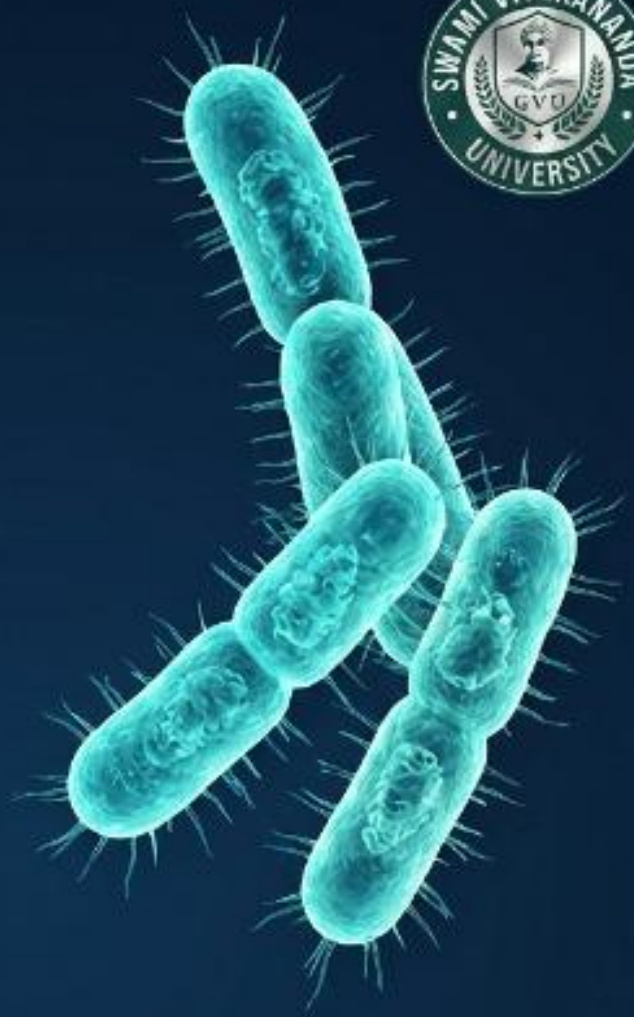

THE MACROWORLD OF THE MICROBES

An Exploration of the Invisible Universe

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Declaration by the Editor

I hereby declare that the book titled **"The Macroworld of the Microbes"** has been edited under my supervision. I have carefully reviewed the manuscript for clarity, coherence, structure, and language, and ensured that it meets the required academic and publishing standards.

To the best of my knowledge, the content of this book is original and has not been published elsewhere in the same form. All sources used by the author(s) have been duly acknowledged, and any necessary permissions have been obtained where applicable.

I confirm that this book is suitable for publication and dissemination.

Preface

The discipline of microbiology has undergone remarkable transformation in recent years, propelled by advances in high-throughput technologies, molecular tools, and integrative research approaches. These developments have significantly expanded our understanding of microbial diversity, physiology, ecology, and their far-reaching implications across environmental, industrial, and biomedical contexts. In this rapidly evolving landscape, there is a growing need for consolidated scholarly resources that critically synthesize emerging knowledge.

The Macroworld of the Microbes presents a curated compilation of review papers that collectively reflect recent discoveries and contemporary developments in microbiology. The chapters included in this volume bring together diverse thematic areas, each offering a comprehensive synthesis of current research, methodological progress, and conceptual advancements. Emphasis has been placed on maintaining scientific rigor while ensuring coherence and continuity across contributions.

This volume is intended to serve as a cohesive academic resource that integrates dispersed findings into a unified framework. By assembling authoritative reviews within a single work, it seeks to facilitate deeper engagement with ongoing research trends and to provide a structured perspective on the dynamic progression of the field.

The completion of this volume has been made possible through the scholarly contributions of the authors, whose expertise and dedication have shaped the intellectual foundation of this work. I express my sincere appreciation to all contributors for their valuable efforts. I am also grateful to colleagues and peers for their insightful discussions and continued encouragement during the preparation of this book.

Every effort has been made to ensure the accuracy and relevance of the material presented. Nevertheless, any inadvertent errors or omissions remain the responsibility of the editor. Constructive feedback from readers will be greatly appreciated and will contribute to the refinement of future editions.

It is hoped that this volume will offer meaningful insight into the expanding frontiers of microbiology and underscore the profound influence of microorganisms on the macroscopic world.

Acknowledgement

The successful completion of *The Macroworld of the Microbes* has been made possible through the support, guidance, and contributions.

I express my sincere gratitude to Swami Vivekananda University for providing the academic environment and institutional support necessary for the development and publication of this volume. The encouragement and resources extended by the University have been instrumental in bringing this work to fruition.

I am deeply indebted to all the contributing authors whose scholarly review papers form the core of this book. Their expertise, dedication, and commitment to advancing the field of microbiology have enriched this volume and ensured its academic rigor and relevance. I also extend my appreciation to my colleagues at the School of Life Sciences for their valuable discussions, insights, and encouragement throughout the editorial process.

I would like to acknowledge the support of my students and research scholars, whose curiosity and engagement with the subject have continually inspired me. Their academic enthusiasm has been a motivating force behind this endeavour.

