

Skin Beautification with an ImmunatuRNA® Complex: A Natural Complex Based on Yeast RNA and Marine Derivatives on the Human Skin Immunity and Microbiome Composition

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Abstract

The skin is the largest organ of human body implicated in vital physiological and immunological processes, including protection against injury, infections, and environmental exposures. Nevertheless, a number of negative external and internal factors, including increased pollution along with modern lifestyle can significantly influence on the skin immunity, promote aging and reduce regenerative capabilities. Cosmetic formulations based on natural ingredients, including plant and marine/algae extracts, and microbial-derived bio-actives may help to maintain healthy skin condition and protect its beautiful appearance.

The ImmunatuRNA® (*The trademark ImmunatuRNA® is a registered trademark in the European Union EUTM 018365297 and Great Britain UK00003630172. Any reference to the registration of the ImmunatuRNA® trademark applies to the designated territories), a signature complex (VRFD SA/VERDILAB Switzerland) based on the Natural RNA molecules obtained from yeasts, with added marine exopolysaccharides and natural hyaluronic acid with potential benefits for antibacterial and antiradical defense and maintaining skin detox homeostasis. Therefore, the main aim of this study is to experimentally assess the ImmunatuRNA® complex capabilities to enhance natural skin antioxidant defenses and protection against oxidative stress, by using human fibroblast cell culture. Additionally, this study will investigate the effects of the skincare products with added ImmunatuRNA® complex on the skin microbiome composition in the group of healthy adults with various skin types. The results obtained from the in vitro studies consistently demonstrated that treatment with an ImmunatuRNA® complex protects human fibroblasts against exposure to pro-oxidants, while at the same time enhances anti-oxidative defenses of these cells, demonstrated as reduced formation

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of free radicals and upregulation of SIRT-1 protein. Furthermore, the cosmetic intervention carried on the group of 10 healthy human volunteers with different skin types (oily, dry, combined, normal) aged between 23 and 65 years old demonstrated that using interchangeably of two cosmetic products-a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask containing the ImmunatuRNA® complex, for 4-weeks led to favorable changes in the composition of skin microbiome observed as a reduction of relative abundance of adverse microorganisms, previously linked to the skin inflammation and infections, such as *Citrobacter koseri*, *Bacillus spp.*, *Enterococcus spp.*, *Alcaligenes spp.*, *Pantoea spp.* and *Staphylococcus aureus*. Based on following results, the use of skincare products containing the ImmunatuRNA® complex can help maintain a healthy skin microbiome while possibly preventing against infections and negative environmental exposures, in particularly against oxidative stress in healthy adults with all types of skin with age range 23-65 years old.

Keywords: Skin care; Skin immunity; Skin microbiome; Skin regeneration; RNA; Marine derivatives; Cosmetic formulation; Oxidative damage; Wound healing; Proliferative activity; Restoration of skin damage.

Abbreviations: CFU:Colony-Forming Unit; DPPH:1,1-diphenyl-2-picrylhydrazyl; DMEM:Dulbecco's Modified Eagle Medium; H₂O₂:Hydrogen Dioxide; HA:Hyaluronic acid; GAGs:Glycosaminoglycans; GLP:Good Laboratory Practice; INCI:International Nomenclature of Cosmetic Ingredients; IFN-γ:interferon gamma; IL:Interleukin; MTT:3,(4,5-dimethylthiazol-2)2,5 difeniltetrazolium bromide; PBS:Phosphate-Buffered Saline solution; RNA:Ribonucleic Acid; ROS:Reactive Oxygen Species; RNS:Reactive Nitrogen Species; SIRT-1:Silent information regulator 1; SD:Standard Deviation; UV:Ultraviolet Radiation.

Introduction

The skin is a complex and active barrier made by interactions between microbial communities, namely skin microbiota, and host tissue associated with immune systems. Skin, being the most outer surface of the human body, is often exposed to various endogenous and exogenous factors, which through influencing on its homeostasis, may potentially cause adverse reactions, and increase the risk of inflammatory skin conditions, allergies or autoimmune diseases [1].

To date, scientific evidence has shown that many environmental factors, such as irritation, chemicals, infection, UV-radiation, temperature as well as exposure to pollutants can have an impact on skin condition and progressively impair its regulatory and repair mechanisms [2].

One of particular factors is oxidative damage, often resultant from increased amount of various pro-oxidants, including reactive oxygen (ROS) and nitrogen species (RNS) in skin cells that, by exceeding capacity of the antioxidant defense systems, results in oxidative stress and chronic inflammation [3].

An increased oxidative stress significantly impairs skin structure, as the consequence of the collagen fibers fragmentation and disorganization [3], also implicated in the skin aging [4] and carcinogenesis [3].

The presence of high level of oxidative stress in the body may exacerbate damage to epithelial barriers, leading to increased levels of inflammation, which serving as an additional source of ROS/RNS exceeding cellular antioxidant capacity, may lead to cell dysfunction, malignant transformation and finally cell death [3].

A persistent oxidative stress resulting in oxidative damage of epithelial skin surfaces significantly affects skin physiological processes observed by changes in its complexion homogeneity, as well as exacerbates aging and melanin production [5] subsequently leading to inflammatory hyperpigmentation and actinic lentigo [6].

