ACTIVE MICRO TECHNOLOGIES

RETHINKING PRESERVATION:

Novel Antimicrobial Peptides as Natural Alternatives for Upholding Product Integrity

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Necessity of Product Preservation

Background

- Cosmetics and personal care products can unintentionally serve as an ideal medium for microbial growth
  - Bacteria, fungi, and mold can cause contamination
- Formulators must develop products that are resistant to microbial contamination for safety and regulatory compliance
- Preservative systems are added to personal care products at relatively low levels to ensure products remain safe and perform as intended over their lifetime

Product Preservation is Key

Market Shift Towards Natural Solutions

- Preservative systems work as an **essential component** of any cosmetic formulation to provide **resistance against microbial contamination**

- The **palette of allowed preservatives**, as listed in Annex V of the EU Cosmetics Directive, **is rapidly diminishing**
  - Due to safety concerns, increased exposure, sensitization

- Cosmetics and Personal Care Industry is on the hunt for new preservative systems to **build consumer confidence**

Product Preservation is Key

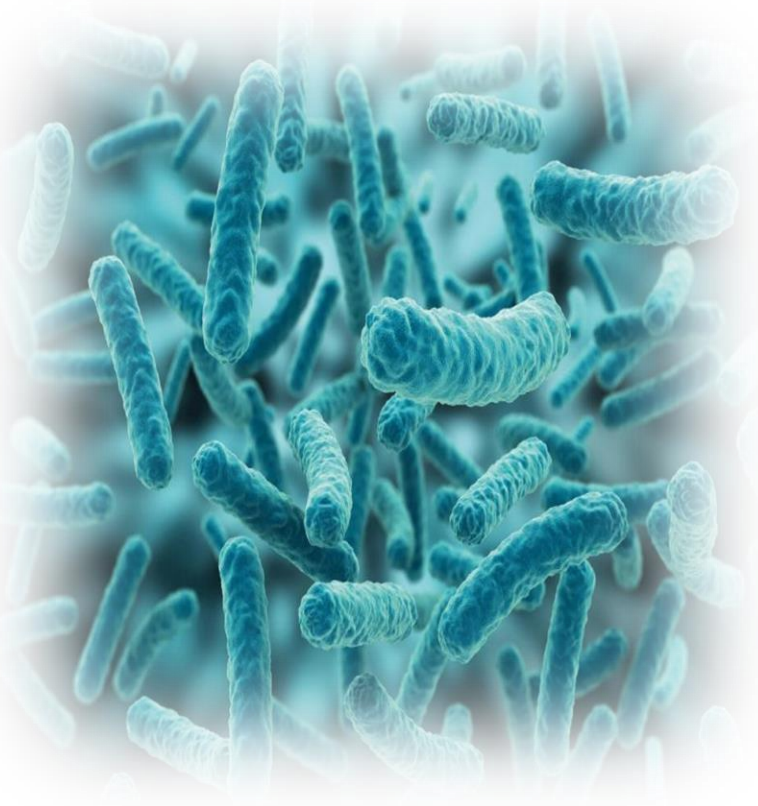
Market Shift Towards Natural Solutions

- Options for formulators to explore have included alcohols, organic acids and salts, multifunctional additives, or natural flavors and fragrance
- These options may have limitations – poor cost performance, potential for irritation, etc.
- Ideal alternative preservation systems should provide broad spectrum activity
- **What if preservation was a secondary benefit from a material's primary activity?**

Natural Product Chemistry

Antimicrobial Peptides Produced through Bacterial Fermentation

- The fermentation of lactic acid bacteria to encourage the production of antimicrobial peptides serves as a solution for alternative preservation
- Peptides function ubiquitously as cellular messengers
- Antimicrobial peptides are relatively short, protein-like compounds that are typically 30 to 60 amino acids in length
- Antimicrobial peptides derived from bacteria, they are typically produced as defense mechanisms to gain a competitive advantage against other microorganisms within their environment



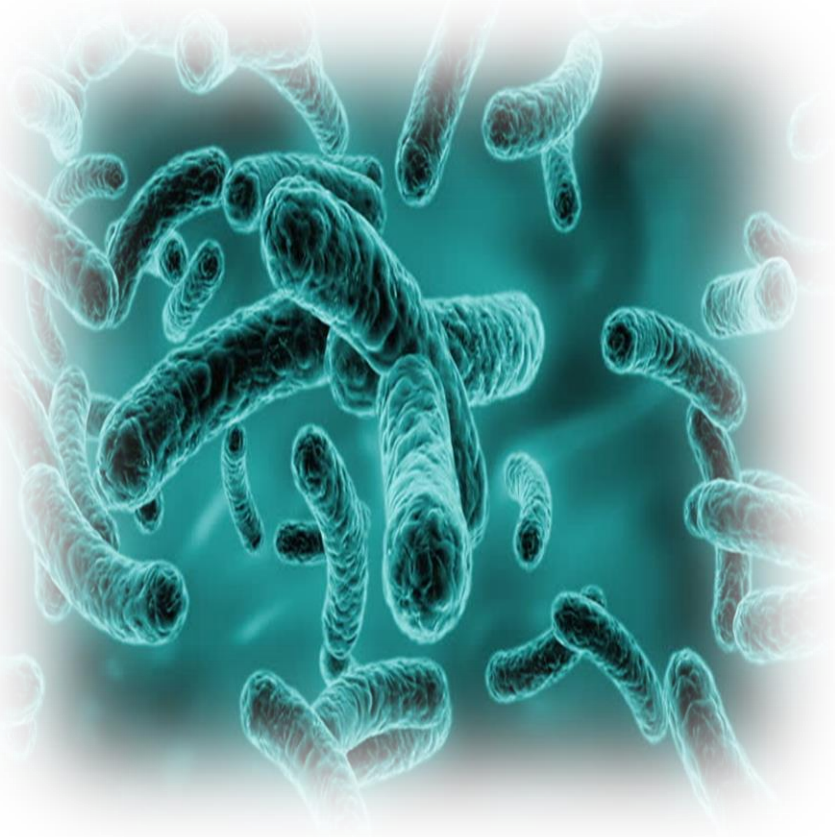
Antimicrobial Peptides

A Long History of Use

- Lactic Acid Bacteria (LAB) group, which includes microorganisms such as *Lactobacillus* sp., *Enterococcus* sp., and *Leuconostoc* sp., produces a variety of antimicrobial peptides

- Nisin produced from *L. lactis*
  - Commercialized in 1953
  - Considered GRAS for some applications

- Antimicrobial peptides are commonly used in the preservation of fermented food products



Antimicrobial Peptides

Use in Fermentation

- Fermented foods represent some of our earliest culinary endeavors
- Represented in every culture
- Is the ability of fermentation to preserve foods more than an issue of pH?
- Microorganisms used for fermentation release active antimicrobial peptides

Severe Acute Respiratory Syndrome
Interest in Antmicrobial Peptide Technology

- Starting in early 2003 SARS began in East Asia and spread to over 2 dozen countries
  - Notably the Korean peninsula was not affected
  - Why?