I also convey my heartfelt thanks to my family and well-wishers for their constant support, patience, and encouragement during the course of this work.

Finally, I extend my gratitude to everyone who has directly or indirectly contributed to the successful completion of this book.

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About the Editor

Dr. Debaprasad Koner is an Assistant Professor in the Department of Biotechnology, School of Life Sciences, Swami Vivekananda University, West Bengal, India. He is involved in teaching and research with a strong research background in oxidative stress biology, redox imbalance, and antioxidant defense mechanisms in aquatic organisms. He earned his Ph.D. in Zoology from North-Eastern Hill University, India, where his doctoral research focused on the effects of zinc oxide nanoparticles on oxidative stress in air-breathing catfish. His work particularly highlighted the role of endogenously produced nitric oxide in mitigating ZnO NPs-induced oxidative damage.

He has served as a Research Associate-II in a SERB-funded project investigating the roles of hypoxia inducible factor (HIF) and mTOR signaling pathways, as well as autophagy, in hypoxia adaptation of air-breathing catfish. Apart from the research, Dr. Koner has also contributed to teaching as a Guest Faculty in Zoology at North-Eastern Hill University, where he taught Genetics to postgraduate students.

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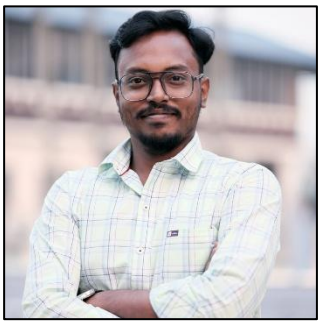

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Dr. Pal's research spans gastrointestinal pathophysiology, chronobiology, neurobiology, and environmental toxicology, with a particular focus on the pleiotropic roles of melatonin as a cytoprotective and antioxidant molecule. His doctoral and postdoctoral investigations have systematically elucidated how melatonin regulates gut function, counters oxidative stress, and modulates inflammatory pathways in response to pharmacological, hormonal, and environmental stressors. His postdoctoral work, supported by the prestigious UGC- Dr. D. S. Kothari Fellowship (2017–2020), examined melatonin's protective effects against adrenaline-induced gastrointestinal injury in Wistar rats. Subsequent post-doctoral research positions at the University of Calcutta (2022-2024) and ICAR-CIFRI (2021-2022) extended his expertise to arsenic-induced neuroinflammation, hippocampal neurogenesis, and the captive breeding of the endangered Hilsa fish, respectively.

To date, Dr. Pal has authored 31 international publications - comprising 21 original research articles, 8 review articles, and 2 book chapters - accumulating 560 citations, an h-index of 13, an i10-index of 18, and a cumulative impact factor exceeding 52. His work has appeared in journals such as Fish Physiology and Biochemistry, Environmental Geochemistry and Health, Food and Chemical Toxicology, Life Sciences, BioFactors, Heliyon, Bioscience Reports, Biological Rhythm Research, and Melatonin Research. He serves as a peer reviewer for high-impact journals of different international publishers including Elsevier, Taylor and Francis, Springer Nature, Portland Press, etc.

Dr. Pal has been recognised with several competitive honours, including the International Travel Award from the North American Society for Comparative Endocrinology (NASCE) and a DBT International Travel Grant for presenting his research at the University of Ottawa, Canada (2015), and the Best Poster Award at the 1st Indian Fisheries Outlook (2022). He is proficient in an extensive array of biochemical and molecular techniques, including atomic absorption spectroscopy, flow cytometry, immunohistochemistry, Western blotting, and primary cell culture. An active member of the Indian Science Congress, Dr. Pal combines rigorous scientific inquiry with a commitment to mentoring the next generation of life scientists.

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Chapter 1

Arsenic Bioaccumulation in Banana

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Abstract

A crucial fruit crop in many parts of the world, bananas (*Musa spp*.) provide calories and important nutrients. However, food safety and human health are increasingly under risk due to the rising infiltration of the deadly metalloid arsenic (As) into agricultural systems. Bananas may accumulate arsenic through the soil, water, or air. Artificial ripening methods that involve calcium carbide (CaC_2), a dangerous but common chemical in underdeveloped nations, also pose a concern. Arsenic toxicity in banana plants causes physiological, biochemical, and metabolic disturbances, including oxidative stress, impaired metabolism of carbon, nitrogen, and sulfur, and decreased yield and nutritional quality. These effects are examined in this review. As's entry into plant systems depends on its interactions with phosphate absorption and transporters. Soil characteristics, plant genotype, and arsenic speciation all have an impact on toxicity. Cadmium, mercury, and arsenic are among the other harmful heavy metals introduced by artificial ripening using CaC_2, which frequently surpasses WHO/FAO safety guidelines. Health risk evaluations indicate that consumers may be exposed to chronic toxicity if Hazard Quotient (HQ) and Hazard Index (HI) values are more than 1.0. Despite advancements, important knowledge gaps about the metabolism of arsenic unique to bananas and its long-term effects on food safety still exist. In order to guarantee safe farming methods and safeguard the public's health, there is a request for stricter regulation of ripening agents and the creation of cultivars that are resistant to arsenic.