Furthermore, there is an accumulating evidence to indicate that environmental factors, such as exposure to pollutants, humidity, temperature, sun radiation [7] and use of cosmetic products [8] may additionally influence skin condition via perturbation of skin microbiome composition [5].

Although the relationship between the skin microbiome, oxidative stress and skin inflammation still needs to be further explored, observations conducted in patients suffering from chronic inflammatory skin conditions, such as psoriasis [9], atopic dermatitis [10] and acne vulgaris [11] demonstrated a connection between microbiota dysbiosis [12] and increased condition severity, which were correlated with increased levels of oxidative stress in the skin of these individuals.

Taken together, these results provide some indications for a close interaction between oxidative status and skin microbiome composition [13].

Due to the important role, that a balanced skin microbiome may play in maintaining optimal skin health, appropriate skin care that limits the overgrowth of pathogenic microbial species while reducing oxidative stress and restoring skin homeostasis appears to be crucial in the prevention and therapy of

dermatologic conditions [3,14]. Not surprisingly, there is an increasing research interest into natural sources of bio-actives with implicated anti-oxidative and protective properties that act synergistically with other active ingredients of the cosmetic formulation and can strengthen skin barrier integrity and its immune defense system.

ImmunatuRNA® is a newly designed, biologically active complex (PCT/IB2022/000231 (in pending), VRFD SA/VERDILAB Switzerland) based on the naturally obtained ingredients including yeast RNA, marine-derived exopolysaccharides and hyaluronic acid (HA) with implicated roles in antibacterial defense and skin detox homeostasis.

Therefore, to confirm the significance of a given complex in improving skin immunity, the main goal of this recent study is the experimental evaluation of the ability of ImmunatuRNA® complex to enhance the natural antioxidant defense of the skin, and to strengthen its barrier integrity *in vitro*. Furthermore, these investigations will allow assessing the impact of skincare products (face cream and face mask) containing ImmunatuRNA® complex on the skin's microbiome composition in the group of healthy adults with various skin types.

Materials and method

Cell culture studies

ImmunatuRNA® complex preparation

ImmunatuRNA® complex was prepared as described in the patent application (PCT/IB2022/000231 (in pending), VRFD SA/VERDILAB Switzerland). The resulting

ImmunatuRNA® complex material was a fluid opaque gel with neutral odour, water-soluble and pH ranging between 4 and 7.

For the series of *in vitro* studies, the samples at various concentrations of ImmunatuRNA® complex were prepared. Prior to the testing, the INCI formulation of ImmunatuRNA® was determined. The concentrate of ImmunatuRNA® complex was made according to the preparation method detailed in PCT/IB2022/000231 (in pending).

Testing solutions, being a diluted version of ImmunatuRNA® complex concentrate, for the purpose of all experiments using cell cultures were prepared by diluting the stock solution (concentrate) of ImmunatuRNA® complex in the deionized purified water to final testing ImmunatuRNA® concentrations, being as follows 8.55%, 5.13% and 0.855%.

These concentrations were selected for the testing owing to its suitability for the cosmetic formulation and applicability in the production of skincare products.

Anti-oxidant properties of ImmunatuRNA® complex

The antioxidant properties of ImmunatuRNA® complex determined by using antiradical scavenging assay. Briefly, the ability of ImmunatuRNA® complex to neutralize the synthetically generated radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined according to the method [15,16]. A 3mL of prepared in methanol solution of DPPH with concentration of 6×10^{-5} M was mixed with 100 µL of testing solutions of ImmunatuRNA® complex being 8.55, 5.13, 0.855%, respectively.

The samples were incubated for 30min at 37 °C in a water bath, and then absorbance variation was measured at 515nm as mean percentage of reduced DPPH. A blank sample containing 100 µL of methanol in the DPPH solution was prepared daily, and its absorbance was measured. The experiment was carried out in triplicates. The results were presented as percentage of reduced DPPH.

Cell viability-MTT assay

To evaluate cell viability the MTT assay was performed. Prior to the assay, the human fibroblast cell culture was maintained in the 25cm² flask with prepared cell medium including DMEM medium supplemented with Fetal Bovine Serum 10%. Cells were incubated at 37 °C and 5% CO₂ until full confluence. Then, cells were plated into 96 well plate and incubated at 37 and 5% CO₂ for 24h. Cells in Go phase were exposed to three concentrations of ImmunatuRNA® complex (8.55, 5.13, and 0.855%) for 24,48 and 72h. The testing MTT solution was prepared by dissolving 15mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent in 30ml of cell medium to achieve a final concentration of 1mg/ml of MTT.

Before the MTT was added to the cell culture, cells were washed with 200 µL of PBS, and then 200 µL of MTT solution was added to the cell culture. Cell culture was incubated and gently shaken on a rotatory plate for 30min in the room temperature, to ensure that all the crystals have dissolved from the cells and have formed a homogeneous solution.

The absorbance was measured by using on a microplate reader at wavelength 540nm. The

results were determined as percentage of cell viability compared to the cell culture without treatment with ImmunatuRNA® complex.

