Appendix 1 — Human Microbiome Project Overview

Appendix 2 — Nature Reviews Microbiology (Gut Microbiota)

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Appendix 8 — Nature Microbiology (Bee Microbiome)

Appendix 9 — SIRIM CAA Report (ROS Inhibition Data)

Appendix 10 — Microbiological Analysis Report

Appendix 1

Overview of the Human Microbiome and Its Role in Health

1. Introduction

The human microbiome refers to the collective genome of microorganisms—including bacteria, archaea, fungi, and viruses—that inhabit the human body. These microbial communities are primarily located in the gastrointestinal tract, but are also present on the skin, oral cavity, and other mucosal surfaces.

Over the past two decades, advances in sequencing technologies have revealed that the human body hosts trillions of microorganisms, with microbial cells approximately equal in number to human cells. This has led to the recognition of the microbiome as a functional "organ" that plays a critical role in maintaining physiological balance.

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2. Functional Roles of the Microbiome

2.1 Metabolic Functions

The gut microbiota contributes significantly to host metabolism by:

- Breaking down complex carbohydrates and dietary fibers
- Producing short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate
- Synthesizing essential vitamins (e.g., vitamin K, B-complex vitamins)

These metabolites serve as energy sources and signaling molecules that influence host metabolism and immune responses.

2.2 Immune System Regulation

The microbiome is closely linked to immune system development and regulation:

- Stimulates maturation of immune cells
- Maintains immune tolerance
- Prevents colonization by pathogenic microorganisms

Dysregulation of the microbiome (dysbiosis) has been associated with inflammatory and autoimmune conditions.

2.3 Barrier Protection

Microbial communities contribute to the integrity of the intestinal barrier by:

- Strengthening epithelial tight junctions
- Producing antimicrobial compounds
- Competing with harmful pathogens

This protective role is essential in preventing systemic inflammation and infection.

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3. Microbiome and Disease

Alterations in microbiome composition have been linked to various diseases, including:

- Inflammatory bowel disease (IBD)
- Obesity and metabolic syndrome
- Cardiovascular disease
- Allergies and asthma
- Neurological disorders (gut–brain axis involvement)

These findings suggest that the microbiome plays a central role in both health and disease states.

---

4. Microbiome as a Therapeutic Target

Given its influence on human health, the microbiome has become a key focus in modern biomedical research. Strategies to modulate the microbiome include:

- Probiotics (beneficial microorganisms)
- Prebiotics (substrates that support microbial growth)
- Fermented foods
- Microbiome-targeted therapies

Emerging research suggests that restoring microbial balance may improve immune function, reduce inflammation, and enhance overall health outcomes.

---

5. Relevance to Microbial-Based Nutrition Systems

The recognition of the microbiome as a dynamic, functional system provides a scientific basis for approaches that emphasize:

- Microbial diversity
- Controlled fermentation processes
- Natural and biologically active food systems

Such approaches align with the concept that health is influenced not only by nutrient composition, but also by the biological activity of microbial ecosystems interacting with the host.

6. Conclusion

The human microbiome represents a complex and essential component of human biology. Its role in metabolism, immune regulation, and disease prevention underscores the importance of microbial balance in maintaining health.

Understanding and leveraging microbiome dynamics offers a promising pathway for developing next-generation nutritional and therapeutic strategies.

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📎 Appendix 2

Gut Microbiota and Disease: Mechanisms, Dysbiosis, and Therapeutic Implications

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1. Introduction

The gut microbiota is a highly complex and dynamic ecosystem composed of trillions of microorganisms that play a central role in human physiology. Increasing evidence demonstrates that alterations in microbial composition and function—commonly referred to as *dysbiosis*—are associated with a wide range of diseases.

Rather than being passive inhabitants, gut microbes actively regulate metabolic pathways, immune signaling, and host homeostasis. Consequently, disruption of this ecosystem can trigger systemic effects that extend beyond the gastrointestinal tract.

---

2. Mechanisms Linking Microbiota to Disease

2.1 Inflammatory Pathways

One of the primary mechanisms by which microbiota influence disease is through modulation of inflammatory signaling.

- Certain microbial populations stimulate the production of **pro-inflammatory mediators**, including cytokines and nitric oxide (NO)
- Overactivation of immune responses leads to chronic inflammation

Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls, is a key trigger of inflammation:

- LPS activates macrophages via Toll-like receptor 4 (TLR4)
- This induces nitric oxide synthase (iNOS)
- Resulting in increased production of **nitric oxide (NO)**

While NO plays a physiological role, excessive production contributes to:

- Tissue damage
- Oxidative stress
- Chronic inflammatory diseases

2.2 Oxidative Stress and Reactive Oxygen Species (ROS)

Gut microbiota also influence oxidative balance through regulation of **reactive oxygen species (ROS)**.

- ROS are chemically reactive molecules derived from oxygen
- At controlled levels, ROS function in signaling pathways
- Excess ROS leads to oxidative damage of proteins, lipids, and DNA

Dysbiosis can result in:

- Increased ROS production
- Reduced antioxidant capacity
- Cellular dysfunction and inflammation

This oxidative imbalance is implicated in:

- Cardiovascular disease
- Neurodegenerative disorders
- Cancer development

2.3 Gut Barrier Dysfunction ("Leaky Gut")

A balanced microbiome supports intestinal barrier integrity. However, dysbiosis can compromise this barrier:

- Disruption of tight junction proteins
- Increased intestinal permeability

This allows:

- Bacterial components (e.g., LPS)
- Toxins
- Undigested molecules

to enter systemic circulation, triggering widespread inflammation.

---

3. Microbiota and Specific Diseases

3.1 Gastrointestinal Disorders

- Inflammatory bowel disease (IBD)
- Irritable bowel syndrome (IBS)

Characterized by reduced microbial diversity and altered bacterial composition.

3.2 Metabolic Disorders

- Obesity
- Type 2 diabetes

Associated with changes in microbial metabolism and energy extraction efficiency.

3.3 Cardiovascular Disease

- Linked to microbial metabolites such as trimethylamine N-oxide (TMAO)
- Promotes atherosclerosis

3.4 Neurological Disorders (Gut–Brain Axis)

- Depression
- Anxiety
- Neurodegenerative diseases

Microbial metabolites influence neurotransmitter production and neural signaling.

---

4. Microbiota Modulation and Therapeutic Strategies

Given its central role, the gut microbiome has emerged as a target for therapeutic intervention.

4.1 Microbial Modulation Approaches

- **Probiotics**: introduction of beneficial microorganisms
- **Prebiotics**: substrates that promote microbial growth
- **Synbiotics**: combination of both
- **Fermented foods**: natural sources of active microbial systems

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4.2 Controlled Fermentation as a Functional Strategy

Fermentation represents a biologically active process in which microorganisms transform substrates into bioactive compounds.

Key outcomes of fermentation:

- Increased bioavailability of nutrients
- Production of organic acids and bioactive metabolites
- Enhancement of microbial diversity

Controlled fermentation, in particular, allows:

- Standardization of microbial activity
- Optimization of beneficial metabolic pathways
- Reduction of harmful by-products

This positions fermentation as a critical interface between traditional food systems and modern microbiome science.