Keywords: Arsenic toxicity, Calcium carbide ripening, Health risk assessment, Oxidative stress

Introduction

Fruits like bananas (*Musa* spp.) are essential to a balanced diet and have numerous health advantages. Berries from the herbaceous blooming plants in the genus "*Musa*" are called bananas (Robinson et al.,1996). A global issue that transcends economic borders is arsenic contamination of the human food chain. The Food and Agricultural Organization (FAO) estimates that 114 million metric tons of bananas are produced worldwide each year from 5.6 million hectares.It has been determined that, aside from tainted drinking water, rice grain is a significant source of human (Meharg et al., 2009).Because of their accumulation and biomagnification, heavy metals—toxic compounds discharged into the environment—have a variety of detrimental impacts on living things in the food chain. There are some contaminants that stay in the environment for a long time, such arsenic (As). Eventually, their accumulation may damage soil physiochemical characteristics, resulting in a decline in agricultural output and soil fertility. If left unchecked, arsenic can cause a series of events that can impede growth, interfere with the respiratory and photosynthetic processes, and increase secondary metabolism. Plants react to As toxicity in a number of ways, such as phytochelation, antioxidant defense, and hyperaccumulation. Symbiosis between arbuscular mycorrhizae and disturbed soils occurs in nearly every habitat and climate.There is mounting evidence that fungi known as arbuscular mycorrhizae may reduce the toxicity of metals and metalloids to their host plants. Here we summarize (1) environmental arsenic speciation and the way As is absorbed by roots and processed by plants, and (2) the function of arbuscular mycorrhizae in reducing agricultural plant toxicity from arsenic (Garg & Singla., 2011). The study assessed the impact of employing calcium carbide (CaC2) as a ripening agent to artificially ripen tropical bananas (musa.spp.). On unripe bananas, sub-lethal doses of CaC2 (0 g/kg, 1 g/kg, 5 g/kg, and 10 g/kg) were applied. The resulting levels of minerals, vitamins, and heavy metals were measured. The proximate analysis revealed a declining trend of: Crude protein (CP) ≥ Carbohydrate (CHO) ≥ Moisture ≥ Dry matter (DM) ≥ Ash (A) ≥ Ether (FAT) ≥ Crude fiber (CF) ≥ Calorific value (CV). Ca ≥ Mg ≥ Na ≥ K ≥ N ≥ P ≥ Mn ≥ Cu ≥ Zn was the order in which the mineral contents declined. The vitamins also dropped in the following order: β-carotene, vitamin C, niacin, thiamine and riboflavin. The average levels of cadmium (0.426 m g/kg) and mercury (0.013 m g/kg) were much higher than the FAO/WHO guidelines (0.01/0.02 m g/kg). The toxic components' Hazard Quotient (HQ) and Hazard Index (HI) were both more than 1, suggesting a possible risk to human health. There were suggestions made (Ubuoh et al., 2022). All soils contain naturally occurring arsenic (As), an environmental hazard (Cullen and Reimer, 1989;

Smedley and Kinniburgh, 2002). Humans are mostly exposed to As in the environment through tainted drinking water, such as in the Indian subcontinent (Nordstrom., 2002). In general, arsenic is poisonous to plants and is not necessary. The first tissue typically exposed to As is the root, where the metalloid prevents root growth and elongation. When As is translocated to the shoot, it can significantly impede plant growth by delaying or stopping biomass buildup and expansion. It can also jeopardize plant reproductive potential by reducing fertility, yield, and fruit production (Garg and Singla.,2011). When As levels are high enough, it can cause mortality by interfering with vital metabolic functions. The majority of plants have systems in place to keep a large portion of their As burden in the roots. Nonetheless, a percentage of the As is translocated to the plant's shoot and other tissues, depending on the genotype. As poisoning can affect many physiological functions. Plants exposed to As experience damage to their cellular membranes, which results in electrolyte leaks (Singh et al., 2006). The importance of oxidative stress in As toxicity is shown by the frequent increase in malondialdehyde, a byproduct of lipid peroxidation, that occurs in conjunction with membrane damage. Exposure to arsenic triggers antioxidant defense systems.Throughout the plant, but especially in the roots, ascorbate, the γ-Glu-Cys-Gly tripeptide glutathione (GSH), and the GSH oligomer ([γ-Glu-Cys]n-Gly) phytochelatin (PC) are synthesized in greater amounts (Schmöger et al., 2000; Li et al., 2004; Geng et al., 2006; Singh et al., 2006; Khan et al., 2009), as anthocyanin builds up in leaves (Catarecha et al., 2007). The strength of plant transpiration can be decreased (Stoeva and Bineva, 2003). In soybeans, a low As burden suppresses the amount of nitrogen-fixing root nodules (Vázquez et al., 2008).Although they are unclear, the chemical processes behind these physiological reactions to As exposure have recently drawn more attention. The fact that plant growth is enhanced at low As concentrations is one of the many intriguing paradoxes surrounding As toxicity (Garg and Singla.,2011). The fact that this phenomenon happens in cultivated plants like *Arabidopsis thaliana* under axenic circumstances suggests that the feature is not based on As interfering with plant-biotic interactions. Rather, it is the consequence of either a contact between As and plant nutrients or a direct interaction between As and plant metabolism (Chen et al., 2010). Although the exact mechanism is uncertain, it has been proposed that stimulation of Pi uptake is the source of the growth benefit (Tu and Ma, 2003). The number of plant species that are naturally As tolerant is quite few. These include a class of plants that hyperaccumulate As, such as Pteris vittata and other Pteridaceae species (Ma et al., 2001; Visoottiviseth et al., 2002; Ellis et al., 2006; Pickering et al., 2006; Zhao et al., 2009). Even when these plants are accumulating very high amounts of As, their growth is unaffected. Hyperaccumulators typically do not limit As to the

