Clinical, Cosmetic and Investigational Dermatology

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CLINICAL TRIAL REPORT

A Randomized Double-Blind, Placebo-Controlled Study to Evaluate the Anti-Skin-Aging Effect of LactoSporin – The Extracellular Metabolite from *Bacillus coagulans* (*Weizmannia coagulans*) MTCC 5856 in Healthy Female Volunteers

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Purpose: There has been a growing interest in the use of probiotics and their products for skin care, over the last decade. LactoSporin is the extracellular metabolite of a spore-forming probiotic *Bacillus coagulans* (*Weizmannia coagulans*) MTCC 5856, with anti-microbial and skin protecting activity.

Patients and Methods: The anti-skin-aging potential of LactoSporin was evaluated in a randomized, double-blinded, placebo-controlled study in healthy female participants (70 screened and 56 randomized). The participants applied either LactoSporin or matched placebo formulation (N=28 in each group) for 10 weeks, and the effects were assessed by dermatological, and non-invasive instrument-based evaluation using Antera, Cutometer, Corneometer, and Tewameter. All the 56 participants completed the study and were included for the analysis.

Results: The regular use of LactoSporin cream for 10 weeks showed a significant reduction in visibility of wrinkles around crow's feet, nasolabial folds, frown lines, and facial fine lines compared to baseline and placebo by dermatological and Antera assessments. LactoSporin showed improvement in skin elasticity and hydration by dermatological assessments, but the effect was not significantly different from placebo when assessed by Cutometer, Corneometer, and Tewameter. No adverse events or skin irritation was observed in any participants during the study.

Conclusion: These results suggest that LactoSporin could be a safe natural ingredient to reduce wrinkles and fine lines in cosmetic formulations.

Keywords: LactoSporin, safety, dermatological assessments, corneometer, tewameter, cutometer

Plain Language Summary

Probiotics and their metabolites are being increasingly used in cosmetic formulations in recent years. Use of live bacteria in cosmetic formulation can pose several challenges. The metabolites produced by probiotics are reported to have several beneficial activities for the skin. LactoSporin is an extracellular metabolite partially purified from *B. coagulans* (*W. coagulans*) MTCC 5856 (a probiotic strain) fermented broth with an INCI name - *Bacillus* Ferment Filtrate Extract. It has antimicrobial activity and was found to be effective in reducing acne in a human clinical study. LactoSporin showed skin protective activity in cell-based studies and hence this study was designed to explore its anti-skin-aging effect in human volunteers. Ten weeks of application of 2% LactoSporin cream was found to significantly reduce wrinkles and fine fines in skin and improve its texture. The result of this clinical study suggests that LactoSporin®could be a safe natural ingredient to be used in anti-aging cosmetic formulations.

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Introduction

Skin aging, a natural and inevitable process, is caused by structural and functional changes in dermal cells due to intrinsic and extrinsic factors. Exposure to pollution, UV radiations, poor nutrition and other environmental factors accelerate the signs of aging by an early appearance of wrinkles, dark spots, and loss of elasticity, accelerating the aging process.[1,2] While intrinsically aged skin has a thin, atrophic, finely wrinkled, and dry epidermis, premature photoaged skin shows a thickened epidermis, mottled discoloration, deep wrinkles, laxity, dullness, and roughness.[3] The reduction in extracellular matrix (ECM), especially collagen content, is a common feature of skin aging.[2] Daily skin care can increase skin regeneration, elasticity, and smoothness and thus help in maintaining good skin conditions. Preserving the ECM proteins like collagen and elastin and reducing skin inflammation and oxidative stress are some strategies to control rapid loss of skin function and appearance.[4]

Skin microbiota, the microbes in the skin, regulate inflammation, help in innate and adaptive immunity, and form a part of the skin barrier.[5] The past few years have seen an explosion in the studies of prebiotics, probiotics, synbiotics, and postbiotics, not only for gastrointestinal diseases but also for skin health.[6] Probiotics, taken orally, can influence the skin by restoring the skin microbiota, apart from its beneficial effect on gut dysbiosis.[7] Topical use of probiotics has shown promising results in the treatment of certain inflammatory skin diseases like eczema and in wound healing and skin cancer by their positive influence on skin flora.[8] A probiotic mist containing live *Nitrosomonas eutropha* could reduce the appearance of wrinkles in a clinical study.[9] However, topical application of live bacteria poses several challenges. Bacterial viability was compromised by more than 99% in formulations containing 20% propylene glycol or ethanol.[10] Moreover, the topical skin care products are non-sterile and may contain antimicrobial preservatives which can affect the viability of probiotic strain that can have a variable influence on skin microbiome.[11]

International Scientific Association for Probiotics and Prebiotics (ISAPP) has defined postbiotics as the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host".[12] Extracellular metabolites are by-products of the metabolic activity of microorganisms in a specific environment, which include exopolysaccharides, biosurfactants, enzymes, peptides, and vitamins.[13] These compounds are slowly replacing conventional chemicals and are finding broader applicability in various cosmetic products.[14] Bacterial metabolites, fragmented cell wall products, and even dead bacteria have been shown to elicit immune responses and improve the skin's barrier function.[15] The topical application of probiotics has shown beneficial effects on acne and erythema in human clinical studies by modifying the skin barrier function and antimicrobial function.[16–19] An extract of *Vitreoscilla filiformis*, applied on skin could strengthen the skin defense and barrier function and help in maintaining good skin condition.[20] The metabolites of the probiotic *Lactobacillus* could improve skin hydration, wrinkles, elasticity, and gloss.[21–23] The microbial metabolites were reported to enhance hyaluronic acid production, generate ceramides, improve skin barrier and immune defense host cells, and thus benefit the skin condition.[16]

