Probacillus Revive
Efficacy Data

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<td><strong>ORAC Assay</strong></td>
<td>Probacillus Revive exhibited antioxidant activity comparable to 200μM Trolox®. The antioxidant capacity of Probacillus Revive increased as the concentration increased, as a result we can assure that its ability to minimize oxidative stress is dose dependent. Probacillus Revive was designed to aid in cell renewal, moisturization, and anti-aging and function as a probiotic. With the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing potent antioxidant benefits when added to cosmetic applications.</td>
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<tr>
<td><strong>Cellular Viability Assay</strong></td>
<td>Probacillus Revive exhibited positive results by increasing cell metabolism. The increase in fluorescent signal indicates an increase in cellular metabolism and viability post Probacillus Revive treatment. For these reasons, we can assume Probacillus Revive is suitable for cosmetic applications designed to increase cell viability and metabolism</td>
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<tr>
<td><strong>IL-6 ELISA Assay</strong></td>
<td>Probacillus Revive did not exhibited anti-inflammatory effects on LPS-treated fibroblasts at higher concentrations. As expected, the changes in IL-6 production using Probacillus Revive appears to be dose dependent and at lower concentrations seems to be moderately anti-inflammatory. Therefore, Probacillus Revive is more suitable for cosmetic applications other than anti-inflammation when higher concentrations are used.</td>
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Cellular Renewal Assay

The results indicate that Probacillus Revive is capable of increasing cellular renewal 13.0% better when compared to the untreated biological control and 9.0% better when compared to the unloaded vehicle. Enhancing cellular renewal can help improve epidermal integrity, therefore enhancing barrier function. This benefit of cellular renewal will ultimately lead to improvements in hydration.

Moisturization Assay

As evidenced in a efficacy study of Probacillus Revive on skin, our findings indicate that Probacillus Revive is 18.0% more effective in moisturizing the skin than glycerin, a known humectant. Furthermore, Probacillus Revive worked 69.0% better at moisturizing the skin than a cost comparable concentration of Hyaluronic Acid. This study was conducted to demonstrate that Probacillus Revive is a more effective approach to moisturization than glycerin and also provides brand differentiation. Additionally, Probacillus Revive is the more economical choice in moisturizing agents when compared to Hyaluronic Acid. Furthermore, the cost contribution of the two when compared shows Probacillus Revive moisturizes much more effectively. Probacillus Revive was designed to provide moisturizing benefits, however with the present study we can confirm that this succulent botanical ingredient is not only capable of providing protective benefits but also ideal for moisturizing and skin hydrating personal care applications.

Increase in Collagen I Production

Collagen I is a major component of the human dermis and provides stability and elasticity to the skin. According to the results, Probacillus Revive shows that all three levels (1%, 2%, and 4%) significantly increase collagen I synthesis in comparison to the control sample. 1% Probacillus Revive increased collagen I synthesis by 46%; 2% Probacillus Revive increased collagen I synthesis by 99%; and 4% Probacillus Revive increased collagen I synthesis by 164%. The data displays that a 4% concentration is the most effective in boosting synthesis of collagen I by fibroblast cells, as the results are dose-dependent. Therefore, we can assume that Probacillus Revive may increase cellular metabolism and in turn enhance collagen I synthesis, improving the overall health of the skin.
Oxygen Radical Absorbance Capacity (ORAC) Assay

Tradename: Probacillus Revive

Code: 16618

CAS #: 9015-54-7

Test Request Form #: 196

Lot #: 27201

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Erica Segura
Principle Investigator: Meghan Darley

Test Performed:
Oxygen Radical Absorbance Capacity (ORAC)

Introduction

Reactive oxygen species (ROS) are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS are detrimental to cellular structures and functional molecules (i.e DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. The oxygen radical absorbance capacity (ORAC) assay is a standard method used to assess antioxidant capacity of physiological fluids, foods, beverages, and natural products. The assay quantitatively measures a sample’s ability to quench free radicals that have the potential to react with and damage cellular components.

Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of Probacillus Revive.