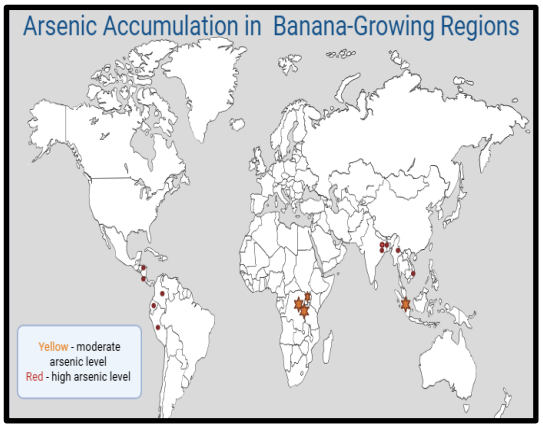
roots, allowing the toxicant to move straight to the shoots, in contrast to As non-hyperaccumulating plants. This probably plays a significant role in the hyperaccumulation phenotype. It is still unclear how these plants manage to prevent As toxicity when As is actively building up to extremely high levels in the leaves, but the methods by which they are able to hyperaccumulate As are being clarified.

A deeper comprehension of the processes underlying plant toxicity and As resistance is required. Better knowledge could lead to the development of As-resistant plants for safe farming and phytoremediation. Remediation of contaminated sites and soil remediation require a broad range of resistant plants that can thrive in a variety of conditions and (hyper)accumulate biomass that can be harvested. As-resistant plants, on the other hand, are necessary for safe cropping in regions where groundwater and/or land are contaminated because they stop As from building up in the harvested flower. Recently, we have made significant progress in understanding how exposure to As affects physiological processes. Additionally, some of the molecular mechanisms behind the interruption of important physiological functions are being better understood. One example of such a biological process is what we know about As as an oxidative stress inducer. The identification of the alterations in gene expression that accompany As stress has been improved in recent years. Finding the cellular metabolic systems most vulnerable to As-induced damage is one area where much more research is needed. The metabolic effects of As exposure on plants are reviewed in this work, along with important areas that require more study (Finnegan & Chen, 2012).

The hazards of arsenic

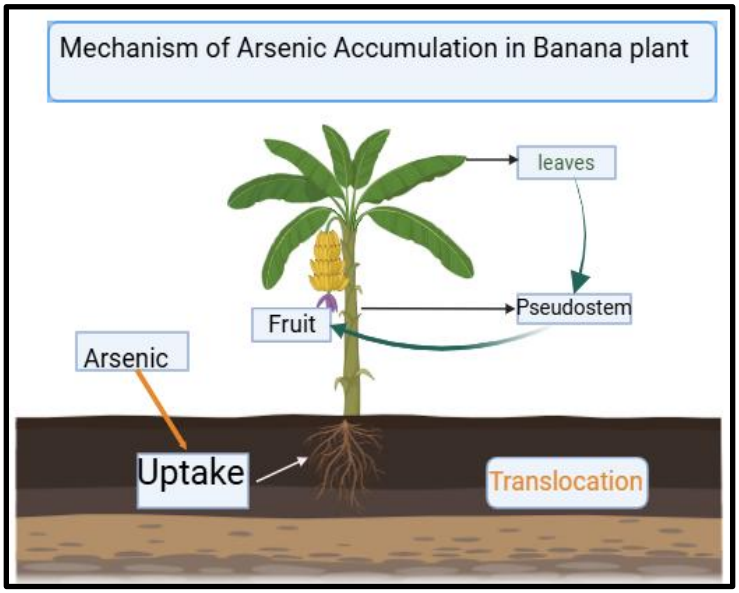
Sivalingam et al. (2017) state that the genus includes up to 50 tropical monocot (pseudo) plants. As a tropical fruit, the banana has several vital nutrients that are needed to stay healthy. Bananas are a staple food and are widely consumed worldwide (Sivalingan et al., 2017 & Haruenkit et al., 2007). Arsenite (As III), arsenate (As V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethyl arsine oxide (TMAO), arsenobetaine (AsB), and other inorganic and organic chemical forms of arsenic are found in the environment (Akter et al., 2005). There is disagreement on which form of As is the most phytotoxic, although the results of several hydroponic tests concur that As phytotoxicity is dependent on the chemical species given to the plant (Meharg, 2008).The potency of externally supplied As on whole plant growth can be best understood through these hydroponic experiments, which remove the complicated and confusing phytoavailability problems caused by variations in the mobility of different As

