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"The development of a new line of cleansing and skincare products based on pro- and prebiotics, which dermo-protective and anti-ageing effects will be enhanced by natural active ingredients derived from fermentation processes"

Summary:

The aim of the study described in the submitted dissertation was to develop a new line of skin cleansing and skin care cosmetics based on pro- pre- and postbiotics, in which the dermo-protective and anti-aging effects would be enhanced by natural active ingredients obtained in fermentation processes. The choice of active substances stemmed from the cosmetic properties attributed to them, which included the effects on microorganisms in the human skin microbiome, as well as anti-aging and skin care properties.

The literature review performed for the purpose of this dissertation indicates a growing interest in the use of fermentation-derived raw materials in skin care preparations. Postbiotics are an innovation that takes advantage of the benefits contained in probiotics. While in the pharmaceutical and food industries, the use of typical probiotics, i.e. live bacterial cultures, is not very difficult, in the cosmetics industry, it is technologically challenging. Postbiotics are an attractive proposition because they eliminate the product safety and shelf life concerns associated with live probiotics and can positively influence the composition of the microbiome. Microbiota skin care is an area that cosmetic manufacturers have only begun to explore. There are many products advertised for their probiotic content. Unfortunately, this is often dishonest advertising. Most cosmetics purporting to have probiotics in their composition actually contain either prebiotics or postbiotics. In companies that distribute cosmetic raw materials, the situation is similar. It is necessary to verify the statements because what a given company calls a probiotic is, for the most part, not. Prebiotics in the form of inulin, glucomannan, and oligosaccharides are found in most cosmetic products. Postbiotics are defined as any substances derived from the metabolic activity of a probiotic microorganism. Bacterial metabolites, cell wall fragments and even dead bacteria induce an immune response in the skin and improve skin barrier function. The use of probiotics in cosmetics is complicated and requires high levels of involvement from manufacturers. Cosmetics contain water, and it is difficult for bacteria to survive in water-based products in the long run. When the packaging begins to lack oxygen, they die. Of great importance is the shelf-life aspect of cosmetics. They have a certain shelf life, reaching up to 30 months. It is impossible to guarantee that any viable bacterial cultures will remain in the cosmetic after such a long time, especially if it is stored at room temperature. This can be compared to probiotics in the food industry. Manufacturers almost always recommend keeping them at low temperatures. In addition, there is the hassle of preservatives, the whole point of which is that they have a bactericidal effect. In addition, how can cosmetics containing live probiotics meet the regulations on the limits of microbiological contamination? The way to get around the above problems is to produce cosmetics completely devoid of water or probiotic capsules. And these two ways are currently being followed by supplement

manufacturers, and in the cosmetics industry. Probiotics are being replaced by postbiotics, whose high efficacy has been demonstrated in our study.. In the presented dissertation also an anti-aging active substance of fermentation-derived γ-aminobutyric acid is characterized.. Having younger skin is a global professional and social status trend, which boosts self-esteem. For this reason, many commercially available products today are advertised to promise improvement of the skin condition.. The cosmetic industry is making discoveries that promise to overcome the merciless effects of time. The effectiveness of aminobutyric acid, which is now positioned as one of the most effective anti-aging ingredients, will be demonstrated by years of use. Aminobutyric acid, more precisely its gamma version, is actually a neurotransmitter that is indirectly responsible for muscle tension, relieves agitation, and has a calming effect. Initially used for people suffering from various diseases, such as premenstrual syndrome, ADHD, and a number of other conditions for which muscle relaxants are needed, the agent is now in high demand in the cosmetic industry. It has been shown to penetrate the skin via ion channels and relax the skin by reversibly deactivating the smallest nerve endings. It also acts as a biological mediator (intermediary), positively influencing cellular metabolism in the epidermis and dermis. It is also active as a growth factor for cells in the dermis and epidermis, that is, it contributes to their rapid renewal. Aminobutyric acid also acts as an enhancer of the skin's immune system and greatly aids skin regeneration after damage.