Protection against oxidative damage

The protective properties of ImmunatuRNA® complex on human skin fibroblasts were evaluated by two assays, including (1) determination of cell viability following exposure to pro-oxidative agent in the presence vs absence of ImmunatuRNA® complex, and (2) ability of ImmunatuRNA® complex to modulate expression of SIRT-1 in the fibroblast cell culture. For that purpose, fibroblast cell culture (ATCC-CRL-2703) was plated on the 96 well plate and maintained in the sterile culture medium with a pH ranging between 7.2 and 7.4, and incubated for 24h at 37 and 5% CO₂ for 24h.

Cell viability was determined by MTT assay upon exposure to the pro-oxidative factor a hydrogen dioxide (H₂O₂) at concentration 150 µM, following treatment with series dilutions of ImmunatuRNA® complex at following concentrations of 8.55%, 5.13% and 0.855%, respectively, after 72h.

The protective capability of ImmunatuRNA® complex were determined as a change in the percentage of cell viability compared to the positive control, which was a cell culture treated only with H₂O₂.

SIRT-1 expression

The efficacy of the modulation of SIRT-1 expression by a ImmunatuRNA® complex, was determined by commercial fluorometric kit in 50 µL of cell homogenate collected after 72h following exposure to H₂O₂ and/or treatment with series of testing

concentrations of ImmunatuRNA® complex. The cells with added a complete cell medium were employed as negative control, whereas cells exposed only to H₂O₂ were a positive control.

Statistical analysis

The results obtained from cell culture studies are presented as mean and standard deviation (SD) of measured concentration. The percentage of change in the mean value was calculated by comparing post-treatment sample with control. The statistical significance of the results was determined through the Student t-test. All the changes with regard to the negative and positive control were considered statistically significant for $p < 0.05$.

Clinical study

Participant recruitment

For the purpose of clinical study assessing the influence of ImmunatuRNA® complex incorporated into skincare products on the human skin microbiome following a long-term use, ten apparently healthy adults were recruited.

Tested group included 7-females and 4-males with age ranging from 23 to 65 years old (mean 42.17 SD 17.15) with various skin types, including dry, oily, combination and normal. The participant recruitment was conducted by the study specialist according to the Declaration of Helsinki of 1964 (with subsequent amendments), Cosmetics Europe directives with use of inclusion and exclusion criteria. The exclusion criteria included being below 18 years of age, having a pharmacological treatment, and in case of

women being pregnant or during breastfeeding.

All procedures involved in the participant recruitment and intervention with the skincare products were conducted in accordance with Regulation of the European Parliament and Council Regulation (EC) No 1223/2009 of 30 November 2009 and No 655/2013 of 10 July 2013 on cosmetics.

Furthermore, the recommendations provided by Cosmetics Europe-The Personal Care Association Guidelines, including Product test guidelines for the Assessment of Human Skin Compatibility 1997 and Guidelines for the Evaluation of the Efficacy of Cosmetic Products 2008 were applied.

Study intervention

Before the study, recruited participants who voluntarily agreed to participate in the study were provided with one package of each of the skin care products containing ImmunatuRNA® complex- a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask.

Participants were instructed to regularly use both products interchangeably for 4 weeks.

During this time, participants were required not to share the cosmetics with others and refrain from using other products with identical or analogous purposes. Each participant also received directions for use of each product. For a probiotic cream use (7 mornings and 3 nights), it was recommended to apply product daily on clean skin of face, neck and around the eyes, by gently patting into the skin; whereas facemask (4-nights) was recommended to use overnight.

Skin surface assessment

The skin microbiome subject was assessed by quantitative and qualitative methods before and after 4-weeks application of the cosmetic regime. The quantitative test was performed by the swabbing method from the participant forehead.

From the collected swabs, microorganisms were released into sterile 0.9% sodium chloride (NaCl) solution, then tenfold dilutions were prepared and seeded onto appropriate microbiological media, including enriched, selective and diagnostic-selective.

The microbial cultures were incubated under aerobic and anaerobic conditions, depending on the medium applied. The quantitative evaluation was performed on media cultured under aerobic conditions. The number of microorganisms was expressed as CFU/cm² of skin.

Identification of the grown microorganisms was carried out based on colony morphology, cell morphology in stained preparations and on the basis of identifying biochemical characteristics.

Result

ImmunatuRNA® complex preparation

ImmunatuRNA® complex which formulation was described in details in patent application (PCT/IB2022/000231 (in pending), VRFD SA/VERDILAB Switzerland) is composed of naturally derived ingredients including sodium RNA, sodium hyaluronate, saccharide isomerate, maris aqua and malus domestica fruit extract with addition of lecithin, glycerin, tocopherol, aqua, phenylene glycol,

sodium hydroxide, benzyl alcohol. For the purpose of the cell culture studies, water solutions of ImmunatuRNA® complex with following concentrations 8.55%, 5.13% and 0.855% were used.

The active ingredients of the complex were yeast RNA sodium salt, exopolysaccharides (saccharide isomerate) from marine microplankton, and medium weight hyaluronic acid (sodium hyaluronate) which concentration was within 5.57%-9,70%. A naturally derived liposome system based on lecithin derived from organic soybean, as well as apple water and seawater were added to the complex formulation in order to achieve better penetration to deeper layers of epidermis and provide additional nutrients to the skin.