Bacillus coagulans MTCC 5856 is a non-Genetically Modified Organism (non-GMO) with US FDA reviewed Generally Recognized as Safe (GRAS) status.[24] The *B. coagulans* was recently reclassified as *Weizmannia coagulans*.[25] LactoSporin is an extracellular metabolite partially purified from *B. coagulans* (*W. coagulans*) MTCC 5856 fermented broth with an INCI name - *Bacillus* Ferment Filtrate Extract.[26] LactoSporin is an antimicrobial compound with beneficial effects in reducing acne in a human clinical study.[27] It could protect cultured dermal cells from UV-induced damage by quenching reactive oxygen species and reducing cell death. LactoSporin could inhibit collagenase activity and suppress glycation while increasing epithelial growth factor (EGF) expression in the dermal cells in vitro, suggesting its potential to prevent premature aging by multiple mechanisms.[27,28] In the present study, we extended our earlier observations by evaluating the clinical benefits of LactoSsporin on age-related changes in the skin of healthy female participants.

Materials and Methods

Investigational Product

LactoSporin was produced from the fermented broth of *B. coagulans* (*W. coagulans*) MTCC5856 culture by a patented process.[26] The cells were centrifuged, and the filtrate was concentrated and precipitated with chilled acetone. The precipitate was centrifuged, and the supernatant was passed through absorbent resin, washed with DM water, concentrated, and filtered through a 0.2-micron filter (Millex, Millipore, India) to get the final product. LactoSporin was

standardized to have 1% (w/v) total protein as estimated by Lowry's method and an antimicrobial activity not less than 200 AU/mL against *Micrococcus Luteus* NCIM 2169.[27]

The base formulation contained the carbomer – 1.4% (Biopol Crystal, Chimica Pomponesco S.p.A, Italy), sorbitan stearate (and) sucrose Cocoate – 6% (Arlacel 2121, Croda, UK), pentaerythrityl *tetra*-di-*t*-butyl hydroxyhydrocinnamate – 0.13% (Tinogard TT, BASF, Germany), phenoxyethanol – 0.9% (Galaxy Surfactants Ltd, India) as preservative, and a fresh dew fragrance – 0.5% (Mane India Pvt. Ltd). Disodium EDTA – 0.1% was used as a chelating agent and sodium hydroxide – 0.16% to adjust the pH. LactoSporin (Sami-Sabinsa Group Limited, India) was added to the base formulation at 2% w/v final concentration in the active formulation, while the base formulation was used as placebo.

Study Design and Ethics

The study was conducted as a randomized, double-blind, placebo-controlled, monocentric, safety, and efficacy trial for 10 weeks, from January to April 2022, at MS Clinical Research Pvt. Ltd., Bengaluru, in compliance with the Helsinki Declaration and the amendments to evaluate the anti-aging benefits of LactoSporin formulation by dermatological assessment, self-assessment, and non-invasive instrument assessment. The study protocol was approved by Clinicom Ethics Committee, an independent ethics committee, on the 10th of January 2022, with a registration number – CTRI/2022/01/039292, 10-01-2022. All the participants signed a written informed consent form.

Sample Size Calculations

The sample size was calculated with a power of 80% at 5% significance. A total of 24 evaluable participants were expected to be sufficient in each group to detect the 0.61 units of change from the baseline. Considering a 10% dropout rate based on previous study data, 56 volunteers were enrolled in the study, with 28 participants in each group.

Inclusion and Exclusion Criteria

The study included adult healthy female participants in the age group of 35–65 years, as determined from a recent medical history of general physical examination and dermatological assessment. The participants having visible fine lines and wrinkles in periorbital area (crow's feet-under eye) and forehead were prescreened from the database available at MS Clinical Research Pvt. Ltd., Bengaluru. All the participants had a Fitzpatrick skin type III to V, most common in Indian population. The included participants were free of excessive hair, acne, cuts, abrasions, fissures, wounds, lacerations, or any other active face skin conditions. All the participants agreed to refrain from using other products/treatment/home remedy on their face during the study period other than the test product, not to undertake bleaching, facial, and other skin treatments, and not to expose themselves to excessive sunlight during the study period.

Participants with any signs of local irritation or skin disease, undergoing any treatment for any skin condition on their face/forearm, taking medication, including food supplements, having a chronic illness, or having undergone major surgery in the last year, were excluded from the study. Participants who had stimulators, hyaluronic acid, and Botox performed were also excluded from the study. Pregnant or nursing females, participants who were not willing to discontinue other topical facial products or having allergies/sensitivity to creams/lotions were also excluded from the study.

Blinding and Randomization

The test and placebo were identical in their shape and size. The randomization code was generated using R-Software and the test product distribution, as per the code, was carried out by a technician not involved in the study. The separation between the investigator, collaborators, and the staff who delivered the intervention were strictly followed as per the established procedures. The study participants, investigators, and sponsors were blinded to the product assignment, and the allocation sequence was kept blinded until data analysis.

Intervention

Post enrolment to the study, baseline assessments were performed which includes Dermatological visual assessment, instrument assessment (Cutometer, Tewameter, Corneometer, Antera-3D) and VISIA images were captured. The participants received the test products as per the randomization and were instructed to apply one fingertip unit for full

face twice daily (after bath in the morning and night before bed) throughout the study. The test products were applied after washing the face with a mild face wash. No functional cleanser was used by the participants. There was no change in the participant's skin care regimen for the entire duration of the study. They continued to use their face wash and stopped all other topical products. The first application of the test product was at the study center under the supervision of the study coordinator. This was followed by the dermatological assessment for skin tolerance.

Outcome Measures

The primary endpoints included improvement of facial fine lines, wrinkles, improvement of skin, hydration, skin appearance in terms of signs of aging as assessed by dermatological and instrument-based assessment. The secondary endpoints were improvement in skin elasticity as assessed by Cutometer, patients' assessments by self-assessment questionnaire, and product safety by dermatologist assessment.