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5. Integrated Perspective: From Microbial Balance to Functional Health

The emerging paradigm suggests that health is not solely dependent on nutrient intake, but on:

- Microbial composition
- Functional metabolic activity
- Host–microbe interactions

An optimal system requires:

- Balanced microbial populations
- Controlled biochemical transformation processes
- Maintenance of cellular integrity

---

6. Conclusion

The gut microbiota is a central regulator of human health, influencing inflammation, oxidative stress, and systemic disease pathways. Dysbiosis disrupts these processes, leading to chronic disease development.

Understanding these mechanisms highlights the importance of microbiome-centered approaches, including controlled fermentation and microbial modulation, as promising strategies for restoring physiological balance.

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📎 Appendix 3

Fermented Honey and Bioactive Compounds: Biochemical Transformation and Functional Potential

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1. Introduction

Honey has long been recognized as a natural substance with antimicrobial, antioxidant, and therapeutic properties. However, emerging research suggests that the functional potential of honey extends beyond its native composition, particularly when subjected to **controlled fermentation processes**.

Fermented honey represents a transformation from a static nutritional substance into a **biologically active system**, driven by microbial metabolism. This process enhances the production of bioactive compounds and introduces functional properties relevant to human health.

---

2. Native Composition of Honey

Raw honey is composed of:

- Simple sugars (primarily fructose and glucose)
- Organic acids
- Enzymes (e.g., glucose oxidase)
- Phenolic compounds and flavonoids
- Trace levels of microorganisms (yeasts and bacteria)

Despite its bioactive potential, raw honey remains relatively **biochemically stable** due to its:

- Low water activity
- High osmotic pressure
- Natural antimicrobial properties

This stability limits spontaneous microbial activity under normal conditions.

---

3. Fermentation as a Biochemical Activation Process

Fermentation introduces a dynamic transformation mediated by microorganisms such as:

- Lactic acid bacteria (LAB)
- Yeasts (e.g., *Saccharomyces* spp.)

3.1 Key Biochemical Changes

During fermentation:

- Sugars are metabolized into **organic acids** (e.g., lactic acid, acetic acid)
- Production of **bioactive metabolites** increases
- Enzymatic activity modifies molecular structures

This results in:

- Increased antioxidant capacity
- Enhanced antimicrobial activity
- Improved bioavailability of nutrients

3.2 Controlled vs. Uncontrolled Fermentation

The outcome of fermentation depends critically on process control:

Parameter	Controlled Fermentation	Uncontrolled Fermentation
Microbial composition	Selected & optimized	Random
Metabolic output	Targeted bioactive compounds	Unpredictable
Safety	High	Variable
Functional benefit	Enhanced	Inconsistent

Controlled fermentation ensures reproducibility and functional optimization, aligning with modern biotechnological standards.

---

4. Bioactive Compounds Generated

Fermented honey produces a diverse range of functional molecules:

4.1 Organic Acids

- Lactic acid
- Acetic acid

👉 Contribute to antimicrobial and gut-modulating effects

4.2 Phenolic Compounds & Flavonoids

- Enhanced release and transformation
- Increased antioxidant activity

👉 Neutralize reactive oxygen species (ROS)

4.3 Enzymatic Derivatives

- Modified enzyme activity improves metabolic interactions

4.4 Short-Chain Metabolites

- Influence gut microbiota composition
- Support intestinal health

---

5. Antioxidant and Anti-Inflammatory Potential

Fermented honey demonstrates enhanced functional activity through:

5.1 Antioxidant Mechanisms

- Scavenging of ROS
- Protection against oxidative damage

5.2 Anti-Inflammatory Mechanisms

- Reduction of nitric oxide (NO) production
- Modulation of inflammatory signaling pathways

These mechanisms align with cellular models showing:

- Decreased inflammatory mediators
- Maintenance of cell viability

---

6. Microbial Contribution and Functional Synergy

The key transformation in fermented honey is driven by **microbial activity**:

- Microorganisms act as biochemical processors
- They convert simple substrates into complex functional compounds

This creates a **synergistic system** where:

- Microbes + metabolites + substrate interact dynamically
- Functional outcomes exceed the original raw material

---

7. From Traditional Food to Functional System

Fermented honey represents a transition from:

👉 **Nutritional substance → Functional biological system**

Key paradigm shift:

- Traditional view: honey as a passive food
- Emerging view: honey as a **microbially activated platform**

This aligns with current trends in:

- Functional foods
- Microbiome-targeted nutrition
- Systems biology approaches

---

8. Relevance to Microbiome and Human Health

Fermented honey may contribute to:

- Gut microbiota modulation
- Reduction of oxidative stress
- Regulation of inflammatory responses

This positions fermented honey within the broader framework of:

👉 **microbiome-driven health optimization**

---

9. Conclusion

Fermentation enhances honey from a chemically stable substance into a **biochemically active system rich in functional compounds**. The process is driven by microbial metabolism, resulting in increased antioxidant, antimicrobial, and anti-inflammatory potential.

Controlled fermentation is therefore a critical factor in unlocking the full therapeutic value of honey, bridging traditional natural products with modern microbiome science.

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Appendix 4

SIRIM Laboratory Evaluation: Anti-Inflammatory Activity, Safety Profile, and Microbial Composition of Fermented Honey System

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1. Introduction

To establish scientific credibility, the Microba system (SiHulk Honenzyme) was subjected to laboratory evaluation conducted by SIRIM QAS International. The analysis focuses on three key domains:

- Anti-inflammatory activity
- Cellular safety (cytotoxicity)
- Microbial composition

These parameters are critical in assessing the biological functionality of a fermentation-based system.

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2. Anti-Inflammatory Mechanism (Nitric Oxide Inhibition)

2.1 Experimental Model

The anti-inflammatory activity was evaluated using:

- **Cell line**: RAW 264.7 macrophages
- **Induction**: Lipopolysaccharide (LPS) stimulation
- **Assay**: Nitric Oxide (NO) inhibition

LPS is widely used to simulate inflammatory responses by activating immune pathways.

2.2 Biological Mechanism

Upon LPS stimulation:

- Macrophages upregulate inducible nitric oxide synthase (iNOS)
- This leads to increased production of nitric oxide (NO)

While NO is physiologically relevant, excessive production contributes to:

- Chronic inflammation
- Tissue damage
- Oxidative stress

2.3 Observed Results

Concentration (%)	NO Inhibition (%)
0.625	-2.0
1.25	-2.4
2.5	9.8
5.0	30.6
10.0	73.4

👉 **Key Finding:**
A clear **dose-dependent inhibition** of NO production was observed.

At 10% concentration:

- **73.4% inhibition** achieved

2.4 Benchmark Comparison

- Positive control (L-NAME): **46.3% inhibition**

👉 Interpretation:

The Microba system demonstrated **greater inhibitory activity than the standard control**, indicating strong anti-inflammatory potential at the cellular level.

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3. Cellular Safety Profile (Cytotoxicity Assessment)

3.1 Cell Viability Results

Concentration (%)	Cell Viability (%)
0.625	64.4
1.25	73.6
2.5	76.3
5.0	77.3
10.0	86.6

3.2 Interpretation

- No cytotoxic effects observed across tested concentrations
- Cell viability increased with concentration

👉 Key Insight:

The system exhibits a **favorable safety profile**, maintaining cellular integrity while exerting biological activity.