Antioxidant properties of ImmunatuRNA® complex

ImmunatuRNA® complex solution Concentration (%)	Percentage of reduced DPPH (%)				
	Measure 1	Measure 2	Measure 3	Mean	SD
0.86%	6.5	4.7	8.2	6.5	0.63
5.13%	24.3	23.8	25.2	24.4	3.13
8.55%	30.7	32.1	29.3	30.7	15.51

Table 1: The reduced DPPH percentage concentration following addition of various testing solutions of ImmunatuRNA® complex.

Cell culture studies

Protective effects against oxidative damage

The experiments conducted to investigate the protective properties of ImmunatuRNA® on

The results of the antioxidant capacity testing of ImmunatuRNA® complex are presented in Table 1.

To evaluate the antioxidant properties of ImmunatuRNA® complex an antiradical scavenging assay using a DPPH was carried out.

The findings presented in this study demonstrated that treatment with ImmunatuRNA® complex resulted in a lead to a dose-dependent reduction of the synthetic radical DPPH in the assessed samples (Table 1).

The means of reduced DPPH by ImmunatuRNA® complex ranged from 6.5% (SD 0.63) for the lowest concentration of 0.855% of ImmunatuRNA® complex to 30.7% (SD 15.51) achieved with 8.50% concentration of ImmunatuRNA® complex.

human skin fibroblasts evaluated by two independent assays, including direct determination of cell viability after exposure to pro-oxidative agent in the presence of ImmunatuRNA®, and ability of ImmunatuRNA® to modify the expression of SIRT-1 in the fibroblast cell culture,

confirmed the ability of ImmunatuRNA® complex to protect against oxidative damage *in vitro*.

The results of the effect of ImmunatuRNA® complex treatment on the protection against oxidative damage *in vitro* are presented in Tables 2 and 3.

Treatment with ImmunatuRNA® complex at all testing concentrations, 8.55%, 5.13% and 0.855% had significantly increased fibroblast viability following the exposure to pro-oxidative agent, when compared to control after 72h (Table 2) in the dose-dependent manner (Figure 1).

Testing conditions		Percentage (%) of cell viability		Protection against oxidative damage (%)
		Mean	SD	
Controls	CTR-	100		
	CTR+	38.4	7.8	
Immunatu RNA® complex	0.86%	86.3	1.5	47.9 [*]
	5.13%	79	0.9	40.7 [*]
	8.55%	69.5	4.6	31.1 [*]

Table 2: Percentages of cell viability and protection in each testing condition.

The protection percentage against the oxidative damage was calculated by intended as cell viability percentage increase compared to the positive control treated with H₂O₂ (150 µM).

CTR- negative control being cell culture added with a complete cell medium; CTR+ positive control being cell culture treated with H₂O₂ (150 µM). Significant results with p-value <0.05 are presented with *.

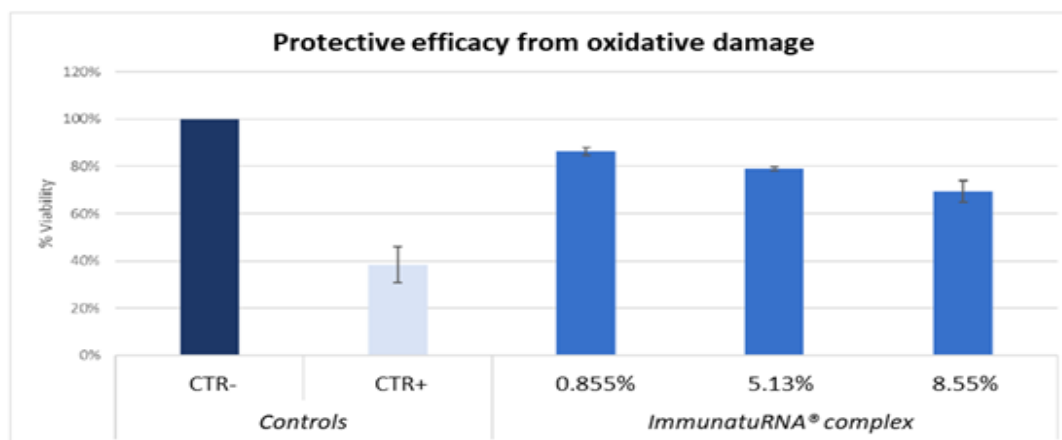


Figure 1: Graph demonstrates percentages of protective efficacy of ImmunatuRNA® complex in the human fibroblast cell culture against an oxidative damage H₂O₂ (150 µM). CTR- negative control being cell culture added with a complete cell medium; CTR+ positive control being cell culture treated with H₂O₂ (150 µM).

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Exposure to oxidative agent led to a significant reduction of SIRT-1 expression ($p < 0.05$) in the human fibroblast cell culture (Figure 2). Among all testing solutions, treatment with ImmunatuRNA® complex at 0.855% concentration showed a significant increase in SIRT-1 level (ng/ml) compared to

the cell culture treated with H_2O_2 alone (positive control) after 72h ($*p < 0.05$), followed by significant upregulation of the fibroblast SIRT-1 expression, relatively to its levels detectable in the cell culture without treatment (negative control) ($*p < 0.05$) (Table 3).