- Kimchi
  - fermented food that mix salted cabbage with seasonings such as chilly powder, garlic, ginger, spring onion, and radish etc. and generate lactic acid at low temperature in a container.
  - Predominately fermented with *Lactobacillus sp*. and *Leuconostoc sp.*

Transforming The Face Of Preservation

Peptide Technology In The Personal Care Industry

- Focusing on natural product chemistry, the fermentation of lactic acid bacteria to encourage the production of antimicrobial peptides serves as a solution for alternative preservation

- **Mechanism of Action**
  - Lactic Acid Bacteria (LAB) family – *Leuconostoc kimchii* produces lactic acid
  - Restricts the growth of microorganisms by acidifying their environment
  - Fermentation of *Leuconostoc* creates bacteriocins (antimicrobial peptides)

- Bacteriocins provide broad spectrum activity and proven conditioning benefits

- **Modulated Activity**
  - Specific lytic agents added to the ferment filtrate to facilitate controlled cell lysis
  - Ensures the release of the bacteriocins for maximized activity

Antimicrobial Peptide (Lactobacillus Ferment)

Peptide Technology In The Personal Care Industry

- Minimum Inhibitory Concentration (MIC)

Organism	MIC (%)
E. coli	0.5
S. aureus	0.5
P. aeruginosa	0.5
C. albicans	0.5
A. Brasiliensis	0.5

Figure 1: MIC Results for Lactobacillus Ferment.

- 4.0% **Lactobacillus Ferment** in Generic Cream Base Challenge Test – pH 5

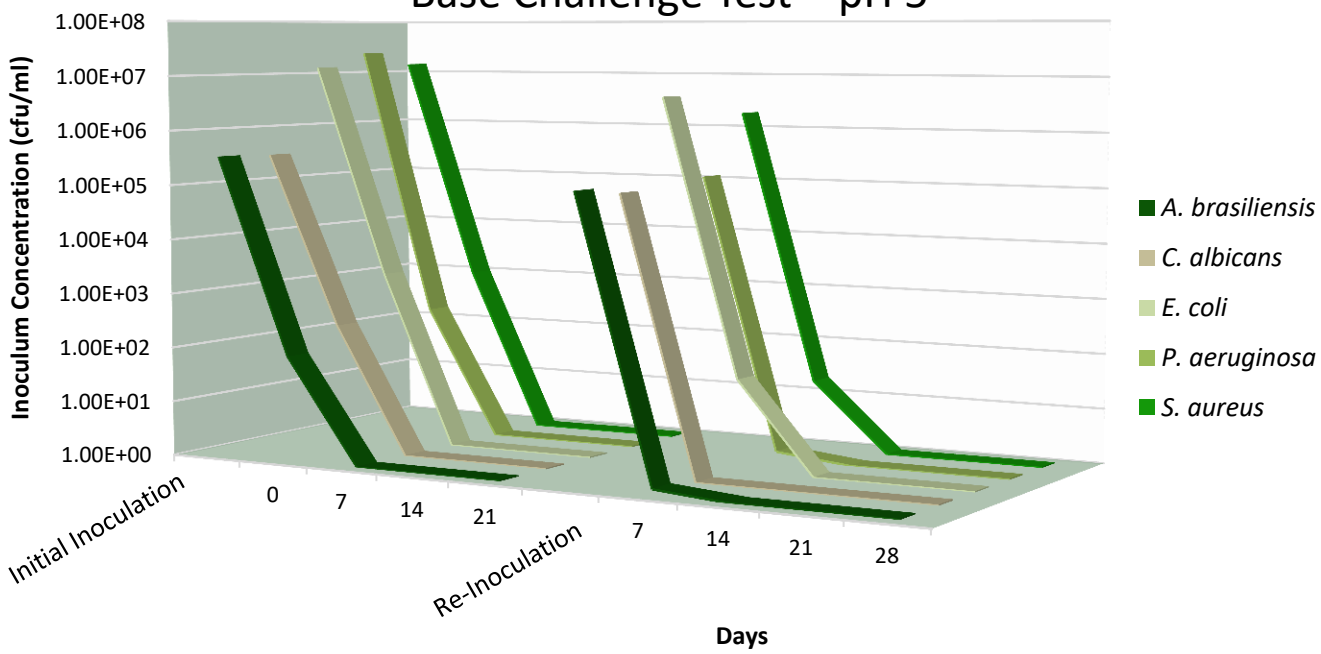


Figure 2: Challenge Test Results for Lactobacillus Ferment.

Antimicrobial Peptide (Lactobacillus Ferment)

Peptide Technology In The Personal Care Industry

- 4.0% **Lactobacillus Ferment** in Generic Cream Base Challenge Test – pH 3

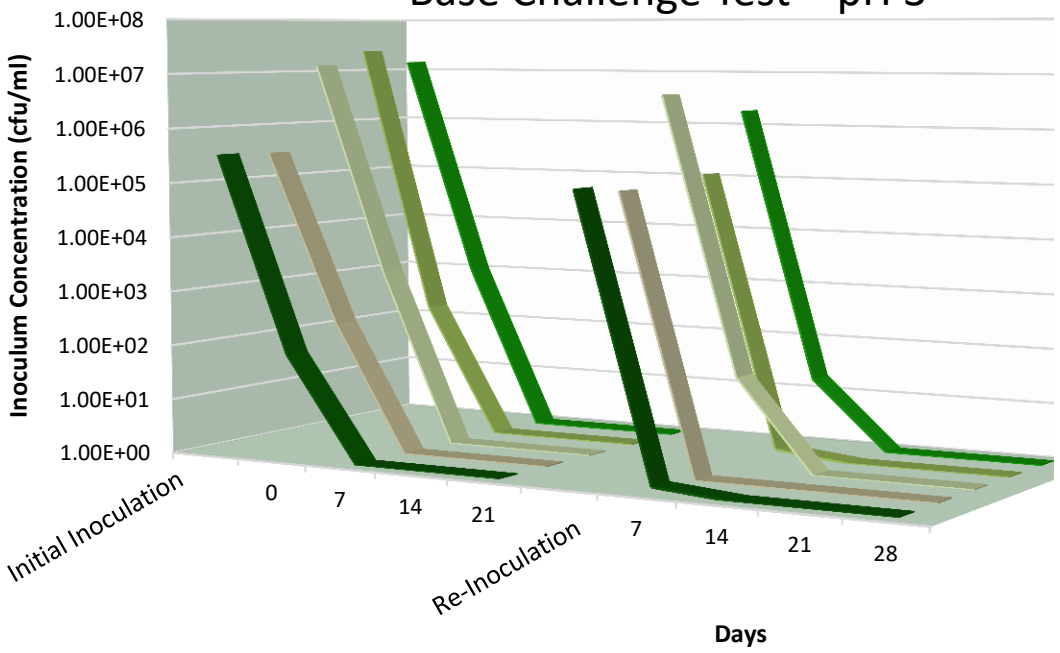


Figure 3: Challenge Test Results for Lactobacillus Ferment.