As part of the experimental part of the submitted dissertation, a series of experimental tasks was were carried out, ultimately allowing achievement of the main objective of this dissertation.

As a result of *in vitro* studies, the types and concentrations of the most-promising raw materials were selected in terms of positive effects on the skin microbiome, and a system that was a combination of two raw materials with positive effects on the skin microbiome was studied. The best promising raw materials, established on the basis of *in vitro* study in terms of positive effects on the skin microbiome and negative effects on the pathogenic microbiome, are Probiotic III at a concentration of 3% and Ecoskin (3%). Based on the collected results, it was shown that, in terms of promoting the growth of the natural skin microbiome, these substances produce a greater increase in the microbiome individually than in a combination. An *in vitro* study of the effect of a combination of the substances: 3% Probiotics III + 3% Lactosporin confirmed that their synergist promoted the skin microbiome development.

The combination of these substances reduced all pathogenic strains as well as the strains of the microbiome tested.

As a result of *in vitro* tests, the types and concentrations of raw materials most promising for the reduction of pathogenic strains of *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* were also selected.

The raw material whose application was found to lead to full reduction (by -100%) of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and partial reduction (by -47%) of *Candida albicans* was Lactosporin in a concentration of 3%.

The raw material at this concentration was the most effective in reduction of pathogenic strains, which is much valuable information when developing a series of, for example, anti-acne products. Based on the results collected, the most promising raw materials in *in vitro* studies in terms of positive effects on the skin microbiome and negative ones on the pathogenic

one, are Probiotic III at a concentration of 3% and Ecoskin (3%). The development of thirteen formulations: in the form of emulsion (E1-E7), gel skin care serum (S1-S4) and a gel facial wash (Ż1-Ż3), in which the active ingredients were the substances most promising for the growth of the natural skin microbiome and non-promoting the growth of pathogenic strains, was the overriding goal of this dissertation. Different types of preservatives were used in the formulations in order to verify the effectiveness of their use and to identify the ones showing the lowest possible interfering effect with the postbiotics added to the formulations on stimulating the growth of mesophilic bacteria of the natural skin microbiome. Preservation of cosmetic products (containing substances that promote the growth of the microbiome) with the following products: Euxyl K 712 (1%), Euxyl PE 9010 (0.7%) and a combination of preservatives with Euxyl K 712 (0.8%) and Euxyl K 903 (0.5%, met the requirements for antimicrobial protection of cosmetic products. The composition of the formulation can have a definite effect on the performance of the active ingredient under study (pro- /anti-microbiome). The preservative whose presence ensured the highest quantitative increase in the natural skin microbiome was Euxyl K 712. It was shown not to reduce the microbiome for up to 72 hours. In view of the above results, the cosmetic formulations with this preservative were selected for *in vivo* studies.

In the next stage, the effects of cosmetic formulations, with the postbiotics selected in earlier stages, on the human skin microbiome were studied. The following samples E1-E7, S1-S3, and Z1-Z3 were tested, and the most promising formulations chosen on the basis of the results of *in vitro* tests as showing positive effects on the microbiome were: E6 and E4. Formulation E6 resulted in an 18% increase in the microbiome compared to that of the base formulation of the test sample of the same mass (i.e., without the addition of microbiome support substances). Formulation E4 resulted in a 17% increase in the microbiome compared to that of the base formulation of the test sample of the same mass (i.e., without the addition of microbiome support substances).

In the course of the study presented in this dissertation, the physicochemical parameters of the formulations submitted for *in vivo* testing were characterized. The obtained pH and stability results indicate that the cosmetic formulations were properly prepared in accordance with technological requirements (preventing contamination of the obtained cosmetic products) and that there were no apparent changes in the stability of the mass of the cosmetic product. Obtaining the viscosity-stable cosmetic formulations during cosmetic storage indicates no tendency to the appearance of changes indicative of instability. The storage period did not affect the obtained viscosity values, regardless of the sample storage temperature.

