rpm. Then, phases A and B were heated until both reached a temperature of 75 °C, with phase B being poured over phase A under constant stirring at 1500 rpm. After cooling the system to 40 °C, phase C was added. pH regulation occurred with the addition of phase D, which was maintained between 6.0 and 7.0. At the end of the process, the formulations were placed in polyethylene (PP) plastic bottles with a capacity of 100 mL and kept under refrigeration for a period of 24 h to carry out subsequent centrifugation tests (pre-stability).

2.6. Pharmacotechnical Characterization of Formulations

2.6.1. Pre-Stability Test

Five grams of each formulation, in triplicate, were placed in 15 mL Falcon tubes and subjected to centrifugation (Baby Centrifuge®, Fanem, Guarulhos, Brazil) for 30 min at 2800 rpm [41].

2.6.2. Organoleptic Tests

According to the Cosmetic Products Quality Control Guide [42], appearance, color, flavor and odor tests were carried out for the O/W formulations developed.

2.6.3. Spreadability

The evaluation of the spreadability of lip balms occurred using the parallel plate method, and the test was carried out in triplicate. One gram of the formulations was transferred onto the central point of a glass plate (20 cm × 20 cm), contained on a graph paper scale. A glass plate of known mass was added to the formulations to determine the surface they covered after one minute of pressure by reading the diameters covered. Subsequently, objects of known mass (2 g, 5 g and 10 g) were added to the glass plate, and after 1 min of adding each object, the diameters covered by the sample were read. Subsequent calculation of the average diameter [43] was performed, and the spreadability of each sample was calculated using Equation (4):

$$Ei = \frac{d^2\,\pi}{4} \tag{4}$$

2.6.4. Moisture Retention

Moisture retention (MR) by lip balm formulations was evaluated using a gravimetric method, according to the methodology proposed by Zhang et al. [44]. Briefly, in a previously tared crucible, 1 g of formulation plus 1 mL of distilled water were added, and the system was homogenized. These were then stored in a sealed humidity desiccator with a saturated solution of K_2CO_3 (43% relative humidity) for a period of 96 h at room temperature. The percentage of MR was calculated according to Equation (5):

$$MR(\%) = \frac{P_T}{P_0} \times 100 \tag{5}$$

where P_T corresponds to the final weight and P_0 to the initial weight, by the percentage of residual water in the samples.

2.6.5. Assessment of the Antioxidant Activity of Formulations

To analyze the antioxidant capacity of lip balm formulations, the same methodology as in Section 2.4. was adopted. The test samples were prepared at a concentration of 400 mg·mL^{-1} with the addition of ethanol and subsequent homogenization in a vortex for 2 min to completely dissolve the formulation in the solvent. Then, the system was filtered through filter paper. It is worth mentioning that to carry out this analysis, all formulations were produced without the addition of BHT in their composition, as this is an excellent antioxidant and could interfere with the results obtained.

2.6.6. Preliminary Stability of Formulations

The preliminary stability test was carried out in accordance with the Cosmetic Products Stability Guide [41], with a duration of 15 days (24 h cycles, alternating temperature); the storage conditions used were oven (40 ± 2 °C) and refrigerator (5 ± 2 °C). The organoleptic and physicochemical parameters (pH, spreadability and moisture retention) were evaluated in parallel, carried out at time zero (24 h after handling the formulations) and after 15 days of thermal stress. The assay was carried out in triplicate, and a reference sample was kept protected from light at room temperature for comparative purposes.

2.7. Response Surface Method (RSM)

Response Surface analysis (Statistica Software version 7.0.0) was performed to define the optimized formulation. The variables analyzed were LEV(x1), SOP(x2) and OCP(x3) in relation to the spreadability, moisture retention and antioxidant activity of the formulations.

2.8. Optimized Formulation Development

O/W emulsion formulations were developed using LEV (0.4%) and SOP (0.8%) as active ingredients based on the optimization proposed by RSM. The formulations were produced as described in Section 2.5. and named test formulation (TF).

2.9. Evaluation of In Vivo Efficacy and Sensory Analysis of Formulations

The study was approved by the Permanent Ethics Committee for Research involving Human Beings at the State University of Londrina, according to Resolution No. 446/2012 of the National Health Council (CAAE 58720522.9.0000.5231).

2.9.1. Study Population

The study population consisted of 61 healthy female volunteers aged between 18 and 37 years (24.6 ± 5.05) who had dry lips [45,46]. These were selected based on a verbal and digital invitation by the research team, and those who met the inclusion criteria were invited to take part in the study and were instructed on its objectives and methods. If accepted, the signing of the Free and Informed Consent Form (FICF) was requested.

Participants were excluded from the sample if they were pregnant or suspected of being pregnant; were breastfeeding; had a history of previously known hypersensitivity to any component of the formulations; were using medication that could alter skin responses; had any chronic, systemic disorder or skin, with signs of skin irritation at the site of application; had used lip, dermatological or aesthetic treatments in the 40 days preceding the study; had participated in similar studies in the previous 30 days; or showed signs of dermatitis or irritation during the evaluation period [2,47].

2.9.2. Study Design

The design used in the study was experimental of the randomized clinical trial type. The effectiveness of two different semi-solid formulations was evaluated against skin hydration. An intensity scaling technique was applied to survey attributes and profiles of the formulations [36]. Acceptance and purchase intention of the formulations were also evaluated through sensory analysis [48,49], as well as their effect (lip regeneration). The equipment used to evaluate biophysical parameters was the Skin Analyzer Digital (SkinUp Beauty Devices, São Paulo, Brazil) [32].

Participants were randomly divided into two treatment groups, namely G1: experimental group, which exclusively used FT, composed of 26 participants; G2: control group, which exclusively used FB, composed of 30 participants. They were "blind" regarding the content of the products applied. Volunteers were asked to only use the cosmetics provided on the lip region during the study period, in order to avoid possible interference.

Participants were instructed on how to apply the products, which should be applied directly to lips previously cleansed with micellar water then rinsed and dried with a soft towel and applied gently and evenly twice a day (morning and night) for a period of

7 days [47]. It was recommended that a wash-out without lip cosmetics or any topical lip therapy be performed 12 h before applying the study products.

2.9.3. Hydration Analysis

Measurements were collected at time zero (baseline value) and 7 days after daily self-application of the lip balm. The lower lip region was selected for measurements [2,3,50], cleaned with micellar water prior to data collection. Six replications were used for hydration analysis. Treatment monitoring was also done through photodocumentation of the lips under standardized lighting, background and positioning conditions. The photographic record took place at time zero and 7 days.

2.9.4. Sensory Analysis

Sensory analysis occurred through affective acceptance tests using a seven-point hedonic scale and purchase intention, intensity scale and evaluation of the effect of the lip balm. This was carried out with untrained evaluators who were potential consumers of the product.

Each participant received two lip balm samples (FT and FB), packaged in eppendorf and previously coded with a three-digit number in random order, as well as evaluation sheets containing method instructions. In the intensity scale test, the attributes evaluated were ease of spreading, absorption, hydration, freshness, formation of a velvety film and fragrance [22,51]. In the acceptance test, the evaluators classified the samples using the 7-point structured hedonic scale: 7—I liked it extremely, 6—I liked it very much, 5—I liked it, 4—I neither liked nor disliked it, 3—I disliked it, 2—I disliked it very much and 1—I really disliked it. In parallel, in the purchase intention test, evaluators were required to indicate their intention to purchase the samples presented, using a 5-point scale: 5—I would certainly buy, 4—I would probably buy, 3—Maybe I would buy, maybe notbuy, 2—Probably wouldn't buy and 1—Certainly wouldn't buy. The attributes description is found in Table 1.

Table 1. Description of the attributes evaluated during sensory analysis.

Attribute	Description
Ease of Spreading	How easy is it to apply the sample to the lips
Absorption	The sample is absorbed through the lips and disappears into the skin
Moisture	The sample hydrates the lips, replenishing their moisture
Freshness	Pleasant sensation produced by the sample in contact with the lips; it refers to something fresh
Velvet Film Formation	The surface of the lips has a soft and smooth texture after applying the sample
Fragrance	The aroma/perfume that the sample presents

The lip balm effect test was carried out after the others. The evaluators received a coded sample in the form of a tube containing the FT of the study (with biomolecules), which they applied daily, twice a day, for a period of 7 days, proceeding with the same precautions indicated for the hydration assessment test. At the end of this period, they evaluated the effect of the product by asking, "After seven days of applying the product, did you notice that there was an improvement in the roughness and dryness of your lips?", choosing one of the alternatives as an answer: yes or no [51].

2.10. Statistical Analysis

Data distribution was analyzed using the Shapiro–Wilk test to verify normality. If distribution was normal, all data were statistically compared using analysis of variance (ANOVA) and, to compare individual differences between means, Tukey test. If the distribution was not normal, the Kruskal–Wallis test and Dunn's test were used. In all cases, a significance level of $p < 0.05$ was accepted to denote significance. For in vivo analyses, as

the distribution was normal, the parametric T test was used to assess statistical differences. To evaluate the intensity of attributes, hedonic scale and purchase intention, it was assumed that the data were parametric, and the T test was used to evaluate statistical differences.

3. Results

3.1. Obtaining LEV and SOP

LEV production by *Bacillus subtilis natto* through the enzyme levansucrase was 42.93 $g \cdot L^{-1}$, while SOP production by *Starmerella bombicola* was 87.10 $g \cdot L^{-1}$, obtained by a fermentation process standardized by the research group of this article.