- 4.0% **Lactobacillus Ferment** in Generic Cream Base Challenge Test – pH 7

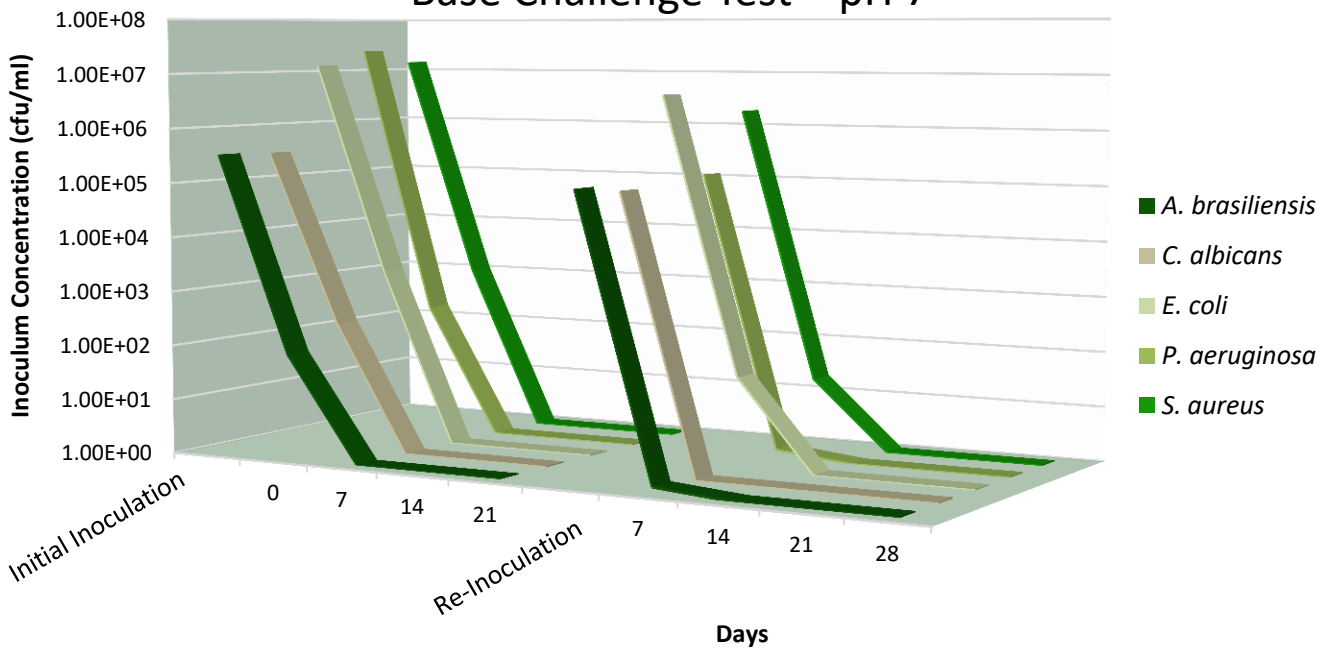


Figure 4: Challenge Test Results for Lactobacillus Ferment.

Antimicrobial Peptide (Lactobacillus Ferment)

Peptide Technology In The Personal Care Industry

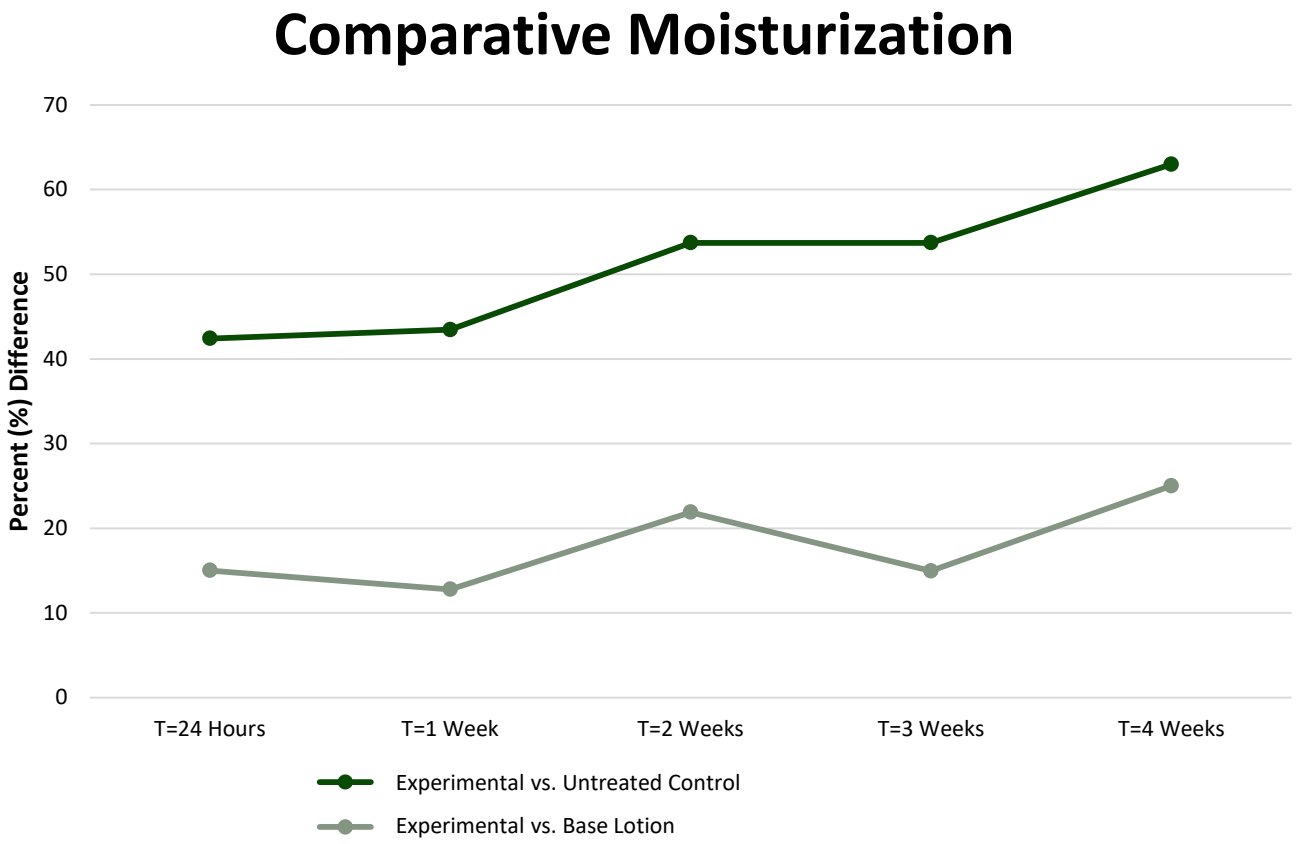


Figure 3: Moisturization Results for Lactobacillus Ferment.