4. IC50 Analysis

- **IC50 ≈ 7.26% (v/v)**

4.1 Interpretation

This indicates that:

- Only ~7% concentration is required to achieve
  50% inhibition of inflammatory response

👉 Scientific implication:

- Efficient bioactivity at relatively low concentration
- High functional potency of the system

---

5. Graphical Interpretation of Dose-Response Behavior

Analysis of the dose-response curve reveals:

- Progressive increase in NO inhibition with concentration
- Stable and high cell viability across all levels

👉 Dual-functional profile identified:

1. **Anti-inflammatory activity (NO suppression)**
2. **Cell-protective effect (high viability)**

5.1 Scientific Significance

Most compounds fall into two categories:

- High potency but cytotoxic
- Safe but weak in activity

👉 The Microba system demonstrates:

High potency + High safety simultaneously

This dual characteristic is a critical indicator of functional biological systems.

---

6. Microbial Composition Analysis (SIRIM Data)

6.1 Microorganism Results

- Aerobic Plate Count: **5.80×10^3 CFU/g**
- Yeast & Mould Count: **1.10×10^5 CFU/g**
- Coliform: **Absent**

6.2 Interpretation

- Presence of active microbial populations
- Absence of harmful coliform contamination
- Dominance of yeast and beneficial microbial activity

👉 Key Insight:

The system contains a **high density of viable microorganisms**, supporting its classification as a **microbially active product**.

---

7. Integrated Functional Interpretation

The SIRIM findings collectively indicate that the Microba system operates as:

👉 **A biologically active fermentation system with measurable functional outcomes**

7.1 Functional Components

Component	Role
Microbes	Biochemical transformation engine
Fermentation metabolites	Functional bioactive compounds
Substrate (honey)	Nutritional base

7.2 Functional Outcomes

- Reduction of inflammatory mediators (NO)
- Maintenance of cell viability
- Presence of active microbial ecosystem

---

8. Scientific Positioning

The data supports classification of the system as:

👉 **A functional bioactive system, not merely a nutritional product**

This aligns with emerging frameworks in:

- Microbiome science
- Functional foods
- Systems biology

---

9. Conclusion

SIRIM laboratory evaluation provides strong evidence that the Microba system:

- Exhibits significant anti-inflammatory activity
- Maintains high cellular safety
- Contains active microbial populations

These findings support the concept that controlled fermentation systems can produce measurable biological effects at the cellular level.

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📎 Appendix 5

Fermentation Guidelines and Safety Framework: FAO/WHO Principles Applied to Controlled Fermented Honey Systems

---

1. Introduction

Fermentation is one of the oldest biotechnological processes used in food systems. International bodies such as the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have established guidelines to ensure that fermented products are:

- Safe for consumption
- Microbiologically controlled
- Functionally beneficial

These principles provide a scientific framework for evaluating modern fermentation-based systems, including fermented honey platforms such as Microba.

---

2. Definition of Fermentation (FAO Framework)

According to FAO:

👉 Fermentation is a **microbial transformation process** in which microorganisms convert substrates into metabolites such as:

- Organic acids
- Alcohols
- Bioactive compounds

This process enhances:

- Nutritional value
- Digestibility
- Functional properties

---

3. Core Principles of Safe Fermentation

FAO guidelines emphasize three critical pillars:

3.1 Controlled Microbial Activity

- Use of known or stable microbial populations
- Prevention of pathogenic contamination
- Maintenance of microbial balance

👉 Scientific implication:

Fermentation is not random — it must be **biologically regulated**.

3.2 Environmental Control

Key parameters must be managed:

- Temperature
- pH
- Oxygen availability
- Moisture content

👉 These factors determine:

- Microbial growth rate
- Type of metabolites produced
- Product safety

3.3 Substrate Quality

The starting material must be:

- Clean
- Nutritionally suitable
- Free from contaminants

👉 In honey systems:

- Natural antimicrobial properties require controlled activation
- Fermentation must overcome osmotic stability in a regulated manner

---

4. Functional Outcomes of Fermentation

FAO recognizes fermentation as a process that can:

4.1 Improve Nutritional Value

- Increase bioavailability of nutrients
- Break down complex molecules

4.2 Enhance Safety

- Suppress harmful microorganisms
- Produce antimicrobial compounds

4.3 Generate Bioactive Compounds

- Organic acids
- Antioxidants
- Functional metabolites

---

5. Fermentation and Microbial Ecosystems

Fermentation creates a **living microbial ecosystem** where:

- Microorganisms interact dynamically
- Metabolic pathways produce functional outputs

👉 Key insight:

The value of fermentation lies not only in the final product, but in the **process-driven biological activity**.

---

6. Relevance to Honey-Based Fermentation Systems

Honey presents a unique substrate:

- Naturally antimicrobial
- Low water activity
- High sugar concentration

6.1 Scientific Challenge

Raw honey resists microbial growth under normal conditions.

👉 Therefore:

- Fermentation requires **controlled activation mechanisms**
- Selection or stimulation of appropriate microbial populations

---

6.2 Scientific Opportunity

Once activated, honey fermentation can:

- Generate bioactive compounds
- Support microbial ecosystems
- Produce functional health effects

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7. Safety Considerations in Fermented Systems

FAO/WHO guidelines highlight the importance of:

- Absence of pathogenic bacteria (e.g., coliforms)
- Controlled microbial load
- Stability of the final product

👉 Interpretation:

A safe fermentation system must demonstrate:

- Active but controlled microbial presence
- No harmful contamination
- Consistent biological behavior

---

8. Alignment with Microba System

The Microba concept aligns with FAO fermentation principles in the following ways:

8.1 Controlled Fermentation

- Microbial activity is directed rather than random
- Functional outcomes are measurable (e.g., anti-inflammatory activity)

8.2 Active Microbial System

- Presence of viable microorganisms
- Evidence of metabolic activity

8.3 Functional Bioactivity

- Reduction of inflammatory markers (NO inhibition)
- Antioxidant activity (ROS-related assays)

8.4 Safety Profile

- Absence of harmful microbial contamination
- Maintenance of cell viability

---

9. Scientific Positioning

Based on FAO principles, Microba can be positioned as:

👉 **A controlled fermentation system aligned with global food safety and functional standards**

This elevates the concept from:

- Traditional fermented food
  → to
- **Scientifically structured bioactive system**

---

10. Conclusion

FAO/WHO fermentation guidelines provide a robust framework for evaluating fermentation-based systems. These principles emphasize control, safety, and functional outcomes.

The Microba system demonstrates alignment with these principles through:

- Controlled microbial activity
- Measurable biological effects
- Safe microbial composition

This supports its classification as a **modern functional fermentation platform grounded in established scientific standards**.

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Appendix 6 — NIH Fermentation & Health Study

Appendix 6

Fermentation, Microbiome Modulation, and Human Health: Translational and Clinical Perspectives

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1. Introduction

Advances in microbiome research have shifted the paradigm of human health from a purely biochemical model to a **host–microbe integrated system**. Increasing evidence indicates that diet-driven microbial modulation plays a critical role in:

- Immune regulation
- Metabolic function
- Inflammatory control

Fermented products, particularly those containing active microbial systems, are now recognized as potential **functional interventions** in maintaining and restoring physiological balance.

