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Care Creations™

Phytofirm® Biotic

Your probiotic-ferment ally for preserving skin youthfulness from head to toe

by Beauty Creations
another Care Creations™ product group Inspired by Life

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SUMMARY FILE

Phytofirm® Biotic	BC10138
Origin - Description	Fermented soybean extract with the probiotic ferment *Lactobacillus plantarum*
Regulatory data	
INCI	Lactobacillus/Soybean Ferment Extract, Pentylene Glycol, Caprylyl Glycol
China	Each component is listed in Inventory of Existing Cosmetic Ingredient in China (IECIC 2015)
CAS#	68607-88-5, 5343-92-0, 1117-86-8
EINECS#	271-770-5, 226-285-3, 214-254-7
Appearance	Yellow to amber liquid
Preservative	None
Halal	Halal certified
Natural labels	None
Naturalness content (ISO 16128)	99.5% from natural origin
Cosmetic use	
Properties	Activates the synthesis of extracellular matrix components (elastin, collagens, glycosaminoglycans). Contributes to improve organization of collagen fibers. Improvement of epidermal thickness. Anti-elastase activity. Contributes to limit fibroblast transformation into myofibroblasts and their contractility. Improves skin elasticity and thickness.
Applications	Anti-aging skincare. Probiotic skin care. Face and body elasticity restoration care. Rejuvenating body and skin care. Stretch marks formation minimization care.
Formulation data	
Concentration of use	2%
Solubility	Soluble in water
Incorporation method	Phytofirm Biotic is incorporated during the final process at a temperature below 30°C
Optimal temperature of use	15 - 30°C
Optimal pH	3 - 9

SKIN YOUTHFULNESS AND BIOTIC INGREDIENTS ARE HIGH CONSUMERS REQUIREMENT

Today, more and more consumers consider fermented product as biobased and natural solutions. In the same time, consumers also consider biotic ingredients as alternative solutions good for the skin and environment-friendly. Indeed, consumers well recognize the health and wellness benefits of biotics, and this is driving more interest to use skincare products with probiotic fermented ingredients (postbiotics).

Why looking for bio-based products and biotic ingredients?

Probiotic means “in favor of life” and is generally used to refer to microorganisms or their derived substances that produce a beneficial effect on our body. Technically speaking, biotics are not invention of the 21st century. In fact, scientists have been harnessing the properties of these materials for over a century.

These health benefits have become increasingly attractive for consumers across markets and particularly for skin and face care products in the cosmetic industry. Products with a biotic claim are associated with health, wellness and eco-friendliness. 47% of US women aged 18-44 who use beauty products already take oral probiotics for general wellbeing, and these consumers are now seeking cosmetic products that contain biotics. 16% of US women aged 18 or over are interested in buying skincare products that have fermented ingredients in them [Tyrell D, 2019]. 28% of all skincare products launched in Europe and the US between December 2018 and November 2019 contained fermented ingredients, while 54% of these products claimed an anti-aging effect.

At the same time, consumers are looking for basic and efficient products that target fundamentals of beauty: collagen and elastin, known as the most important elements of skin structure. They want to feel better with their skin and thus maintain as long as possible skin beauty, and youthfulness.

Indeed, human skin undergoes natural aging process as a consequence of time which goes on. In addition, human skin continuously experiences harmful stress and damage from environmental sources such as solar UV irradiation. As skin changes are among the most visible signs of aging, skin is central in the social and visual experience. As such, skin appearance has a significant emotional and psychological impact on our life quality [Shao *et al*, 2017].

To answer consumers request of product maintaining skin youthfulness whatever skin age, BASF Beauty Care Solutions combined a non-GMO soybean extract with the performance of a fermentation process, thanks to a famous *Lactobacillus bacterium*.

We obtained the perfect probiotic-ferment or postbiotic ally for preserving youthfulness from head to toe.

SKIN ALTERATIONS DURING AGING PROCESS

Human skin offers protection to the body against external assaults and mechanical trauma, via the reversible deformation of its structure, but with aging skin protection capacity is altered.

The first signs of aging are related to individual characteristics (chronological aging) but are accelerated by external factors such as sunlight, cigarette smoke, or pollution. Clinical manifestations of aged skin include dryness, laxity, wrinkles, slackness, thinning, and even stretch-marks can occur [Bonte *et al*, 2019]. Skin aging is characterized by changes in its different layers.

The epidermis becomes thinner and its junction with dermis is flattened [Bonte *et al*, 2019l]. This age-related thinning of the epidermis significantly impairs skin protective functions against environmental insults [Shao *et al*, 2017].

The dermis, made of extracellular matrix (ECM) and cells such as fibroblasts also shows dramatic changes. The dermal matrix becomes less elastic, less dense, and thinner with age, so that alterations in the dermis are one of the main causes of the appearance of old skin [Pageon *et al*, 2019]. At the cellular level, alterations of dermis are mainly due to fibroblast decrease in number and function defects. Thus, with aging, the level of collagen, elastin and GAGs are reduced in the dermis; their organization is impaired [Binic *et al*, 2013].

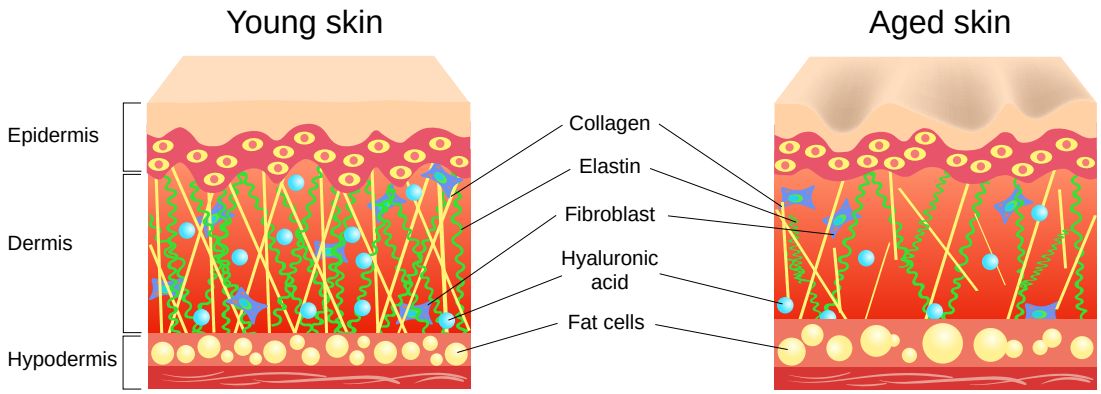


Figure 1 - Schematic representations of young and aged skin structures.

Several studies have shown that aging processes affect the enzymatic activities related to synthesis, remodeling, and catabolism of the ECM components of the dermis (collagen, elastin, and proteoglycans made of glycosaminoglycans linked to a specific protein core). As a result, not only does the aging processes induce a reduction of the ECM density but its quality is also affected [D'Aloiso *et al*, 2020].

Fibroblasts are the major ECM-producing cells in skin. Hallmarks of fibroblast aging are multiple: DNA damage accumulation, disruption of transcriptional processes, and disruption of protein homeostasis. Most phenotypic changes in extrinsically aged skin, such as wrinkle formation or slackness, are linked to dysfunctions of dermal fibroblasts and corresponding remodeling of the dermal ECM [Tigges *et al*, 2014].

In aged dermal fibroblasts there is an up-regulation of proteolytic enzymes secretion [Quan *et al*, 2012]. Enhanced expression of proteolytic enzymes like matrix metalloproteinases (MMPs), elastase, and decrease in expression of TIMPs (Tissue Inhibitor of Metalloproteinase) contributes to the destruction of the dermal fibrous collagen-elastin matrix [Panwar *et al*, 2020].

Production of ECM proteins by dermal fibroblasts is primarily regulated by the TGFβ pathway. In aged human skin, several components of the TGFβ pathway are reduced. Impairment of this TGFβ pathway accounts, at least in part, for diminished ECM component production that is observed in aged skin [Qin *et al*, 2018].

Collagens

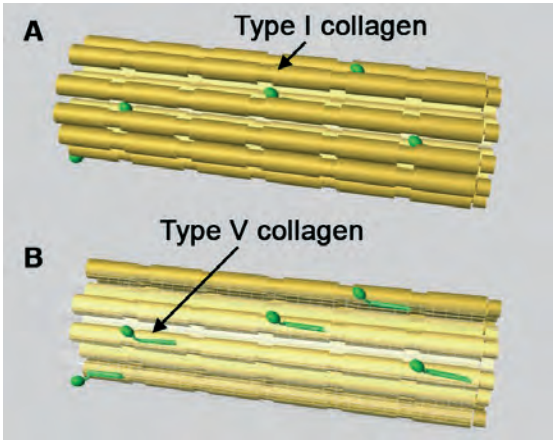


Figure 2 - Proposed model of heterotypic type I and V collagen.
A - complete view. **B** - view in transparency.

Skin is a tissue containing many collagen types. Several collagens which are minor in terms of abundance are restricted at the dermo-epidermal junction, contrarily to others like collagen I, III and V present throughout the dermis.
Collagen I, is the most prominent constituent of ECM molecules distributed throughout the interstitium, making up to 90% of the total connective tissue. Collagen I, as the most abundant ECM molecule, contributes to mechanical properties of the dermis, namely resistance to shock and thus tensile strength of the skin. It is known to provide a structural scaffold for cell attachment with impacts on tissue organization and tissue homeostasis by affecting cell growth, motility, viability, and differentiation [Plant *et al*, 2009]. Zoller and coworkers have shown that collagen I is able to modulate lipogenesis and adiponectin expression and therefore may contribute to metabolic dysfunctions associated with aging [Zôller *et al*, 2019].

Collagens also exist at subcutaneous level. The subcutaneous network of collagenous fibers is known as the retinacula cutis (RC). Sparse RC structure contributes to a reduction in the elasticity of subcutaneous tissue, resulting in a greater degree of sagging facial skin [Sakata *et al*, 2018].
During aging, collagen fibrils become fragmented, less dense and disorganized. Decrease in collagen I with age is due to the loss of type I collagen expression in aged fibroblasts, partly through downregulation of TGFβ1 at local level. Decrease in age-induced collagen expression might be also linked to an increase in nuclear factor-κB (NF-κB) DNA-binding activity. With aging, fibroblasts also synthesize more metalloproteinase (MMP-1) through an elevation of the transcription factor activator protein-1, leading to fragmentation of collagen fibrils with consequent reduction of the mechanical tension normally observed in young skins [Brun *et al*, 2016].

Besides collagen I, collagen V is a minor collagen in terms of abundance but necessary for the development of a functional skin matrix [Chanut-Delalande *et al*, 2004], as it acts as a regulator of the collagen fibrils formation (Figure 2). One of its subtypes serves as a bridging molecule that contributes to the stabilization of the epidermal-dermal interface. Collagen V defects lead to a connective tissue disorder typically characterized by skin fragility and abnormal wound healing [Bonod-Bidaud *et al,* 2012].

In skin, collagen associates with elastin, another major ECM fiber.

Elastin

Elastic fibers representing 3 to 4% of ECM of the dermis are tight, three-dimensionally interlaced of fibrils, intimately linked with collagen fibers.
Elastic fibers, composed of an elastin core (90%) surrounded by fibrillin-rich microfibrils (10%), are essential extracellular matrix (ECM) macromolecules endowing extensible tissues with critical mechanical properties such as elasticity, flexibility and resilience [Fhayli *et al*, 2020]. Elastic fibers provide recoil to tissues that undergo repeated stretch. Importantly, elastin stretch is crucially limited by tight association with collagen fibrils [Wise & Weiss, 2009]. Secreted tropoelastin (the precursor of elastin) molecules assemble into fibers and become highly crosslinked to one another via their lysine residues by members of the lysyl oxidase (LOX) enzyme family, which include LOX and LOXL [Lucero and Kagan, 2006]. Elastin fibers are covered by glycoprotein microfibrils, mainly fibrillins, which are also essential for the integrity of the elastin fibers [Wise & Weiss, 2009].

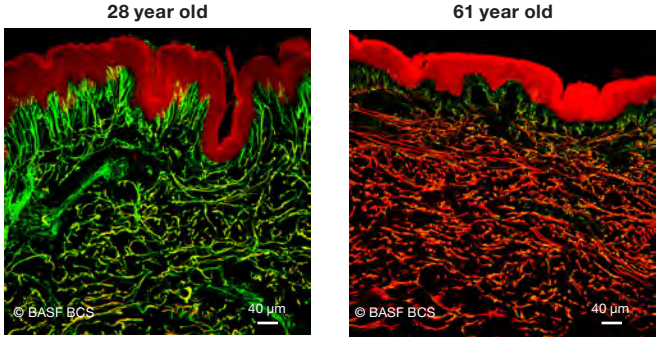


Figure 3 - Visualization of elastic fibers in young skin (28 yo) *vs* aged skin (61 yo) in the dermis by immunostaining (green) and confocal microscopy on skin biopsies. Scale bar: 40µm.