H ₂ O ₂ 150 µM protection		SIRT-1 expression		Protection %
		Mean (ng/ml)	SD	
Control	CTR-	4.52	0.59	
	CTR+	1.18	0.71	
ImmunatuRNA™ complex	0.86%	3.19	0.7	44.6%*
	5.13%	2.22	0.76	23.00%
	8.55%	2.12	0.94	20.90%

Table 3: The mean (SD ±) concentrations (ng/ml) of expressed SIRT-1 in the human fibroblast cell culture with after 72h following exposure to oxidative stress along with calculated percentage of protection following treatment with ImmunatuRNA® complex.

CTR- negative control being cell culture added with a complete cell medium; CTR+ positive control being cell culture treated with H_2O_2 (150 µM).

*Indicates statistically the significant change ($p < 0.05$) in the mean concentrations (ng/ml) of SIRT-1 when compared to control.

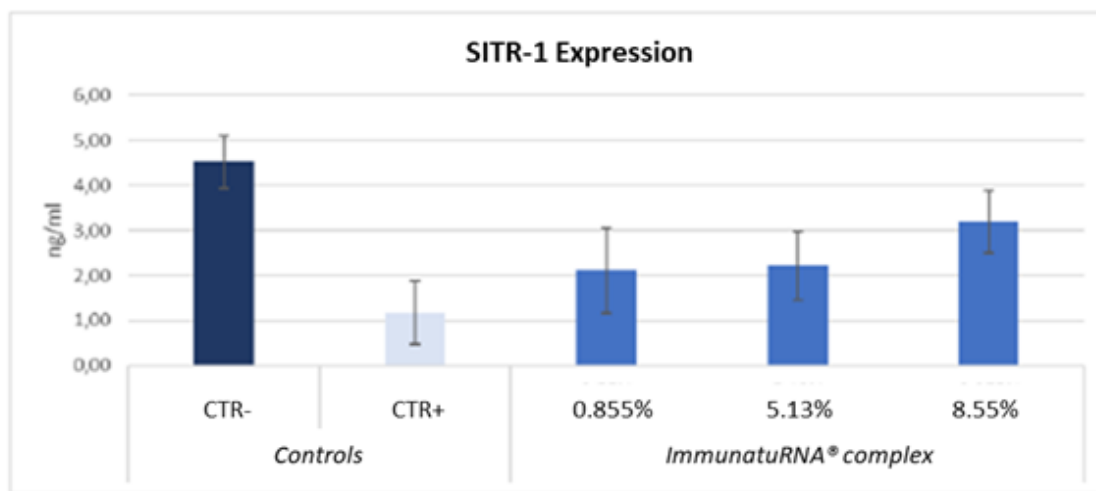


Figure 2: Graph representing detectable concentrations (ng/ml) of expressed SIRT-1 in the human fibroblast cell culture after 72h following exposure to oxidative stress and treatment with ImmunatuRNA® complex. CTR- negative control being cell culture added with a complete cell medium; CTR+ positive control being cell culture treated with H_2O_2 (150 µM).

Clinical study

Investigations of ImmunatuRNA® complex on the human skin microbiome

The aim of this study was to assess the effect of ImmunatuRNA® complex in the form of skincare products on the human skin

microbiome composition following a long-term regular use. For this purpose, ImmunatuRNA® complex was incorporated into formulation of two skin care products, a Natural Moisturizer Probiotic Cream and Natural Detox Face Replenishing Mask. The exact formulation of products used in this study are presented in Table 4.

Skin care product	INCI
Natural Moisturizer Probiotic Cream	Ascophyllum Nodosum Extract, Glycerin, Aqua (Water), Propanediol, Centaurea Cyanus Flower Water, Cetearyl Alcohol, Pentylene Glycol, Malus Domestica Fruit Extract, Hydrogenated Vegetable Glycerides, Olus Oil (Vegetable Oil), Decyl Oleate, Coco-Caprylate/Caprate, Caprylic/Capric Triglyceride, Cetearyl Glucoside, Glyceryl Stearate, Maris Aqua (Sea Water), Sodium Levulinate, Alpha-Glucan Oligosaccharide, Xylitylglucoside, Butylene Glycol, Squalane, Anhydroxylitol, Parfum (Fragrance), Lecithin, Lysolecithin, Sclerotium Gum, Sodium Anisate, Sodium Rna, Betaine, Polymnia Sonchifolia Root Juice, Xylitol, Pullulan, Xanthan Gum, Sodium Hyaluronate, Aloe Barbadensis Leaf Juice Powder, Maltodextrin, Tocopherol, Helianthus Annuus (Sunflower) Seed Oil, Glycyrrhiza Glabra (Licorice) Root Extract, Hydrolyzed Algin, Leuconostoc/Radish Root Ferment Filtrate, Limonium Gerberi Extract, Phytic Acid, Tremella Fuciformis Sporocarp Extract, Ci 77288 (Chromium Oxide Greens), Cryptomeria Japonica Bud Extract, Phenethyl Alcohol, Alteromonas Ferment Extract, Silica, Glucose, Lactobacillus, Saccharide Isomerate, Laminaria Digitata Extract, Glyceryl Caprylate, Glyceryl Undecylenate, Fucus Vesiculosus Extract, Chlorella Vulgaris Extract, Sodium Hydroxide, Camellia Sinensis Leaf Extract, Hydrogenated Palm Glycerides Citrate.
Natural Detox Replenishing Face Mask	Ascophyllum Nodosum Extract, Glycerin, Aqua (Water), Pentylene Glycol, Sempervivum Tectorum Leaf Juice, Caprylic/Capric Triglyceride, Sclerotium Gum, Alpha-Glucan Oligosaccharide, Sodium Levulinate, Xylitylglucoside, Cocos Nucifera (Coconut) Oil, Parfum (Fragrance), Anhydroxylitol, Malus Domestica Fruit Extract, Sodium Anisate, Polymnia Sonchifolia Root Juice, Betaine, Maris Aqua (Sea Water), Ci 77019 (Mica), Xylitol, Ci 77891 (Titanium Dioxide), Maltodextrin, Aloe Barbadensis Leaf Juice Powder, Citric Acid, Ci 77288 (Chromium Oxide Greens), Lecithin, Glycyrrhiza Glabra (Licorice) Root Extract, Hydrolyzed Algin, Limonium Gerberi Extract, Sodium Rna, Tremella Fuciformis Sporocarp Extract, Sodium Hyaluronate, Ci 77491 (Iron Oxides), Glucose, Lactobacillus, Cinnamomum Cassia Bark Extract, Ci 77510 (Ferric Ferrocyanide), Menyanthes Trifoliata Leaf Extract, Phenethyl Alcohol, Laminaria Digitata Extract, Glyceryl Caprylate, Glyceryl Undecylenate, Fucus Vesiculosus Extract, Salicornia Herbacea Extract, Saccharide Isomerate, Chlorella Vulgaris Extract, Camellia Sinensis Leaf Extract, Sodium Hydroxide, Tocopherol.