3.2. Antimicrobial Activity of Biomolecules

SOP and OCP inhibited the growth of the tested microorganisms, with MIC values ranging from 0.012 to 0.048 $mg \cdot mL^{-1}$ and from 10.44 to 41.75 $mg \cdot mL^{-1}$, respectively. The combination of both biomolecules reduced their MIC when compared to them individually, resulting in aditism interaction (Table 2).

Table 2. Effect of sophorolipids (SOPs) and *Citrus paradisi* essential oil (OCP) against the microorganisms *S. aureus*, *S. epidermidis* and *S. mutans*.

	SOP ($mg \cdot mL^{-1}$)			OCP ($mg \cdot mL^{-1}$)			FICI	Interaction
	MIC	MICc	FIC	MIC	MICc	FIC		
S. aureus	0.012	0.006	0.5	10.44	5.22	0.5	1.0	Aditism
S. epidermidis	0.048	0.024	0.5	41.75	20.88	0.5	1.0	Aditism
S. mutans	0.012	0.006	0.5	20.88	10.44	0.5	1.0	Aditism

Minimum Inhibitory Concentration (MIC) of isolated active ingredients, in combination (MICc) and Fractional Inhibitory Concentration Index (FICI) obtained by the Fractional Inhibitory Concentration (FIC) of SOP and OCP. FICI, which depends on the FIC, is interpreted as follows: synergistic ($\leq$0.5), additive (>0.5 and $\leq$1), indifferent (>1 and <4) or antagonistic ($\geq$4). FIC is determined by the equation: $FIC = MIC_{combination}/MIC_{individual}$. FICI is determined by the equation: $FICI = FIC_{SOP} + FIC_{OCP}$.

3.3. Assessment of the Antioxidant Activity of Actives

The DPPH assay showed that OCP has low antioxidant potential, with radical scavenging activity below 12.75%, at a concentration range of 1.0–50 $\mu L \cdot mL^{-1}$. SOP showed antioxidant potential between 33.87% and 61.53% at a concentration range of 5.0–40 $mg \cdot mL^{-1}$, respectively. LEV had an antioxidant potential between 22% and 56.1% at a concentration range of 1.0–100 $mg \cdot mL^{-1}$, respectively.

3.4. Development of Cosmetic Formulations

The choice of concentration of active ingredients (variables) used in experimental planning was based on antioxidant and antimicrobial activity data obtained in this study and information from the literature. The maximum chosen concentrations were defined as 0.3% (2.5 $mg \cdot mL^{-1}$) for OCP, 1.0% for SOP and 2.0% for LEV (Table 3).

Table 3. Concentration of active ingredients used in formulations according to simplex-centroid experimental design.

Formulations	LEV	SOP	OCP	Actives Concentration (%/100 g)
F1	1	0	0	2.0% LEV
F2	0	1	0	1.0% SOP
F3	0	0	1	0.3% OCP
F4	$1/2$	$1/2$	0	1.0% LEV + 0.5% SOP
F5	0	$1/2$	$1/2$	0.5% SOP + 0.15% OCP
F6	$1/2$	0	$1/2$	1.0% LEV + 0.15% OCP
F7	$1/3$	$1/3$	$1/3$	0.66% LEV + 0.1% OCP + 0.33% SOP
FB	0	0	0	No

Levan: LEV; Sophorolipids: SOPs; *Citrus paradisi* essential oil (OCP).

3.5. Pharmacotechnical Characterization of Formulations

In the centrifugation test, the base formulation (FB) and other formulations (F1, F2, F3, F4, F5, F6 and F7) did not show any signs of instability, such as phase separation, sedimentation or creaming (Table 4). For organoleptic tests, all formulations, including FB, presented white color, slightly sweet flavor (normal) and characteristic odor, which was slightly minty due to the presence of menthol in the formulation (normal), except the formulations containing OCP in their composition, which presented a characteristic odor of this essential oil. Regarding appearance, all formulations were homogeneous and viscous. The formulations containing SOP appeared to be slightly less viscous due to the incorporation of this molecule; however, they showed better spreadability when compared to the base.

Table 4. Pharmacotechnical characterization of the formulations.

Formulations	PS	Aspect	Color	Odor	Flavor	Spreadability (mm^2)	Moisture Retention (%)
FB	NPS	V	White	N	N	423.0 ± 42.6	97.92 ± 0.38
F1	NPS	LV	White	N	N	415.3 ± 21.5	97.70 ± 0.54
F2	NPS	V	White	CPC	N	909.6 ± 41.1 *	96.29 ± 1.00
F3	NPS	LV	White	N	N	422.5 ± 20.8	97.32 ± 1.61
F4	NPS	LV	White	CPC	N	1170.0 ± 14.1 *	95.30 ± 0.56 *
F5	NPS	V	White	CPC	N	1217.1 ± 14.3 *	97.71 ± 0.87
F6	NPS	LV	White	CPC	N	469.2 ± 5.1	97.42 ± 0.30
F7	NPS	V	White	N	N	1061.2 ± 7.4	97.56 ± 0.16

PS: pre-stability assay (centrifugation). NPS: no phase separation. V: viscous. LV: less viscous. N: normal. CPC: *Citrus paradisi* characteristic odor. mm^2: square millimeters. %MR: moisture retention percentage. * significant statistical difference in comparison to FB ($p < 0.05$), ANOVA, Dunnett's multiple comparison test. F1: 2% of LEV; F2: 1% of SOP; F3: 0.3% of OCP; F4: 1% of LEV and 0.5% of SOP; F5: 0.5% of SOP and 0.15% of OCP; F6: 1% of LEV and 0.15% of OCP; F7: 0.66% of LEV, 0.1% of OCP and 0.33% of SOP.

All formulations had pH between 6 and 7. In terms of spreadability, formulations FB, F1, F3 and F6 presented results between 415.3 and 469.2 mm^2, while formulations containing SOP (F2, F4, F5 and F7) presented results between 909.6 and 1217.1 mm^2. All formulations presented moisture retention values between 95.30% and 97.92%.

3.6. Preliminary Stability Data of Formulations

For all formulations, the color and odor remained unchanged after 15 days under thermal stress during a preliminary stability study. For appearance and flavor, some formulations showed slight changes, such as viscosity decrease and reduction in sweet taste; however, the changes were not relevant to eliminate these formulations from this study. The pH of the formulations did not change, remaining between 6 and 7.

For spreadability, after 15 days of preliminary stability study, all formulations showed variation in their spreading capacity when compared to time zero (before thermal stress), denoting statistical significance ($p < 0.05$) (Figure 1). This parameter indicated that the formulations submitted showed improvement in their spreadability over time.

For moisture retention (Figure 2), all formulations before and after 15 days of stability assay maintained their level of hydration, since they did not show a statistically significant difference ($p < 0.05$) for this parameter.

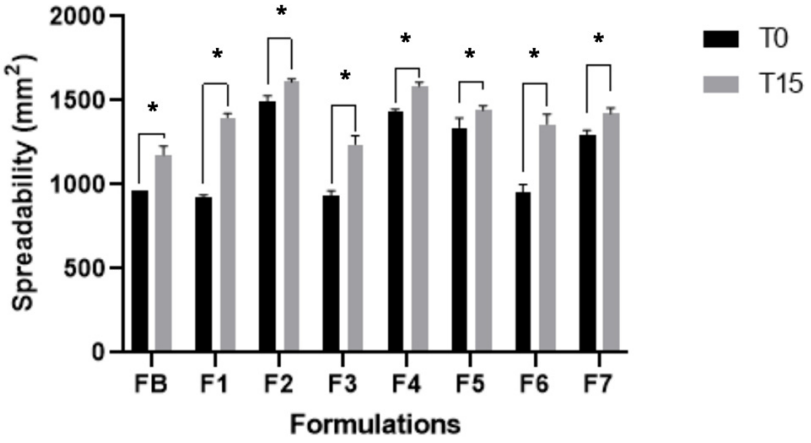

Figure 1. Formulations spreadability during the stability test. T0: time zero; T15: time 15 days; mm^2: square millimeters; * significant statistical difference ($p < 0.05$). F1: 2% of LEV; F2: 1% of SOP; F3: 0.3% of OCP; F4: 1% of LEV and 0.5% of SOP; F5: 0.5% of SOP and 0.15% of OCP; F6: 1% of LEV and 0.15% of OCP; F7: 0.66% of LEV, 0.1% of OCP and 0.33% of SOP.

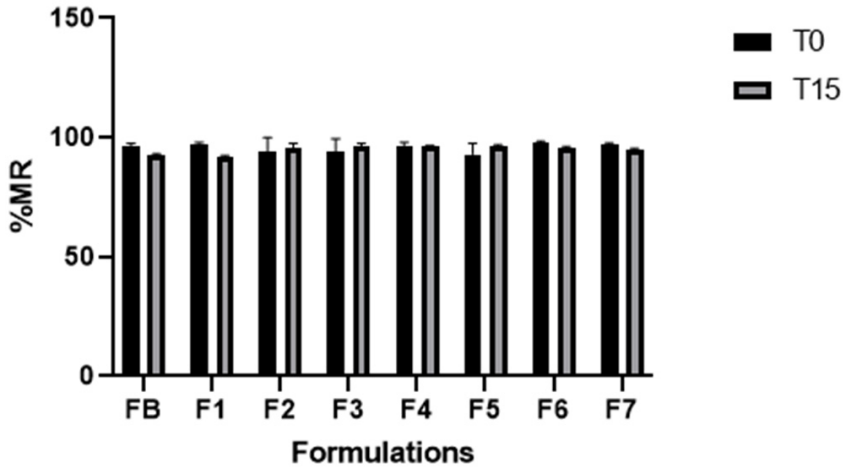

Figure 2. Moisture retention presented by the formulations before and after the stability study. T0: time zero, before stability study; T15: 15 days after stability study; %MR: Moisture retention percentage. F1: 2% of LEV; F2: 1% of SOP; F3: 0.3% of OCP; F4: 1% of LEV and 0.5% of SOP; F5: 0.5% of SOP and 0.15% of OCP; F6: 1% of LEV and 0.15% of OCP; F7: 0.66% of LEV, 0.1% of OCP and 0.33% of SOP.