Elastin, the major component elastic fibers, has been shown to decrease during aging (Figure 3). Elastin's overall quantitative and qualitative decline, including the loss of structural integrity due to proteolytic enzymatic activity and lack of production by fibroblasts, contributes to the aging of skin [Panwar *et al*, 2020].
Elastin can be cleaved by several proteases including serine proteases (leukocyte elastase, proteinase-3), and cathepsin (Cat) G, matrix metalloproteases (MMP-2, MMP-9, MMP-12 and MMP-14), and cysteine proteases (CatK, S, and V) [Frantz *et al*, 2010].

Glycosaminoglycans (GAGs)

The GAGs chains are unbranched polysaccharide chains composed of repeating disaccharide units [sulfated N-aceltylglucosamine or N-acetylgalactosamine, D-glucuronic or L-iduronic acid and galactose (–4 N-acetylglucosamine-β1,3-galactose-β1) that can be divided further into sulfated (chondroitin sulfate, heparan sulfate and keratan sulfate) and non-sulfated (hyaluronic acid or HA) GAGs [Frantz *et al*, 2010]. Glycosaminoglycans (GAGs), especially hyaluronic acid (HA), are of high importance. HA is a high molecular weight polymer consisting of disaccharide repeats of D-glucuronic acid and N-acetyl-D-glucosamine. HA by virtue of its viscosity, elasticity, and other rheological properties, acts as a lubricating and shock absorbing fluid in skin [Ciccone *et al*, 2019]. In addition, HA has been implicated as a regulator of cell proliferation and locomotion. Two cell-associated receptors, CD44 and receptor for HA-mediated motility (RHAMM) mediate the biological effects of HA [Tzellos *et al*, 2009].

Stretch-marked skin: aged skin shares some common defects with stretch marks

Stretch marks or striae distensae (SD) are common and permanent skin lesions caused by mechanical stress, namely over stretching to the dermis. At first, SD appear as pink-red lines on skin that turn with time into white lines reflecting an atrophic skin. Stretch marks can appear during aging but are more frequent in young people. Among young people, the main causes of SD are a rapid growth or a weight gain. SD are also very frequent (around 90%) among pregnant women on abdomen or breast [Casabona *et al*, 2017].

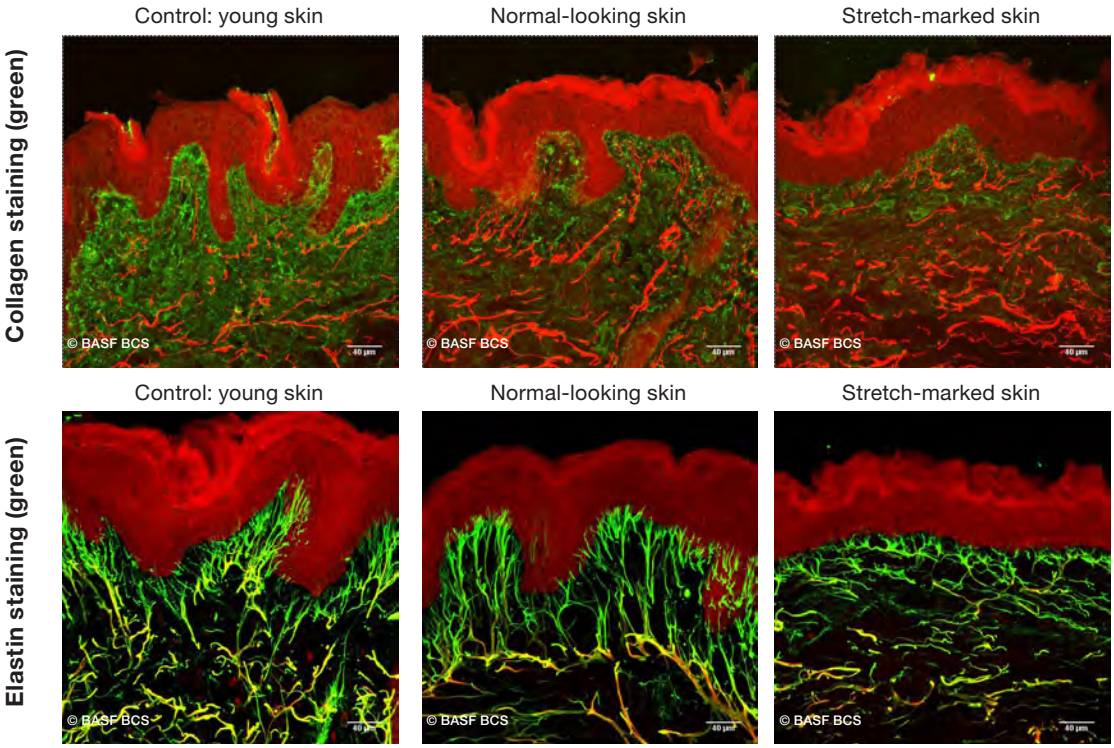


Figure 4 - Collagen and elastin immunostaining (green) in young skin *vs* normal skin and stretch-marked skin of the same volunteer.

Skin with stretch marks shows thinner epidermis [Pierard -Franchimont *et al*, 2014], decrease in collagen and elastic fiber network (Figure 4), increase in elastolysis [Ud-Din *et al*, 2016], as well as increase in protease production. In addition, fibroblasts functionality is also impaired; they tend to change their phenotype into myofibroblasts and become more contractile.

Skin with stretch marks has reduction and destructuration of collagen fibers linked to disturbance in the maintenance of collagen secondary structure. Water molecules form inter and intrachain bridges through hydrogen bonds with the backbone carbonyl groups and hydroxyl group of hydroxyproline. Thus, a decrease in the hydroxyproline proportion would result in a loss of these bonds and consequently in disorders in the molecular organization of collagen [Schuk *et al,* 2020].

A decrease of fibrillin-1, a protein necessary to elastic fiber formation (termed elastogenesis), and a disorganized elastic fiber network in stretch-marked skin are largely established by Beauty Care Solutions and other scientists [Watson *et al*, 1998; Jeanmaire *et al*, 2013; Bogdan *et al*, 2017].

In fact, fibrillin decrease can cause an increase of TGFβ1 bioavailability which alters locally the ECM homeostasis [Doyle *et al*, 2012]. An excessive TGFβ1 signaling promotes transformation of fibroblasts into myofibroblasts [Doyle JJ *et al*, 2012] which express smooth muscle actin (SMA) and have a more contractile phenotype [Viennet *et al*, 2005] than an extracellular matrix component producer. This creates an altered cell environment [Choi *et al*, 2009] which contributes to human skin aging [Quan *et al*,2015].

Alteration of skin quality and biomechanical properties impact skin youthfulness. This can occur during aging or at specific period of life like pregnancy, adolescence and after life habit changes which will affect skin (weight gain or loss after diet or intensive sport practice).

Skin youthfulness is a high requirement for cosmetic products, particularly the recovery in skin elasticity.

This prompted BASF BCS scientists to design and develop a probiotic solution which will target stimulation of the synthesis of key ECM components like elastin, collagen, and GAGs. Overall protection of skin fibroblast functionality will be also targeted to keep them producing ECM components for a youthful skin appearance.

PHYTOFIRM BIOTIC A PROBIOTIC FERMENT FROM *LACTOBACILLUS PLANTARUM*

Phytofirm Biotic is obtained by fermentation of soybean extract by a probiotic strain: *Lactobacillus plantarum*

Description of the starting material and the probiotic strain

Glycin max or Soybean

Family: Fabacea

Species: glycin max

Common names: Soybean, Soya bean

Soybean is an erect, bushy, hairy, annual legume that grows under temperate and tropical climates. Soybean is the world's most important legume crop and ranks sixth of all cultivated crops in terms of total harvest. More than 90% of the soy produced today all over the world is genetically modified (GMO) (Acreage NASS 2010).

The soybean used for Phytofirm Biotic is non GMO, and cultivated in Europe.

Soybean uses [Chen *et al*, 2012]:

Soybean have been used for centuries for nutritional and health purposes. Soybean was first considered as sacred for its use in crop rotation as a method of nitrogen fixation. Today, soybean is a key plant-based protein source used in foods, beverages, supplements and in beauty products. The high nutritional value of soybean comes from its nutrients: proteins, lipids, vitamins and minerals. Well known as one of the five sacred grains, a staple of ancient Chinese nutrition, it represents a good source of organic compounds and antioxidants, which further help in boosting your health. Soybeans are also known to improve metabolism, help in healthy weight gain, protect the heart, decrease risk of diabetes, and improve microcirculation.

***Lactobacillus plantarum* probiotic strain uses**

Among probiotics, *L. plantarum* is pointed out as an industrially important microorganism that can be found and isolated from dairy product, fermented products, environments (silage, sewage, etc…), human mouth and intestinal tract and is considered as safe. This specie has been widely used for the development of functional food and potential oral vaccine [Meixiu *et al*, 2016]. *L. plantarum* has one of the largest genomes known among lactic acid producing bacteria, offering so, wide capacity of substrate transformation during fermentation and diverse benefits.

Fermentation has recently re-emerged as an approach for improved functionality of food products in addition to the traditional roles such as shelf life, taste, and texture increase [Lee *et al*, 2019]. *L. plantarum* fermentation increases functionality of some food ingredient:

- *L. plantarum* fermentation increased antioxidant compounds and antioxidant activity in some fruits [Zhou *et al*, 2020],
- *L. plantarum* fermentation increased nutritional potency of some plants like Coix seed by increasing nutrients availabilities [Yin *et al*, 2019].

L. plantarum has also been successfully used to treat patient with specific phenotype of atopic dermatitis [Kim *et al*, 2017]. Some *L. plantarum* strains have shown anti-inflammatory properties [Schmitter *et al*, 2018].

The beneficial effects of probiotics are now widely reported, although there are only a few studies on their anti-aging effects. Lee and coworkers have found that *Lactobacillus plantarum* HY7714 improves skin hydration and has anti-photoaging effects, *in vitro* and *in vivo* [Lee *et al*, 2015].

Phytofirm Biotic, a sharply controlled and reproductible *Lactobacillus*/ soybean ferment extract for higher efficacy

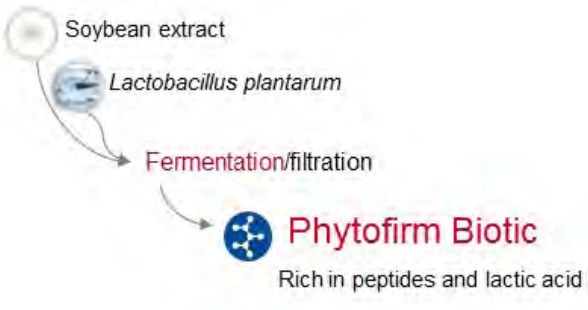


Figure 5 - Phytofirm Biotic obtention process

Fermentation using *L. plantarum* strain machinery under optimal condition of temperature, pH for total consumption of nutrients was set up to transform soybean extract into a specific mixture of peptides, aminoacids and lactic acid with beneficial effects on skin. After removal of *L. plantarum* biomass and mixing with adjuvants to ensure its stability, we obtained Phytofirm Biotic as a liquid solution (INCI: *Lactobacillus*/Soybean Ferment Extract, Pentylene Glycol, Caprylyl Glycol) titrated in peptides (0.4 - 0.8%) and lactic acid (0.5 - 1.5%).

Phytofirm Biotic characteristics

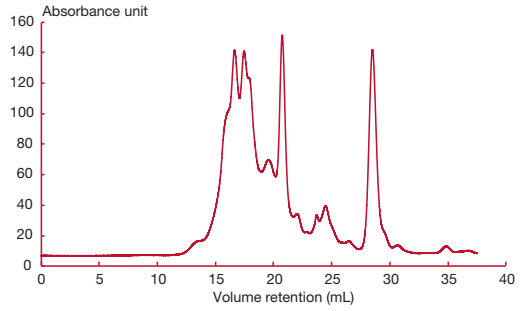


Figure 6 - Molecular weight profile of Phytofirm Biotic (gel permeation chromatography).

Phytofirm Biotic mainly contains peptides and lactic acid generated by *L. plantarum* fermentation. Lactic acid is a postbiotic (probiotic metabolite) naturally present in the skin. It is commonly used in cosmetics to gently accelerate cell renewal and to give radiance to the skin; some authors have shown that lactic acid participates to synthesis of epidermal and dermal compounds for skin rejuvenation [Yamamoto *et al*, 2006, Smith *et al*, 1996]. Finally, lactic acid helps to increase production of ceramides in keratinocytes thus reinforcing skin barrier and preserving skin moisturization [Rawlings *et al*, 1996].
Peptides are mainly of low molecular weight with an average MW ~5 kDa.