Assessments

During the visit to the site, the participants were asked to wash their face with water under controlled temperature at ~22°C±2 °C and relative humidity of 45±5%. The assessment sequence shown in Figure 1. Full face digital images were taken using VISIA-CR Blue 4.3 Camera Imaging System (Canfield Imaging Systems, Fairfield, NJ, USA). These images were used only for documentation and visual representation. VISIA CR images were not used for any assessments.

Dermatological Assessment

The study dermatologist conducted the dermatological visual assessment for forehead wrinkles, crow's feet, nasolabial folds, frown lines, and under-eye fine lines. The visual assessment also included skin texture, firmness and elasticity, and skin dryness. A photo numerical scale was used to quantify visible fine lines and wrinkles.[29]

Wrinkles–Crow's Feet: The efficacy parameters were assessed on the scale of 0 to 9, where 0 = none (best possible condition), 1–3 = mild, 4–6 = moderate and 7–9 = severe (worst possible condition). A photographic representation of the assessment is given in Figure S1 in the Supplementary Section.

Nasolabial folds: A score of 0 = none (no folds), 1–3 = mild, 4–6 = moderate and 7–9 = severe (deep and long folds).

Frown line: A score of 0 = none (no frown line), 1–3 = mild, 4–6 = moderate and 7–9 = severe.

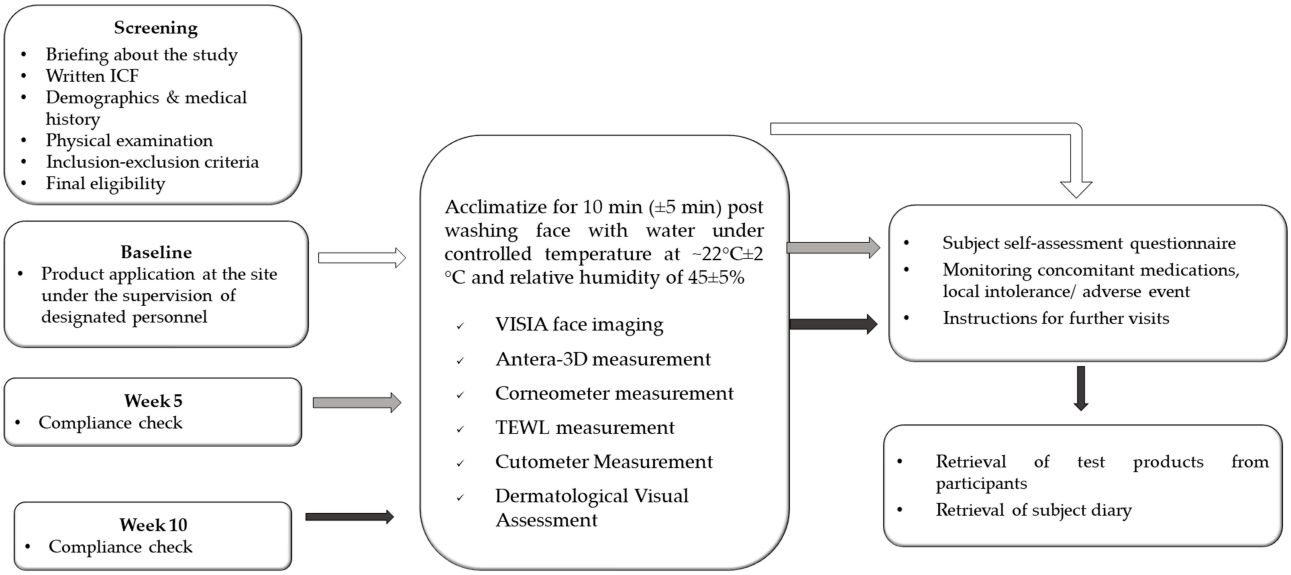

Figure 1 Participant flow chart at each visit. A flow chart showing the activities carried out on each visit during the study. Open arrows: baseline, grey arrows: Week 5 and black arrows represent week 10.

Facial fine lines: The efficacy parameters were assessed on the scale of 0 to 9, where a score of 0 = none (best possible condition), 1–3 = mild, 4–6 = moderate and 7–9 = severe. Photographic representation of the assessment is provided in Figure S2 in the Supplementary Section.

Skin texture: is the condition of the skin surface -A score of 0 = highly smooth skin, 1–3 = mild, 4–6 = moderate and 7–9 = rough skin.

Skin firmness/elasticity is skin's ability to stretch and snap back to its original shape and was assessed on a scale of 0–9. A score of 0 = highly firm skin, 1–3 = mild, 4–6 = moderate and 7–9 = very loose skin

Skin Hydration was assessed on a scale of 1–5, where a score of 1=very dry, stretchy, scaly peeling skin, 2=stretchy, scaly but not peeling, 3=normal, 4=mildly hydrated, 5=hydrated. Photographic representation of the assessment is provided in Figure S3 in the Supplementary Section.

Instrumental Assessments

Antera 3D (version 3.1.0, Miravex, Dublin, Ireland) was used for the evaluation of wrinkles, fine lines, and texture. Seven images were taken in the center of the forehead, two images each for Crow's feet area, undereye, and nasolabial area, including the right and left cheek area. The images were analyzed using Antera 3D software. The same predetermined areas were assessed on every visit.

Corneometer (CM-825, Courage & Khazaka Electronic GmbH, Cologne, Germany) was used to measure hydration. The results were expressed in arbitrary units. The measurements were performed by applying the probe to the skin surface. Upon contact, an electric field passes through the stratum corneum, and the dielectric constant was recorded. The changes in water content of the stratum corneum are converted into arbitrary units of hydration.

Tewameter (TM-300, Courage & Khazaka Electronic GmbH, Cologne, Germany) was used for water barrier function on the right and left sides of the cheek. The probe head of Tewameter was placed perpendicular without pressure on the skin with the surface area in a horizontal position. The density gradient of the water evaporation from the skin was measured by two pairs of sensors located at different heights and the results expressed in $g/m^2/h$.