According to the preliminary stability study, the formulations remained stable when subjected to stress conditions. Therefore, there was no exclusion of any of them in the next tests.

3.7. Antioxidant Activity of Formulations

For DPPH assay, the formulations showed free radical inhibition rates between 45.71% and 53.28%, as shown in Table 5. Only formulations F3 and F6 showed a statistically significant difference ($p < 0.05$) in relation to FB, with a decrease in their antioxidant capacity.

Table 5. Antioxidant activity of formulations according to the percentage of inhibition of DPPH assay.

Formulation	% inhibition
F1	49.14 ± 2.38
F2	53.28 ± 0.38
F3	45.71 ± 0.38 *
F4	52.70 ± 2.57
F5	47.60 ± 0.40
F6	46.05 ± 1.97 *
F7	51.48 ± 1.99
FB	51.32 ± 1.03

* significant statistical difference in relation to FB ($p < 0.05$), one-way ANOVA, Dunnett's multiple comparison test. F1: 2% of LEV; F2: 1% of SOP; F3: 0.3% of OCP; F4: 1% of LEV and 0.5% of SOP; F5: 0.5% of SOP and 0.15% of OCP; F6: 1% of LEV and 0.15% of OCP; F7: 0.66% of LEV, 0.1% of OCP and 0.33% of SOP.

3.8. Response Surface Method (RSM)

Table 6 shows the spreadability, antioxidant activity and moisture retention data of formulations prepared based on simplex-centroid planning. Such results were statistically analyzed by the Response Surface method to determine the optimized formulation in terms of SOP, LEV and OCP concentrations.

Table 6. Antioxidant effect, spreadability and moisture retention of formulations developed based on simplex-centroid planning.

Essay	x1	x2	x3	Antioxidant Activity (%)	Spreadability (mm^2)	Moisture Retention (%)
1	1.000	0.000	0.000	49.14	415.30	97.70
2	0.000	1.000	0.000	53.28	909.60	96.29
3	0.000	0.000	1.000	45.71	422.50	97.32
4	0.500	0.500	0.000	52.70	1170.00	95.30
5	0.000	0.500	0.500	47.60	1217.00	97.71
6	0.500	0.000	0.500	46.05	469.20	97.42
7	0.333	0.333	0.333	52.57	1061.20	96.20
8	0.333	0.333	0.333	52.70	1084.90	96.34
9	0.333	0.333	0.333	49.18	1056.50	97.56
10	0.333	0.333	0.333	51.48	1075.80	96.78

x1: LEV, x2: SOF e x3: OCP. mm^2: square millimeters.

3.8.1. Spreadability

The response surface and the profile of predicted value and desirability for spreadability are presented in Figure 3. According to statistical analyses, the formulation composed of 0.5 of SOP (0.5%) and 0.5 of OCP (0.15%) would show the best spreadability. The model estimates are presented in Table 7. LEV, SOP and OCP were significant ($p < 0.05$) for spreadability. The formulations with binary-combinations containing SOP plus LEV and SOP plus OCP showed the best spreadability (2030.20 and 2204.20 mm^2, respectively). The ANOVA of the model obtained to describe spreadability showed a coefficient of determination (R^2) of 0.9997, and it was significant at the 5% level.

Table 7. Model estimates for the spreadability of the formulations.

Factors	Estimates	Standard Error	T-Value	*p*-Value
Levan (x1)	415.30	11.96	34.74	0.000828 *
Sophorolipid (x2)	909.60	11.96	76.08	0.000173 *
Citrus paradisi essential oil (x3)	422.50	11.96	35.34	0.000800 *
x1x2	2030.20	58.57	34.66	0.000831 *
x1x3	201.20	58.57	3.44	0.075299
x2x3	2204.20	58.57	37.63	0.000705 *
x1x2x3	−36.30	316.77	−0.11	0.919236

R^2: 0.9997, R^2ajusted: 0.9987; Lack-of-fit: $p = 0.919236$; * $p < 0.05$, the factor was significant for the spreadability response.

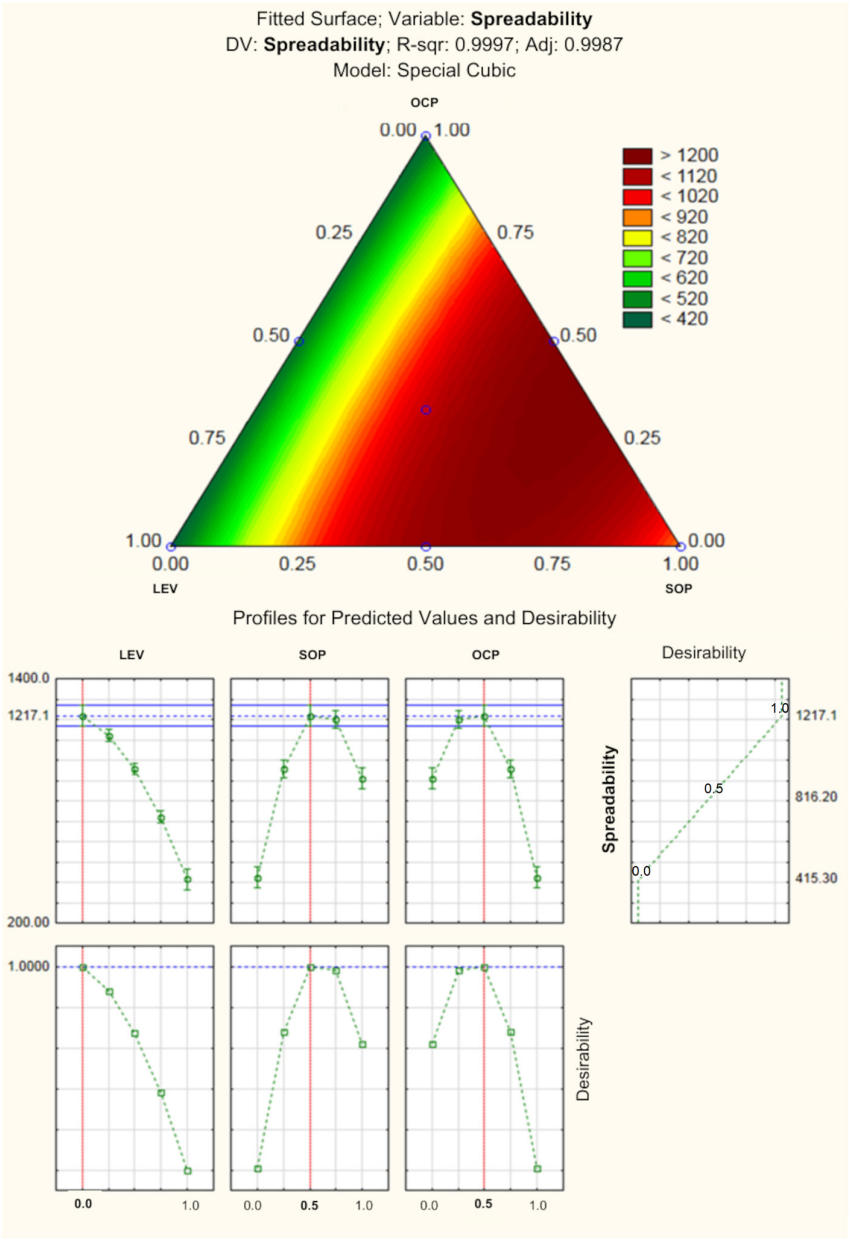

Figure 3. Response surface and profiles for predicted values and desirability for the spreadability of formulations.

3.8.2. Antioxidant Activity

The response surface and the profile for predicted values and desirability for antioxidant activity are presented in Figure 4. According to statistical analyses, the formulation composed of 0.25 (0.5%) of LEV and 0.75 (0.75%) of SOP would show the best antioxidant activity. The model estimates are presented in Table 8. LEV, SOP and OCP were significant ($p < 0.05$) for antioxidant activity, showing a positive effect. The combination of SOP, LEV and OCP had the best effect on the antioxidant activity of the formulations (98.94%). Negative effects, including a reduction in antioxidant activity, were seen when combining OCP with SOP or LEV (−7.58% and −5.50%, respectively). The ANOVA of the model obtained to describe the antioxidant activity showed a coefficient of determination (R^2) of 0.9879, and it was significant at the 5% level.