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2. The Human Microbiome as a Functional System

The human body hosts trillions of microorganisms, collectively known as the microbiome, which:

- Regulate digestion and nutrient absorption
- Modulate immune responses
- Influence metabolic pathways

2.1 Functional Roles

System	Microbial Function
Gastrointestinal	Digestion & fermentation
Immune	Regulation of inflammation
Metabolic	Energy balance & nutrient synthesis
Neurological	Gut–brain signaling

---

3. Dysbiosis and Disease Development

Disruption of microbial balance (*dysbiosis*) is associated with:

- Chronic inflammation
- Oxidative stress
- Immune dysfunction

3.1 Clinical Implications

Dysbiosis has been linked to:

- Inflammatory bowel disease (IBD)
- Obesity and metabolic syndrome
- Type 2 diabetes
- Cardiovascular disease
- Neurodegenerative conditions

---

4. Fermented Systems as Therapeutic Modulators

Fermented foods introduce:

- Live microorganisms
- Bioactive metabolites
- Enzymatically transformed compounds

4.1 Mechanisms of Action

Fermented systems influence human physiology through:

1. **Microbial interaction**
    - Introduction of beneficial microorganisms
2. **Metabolic modulation**
    - Production of organic acids and signaling molecules
3. **Immune regulation**
    - Reduction of pro-inflammatory mediators
4. **Oxidative balance**
    - Reduction of reactive oxygen species (ROS)

---

5. Clinical Evidence for Fermented Foods

Recent human studies demonstrate that fermented food consumption can:

- Increase microbiome diversity
- Reduce inflammatory biomarkers
- Improve immune system responsiveness

5.1 Key Findings (Human Studies)

- Increased microbial diversity correlates with improved health outcomes
- Reduced levels of inflammatory cytokines

- Enhanced gut barrier integrity

👉 Implication:

Fermented systems can act as **non-pharmaceutical interventions** in chronic disease management.

---

6. Controlled Fermentation as a Precision Approach

Traditional fermentation is often variable. In contrast:

👉 **Controlled fermentation enables:**

- Standardization of microbial activity
- Reproducibility of functional outcomes
- Optimization of beneficial metabolites

6.1 Clinical Relevance

Controlled systems allow:

- Targeted modulation of inflammation
- Predictable antioxidant effects
- Consistent microbiome interaction

---

7. Integration with Cellular Evidence (SIRIM Context)

Cellular-level findings (Appendix 4) demonstrate:

- Reduction of nitric oxide (NO) → anti-inflammatory effect
- Maintenance of cell viability → safety
- Active microbial presence → functional system

7.1 Translational Interpretation

👉 Cellular findings suggest:

Potential downstream human effects:

- Reduced inflammatory burden
- Improved immune regulation
- Enhanced cellular resilience

---

8. Human Application Model

A conceptual framework for Microba application:

8.1 Step-by-Step Functional Model

1. **Ingestion**
   → Intake of fermented bioactive system
2. **Microbial Interaction**
   → Interaction with gut microbiota
3. **Metabolic Transformation**
   → Production of functional metabolites
4. **Systemic Effect**
   → Modulation of inflammation and oxidative stress

8.2 Expected Outcomes

- Improved microbiome balance
- Reduction in inflammatory markers
- Enhanced metabolic efficiency

---

9. Limitations and Future Directions

While cellular and biochemical evidence is strong:

- Clinical trials are required for validation in human populations
- Dose-response relationships need further exploration
- Long-term safety and efficacy must be evaluated

---

10. Scientific Positioning

Based on current evidence, fermentation-based systems such as Microba can be positioned as:

👉 **Microbiome-modulating functional systems with translational potential in human health**

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11. Conclusion

The integration of microbiome science and controlled fermentation provides a promising pathway for developing functional systems that influence human health.

Evidence suggests that:

- Microbial balance is central to physiological stability
- Fermentation enhances bioactive potential
- Controlled systems enable reproducible outcomes

These insights support the development of microbiome-based approaches as complementary strategies in modern healthcare.

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Appendix 7

Bee Gut Microbiota: Natural Biotechnological Systems and Functional Implications

---

1. Introduction

The honey bee (*Apis mellifera*) is not only a pollinator but also a carrier of a highly specialized and stable gut microbiota. This microbial community plays a critical role in:

- Nutrient transformation
- Pathogen defense
- Production of bioactive compounds

Recent studies identify the bee gut microbiome as a **natural biotechnological system**, capable of converting raw plant substrates into functionally enhanced products, including honey.

---

2. Composition of Bee Gut Microbiota

The bee gut microbiota is relatively simple but highly specialized, dominated by a few core bacterial taxa:

- *Gilliamella apicola*
- *Snodgrassella alvi*
- *Lactobacillus* spp.
- *Bifidobacterium* spp.

2.1 Functional Roles

Microorganism	Function
Gilliamella	Carbohydrate metabolism
Snodgrassella	Gut barrier stability
Lactobacillus	Acid production & pathogen inhibition
Bifidobacterium	Nutrient processing

---

3. Microbial Transformation in Honey Production

During nectar collection and processing:

- Bees ingest plant-derived sugars
- These substrates interact with gut microbes
- Enzymatic and microbial transformation occurs

3.1 Biochemical Processes

- Breakdown of complex sugars
- Production of organic acids
- Modification of phytochemicals

👉 Result:

Honey is not merely plant-derived — it is **microbially transformed**.

---

4. Bee Gut as a Natural Fermentation Chamber

The bee digestive system functions as a **biological fermentation reactor**:

- Controlled internal environment
- Stable microbial population
- Continuous metabolic activity

4.1 Key Characteristics

- Selective microbial ecosystem
- Efficient biochemical conversion
- Low pathogenic contamination

👉 Insight:

The bee gut represents a **naturally optimized fermentation system**.

---

5. Bioactive Outcomes of Bee-Mediated Transformation

Microbial processing contributes to:

- Enhanced antimicrobial activity
- Increased antioxidant compounds
- Production of bioactive metabolites

5.1 Antimicrobial Effects

- Organic acids inhibit pathogen growth
- Microbial metabolites create hostile environments for harmful bacteria

---

5.2 Antioxidant Enhancement

- Transformation of phenolic compounds
- Increased capacity to neutralize ROS

---

6. Ecological and Habitat Influence

Bee microbiota is influenced by:

- Floral diversity
- Environmental conditions
- Geographic habitat

6.1 Implication

Different environments lead to:

- Variation in microbial composition
- Differences in metabolic output
- Diversity in honey characteristics

👉 Key Insight:

Quality is not solely determined by origin label, but by **microbial ecology**.

---

7. From Natural System to Human Application

The bee microbiome provides a model for:

👉 **Microbe-driven biochemical transformation**

7.1 Translational Concept

- Bees = natural microbial processors
- Honey = transformed bioactive substrate
- Microbes = functional drivers

---

8. Relevance to Microba Concept

The Microba system reflects this natural paradigm:

8.1 Core Alignment

Natural System (Bee)	Microba System
Bee gut microbiome	Controlled microbial system
Nectar transformation	Substrate fermentation
Bioactive honey output	Functional fermented product

8.2 Key Principle

👉 It is not the raw material alone that defines functionality, but:

The microbial system that processes it

---

9. Scientific Positioning

The bee microbiome supports the concept that:

👉 Biological transformation systems driven by microbes can produce functional outcomes beyond the original substrate.

This aligns with:

- Microbiome science
- Systems biology
- Functional nutrition

---

10. Conclusion

The honey bee gut microbiota represents a naturally evolved biotechnological system capable of transforming plant-derived substrates into functionally enriched products.

Understanding this system provides a foundational model for developing controlled microbial processes, such as those applied in fermentation-based platforms like Microba.