Aminogram i.e aminoacid analysis after complete hydrolysis of Phytofirm Biotic peptides

Amino acids *	**Phytofirm Biotic** (sample without preservative - expressed as relative %)
Cystein + Cystine	3.30
Alanine	5.80
Aspartic acid	11.60
Arginine	5.80
Glutamic acid	18.20
Glycine	5.29
Histidine	3.05
Isoleucine	5.42
Leucine	7.85
Lysine	6.82
Phenylalanine	4.14
Proline	5.89
Serine	3.91
Tyrosine	3.25
Valine	5.94
Threonine	3.74

Table 1 - Typical composition of Phytofirm Biotic in amino acids (after total hydrolysis).
(*) by liquid chromatography after acid hydrolysis according to ISO 13903:2005

Phytofirm Biotic contains amino acid like Glycine and Proline involved in the synthesis of ECM component like Collagen I.

Comparison of the activity of two extracts obtained by fermentation (Phytofirm Biotic) and enzymatic hydrolysis

We used a fermentation process to achieve transformation of soybean extract into a valuable mixture of peptides and lactic acid for anti-aging properties. The efficacy of Phytofirm Biotic on the synthesis of the major ECM component collagen I was compared to a soybean protein hydrolysate obtained by enzymatic hydrolysis.

The experiment was validated using Vit C at 50 µM as positive control of stimulation (128% ± 11). The results showed that Phytofirm Biotic at 2% stimulated significantly collagen I synthesis in fibroblast while soy protein enzymatic hydrolysate didn't show a significant stimulation compared to untreated control. Moreover, Phytofirm Biotic efficacy was statistically higher than that of soy protein hydrolysate obtained by enzymatic hydrolysis, evidencing thus the advantage of the fermentation by *Lactobacillus plantarum vs* enzymatic hydrolysis for skin anti-aging application.

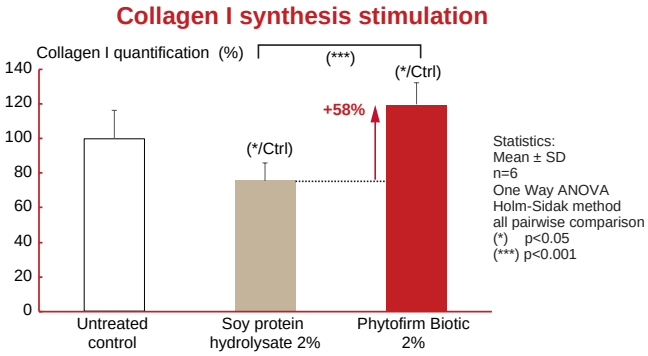


Figure 7 - Comparison of Phytofirm Biotic and Soy protein hydrolysate effect on collagen I synthesis in fibroblasts.

MATERIALS&METHODS

Cell culture

Normal human dermal fibroblasts obtained from an abdominal biopsy from a 63 year old donor were cultured in monolayers and grown to confluence in 96 well-plates, thereafter incubated 48h at 37°C and 5% CO_2 in the presence of the ingredients to test or without (untreated control)

Test products

Test ingredients were vitamin C (ascorbic acid) at 50 µM as positive control, Lactobacillus/Fermented Soybean Extract without additives corresponding to Phytofirm Biotic at 2% and soy protein hydrolysate at 2%.

Assay

Collagen was assayed using a method developed by BASF Beauty Creations (Immunoassay method for *in vitro* measurements). The cells were incubated again for 48h in the presence of the products to be assayed. The medium was then discarded and a dedicated lysis solution was added. This solution allows to disrupt cell membranes without solubilizing the deposited matrix. Then, the lysis buffer was removed and replaced by a PBS/BSA saturation solution (Perkin Elmer, Courtaboeuf, France). The primary anti-collagen I antibody (Interchim, Montluçon, France) were added. After rinsing in PBS, the Europium-conjugated secondary antibody (Perkin Elmer) was added. Finally, a specific enhancement solution (Perkin Elmer) was added. Fluorescence intensity was read (λexc. 340 nm / λem. 615 nm) using a Victor V² plate reader (Perkin Elmer).

Results and statistics

Results are expressed as mean % of collagen I *vs*. untreated control. The statistical comparison was performed running Student's t test for vitamin C and untreated control and using One way ANOVA followed by Holm Sidak method (all pairwise comparison).

Safety / tolerability of the product

Phytofirm Biotic was tested to ensure its safety under the recommended conditions of use. Phytofirm Biotic does not irritate the eyes or skin and no indication of skin sensitization was observed.
Phytofirm Biotic is a preservative-free probiotic ferment titrated in peptides and lactic acid.

Description

INCI name: Lactobacillus/Soybean Ferment Extract, Penthylene Glycol, Caprylyl Glycol

Naturalness content (according to ISO 16128): 99.5% from natural origin

Dose of use: 2%

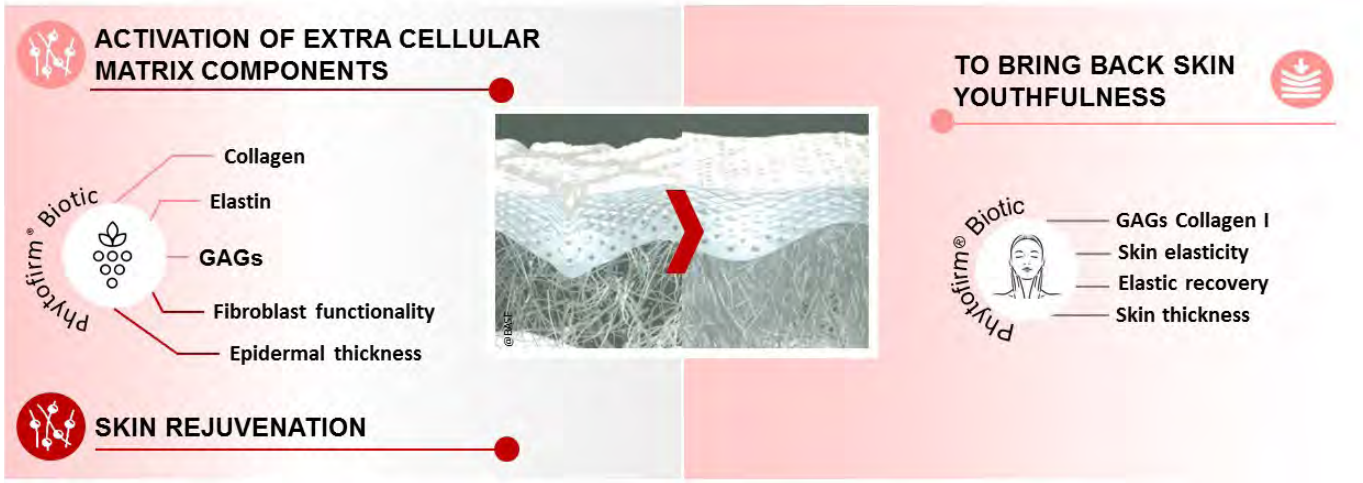
China IECIC listed

** Vegan Friendly

Example: illustration of a commercial sample of Phytofirm Biotic, with an emulsion and hydrogel containing it at 2%.

**No involvement of animal genes or animal-derived substances; unlikely cross-contamination from animal substances; no animal testing has been carried out by or on behalf of BASF on the ingredients of the product after 11th March 2009 and/or after the extended deadline 11th March 2013 for the purposes of the Cosmetic Regulation (EU) 1223/2009 (statement available upon request)

DEMONSTRATED EFFICACY



Phytofirm Biotic's abilities to rejuvenate the skin has been evaluated *in vitro* and *in vivo*.

***In vitro* performance**

The abilities of Phytofirm Biotic to activate the synthesis of extracellular matrix components was evaluated using several models:

- it increased elastin synthesis by fibroblasts, contributing thus to the improvement of skin elasticity and resilience,
- it boosted collagen I and GAGs synthesis by fibroblasts, in monolayer culture and 3D model, helping thus to replenish skin ECM,
- it boosted collagen V synthesis by fibroblasts, contributing thus to a better organization of collagen fibers network in ECM,
- it participates in the protection of the ECM component elastin by inhibiting elastase activity,
- it preserved fibroblast functionality by limiting TGFβ1-induced transformation of fibroblast into myofibroblast and by limiting their contraction in a collagen lattice model,
- it also showed its ability to increase epidermis thickness in a 3D model, helping thus to rejuvenate the epidermis.

***In vivo* performance**

Phytofirm Biotic demonstrated its capacity:

- to increase GAGs and collagen I level within skin,
- to improve skin elasticity and elastic recovery as well as to increase skin thickness on healthy volunteers.

EFFICACY

Stimulation of skin extracellular matrix components

Stimulation of elastin, collagen, hyaluronic acid and chondroitine sulfate GAGs synthesis

in vitro

OBJECTIVE

During aging, ECM components decrease in terms of quantity and their organization is impaired. Elastin, collagen and GAGs all involved in the maintenance of dermis and skin biomechanical properties are decreased; elastin and collagens, become more susceptible to degradation.

The aim of this study was to assess Phytofirm Biotic capacity to enhance elastin, collagens, and GAGs synthesis in dermal fibroblasts monolayers using conventional colorimetric and radiolabeling technics.

RESULTS & DISCUSSION

The results presented in Figure 8 show that Phytofirm Biotic significantly stimulated fibroblast synthesis of elastin by 34%. The activity of Phytofirm Biotic on fibroblast synthesis of collagens by fibroblasts was evaluated using a sensitive technique based on radioactivity.

The results presented in Figure 9, show that Phytofirm Biotic increased significantly synthesis of collagens by 59%.

Finally, hyaluronic acid and chondroitin 4 sulfate GAGs synthesis by fibroblasts was assessed by a radiolabellig assay.

The results presented in Figure 10 show that Phytofirm Biotic significantly stimulated the fibroblast synthesis of hyaluronic acid (HA) and of chondroitin 4 sulfate (C4S) by +25 and +28%, respectively

Elastin stimulation in dermal fibroblasts

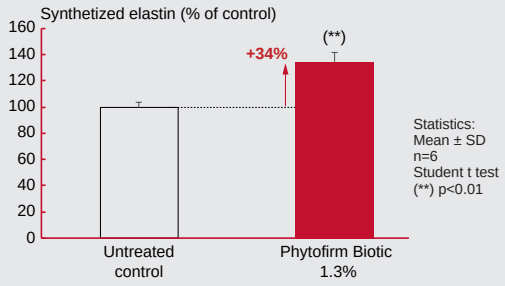


Figure 8 - Effect of Phytofirm Biotic on elastin synthesis in dermal fibroblast monolayer.

Collagen stimulation on skin fibroblasts

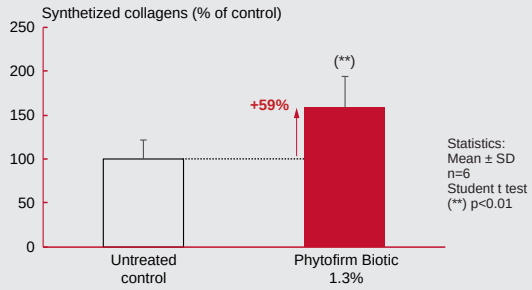


Figure 9 - Effect of Phytofirm Biotic on collagen synthesis in dermal fibroblast monolayer.

Stimulation of GAGs synthesis in fibroblasts

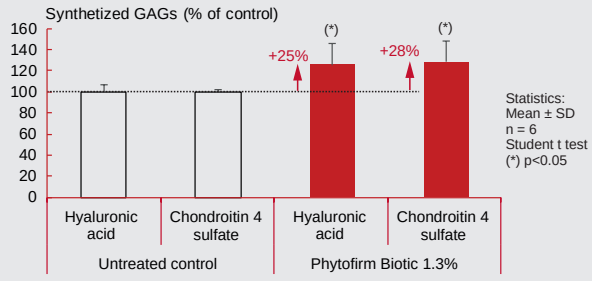


Figure 10 - Effect of Phytofirm Biotic on GAGs synthesis in dermal fibroblast monolayer.

These 3 experiments evidenced the capacity of Phytofirm Biotic to stimulate the production of elastin, collagens, and GAGs which are key components of ECM. These Phytofirm Biotic capacities will be further evaluated using 3D models.

CONCLUSION

In vitro Phytofirm Biotic showed a capacity to boost the synthesis of key components of ECM, collagens, elastin, and the GAGs hyaluronic acid and chondroitin 4 sulfate, and thus its potential to regenerate the ECM.

MATERIALS&METHODS

Elastin quantification

Normal human dermal fibroblasts were cultured for 3 days in DMEM containing 10% of bovine fetal serum (BFS). The cells were then cultured for 3 additional days in DMEM supplemented with 2% BFS in the absence (untreated control) or in the presence of the Lactobacillus/Soybean Ferment Extract at a concentration corresponding to Phytofirm Biotic at 1.3%.
At the end of the culture period, the elastin contained in cell incubation media was quantified using a dosage kit based on the method described by Winkelman & Spicer, 1962.