Protocol

- **Equipment:** DermaLab Combo
- **Principle of measurement:** Conductance, single frequency
- **Subjects:** 10 (m/f)
- **Test area:** Volar forearms
- **Concentration of active used:** 2.0%
- **Frequency of application:** Twice Daily

Preservation and the Skin Microbiome

Peptide Technology In The Personal Care Industry

- Product preservation is crucial to prevent microbial contamination in a product during its foreseeable life in use by the end consumer

- The different microorganisms which have been found to grow in cosmetics are also resident commensal microorganisms found on our skin
  - Traditional preservatives may destroy pathogenic & commensal bacteria

- Protective microbiome should be considered
    - Could unintentionally alter the skin's natural defenses

- This principle can help guide appropriate use of potential topical probiotics
  - **Promote the delicate balance of the microflora!**

Segre, J. and Belkaid, Y. (2014). Dialogue between skin microbiota and immunity. Science. 612: 954-955

Preservation and the Skin Microbiome

In an Ideal World…

- **Cosmetics preservatives and biocides would prevent microbial growth within personal care products without affecting the skin's natural microbiome**

- How can we evaluate the effect of an antimicrobial on the microbiome?

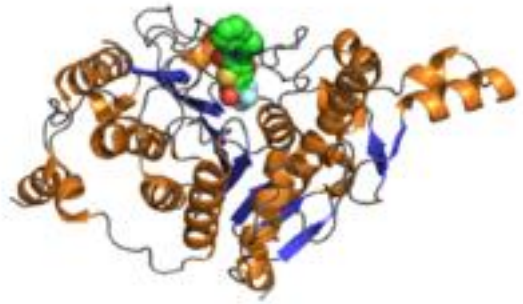
Preservation and the Skin Microbiome

HDAC: Marker of Microflora Balance

- The selective activity of natural antimicrobials and traditional preservatives has been evaluated through the analysis of Histone Deacetylaces (HDAC)

- HDAC are a class of enzymes expressed in skin cells
  - HDAC maintains healthy skin by removing acetyl groups from histones, allowing histones to condense and organize DNA for easy replication

- **HDAC serves as an innovative marker for microflora balance**
  - When the enzymes function properly, the microbial population of healthy skin remains intact
  - Preserving skin's integrity and natural barrier function



E Robertson et al, ARNT controls the expression of epidermal differentiation genes through HDAC-and EGFR-dependent pathways, Journal of Cell Science 125, 3320-3332 (2012)
T Alenghatet al, Histone deacetylase 3 coordinates commensal-bacteria-dependent intestinal homeostasis, Nature 504, 153-157 (2013)
K Youngmiet al, Histone Deacetylase 3 Mediates Allergic Skin Inflammation by Regulating Expression of MCP1 Protein, The Journal of Biological Chemistry 287(31), 25844-25859 (2012)

Preservation and the Skin Microbiome

HDAC: Marker of Microflora Balance

- HDAC3 is most prominently expressed in N-TERT human keratinocyte cells
- HDAC3 expression is essential to maintain healthy skin
  - Regulates the relationship between commensal bacteria and cell function
- HDAC expression within multiple tissue systems such as the digestive tract and the skin is an essential factor in maintaining organ health and function
- **When HDAC is altered or reduced, the skin's commensal bacteria is no longer as effective against unwanted microbes**
  - Leads to compromised immune system and reduced skin health
- HDAC is extremely sensitive to environmental and intrinsic factors such as preservatives and biocides

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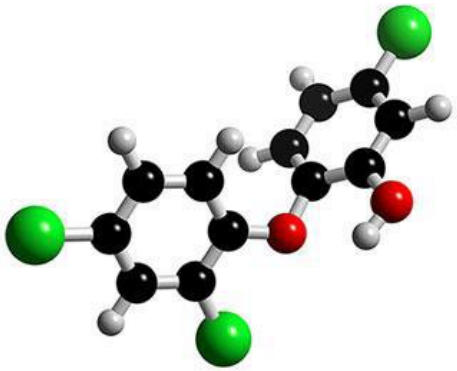
Preservation and the Skin Microbiome

Triclosan

- Triclosan is a bactericidal broad-spectrum agent developed over 40 years ago and first introduced as a surgical scrub
- Primarily used as a topical biocide more so than a cosmetic preservative
- Both biocides and preservatives affect the skin microbiome
- Over the last 20 years its use has **grown rapidly in personal care products** including soap, hand sanitizer, cosmetics, toothpaste, as well as household products such as odor fighting socks and germ resistant sponges, kitchenware, and bedding



C Cooney, Triclosan Comes under Scrutiny, Environmental Health Perspectives, 118(6), A242 (2010).

Preservation and the Skin Microbiome

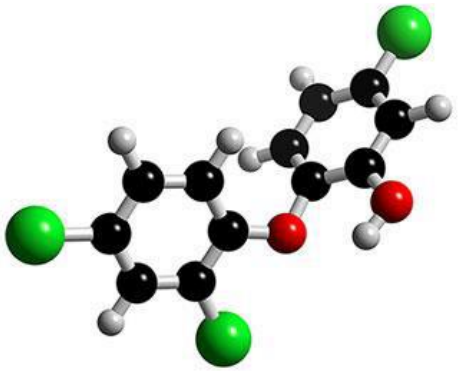
Triclosan

- Causes disruption of bacterial cell walls in nonspecific targets

- Results in disturbance of skin's microflora balance
  - **Pathogenic and commensal bacteria are killed**
  - **Skin left defenseless against new destructive microorganisms**

- Can also cause dangerous antimicrobial resistance to vital medicines
  - Growing threat to overall healthcare

- **Decreases HDAC expression in skin keratinocytes**
  - Leads us to consider natural, effective alternatives



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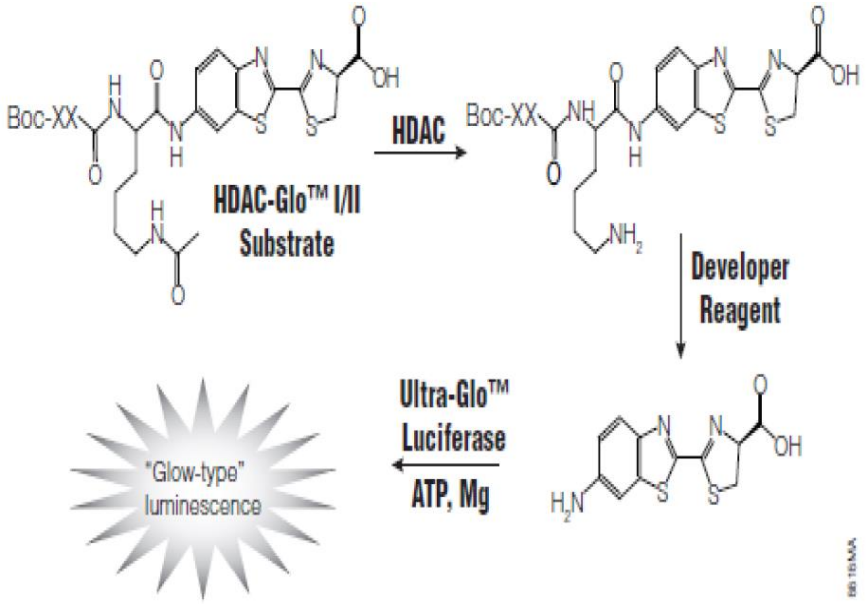
Preservation and the Skin Microbiome
HDAC Assay