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Appendix 8 — Nature Microbiology (Bee Microbiome)

📎 Appendix 8

Bee Microbiome as a Model of Functional Symbiosis: Insights from Nature Microbiology

---

1. Introduction

The honey bee microbiome represents one of the most well-characterized examples of **host–microbe symbiosis** in nature. Unlike highly diverse mammalian microbiomes, the bee gut microbiota is:

- Structurally simple
- Highly conserved
- Functionally specialized

This makes it an ideal biological model for understanding how microbial systems can be optimized to perform specific biochemical functions.

---

2. Core Concept: Functional Symbiosis

In the bee system:

👉 Host (bee) + Microbiome = Integrated biological unit

This relationship is not incidental but **evolutionarily optimized**, where:

- The host provides a controlled environment
- Microbes perform specialized biochemical tasks

2.1 Key Features of Symbiosis

- Stability across individuals
- Functional redundancy
- High metabolic efficiency

👉 Insight:

The system prioritizes **function over diversity**

---

3. Spatial Organization of Bee Microbiome

The bee gut is compartmentalized:

- Crop (honey stomach)
- Midgut
- Hindgut

Each region supports different microbial activities.

3.1 Functional Zonation

Region	Function
Crop	Initial processing of nectar
Midgut	Enzymatic digestion
Hindgut	Microbial fermentation

👉 This organization allows **sequential biochemical transformation**

---

4. Metabolic Capabilities of Bee Microbiota

Bee-associated microbes exhibit highly specialized metabolic pathways:

4.1 Carbohydrate Metabolism

- Breakdown of complex plant sugars
- Conversion into simpler bioavailable compounds

4.2 Detoxification

- Degradation of plant toxins
- Protection against harmful compounds

4.3 Bioactive Compound Production

- Organic acids
- Antimicrobial substances
- Functional metabolites

---

5. Stability and Resilience of the System

The bee microbiome is:

- Highly stable across environmental changes
- Resistant to pathogen invasion

- Rapidly recoverable after disturbance

5.1 Mechanisms of Stability

- Competitive exclusion of pathogens
- Cooperative microbial interactions
- Host regulation of microbial composition

---

6. Implications for System Design

The bee microbiome provides a blueprint for designing functional systems:

6.1 Key Design Principles

1. **Simplicity with specialization**
2. **Stable microbial composition**
3. **Controlled environment**
4. **Efficient metabolic pathways**

---

7. Translation to Human and Industrial Systems

Insights from bee microbiome research can be applied to:

- Functional food design
- Controlled fermentation systems
- Microbiome engineering

7.1 Conceptual Translation

Bee System	Applied System
Natural microbiome	Engineered microbial system
Nectar transformation	Substrate fermentation
Bioactive honey	Functional product

---

8. Relevance to Microba System

The Microba system reflects these principles:

8.1 Functional Alignment

- Microbial-driven transformation
- Controlled biochemical environment
- Targeted functional outcomes

8.2 System-Level Perspective

👉 Microba is not a single compound or ingredient

👉 It represents a:

“Designed microbial ecosystem inspired by natural symbiotic systems.”

---

9. Beyond Composition: Function Over Material

A critical insight from Nature Microbiology studies:

👉 The value of biological systems lies not in their components alone, but in their **functional interactions**

9.1 Implication

- Raw material (honey) is only the substrate
- Function emerges from microbial activity

---

10. Conclusion

The bee microbiome demonstrates how a stable and specialized microbial system can perform highly efficient biochemical transformations.

This model provides a foundation for:

- Designing controlled fermentation systems
- Developing microbiome-driven functional products
- Understanding biological systems as integrated networks

The Microba concept aligns with this paradigm, translating natural symbiotic intelligence into applied functional systems.

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Appendix 9 — SIRIM CAA Report (ROS Inhibition Data)

Appendix 9

Cellular Antioxidant Activity (CAA) and Reactive Oxygen Species (ROS) Inhibition: SIRIM-Based Functional Evaluation of Fermented Honey System

---

1. Introduction

Oxidative stress is a fundamental mechanism underlying many chronic diseases. It is primarily driven by the accumulation of **reactive oxygen species (ROS)**, which are chemically reactive molecules capable of damaging cellular structures.

The Cellular Antioxidant Activity (CAA) assay is a widely used method to evaluate the ability of compounds to:

- Neutralize intracellular ROS
- Protect cells from oxidative damage

This appendix presents the interpretation of SIRIM CAA findings in evaluating the antioxidant capacity of the Microba fermented honey system.

---

2. Reactive Oxygen Species (ROS) and Cellular Damage

2.1 Definition

Reactive Oxygen Species (ROS) include:

- Superoxide anion (O_2^-)
- Hydrogen peroxide (H_2O_2)
- Hydroxyl radicals (•OH)

2.2 Biological Impact

At physiological levels:

- ROS participate in signaling pathways

At elevated levels:

- Oxidative stress occurs
- Cellular components are damaged

2.3 Disease Association

Excess ROS is linked to:

- Chronic inflammation
- Cardiovascular disease
- Neurodegenerative disorders
- Cancer

---

3. Cellular Antioxidant Activity (CAA) Assay

The CAA assay evaluates antioxidant activity within living cells, providing a more biologically relevant measurement compared to chemical assays.

3.1 Assay Principle

- Cells are exposed to oxidative stress inducers
- Fluorescent probes detect intracellular ROS
- Reduction in fluorescence indicates antioxidant activity

3.2 Scientific Significance

CAA reflects:

- Cellular uptake of compounds
- Intracellular antioxidant action
- Functional biological activity

---

4. SIRIM CAA Findings (Interpretation)

Based on SIRIM analysis:

👉 The Microba system demonstrates:

- Significant intracellular ROS inhibition
- Dose-dependent antioxidant activity

4.1 Key Observations

- Increased concentration → increased antioxidant activity
- Effective ROS suppression within cellular environment

5. Mechanisms of Antioxidant Action

The antioxidant activity observed can be attributed to:

5.1 Direct ROS Scavenging

- Neutralization of free radicals
- Reduction of oxidative burden

5.2 Indirect Cellular Protection

- Stabilization of cellular membranes
- Protection of DNA and proteins

5.3 Microbial Contribution

Fermentation enhances antioxidant capacity through:

- Production of bioactive metabolites
- Transformation of phenolic compounds

6. Integration with Anti-Inflammatory Pathways

ROS and inflammation are closely linked:

- ROS promotes inflammatory signaling
- Inflammation further increases ROS production

6.1 Dual Modulation

The Microba system demonstrates:

- ROS inhibition (antioxidant effect)
- NO suppression (anti-inflammatory effect; Appendix 4)

👉 This indicates a **dual-function biological system**:

Antioxidant + Anti-inflammatory

7. Safety and Cellular Compatibility

CAA findings must be interpreted alongside cytotoxicity data:

- High antioxidant activity
- Maintained cell viability

👉 This confirms:

- Functional activity without cellular damage

---

8. Scientific Interpretation

The SIRIM data supports classification of the system as:

👉 **A bioactive antioxidant system operating at the cellular level**

8.1 Key Insight

Unlike simple antioxidants:

- Activity occurs within living cells
- Indicates true biological interaction

---

9. Functional Implications

The observed antioxidant activity suggests potential roles in:

- Reducing oxidative stress
- Supporting cellular health
- Modulating inflammatory pathways

---

10. Conclusion

The SIRIM CAA assay demonstrates that the Microba system exhibits:

- Significant intracellular antioxidant activity
- Effective ROS inhibition
- Compatibility with living cells

These findings reinforce the concept that controlled fermentation systems can generate functionally active compounds capable of interacting with biological systems at the cellular level.