Table 4: Detailed INCI formulation for skin care products, a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask used in the study.

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Participant characterization

The detailed information on participant characteristics, including age, gender, skin type as well as composition of skin microbiota assessed before and after intervention are presented in Table 5.

Tested group recruited to assess impact of cosmetic products with added ImmunatuRNA® complex included 7 females

and 4 males with age ranging from 23 to 65 years old (mean 42.17 SD 17.15). In this group, 5 participants had a dry skin type (4 females and 1 male), 3 individuals had a combined skin type (1 female and 2 males) whereas 2 participants had a normal skin (2 females).

During and after intervention with cosmetic products, individuals participating in the study did not report any unexpected effects related to the cosmetics use.

Participant characteristics			Number of microorganisms on the skin	
Age	Gender	Skin type	Before cosmetic use	After cosmetic use
62	Female	Dry	1,30x10 ¹	2,00x10 ¹
65	Male	Combination	6,80x10 ¹	8,00x10 ¹
55	Male	Combination	1,00x10 ¹	3,00x10 ¹
64	Female	Dry	4,50x10 ¹	44,00 x10 ²
55	Female	Dry	1,00x10 ²	4,00x10 ³
34	Female	Normal	3,90x10 ¹	3,30x10 ³
23	Female	Combination	3,00x10 ¹	9,00x10 ²
24	Male	Dry	2,24x10 ²	6,00x10 ²
42	Female	Dry	15,5x10 ²	20,00x10 ²
35	Female	Normal	8,10x10 ²	7,75x10 ²

Table 5: Microbial cell count before and after cosmetic application. Number of microorganisms on the skin are presented as CFU/cm² detected on the skin sample (forehead).

Assessment of changes in skin microbiota composition

The changes in the skin microbiota composition on the forehead skin before and after 4-week long intervention with the cosmetics containing ImmunatuRNA® complex are presented in the Table 6. The main findings have been shown that after the

time of 4 weeks of the regular use of skincare products, both a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask with the ImmunatuRNA® complex in the formulation, did not reduce the number of microorganisms on the selected from the forehead skin sample, in respect of the skin type.

Participant characteristics			Microorganism name	Microorganism detection	
Age	Gender	Skin type		Before cosmetic use	After cosmetic use
62	Female	Dry	<i>Staphylococcus epidermidis</i>	+	+
			<i>Micrococcus spp.</i>	+	+
			<i>Staphylococcus xylosus</i>	+	+
65	Male	Combination	<i>Staphylococcus epidermidis</i>	+	+
			<i>Staphylococcus aureus</i>	+	-
			<i>Bacillus spp.</i>	+	-
			<i>Cutibacterium acnes</i>	+	+
55	Male	Combination	<i>Staphylococcus epidermidis</i>	+	+
			<i>Staphylococcus capitis</i>	+	+
			<i>Micrococcus spp.</i>	+	+
			<i>Cuibacterium acnes</i>	+	+
64	Female	Dry	<i>Staphylococcus epidermidis</i>	+	+
			<i>Neisseria spp.</i>	+	-
			<i>Cutibacterium acnes</i>	+	+
55	Female	Dry	<i>Staphylococcus epidermidis</i>	+	+
			<i>Staphylococcus hominis</i>	+	+
34	Female	Normal	<i>Staphylococcus epidermidis</i>	+	+
			<i>Micrococcus spp.</i>	+	+