The skin firmness/elasticity on the right and left cheeks was measured using Cutometer MPA580 (Courage + Khazaka Electronic GmbH, Cologne, Germany) with a 2-mm diameter probe at a reduced pressure of 400 mbar with 2 s of suction followed by 2 s of release. The resistance of the skin to the negative pressure (firmness) and its ability to return to its original position (elasticity) are displayed as real-time measurement curves (penetration depth in mm/time). The following parameters were considered. R2, also called gross elasticity, is the expression of visco-elasticity (the ratio of the skin resistance to the suction phase and ability to return during the relaxation step; R5 defines the net elasticity (the elastic portion of the suction phase versus the elastic portion of the relaxation phase, and R7 is the ratio of elastic recovery to the total deformation.[30] The value of 1 (100%) represents elastic skin. Results were expressed in mm. Details of instrument assessments are represented Figure S4 in the Supplementary Section.

Self-Assessment Questionnaire

The participants performed self-assessment by viewing their faces in the mirror under controlled light conditions, with a set of questionnaires after 30 min of product application on their faces at week 5 and week 10. The participants gave their responses to each question with respect to the 5-point response scale (1, strongly disagree; 2, disagree; 3, neither agree nor disagree; 4, agree; 5, strongly agree).

Safety Assessments

The safety assessments were carried out by dermatological evaluation based on the following parameters: erythema, dryness, edema, urticaria, and allergic reactions. The patients were given a form to record any adverse events or localized skin irritation that were observed during the study period.

Statistical Analysis

The normality of the data was checked by Shapiro–Wilk Test. Normally distributed data were compared by paired *t*-test. Data not following the normal distribution were analyzed by non-parametric test – Wilcoxon signed rank or Pratt Lehman to compare each visit with baseline. For the comparison active and placebo, an unpaired *t*-test/Mann–Whitney

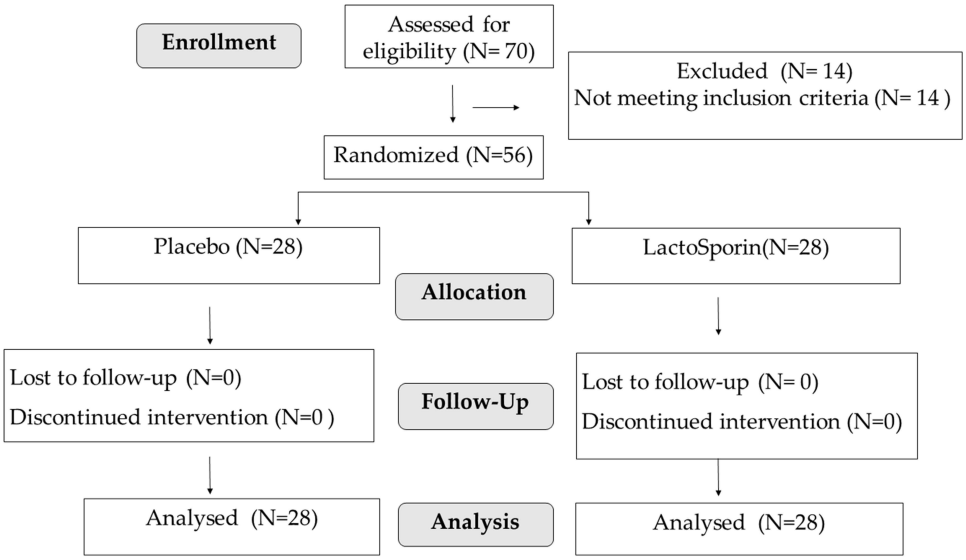

Figure 2 Consort diagram.

U-test was performed on change from baseline values based on normality. All statistical tests have used a significant level of $\alpha \leq 0.05$. A two-tailed test was performed for all analyses.

Results

Demographic Details

A total of 70 volunteers were prescreened based on the inclusion criteria from the database available at MS Clinical Private Limited. From these, 56 participants with an average age of 43.83±6.61 were recruited for the study (Figure 2). The mean age of participants in LactoSporin (44.17 ± 6.6) and Placebo (43.50 ± 6.4) groups were comparable (Table 1). The participants were not using any special skin care regimen before inclusion into the study. None of the participants had any untoward event that required clinical intervention or rescue medication.

Safety

No adverse events or serious adverse events were reported in any of the participants during the study. None of the participants experienced any skin irritation, and dermatological evaluations did not observe any allergic skin reactions in any of the participants. LactoSporin 2% formulation was earlier tested for safety in a Primary Skin Irritation Patch Test (PIPT) and was found to be dermatologically safe.[27] The same dose was used in the present study as well.

Table 1 Baseline Demographics

Parameter	Placebo (N=28)	LactoSporin (N=28)	Total (N=56)
Age (Mean ± SD)	43.50 ± 6.4	44.17 ± 6.6	43.83 ± 6.6
Fitzpatrick Skin type (N)			
III	3	10	13
IV	11	12	23
V	14	6	20

Note: Demographic details of the study participants.
Abbreviation: SD, Standard deviation.