- Screen each product for its effect on HDAC activity and microflora balance
- Used to determine histone deacetylase activity in cell-based or biochemical formats, providing accurate and efficient inhibitor profiling
- Bioluminescence-based detection so the light output or luminescence correlates to the amount of HDAC activity
- **Less HDAC inhibition = higher light output**



Promega HDAC-Glo™ I/II Assay and Screening System Technical Manuel, INSTRUCTIONS FOR USE OF PRODUCTS G6420, G6421, G6422, G6430, G6431, G6550, G6560 AND G6570.

Preservation and the Skin Microbiome

HDAC Assay

- More HDAC inhibition yields a lower luminescence value
  - Denotes the most damaging antimicrobial

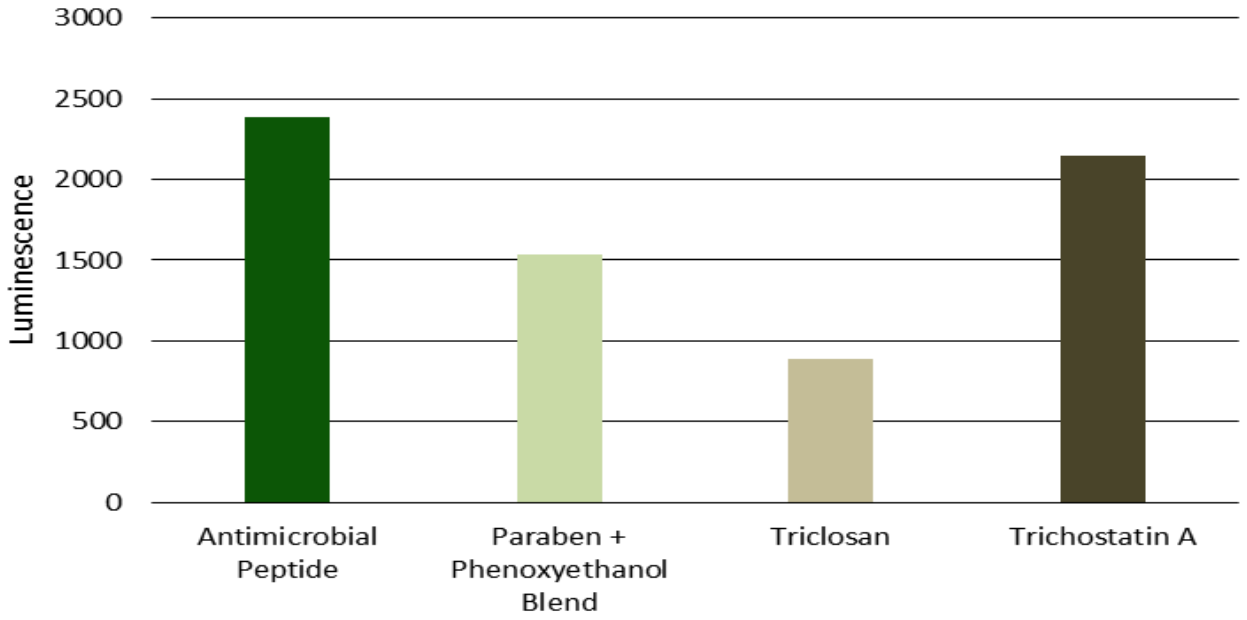


Figure 4. HDAC Assay Results

Product	Conc/Dilution	Luminescence
Peptide	32	2388
Paraben + Phenoxyethanol Blend	32	1539
Triclosan	32	889.35
Trichostatin A	1.56	2132

Figure 5. HDAC Assay Results

Preservation and the Skin Microbiome

Antimicrobial Peptides and the Microbial Population of the Microbiome

- HDAC assay has concluded that some naturally derived antimicrobials are able to destroy pathogenic bacteria while maintaining commensal microflora on the skin
  - Supporting the balance of the microbiome and promoting overall skin health

- While this research suggested HDAC is channel of communication between microflora and the skin, the effects on the population of **species** of the microbiome was not analyzed

- 16S ribosomal RNA (rRNA) analysis has been used to investigate variations in the population of microbial species after the application of antimicrobial peptides

Preservation and the Skin Microbiome

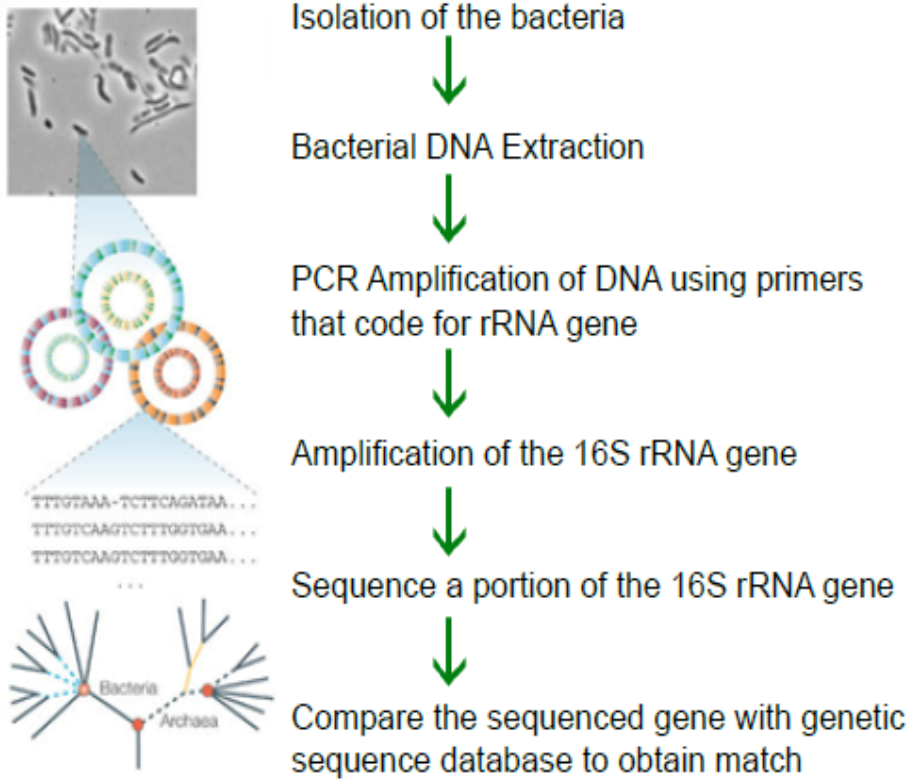
Metagenomics Analysis

- In this study, a more conventional approach was taken to analyze the effects of the population of species in the skin microbiome
- The effect of the microbial population present on the skin with the application of an antimicrobial peptide was compared to water (negative control) and Triclosan (positive control)
- Microbiome population was determined by DNA extraction, 16S ribosomal RNA (rRNA) polymerase chain reaction (PCR) amplification and sequencing
- Every person has their own unique microbiome
  - Examining the nasolabial folds of each subject isolates the geographic location
  - Person-to-person variation is uncontrollable
  - Patterns in microbial change were evaluated individually