Collagens and GAG quantification

Cell culture

Normal human dermal fibroblasts were cultured for 7 days in the DMEM culture medium containing 10% of BFS. The cells were then cultured for 7 more days in DMEM medium supplemented with 5% BFS in the absence (untreated control) or in the presence of the Lactobacillus/Soybean Ferment Extract at a concentration corresponding to Phytofirm Biotic at 1.3%.

Collagen quantification

On the thirteenth day of culture, 5 µCi/ml of tritiated proline were added to the fibroblasts incubation medium. After an additional 24 hours of incubation, synthesized collagens were quantified in the cell layer by means of radioactivity counting and the total proteins were dosed by a method using bicinchoninic acid (data not shown)

In order to evaluate the specificity of the effects of the active ingredients on the synthesis of collagens, we could not take into account the stimulating activity of these components on cell proliferation. Quantities of synthesized collagens in response to the various treatments were therefore compared to total proteins contained in the culture wells [Smith *et al*, 1985].

GAG quantification

At the end of the culture period, GAGs contained in the culture media were separated by gel electrophoresis after alkaline digestion and ethanolic precipitation. This method enabled, by colorimetric densitometry, identification and quantification of the various types of GAGs (hyaluronic acid, chondroitin 4 sulfate) by comparison with standards co-separated during the same electrophoresis.

Results and statistics

The results are expressed in percentage, as the mean ± standard deviation (SD) compared to the untreated control standardized to 100%. Each condition was carried out in 6 replicates (n=6). Statistical analysis *vs* the untreated control was done after normal distribution comparison of the values (Shapiro-Wilk test) following Sigmaplot software recommendations (Systat Software Inv. USA) using Student t test. The threshold of significance was set to 5% ($p<0.05$).

EFFICACY

Stimulation of skin extracellular matrix components:

Stimulation of collagens, hyaluronic acid and chondroitin 4 sulfate GAGs synthesis in reconstructed dermis

in vitro

OBJECTIVE

The aim of these experiments was to confirm in reconstructed dermis 3D model the stimulatory effect of Phytofirm Biotic on ECM components collagens and GAGs.

RESULTS & DISCUSSION

The results presented in Figure 11 below show that Phytofirm Biotic at 1.3% significantly increased fibroblast synthesis of collagens in reconstructed dermis: +65%.

Worth to note that this effect may be considered as specific since its extent greatly exceeds the activity of Phytofirm Biotic on the neo-synthesis of total proteins (+ 8%; data not shown).

The results presented in Figure 12 show that Phytofirm Biotic at 1.3% significantly increased the synthesis of chondroitin 4 sulfate (C4S) and hyaluronic acid (HA): +39 and +68%, respectively (Figure 12).

Synthesis of these two GAGs which are particularly involved in the hydration of the dermis, decreases rapidly in the dermis with aging [Binic *et al*, 2013].

Collagen synthesis stimulation in 3D model

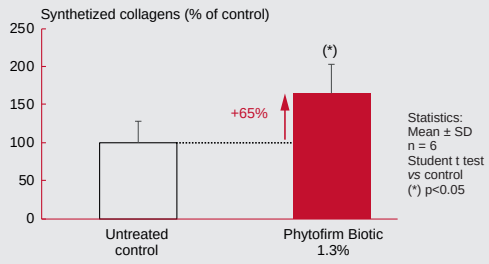


Figure 11 - Effect of Phytofirm Biotic on collagen synthesis in reconstructed dermis model.

GAG synthesis in 3D model

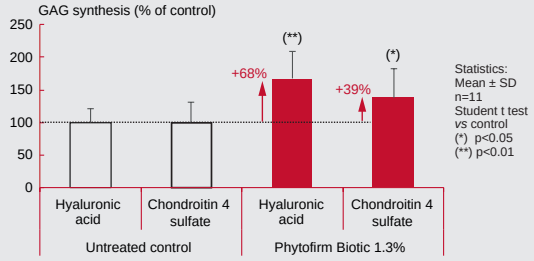


Figure 12 - Effect of Phytofirm Biotic on GAG synthesis in reconstructed dermis model.

This second series of experiments therefore confirmed that Phytofirm Biotic presents a stimulating effect on the synthesis of some GAGs and a specific action on the production of collagens by fibroblasts, which are essential components of the extracellular matrix.

CONCLUSION

The stimulation of collagen and GAG syntheses have been confirmed on 3D reconstructed dermis model, confirming thus the capacity of Phytofirm Biotic to participate in the regeneration of skin ECM.

MATERIALS&METHODS

Preparation of reconstructed dermis

Normal human dermal fibroblasts were seeded on collagenic matrixes Mimedisc at density of 100,000 cells per cm². After 21 days of culture in DMEM supplemented with 10% of Fetal Bovine Serum (FBS), the reconstructed dermis Mimederm were incubated for 5 (for GAGs) or 8 days (collagens) in DMEM + BFS 5%, in the absence (untreated control) or presence of the Lactobacillus/Soybean Ferment Extract at a concentration corresponding to Phytofirm Biotic at 1.3%. The syntheses of total proteins, collagens and GAGs, were quantified using specific, sensitive methods.

Assays protocol

Collagen Assay

On the seventh day, 5 µCi/ml of tritiated proline were added to the medium. After additional 24 hours incubation, total synthesized proteins were quantified in culture media by measurement of the radioactivity present. Radioactivity measurements performed in the same media after action of the collagenase allowed the quantification of synthetized collagens [Diegelmann & Peterkofsky, 1972].

GAG Assay

After 5 days of incubation, GAGs were quantified in culture media after electrophoretic separation on cellulose acetate gels using the method described by Cappeletti *et al*, 1979.

Briefly, after migration, GAGs were stained by Alcian Blue in the electrophoresis gels. After discoloration and drying of the latter, GAGs were quantified by colorimetric densitometry. Interestingly, this method enables the identification and differential quantification of the various types of synthesized GAGs (hyaluronic acid, chondroitin 4 sulfate) by comparison with co-separated standards on the same electrophoresis gel.

Results and statistics

The results are expressed in percentage, as the mean ± standard deviation (SD) compared to the untreated control standardized to 100%. Each condition was carried out in 6 replicates (n=6) for collagens and 11 replicates (n=11) for GAGs. Statistical analysis *vs* the untreated control was done after normal distribution comparison of the values (Shapiro-Wilk test) following Sigmaplot software recommendations (Systat Software Inv. USA) using Student t test. The threshold of significance was set to 5% ($p<0.05$).

EFFICACY

Stimulation of skin extracellular matrix components:

Stimulation of collagen I and collagen V synthesis and deposition

in vitro

OBJECTIVE

The aim of this study was to measure the ability of Phytofirm Biotic to increase synthesis and the deposition of collagen I and V in the extracellular medium in an *in vitro* model of cultured dermal fibroblasts using immunohistochemistry method.

RESULTS & DISCUSSION

The method used in this experiment is based on the Delfia method (Perkin Elmer), which quantify extracellular deposited collagen I. Using the unique properties of lanthanide chelates, Delfia allows reducing background noise and increasing signal intensity for highly specific and sensitive measurements. The proteins (here collagen I and V) that have deposited on the culture plate can be measured very precisely. Vitamin C (ascorbic acid) at 50 µM, a well-known enhancer of procollagen hydroxylation and secretion, significantly increased collagen I and V deposition by 31% ($p<0.01$) and 34% ($p<0.05$) respectively, which validated the experiment.

Results obtained show a dose related efficacy of Phytofirm Biotic on the synthesis of deposited collagen I up to +23% ($p<0.05$) at 2.1% dosage (Figure 13).

Phytofirm Biotic turned out to have an even greater effect on collagen V production (Figure 14). Collagen V plays a crucial role in the 3D organization of collagen I and is thus of major importance for collagen network functionality and as a consequence, for skin biomechanical properties also.

Here again, a dose-related stimulatory effect of Phytofirm Biotic on collagen V deposition was observed. A significant stimulation was observed from 1.3% (+16%, $p<0.05$) and a maximum increase was obtained at 2.1% (+51%, $p<0.05$).

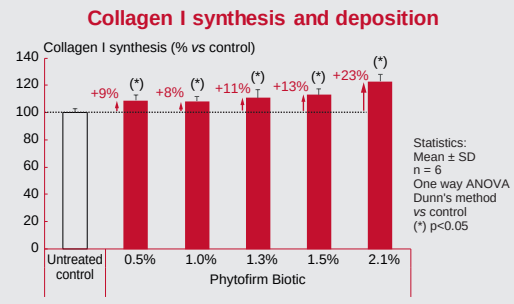


Figure 13 - Effect of Phytofirm Biotic on collagen I synthesis and deposition in the extracellular media of dermal fibroblast monolayer.

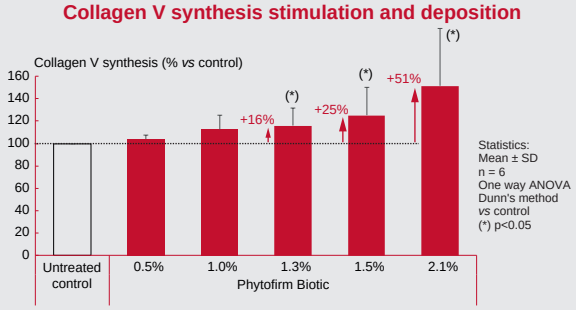


Figure 14 - Effect of Phytofirm Biotic on collagen V synthesis and deposition in the extracellular media of dermal fibroblast monolayer.

CONCLUSION

These results shows the ability of Phytofirm Biotic to increase the synthesis and deposition of collagen I and V, and thus its potential to improve collagen fibrillogenesis and ultimately ECM structure and quality.

MATERIALS&METHODS

Cell culture

Normal human dermal fibroblasts obtained from an abdominal biopsy from a 63 year old donor were cultured in monolayers and grown to confluence in 96 well-plates, thereafter incubated 48h at 37°C and 5% CO_2 in the presence of the ingredients to test.

Treatments

Test ingredients were vitamin C (ascorbic acid) at 50µM, a positive control and the Lactobacillus/Soybean Ferment Extract corresponding to Phytofirm Biotic at (0.5; 1; 1.3; 1.5 and 2.1%). Cells grown in the cultured medium alone were used as untreated control.

Delfia method

Collagen was quantified using a method developed by BASF Beauty Care Solutions (patented immunoassay method for *in vitro* measurements). The cells were incubated again for 48h in the presence of the products to be assayed. The medium was then discarded and a dedicated lysis solution was added. This solution allows to disrupt cell membranes without solubilizing the deposited matrix. Then, the lysis buffer was removed and replaced by a PBS/BSA saturation solution (Perkin Elmer, Courtaboeuf, France). The primary anti-collagen I or anti-collagen V antibodies (Interchim, Montluçon, France) were added. After rinsing in PBS, the Europium-conjugated secondary antibody (Perkin Elmer) was added. Finally, a specific enhancement solution (Perkin Elmer) was added. Fluorescence intensity was read (λexc. 340 nm / λem. 615 nm) using a Victor V² plate reader (Perkin Elmer).

Results and statistics

Results are expressed as mean % of collagen I or collagen V synthesis *vs*. untreated control. The statistical comparison was performed running Student's t test for vitamin C and untreated control comparison and using One way ANOVA followed by Dunn's procedure for Phytofirm Biotic and untreated control comparison.

EFFICACY

Stimulation of skin extracellular matrix components:

Improvement of quality of collagen I and collagen V synthetized in fibroblasts culture

in vitro

OBJECTIVE

The aim of this study was to evaluate the effect of Phytofim Biotic on the quality of collagen I and V synthetized in dermal fibroblasts. In order to assess the quality, we have better characterized the network of neosynthesized collagen I and V fibers, by measuring the length and preferred orientation of collagen fibers synthetized.