Assay Principle

This assay is based upon the effect of peroxyl radicals generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The degree of change is indicative of the amount of radical damage and the presence of antioxidants results in an inhibition in the free radical damage to the fluorescein. The antioxidant protection of the sample can be calculated by comparing it to a set of known standards. Trolox®, a water soluble vitamin E analog, with known antioxidant capabilities is used in this ORAC assay as the standard for measuring the antioxidant capacity of unknown substances. ORAC values, expressed in µM of Trolox® equivalents (TE), are calculated using the area under the curves (AUC) of the test product, Trolox®, and the control materials. Trolox equivalency is used as the benchmark for antioxidant capacity of mixtures since it is difficult to measure individual components.
Oxygen Radical Absorbance Capacity (ORAC) Assay

Materials

A. Equipment: Synergy H1 Microplate reader (BioTek Instruments, Winooski, VT); Gen5 software (BioTek Instruments, Winooski, VT); Pipettes
B. Buffers: 75mM Potassium Phosphate (pH 7.4); Deionized H₂O
C. Reagents: 2,2’-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)
D. Preparation: Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.
E. Microtitre Plates: Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of Probadillus Revive and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared at concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37°C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

\[
\text{AUC} = 0.5 + \frac{R_2}{R_1} + \frac{R_3}{R_1} + \frac{R_4}{R_1} + \ldots + \frac{R_n}{R_1} \rightarrow \text{Where } R \text{ is fluorescence reading}
\]

\[
\text{Net AUC} = \text{AUC_{sample}} - \text{AUC_{blank}}
\]

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample’s Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents (µMTE), where 1 ORAC unit is equal to 1 µMTE.
Results

**Probacillus Revive** began exhibiting antioxidant activity at a 0.1% concentration.

![16618 Probacillus Revive ORAC](image)

Figure 1: Antioxidant capacities

**Discussion**

As shown in figure 1, **Probacillus Revive** exhibited antioxidant activity comparable to 200µM Trolox®. The antioxidant capacity of **Probacillus Revive** increased as the concentration increased, as a result we can assure that its ability to minimize oxidative stress is dose dependent.

**Probacillus Revive** was designed to aid in cell renewal, moisturization, and anti-aging and function as a probiotic. With the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing potent antioxidant benefits when added to cosmetic applications.
**Tradename:** Probacillus Revive

**Code:** 16618

**CAS #:** 9015-54-7

**Test Request Form #:** 181

**Sponsor:** Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

**Study Director:** Erica Segura

**Principle Investigator:** Meghan Darley

**Test Performed:**
Cellular Viability Assay

**Introduction**

The cellular viability assay is useful for quantitatively measuring cell-mediated cytotoxicity, cell proliferation and mitochondrial metabolic activity. Increased metabolism in a cell indicates ample cellular respiration and adenosine triphosphate (ATP) production. ATP is the molecular energy of cells and is required in basic cell function and signal transduction. A decrease is ATP levels indicates cytotoxicity and decreased cell function while an increase in ATP levels indicates healthy cells.

The cellular viability assay was conducted to assess the ability of **Probacillus Revive** to increase cellular metabolic activity in cultured dermal fibroblasts.

**Assay Principle**

The assay utilizes a nonfluorescent dye, resazurin, which is converted to a fluorescent dye, resorufin, in response to chemical reduction of growth medium from cell growth and by respiring mitochondria. Healthy cells that are in a proliferative state will be able to easily convert resazurin into resorufin without harming the cells. This method is a more sensitive assay than other commonly used mitochondrial reductase dyes such as MTT. An increase in the signal generated by resazurin-conversion is indicative of a proliferative cellular state.

This information is presented in good faith but is not warranted as to accuracy of results. Also, freedom from patent infringement is not implied. This information is offered solely for your investigation, verification, and consideration.
Materials

A. Kit: PrestoBlue™ Cell Viability Reagent (Invitrogen, A13261)
B. Incubation Conditions: 37°C at 5% CO₂ and 95% relative humidity (RH)
C. Equipment: Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Pipettes
D. Cell Line: Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers: Dulbecco's Modified Eagle Medium (DMEM); Penicillin-Streptomycin (50U-50mg/mL); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS)
F. Culture Plate: Falcon flat bottom 96-well tissue culture treated plates
G. Reagents: PrestoBlue™ reagent (10X)
H. Other: Sterile disposable pipette tips

Methods

Human dermal fibroblasts were seeded into 96-well tissue culture plates and allowed to grow to confluence in complete DMEM. A 10-fold serial dilution was performed resulting in Probacillus Revive concentrations on 1%, 0.1%, and 0.01% in complete DMEM and incubated with fibroblasts for 24 hours.