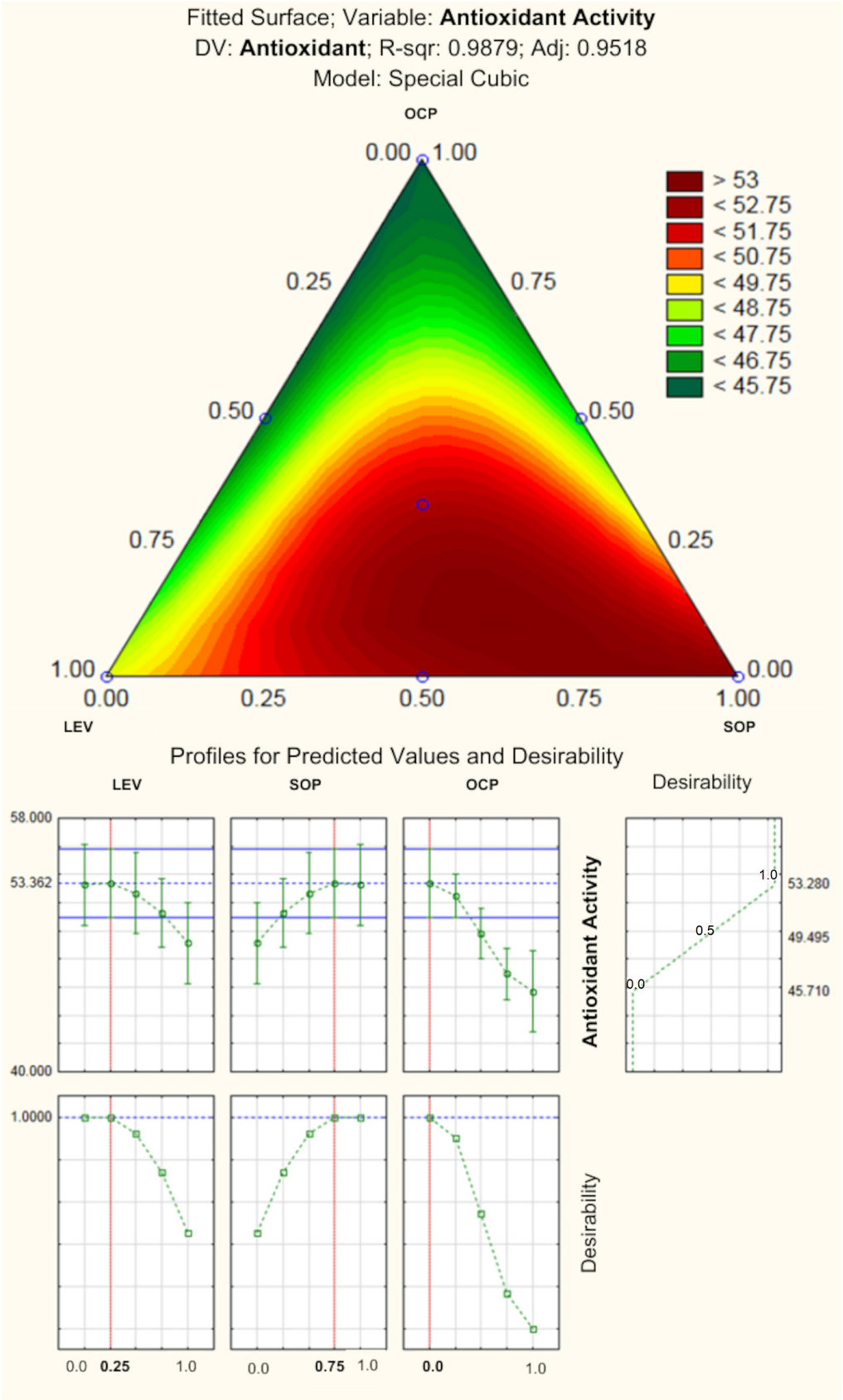

Figure 4. Response surface and profiles for predicted values and desirability for the antioxidant activity of the formulations.

Table 8. Model estimates for the antioxidant activity of the formulations.

Factors	Estimates	Standard Error	T-Value	*p*-Value
Levan (x1)	49.14	0.67	73.34	0.000186 *
Sophorolipid (x2)	53.28	0.67	79.52	0.000158 *
Citrus paradisi essential oil (x3)	45.71	0.67	68.22	0.000215 *
x1x2	5.96	3.28	1.82	0.211055
x1x3	−5.50	3.28	−1.67	0.235796
x2x3	−7.58	3.28	−2.31	0.147203
x1x2x3	98.94	17.75	5.57	0.030716 *

R^2: 0.9879, R^2ajusted: 0.9518; Lack-of-fit: *p*: 0.2358; * $p < 0.05$, the factor was significant for antioxidant activity response.

3.8.3. Moisture Retention

The response surface and the profile for predicted values and desirability for moisture retention are presented in Figure 5. According to statistical analyses, the formulation composed of 0.5 of SOP (0.5%) and 0.5 of OCP (0.15%) would show the best moisture retention. The model estimates are presented in Table 9. LEV, SOP and OCP were significant ($p < 0.05$) for moisture retention, showing a positive effect, i.e., high moisture retention. Negative effects, such as lack of moisture retention, were seen when combining LEV with SOP or OCP (−6.78% and −0.36%, respectively), as well as when combining the three active ingredients (−7.35%). A positive effect, presence of moisture retention, was found for the formulation containing the mixture of SOP and OCP (3.62%); however, this was not significant. The ANOVA of the model obtained to describe moisture retention exhibited a coefficient of determination (R^2) of 0.9659, and it was significant at the 5% level.

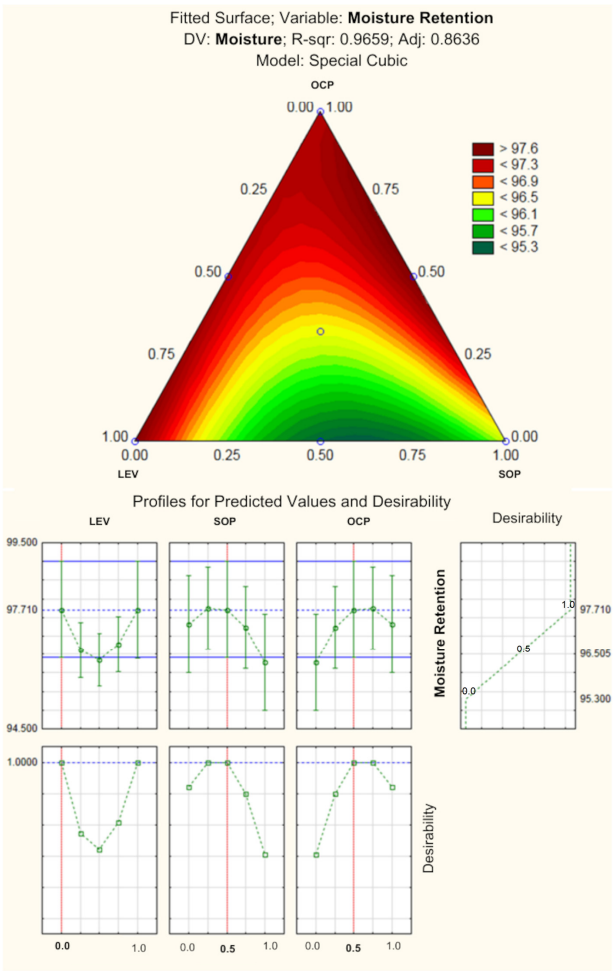

Figure 5. Response surface and profiles for predicted values and desirability for moisture retention of formulations.

Table 9. Model estimates for the moisture retention of the formulations.

Factors	Estimates	Standard Error	T-Value	*p*-Value
Levan (x1)	97.70	0.30	322.81	0.000010 *
Sophorolipid (x2)	96.29	0.30	318.15	0.000010 *
Citrus paradisi essential oil (x3)	97.32	0.30	321.55	0.000010 *
x1x2	−6.78	1.48	−4.57	0.044646 *
x1x3	−0.36	1.48	−0.24	0.830790
x2x3	3.62	1.48	2.44	0.134684
x1x2x3	−7.35	8.02	−0.92	0.456121

R^2: 0.9659, R^2ajusted: 0.8636; Lack-of-fit: *p*: 0.830790; * $p < 0.05$, the factor was significant for moisture retention response.

3.8.4. Formulation Selection Based on Response Surface Analysis

The response surface analysis allowed prediction of the optimized formulation (Figure 6), which is composed of 0.2 of LEV (0.4%) plus 0.8 of SOP (0.8%), in order to obtain the best responses for spreadability (1135.6 mm^2), antioxidant activity (53.41%) and moisture retention (95.49%). The concentration of active ingredients in the optimized formulation was based on the initial concentration used for each one (2% LEV, 1% SOP and 0.3% OCP), with a proportionality calculation being carried out for values 0.2 of LEV and 0.8 of SOP, which sum corresponds to 1.0 (or 100%) in the statistics.