RESULTS & DISCUSSION

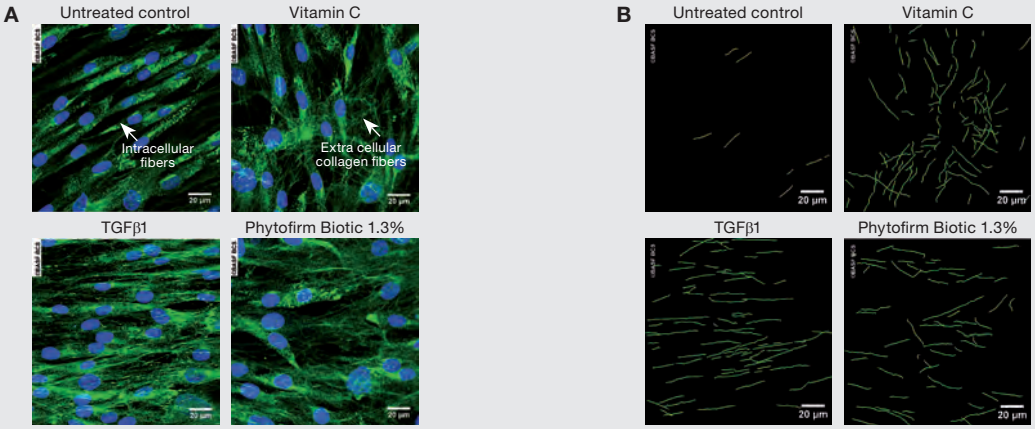


Figure 15 - **A** - Expression of collagen I in monolayer culture of fibroblasts. Collagen I in green, nuclei in blue. **B** - Orientation and length of the network of type I collagen fibers in monolayer culture of fibroblasts.

Phytofirm Biotic at 1.3% increased collagen I deposition outside the cell and the formation of collagen fibers as the positive control vitamin C (50 µM) and TGFβ1 (1 ng/ml).
In contrast, (pro)collagen I is mainly expressed in the cytoplasm of cells in the untreated condition (Figure 15A) showing a delay in the secretion and then in the maturation of the collagen I.
Phytofirm Biotic at 1.3% provided a network of entangled collagen I fibers as the positive control Vitamin C (50 µM). In contrast, the orientation of network of collagen I fibers are totally longitudinal after treatment with the wound healing marker TGFβ1. Very few fibers of collagen I are observed in the untreated condition (Figure 15B)

All treatments allowed a large and significant improvement in fiber length as compared to the untreated control, of 7.2 fold. Phytofirm Biotic at 1.3% increased collagen I fibers formation which are as long as those produced by the positive control TGFβ1 (Figure 16).

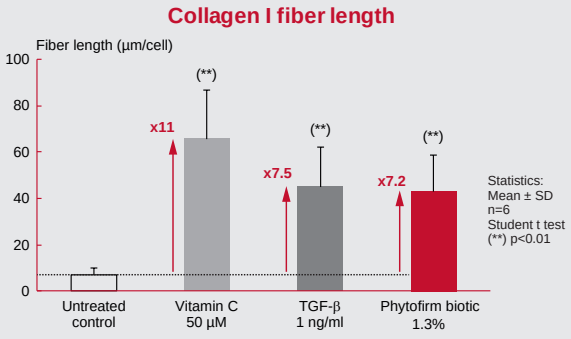


Figure 16 - Effect of Phytofirm Biotic on collagen I fibers length in monolayer culture of fibroblasts.

in vitro

Phytofirm Biotic at 1.3% increased collagen V deposition outside the cell and the formation of collagen fibers as the positive control vitamin C (50 µM) (Figure 17A). In contrast, when TGFβ1 (1 ng/ml) is applied (pro)collagen V is mainly expressed in the cytoplasm of cells as in the untreated condition, showing a delay in the secretion and then in the maturation of the collagen V.

Phytofirm Biotic at 1.3% provided a network of entangled collagen V fibers as the positive control vitamin C (50 µM). In contrast, a longitudinal network of intracellular collagen V fibers was observed with healing marker TGFβ1 and in the untreated condition (Figure 17B).

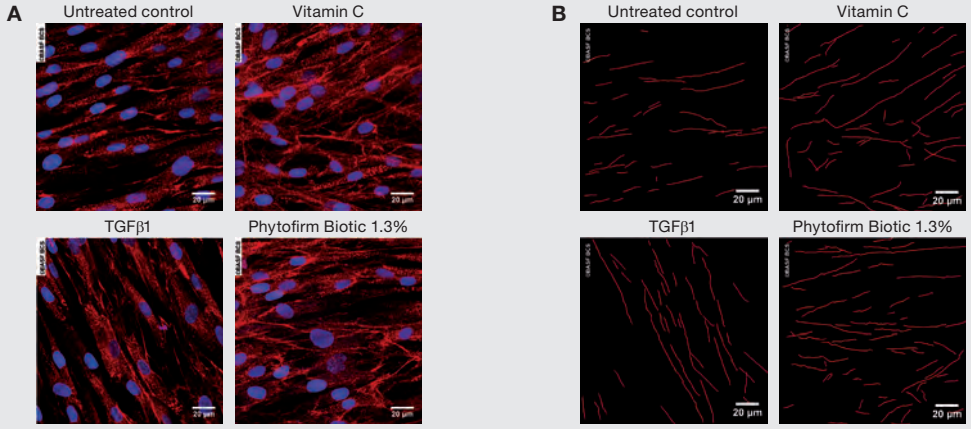


Figure 17 - **A** - Expression of type V collagen in monolayer culture of fibroblasts. Type V collagen in red, nuclei in blue.
B - Orientation of the network of type V collagen fibers in monolayer culture of fibroblasts.

Only vitamin C and Phytofirm Biotic at 1.3% treatment increased the length of collagen fibers organized within collagen V. Both treatments allowed a significant increase *vs* the untreated control.

Phytofirm Biotic at 1.3% increased type V collagen fibers formation which are longer than those produced by the positive control TGFβ1 (Figure 18).

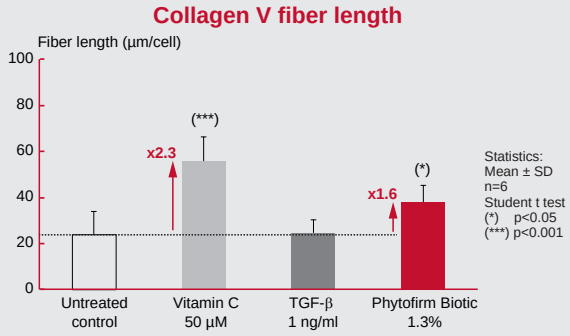


Figure 18 - Effect of Phytofirm Biotic on length of type V collagen fibers in monolayer culture of fibroblasts.

CONCLUSION

We showed that Phytofirm Biotic at 1.3% allowed the formation *in vitro* of network of collagen I and V fibers displaying an entangled organization and elongated fibers. When used at 1.3%, Phytofirm Biotic clearly demonstrated its ability to promote the extracellular deposition and organization of collagen I and V fibers by fibroblasts, and thus its potential to improve the ECM quality.

MATERIALS&METHODS

Cell culture

Normal human fibroblasts were seeded in culture chambers (LabTek Chamber Slide System, Fisher Bioblock Scientific, Illkirch, France) and cultivated until confluence in FGM medium.

Treatments

At cell confluence, fibroblasts were treated for 3 additional days with either culture medium (untreated control), or vitamin C 50 µM, TGFβ1 1 ng/ml or the Lactobacillus/Soybean Ferment Extract at a dose corresponding to Phytofirm Biotic at 1.3%.

Immunohistochemistry

After treatments, the cell cultures were fixed in methanol, and then saturated in a PBS/BSA solution. The anti-collagen I and V antibodies (Novotec, Lyon, France) were then added. After rinsing, the Alexa Fluor 488 conjugated secondary antibody (Invitrogen) was added.

Microscope and Image analysis

The labeling of collagens I and V was observed using a confocal microscope LSM700 (Zeiss, Le Pecq, France). Detection was made using a MBF ImageJ plugin (NeuronJ, Erik Meijering). 6 images per experimental condition were quantified. Data were normalized to the cell number and were expressed in µm/cell

Results and Statistics

Fibers length data were normalized to the cell number and were expressed in µm/cell. The statistical comparison was performed running Student t test for vitamin C, TGFβ1 and Phytofirm Biotic.

EFFICACY

Protection of skin extracellular matrix: effect of Phytofirm Biotic on human elastase activity

in vitro

OBJECTIVE

In the course of skin aging, elastin synthesis is reduced, and elastase activity is accelerated, resulting in skin sagging and reduced skin elasticity [Zhao *et al*, 2009]. Under inflammatory conditions like in stretch marks, Human Leukocyte Elastase (HLE) is an enzyme present in granules of neutrophil [Warren *et al*, 2001]. Once released from activated leukocytes, HLE can degrade by proteolysis various ECM structural components such as collagens, proteoglycans and more particularly elastin. We aimed to assess the effect of Phytofirm Biotic on the human elastase activity for a protection of ECM from degradation.

RESULTS & DISCUSSION

In this assay, the reference ingredient, alpha-1-antitrypsin inhibited significantly the activity of Human Leukocyte Elastase (HLE, 82 ± 2%). This result was expected and validated the assay on HLE.
Phytofirm Biotic at 0.1 and 0.5% has significantly inhibited the enzymatic activity of HLE by respectively 21% and 48% (Figure 19).

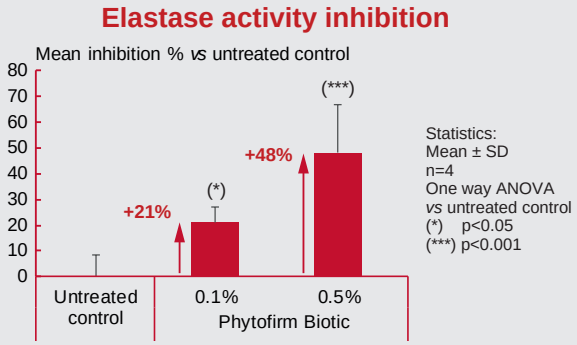


Figure 19 - Phytofirm Biotic effect on human elastase activity.

CONCLUSION

Phytofirm Biotic starting at 0.1% has significantly inhibited the enzymatic activity of human leucocyte elastase. Therefore, it has the potential to protect the skin against ECM destructuring, and more particularly elastic fibers to maintain skin elasticity.

MATERIALS&METHODS

Assay

The enzyme elastase (human leukocytes, SIGMA E8140) at 0.5 mU, and active ingredients Phytofirm Biotic (at 0.1% and 0.5%) and alpha-1-antitrypsin at 0.0003 mg/mL were diluted in a TRIS-HCl buffer (Sigma T3253) containing Triton X-100 (Sigma T8532) at 0.1% and balanced at pH=7.5. In the same time, the enzymatic substrate N-Metoxysuccinyl-(Ala)2-Pro-Val-pNA (SIGMA M4765) was dissolved in DMSO. After an incubation of 60 minutes, the quantity of hydrolyzed substrate was measured by recording the optical density at 405 nm.

Statistics

The results were calculated in % of inhibition *vs* the untreated control (results of enzymatic activity without any ingredient) and presented as a mean ± Standard deviation (SD). Statistical evaluations were based on 1 assay made in quadruplate and using One Way ANOVA according to Sigmaplot software recommendation.

EFFICACY

Stretch marks formation minimization: effect of Phytofirm Biotic on skin fibroblasts transformation into myofibroblasts

in vitro

OBJECTIVE

In striae distensae (SD) or stretch marks a disorganized elastic fiber network has been evidenced and a local increase in TGFβ1 bioavailability, which alters locally the ECM homeostasis [Doyle *et al*, 2012]. An excessive TGFβ1 signaling promotes transformation of fibroblasts into myofibroblasts [Doyle *et al*, 2012] which express alpha smooth muscle actin (α-SMA) and have a more contractile phenotype [Viennet *et al*, 2005] than an extracellular matrix component producer. The aim of this study was to assess Phytofirm Biotic effect on fibroblast transformation into myofibroblast by staining and quantification of alpha smooth muscle actin (α-SMA) expression.

RESULTS & DISCUSSION

TGFβ1 at 0.1ng/mL has highly and significantly stimulated the expression of α-SMA in fibroblasts. Dexamethasone tested at 1µM, has significantly inhibited TGFβ1 effect on fibroblasts and decreased expression of α-SMA (-71%; $p<0.05$; Figure 20B). This inhibition was expected and thus validated the experiment.

In these experimental conditions, Phytofirm Biotic at 1% had significantly inhibited by 72% ($p<0.05$) the stimulation induced by TGFβ1 on α-SMA expression in fibroblasts (Figure 20 A and B). This effect is similar to the effect observed with the positive control dexamethasone.

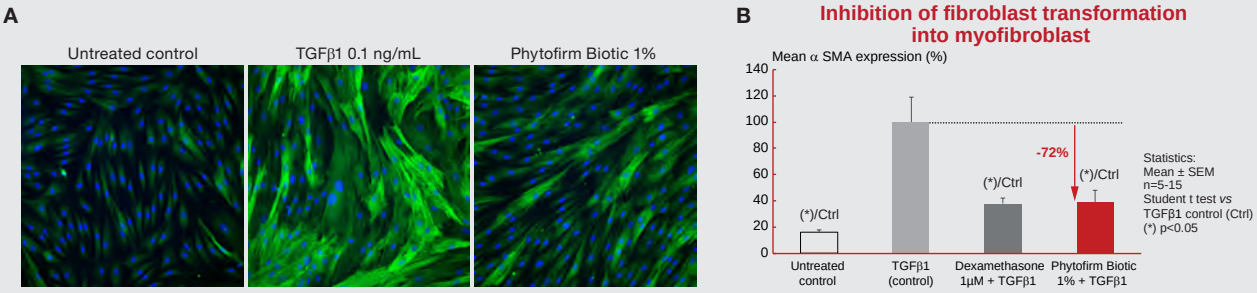


Figure 20 - **A** - Visualization of Phytofirm Biotic effect on α-SMA expression in fibroblasts. α-SMA in green and nuclei in blue. **B** - Effect of Phytofirm Biotic on α-SMA expression in fibroblasts

CONCLUSION

Phytofirm Biotic inhibited transformation of fibroblast into myofibroblast preserving thus their phenotype and their functionality regarding ECM components synthesis. Through this activity, Phytofirm Biotic participates in minimizing stretch marks appearance.