A key step was to study the effect of postbiotic products on the skin microbiome of volunteers (*in vivo* study). The effects of cosmetic formulations with postbiotics E3, E5, E6, Z1, Z3, S2, S4 and the combination of S1 + S4 products on the human skin microbiome, selected in earlier stages, were evaluated. For all probands, a reduction in microbiome was observed. The two-step treatment with the S1 + S4 products resulted in a reduction in the microbiome, as it negatively affected the physiological natural skin microflora, despite the previously proven pro-microbiome effect of the S3 formulation, as the S1 formulation still shows strong antimicrobial activity 15 minutes after application. In future, the effect of the length of the interval between application of the products on the promicrobiome effect of the second formulation would be important to establish. The formulation (E6) containing the

postbiotic showing the strongest microbiome promoting properties of all the probands, after 14 days of application to the skin, resulted in an increase in the microbiome (T14) relative to baseline (T0) of 59% to 1536%. After 14 days of application, the increases in the microbiome were recorded for all probands between the baseline formulation and the formulation (E6) with the addition of the promicrobiome substance. The increase in microbiome after application of the E6 formulation ranged from 14% to 546%.

The effectiveness of market products with postbiotics on the skin microbiome of volunteers was also investigated (*in vivo* study). The results indicated very high efficacy of both probiotic and postbiotic-based cosmetics.

Oil for redness BAK (K1) with a probiotic after 14 days of application to the skin caused an increase in the microbiome from 356% to 1208%, depending on the proband. After 14 days of application, increase in the microbiome was observed for all probands treated with the products containing a promicrobiome substance relative to the effect of the base formulation. For K1 formulation, the microbiome increase ranged from 10% to 912%, depending on the proband.

An important part of the study presented in the submitted dissertation was determination of the effect of substrate type on the release of γ-aminobutyric acid (GABA). It was found that γ-aminobutyric acid (GABA) at a concentration of 2% was better released from the gel substrate as the cream substrate does not facilitate the diffusion of this substance. Thus, the gel medium provides better bioavailability of this active substance. At a concentration of 10%, γ-aminobutyric acid (GABA) was released at a comparable rates from cream and gel medium. Based on the results obtained, the emulsion containing 10% γ-aminobutyric acid in its composition was selected for further studies.

In the next stages, the effect of the cosmetic formulation with GABA on selected skin parameters was evaluated. The effectiveness of the obtained cosmetic formulations determined using noninvasive testing methods was carried out to verify the beneficial effects of the cosmetic product declared by the producer. It was based on the results of objective measurements of skin parameters. A cream with 10% γ-aminobutyric acid (GABA) was developed as a cosmetic to reduce the appearance of wrinkles associated with the aging process. *In vivo* studies were conducted to evaluate the effectiveness of selected cosmetic formulations also by examining stratum corneum hydration and transepidermal water loss, skin topography, and macrosculpture. The tested product improved the appearance of facial wrinkles and increased hydration. This improvement was associated with objective changes in several skin parameters. Corneometric measurements showed a 14.2% increase in skin hydration after one month of cream application. Further tests showed a 17.3% decrease in TEWL values 10 minutes after the cream application. After one month of the cream application, TEWL decreased by 7.9%. Analysis of the skin surface showed a reduction in the depth of the wrinkles. A time-dependent reduction in the average depth and length of wrinkles was observed. These data indicate a strong effect of the cream with 10% ϒ-aminobutyric acid and postbiotics on wrinkle reduction. After 10 minutes of application, the average wrinkle length was reduced by 7.14%, and after 1 month of application, this reduction was 1.43%. The average depth of the wrinkles 10 minutes after application was reduced by 3.62%, while after one month of application there was a reduction of 0.52%. On the other hand, the maximum depth of the wrinkles decreased by 7.80% in 10 minutes after application, while there was a reduction of 4.26% after one month of