Dermatological Assessments

Dermatological assessments were carried out on the entire face by a qualified dermatologist, using a photo numerical scale (PNS) as described in the methods section. The regular usage of LactoSporin formulation showed a significant reduction in visibility of wrinkles around the crow's feet area (6.0 ±2.01 at baseline to 5.04±1.91 at week 5 and 4.67±1.96 at week 10, p<0.0001 respectively) compared to (5.63±1.49 to 5.70±1.52 and 5.74±1.59 at baseline, week 5 and week 10) placebo. The reduction was 16% and 22.16% at week 5 and week 10 respectively, for LactoSporin formulation, which was significantly better than the placebo (Table 2). The wrinkles around nasolabial folds slightly increased in placebo but showed significant reduction with LactoSporin formulation application at week 5 (11.2%) and week 10 (13.7%). Frowning leads to deep wrinkles between the eyes, resulting in the tissues between the eyebrows developing into a fold. These wrinkles in the skin appear on the bridge of the nose, directly between the eyebrows. Frown lines were reduced by 18.9% (5.39±2.38 to 4.38±2.23) in participants using LactoSporin formulation at week 10, while an increase was registered in the placebo group (4.75±1.86 to 5.13±1.83) (Table 2).

As the skin begins to age, fine lines start appearing around the eyes and other areas on the face. These shallow lines tend to form along expression lines that appear during a smile, laugh, or frown. Fine lines showed a decrease from 7.32 ±1.82 to 5.75±1.82, p<0.001 in LactoSporin formulation, while there was an increase in placebo (7.07±1.25 to 7.4±1.41). Skin texture (5.07±1.36 to 4.35±1.48, 14.2%, p<0.001), firmness and elasticity (5.86±1.67 to 5.15±1.62, 12.1%, p<0.001) and hydration (3.5±0.64 to 4.38±0.41, 25.1%, p<0.001) were also found to improve on regular use of LactoSporin formulation (Table 2).

Table 2 Dermatological Assessment of Facial Skin for Signs of Ageing

Assessments	Placebo Formulation			LactoSporin Formulation			#p-value for Change
	Baseline	Week 5	Week 10	Baseline	Week 5	Week 10	
Wrinkles – crow's feet	5.63±1.49	5.70±1.52	5.74±1.59	6.00±2.01	5.04 ± 1.91*	4.67 ± 1.96*	
Change		0.07	0.11		−0.96	−1.33	<0.001
Nasolabial folds	4.98±2.14	5.18±2.1	5.19±2.08	5.09±2.29	4.52±2.18*	4.39±2.18*	
Change		0.20	0.21		−0.57	−0.70	<0.001
Frown lines	4.75±1.86	5.02±1.77	5.13±1.83	5.39±2.38	4.61±2.32*	4.38±2.23*	
Change		0.27	0.38		−0.79	−1.02	<0.001
Fine lines	7.07±1.25	7.39±1.08	7.4±1.41	7.32±1.82	6.45±1.77*	5.75±1.82*	
Change		0.32	0.33		−0.88	−1.57	<0.001
Skin texture	4.93±1.05	4.96±1.07	5.13±1.2	5.07±1.36	4.66±1.42*	4.35±1.48*	
Change		0.04	0.20		−0.41	−0.72	<0.001
Skin firmness /elasticity	5.25±1.46	5.38±1.44	5.54±1.49	5.86±1.67	5.45±1.65*	5.15±1.62*	
Change		0.13	0.29		−0.41	−0.71	<0.001
Skin hydration	3.29±0.6	3.51±0.62*	3.87±0.51*	3.5±0.64	4.11±0.55*	4.38±0.41*	
Change		0.22	0.58		0.61	0.88	<0.001

Notes: Dermatological assessments were carried out at baseline, week 5 and week 10. The values in photo numeric scale are represented as Mean±SD and the change in the photo numerical scale readings from baseline to week 5 and week 10 are presented. For crow's feet, fine lines, frown lines, nasolabial folds, skin texture, and firmness, the scale was from 0 to 9 and lower mean values indicate better condition. An increase in skin hydration is measured as the higher mean value. The #P value was computed for the change in values from baseline and week 10 in LactoSporin and Placebo groups. *P<0.05 between baseline and week 5 and week 10 within the group.
Abbreviation: PNS, Photo Numeric Scale.

Instrument Assessments

Antera 3D® allows the users to analyze and measure topographic features of the skin. The wrinkles on the forehead, forehead texture, crow's feet, nasolabial folds, and under-eye fine lines showed significant improvements in both LactoSporin and placebo groups at week 10 compared to baseline. Regular use of LactoSporin formulation reduced forehead wrinkles (11.3% vs 5.8%, p=0.02) and improved its texture (11.4% vs 8.7%, p=0.04) compared to placebo at the end of 10 weeks. The wrinkles around the crow's feet area were reduced by 8.3% in the LactoSporin group compared to 5.2% in the placebo (p=0.04), while the nasolabial folds were reduced by 9.9% vs 7.4% (p=0.04). The fine lines under the eye showed a better reduction with LactoSporin formulation application (11.9%) compared to placebo formulation (8.2%, p=0.07) (Table 3). The Antera 3D® images showing the wrinkles as blue marks within the area of interest are represented in Figure 3. A lower value indicates less severity and an improvement.

Skin elasticity parameters *R2* (gross elasticity), *R5* (net elasticity), and *R7* (recovery after deformation) were recorded using the Cutometer. All three parameters of elasticity increased significantly in both placebo and LactoSporin groups after 10 weeks of application (p<0.001). The extent of improvement in skin elasticity was comparable (R2=30%, R5=16% and R7=30%) between the two groups. Skin hydration (30%) and Trans epidermal water loss (TEWL)