23	Female	Combination	<i>Staphylococcus epidermidis</i>	+	+
			<i>Staphylococcus caprae</i>	+	+
			<i>Enterococcus spp.</i>	+	-
			<i>Neisseria spp.</i>	-	+
24	Male	Dry	<i>Staphylococcus epidermidis</i>	+	+
			<i>Micrococcus spp.</i>	+	+
42	Female	Dry	<i>Staphylococcus epidermidis</i>	+	+
			<i>Micrococcus spp.</i>	+	+
			<i>Bacillus spp.</i>	+	-
			<i>Citrobacter koseri</i>	+	-
			<i>Alcaligenes spp.</i>	+	-
35	Female	Normal	<i>Staphylococcus epidermidis</i>	+	+
			<i>Staphylococcus sciuri</i>	+	+
			<i>Staphylococcus xylosus</i>	+	+
			<i>Micrococcus spp.</i>	+	+
			<i>Pantoea spp.</i>	+	-

Table 6: Composition of skin microbiome before and after intervention with cosmetics, a facemask and probiotic cream. Changes in the presence of certain microorganism in the skin microbiome composition are highlighted in green (absence of microorganism after cosmetic use) and yellow (absence of microorganism before cosmetic use).

The further analysis of skin microbiota composition following an intervention with the Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask both

containing an ImmunatuRNA® complex, indicated a subtle, however inconsistent changes in the presence of certain microbial species on the forehead skin samples. These

changes included elimination of the *Staphylococcus aureus* (1 participant), *Bacillus spp.* (2 participants), *Enterococcus spp.* (1 participant), *Citrobacter koseri* (1 participant), *Alcaligenes spp.* (1 participant) and *Pantoea spp.* (1 participant). Furthermore, analyses have shown that following cosmetic intervention resulted in the removal of *Neisseria spp.* in the sample provided by female participant with dry skin type; whereas in another female participant with combination skin type, these bacteria was introduced/appeared after the treatment (Table 6).

Discussion

The results obtained from both *in vitro* and clinical studies consistently demonstrated that treatment with ImmunatuRNA® complex has potential to protect human skin cells against exposure to oxidative stress and promote favorably impact on the skin microbiome composition in different skin types.

Findings obtained from the *in vitro* studies utilizing fibroblast cell culture consistently demonstrated anti-oxidative properties of ImmunatuRNA® formulation. Results obtained from the pre-eliminary study using an antiradical scavenging assay using a DPPH indicated that ImmunatuRNA® complex has capability to reduce synthetic radicals, in this case DPPH, in the dose-dependent manner. Further investigations conducted to characterize antioxidant properties of ImmunatuRNA® complex *in vitro* in the human fibroblast cell culture consistently have shown that treatment with bio-actives being a part of ImmunatuRNA® complex

formulation significantly increased skin cells viability following the exposure to oxidative stress, when compared to control after 72h, as well as increased expression of SIRT-1 protein, a member of the NAD⁺-dependent deacetylase family, implicated in regulating many vital physiological functions in humans, including DNA repair, aging, gene expression, and apoptosis [17].

Previous studies have consistently shown that exposure to oxidative stress can induce expression of Sirtuin family proteins, including SIRT-1, thereby leading to increased proliferation and protection against oxidative stress-induced cell death, potentially through suppressing the transcriptional activity of p53 [18]. Interestingly, SIRT-1 activity appears to be modulated not only by energy restriction by also, by certain natural bio-actives, including resveratrol [19], berberine [20], as well as microRNA [21]. Therefore, derived from yeast sodium RNA present in the formulation of ImmunatuRNA® complex, might be implicated in the modulation of SIRT-1 activity, particularly in the context of inflammation, oxidative stress and carcinogenesis, thereby prevent against progression of age-related diseases and aging [21].

Daily application of cosmetics, depending on the product type, as well as duration may influence on the skin microbiome composition [22].

Intervention with cosmetics containing ImmunatuRNA® complex conducted in the group of human volunteers with different skin types (oily, dry, combined, normal) aged between 23 and 65 years of age demonstrated

that both Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask after 4 weeks can result in beneficial changes in the composition of skin microbiome. These changes have been observed as reduction of relative abundance of potentially pathogenic microorganisms implicated in skin inflammation and infections. In addition, a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask did not compromise the skin's microbiome (Table 8), as the average number of detectable microorganism on the skin were within the expected range between from 10^3 to 10^4 CFU/cm², with possible exception for the forehead, where about 10^6 CFU/cm² can be found [7]. Further investigations focused on the analysis of skin microbiota composition (results presented in Table 9) indicated that use of cosmetics with added ImmunatuRNA® complex lead to changes in the presence of certain species in the forehead skin samples. These changes included elimination of the gram-negative bacilli, in particular *Citrobacter koseri* and *Enterococcus spp. (faecal streptococci)*, which presence was reported in the patients with secondary infections like ulcer/decubitus [23] as well as individuals with acne [24] and scalp folliculitis [25]. Furthermore, use of cosmetics containing ImmunatuRNA® lead to subsequent reduction of aerobic gram-positive rod-shaped bacteria including genus *Bacillus*, *Alcaligenes* and *Pantoea*, being implicated in the wound infections [26,27] and skin lesions in the post-operative patients [28]. Most importantly, use of skincare products containing an ImmunatuRNA® complex also lead to elimination of the *Staphylococcus aureus*, which is a