species through the different growth substrates. The hydroponic survey of 46 distinct plant species and the studies largely concurs that the order of As intake by plants is AsIII > AsV > MMV > DMAV, but the order of translocation from the roots to the rest of the plant is DMAV > MMV > AsV ≥ AsIII. But it doesn't seem that any one As form is regularly the most phytotoxic. AsIII > AsV ≈ MMAV > DMAV in two Spartina species, but DMAV ≈ MMAV > AsIII ≈ AsV in the order of phytotoxicity (Raab et al., 2007a). This would imply that DMAV had the greatest harmful impact on the plant due to its limited absorption and high phytotoxicity. However, the order of uptake in rice was AsIII > MMV > AsV > DMAV, which is comparable to the order of phytotoxicity, which was MMAV > AsIII > AsV = DMAV. Lastly, AsV > AsIII > DMAV was the order of phytotoxicity in maize, a species with the usual order for absorption (Abbas & Meharg, 2008). Most likely, a Pi transporter will be the first enzyme that AsV will compete with when it comes into touch with a cell surface inside the plant root. Plants have Pi transport systems with low and high affinity. PHT1 proteins are the mediators of high-affinity transport. A low-affinity activity is also present in certain PHT1 proteins, although the protein that causes the low-affinity transport is unclear. Monocots and dicots, as well as As-hyperaccumulators and non-hyperaccumulators, have all been shown to exhibit competition between AsV and Pi for entry into the cell through both of these transport routes (Esteban et al., 2003). High Pi conditions reduce AsV toxicity because Pi and AsV compete for uptake. In contrast, AsV might outcompete Pi for uptake in low Pi environments, making Pi deprivation worse (Tu and Ma, 2003). Due to its high toxicity, contamination, numerous pollution sources, non-biodegradability, and high concentrations and accumulation in the environment, arsenic (As) poses a serious threat to the environment. It is also an element that is very expensive and challenging to recover and remove from its surroundings (Han et al.,2019). Investigating and cleaning up arsenic pollution in various settings is crucial because of this. Arsenic is commonly removed from water using conventional methods such as oxidation, coagulation–flocculation, and membrane techniques (Nicomel et al.,2015).



Transport and acquisition of arsenic in banana plant

A non-essential element, arsenic builds up in plant by oxidative and/or genotoxic processes, depending on a number of variables. It can enter the food chain through plants' soil, water, and air, where it accumulates and undergoes biomagnification (Garg & Singla., 2011).Furthermore, as a non-essential element, arsenic also has negative effects on crops, including decreased biomass and yield productivity, toxicity symptoms, a surge of reactive oxygen species (ROS), photosynthetic efficiency, damage to lipids, proteins, carbohydrates, and nucleic acids, and interference with various metabolic pathways through the inactivation of enzymes or competitive inhibitors of phosphate (Pi) (Abbas et al., 2018).Proline is used exogenously or enzyme activity is increased to reduce oxidative stress. The antioxidant defense system, phyto-chelation, and hyperaccumulation are some of the ways that plants react to this toxicity. One of the main mechanisms for detoxifying arsenate is its conversion to As(iii) and subsequent sequestration in the vacuole in plant roots (Martínez-Castillo et al., 2022). The enzymes from other systems, such as glyceraldehyde-3-phosphate dehydrogenase and mitochondrial F1Fo ATP-synthase, could also decrease As(v) in addition to As reductase enzymes. Furthermore, these enzymes create arseno-esters by incorporating As(v) into biological molecules in place of inorganic phosphate (Bakhat et al., 2019). Numerous factors, including the development of iron plaque, phosphate status, soil oxidation/reduction status, and inter-conversion between organic and inorganic As species, influence the dynamics of As in the rhizosphere with regard to root uptake from the soil. Additionally, the type and concentration of clay and the parent material of the soil are important factors in controlling the amount of As in the soil solution. Different varieties of plants have different capacities for absorbing As (Bakhat et al., 2019). Arsenic concentration in fruits has been reported between 0.01 to 0.10 mg kg-1 (Patel et al., 2015).



Bioremediation and phytoremediation methods that rely on reducing As with bacteria (*Pseudomonas, Psychrobacter, Citrobacter, Bacillus, Bosea, Vibrio, and Enterobacter*), oxidizing As with *archaebacterium Sulfolobus acidocaldarius* strain BC, *Alcaligenes faecalis, Shewanella algae*, β-proteobacteria strain UPLAs1, *Alcaligenes faecalis, Comamonas terrae* sp.), and methylating As with *Salmonella, Yersinia, Haemophilus*, and *Brucella*, as well as bioaccumulating As with free-floating and aquatic rooted plants (water hyacinth, *Agrostis sp., Pteris vittata, Pteris cretica*, etc.) are widely recognized techniques (Dwivedi et al., 2015).*Baccharis neglecta, Scirpus holoschoenus, Atriplex lentiformis, Rhododendron tomentosum, Lupinus albus, Detama sphaerocarpa, Ulex europaeus, Eucalyptus cladocalyx, Leptospermum scoparium, Fuchsia excorticate, Anthoxanthum odoratum,* and *Agrostis capillaries* are a few examples of plants. Plants from the Asteraceae, Cyperaceae, Chenopodiaceae, Ericaceae, Fabaceae, Griseliniaceae, Myrtaceae, Onagraceae, Poaceae, Scrophulariaceae, Salicaceae, and Violaceae families—*Veronica beccabunga, Salix atrocinerea, Viola allchariensis, Viola arsenica, and Viola macedonia*—have been reported to phytostabilize As in their roots (Bundschuh et al., 2014).Since they accumulate As in shoots after a particular threshold limit, where the BF (bioaccumulation factor, soil to plant metal transfer) and TF (translocation factor, root to shoot metal transfer) should be greater than one (>1), the majority of them are hyperaccumulators.Rhizodegradation (degradation of the contaminant under the sphere of plant roots), phyto-stabilization (elimination of the bioavailability of metal in soil), rhizo-filtration (removal of metals from water), phyto-degradation (degradation of the contaminant), and phytovolatilization (uptake of the

contaminant by plant roots and its conversion to a volatile form) mechanisms are all part of the phyto-remediation technology (Tanghau et al., 2011).