MATERIALS&METHODS

Cell culture and treatment

Fibroblasts were seeded in 96 well plates and grown in DMEM medium supplemented with 10% FCS (fetal calf serum) at 37°C under 5% of CO_2 and 95% of relative humidity for 24h. The culture medium was then removed and replaced by DMEM supplemented with 1% FCS containing TGFβ1 0.1ng/mL alone, TGFβ1 0.1 ng/mL with the active ingredients: Lactobacillus/Soybean Ferment Extract used at a concentration corresponding to Phytofirm Biotic 1% or Dexamethasone 1µM. The culture medium alone was used as untreated condition.

Fibroblast treated with active ingredients and controls were incubated 72hours. All conditions were treated in triplicate.

α-SMA immunostaining

After incubation the medium was discarded, and cells rinsed and fixated. Cells were then incubated with primary anti-α-SMA antibody followed by secondary antibody coupled to a fluorochrome (GAM-Alexa 488). In parallel nuclei were colored with Hoechst 33258 (bis-benzimide).

Microscope observation

Images aquisition were made using a high resolution imagery system INCell Analyzer 2200 (GE-Healthcare). For each well, 5 images were taken (magnification X20).

Stainings were quantified by measuring the area of fluorescence of α-SMA normalized to the nuclei number identified by Hoechst 33258 coloring (numeric data were integrated in software Developet Toolbox 1.5, GE Healthcare).

Results and statistics

Results were expressed as mean percentage expression of α-SMA ± standard error on the mean

TGFβ1 was used as positive control of SMA expression and set up at 100%. Statistical comparison to TGFβ1 control were made with minimum 5 images of the same condition using Student t test. Threshold of significance was set at 5%.

EFFICACY

Stretch marks formation minimization: effect of Phytofirm Biotic on skin fibroblasts contraction

in vitro

OBJECTIVE

The purpose of this test was to evaluate the potential of Phytofirm Biotic to decrease the TGFβ1-stimulated contraction of Fibroblast Populated Collagen Lattice (FPCL). In fact, it has been demonstrated that fibroblasts from early SD have the highest content of alpha-smooth muscle actin and have a more contractile phenotype [Viennet *et al*, 2005], a trait they have in common with fibroblasts from scarred skin [Chen *et al*, 2005]. These characteristics of dermal fibroblasts of stretch marks could be due to a local excess of TGFβ1 activity [Doyle *et al*, 2012].

RESULTS & DISCUSSION

FPCL is an *in vitro* model based on the potential of fibroblasts to condensate a collagen gel into a lattice with a dermis-like structure [Bell *et al*, 1979]. The condensation of free floating FPCL result in mechanically relaxed tissue with fibroblasts displaying an aged phenotype [Grinnell 1994].
In this study, TGFβ1 at 10 ng/mL has significantly increased the contraction of FPCL by 67%. This result was expected and thus validated the experiment.
Phytofirm Biotic at 1% has significantly increased by 33 % ($p<0.001$) the collagen gel area of TGFβ1-treated FPCL. This corresponds to an inhibition by 50% of the retraction induced by TGFβ1 (Figure 21 A and B).

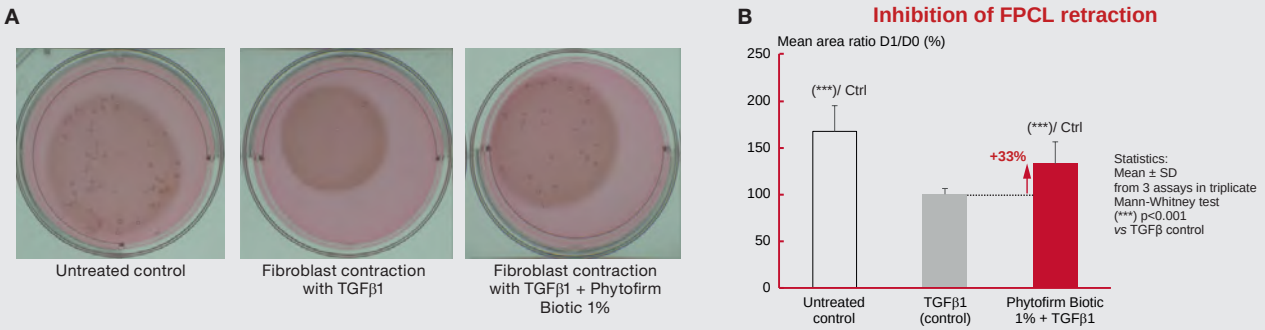


Figure 21 - **A** - Visualization. **B** - Effect of Phytofirm Biotic on TGFβ1-induced FPCL retraction (area measurements).

CONCLUSION

By inhibition of FPCL retraction induced by TGFβ1, Phytofirm Biotic contributes in delaying the formation of stretch marks and in improving overall skin aspect under mechanical tension.

MATERIALS&METHODS

Assay

FPCL building

Cell suspensions of human dermal fibroblasts from different donors (from 40 yo to 69 yo) seeded in 6 well plates were mixed in basal medium (EMEM + fetal calf serum FCS at 2%) containing TGFβ1 (Sigma France) at 10ng/mL with collagen solution (Sigma France) to obtain a gel of 2.5 mL involving 0.1 billion of fibroblasts and 0.45 mg/mL of collagen. The product Phytofirm Biotic at 1% was included from the beginning in cell suspension for lattice preparation. Then collagen gels were incubated for one day at 37°C, CO_2 at 5% and 95% of relative humidity.

FPCL analysis

Cell surface of fibroblasts populated collagen lattices (FPCL) was recorded by a CCD camera (Biorad France) and quantified by Image J software. Each condition was tested in triplicate, the results were expressed in % *vs* control (FPCL) with basal medium containing TGFβ1) and presented as a Mean ± standard deviation (SD).
Statistic evaluation were based on Student t test according to Sigmaplot software recommendations.

EFFICACY

Skin rejuvenation effect: effect of Phytofirm Biotic on epidermis thickness in 3D reconstructed skin

in vitro

OBJECTIVE

The goal of this study was to assess the effect of Phytofirm Biotic on epidermis thickness which decreases during aging and in stretch marks, using a 3D reconstructed skin model.

RESULTS & DISCUSSION

Phytofirm Biotic at 1.3% significantly increased by 2.2-fold (Figure 22) the thickness of viable epidermal layers in the 3D reconstructed skin as compared to the untreated condition.

Phytofirm Biotic through this action on epidermal thickness will contribute to protect the skin from visible aging signs and particularly the epidermis part from the thinning caused by aging.

Phytofirm Biotic which has demonstrated its ability to promote the fibroblast proliferation (data not shown) did not disrupt the number of Ki-67 positive-keratinocytes in the epidermis. This prove that Phytofirm Biotic at 1.3% did not impact the keratinocytes renewal rate (Figure 23). The thickening of the epidermis observed Figure 22 could be in part linked to rebalancing effect of Phytofirm Biotic on the expression of differentiation marker Transglutaminase 1 (data not shown).

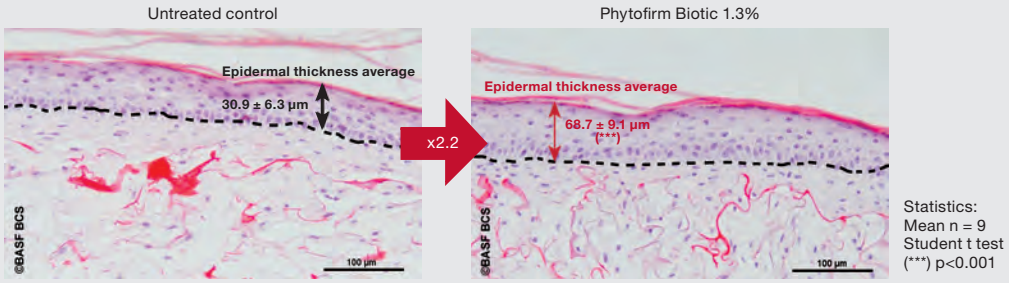


Figure 22 - **A** - Visualization and measurement of epidermal thickness in hematoxylin-eosin stained 3D reconstructed skin.

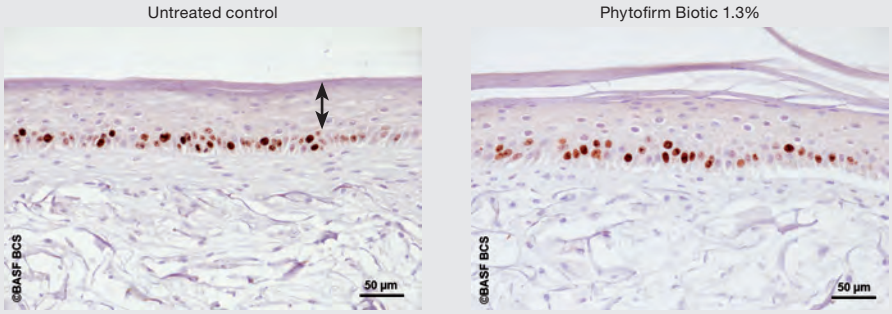


Figure 23 - Expression of Ki67 (in brown) in the 3D reconstructed skin.

CONCLUSION

We showed that Phytofirm Biotic 1.3% increases *in vitro* the epidermal thickness on 3D reconstructed skin. It can thus contribute to epidermal rejuvenation.

MATERIALS&METHODS

Preparation of Skin Equivalents (SE)

Dermal equivalent preparation: normal human dermal fibroblasts (20 years old) were seeded on the top of dermal substrate composed of collagens, glycoaminoglycan and chitosan at the density of 500,000 cells per cm^2.
All equivalents were cultivated in immerged condition for 28 days in fibroblast medium containing 10% of Fetal Bovine Serum (FBS) and 50 µg/ml vitamin C. The dermal equivalents were fed daily.

3D reconstructed skin preparation (Mimeskin): normal human keratinocytes were plated on dermal equivalents at the density of 250,000 cells per cm^2 and were cultivated in immerged condition for one week in keratinocyte medium containing 10% of FBS and 50 µg/ml vitamin C. The cultures were then elevated to the air-liquid interface for 2 additional weeks in keratinocyte medium containing 8 mg/ml of bovine serum albumin (BSA) and 50 µg/ml vitamin C.
Treatment: Lactobacillus/Soybean Ferment Extract at a dose equivalent to Phytofirm Biotic 1.3% was added during the whole culture except when fibroblasts and keratinocytes were seeded. Condition with the cultured medium alone was used as untreated control.

Protocol assay

Epidermal thickness

Histology: Mimeskin samples were stained in hematoxylin-eosin to measure epidermal thickness. Specimens were observed using a Zeiss Axioskop 2 Plus optical microscope (Zeiss) and images were captured using DS-Ri1 CCD camera (Nikon, Champigny-sur-Marne, France) and NIS-Elements software (Nikon).
Image analysis: epidermal thickness was obtained with an Euclidean distance map. Pixels corresponding to the epidermis were selected from other pixels.
Images corresponding to the epidermis were converted to a distance map. For each epidermal pixel a distance is measured to the basal layer. Average distance between the basal line and the stratum corneum is then calculated. 9 images (3 images per Mimeskin n=3 Mimeskin) per experimental condition were quantified. Data are expressed in µm per observed field.

Ki-67 immunostaining

Immunohistochemistry: Mimeskin samples were fixed in neutral buffered formalin 4% (Thermo Shandon, Pittsburgh, US) for 24 hours and then embedded in paraffin. Paraffin-embedded formalin-fixed samples were then cut into 6 µm sections.
After heat-mediated antigen retrieval treatment, tissue sections were incubated in peroxidase-blocking solution (Dako, Denmark) to inactivate endogenous peroxidase.
Non-specific binding was blocked in PBS containing 3% of BSA. Sections were then incubated with the Ki-67 primary antibody (clone MIB-1, Dako) diluted in PBS / BSA 3% for 2 hours at room temperature. After incubation for 1 hour with the peroxidase-conjugated secondary antibody (Envision, Dako), the antigen was detected with 3,3-diaminobenzidine tetrahydrochloride (Dako) as the substrate.
Mimeskin sections were subsequently counterstained using Gill's hematoxylin (Microm Microtech, France) for 1 minute. As a negative control, primary antibody was replaced by the corresponding isotype control.
Image acquisition: images were performed using an Axioskop 2 Plus optical microscope (Carl Zeiss, Le Pecq, France) with a Ds-Ri1 CCD camera (Nikon, Champigny-sur-Marne, France).