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📎 Appendix 10

Microbiological Analysis and Safety Profile: SIRIM-Based Evaluation of Microbial Load and Composition

---

1. Introduction

Microbiological analysis is a critical component in evaluating the safety, stability, and functional classification of fermentation-based products. For systems such as Microba, which rely on active microbial processes, it is essential to distinguish between:

- Beneficial microbial presence
- Harmful contamination

This appendix presents the interpretation of SIRIM microbiological findings, focusing on microbial load, composition, and safety indicators.

---

2. Microbial Enumeration Results

Based on SIRIM laboratory analysis:

Parameter	Result
Aerobic Plate Count (APC)	5.80×10^3 CFU/g
Yeast & Mould Count	1.10×10^5 CFU/g
Coliform	Not Detected

---

3. Interpretation of Microbial Load

3.1 Aerobic Plate Count (APC)

APC reflects the total population of viable aerobic microorganisms.

👉 Interpretation:

- Moderate microbial presence
- Indicates an **active biological system**
- Within acceptable range for fermented products

3.2 Yeast and Mould Count

Yeasts are key drivers of fermentation processes.

👉 Interpretation:

- High yeast presence indicates **ongoing or established fermentation activity**
- Yeast contributes to:
    - Sugar metabolism
    - Production of organic acids
    - Generation of bioactive compounds

---

4. Absence of Pathogenic Indicators

4.1 Coliform Analysis

Coliform bacteria are commonly used as indicators of contamination.

👉 Result:

- **Not detected**

4.2 Interpretation

- No evidence of fecal or environmental contamination
- Confirms microbiological safety

---

5. Functional Classification of Microbial System

The microbial profile suggests that the system is:

👉 **Microbially active but controlled**

5.1 Key Characteristics

- Presence of viable microorganisms
- Dominance of fermentation-associated microbes
- Absence of pathogenic indicators

---

6. Microbial Role in Functional Activity

Microorganisms within the system contribute to:

6.1 Biochemical Transformation

- Conversion of sugars into metabolites
- Production of organic acids

6.2 Bioactive Compound Generation

- Antioxidants
- Antimicrobial compounds
- Functional metabolites

6.3 System Stability

- Competitive exclusion of harmful microbes
- Maintenance of microbial balance

---

7. Safety vs Activity: A Dual Perspective

A key challenge in microbial systems is balancing:

- Biological activity
- Safety

7.1 Conventional Systems

- High microbial activity → potential safety risks
- Low microbial activity → reduced functionality

7.2 Observed Profile

👉 The Microba system demonstrates:

- Active microbial presence
- Absence of harmful contamination

👉 This indicates:

Safe + Functional microbial system

---

8. Regulatory Perspective

From a food safety standpoint, acceptable microbial systems must demonstrate:

- Absence of pathogenic organisms
- Controlled microbial levels
- Consistent product behavior

👉 SIRIM findings support:

- Compliance with basic microbiological safety criteria
- Suitability for further functional classification

---

9. Integration with Functional Data

When combined with previous appendices:

- Appendix 4 → Anti-inflammatory (NO inhibition)
- Appendix 9 → Antioxidant (ROS inhibition)

👉 Microbial data (Appendix 10) provides:

The biological source of functional activity

---

10. Scientific Interpretation

The system can be interpreted as:

👉 **A controlled microbial ecosystem generating measurable functional outcomes**

10.1 Key Insight

- Microbial presence is not contamination
- It is a **functional driver**

---

11. Conclusion

The SIRIM microbiological analysis demonstrates that the Microba system:

- Contains active microbial populations
- Shows no evidence of harmful contamination
- Maintains a balance between safety and biological activity

These findings support its classification as a **functional fermentation-based system with controlled microbial composition**.

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A. Anti-inflammatory (NO inhibition) Report No: R0449/22/B19/33

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TEST REPORT

REPORT NO: R0448/22/B19/33	PAGE: 1 of 6

Applicant	:	Abdul Rahim Manan
Manufacturer / Company	:	Nusaraya Resources Sdn.Bhd No 17 Jalan Sri Purnama 2/3, Kawasan Perindustrian Sri Purnama, 81100 Johor Bahru, Johor
Sample	:	Received 1 (one) sample with the following identification for the analysis: Sample name: SiHULK Microbic
Reference standard / Method of Test	:	LWI-238-76: Cellular Antioxidant Assay
Date Received	:	20th May 2022
Job No.	:	J0448/22
Job Complete	:	20th July 2022
Issue Date	:	22nd July 2022

Approved signatories,

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In-House Antioxidant Assay : Cellular Antioxidant Activity (CAA)

1.0 OBJECTIVE

To determine the antioxidant properties of samples as reactive oxygen species (ROS) inhibitor.

2.0 PRINCIPLE

Accumulation of reactive oxygen species (ROS) coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease states. The role of oxidative in several diseases such as cancer, inflammatory disease and diabetes has been well established. Free radicals and other reactive species are constantly generated in vivo and cause oxidative damage to biomolecules, a process held in check by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lipids. Measuring the effect of antioxidant therapies and ROS activity intracellularly is important to suppressing or treating oxidative stress inducers.

In-House Antioxidant Assay: Cellular Antioxidant Activity (CAA) is a cell-based assay for measuring antioxidant or reactive oxygen species (ROS) activity within a cell. The assay employs the cell-permeable fluorogenic probe 2', 7'-Dichlorodihydrofluorescin di-acetate (DCFH-DA). In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) by ROS. The fluorescence intensity is proportional to the ROS levels within the cell. The effect of antioxidant or free radical compounds on DCF-DA can be measured against the fluorescence of the provided DCF standard.

3.0 ACCEPTANCE CRITERIA

Epigallocatechin gallate (EGCG) was used as a standard. The value of EGCG (500 µM) shall indicate at least 50% inhibition of ROS release with cell viability more than 90%.

4.0 METHOD

4.1 Sample Preparation

SiHULK Microbic was dissolved in aqueous with a final concentration range from 0.78 % to a maximum of 25 %, the selected concentration is a non-toxic concentration.

4.2 Measurement of reactive oxygen species (ROS)

Specifically, Human keratinocytes cells line (HaCaT) were seeded into 96 well plates and cultured until they reached 90% confluence. After reached the 90% confluence, the media from all wells were carefully removed and discard. Cells were then washed gently 2 times with phosphate buffer saline (PBS) and remove the last wash and discard. A DCFH-DA Probe solution, samples and standard were then added to all wells with confluent cells to be tested. The cells were then incubated for 2 hours in a dark condition. After 2 hours incubation, the solution was removed, and the cells were then washed with PBS. A free radical initiator solution tert-Butyl hydroperoxide (tBuOOH) was added to all wells. The supernatant was collected and immediately read with a fluorescent microplate reader at 37°C with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

The percentage of ROS inhibition calculation:

$$\frac{ROS_{ref} - ROS_{sample}}{ROS_{ref}} \times 100\%$$

ROS_{ref} : Cells exposed with (tBuOOH) only (without sample).
ROS_{sample} : Cells exposed with (tBuOOH) and treated with sample / standard

The half maximal inhibitory concentration (IC_{50}) calculation based on the log/linear equation derived from the graph of ROS Inhibition (%) vs sample concentration, with R^2 >0.9 :

$$Y = mx + c$$

Y: ROS Inhibition (%)
m: slope
c: intercept
X: Concentration sample

4.3 Cell Viability

The viability of the cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 0.5mg/mL MTT solution was added into each well and cells incubated for 4 hours at 37°C in a humidified incubator containing 5% CO_2. Cells then centrifuged and dissolved with dimethyl sulfoxide (DMSO). Absorbance was measured using spectrophotometer at 630 nm.