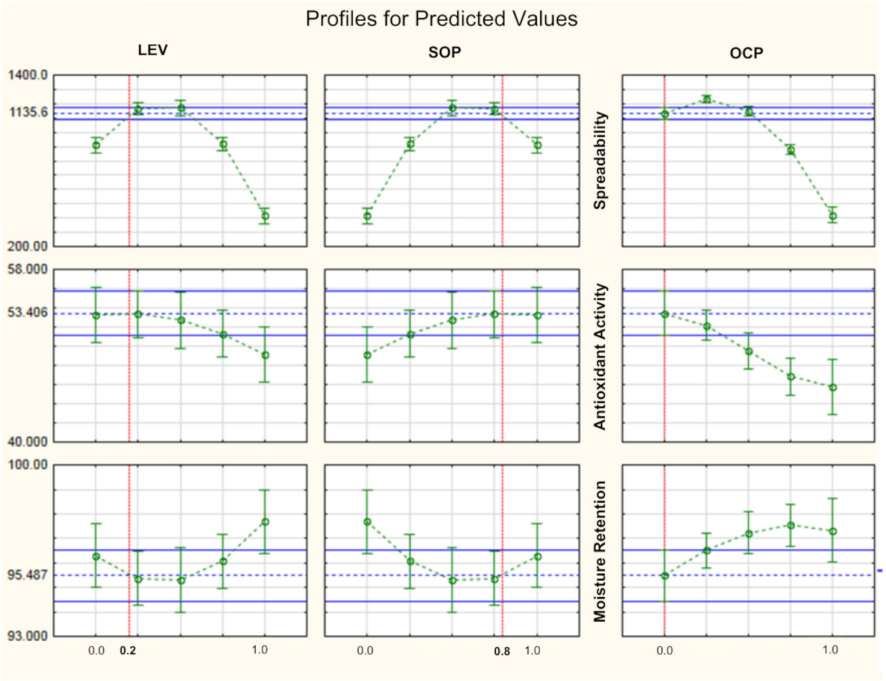

Figure 6. Joint optimization of actives to select the optimal formulation of the study, which was: 0.4% of LEV and 0.8% of SOP.

3.9. In Vivo Efficacy and Sensory Analysis of Formulations

This study was carried out from April to May 2023 at the Sensory Analysis Laboratory, in the Department of Food Science and Technology—DCTA from the State University of Londrina, Paraná, Brazil. In Figure 7, it is possible to observe the diagram of the study that was developed. At the beginning of the research, 61 participants expressed interest in participating in the study. After applying a checklist on the inclusion and exclusion criteria, 5 participants were excluded: 1 participant was over 40 years old, and 4 participants were taking thyroid medication. Therefore, only 56 participants were able to participate. These were randomized into two groups; G1 consisting of 26 participants, who performed the FT application, and G2 consisting of 30 participants, who performed the FB application.

After 7 days of daily application of lip balms, 5 participants from G1 and 5 participants from G2 did not return for the final hydration assessment. Furthermore, 5 participants reported not having applied the products correctly (G1: n = 3; G2: n = 2), thus totaling 18 able participants in G1 and 23 fit participants in G2. The sensory and effect analysis of the formulation was carried out with the same study participants. Of the 56 participants initially eligible, only 46 returned to carry out the sensory tests and evaluation of the effect of FT; in addition, 4 participants withdrew due to the fact that the test lasted another 7 days, and 2 did not perform correct application of the product during this period, resulting in 40 eligible participants.

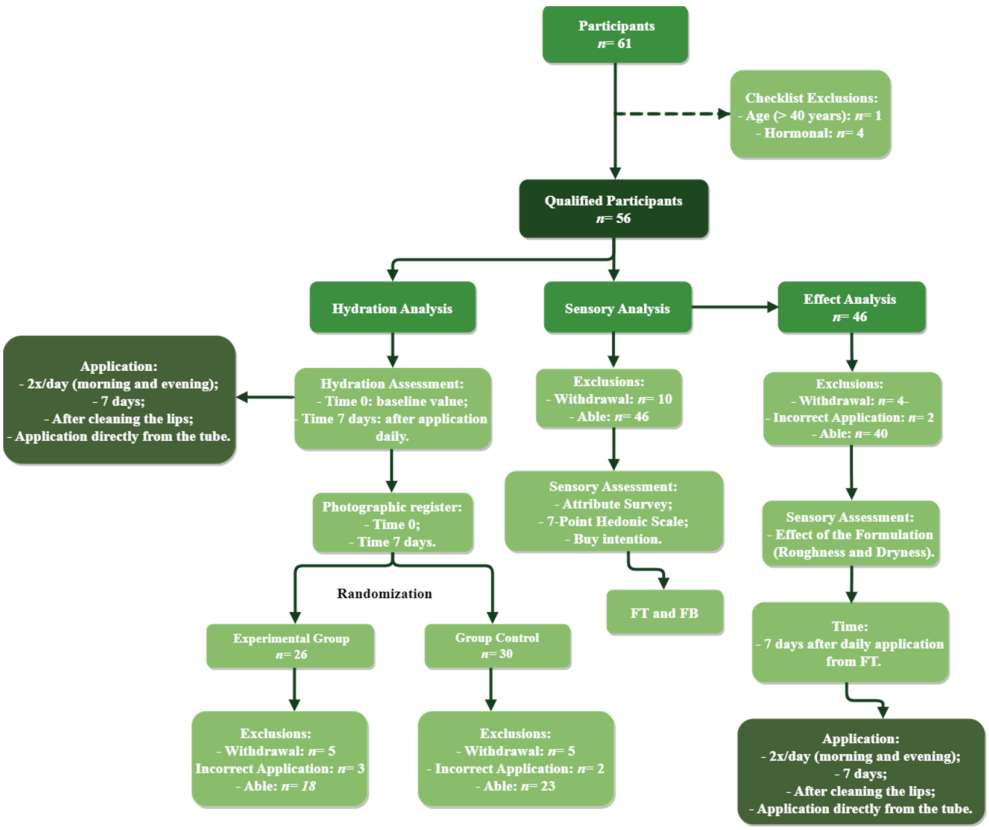

Figure 7. Study diagram covering the exclusions and inclusions of participants obtained throughout the in vivo study.

The results for hydration and oiliness of the lip region of the study participants are described in Table 10. At time zero, there was no statistically significant difference between hydration (p = 0.9089) and oiliness (p = 0.8082) of G1 participants in comparison to G2 participants. G1 showed hydration and oiliness of 41.93 ± 11.4% and 28.61 ± 4.40%, respectively; they were 1.53 ± 10.6% and 28.95 ± 4.64% for G2, respectively. After applying the lip balm for 7 days, there was no statistically significant difference for hydration (p = 0.8520) and oiliness (p = 0.2552) between G1 and G2; G1 showed hydration and oiliness of 45.15 ± 9.8% and 25.95 ± 4.04%, respectively, and they were 44.55 ± 10.3% and 27.87 ± 6.03% for G2, respectively. There was no statistically significant difference ($p > 0.05$) also between times 0 and 7, for hydration and oiliness, both in G1 and G2. According to the Heinrich score [40], which classifies the skin according to hydration, based on the hydration averages obtained for G1 and G2 at time 0 and time 7, it was verified that the participants had skin normal (hydration > 40) in the lip region.

Table 10. Hydration and oiliness of the lip region of participants at times 0 and 7 days after application of formulation.

	G1		G2	
	Moisture (%)	**Oiliness (%)**	**Moisture (%)**	**Oiliness (%)**
Time 0	41.93 ± 11.4	28.61 ± 4.40	41.53 ± 10.6	28.95 ± 4.64
Time 7	45.15 ± 9.80	25.95 ± 4.04	44.55 ± 10.3	27.87 ± 6.03

Figure 8 shows photographic records of the lip region of participants from G1 and G2 taken at times 0 and 7 days after formulation application. The appearance of dryness improved visually, with a reduction in cracks and wounds.

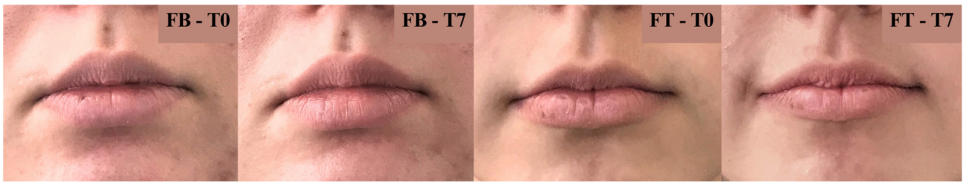

Figure 8. Photo documentation of the labial region during the study development period. FB refers to participants who applied the control formulation and FT to those who applied the test formulation of the study. T0 corresponds to the period prior to application of lip balms and T7 after their daily use.

The frequency of using the lip balms by study participants is shown in Figure 9. Of the 46 participants who carried out sensory analyses, 23 (50%) used lip balms more than once a day, 8 (17%) used it daily, 5 (11%) used it several times during the week, 3 (7%) used it weekly, 5 (11%) used it less than once a month and 2 (4%) participants never used the lip balm.

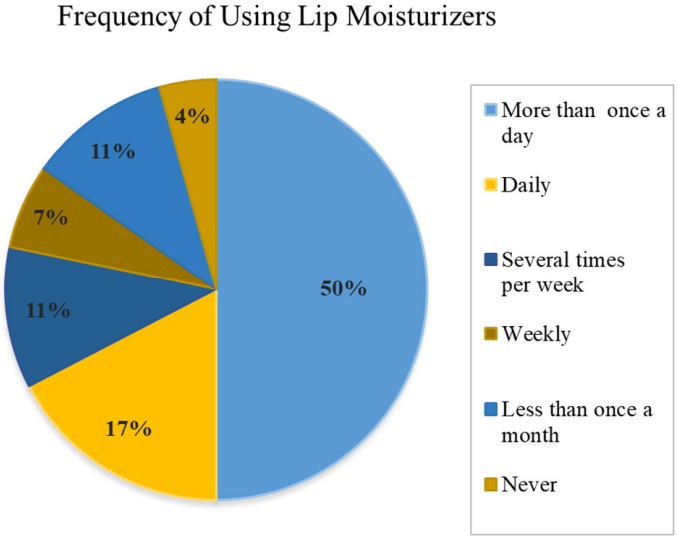

Figure 9. Frequency of using lip balms by study participants.