Arsenic's direct metabolic effects

Phosphate (Pi) can be replaced by arsenate (AsV), and plant metabolism is disrupted by arsenite (AsIII), which binds to enzymes and produces ROS. These variables most likely impact the sulfur, nitrogen, and carbon pathways in *Musa* spp. (banana). In addition to raising the need for antioxidant metabolites, arsenic-induced ROS also modifies metabolism indirectly. However, just a small number of studies explicitly look at these effects in bananas, and results fluctuate depending on factors including growth conditions, plant age, nutrition, and sampling schedules. Interpreting certain reactions, including variations in chloroplast ATPase, Rubisco activity, and quantum yield, can be challenging since they can be fleeting or species-specific. In order to determine which metabolic pathways are most susceptible to As and if the changes seen are transient or permanent stress responses, time-course investigations under controlled settings are necessary for *Musa* spp. (Wang et al.,2012).

Carbon metabolism: In plants, arsenic (As) in the forms AsV and AsIII interferes with carbon metabolism; *Musa* spp. (bananas) are probably affected in a similar way. By decreasing ATP/NADPH synthesis, photosystem II activity, and chlorophyll content, it inhibits photosynthesis. It may also affect Rubisco levels, which hinder carbon fixation. By targeting enzymes like amylase and GAPDH, arsenic prevents the digestion of starch and glycolysis, which lowers the amount of sugar available and the amount of ATP produced. Additionally, it decouples ATP generation through phosphate mimicry and impairs mitochondrial respiration by deactivating enzymes that contain lipoamides. Arsenic causes oxidative stress in maize, which causes mitochondrial enzymes to degrade. Bananas may also experience this reaction. All things considered, exposure to arsenic probably hinders *Musa* spp. growth and energy flow by altering important carbon metabolism processes (Millar et al.,2011).

Nitrogen metabolism: *Musa* spp. (bananas) are probably affected in a similar way to how arsenic exposure alters nitrogen metabolism in plants. Despite not using nodules to fix nitrogen, bananas rely on the soil's nitrate and ammonium. Potassium uptake and assimilation in bananas may be hampered by arsenate (AsV), which decreases the expression of nitrate and ammonium transporter genes and enzymes such as glutamine synthetase and nitrate reductase, found in rice. In addition to lowering the overall protein content, arsenic also encourages the breakdown of nitrogen-rich proteins, such as Rubisco, which lowers the availability of amino acids. Due

to protein degradation, this may cause abnormal amino acid pools in bananas, particularly when there is a decreased metabolism of carbohydrates. Furthermore, AsV suppresses genes that transport amino acids, which may limit the flow of nitrogen compounds throughout tissues (Pavlik et al.,2010).

Sulphur metabolism: Similar to other plants, sulfur metabolism in *Musa* spp. is probably involved in arsenic detoxification, specifically through the manufacture of glutathione (GSH) and phytochelatins (PC). These non-protein thiols help sequester arsenic by chelating it. One important precursor of GSH and PC, cysteine (Cys), may be a limiting factor, particularly when arsenic toxicity is present. Increased sulfate absorption and subsequent Cys assimilation through enzymes such as OAS-TL (O-acetylserine (thiol)-lyase) may increase the effectiveness of detoxification. As seen in model plants like Arabidopsis and Brassica juncea, it is conceivable that boosting sulfur assimilation and GSH/PC production would improve arsenic tolerance, notwithstanding the paucity of particular studies in bananas. These routes in *Musa* spp. require further investigation (Thorsen et al., 2007).

Heavy metal content of calcium carbide-ripened banana fruit and control banana.

Bananas and other fruits undergo a biochemical process called ripening, which alters their physiological makeup and, as a result, their color, flavor, texture, and aroma. A widespread and easily accessible meal in Asia and Sub-Saharan Africa, ripe bananas are often eaten uncooked and contain starch. It is often consumed raw when ripe and is a primary starchy meal that supplies over 25% of the body's carbohydrate needs for all age groups in Asia and sub-Saharan Africa (Sogo-Temi et al.,2014 & Adeyemi et al.,2018). A significant financial problem during the post-harvest period is the perishable quality of ripe bananas. This is due to the possibility of 20–50% losses for farmers and fruit dealers. Furthermore, the majority of banana fruit vendors are overly ambitious and seek to make quick cash (Asif, 2012 & Zweter et al.,2012). Therefore, some vendors (the ambitious ones), particularly in Nigeria and other Sub-Saharan Africa, utilize chemicals to quickly artificially ripen green, unripe mature bananas in an effort to lower post-harvest losses (Ali, 2011). Since commercial ripening of fruits is unavoidable in the fruit industry, fruit dealers gather unripe fruits and use various techniques to accelerate the ripening process. Common ripening agents include calcium carbide, ethylene glycols, ethephon/ethrel (2-chloro ethyl phosphoric acid), and ethylene (Rabaya et al.,2017).