The viability of cells (MTT assay) calculation:

$$\frac{B}{A} \times 100\%$$

A: Cells exposed with (tBuOOH) only (without sample).
B: Cells exposed with (tBuOOH) and treated with sample / standard

5.0 RESULTS

5.1 Positive control: Epigallocatechin gallate (EGCG)

The acceptance criteria of the test were met, and the test was considered valid as shown in Table 1. For this experiment, 500 µM of EGCG inhibited (76.0±5.1) % of ROS production with cell viability (98.3±2.67) %. This indicates integrity of the assay.

Table 1: The table indicates the effects of EGCG on the inhibition of reactive oxygen species (ROS) release and cell viability.

EGCG (µM)	ROS Inhibition (%)					Cell Viability (%)				
	R1	R2	R3	**Ave**	StDev	R1	R2	R3	**Ave**	Stdev
500	79.6	70.2	78.2	**76.0**	5.1	97.4	101.3	96.2	**98.3**	2.67

*Ave = Average

5.2 Sample : SiHULK Microbic

The Reactive Oxygen Species (ROS) inhibition was determined by measuring the ROS generation relative to non-treated medium control. Free radical initiator solution tert-Butyl hydroperoxide (tBuOOH) significantly increased ROS generation and the cells treated with SiHULK Microbic inhibited tBuOOH -induced ROS generation as shown in Table 2.

Table 2: The table indicates the effects of SiHULK Microbic at selected concentration inhibiting the reactive oxygen species (ROS) release and cell viability relative to non-treated medium control.

Sample	Conc. (%)	ROS Inhibition (%)					Cell Viability (%)				
		R1	R2	R3	**Ave**	StDev	R1	R2	R3	**Ave**	Stdev
SiHULK Microbic	0.78	8.6	2.3	5.0	**5.3**	3.2	82.1	80.8	93.6	**85.5**	7.06
	1.56	22.9	14.8	16.6	**18.1**	4.3	92.3	85.9	89.7	**89.3**	3.23
	3.13	32.1	28.7	22.4	**27.7**	4.9	88.5	87.2	85.9	**87.2**	1.28
	6.25	32.4	38.4	35.9	**35.6**	3.0	100.0	97.4	92.3	**96.6**	3.92
	12.50	52.7	55.0	61.6	**56.4**	4.62	106.4	100.0	119.2	**108.5**	9.79
	25.00	66.7	61.5	56.9	**61.7**	4.92	109.0	124.4	110.3	**114.5**	8.54

Ave= Average

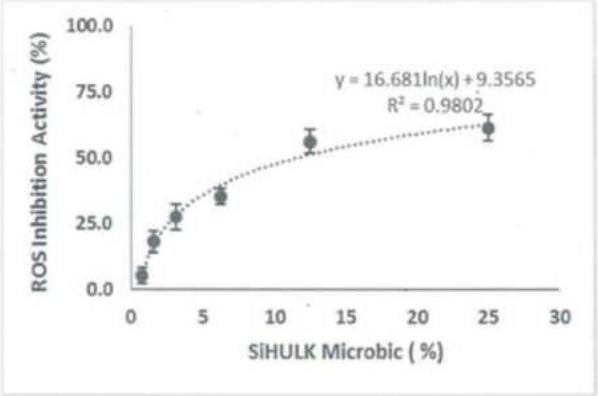

Figure 1: Effects of SiHULK Microbic on reactive oxygen species (ROS) release in human keratinocytes cell irradiated with tert-Butyl hydroperoxide.

6.0 DISCUSSION

Based on data in Table 2, SiHULK Microbic shows no cytotoxicity effect against human keratinocyte cells at the concentration of 0.78 % to a maximum of 25 %, with more than 50% cell viability. SiHULK Microbic promotes ROS scavenging activity at dose dependent manner with IC_{50} value 11.4±0.63 %.

7.0 CONCLUSION

Based on the result, SiHULK Microbic showed potential in anti-oxidant activity by inhibiting the reactive oxygen species (ROS) production.

*** This finding partially supported the ROS scavenging activity by SiHULK Microbic, where further studies are recommended.

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B. **Nutritional + Microbiological Analysis Report No: 2020CE0358**

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TEST REPORT

REPORT NO: R0449/22/B19/33	PAGE: 1 of 6

Applicant	:	En Abdul Rahim Manan
Manufacturer / Company	:	Nusaraya Resources Sdn Bhd. No 17, Jalan Sri Purnama 2/3, Kawasan Perindustrian Sri Purnama, 81100 Johor Bahru, Johor
Sample	:	SiHULK Microbic
Reference standard / Method of Test	:	Cellular Anti Inflammatory Assay: Nitric Oxide (NO) Inhibition Assay
Description of Sample	:	Received 1 (one) sample with this identification: SiHulk Microbic – Light yellow liquid
Date Received	:	19th May 2022
Job No.	:	J0449/22
Issue Date	:	8th September 2022

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Cellular Anti Inflammatory Assay: Nitric Oxide (NO) Inhibition Assay

1. Principle of the assay

Inflammation is the normal response of a living tissue to injury caused by physical or noxious chemical stimuli or microbiological toxins. Macrophages are the main pro-inflammatory cells responsible for invading pathogens by releasing many pro-inflammatory molecules such as nitric oxide (NO), prostaglandin E2 (PGE2), and of cytokines, like interleukin-6 (IL-6) and tumor necrosis factor-a (TNF-a). Among these, NO is a short-lived biomolecule that mediates many biological functions, including host defense, vasoregulation, platelet aggregation and neurotransmission. However, overproduction of NO is related to the development of various inflammatory diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis. Hence, this pro-inflammatory mediator, NO has been considered as an important target in the development of anti-inflammatory drugs.

Cellular Anti Inflammatory Assay is a cell based assay to evaluate the inhibition of NO production in RAW 264.7 cells activated with lipopolysaccharide. NO production is measured as nitrite concentration in the culture medium. The effect of anti- inflammatory agents can be measured through the inhibition of NO production.

2. Method

2.1 Sample preparation

The sample, SiHULK Microbic solution with various concentrations were prepared and filtered with 0.2 µm membrane filter prior treatment. Final tested concentrations of the sample were prepared ranging from 0.625 to 10 %, v/v.

2.2 Measurement of Nitric Oxide (NO) Inhibition

Murine macrophage, RAW 264.7 (ATCC TIB-71) were seeded at 2.5 x10^5 cells/ml in 24 well plate incubated for 24hours followed by incubation at 37°C and 5% CO_2. After 24 hours, the medium was removed and replaced with FBS-free DMEM media and then the cells were treated with various concentrations of SiHULK Microbic ranged from 0.625 to 10 (%, v/v), positive control Nitro-L-arginine methyl ester (L-NAME) with final concentration of 50 µg/mL and blank (medium without treatment). The cells were allowed to incubate at 37°C for 1.0 hour. After incubation, the cells were stimulated with 0.1 µg/mL of Lipopolysaccharide (LPS) derived from *E. coli* for 24 hours. The medium were collected to determine the presence of nitrite in culture media by adding 100uL of Griess reagent into 100uL of culture media. The absorbance was measured at 542 nm using a microplate reader (FLOUstar Omega, BMG

Labtech, Germany). The amount of nitrite content in the media was calculated based on sodium nitrite ($NaNO_2$) standard curve.

