NATURAL ANTIMICROBIALS

The Ability to Maintain Skin’s Microflora Balance vs. the Damaging Effects of Traditional Synthetic Preservatives
OUTLINE

I. Importance of Preservation

II. Bacterial Symbiosis: Resistant, Transient, and Commensal

III. Traditional Biocides

IV. Natural Antimicrobials

V. Ideal Balance: Eliminating “Bad” & Promoting “Good” Bacteria
IMPORTANCE OF PRESERVATION

What is a Preservative?

• Historically, preservatives have been substances added to cosmetic products for the primary purpose of inhibiting the growth of microorganisms

• The PCPC defines a “preservative system” as the agent(s) incorporated into a product to reduce or prevent microbial growth

• The goal is to ensure product stability and consumer protection
IMPORTANCE OF PRESERVATION

The Skin

• The body’s largest organ and one that is constantly exposed to the environment

• It protects the body from pathogens while sustaining microorganisms that influence human health and disease.

• An ideal location for the controlled growth of bacteria

• Consists of various cutaneous antimicrobial defence mechanisms:
  • Stratum Corneum
  • Stratum Corneum Lipids
  • Production of Lysozyme
  • Acidity
  • Defensins
  • Commensal Bacteria
IMPORTANCE OF PRESERVATION

The Skin

- Dry environment unfavourable for bacterial replication
- Dead keratinocytes will slough and physically remove colonizing bacteria
- Cooler temperature than normal body and slightly acidic
- However many organisms still evade cutaneous host defences → leads to a need for antimicrobial protection
IMPORTANCE OF PRESERVATION

Cosmetic Preservatives

• The proper use of preservatives and biocides are to prevent microbial contamination without altering the skin microbiome

• Gram negative and Gram positive bacteria, yeasts, and molds have all been found to grow in various cosmetic products

• Yet, some of these microbes are also resident commensal bacteria on the skin

• Active biocides must eliminate the bad, yet promote the good bacteria

• Some traditional preservatives effective at reducing cosmetic contamination:
  • Parabens
  • Formaldehyde donors
  • Halogenated compounds

• But are they safe?
Skin Microbiome

- The skin is an ecosystem, harboring microbial communities that live in a range of physiologically and topographically distinct niches.

- Bacterial growth on the skin includes:
  - **Resident & Transient Pathogenic Bacteria** - capable of invading and causing harm
  - **Commensal Bacteria** - protects the host from these pathogens

- A 2009 NIH microbiome study analyzed ribosomal RNA gene sequences obtained from 20 distinct skin sites of healthy humans:
  - Revealed that physiologically comparable sites harbor similar bacterial communities
  - The complexity and stability of the microbial community are dependent on the specific characteristics of the skin site and extrinsic environmental factors
  - Examined the microbial interdependencies required to maintain healthy skin
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

Skin Microbiome

Diagram showing the distribution of bacterial species on different skin locations.
Recent research has begun to document how skin commensals interact with one another, with pathogenic microbes, and with human cells. Examples:

- *Staphylococcus epidermidis* secretes antimicrobial substances that help fight pathogenic invaders
- *P. acnes* uses the skin’s lipids to generate short-chain fatty acids that can similarly ward off microbial threats

“I think anything we can do to restore more balance or more appropriate microbe composition in the skin, as in all the different tissues, is extremely important.”

- Yasmine Belkaid of the National Institute of Allergy and Infectious Disease

These and other skin microbes can impact the local molecular environment, and may be able to alter the behavior of human immune cells
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

Role of the HDAC

• **Histone Deacetylases (HDAC’s)** are a class of enzymes expressed in skin cells

• These enzymes help maintain healthy skin by balancing acetylation activities of histone acetyltransferases on chromatin remodeling and also play essential roles in regulating gene transcription

• Can be responsive to many different environmental signals

• Specifically, HDAC3 which is the most prominently expressed histone deacetylase in N-TERT human keratinocyte cells, has various inflammatory and metabolic roles
Research has found that HDAC3 is a key mediator in maintaining the overall integrity and function of human organs such as the intestines and skin, while in the presence of commensal “friendly” bacteria.

HDAC3 also plays a key role in the relationship between microbiota and inflammation.

A recent study found that there were major differences between the microbial population diversity within normal mice compared to mice deficient in HDAC3.