The inexpensive cost of compounds like calcium carbide makes them favored over ethylene glycol, ethephon, and other chemicals in the majority of Sub-Saharan African developing nations (Hakim et al.,2012).As a result, calcium carbide is typically the chemical of choice for artificial ripening, regardless of other recommended methods, simply due to its accessibility. However, because calcium carbide is recognized to be harmful, it is a red-flagged substance. The green, unripe bananas are supplied to the general public for human consumption after being pushed to ripen by a hazardous chemical reaction with calcium carbide (Hazardous Substance Fact Sheet, 2016 & Igbinaduwa et al.,2016). Acetylene is created when a banana treated with calcium carbide is sprayed with water (Gupta,2016).

$$CaC_2\,(s) + 2H_2O\,(l) \rightarrow Ca(OH)_2\,(aq) + C_2H_2\,(g)$$

Individuals eat fruits that have been artificially ripened with calcium carbide without being aware of the harmful health implications. There are numerous possible risks to human health while using calcium carbide (Gandhi et al.,2016 & HSI,2015). Quantifying the risk level, which is expressed as a carcinogenic or non-health risk, may be the basis for evaluating the health risk of hazardous metals. The reference dose (RfD) for non-carcinogenic risk characterization is used to calculate the possible danger to human health associated with consuming hazardous fruits. The Hazard quotient (HQ) and Hazard index (HI) indices can be used to evaluate this risk ((Kacholi & Sahu, 2018 & Adamu et al. ,2015). The practice of artificially ripening fruits is considerably more common in Nigeria, particularly among rural and urban women who rely entirely on fruit selling, such as bananas, as a source of income in the face of the country's economic collapse and suffering. Artificial agents may produce more aesthetically pleasing colors than naturally ripened fruits, but they may also make food ingredients more hazardous. The institution in charge of guaranteeing the safety of all foods and medications produced and brought into the nation is the Nigerian National institution for Food, Drugs Administration, and Control. The government has, however, failed to monitor and regulate the use of chemicals and pesticides for fruit and other food preservation and ripening, which has resulted in a gap in that area. The physicochemical differences and potential toxicity between naturally ripened bananas and artificially ripened bananas using ripening agents like calcium carbide, which are widely found in major markets throughout South Eastern Nigeria, must therefore be established through experimentation ((Hakim et al., 2012). AAS, or the Atomic Absorption Spectrophotometer, was used to analyze the levels of heavy metals in the banana sample discussed below. Specific wavelengths were employed in a double beam and deuterium

background hollow cathode lamps of Cu, Hg, Cd, Cr, Pb, Zn, Mn, As, and Ni. Three duplicates of each sample were made (Uroko et al., 2019).

The range of the cadmium (Cd) value in bananas was 0.036 ± 0.01 at control and 0.690 ± 0.03 at 5 gkg−1(C) calcium carbide treated banana. This value was exceeding the FAO/WHO Standard (0.05 mg/kg) and falling below the 0.6–14.0 range. According to reports, fruits have a maximum cadmium content of 0.05 mg/kg (Ubuoh., 2022 & NSCMLCF, 2005). The amount of chromium (Cr) was less than the FAO/WHO Standard of 0.10 mg/kg and ranged from 0.003 ± 0.01 to 0.080 ± 0.01 (FAO/WHO, 2010). The range of lead (Pb) was 0.002 ± 0.02 to 0.052 ± 0.04, which was less than the 0.10 mg/kg FAO/WHO Standard. Although different amounts of calcium carbide-treated bananas included lead, the concentration was below the National Standard of China on Maximum amounts of Contaminants in Foods' maximum safe level of 0.10 mg/kg in fruits (FAO/WHO, 2010 & NSCMLCF, 2005).

As the amount of calcium carbide treatments increased, iron (Fe) varied from 0.043 ± 0.01 to 0.125 ± 0.03. The iron content of naturally ripened bananas was 1.65 mg/100 gm, whereas the iron content of bananas treated with ripening agents was 1.44 mg/100 gm at 4 ml and 0.96 mg/100 gm at 8 ml, respectively. These findings were in conflict with those of the previous study. He found that when the concentration of artificial ripening agent increased, the amount of iron reduced (Taposhe et al., 2017).