Ten microliters of viability reagent was added to 90µL of cell culture media in culture wells.
Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

**Probacillus Revive** at all concentrations is able to increase cellular metabolism compared to the control.

Cellular metabolism results are expressed as a percentage of the control.

![Graph showing cellular metabolism results for Probacillus Revive](image)

**Figure 1**: Cellular Metabolism of **Probacillus Revive**-treated fibroblasts expressed in terms of percent of control.

Discussion

As shown in figure 1, **Probacillus Revive** exhibited positive results by increasing cell metabolism. The increase in fluorescent signal indicates an increase in cellular metabolism and viability post **Probacillus Revive** treatment. For these reasons, we can assume **Probacillus Revive** is suitable for cosmetic applications designed to increase cell viability and metabolism.
Tradename: Probacillus Revive

Code: 16618

CAS #: 9015-54-7

Test Request Form #: 208

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Erica Segura
Principle Investigator: Meghan Darley

Test Performed:
Interleukin (IL)-6 Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Interleukin-6 is a proinflammatory cytokine known to play an active role in inflammation, immunology, bone metabolism, reproduction, arthritis, neoplasia, and aging. IL-6 signals through the nuclear factor-kappa B (NF-κB) pathway that results in the transcription of inflammatory mediators, including matrix metalloproteinase-1 (MMP-1). MMP's are responsible for breaking down the extracellular matrix and collagen in the skin leading to wrinkles, fine lines, and loss of skin elasticity. Reducing the level of IL-6 and other inflammatory mediators is believed to slow down degradation of the skin matrix and, possibly, stimulate its replenishment.

Interleukin-6 ELISA was conducted to assess the changes in IL-6 levels in Probacillus Revive-treated in vitro cultured human dermal fibroblasts.

Assay Principle

This ELISA utilizes a colorimetric reaction employing antibodies with antigen specificity to human IL-6. Monoclonal antibodies specific for IL-6 epitopes are coated on a microtiter plate. In positive samples, IL-6 will bind to these antibodies and are tagged a second time with another IL-6-specific antibody labeled with horseradish peroxidase (HRP). The addition of the chromagen solution, containing 3,3',5,5'-tetramethylbenzidine, provides the colorimetric reaction with HRP that is quantitated through optical density (OD) readings on a microplate spectrometer. The standard curve provides a reference from the OD readings for the amount of collagen in each sample.
Materials

A. Kit: IL-6 ELISA Kit (Biosource; KAC1261)
B. Incubation Conditions: 37°C at 5% CO$_2$ and 95% relative humidity (RH)
C. Equipment: Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes
D. Cell Line: Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers: Dulbecco's Modified Eagle Medium (DMEM); Penicillin-Streptomycin (50U-50mg/mL); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Amphotericin (45pg/mL)
F. Culture Plate: Falcon flat bottom 12-well tissue culture treated plates
G. Reagents: Lipopolysaccharide (LPS) (1µg/mL)
H. Other: Sterile disposable pipette tips; wash bottles

Methods

Human dermal fibroblasts were seeded into 12-well tissue culture plates and allowed to grow to confluence in complete DMEM. 1%, 0.1%, 0.01% concentrations of Probacillus Revive were added to complete DMEM containing 1µg/mL LPS and incubated with fibroblasts for 24 hours. Complete media containing 1µg/mL LPS was used as the positive controls and complete DMEM was used as a negative control.