The attributes evaluated in the sensory analysis were as follows: ease of spreading, absorption, hydration, freshness, formation of a velvety film and fragrance. There was no verified significant statistical difference between FT and FB for all attributes evaluated, as shown in Figure 10, with the p values being as follows: ease of spreading, 0.9234; absorption, 0.7626; hydration, 0.8752; freshness, 0.2957; velvety film formation, 0.2047; and fragrance, 0.9840.

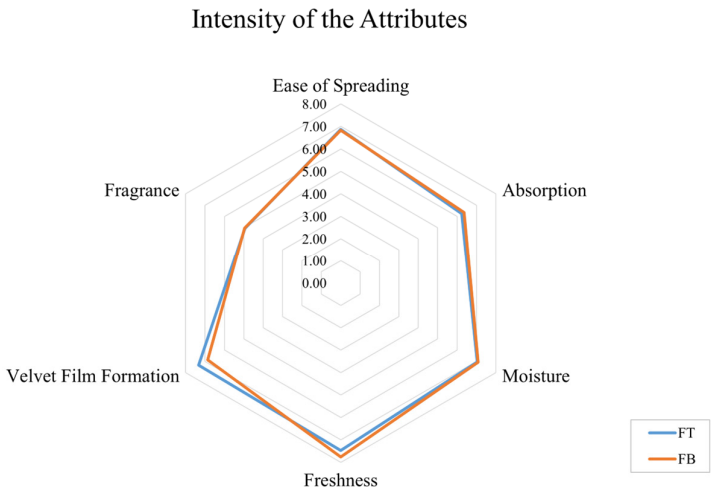

Figure 10. Radar graph to compare the intensity verified for each attribute by the study participants. Orange line: FB; blue line: FT.

According to the 7-point hedonic scale that was applied to verify acceptance of the FT and FB formulations by evaluator, for FT the score was 5.935 ± 0.83, which represents an acceptance rate of 84.71%; for FB, it was 5.522 ± 1.00, which represents an acceptance rate of 78.86%. A statistically significant difference was found between FT and FB (p = 0.0341), showing that there was greater acceptance of FT by the evaluators.

For purchase intention of the formulations, the score was 4.087 ± 0.78 for FT, which represents a purchase rate of 81.8%; the score was 3.848 ± 0.87 for FB, which represents a purchase rate of 77.0%. There was no statistically significant difference (p = 0.1691) between FT and FB regarding this parameter.

The effect of the FT formulation in helping with dryness and roughness of the lips was verified through sensory analysis, and the results are shown in Figure 11. Of the 40 participants able to carry out this test, 34 (85%) realized that the FT helped to hydrate the lips, while 6 (15%) described that they did not observe a moisturizing effect or help with dry lips. For the latter, the limited number of applications (twice a day) hindered the verification of the moisturizing properties of the lip balm, as, throughout the day, the lips ended up drying out due to ingestion of food and drinks. Another determining factor was the change in climate, with abrupt drops in temperature, which helped with dry lips.

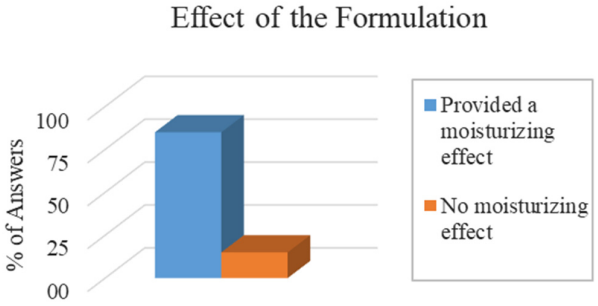

Figure 11. Evaluation of the moisturizing effect of the test formulation (FT) by study participants after 7 days of daily application.

4. Discussion

The search and development of natural, sustainable and biodegradable products have emerged in the cosmetic and pharmaceutical markets as an alternative to synthetic compounds. Molecules obtained by biotechnology, such as LEV from *B. subtilis natto* and SOP from *S. bombicola*, which were obtained through fermentative processes, are examples of active ingredients that meet this trend [12]. For the first time, a lip moisturizer containing LEV and SOF in combination was developed, resulting in an innovative product.

In this study, the production of LEV was 42.93 $g \cdot L^{-1}$, which was higher than that reported by other studies. Pei et al. [17] described a production of 4.82 $g \cdot L^{-1}$ of LEV from *B. megaterium*. Bouallegue et al. [19] reported a production of 2.85 $g \cdot L^{-1}$ of LEV from *B. subtilis* A17. Gojgic-Cvijovic et al. [52] reported a production of LEV by *B. licheniformis* NS032 between 6.53 and 52.85 $g \cdot L^{-1}$, varying according to the fermentative parameters. All these studies produced LEV by the microorganism and not by the enzyme. LEV production in the present study was high due to previous studies carried out by our research group related to fermentative parameters [53,54] and the use of the enzyme levansucrase for its production. Other studies that used levansucrase for the production of LEV presented higher production than those that used only the microorganism, such as reported by Ko et al. [55], with a production of 76 $g \cdot L^{-1}$, and Wang et al. [56], with a production of 30.6 $g \cdot L^{-1}$. As seen, enzymes derived from microorganisms are important tools in the biotechnological process, being more useful than naturally occurring enzymes in microorganisms [57], probably because in microbial cells there are other enzymatic routes that interfere in the best yield of the levansucrase route.

SOP production was 87.10 $g \cdot L^{-1}$, which was superior to that described by other studies. Silveira et al. [37] reported a production of 69.83 $g \cdot L^{-1}$ of SOP from *S. bombicola*; Hipólito et al. [22] showed a production of 67.0 $g \cdot L^{-1}$, which was lower than that described by Caretta et al. [58] who reported SOP production of 111.25 $g \cdot L^{-1}$. All these studies used glucose and oleic acid as substrates, and it was shown in previous studies that the use of them allows the production optimization of this biosurfactant [22,26,37,58]. The production of SOP by Intasit and Soontorngun [59] using as co-subtracts glucose and palm oil was 27.87–30.78 $g \cdot L^{-1}$, while the production of SOP by Kim et al. [60] was 24.1 $g \cdot L^{-1}$ using as substrates glucose, rapeseed oil, ammonium nitrate and yeast extract. Both studies presented a lower production of SOP compared to those that used glucose and oleic acid as substrates. Furthermore, glucose and oleic acid favored the production of lactonic SOP, which has been recognized in the literature as a potent antimicrobial agent [61].

The antimicrobial tests were carried out with the active ingredients SOP and OCP, which have reports in the literature of their antimicrobial properties. The microorganisms used to carry out the analyzes are related to infections caused in the lip region [62–65], in addition to being part of the microbiota of this region and the oral cavity. Regarding antimicrobial activity (Table 1), SOP showed an MIC range of 0.012–0.048 $mg \cdot mL^{-1}$ for *S. aureus*, *S. epidermidis* and *S. mutans*, while OCP presented an MIC range of 10.44–41.75 $mg \cdot mL^{-1}$ for the same microorganisms. Da Fontoura et al. [66] reported that SOPs from *S. bombicola* presented an MIC value of 500 $\mu g \cdot mL^{-1}$ for *S. aureus* ATCC 6336 and *S. mutans* ATCC 25175, while Filipe et al. [26] found that SOPs from *S. bombicola* presented an MIC of 31.25 $\mu g \cdot mL^{-1}$ for *S. aureus* and 125 $\mu g \cdot mL^{-1}$ for *S. epidermidis*. The action of SOP has been reported in other studies [37,58,67,68]; its antimicrobial activity occurs mainly due to destabilization or alteration of the cell membrane of pathogens, which leads to changes in its permeability, inducing loss of cytoplasmic content and, consequently, death. This antimicrobial mechanism of SOPs is related to their surfactant effect caused by the amphiphilic nature of their molecule, which allows interactions to occur between the sugar (sophorose) and the lipid portion, resulting in damage to the bacterial envelope. SOPs have action against Gram-negative and Gram-positive, but their effect is more noticeable against this last bacterial group, which indicates that SOP antimicrobial action is influenced by the composition of the bacterial cell wall [26,37,58,66].

In this study, OCP showed lower antimicrobial activity than SOP against *S. aureus*, *S. epidermidis* and *S. mutans* once OCP MIC values were higher than SOP MIC values. Denkova-Kostova et al. [69] reported an OCP MIC of 6 ppm (0.006 $mg \cdot mL^{-1}$) against *S. aureus*, while Deng et al. [70] showed a value of 6.25 $\mu L \cdot mL^{-1}$ (5.21 $mg \cdot mL^{-1}$). Filoche, Soma and Sissons [71] reported that OCP MIC was greater than 10 $mg \cdot mL^{-1}$ against *S. mutans*. The effect of OCP against various microorganisms has already been proven by several studies [61,72–74]. The antimicrobial property is related to the chemical composition (secondary metabolites) and hydrophobicity of the essential oil. Limonene is the most

abundant component in OCP, as well as in most essential oils from *Citrus* species, which is related to its antibacterial and antifungal activities. Flavonoids and phenolic compounds may help the antimicrobial effect of limonene. The hydrophobicity allows the essential oil to interact with the bacterial cell membrane, causing changes in this structure that make it more permeable, leading to loss of cytoplasmic material and cell death [61,69,72,73].