Preservation and the Skin Microbiome

Metagenomics Analysis

- 16S rRNA sequencing is a common amplicon sequencing method used to identify and compare bacteria present within complex microbiomes and environments
- The analysis of rRNA genes begins with isolating a sample of bacteria, followed by the extraction of bacterial DNA
- The bacterial DNA undergoes PCR amplification using primers that specifically code for the 16S rRNA gene fragment. Amplification produces a population of rRNA gene fragments of equal size, determined by the specific primers used. The population of rRNA gene fragments is considered to be representative of the natural microbial population.



Preservation and the Skin Microbiome

Metagenomics Analysis

- Participants separated into blind treatment groups with each group having one of the following applied to the lateral nasal folds
  - 4.0% Antimicrobial Peptide
  - 1.0% Triclosan
  - Water

- Treatments were applied twice a day for a period of 2 weeks and new samples were taken from each participant to analyze population differences after product applications

- Samples were submitted to the Genomics Laboratory at the David H. Murdoch Research Institute (DHMRI) for DNA extraction, 16S rRNA PCR amplification and sequencing analysis

Preservation and the Skin Microbiome

Metagenomics Analysis

- DNA extracted from the samples shows a diversity population of
  - *Staphylococcus* sp., *Corynebacterium* sp., *Propionibacterium* sp., *Streptococcus* sp., *Aerobacillus* sp.

- As well a different populations known as transient and/or opportunistic invaders, such as
  - *Escherichia* sp, *Pseudomonas* sp., *Vibrio* sp., *Clostridium* sp., *Neisseria* sp.

Name	Taxonomy
HM267149.1.1374	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Bacillales, D_4__Staphylococcaceae, D_5__Staphylococcus, D_6__uncultured bacterium
JF144078.1.1370	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Bacillales, D_4__Staphylococcaceae, D_5__Staphylococcus, D_6__uncultured bacterium
DQ870740.1.1288	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Bacillales, D_4__Staphylococcaceae, D_5__Staphylococcus, D_6__Staphylococcus epidermidis
EF509212.1.1332	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Streptococcaceae, D_5__Streptococcus, D_6__uncultured bacterium
JF172400.1.1363	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Pasteurellales, D_4__Pasteurellaceae, D_5__Haemophilus, D_6__uncultured bacterium
FN908168.1.1419	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Streptococcaceae, D_5__Streptococcus, D_6__Streptococcus sp. 183-08
JF239161.1.1368	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Streptococcaceae, D_5__Streptococcus, D_6__uncultured bacterium
AJ276512.1.1499	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Aerococcaceae, D_5__Aerococcus, D_6__Aerococcus sanguinicola
HQ450584.1.1399	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Streptococcaceae, D_5__Streptococcus, D_6__uncultured bacterium
DQ805513.1.1407	D_0__Bacteria, D_1__Firmicutes, D_2__Erysipelotrichia, D_3__Erysipelotrichales, D_4__Erysipelotrichaceae, D_5__Incertae Sedis, D_6__uncultured bacterium
EF653422.1.1493	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Lactobacillaceae, D_5__Lactobacillus, D_6__uncultured bacterium
FM996743.1.1462	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Actinomycetales, D_4__Actinomycetaceae, D_5__Actinomyces, D_6__uncultured bacterium
FJ557743.1.1389	D_0__Bacteria, D_1__Firmicutes, D_2__Clostridia, D_3__Clostridiales, D_4__Lachnospiraceae, D_5__Stomatobaculum, D_6__uncultured bacterium
FJ558013.1.1408	D_0__Bacteria, D_1__Bacteroidetes, D_2__Bacteroidia, D_3__Bacteroidales, D_4__Prevotellaceae, D_5__Prevotella, D_6__uncultured bacterium
GU940721.1.1398	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Actinomycetales, D_4__Actinomycetaceae, D_5__Actinomyces, D_6__uncultured bacterium
FJ557924.1.1338	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Corynebacteriales, D_4__Corynebacteriaceae, D_5__Corynebacterium, D_6__uncultured bacterium
HQ855619.1.1284	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Corynebacteriales, D_4__Corynebacteriaceae, D_5__Corynebacterium, D_6__uncultured bacterium
GQ069781.1.1371	D_0__Bacteria, D_1__Firmicutes, D_2__Bacilli, D_3__Lactobacillales, D_4__Leuconostocaceae, D_5__Leuconostoc, D_6__uncultured bacterium
JF142155.1.1344	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Corynebacteriales, D_4__Corynebacteriaceae, D_5__Corynebacterium, D_6__uncultured bacterium
HQ452545.1.1417	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Corynebacteriales, D_4__Corynebacteriaceae, D_5__Corynebacterium, D_6__uncultured bacterium
AEQO01000237.30.1459	D_0__Bacteria, D_1__Bacteroidetes, D_2__Bacteroidia, D_3__Bacteroidales, D_4__Prevotellaceae, D_5__Prevotella, D_6__Prevotella salivae DSM 15606
HQ804831.1.1450	D_0__Bacteria, D_1__Actinobacteria, D_2__Actinobacteria, D_3__Micrococcales, D_4__Micrococcaceae, D_5__Rothia, D_6__uncultured organism
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FJ470489.1.1508	D_0__Bacteria, D_1__Firmicutes, D_2__Negativicutes, D_3__Selenomonadales, D_4__Veillonellaceae, D_5__Selenomonas, D_6__uncultured bacterium
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GQ061522.1.1348	D_0__Bacteria, D_1__Firmicutes, D_2__Clostridia, D_3__Clostridiales, D_4__Family XI, D_5__Anaerococcus, D_6__uncultured bacterium
GQ006276.1.1348	D_0__Bacteria, D_1__Firmicutes, D_2__Clostridia, D_3__Clostridiales, D_4__Family XI, D_5__Anaerococcus, D_6__uncultured bacterium
EU375190.1.1218	D_0__Bacteria, D_1__Proteobacteria, D_2__Alphaproteobacteria, D_3__Sphingomonadales, D_4__Erythrobacteraceae, D_5__uncultured, D_6__uncultured Porphyrobacter sp.
AY860251.1.1438	D_0__Bacteria, D_1__Proteobacteria, D_2__Betaproteobacteria, D_3__Burkholderiales, D_4__Burkholderiaceae, D_5__Cupriavidus, D_6__Cupriavidus taiwanensis
CP000507.436076.437612	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Alteromonadales, D_4__Shewanellaceae, D_5__Shewanella, D_6__Shewanella amazonensis SB2B
AB845250.1.1210	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Enterobacteriales, D_4__Enterobacteriaceae, D_5__Enterobacter, D_6__Enterobacter sp. BD6
KC337225.1.1448	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Oceanospirillales, D_4__Halomonadaceae, D_5__Halomonas, D_6__uncultured Halomonas sp.
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HQ467996.1.1398	D_0__Bacteria, D_1__Proteobacteria, D_2__Betaproteobacteria, D_3__Neisseriales, D_4__Neisseriaceae, D_5__Kingella, D_6__uncultured bacterium
HQ681963.1.1488	D_0__Bacteria, D_1__Proteobacteria, D_2__Betaproteobacteria, D_3__Burkholderiales, D_4__Comamonadaceae, D_5__Comamonas, D_6__uncultured bacterium
GU272313.1.1510	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Xanthomonadales, D_4__Xanthomonadaceae, D_5__Stenotrophomonas, D_6__uncultured bacterium
DQ813307.1.1471	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Pseudomonadales, D_4__Pseudomonadaceae, D_5__Pseudomonas, D_6__Pseudomonas sp. IBUN MAR1
FM163487.1.1535	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Enterobacteriales, D_4__Enterobacteriaceae, D_5__Salmonella, D_6__Achromobacter xylosoxidans
JF830196.1.1513	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Pseudomonadales, D_4__Moraxellaceae, D_5__Acinetobacter, D_6__uncultured bacterium
DQ192213.1.1346	D_0__Bacteria, D_1__Proteobacteria, D_2__Gammaproteobacteria, D_3__Pseudomonadales, D_4__Moraxellaceae, D_5__Enhydrobacter, D_6__Moraxella sp. L70
FJ375496.1.1483	D_0__Bacteria, D_1__Proteobacteria, D_2__Betaproteobacteria, D_3__Burkholderiales, D_4__Oxalobacteraceae, D_5__Massilia, D_6__uncultured bacterium
HQ456596.1.1360	D_0__Bacteria, D_1__Fusobacteria, D_2__Fusobacteriia, D_3__Fusobacteriales, D_4__Fusobacteriaceae, D_5__Fusobacterium, D_6__uncultured bacterium