Standards were prepared in concentrations ranging from 2476pg/mL to 0pg/mL. 50µL of Solution B was added to wells for standards and assay controls and 50µL of Solution A was added to experiment wells. 100µL of standards, controls, and samples were added to appropriate wells. After a one hour incubation at room temperature and washing, 50µL Solution A and 100µL anti-IL-6 conjugate was added to all wells. Following a one hour incubation and washing, 100 µL chromagen solution was added for the colorimetric reaction. One-hundred µL stop solution was added to stop the reaction after 15 minutes. The optical density was read at 450nm on the Synergy HT Microplate Reader.

A standard curve was created by reducing the data and generating a linear curve fit. The IL-6 concentration of Probacillus Revive treated-fibroblasts was determined by extrapolation from the standard curve and expressed in pg/mL.
Results

The data obtained from this study met criteria for a valid assay and the positive and negative controls performed as anticipated.

**Probacillus Revive** was not able to decrease IL-6 production compared to our positive control at higher concentrations but at low concentrations has some anti-inflammatory activity.

IL-6 levels are expressed by the following formula:

\[ \text{IL-6 Concentrations} \times \text{Dilution Factor} \]

![16618 Probacillus Revive IL-6 ELISA](image)

**Figure 1: Probacillus Revive**-treated fibroblasts IL-6 concentrations
IL-6 production percent decrease is calculated by the following formula:

\[
\frac{\text{Positive Control Avg.Concentration} - \text{Sample Avg.Concentration}}{\text{Positive Control Avg.Concentration}} \times 100
\]

Discussion

As shown in figure 1, Probacillus Revive did not exhibit anti-inflammatory effects on LPS-treated fibroblasts at higher concentrations. As expected, the changes in IL-6 production using Probacillus Revive appears to be dose dependent and at lower concentrations seems to be moderately anti-inflammatory. Therefore, Probacillus Revive is more suitable for cosmetic applications other than anti-inflammation when higher concentrations are used.
Abstract

Probacillus Revive was evaluated for its ability to accelerate cell renewal by means of a traditional Dansyl Chloride protocol. In this study, an increase in cell renewal or total exfoliation was determined by comparing the number of days required for the Dansyl chloride to disappear from the volar forearm.

Methods & Materials

A 5% Dansyl Chloride was prepared by dispersing Dansyl Chloride 95% (Sigma) in petrolatum. 10.0% Probacillus Revive was added to an O/W emulsion, the exfoliating properties of this product were compared to the unloaded test vehicle and an untreated control. 12 (M/F) subjects between the ages of 20 and 45 applied the control, positive control and test material to their volar forearm. The material was allowed to remain in place for 24 hours at which time any excess ointment was removed.

The control, positive control and test products were evaluated, with the remaining untreated site serving as the biological control. The products were applied in a randomized fashion. In this study, an increase in cell renewal or total exfoliation was determined by comparing the number of days required for the Dansyl chloride to disappear from the volar forearm. The sites were then examined daily under ultraviolet light (SL-3660 Long Wave Ultra Violet, Black Light Eastern Corp., Westbury, Long Island, NY) for fluorescence. The test was continued until no fluorescence was detectable at any site. The values listed reflect the average time for each product.

Results:

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>N/A</td>
<td>23</td>
</tr>
<tr>
<td>Unloaded Vehicle</td>
<td>N/A</td>
<td>22</td>
</tr>
<tr>
<td>Glycolic Acid</td>
<td>4.0%</td>
<td>17</td>
</tr>
<tr>
<td>Probacillus Revive</td>
<td>10.0%</td>
<td>20</td>
</tr>
</tbody>
</table>
Cellular Renewal Assay

Discussion

The results indicate that **Probacillus Revive** is capable of increasing cellular renewal 13.0% better when compared to the untreated biological control and 9.0% better when compared to the unloaded vehicle. Enhancing cellular renewal can help improve epidermal integrity, therefore enhancing barrier function. This benefit of cellular renewal will ultimately lead to improvements in hydration.
Moisturization/ Hydration Assay

Tradename: Probacillus Revive

Code: 16618

CAS #: 9015-54-7

Test Request Form #: 963

Lot #: 37886

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092
Study Director: Erica Segura
Principle Investigator: Meghan Darley

Test Performed:
Moisturization/ Hydration Assay

Introduction

An in-vivo study was conducted over a period of three weeks to evaluate the moisturization benefits of Probacillus Revive. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly increasing moisturization compared to the control.