Some bacterial species were actually over-populated when HDAC3 was reduced.
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

HDAC3

• Research proved that there is a fundamental change in the relationship between commensal bacteria and their mammalian hosts following deletion of HDAC3

• HDAC3 not only influences the bacterial population, but the bacteria in turn influence cells’ behaviour as a result, which includes gene expression
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

Role of HDAC

- A 2012 study from the Journal of Cell Science investigated HDAC protein levels and total activity dependence on Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) in N-TERT epidermal keratinocytes cells
- An HDAC activity assay was employed
- HDAC3 appeared to be the most prominently expressed histone deacetylase in these cells
- At the protein level, depletion of ARNT lead to a significant increase of HDAC3
- In ARNT-depleted HaCaT cells, there was also a prominent increase in HDAC3 protein levels
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

Role of HDAC

- The Journal of Biological Chemistry published a study investigating HDAC3 mediation of skin inflammation
- The role of HDAC3 in allergic skin inflammation primarily remains unknown
- Their results indicate that HDAC3 interacts with FcεRI and regulates expression of MCP1 through Sp1 and c-Jun to mediate allergic skin inflammation
- Concluded that HDAC3 mediates allergic skin inflammation in relation with angiogenesis by regulating MCP1
- HDAC3 serves as a target for development of allergy therapeutics
BACTERIAL SYMBIOSIS: RESISTANT, TRANSIENT & COMMENSAL

Role of HDAC

• HDAC expression within multiple tissue systems such as the digestive tract and skin, is an **integral factor in organ health and function**

• This enzyme is extremely sensitive to environmental and intrinsic factors such as preservatives and biocides, i.e.:
  • Triclosan
  • Parabens

• Expression of HDAC is an **essential component** of how we regulate the relationship between commensal bacteria and cell function to maintain healthy skin
Role of HDAC

- When HDAC is altered or reduced then the skin’s commensal bacterial, which protects against unwanted microbes, is no longer as effective.

- Our research has shown most traditional biocides decrease HDAC expression in human keratinocytes.

- This leads to a compromised immune defence system and reduced skin health.

- What if biocides could only eliminate the “bad” without altering the “good” commensal microbes, while still not affecting HDAC activity?

- Could traditional preservatives do this or do natural antimicrobials hold the key?
TRADITIONAL BIOCIDES

What are they?

• Biocides are compounds used to preserve cosmetics and personal care products, foodstuffs and medicine for both longevity of preparations and to maintain sterility

• Cosmetic preservatives are just one example of a diverse class of biocides, intended to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means

• Other examples include disinfectants, antiseptics, pesticides, herbicides, fungicides and insecticides

• These compounds vary greatly in their chemical structures

• The precise mechanism(s) of action often reflects this diversity in structure, although the final damage, when high or lethal concentrations are used, may show considerable similarity
TRADITIONAL BIOCIDES

Targets

- Biocide and antimicrobial activity varies greatly between different types of micro-organisms and it might also differ between different strains of the same species.

- Classification of micro-organisms according to their sensitivity to biocides.

- Chart does not attempt to explain how various micro-organisms may react to certain biocides.

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Fig. 1: Classification of micro-organisms according to their sensitivity to biocides. CJD, Creutzfeldt–Jacob disease agent; BSE, bovine spongiform encephalopathy agents; MAI, Mycobacterium avium intracellulare; HIV, human immunodeficiency virus. Adapted from Russell et al. (1997).
TRADITIONAL BIOCIDES

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 product preservative, such as parabens

• 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, and toothpaste, as well as household products such as odor-fighting socks and germ-resistant sponges, kitchenware, and bedding
TRADITIONAL BIOCIDES

Triclosan

Potentially Harmful Effects:

• In 2009, the European Union’s Scientific Committee on Consumer Products, wrote that the toxicologic data suggests “the continued use of triclosan as a preservative at the current concentration limit of maximum 0.3% in all cosmetic products is not safe for the consumer because of the magnitude of the aggregate exposure.”

• However, the committee noted that continued use in specific subcategories including toothpaste, soap, deodorant, face powder, and blemish concealer is considered safe

• In 2013, the FDA announced a draft rulemaking process that would require manufacturers to demonstrate triclosan’s safety and efficacy for use in soaps and body washes. This process will not be finalized until 2015 and does not include hospital-based use.
TRADITIONAL BIOCIDES

Triclosan

• Can cause disruption of bacterial cell walls in nonspecific targets

• **Decreases HDAC expression in skin keratinocytes**

• Results in disturbance of the 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 healthcare as a whole

• May enhance the production of chloroform, which is classified by the EPA as a probable human carcinogen.
  * A 2007 study illustrated that, under some circumstances, triclosan triggered production of chloroform in amounts up to 40% higher than background levels in chlorine-treated tap water.
NATURAL ANTIMICROBIALS

New Formulation Approach

- Some natural antimicrobials are equipped to kill pathogenic bacteria while still maintaining a vigorous commensal microflora on the skin.
- Other natural antimicrobials will not have a negative or inhibitory effect on HDAC expression.
- Therefore not affecting the commensal microbiota which protects and strengthens overall skin health.
- Help achieve the goal of developing natural antimicrobial cosmetic products which are not harmful under normal or foreseeable conditions of use.
Antimicrobial Peptides