microorganism widely distributed in the environment, including water and soil [29], as well as on the mucosal and skin surfaces of ~30% population and transiently in up to 60-80% of the individuals [30]. That progressively increased colonization with *S. aureus* associated with risk of skin infections [31], as well as other inflammatory conditions such as atopic dermatitis [32]. Interestingly, super-antigens produced by *S. aureus*, by interacting with T lymphocytes deposited in the skin can promote skin inflammation and increase expression of potent pro-inflammatory mediators, such as IL-36 α in keratinocytes [32], a cytokine previously implicated in development of psoriasis [33]. These results may therefore indicate that regular use of both a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask containing ImmunatuRNA® complex in their formulas, by influencing on the skin microenvironment, is most likely to create unfavorable conditions for the persistence of the potentially pathogenic microorganisms, limiting the risk of developing adverse skin conditions. The reported immunomodulatory properties of ImmunatuRNA® complex formulation may be attributed to the presence of yeast-derived sodium RNA salt have potential to protect against pathogenic microorganisms, while promoting beneficial activities of beneficial skin microorganisms to improve skin defense systems and prevent against inflammation and infections. For instance, addition of supplement containing processed yeast and nucleotides (0.05%) to boiler chicken diet increased animal humoral immunity by increasing immunoglobulin IgA level as well as upregulated expression of

cytokines involved in T helper (Th) cells 2 induced antibody production, including IL-10, IL-4, and IL-13, thereby suggesting enhanced immune protection upon pathogen challenge [34]. Similarly, an *in vitro* study evaluating potential of RNA aptamers to inhibit *Candida albicans* biofilm formation demonstrated that RNA aptamers Ca-apt-1 was able to selectively recognize *C. albicans* isolated from clinical samples, while ignoring other beneficial microorganisms (i.e., *Streptococcus mutants* and *Saccharomyces cerevisiae*), thereby leading to selective inhibition of hyphal formation of *C. albicans* [35]. Interestingly, use of small noncoding micro-RNAs (miRNAs) have been shown a potent regulators of skin physiological processes associated with skin cell proliferation, angiogenesis, metastasis, apoptosis, immune response with additional role in the treatment of epithelial skin tumors [36]. Interestingly, in this study use of a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask with added ImmunatuRNA® complex demonstrated potential differential effects on the presence of *Neisseria spp.*, on the forehead skin sample. The findings have shown that *Neisseria spp.* was eliminated in the individual with a dry skin type, whereas in the participant with combination skin type these bacteria appeared after the intervention (Table 9). *Neisseria* is a large genus including gram-negative coccoid bacteria, which may have both pathogenic and non-pathogenic effects [37]. Among species found in humans, only two, *N. meningitidis* and *N. gonorrhoeae* are pathogens, whereas the rest of them are considered as commensals, often found in the mucosal surfaces [38]. For example, *N.*

lactamica, lactose-fermenting human bacteria, has potential to protect against *N. meningitidis* through natural immunity (from carriage) [39]. Therefore, to assess the potential health consequences the more detailed characterization of the *Neisseria* is needed. Additionally, it should be noted that, observed inconsistencies can be explained by individual characteristics, such as skin type, gender [40], specific lifestyle habits [41] and environmental exposures [42] that may modify composition of the skin microbiome.

Conclusion

Series of the experiments using an *in vitro* model of human skin cells demonstrated that ImmunatuRNA® complex has potent antioxidant properties, which significantly protect human fibroblasts against oxidative damage by scavenging free radicals efficiently.

Additionally, evaluation of SIRT-1 expression in human fibroblasts exposed to oxidative stress (H₂O₂) indicated that use of ImmunatuRNA® complex at 0.855% may favorably modulate skin cell response to oxidative stress resulted in higher SIRT-1 levels, suggesting increased activity and longevity of the human skin cells. Additionally, the clinical study investigating the effect of ImmunatuRNA® complex in the form of skincare products, a Natural Moisturizer Probiotic Cream and Natural Detox Replenishing Face Mask on the human skin microbiome composition confirmed that ImmunatuRNA® complex had a protective effect on the skin microbiome demonstrated by elimination of potential the adverse microorganisms, including *Citrobacter koseri*,

Bacillus spp., *Enterococcus spp.*, *Alcaligenes spp.*, *Pantoea spp.* and *Staphylococcus aureus*; with no disruptive effects on skin microbiome abundance and composition determined in the forehead of adults with oily, dry, combined and normal skin types.

In conclusion, use of ImmunatuRNA® complex alone as well as active functional ingredient of everyday use skincare products have positive influence on the human skin immunity and microbiome.

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Conflict of interest

None to report.

Declaration of interest

Dr. Joanna Michalina Jurek was a main person involved in the manuscript preparation and submission to the Journal. This work was carried out as own initiative independent of the employment and conducted in the spare time based on the research collaborative agreement with VRFD SA/VERDILAB Switzerland. Victoria Neymann is a founder of VERDILAB Switzerland/VRFD SA, as well as the main owner of the intellectual property and chief lead on conducted research including conceptualization, methodology, formal analysis, investigation and data curation. The research activities described in the manuscript was conducted in collaboration with Comp life Group and Dr Koziej Institute of Cosmetic Research. The authors bear primary responsibility for the accuracy of made statements and for the content and writing of the paper.

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