Calculation:

The NO inhibition (%):

$$\frac{NO_{ref} - NO_{sample}}{NO_{ref}} \times 100\%$$

NO_{ref} : Nitrite content in cells stimulated with LPS only.
NO_{sample} : Nitrite content in cells stimulated with LPS and treated with sample

The half maximal inhibitory concentration (IC_{50}) calculation based on the log/linear equation derived from the graph of NO Inhibition (%) vs sample concentration:

$$Y = mX + c \text{ with } R^2 > 0.9$$

Y : NO Inhibition (%)

m : slope

c : intercept

X : Concentration of SiHULK Microbic

2.3 Cell Viability

The viability of the cells was determined using MTT cell viability assay. 5mg/mL of MTT was added into each well and the cells were incubated for 4 hours at 37°C and 5% CO_2. The medium was then removed and the formazan precipitate was solubilized with DMSO. The Absorbance was measured using at 630 nm using a microplate reader (FLOUstar Omega, BMG Labtech, Germany).

Calculation:

The viability of cells (%):

$$\frac{B}{A} \quad X \quad 100\%$$

A: Absorbance of reference (Cells stimulated with LPS only)
B: Absorbance of test (Cells stimulated with LPS and treated with sample)

Results

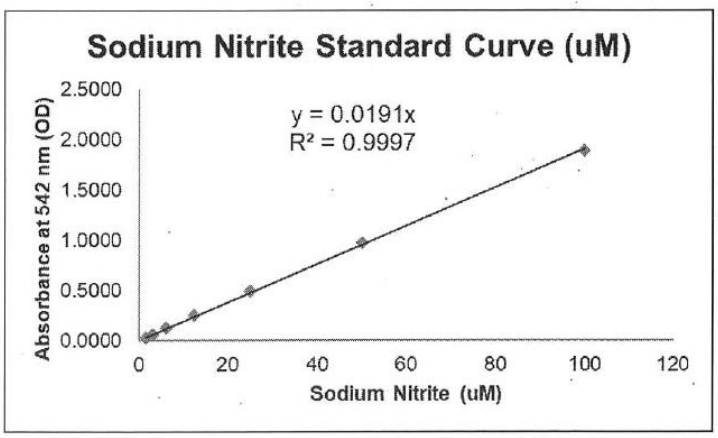

Figure 1: The sodium nitrite standard curve used in this experiment.

2.4: Nitrite Concentration (µM)

SAMPLE	Nitrite Concentration (µM)			Average	StDev
	R1	**R2**	**R3**		
LPS (0.1 µg/ml)	23.9	22.9	22.4	23.1	0.8
L- NAME (50µg/ml)	12.5	12.8	12.0	12.4	0.4
SiHULK Microbic (%, v/v)	Nitrite Concentration (µM)			Average	StDev
0.625	23.2	21.8	25.8	23.6	2.0
1.25	22.7	25.8	22.5	23.7	1.9
2.5	21.9	21.8	18.8	20.8	1.8
5.0	15.7	15.6	16.7	16.0	0.6
10.0	6.4	5.9	6.1	6.1	0.3

Table 1: The effects of SiHULK Microbic on NO production by LPS-stimulated RAW 264.7 cells. The amount of nitrite in the media was calculated based on sodium nitrite standard curve (Refer to Figure 1).

2.5: Nitric Oxide (NO) Inhibition %

	NO Inhibition (%)					Viability (%)				
Sample	R1	R2	R3	**Average**	StDev	R1	R2	R3	**Average**	StDev
L-NAME (50µg/mL)	46.1	44.8	48.2	**46.3**	1.7	62.8	64.9	67.8	**65.2**	2.5
SiHULK Microbic (%, v/v)	R1	R2	R3	**Average**	StDev	R1	R2	R3	**Average**	StDev
0.625	-0.3	5.8	-11.5	**-2.0**	8.8	68.2	62.0	66.8	**64.4**	3.4
1.25	1.8	-11.7	2.5	**-2.4**	8.0	73.9	78.7	68.4	**73.6**	5.2
2.5	5.1	5.5	18.6	**9.8**	7.7	78.7	73.9	77.7	**76.3**	3.4
5.0	32.0	32.3	27.5	**30.6**	2.7	72.9	81.3	81.6	**77.3**	6.2
10.0	72.2	74.6	73.5	**73.4**	1.2	87.7	85.6	86.0	**86.6**	1.5

Table 2: The effect of SiHULK Microbic at selected concentration on the cell viability and inhibition of NO production. NO inhibition (%) was calculated based on the method in 2.2.

Treated with L-NAME prior to LPS stimulation

Treated with sample prior to LPS stimulation

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Assay integrity: Acceptable: R^2 >0.9

L-NAME was used as a positive control. The value of L-NAME (50µg/mL) shall indicate at least 50% inhibition of NO production. For this experiment, the inhibition was at 46.3 ± 1.7 % with concentration of 50µg/mL.

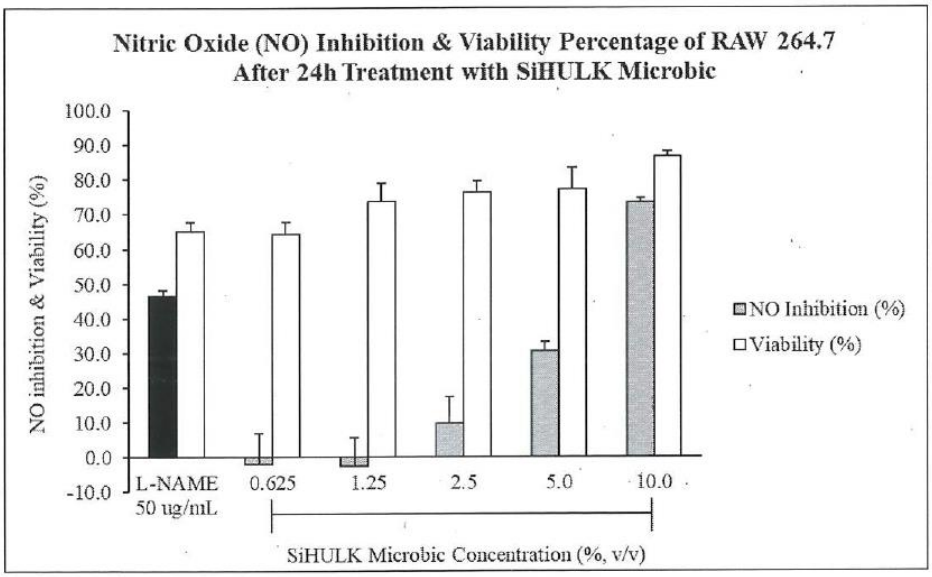

Figure 2: The figure indicates that the SiHULK Microbic (at selected concentrations) consistently inhibit NO production while maintaining cell viability of more than 50%.

*StDev is the standard deviation of the sampling distribution.
**L-NAME is the positive control.
*** LPS is Lipopolysaccharide an activator of inflammation.

Conclusion

SiHULK Microbic showed safe and effective concentration (IC_{50}) against in-vitro inflammation condition (by nitric oxide) at 7.262 ± 0.076 (%, v/v) with cell viability of 86.6 ± 1.5% at the sample highest prepared concentration.

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4.0 References

1. Taewoo.J et. all (2014) . Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila* L. Saudi Journal of Biological Sciences (2014) 21, 427-