In addition to the antimicrobial effect, OCP is described in the literature for its antioxidant activity, which varies according to the extraction method used to obtain the oil. Denkova-Kostova et al. [69] reported that the OCP obtained by distillation had an antioxidant potential of 87.5% at a concentration of 1.0 mg·mL^{-1}, according to DPPH assay. Ou et al. [75] reported that OCP obtained by distillation had better antioxidant potential (51.24%, at a concentration of 40 mg·mL^{-1}) than OCP oil obtained by cold pressing (7.75%, at the same concentration). Based on a DPPH test, Lin et al. [76] reported that OCP obtained by cold compression presented antioxidant activity of 6.3% at a concentration of 5.0 mg·mL^{-1}. Yang et al. [77] reported in their study that the OCP showed low antioxidant potential (18.3%, DPPH assay) at a concentration of 5.0 mg·mL^{-1}. Essential oils rich in monoterpenes (limonene and α-pinene), such as OCP, have significant antioxidant activity due to the fact that these secondary metabolites are oxygenated monoterpenes, which have strongly active methylene groups in their molecule [54,75]. In our study, OCP did not show significant antioxidant activity, which was concentration-dependent. The low antimicrobial activity and the lack of antioxidant activity observed in our study may have occurred due to factors that influenced the OCP's composition, which is fundamental for those activities, like the extraction method, which was cold pressing, the part of the plant used, the vegetative age and the origin of the plant [61,73].

The SOP obtained in our study presented a medium antioxidant potential, which was concentration-dependent. The antioxidant activity of SOPs is poorly described in the literature. Filipe et al. [26] reported that SOP obtained from *S. bombicola* presented low antioxidant potential (28.31%) at a concentration range of 2.0–6.0 mg·mL^{-1}. Kumari et al. [78] demonstrated in their study the antioxidant activity of SOPs (at 10 mg·mL^{-1}) from *Metschnikowia churdharensis*, which was 62.98%. Costa et al. [25] showed that SOP (at 10 mg·mL^{-1}) obtained from *Starmerella bombicola* had an antioxidant capacity of 59.40%, based on DPPH assay. Antioxidant activity of SOPs is due to their ability to donate hydrogens and stabilize free radicals such as DPPH [78]. This antioxidant action may help delay skin aging, which is strongly related to the cumulative effect of oxidative damage [26].

Several authors have already described in the literature the antioxidant activity of levan based on DPPH assay. Pei et al. [17] reported in their study that LEV from *Bacillus megaterium* PFY-147 presented antioxidant activity of 35.34% and 94.78% at 0.5 mg·mL^{-1} and 5.0 mg·mL^{-1}, respectively; Srikanth et al. [40] showed that LEV from *Acetobacter xylinum* NCIM2526 presented antioxidant activity of 81.26% at 1.0 mg·mL^{-1}. Domżał-Kędzia et al. [18] reported that LEV from *B. subtilis natto* KB1 presented antioxidant activity of 31.70% at 0.1 mg·mL^{-1}. The antioxidant property presented by exopolysaccharides depends on their structural factors, such as their molecular weight, monosaccharide content and the configuration of glycosidic bonds [19]. The antioxidant activity of LEV may be related to the presence of many hydroxyl groups in its structure, which can react with free radicals and generate chain reactions [79,80]. In our study, the antioxidant activity presented by LEV was medium and concentration-dependent.

The choice of concentration of active ingredients (variables) used in experimental planning was based on data of antioxidant and antimicrobial analysis and information from the literature. OCP did not present relevant antioxidant activity at the tested concentrations. The OCP MIC values were very high (above 10.44 mg·mL^{-1} or above 5.22 mg·mL^{-1} when alone or combined withSOP, respectively). However, as the oil would be applied to a lip product, OCP at 0.3% (2.5 mg·mL^{-1}) was chosen to avoid undesirable taste and odor. To reduce the risk of allergic reactions, the highest concentration of essential oil used in cosmetics is usually about 2% [81]. SOP demonstrated excellent antimicrobial activity against the tested microorganisms, with the highest MIC value of 0.048 mg·mL^{-1} (0.0048%).

In general, the active ingredient is incorporated into cosmetic products at a concentration ten times greater than the minimum concentration of its activity, generally to guarantee its effectiveness in the formulation (i.e., 0.048%). However, as SOP showed no toxicity at concentrations up to 25 $mg \cdot mL^{-1}$ (2.5%) and its antioxidant activity was close to 40% at 10 $mg \cdot mL^{-1}$ (1.0%), the concentration of SOP chosen to be used in the lip balm was 1%. In addition, our research group previously developed another product containing SOP at 1% [25]. LEV showed medium antioxidant activity; it did not show any difference in terms of DPPH radical scavenging at 1.0% and 2.0% (33.63 and 34.37%, respectively). LEV also has excellent moisturizing activity [15], which is similar to the hyaluronic acid effect. As LEV is not cytotoxic [18] and there are no contraindications of concentrations for its use, it was decided to use 2% of it in the formulations.

The eight formulations developed based on simplex-centroid experimental design were subjected to pharmacotechnical characterizations and remained stable in relation to the analyzed parameters, as shown in Table 3. The incorporation of SOP in formulations F2, F4, F5 and F7 statistically improved ($p < 0.05$) their spreadability in comparison to the base. The formulations F1, F3 and F6 did not show statistically significant differences in terms of spreadability compared to the base. SOPs are formed by a hydrophilic portion (sophorose) and a hydrophobic tail, so they are biomolecules capable of modifying the physicochemical characteristics of formulations, such as spreadability, by reducing the surface and interfacial tension of the system, increasing dissolution of hydrocarbons and facilitating the solubilization and absorption of compounds [68]. For moisture retention, F4 was the only formulation to present a significant statistical difference ($p < 0.05$) compared to FB. All formulations showed excellent moisture retention capacity, which can help in maintaining moisture and hydration levels of the labial SC. After carrying out the pharmacotechnical characterizations, we subjected all formulations to preliminary stability testing over a period of 15 days; they remained stable after being subjected to stress conditions.

The study formulations were also subjected to the antioxidant test by scavenging the DPPH radical. BHT, which is an excellent antioxidant, was not incorporated into the formulations subjected to the DPPH test. As can be seen, even FB showed good antioxidant capacity, which is quite unusual (Table 4); they contained emollients of natural origin, such as shea butter and castor oil, which have antioxidant properties due to the presence of tocopherols, carotenoids and phenolic compounds in their composition [82,83]. The use of the active ingredients SOP, LEV and OCP as antioxidant agents did not change the antioxidant capacity of the formulations compared to FB.

This study optimized the formulation employing response surface methodology (RSM) (Table 6). Most of the studies available in the literature about the development of lip cosmetic products do not employ statistical tools to assist in optimizing the formulation [84–87], as demonstrated in this manuscript. Some exceptions are the study developed by Kamairudin et al. [88], in which the authors optimized the production of a lipstick based on Pitaya seed oil using D-optimal mixture design, and the study conducted by Poomanee et al. [89], in which they optimized the formulation of colored lipstick using factorial experimental design. The RSM corresponds to mathematical and statistical techniques that are used in the development of relationships between a response of interest and the variables studied, which may help in the optimize the process by reducing the number of test formulations developed, reducing the number of experiments carried out to test the effectiveness of the product under development, reducing the cost of raw materials and making product development faster, among others [90,91].

The spreading capacity of a product is related to the area that it covers when spread during its application on the skin [92]. According to Table 6 (item 3.8), tests 1, 3 and 6 (containing LEV alone, OCP alone or a combination of both, respectively) showed low spreading capacity compared to the other tests containing SOP, which showed excellent response for this parameter. Assay 5 (containing both OCP and SOP) showed the highest spreadability value. The incorporation of SOP in formulations helped to improve the spreading capacity of the formulations, as this biosurfactant has the ability to reduce the

surface and interfacial tension of the system, modifying its physicochemical characteristics [68]. The response surface and the profile of prediction value and desirability are presented in Figure 3. According to statistical analyses, the formulation composed of 0.5 of SOP (0.5%) and 0.5 of OCP (0.15%), without LEV, would be ideal to obtain the lip balm showing the best spreadability. In fact, the incorporation of SOP and OCP could improve this parameter, as they are substances composed of hydrophobic portions or in their entirety, as is the case of OCP, which would act by reducing the interfacial and surface tension of the system, in addition to being active emollients, facilitating the spreading of the formulations. In general, emollients have a great impact on the physical-chemical characteristics of products, such as spreadability; they reduce the formulation's coefficient of friction, modifying its performance during spreading, in addition to influencing its final consistency [93].

According to Table 6, all formulations showed good antioxidant capacity; trial 2 (containing only SOP) showed the best response (53.28%) for this parameter. In this study, SOPs presented low antioxidant activity compared to the literature; however, our research group has already carried out studies showing 59.40% inhibition of the DPPH radical by these biomolecules at 10 mg·mL^{-1} [25]. This property can be attributed to the fact that SOPs donate hydrogens to reactive species, stabilizing them [78]. The response surface and the profile of prediction value and desirability are presented in Figure 4. According to statistical analyses, the formulation composed of 0.25 (0.5%) of LEV and 0.75 of SOP (0.75%), with no OCP, would be ideal to obtain the lip balm showing the best antioxidant activity. In fact, the incorporation of LEV and SOP could increase this response, as their antioxidant properties are already described in the literature [17,18,25,40].