Table 3 Instrument Analysis of Facial Skin for Signs of Ageing

Assessments	Placebo Formulation			LactoSporin Formulation			#p-value for Change
	Baseline	Week 5	Week 10	Baseline	Week 5	Week 10	
Forehead wrinkles (mm)	18.42 ± 5.3	17.71 ± 5.3*	17.34 ± 5.2*	16.44 ± 4.9	15.10 ± 4.8*	14.58 ± 4.7*	
Change		−0.7	−1.07		−1.34	−1.86	0.022
Forehead texture (μm)	13.40 ± 2.8	12.69 ± 2.7*	12.23 ± 2.7*	13.45 ± 2.4	12.61 ± 2.1*	11.91 ± 2.1*	
Change		−0.71	−1.17		−0.84	−1.54	0.049
Crow's feet (mm)	17.71 ± 4.8	17.32 ± 4.8*	16.79 ± 4.7*	17.03 ± 3.9	16.33 ± 3.9*	15.61 ± 3.8*	
Change		−0.39	−0.92		−0.7	−1.42	0.042
Nasolabial folds (mm)	15.66 ± 3.8	15.00 ± 3.9*	14.49 ± 3.7*	14.84 ± 4.0	14.06 ± 3.8*	13.36 ± 3.8*	
Change		−0.65	−1.17		−0.77	−1.47	0.047
Under eye-fine lines (mm)	12.58 ± 2.7	12.09 ± 2.6*	11.54 ± 2.5*	12.26 ± 2.2	11.66 ± 2.1*	10.90 ± 2.1*	
Change		−0.49	−1.04		−0.6	−1.36	0.078
Skin hydration (AU)	35.91 ± 2.5	41.53 ± 2.4*	47.05 ± 2.5*	36.56 ± 2.2	41.94 ± 2.5*	47.36 ± 2.3*	
Change		5.61	11.13		5.38	10.8	0.15
Transepidermal water loss (g/m^2h)	10.26 ± 1.6	9.57 ± 1.5*	9.04 ± 1.6*	10.05 ± 2.6	9.42 ± 2.6*	8.85 ± 2.5*	
Change		−0.69	−1.22		−0.63	−1.2	0.52
R2 – Skin elasticity (mm)	0.58 ± 0.3	0.68 ± 0.1*	0.74 ± 0. 2*	0.55 ± 0.1	0.66 ± 0.1*	0.72 ± 0.1*	
Change		0.09	0.16		0.1	0.17	0.73
R5 – Skin elasticity (mm)	0.64 ± 0.2	0.71 ± 0.2*	0.76 ± 0.2*	0.58 ± 0.2	0.68 ± 0.1*	0.67 ± 0.1*	
Change		0.07	0.12		0.1	0.09	0.39
R7 – Skin elasticity (mm)	0.41± 0.1	0.47 ± 0.1*	0.54 ± 0.1*	0.37 ± 0.1	0.44 ± 0.1*	0.48 ± 0.1*	
Change		0.06	0.13		0.07	0.11	0.34

Notes: The values for instrument-based assessments carried out at baseline, week 5 and week 10 are represented at Mean ± SD, and the change from baseline to week 5 and week 10 is presented. The #P value represents the difference between LactoSporin and Placebo groups at week 10. Forehead wrinkles, texture, nasolabial folds, crow's feet, and under eye fine lines were assessed by Antera- 3D, Skin hydration was by Corneometer, barrier function by Tewameter and elasticity by Cutometer. *P<0.05 between baseline and end of the study within the group.
Abbreviation: AU, arbitrary units.