application. The results obtained may indicate that ϒ-aminobutyric acid present in the cream is more effective against wrinkles when it is present on the skin, as a higher percentage of wrinkle reduction was observed in 10 minutes after application of the cream than after a month of using the cosmetic. The final stage of the research conducted was an application-use study. A home survey in a group of volunteers confirmed the results of the apparatus tests. The volunteers evaluated the properties of the cosmetics very well. The results of the study show that daily use (for 28 days) of the product with γ-aminobutyric acid and postbiotics Ecoskin and Probiotics III has clearly improved the condition of the skin and reduced the appearance of wrinkles. The study presented in this dissertation allowed a design and obtaining of cosmetic formulations with postbiotics and -aminobutyric acid, optimized in terms of physicochemical parameters and application parameters, confirmed by apparatus studies. Taking into account the result of *in vivo* studies, the high efficacy of the cosmetics with probiotics and postbiotics on the growth of microorganisms included in the skin microbiome was indicated. The growth of the microbiome varied for different probiotics and the results of the study may have been influenced by age, sex, weight, lifestyle, chronic diseases, dietary habits, stress exposure, work activity, pet ownership, antibiotic therapy, allergies, and personal hygiene products used. Creating a cosmetic formulation with a probiotic that ensures the survival of the microorganisms and meets other performance requirements, including consumer safety, is difficult and challenging, so postbiotics are an attractive alternative because they eliminate the product safety and shelf life concerns associated with live probiotics and can positively influence the composition of the microbiome. Comparison of the efficacy of postbiotics and probiotics is a new and innovative topic that requires further and extensive research. A better understanding of the function and dynamics of the skin microbiome and its important role in skin health represents a major opportunity for cosmetic manufacturers. It is interesting to take a holistic approach to product development and address the skin microbiome. The skin microbiome is an exciting area for the cosmetics industry, leading to a new generation of products and marketing opportunities. Consumer interest in the beauty microbiome has increased after the Covid-19 pandemic and consumer concerns about their skin as a first line of defense by protecting and rebalancing the skin microbiota. Latech plans to continue the research presented in this dissertation in future projects.

Table 1. List of formulations presented in this dissertation.

Formulation label	Formulation Type
E1	Emulsion o/w without postbiotic with preservative Euxyl K 712 (0.8%) and Euxyl K 903 (0.5%)
E2	Emulsion o/w without postbiotic with preservative Euxyl PE 9010 (0.7%)
E3	Emulsion o/w without postbiotic with preservative Euxyl K 712 (1%)
E4	Emulsion o/w with Ecoskin (3%) and Probiotics III (3%) with preservative Euxyl K 712 (1%)

Formulation label	Formulation Type
E5	Emulsion o/w with Probiotics III (3%) and preservative Euxyl K 712 (1%)
E6	Emulsion o/w with Probiotics III (3%) and Ecoskin (3%) and with the preservative Euxyl K 712 (0.8%) and Euxyl K 903 (0.5%)
E7	Emulsion o/w with Probiotics III (3%) and Ecoskin (3%) and with the preservative Euxyl PE 9010 (0.7%)
S1	Gel serum with Lactosporin (3%) and with the preservative Euxyl K 712 (1%)
S2	Gel serum without postbiotics and with the preservative Euxyl K 712 (1%)
S3	Gel serum with Lactosporin (3%) and Probiotics III (3%) and with the preservative Euxyl K 712 (1%)
S4	Gel serum with Probiotics III (3%) and with preservative Euxyl K 712 (1%)
Ż1	Facial cleansing gel without postbiotics, with preservative Euxyl K 712 (1%)
Ż2	Facial cleansing gel with Probiotics III and Ecoskin and with the preservative Euxyl K 712 (1%)
Ż3	Facial cleansing gel with Probiotics III and with the preservative Euxyl K 712 (1%)
K1	Market cosmetic with probiotic "Oil for Redness" by BAK
K2	Market cosmetic in biphasic form with probiotic