According to data presented in Table 6, all formulations showed good moisture retention (above 95%); tests 1 (containing only LEV) and 5 (containing SOP and OCP) showed the best response (97.70% and 97.71%, respectively) for this parameter. The moisturizing effect presented by LEV has already been studied by some authors; due to the hydrogen bonds present in its molecule, LEV can retain a vast amount of water, presenting moisturizing activity similar to that of hyaluronic acid [15]. SOPs are biosurfactants that also have potential effects on skin, especially in terms of hydration; these biomolecules can maintain skin functions due to their lipid portion, which allows their greater penetration into the skin [94]. The association of SOP with OCP showed good moisture retention. According to statistical analyses, the formulation without levan and composed of 0.5 of sophorolipid (0.5%) and 0.5 OCP (0.15%) would be ideal to obtain the lip balm showing the best moisture retention.

Based on the results obtained for the response surface analysis, it was possible to predict the optimized formulation of the study, which is composed of 0.2 of LEV (0.4%), 0.8 of SOP (0.8%) and without OCP (Figure 6). As reported in this study, OCP did not show good antioxidant and antimicrobial activities, and it would only be used with the intention of providing fragrance for the formulation, unlike SOP and LEV, which presented slight antioxidant activity and excellent antimicrobial effects. Although OCP helped with spreadability and moisture retention responses when in combination with the other active ingredients, its isolated effect was inferior to SOP and LEV for all responses, in addition to having demonstrated a negative effect when combined with SOP and LEV in the antioxidant activity response (lack of antioxidant activity). In addition to these factors, although OCP is described in the literature as GRAS, some review studies reported it as phototoxic, due to the non-volatile compounds present in its composition, even though the risk is considered low [30]. In this way, the optimized formulation developed in the study was composed only of LEV and SOP.

Verifying the effectiveness of cosmetic formulations is extremely important, as it involves confirming the claims being proposed by the product, such as aiding hydration, reducing fine lines and retaining oil, among others. Several techniques can be used with this objective, among them, non-invasive biophysical analysis through instrumental evaluation, which are safe, do not harm the participants' skin and can simulate situations of real use

of the formulation [95], in addition to being an alternative to animal efficacy studies [96]. The present manuscript shows a clinical study in which lip hydration and oiliness were checked, for the first time, using non-invasive, cheap and portable Skin Analyzer Digital equipment (SkinUp® Devices), which is based on the bioimpedance method. It is highly sensitive equipment that shows a good correlation with the Corneometer®, which is widely used for skin hydration analyses, and demonstrates good data reproducibility [32], being efficient for verifications such as those proposed in this study.

According to the results described in Table 2, it is possible to observe that participants in G1 and G2 had good lip hydration before application of lip balm (time 0), showing hydration levels above 41%, which are described by the Heinrich score [97] as normal. The same was true for oiliness, whose levels were above 28%. After 7 days of applying the FT and FB formulations, there was a slight increase in lip hydration, whose levels were close to 45%; however, there was no statistical difference compared to time 0. Oiliness was reduced, maintaining its level close to 25%, but no statistical significance was found either in comparison to time 0. When comparing G1 and G2, after applying the lip balms, there was no statistical difference in the hydration and oil content of participants' lips; both measurements were very close. No adverse effects or irritability were described by participants throughout the study.

Some factors influenced negatively on results found in this study, such as the limited number of formulations' daily applications, as described by several participants, which allowed periods of dryness to occur throughout the day, because of the ingestion of food/drinks and the non-reapplication of the product and the temperature changes in the months of April and May (from 30 °C to 15 °C, for example) that favored dry lips, with the appearance of cracks and wounds. To overcome these problems, a greater number of daily applications would be ideal, such as three to four, which would allow the product to form a protective barrier on the lips throughout the day. However, even with these events, it is possible to observe that the use of the formulations developed in this study helped to maintain the lip hydration and oiliness already exhibited by the participants, which is a very promising result.

Studies using the Skin Analyzer Digital device as a technique for evaluating lip hydration and oiliness are not described in the literature, as it is a recent method. In this way, the present study may help in the development of future studies in the field of cosmetology on lip hydration and evaluation of the effectiveness of lip cosmetics using a portable, cheap, sensitive and reproducibility device like the one from SkinUp® Beauty Device. There are reports in the literature involving other instrumental methods in studies of the effectiveness of lip products, such as the Corneometer®, which is a non-portable and more expensive device; Gfeller et al. [1] developed a lip cream containing micro repair technology that improved dryness compared to the untreated group; Bielfeldt et al. [3] developed a lip cosmetic containing natural emollients that improved hydration, as well as reducing transepidermal water loss from the lips. Furthermore, there are no studies in the literature that report the development of lip products containing LEV and SOP in combination, which makes the cosmetics developed in this study innovative.

Sensory analysis is a useful and highly important tool in the cosmetic industry, as it helps in the development of products, ensuring their quality, and in aggregate marketing, in addition to allowing the evaluation of product acceptance among the consumer public [98]. In the present study, untrained evaluators but potential consumers of lip products participated in the development of sensory analyses, which can be verified through Figure 9; 50% of these participants used it daily, several times a day, 17% of participants used lip balms daily, once a day and 7% used lip balms weekly.

According to the intensity of the attributes (Table 3), it is possible to verify that there was no significant statistical difference between FT and FB for all parameters evaluated, with values above 6.2 (tending to "very intense"), with the exception of fragrance, which presented values close to 4 ("neither too intense nor too little intense") due to the non-incorporation of essential oils or aromas, maintaining the characteristic odor of the

formulations. The standard deviations ranged from 1.28 to 2.68, which indicates a great variability among the evaluators' response; it probably occurred due to the difficulty in describing and discriminating aspects of both formulations, like aroma, spreadability and freshness, that are very similar [36]. The non-statistical significance obtained in this analysis only confirms the difficulty of differentiating between FB and FT, which demonstrates that the incorporation of LEV and SOP actives does not modify the evaluators' perception or result in sensory changes in formulation (when comparing FT to the control).

The formulations showed good acceptance rates, which were 84.71% for FT and 78.86% for FB, being qualitatively shown as "I liked" and "I really liked". The results obtained using the hedonic scale showed statistical significance, demonstrating that there was preference for FT when compared to FB, even with the difficulties faced in differentiating the formulations in relation to their attributes. In fact, the hedonic scale allows evaluators to choose the answers that suit their preferences and that reflect their opinion regarding the products tested, without the need to form a trained panelist group, thus helping in the development of several studies in which it is intended to know a preference sample [49]. A lip balm development study conducted by Azmin, Jaine and Nor [85] used a hedonic scale to evaluate the spreadability, color, odor and general acceptance of different samples, comparing the results with previously carried out instrumental analyses. They verified that there was no significant difference between the attributes evaluated, so all the lip balms produced could be commercialized. A study developed by Esposito and Kirilov [99] used a 9-point hedonic scale to evaluate spreadability, hardness, opacity, gloss effect and oiliness of different lipstick samples. They verified, for example, that greasiness and glossiness presented a significant difference among the formulations, because of the composition of lipsticks (concentration of vaseline), while the spreadability was good for all samples, without significant difference.

The acceptance rates were confirmed through purchase intention, which were 4.087 ± 0.78 for FT and 3.848 ± 0.87 for FB, being qualitatively shown as "maybe I would buy, might not buy" for FB and "probably I would buy" for FT. The probability of consumers purchasing a cosmetic product is mainly determined by its sensoriality; regarding lip products, it is also determined by the sensation felt during application [100]. All attributes (ease of spreading, absorption, hydration, freshness, formation of a velvety film and fragrance) evaluated in this study were well accepted by the evaluators, which consequently influenced the positive purchase intention of the present lip balm.

After a period of 7 days of applying the FT lip balm daily, a self-assessment test was submitted to the participants to verify the long-term effectiveness of the product [51]. For improvement in lip dryness and roughness, 85% of participants reported that the formulation helped to hydrate their lips, while 15% did not observe this effect (Figure 11). This is a promising result, as it demonstrates that the hydration attribute may not be perceived immediately after application; however, over the days, it promotes an effect on dryness and roughness of the lips.

As can be seen in this study, sensory analysis is a tool that assists in the development and evaluation of cosmetic products. Several studies on lip products, such as those carried out by Abidh et al. [100], Kasparaviciene et al. [101] and Rafferty et al. [102], demonstrate the importance of this science in determining and considering the properties and attributes of cosmetics intended for the lips.

5. Conclusions

Multifunctional lip balm formulations containing LEV from *Bacillus subtilis natto*, SOP from *Starmerella bombicola* and OCP were developed in this study. Through statistical analysis of the Response Surface, the optimized formulation (FT), composed of 0.4% LEV and 0.8% SOP, was selected based on its spreadability, moisture retention and antioxidant activity. Efficacy evaluation and sensory analysis of FT helped to identify the attributes and acceptance of the product by consumers, in addition to allowing the evaluation of the effect on lip hydration and oiliness before and after applications. The lip balm helped

to maintain the hydration and lip oiliness already presented by the participants; this was corroborated by sensorial analysis carried out over 7 days, which showed 85% positive responses, in addition to showing good acceptance. A high level of purchase intention was also confirmed. In this way, the lip balm developed in this work has market potential and corresponds to an innovative product, with sustainable and natural characteristics, and good acceptance by consumers. Furthermore, this work can assist in the development of future studies on the sensorial aspects of cosmetic products and in the evaluation of their clinical efficacy through the Skin Analyzer Digital device, which is poorly described in the literature but presents high sensitivity and low cost.

6. Patents

Patent deposit was made on 2 May 2023, at INPI (Instituto Nacional da Propriedade Industrial, Brazil), with process number BR 10 2023 008390 0.

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Institutional Review Board Statement: All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of State University of Londrina (CAAE 58720522.9.0000.523, 17 June 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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