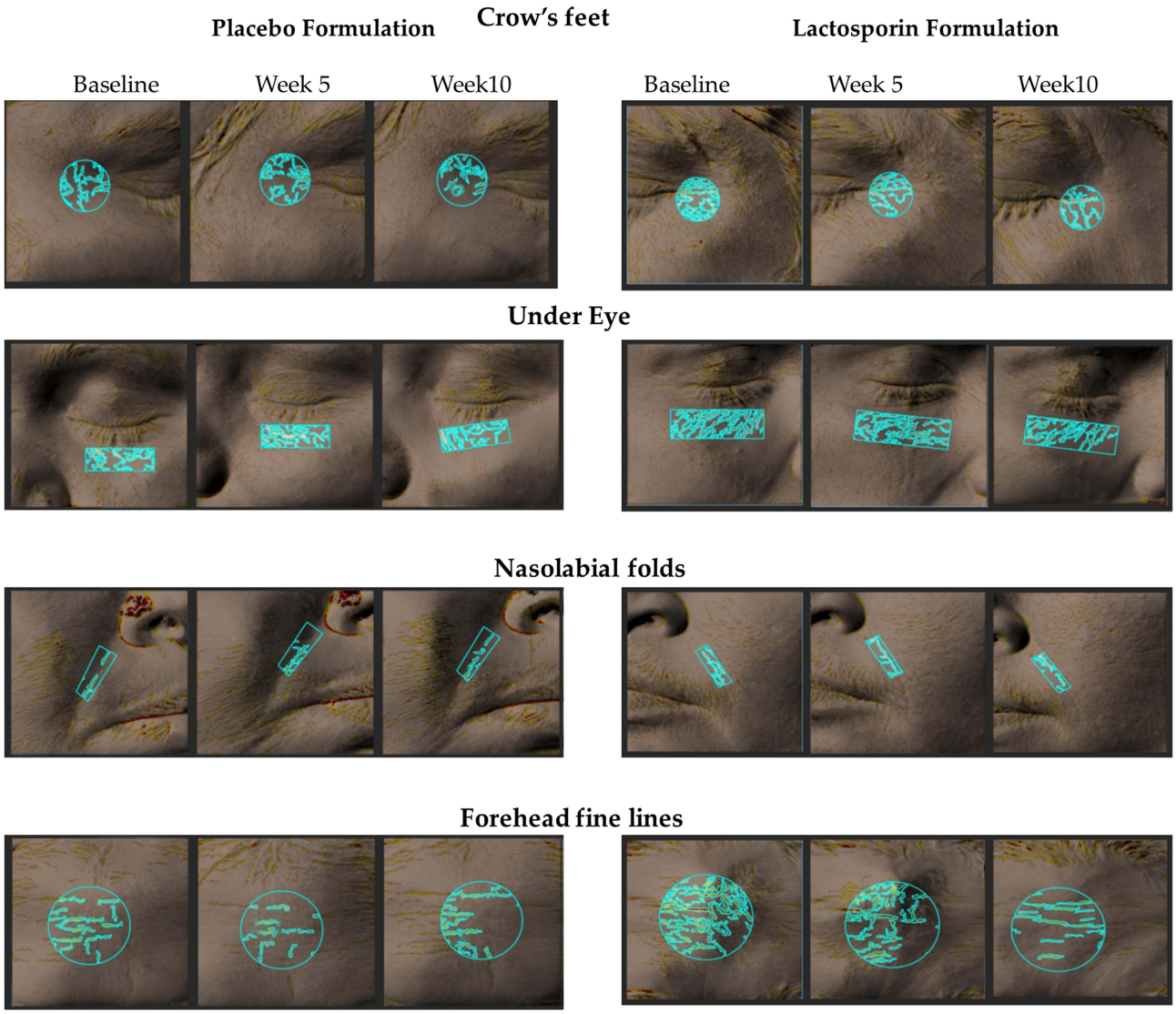

Figure 3 Analysis of topographical features of the skin by Antera 3D. Representative images from Antera 3D, showing the changes in crow's feet, under-eye fine lines, nasolabial folds, and forehead fine lines in placebo and LactoSporin at baseline, week 5 and week 10. The blue marks represent wrinkles within the area of interest. A lower value indicates less severity and an improvement.

(11.9%) also showed a similar trend as both parameters showed an improvement over baseline in both groups, while the extents of improvements were comparable between the groups (Table 3). The representative VISIA images taken at baseline week 5 and week 10 for 4 participants are shown in Figure 4.

Self-Assessment Questionnaire

All 56 participants completed a self-assessment questionnaire after 30 min of product application on their faces with respect to the 5-point response scale. A total of 27 out of 28 participants (96.4%) agreed that regular use of LactoSporin makes their skin firmer, improves fine lines and wrinkles, and keeps their skin moisturized, compared to 26/28 (92.8%) in placebo who felt that their skin was firmer without wrinkles, 25/28 (89.29%) believed that their skin looks moisturized (Table 4).

In the present study, we observed a positive effect of a cosmetic formulation containing 2% w/v.

Figure 4 VISIA representative images. Representative VISIA images of two participants each from A -LactoSporin and B- Placebo group taken at baseline, week 5 and week 10. Full face digital images were taken using VISIA-CR Blue 4.3 Camera Imaging System on day 0, day 35 and at day 70 for all the participants. The representative VISIA images of two participants each from LactoSporin (**A**)and Placebo (**B**) taken at day 01, day 35 and day 70 are depicted.

Discussion

In the present study, we observed a positive effect of a cosmetic formulation containing 2% w/v LactoSporin in reducing age-related wrinkles and fine lines by dermatological and Antera 3D instrumental assessments in female volunteers compared to placebo. The improvements in skin elasticity, hydration and trans-epidermal water loss, evaluated by Cutometer, Corneometer and Tewameter, were not significantly different in LactoSporin formulation compared to placebo, although dermatological assessments showed a significant improvement. Our results showed that 2% LactoSporin cream was safe with no skin irritation or adverse events reported during the 10 weeks of study.

Table 4 Participant Response to Self-Assessment Questionnaire

Questions	Placebo Formulation		LactoSporin Formulation	
	Week 5	Week 10	Week 5	Week 10
Does your skin look firmer	17 (60.71%)	26 (92.86%)	19 (67.86%)	27 (96.43%)
Does your skin look free of fine lines/wrinkles on your face?	10 (35.71%)	26 (92.86%)	16 (57.14%)	27 (96.43%)
The test products make my skin moisturized.	16 (57.14%)	25 (89.29%)	18 (64.29%)	27 (96.43%)

Notes: Response recorded by participants on their perception of the skin condition. The number and percentage of participants who agreed (4 or 5 in the response scale) that they felt an improvement in skin condition is represented as number and percentage.

The anti-aging activities of probiotics and their metabolites have been increasingly explored in recent studies.[31] Normal skin is generally acidic, which helps to prevent the growth of pathogenic bacteria, inhibits protease activity and helps to maintain skin moisture.[32] However, with age the pH increases, stimulating the protease activity leading to ECM degradation. Several probiotics produce acidic metabolites like lactic acid, which can maintain the acidic skin environment and may also improve the skin tone.[33]

Microorganisms produce extracellular metabolites as by-products of metabolic activity in a specific environment.[13] These fermented by-products from probiotics are being promoted as skin-friendly ingredients in various cosmetic products in recent years.[14] The lysates of the probiotic strains of *Lacticaseibacillus rhamnoses* GG and *Bifidobacterium longum* were shown to increase tight-junction barrier resistance in vitro by modulating protein components.[34] The lysate from the probiotic *B. longum* reuter strain was shown to decrease vasodilation, inflammation, and improve trans-epidermal water loss, and thus was reported to be beneficial for reactive skin.[35] Cell-free extract of the probiotic, lactic acid bacteria, was found to exert antimicrobial and immunomodulatory activities.[15] In a recent study, blackberry fermented with *Lactobacillus plantarum* JMBI-F5 was shown to inhibit UV-induced skin wrinkles and increase collagen fiber accumulation and epidermal thickness of the skin. Oral consumption of this product was claimed to result in reduced wrinkles in human participants.[36,37] The extracellular polysaccharides from *B. coagulans* (*W. coagulans*) RK-02 was found to have antioxidant and free radical scavenging activities in vitro.[38] A clinical study with skin aging formulations containing (Lactobacillus/Kelp Ferment Filtrate), along with several other actives was demonstrated to produce significant improvements in skin hydration, TEWL, firmness, and skin elasticity with associated improvements in facial skin appearance.[39] In another clinical study, a cosmetic lotion containing heat-treated *Lactobacillus johnsonii* NCC 533 was found to be beneficial for atopic dermatitis patients.[40]