• Example of a natural antimicrobial equipped to kill pathogenic bacteria while still maintaining a vigorous commensal microflora on the skin are antimicrobial peptides

• Derived from fermentation with proven efficacy

• Typically short chains of less than 50 amino acids

• Synthesized by a number of organisms as protective or competitive advantage mechanisms

• Via fermentation technology, these peptides are designed to provide:
  • Superior antimicrobial efficacy
  • Promotion of HDAC activity and commensal bacteria balance
Peptide MIC results demonstrate potential effectiveness at product addition rates of 1 to 3%.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Minimum Inhibitory Concentration (ppm in final product)</th>
<th>Minimum Inhibitory Concentration (% in final product)</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td>$1.56 \times 10^4$</td>
<td>1.6</td>
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<tr>
<td><em>S. aureus</em></td>
<td>$3.13 \times 10^4$</td>
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<tr>
<td><em>P. aeruginosa</em></td>
<td>$1.56 \times 10^4$</td>
<td>1.6</td>
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<tr>
<td><em>C. albicans</em></td>
<td>$7.81 \times 10^3$</td>
<td>0.8</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>$7.81 \times 10^3$</td>
<td>0.8</td>
</tr>
<tr>
<td><em>Bacillus spp.</em></td>
<td>$6.25 \times 10^3$</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>$6.25 \times 10^3$</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>$6.25 \times 10^3$</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Vibrio spp.</em></td>
<td>$3.12 \times 10^3$</td>
<td>0.3</td>
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Challenge Test results show successful protection of a generic cream formulation

<table>
<thead>
<tr>
<th></th>
<th>S. aureus (Initial)</th>
<th>E. coli (Initial)</th>
<th>P. aeruginosa (Initial)</th>
<th>C. albicans (Initial)</th>
<th>A. niger (Initial)</th>
<th>K. pneumoniae (Initial)</th>
<th>B. cepacia (Initial)</th>
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<td>Day 0</td>
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<td>5.485%</td>
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<td>39.535%</td>
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<td>&gt;99.999%</td>
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<td>1.23E+06</td>
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<tr>
<td>(re-inoculated)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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</table>
Peptide HDAC Assay

**Protocol: HDAC-Glo™ Assay and Screening System**

- A single-reagent-addition, homogeneous, luminescent assay that measures the relative activity of HDAC enzymes from cells

- Uses an acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC activities.

- Deacetylation of the peptide substrate is measured using a coupled enzymatic system in which a protease in the Developer Reagent cleaves the peptide from aminoluciferin.

- This is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase.

- The HDAC-mediated luminescent signal is persistent and proportional to deacetylase activity, which allows for processing of multiwell plates.
Peptide HDAC Assay

Protocol: HDAC-Glo™ Assay and Screening System

1. Human Keratinocytes plated on Day 1
2. Cells incubated on growth media overnight
3. Day 2 – Begin 1:2 serial dilutions on separate multiwell plate
4. HDAC Buffer added to each well on dilution plate
5. Media discarded from cell plate to replace with fresh media
6. Dilutions with standard transferred onto cell plate
7. Mix for one hour to homogenize
8. Incubated for 30 minutes to allow HDAC inhibitor to begin
9. Luminescent reagent prepared
10. Reagent added to cell plate
11. Incubated for 30 minutes at room temp (23-25°C)
12. HDAC activity measured on colorimetric plate reader
# Antimicrobial Peptide: HDAC Data

## Peptide HDAC Assay

### Results

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<th>Name</th>
<th>Conc/Dil</th>
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<td>Peptide</td>
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<td>Phenonip</td>
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<tr>
<td>Triclosan</td>
<td>32</td>
<td>889.35</td>
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</table>
Peptide HDAC Assay

RESULTS

HDAC Assay

Luminescence

Peptide: HDAC Data

1.56nM
Peptide HDAC Assay

**Results**

- Demonstrates successful relationship between antimicrobial peptide and skin microflora.

- Although the antimicrobial peptide still has slight effect on HDAC activity, it does not inhibit or reduce HDAC nearly as much as triclosan or parabens.

- Traditional preservatives and biocides are show to drastically inhibit HDAC activity, which negatively affects skin’s natural microflora balance.

- With natural antimicrobial peptide, the skin’s commensal bacterial, which protects against unwanted microbes, remains effective and intact.
The history of the cosmetic industry over the past 30 years has created increasing pressure on conventional preservatives and biocides. This will continue to intensify unless we find alternative approaches to the preservation of cosmetics and personal care products. There are effective natural alternatives to conventional preservation methods that only target microbes but not the underlying enzymes and cell functions they affect, such as HDAC. Provides a solution to finding the ideal balance between eliminating bad and promoting good bacteria.