Results and statistics

For epidermal thickness results were expressed as average thickness expressed as mean ± SD in µm.
The statistical comparison was performed running Student's t test.

Conclusion *in vitro*

We demonstrated *in vitro* the antiaging and rejuvenating properties of Phytofirm Biotic.

Activation of extracellular matrix component synthesis through:
- increase in elastin synthesis in fibroblats monolayer (+34%),
- stimulation of collagen I (+23%) and V (+51%) production in fibroblasts monolayer, and of collagens (+65%) in reconstructed dermis,
- improvement of the length and organization of collagen I (x7.2) and V (x1.6) fibers,
- increase in GAG synthesis in fibroblast monolayer and in reconstructed dermis (Hyaluronic acid +68%; Chondroitin 4 sulfate +39%).

Protection of ECM and fibroblast functionality through:
- inhibition of elastase activity (-48%),
- inhibition of TGFβ1-induced transformation of fibroblast into myofibroflast (-72%) and contractile phenotype (-50%).

Rejuvenation of epidermis via:
- increase in epidermis thickness (x2.2) in reconstructed skin.

Altogether, Phytofirm Biotic showed capacities in rejuvenating skin by increasing production of extracellular matrix components, epidermis thickness, and by protecting ECM from destruction by inhibiting elastase activity. Moreover Phytofirm Biotic also showed abilities in preserving skin youthfulness and preventing stretch mark by avoiding the transformation of fibroblasts into overcontractile myofibroblasts.

EFFICACY

Stimulation of GAGs and Collagen I synthesis on healthy volunteers

in vivo

OBJECTIVE

The purpose of this study was to evaluate the stimulating efficacy on dermal GAGs and collagen I synthesis of Phytofirm Biotic formulated at 2%. The study was a half-face, placebo-controlled study with 9 female volunteers aged from 42-67 years old, about to undergo a face lift. After the face lift, GAGs and type I collagen were quantified in the surgical leftovers (Figure 24).

Volunteers applied Phytofirm Biotic at 2% on one face side and the placebo on the other side twice a day on each half face

Phytofirm Biotic  Placebo

After 4 weeks of application (quantification of GAGs and Collagen I) using colorimetry and RIA (RadioImmunoAssay) method on skin leftovers

Figure 24 - Clinical study on Phytofirm Biotic stimulating efficacy on GAGs and collagen I synthesis.

RESULTS & DISCUSSION

The results presented in Figure 25 show that after one month of the daily use of a formulation containing 2% of Phytofirm Biotic, there was an increase in the GAGs content in the dermis compared to the use of a placebo formulation.
The results presented in Figure 26 show that after one month of the daily use of a formulation containing 2% of Phytofirm Biotic, there was an increase in the quantity of collagen I in the dermis compared to the use of a placebo formulation.

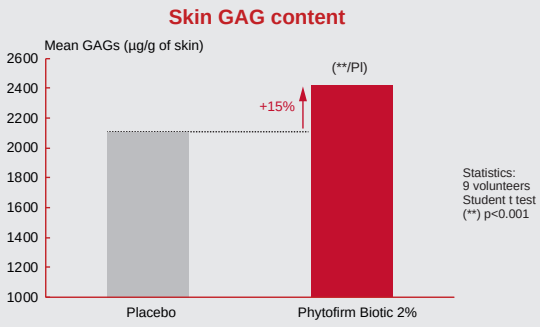


Figure 25 - Effect of Phytofirm Biotic on dermal synthesis of GAGs.

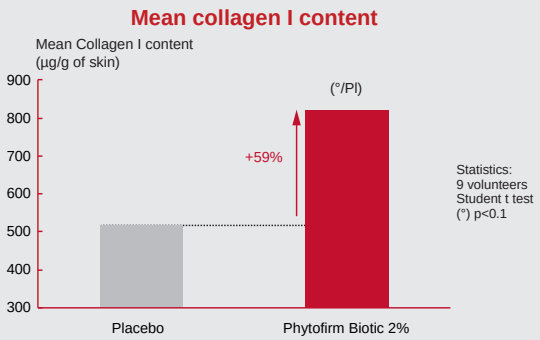


Figure 26 - Effect of Phytofirm Biotic on dermal synthesis of collagen I.

CONCLUSION

The 2% Phytofirm Biotic treatment improved skin production of GAGs and collagen I, thus contributing to its effectiveness in redesigning, rejuvenating skin face.

MATERIALS&METHODS

Study design

The clinical study was carried-out as a half-face placebo controlled study. The efficacy of the formulation containing Phytofirm Biotic at 2% was compared to that of a placebo. The study was conducted in France, in the winter season for a period of 28 days (one month), with measurement at D28.

Inclusion criteria

The study was done on a group of 9 healthy female volunteers, aged 42 to 67 years old due to undergo a face lift.

Application modality

The volunteers applied themselves under normal conditions of use, the products twice a day (once in the morning and once in the evening) for 28 days. Phytofirm Biotic and the placebo formula were applied to the right and the left half face.

Evaluation methods

Quantification of skin GAGs

The GAGs were extracted from the skin biopsies by alkali treatment. Recovery of the GAGs is processed from proteolytic digests by an ethanolic precipitation. GAGs are identified by cellulose acetate electrophoresis (comparison with hyaluronic acid, chondroïtin-4-sulfate, dermatan sulfate and heparan sulfate standards). GAGs are quantified by alcian blue colorimetric method [Gold E. W., 1979].

Collagen quantification

Collagen extraction is performed by pepsin treatment of the skin biopsies. The collagen I is processed by a RIA method using specific anti-human collagen type I (Polyclonal antibodies, IgG fraction - Pasteur Institute, France).

Results and statistics

The results are expressed as the mean quantity of GAGs and collagen I in skin biopsies percentage (in µg/g of skin).
The statistical analysis of the parameters has been done after checking the normality of distribution using Shapiro-Wilk test.
The statistical analysis of the change in the studied parameters for each product over time was performed as follows:
- validation of the normality of the studied parameters: paired t test,
- invalidation of the normality of the studied parameters: Wilcoxon test.
The statistical analysis of the differences in the studied parameters between the treatment groups was performed as follows:
- validation of the normality of the studied parameters: paired t test.

EFFICACY

Improvement of skin elasticity and skin thickness with Phytofirm Biotic for a youthful skin

in vivo

OBJECTIVE

The purpose of this study was to evaluate the skin rejuvenating efficacy on the jaw area of Phytofirm Biotic formulated at 2%. The study was a full-face, double-blind, placebo-controlled and randomized study with 42 female volunteers aged from 55-63 years old, considering themselves to have a loss of skin elasticity and firmness specifically in the jaw area (Figure 27).

RESULTS & DISCUSSION

Elasticity improvement

Figures 28-31 show the results obtained with Phytofirm Biotic and the placebo on the various skin elasticity parameters measured over the treatment time: net elasticity (R5) elasticity recovery (R7), gross elasticity (R2) all reported to decrease with age [Krueger, 2011] and viscous recovery (Q2). These parameters were measured after 2 months (D56), 3 months (D84) and 4 months (D112).

Net elasticity (R5) (Figure 28)

- Compared to their baseline, Phytofirm Biotic at 2% showed a 8%, 10%, and 65% significant increase ($p<0.05$) in net elasticity (R5) at D56, at D84 and at D112 respectively, while the placebo showed an effect only at D112.
- Compared to the placebo, Phytofirm Biotic showed a 19% and 31% significant improvement ($p<0.05$) in net elasticity (R5) at D56 and D112 respectively.

Elasticity recovery (R7) (Figure 29)

- Compared to their baseline, Phytofirm Biotic showed a 6% and 53% significant increase ($p<0.05$) in elasticity recovery (R7) at D56 and at D112 respectively, while the placebo showed a positive effect only at D112 (+31%).
- Compared to the placebo, Phytofirm Biotic showed a 18% and 22% significant improvement ($p<0.05$) in elasticity recovery (R7) at D56 and D112 respectively; and a 5% improvement ($p<0.1$) at D84.

Gross elasticity (R2) (Figure 30)

- Compared to their baseline, the Phytofirm Biotic showed a 22% significant increase ($p<0.05$) in R2 at D112, while the placebo showed a lower effect (8%, $p<0.1$) at the same time.
- Compared to the placebo, the Phytofirm Biotic showed significant improvement of the skin gross elasticity (R2): +15% ($p<0.05$) at D56 and +14% ($p<0.1$) at D112.

Viscous recovery (Q2) (Figure 31)

- Compared to their baseline, Phytofirm Biotic showed a 8% ($p<0.05$) and 14% ($p<0.01$) significant increase in viscous recovery (Q2) after 2 months (D56) and 4 months (D112) respectively, while the placebo did not demonstrate any positive antiaging effect.
- Compared to the placebo, the Phytofirm Biotic showed significant improvement of the elastic recovery of the skin (Q2): +16% ($p<0.01$) at D56 and +14 ($p<0.1$) at D112.

These above results on the different skin elasticity parameters demonstrate an improvement of the jaw skin elasticity due to the Phytofirm Biotic treatment.

Volunteers were divided in 2 groups of 21 volunteers,
applying either Phytofirm Biotic at 2% or placebo, twice a day

Skin elasticity measurement on the jaw (R2, R5, R7and Q2) using Cutometer
Skin thickness evaluation using DUB SkinScanner
Time points: baseline (D0), D56, D82 and D112

Figure 27 - Clinical study on Phytofirm Biotic rejuvenating efficacy.

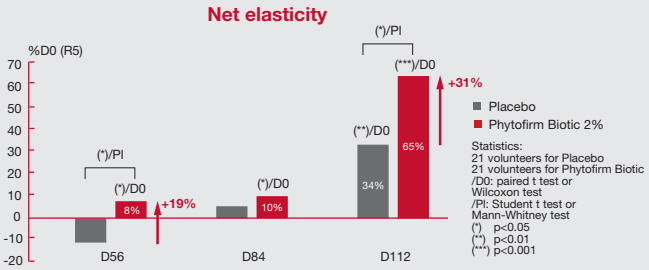


Figure 28 - Effect of Phytofirm Biotic on skin net elasticity (R5, Ur/Ue) of the jaw.

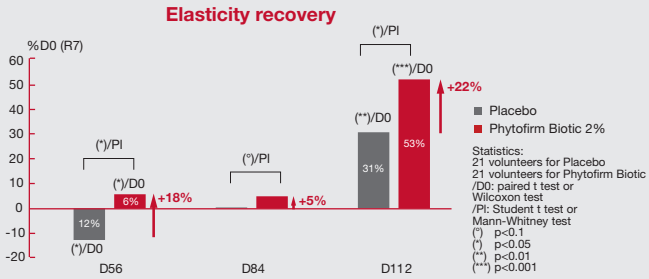


Figure 29 - Effect of Phytofirm Biotic on skin elasticity recovery (R7, Ur/Uf) of the jaw.

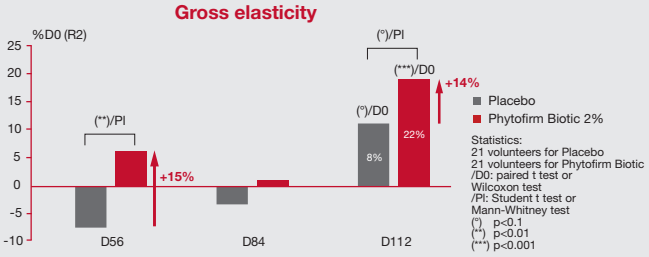


Figure 30 - Effect of Phytofirm Biotic on skin gross elasticity (R2, Ua/Uf) of the jaw.

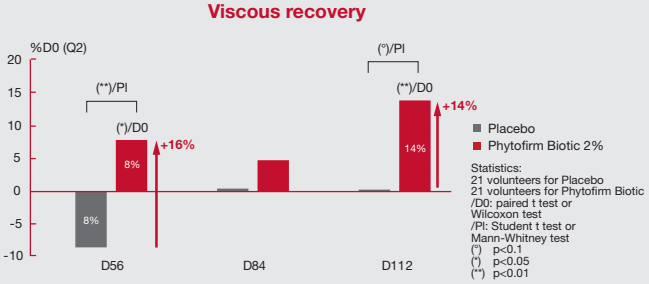


Figure 31 - Effect of Phytofirm Biotic on skin viscous recovery (Q2) of the jaw.

in vivo

RESULTS & DISCUSSION

Skin thickness improvement

Figure 32 shows the results obtained with Phytofirm Biotic and the placebo on skin thickness of the jaw area over treatment time.