Preservation and the Skin Microbiome

Metagenomics Analysis

- The antimicrobial peptide increased the beneficial bacteria in the participants' skin area studied, while decreasing the presence of *Propionibacterium* sp.

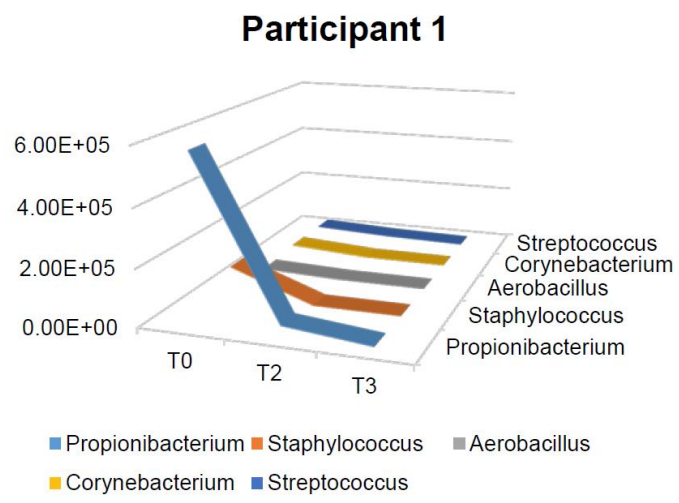


Figure 9. Antimicrobial Peptide Results

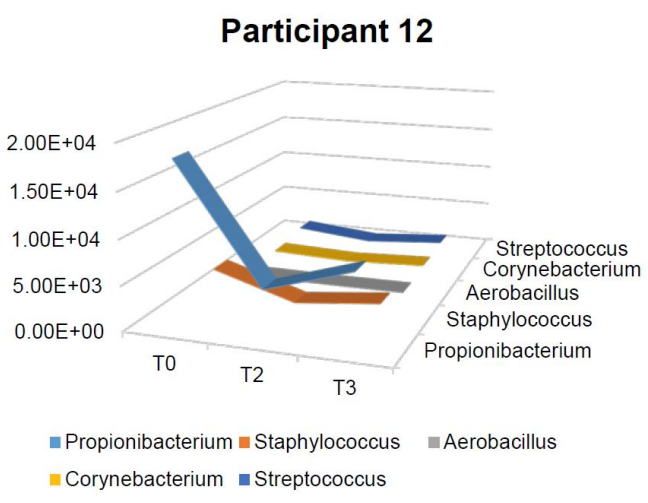


Figure 10. Triclosan Results

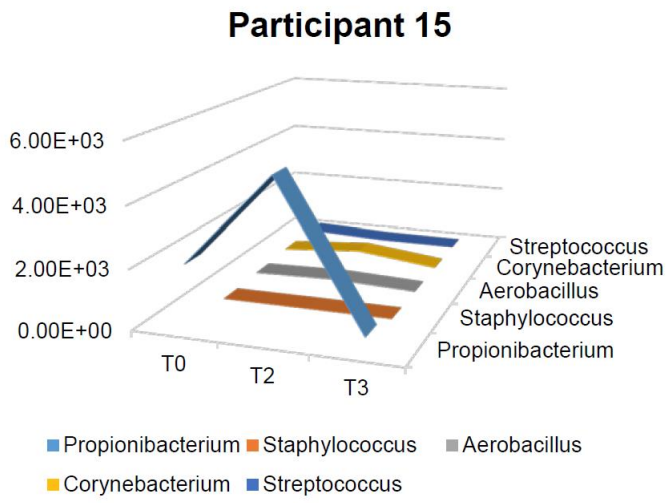
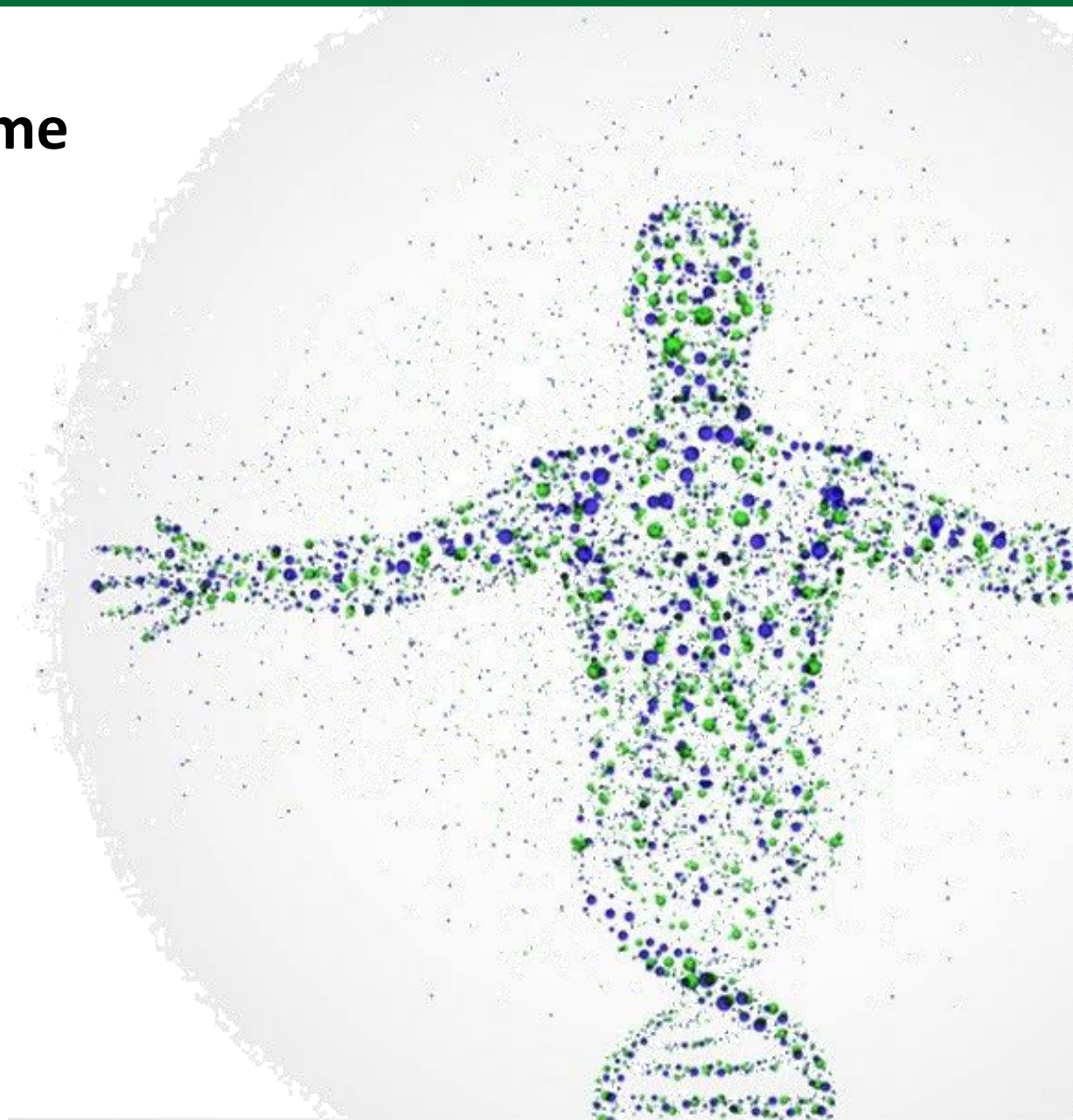


Figure 11. Water Results

Preservation and the Skin Microbiome

Metagenomics Analysis

- By increasing the populations of beneficial bacteria and decreasing the population of *Propionibacterium* sp. this current study demonstrates the potential of natural antimicrobial peptides to **promote a balanced skin microbiome**



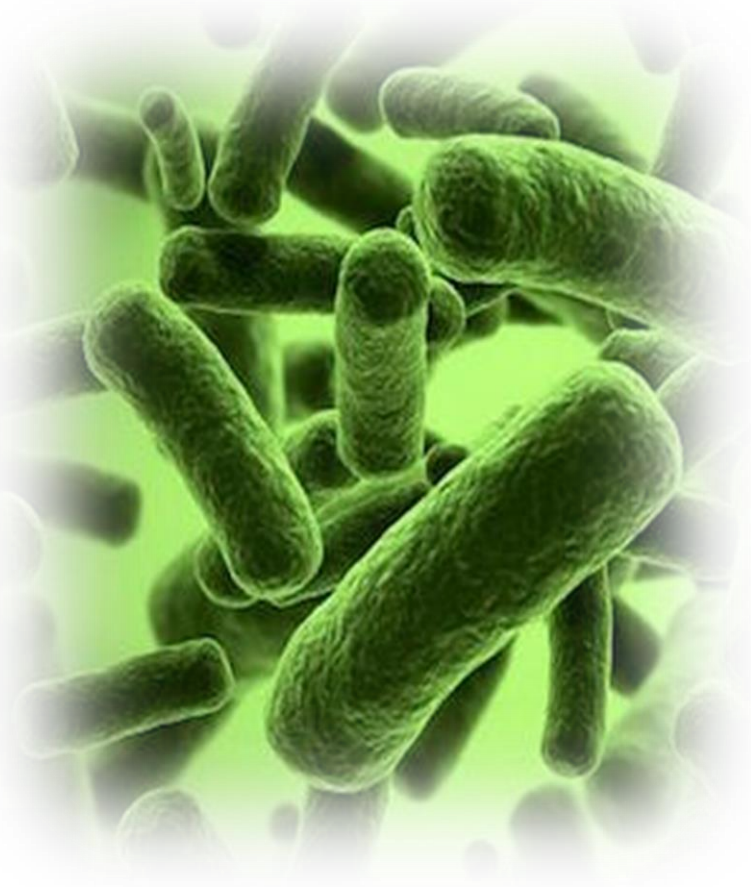
Antimicrobial Peptides
Versatility in Formulation

- Unlike more complex proteins and enzymes, antimicrobial peptides are much less susceptible to temperature and pH extremes
- Temperatures well above 40°C are typically tolerated, as are the range of pH values commonly found in cosmetic products
- Antimicrobial peptides produced by bacterial fermentation typically impart neither color nor odor to the final formulation
- These characteristics of antimicrobial peptides provide the flexibility needed to be effective in a wide variety of cosmetic and personal care formulations

Rethinking Preservation

Conclusion

- Antimicrobial peptides produced through bacterial fermentation allow cosmetic chemists to approach formulating in a more holistic manner
- Instead of adding preservatives as a final thought to the formulation the entire process of formulating and production will have to be considered, choosing bases and actives specifically to help deter microbial growth
- The use of antimicrobial peptides produced by lactic acid bacteria serves as a solution for alternative preservation



ACTIVE MICRO TECHNOLOGIES

RETHINKING PRESERVATION:

Novel Antimicrobial Peptides as Natural Alternatives for Upholding Product Integrity

Presented By: Tia Alkazaz