The Moisturization Assay was conducted to assess the moisturizing ability of Probacillus Revive.

Materials

A. Equipment: 9003 DPM Novameter

Methods

An in vivo moisturization study was performed using a 9003 DPM Novameter to quantitatively determine improvements in hydration following treatments on the volar forearm with 10% Probacillus Revive, 10% glycerin and 0.05% hyaluronic acid in o/w emulsions. The concentration of hyaluronic acid tested was chosen based on comparable cost contribution to a formula when compared to the other ingredients. For added perspective, measurements of an untreated test site and a site treated with the unloaded vehicle were also recorded.

Results

Probacillus Revive showed very high moisturizing capabilities at a 10.0% concentration. Please note, each value is an average of three consecutive readings per test site.

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Moisturization/ Hydration Assay

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Figure 1. Percent Increase in skin hydration following the application of test and control materials.

Discussion

As evidenced in a efficacy study of Probacillus Revive on skin, our findings indicate that Probacillus Revive is 18.0% more effective in moisturizing the skin than glycerin, a known humectant. Furthermore, Probacillus Revive worked 69.0% better at moisturizing the skin than a cost comparable concentration of Hyaluronic Acid. This study was conducted to demonstrate that Probacillus Revive is a more effective approach to moisturization than glycerin and also provides brand differentiation. Additionally, Probacillus Revive is the more economical choice in moisturizing agents when compared to Hyaluronic Acid. Furthermore, the cost contribution of the two when compared shows Probacillus Revive moisturizes much more effectively.

Probacillus Revive was designed to provide moisturizing benefits, however with the present study we can confirm that this succulent botanical ingredient is not only capable of providing protective benefits but also ideal for moisturizing and skin hydrating personal care applications.
Probacillus Revive
Code: 16618

Abstract

The purpose of this study is to determine the effects of Probacillus Revive on collagen I synthesis. Collagen I is a major component of the dermis and provides structure and elasticity to the skin. Our technical staff conducted a series of tests in-vitro within an amino acid deficient medium to determine the supplemental effects of Probacillus Revive on fibroblast cellular metabolism and collagen I synthesis. Three dose levels were compared to a control, and the results were determined by an ELISA assay. These results showed that all dose levels are effective at increasing collagen I synthesis, though it is most effective at 4% because Probacillus Revive is dose-dependent.

Materials and Methods

Human fibroblasts were grown in a medium containing 10% fetal calf serum and inoculated at a concentration of approximately 6000 cells per dish. They were incubated in a humid atmosphere containing 5% CO₂ at 38°C for 24 hours. These cells were then removed and placed into an amino acid deficient medium and supplemented with Probacillus Revive at levels of 1%, 2%, and 4%. These samples were compared to a control that was placed in an amino acid deficient medium and given no supplement. Collagen I synthesis was then checked 48 hours later. Collagen I synthesis was determined by immunolabeling with primary and secondary antibodies (murine anti-collagen I monoclonal antibody and murine anti-IgG antibody) in a peroxidase/ TMB substrate (3,3′,5,5′-tetramethylbenzidine) visualization system.

Results

Increase in collagen I synthesis is expressed by the following formula:

\[(\text{Probacillus Revive} - \text{Control}) \times 100 = \% \text{ Increase in Collagen I} \]

\(\text{Control}\)
Collagen Production

Collagen I is a major component of the human dermis and provides stability and elasticity to the skin. According to the results, Probacillus Revive shows that all three levels (1%, 2%, and 4%) significantly increase collagen I synthesis in comparison to the control sample. 1% Probacillus Revive increased collagen I synthesis by 46%; 2% Probacillus Revive increased collagen I synthesis by 99%; and 4% Probacillus Revive increased collagen I synthesis by 164%. The data displays that a 4% concentration is the most effective in boosting synthesis of collagen I by fibroblast cells, as the results are dose-dependent. Therefore, we can assume that Probacillus Revive may increase cellular metabolism and in turn enhance collagen I synthesis, improving the overall health of the skin.

Discussion

Figure 1. Increased collagen I synthesis due to the application of Probacillus Revive.