- Compared to their baseline, Phytofirm Biotic showed a 9% and 10% significant increase ($p<0.05$) in skin thickness at D84 and at D112, respectively while the placebo showed lower effects at the same times ($p<0.1$).
- Compared to the placebo, Phytofirm Biotic showed a 7% improvement ($p<0.1$) at D112.

The above results indicate an improvement of the jaw skin thickness due to the Phytofirm Biotic treatment.

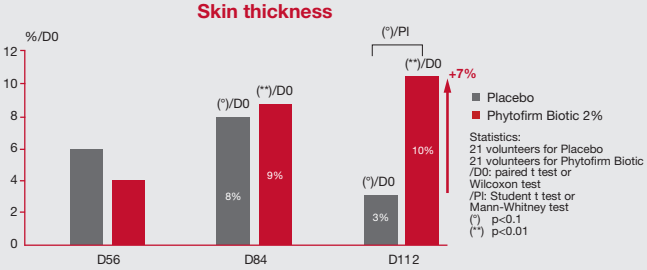


Figure 32 - Effect of Phytofirm Biotic on jaw skin thickness.

CONCLUSION

The 2% Phytofirm Biotic treatment improved skin elasticity and skin thickness of the jaw area, thus demonstrating its effectiveness in redesigning, rejuvenating face skin and bringing back its youthfulness.

MATERIALS&METHODS

Study design

The clinical study was carried-out as a randomized, double-blind study on full face *vs* placebo. The efficacy of the formulation containing Phytofirm Biotic at 2% was compared to the baseline (before treatment, D0) and to the placebo. The formulation is detailed in Annex 2. The study was conducted in New York, USA, in the winter season for a period of 112 days (4 months), with check points at D0, D56, D84 and D112.

Inclusion criteria

The study was done on 2 groups of 21 healthy female volunteers, Fitzpatrick skin type I, II and III, aged 55 to 63 considering themselves to have a loss of skin elasticity and firmness, especially in the lower face area.

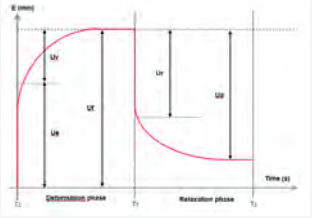
Application modality

The volunteers applied themselves under normal conditions of use, the products twice a day (once in the morning and once in the evening) for 112 days. Five pumps of either the Phytofirm Biotic or the placebo formula were applied to the entire face and neck. The last application was made the night before the last visit.

Evaluation methods

Skin elasticity: Cutometer

The Cutometer dual MPA 580 is used to measure elasticity of the upper skin layer. By using negative pressure, it deforms the skin mechanically. The measuring principle is based on the suction method. Negative pressure is created in the device and the skin is drawn into the aperture of the probe and after a defined time it is released again. Inside the probe, the penetration depth is determined by a non-contact optical measuring system. This optical measuring system consists of a light source and a light receptor, as well as two prisms facing each other, which project the light from the transmitter to the receptor. The light intensity varies due to the penetration depth of the skin. The resistance of the skin to the negative pressure (firmness) and its ability to return into its original position (elasticity) are displayed as curves (penetration depth in mm/time) in real time during the measurement. This measurement principle allows getting information about the elastic and mechanical properties of skin surface and enables to objectively quantify skin aging. The measurements were performed in the jaw area.



Para-meters	Ratio	Name	Phase	Interpretation *vs* age
R2	Ua/Uf	Viscoelastic recovery index **Gross elasticity**	Recovery phase	↘
R5	Ur/Ue	Net elasticity (without viscous deformation) **Immediate elasticity**	Recovery phase	↘
R7	Ur/Uf	Elastic recovery to distensibility ratio **Elasticity recovery**	Recovery phase	↘
Q2	Q_E/Q_0	Viscous recovery	-	-

Figure 33 - Age-related changes of skin elaticity parameters measure by a cutometer. Adapted from Krueger *et al*, 2011.

Skin thickness: DUB SkinScanner (Ultrasound) (22 MHz Probe)

The DUB SkinScanner is a high-resolution diagnostic ultrasound system. The basic principal of how the system works is that ultrasound impulses pass through the tissue with the sound velocity. The sender of the system generates a high voltage velocity which is passed on to the transducer. At the same time the transducer will move inside the applicator a specific distance. With the high speed the electrical impulses come back from the transducer is a digital form, thus producing the images. The high-frequency ultrasound allows the assessment of skin thickness [Crisan *et al*, 2012]. The thickness of the skin can be obtained by measuring the distance between the epidermis and dermo-hypodermic junction at three different sites and by establishing the mean of the three values [Crisan *et al*, 2012].

Statistics

The results are expressed as the mean percentage. The statistical analysis of the parameters has been done after checking the normality of distribution using Shapiro-Wilk test.

The statistical analysis of the change in the studied parameters for each product over time was performed as follows:

- validation of the normality of the studied parameters: paired t test,
- invalidation of the normality of the studied parameters: Wilcoxon test.

The statistical analysis of the differences in the studied parameters between the treatment groups was performed as follows:

- validation of the normality of the studied parameters: Student t test,
- invalidation of the normality of the studied parameters: Mann-Whitney test.

GENERAL CONCLUSION

A probiotic ferment (postbiotic) for preserving skin youthfulness

- Phytofirm Biotic is a nature-based fermented ingredient for anti-aging skincare products.
- It is proven *in vitro* to improve the synthesis of three extracellular matrix (ECM) components, and *in vivo* to boost two of them. Moreover, in a clinical test it has been shown to improve skin elasticity and thickness to keep skin youthfulness.
- Phytofirm Biotic enables cosmetics manufacturers to develop next-generation skin care solutions that respond to the probiotic trend.

Skin youthfulness, particularly skin elasticity, is one of the most important requirement. Women are looking for simple, safe, and efficient products that target fundamentals of beauty like key ECM components, known as the most important elements of skin structure, resilience, and elasticity. In the same time, consumers also recognize the health and wellness benefits of biotics; this is driving the increasing attractiveness of skincare products with probiotics in general and probiotic fermented ingredients (postbiotics).

To meet this consumer need, BASF BCS has developed Phytofirm Biotic as a probiotic fermented ingredient as a part of this biotic category of product and which fulfills the quest of efficiency. It is obtained by fermenting soybean extract from non-GMO traceable European soybeans, using the *Lactobacillus plantarum* strain. This fermentation process generates an extract titrated in peptides and lactic acid, offering manufacturers worldwide an effective alternative to soy protein hydrolysate when making probiotic skin care products.

In vitro tests have proven that this innovative postbiotic has potent capacities to increase the production of elastin, collagen I, collagen V, and GAGs, specifically hyaluronic acid, in several experimentations using different cellular and reconstructed dermis models. In addition, neosynthesized fibers of collagen I and V were longer and better organized, for an improved ECM quality. At the same time, Phytofirm Biotic also protects ECM components from degradation through the inhibition of elastase activity. Phytofirm Biotic also preserved fibroblasts functionality by limiting the fibroblast transformation into myofibroblasts and their contractility, contributing thus to maintain their capacity to produce ECM components.

Beyond these activities toward ECM, these rejuvenating capacities have been evidenced also on epidermis with an increase of its thickness in a reconstructed skin model.

Finally, two clinical investigations in a placebo-controlled study revealed the potential of Phytofirm Biotic to increase GAGs and Collagen I quantity in the skin; to bring back skin youthfulness by improving skin elasticity and increasing skin thickness for a restored and rejuvenated skin.

Phytofirm Biotic – Lactobacillus/Soybean ferment extract is a probiotic ferment (postbiotic) answering consumers quest for safe, bio-based, and effective active ingredients to preserve the youthful aspect of face and body skin by maintaining and improving skin elasticity, and thickness.

ANNEXES

Annex 1 - Technical data - Available upon request

- Quality and Regulatory Product Information
- Information on toxicological data
- Composition sheet
- Specifications
- Formulation Data Sheet
- Natural content (according ISO 16128)
- Vegan statement

Annex 2 - Clinical test formula

Trade name	INCI name	Placebo formulation %	Phytofirm Biotic fomulation %
Cetiol Sensoft	Propylheptyl caprylate	4.00	4.00
Cetiol CC	Dicaprylyl carbonate	4.00	4.00
Cosmedia SP	Sodium Polyacrylate	0.70	0.70
Hispagel 200 NS	Glycerin, Glyceryl Polyacrylate	10.00	10.00
Butanediol-1,3	Butylene Glycol	5.00	5.00
Elestab 388	Propylene Glycol, Phenoxyethanol, Chlorphenesin, Methylparaben	2.50	2.50
Edeta BD	Disodium EDTA	0.05	0.05
Hydrasensyl Glucan	Aqua, beta-Glucan	2.00	2.00
Phytofirm Biotic	Lactobacillus/Soybean Ferment Extract, Pentylene Glycol, Caprylyl Glycol	-	2.00
Water	Aqua	qsf 100	qsf 100

Annex 3 - Formulation examples

Body milk mist (SC FR 20 BC 50901 02)

Phase	Ingredients	INCI	% by weight	Function
A	Water, demin.	Aqua	73.75	
	Edeta® BD	Disodium EDTA	0.05	Complexing agent
	Preservative		q.s.	Preservative
	Glycerin	Glycerin	2.00	Humectant
	Sorbitol Solution 70% USP *(Escuder)*	Sorbitol	2.00	Humectant
	Cosmedia® SP	Sodium Polyacrylate	0.50	Rheology modifier
	Tinovis® GTC UP	Acrylates/Beheneth-25 Methacrylate Copolymer	0.70	Rheology modifier
	Sodium Hydroxide (25% solution)	Sodium Hydroxide	q.s.	pH Adjustment
B	Plantapon® LGC SORB	Sodium Lauryl Glucose Carboxylate (and) Lauryl Glucoside	1.50	Emulsifier (O/W)
C	Dehymuls® PGPH	Polyglyceryl-2 Dipolyhydroxystearate	2.00	Emulsifier (W/O)
	Lanette® 22	Behenyl Alcohol	0.50	Consistency agent
	Xiameter PMX-200 Silicone Fluid 100CS *(Dow Corning)*	Dimethicone	1.00	Emollient
	Myritol® 331	Cocoglycerides	2.00	Emollient
	Cetiol® RLF	Caprylyl Caprylate/ Caprate	4.00	Emollient
	Cetiol® C 5	Coco-Caprylate	3.00	Emollient
D	Phytofirm™ Biotic BC10138	Lactobacillus/Soybean Ferment Extract, Pentylene Glycol, Caprylyl Glycol	2.00	Active ingredient
E	Syniorage™ PW LS 9847	Mannitol, Acetyl Tetrapeptide-11	1.00	Active ingredient
	Water, demin.	Aqua	4.00	
F	Perfume	Parfum	q.s.	Fragrance

Specifications	
pH value (20°C)	6.65
Viscosity (Brookfield; RVT; spindle 5; 10 rpm; 20°C)	5000 mPa s

Night Recovery cream (SC FR 20 BC 50902 02)

This formulation concerns IP rights of L'Oréal. BASF has a License Agreement with L'Oréal. Please contact us for details.

Phase	Ingredients	INCI	% by weight	Function
A	Water, demin.	Aqua	58.50	
	Glycerin	Glycerin	5.00	Humectant
	Preservative		qs	Preservative
B	Cosmedia® SP	Sodium Polyacrylate	1.00	Rheology modifier
C	Eumulgin® VL 75	Lauryl Glucoside, Polyglyceryl-2 Dipolyhydroxystearate, Glycerin	3.00	Emulsifier (O/W)
	Eumulgin® SG	Sodium Stearoyl Glutamate	0.50	Emulsifier (O/W)
	Cutina® HVG	Hydrogenated Vegetable Glycerides	2.00	Consistency agent
	Cutina® PES	Pentaerythrityl Distearate	2.00	Consistency agent
	Cetiol® SB 45	Butyrospermum Parkii Butter	5.00	Emollient
	Cegesoft® PS 6	Olus Oil [EU], Vegetable Oil [CTFA]	8.00	Emollient
	Myritol® 331	Cocoglycerides	6.00	Emollient
	Cetiol® C 5	Coco-Caprylate	4.00	Emollient
D	Cetiol® Ultimate	Undecane, Tridecane	3.00	Emollient
E	Phytofirm™ Biotic BC10138	Lactobacillus/Soybean Ferment Extract, Pentylene Glycol, Caprylyl Glycol	2.00	Active ingredient
F	Perfume	Parfum	qs	Fragrance

Specifications	
pH value (20°C)	6.3-6.5
Viscosity (Brookfield; DV-I+; spindle TD, Helipath; 10 rpm; 20°C)	70 000 - 90 000 mPa s

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