Topical formulations that retain probiotic bacterial viability are challenging as the presence of moisture in water-based formulations would allow the dried organisms to hydrate and multiply or die and the use of oil-based formulation may not be conducive for the metabolic activity of live probiotics.[11] Compared to live probiotics, the metabolites are easier to formulate as they have better stability and are becoming increasingly popular for topical applications. LactoSporin, the extracellular metabolite from the probiotic strain *B. coagulans* (*W. coagulans*) MTCC5856 has shown antimicrobial and anti-inflammatory activities in earlier studies. The metabolite is thermostable and retains its activity in an acidic condition. The product was clinically effective in treating volunteers with mild-to-moderate acne vulgaris.[27] Preclinical safety studies as per the Organization for Economic Co-operation and Development (OECD) guidelines reported the safety of LactoSporin for ocular, dermal irritation and environmental safety and is registered with European Union under the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH). In the in vitro studies, LactoSporin showed significant antioxidant, and skin protecting activities.[28] Based on the anti-aging activity of LactoSporin in vitro, the present study evaluated the clinical anti-aging effect of the ingredient.

Crow's feet are wrinkles etched in the corner of the eye, which appear deeper and more pronounced on the skin. These facial wrinkles and fine lines appear due to a decline in the extracellular matrix, affecting the normal organization of the skin and its capacity to repair.[41] Both dermatological evaluation and Antera-3D assessments of skin topography showed a significantly better effect of LactoSporin formulation on reducing wrinkles and fine lines. Exposure to ultraviolet radiation, pollution, and oxidative stress accelerate the manifestation of wrinkles on the skin. The presence of an antioxidant in skin care products helps to reduce the free radicals and oxidative stress. LactoSporin showed antioxidant activity, as it could reduce reactive oxygen species and scavenge free radicals in vitro. Further, LactoSporin was also shown to protect skin cells against ultraviolet (UV)-induced apoptosis and cell death, suggesting that the product could act as an antioxidant to protect the skin against oxidative stress.[28] In the same study, we also observed the anti-glycation and collagenase inhibiting activity of LactoSporin, which could have contribute to its effect in the clinical study.[28]

Besides wrinkles and fine lines, skin barrier functions, hydration, biomechanical properties, firmness, and elasticity decrease with age.[41] To understand the effect of cosmetic products on moisturizing effects, the increase in the water content of the stratum corneum as well as the decrease in TEWL are evaluated.[42] Our results showed a decrease in TEWL values for both placebo and LactoSporin formulations. The values were comparable between the groups, suggesting that LactoSporin may not contribute to water retention on the skin.

The improvements in skin elasticity and firmness were comparable between placebo and LactoSporin formulations in Cutometer evaluations. These parameters were better for LactoSporin formulation as per the assessment by dermatologists. We observed a difference in the results obtained by dermatological and instrument-based evaluation for skin elasticity and hydration. Although a positive correlation has been reported between instrumental and clinical assessments, few studies show also observed a weaker correlation for skin hydration[43,44] In our study, the dermatological assessments were focused on the entire face while the instrument analysis was carried out on a few selected areas. The lack of correlation between the two results could be due to the selected areas used in instrument analysis which warrants further validation in future studies.

Further, the placebo effect observed in the instrumental analysis could be due to multiple reasons. The participants were not using any skin care regimen prior to enrollment in the study and used the formulations diligently for 10 weeks. Regularity in the use of face cream might have resulted in skin improvements observed in placebo.

Visibly thin skin, fine lines and wrinkles are the first signs of collagen reduction in the skin due to the aging process. LactoSporin was effective in reducing these changes in the skin significantly better than placebo by dermatological and instrument-based assessments. Although we were unable to differentiate LactoSporin formulation from placebo for skin hydration and elasticity by Corneometer and Cutometer in this study, extending the study for longer duration might help in increasing skin elasticity which can be explored in future studies. An active anti-aging ingredient should influence the visible signs of skin aging through wrinkle reduction, firmness improvement, and moisturization of dry skin. Most of the marketed anti-aging cosmetic formulations generally include several active ingredients having antioxidants, moisturizing, and skin repair effect. LactoSporin could be used as one of the active ingredients in anti-aging formulations for reducing wrinkles.

The strengths of this study include the randomized, double-blind, placebo-controlled design and use of dermatological and instrument analysis to evaluate the effect of LactoSporin. Few limitations could be the single-center design, shorter duration and the relatively few participants. The instrument analysis was used in selected areas, which can be considered as a limitation. The average age of the study participants was 43 years, which can still be considered younger for an anti-aging study.

Conclusion

In conclusion, the results of the study suggest an improvement in wrinkles and skin texture with a skin formulation containing 2% LactoSporin. Thus, LactoSporin could be an effective natural ingredient for reducing fine lines and wrinkles in human participants and could be a useful addition in cosmetic formulations. With no negative skin reactions, LactoSporin is also a safe ingredient for skin-formulations. Future studies in participants from various parts of the country/globe will be valuable in exploring the skin-benefits of this ingredient in various formulations.

Abbreviations

ECM, extracellular matrix; EGF, epithelial growth factor; GRAS, Generally Recognized as Safe; OECD, Organization for Economic Co-operation and Development; PIPT, Primary Skin Irritation Patch Test; PNS, Photo Numerical Scale; REACH, Registration, Evaluation, Authorization, and Restriction of Chemicals; TEWL, transepidermal water loss; UV, ultraviolet.

Data Sharing Statement

All the data pertaining to the manuscript are included in the manuscript and the Supplementary Section. Any request for data can be directed to the corresponding author (lakshmi@sami-sabinsagroup.com)

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Disclosure

All the authors are employees of Sami-Sabinsa Group Limited or Sabinsa Corporation. Dr Kalyanam Nagabhushanam has a patent US11123382 issued to Sami-Sabinsa Group. The authors report no other conflicts of interest in this work.

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