Bananas ripened with varying amounts of calcium carbide also included arsenic (As) residues, which ranged from 0.020 ± 0.02 to 0.083 ± 0.01 mg/kg. This was higher than the 0.013 ± 0.07 mg to 0.029 ± 0.06 mg of arsenic concentration discovered in Bangladeshi banana cultivars. However, ripe fruits bought from the market had a higher level of arsenic than the 0.066 ± 0.2 limit. According to WHO (2010), the daily maximum amount of arsenic that can be tolerated is 3.0 μg/kg body weight. While the naturally ripened banana had no arsenic residue, the banana ripened with CaC2 had the maximum concentration of arsenic residue (0.083 ± 0.01 mg/kg) at 10 g/kg. This shows that arsenic entered the body only by CaC2 (Sajib et al., 2014).Since fruits were not exposed to a lot of calcium carbide or direct touch with it, there was no arsenic in the fruits that were ripened in a lab. According to Food Safety and Standards FSSA (2011), the range of arsenic residual in calcium carbide-ripened fruit is 0.020 ± 0.02 to 0.083 ± 0.01—well below the top limit. Cd ≥ Fe ≥ Cr ≥ As ≥ Pb ≥ Hg ≥ Ni is the concentration pattern of the chosen hazardous elements. At varying concentrations, the average concentrations of the chosen hazardous metals in bananas treated with CaC2 are 0.013 (Hg), 0.426 (Cd), 0.042 (Cr), 0.021 (Pb), 0.080 (Fe), 0.040 (As), and 0.006 (Ni) μg/g (Igbinaduwa et

al., 2018). Research has shown that the risk estimation method is valid, reliable, and helpful. However, this Hazard Quotient (HQ) technique ignores additional routes such as ingestion, cutaneous contact, etc. and only takes into account exposure to naturally ripened bananas and bananas treated with calcium carbide. For every toxic ingredient chosen, the HQs for bananas treated with CaC2 were all higher than 1.0. From 2.30 to 8.4; from 1.87 to 27.21; from 0.017 to 12.2; from 0.24 to 4.4; from 1.96 to 12.6; from 13.34 to 70.87; and from trace to 0.03 were the average HQ values for Hg, Cd, Cr, Pb, Fe, As, and Ni, respectively (Oguh et al., 2019).

Conclusion

The dual threats of environmental arsenic contamination and artificial fruit ripening methods in the banana industry are critically highlighted in this review. Despite being naturally occurring, arsenic poses a major threat to plant health and human safety because it enters agricultural systems through human activity. Arsenic uptake in banana plants damages essential metabolic pathways, affects physiological processes, and lowers fruit output and quality. The use of calcium carbide, a widely available but extremely harmful ripening chemical, exacerbates the issue by causing treated fruits to contain higher concentrations of heavy metals like cadmium, mercury, lead, and arsenic.The substantial effects of arsenic stress on the carbon, nitrogen, and sulfur pathways are demonstrated by scientific evaluations of mineral content, antioxidant responses, and metabolic alterations. Furthermore, assessments of the Hazard Quotient and Hazard Index verify that bananas ripened with CaC_2 surpass safe exposure limits, posing a significant risk to consumers. The problem is made worse by the lack of knowledge and regulation around these activities, particularly in areas that are economically weak.The report does, however, also suggest workable remedies. Promising pathways for bioremediation are provided by hyperaccumulator species and arbuscular mycorrhizal fungus. Agronomic and genetic treatments targeted at improving Musa spp. resistance to arsenic may open the door to safer agriculture in polluted soils. The protection of food quality and public health ultimately depends on closing information gaps about the transport, metabolism, and detoxification of arsenic in banana plants as well as tougher regulations on ripening chemicals. This comprehensive strategy, which combines regulatory action, plant physiology, and environmental science, provides the best way to address the risks associated with arsenic in banana production.

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Chapter 2

Targeting Toxicity: A Comprehensive Review of Highly Specific Lateral Flow Biosensors for Aflatoxin B1 Detection in Food Crops

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Abstract

This study thoroughly investigates lateral flow biosensors (LFBs) for the detection of aflatoxin B1 (AFB1) in food crops, a crucial topic given AFB1's severe carcinogenic effects. It begins by discussing the global concern for food safety and the function of mycotoxin monitoring, focusing on the toxicity and prevalence of AFB1 in diverse commodities. The review examines the ideas, components, and historical evolution of biosensors, with a focus on LFBs as point-of-care testing methods. Different types of biosensors, such as electrochemical, piezoelectric, and optical, are examined for their ability to detect AFB1, highlighting their advantages and limitations. Recent advances in nanomaterials, luminous reporters, and bioreceptor technologies are presented, demonstrating their potential to improve the sensitivity, selectivity, and cost-effectiveness of long-frequency filters. It also addresses the barriers to commercialising biosensors, including manufacturing costs, regulatory constraints, and the need for interdisciplinary collaboration. Finally, it discusses future developments, such as the utilisation of smartphones, IoT, AI, and blockchain technologies to develop automated, high-throughput, and user-friendly AFB1 detection systems for improved food safety surveillance, particularly in resource-constrained environments. The conclusion highlights the transformational potential of biosensors in creating a safer and more secure global food supply chain.

Keywords: Aflatoxin (AFB1), Lateral Flow Biosensor (LFB), Mycotoxin Detection, Nanomaterials, Luminous reporters, Biosensor technologies, Point-of-Care Testing.

Introduction

Nutrition is crucial for every living organism. Typically, humans consume grains, fruits, nuts, vegetables, meats, and dairy items, such as milk (Aziz et al., 2024). People throughout the world are becoming increasingly aware of the importance of safe and healthy food, which is driving the development of systems to identify and alert to the presence of harmful substances. The European Union's food safety strategy incorporates the Rapid Alert System for Food and Feed (RASFF). The RASFF system generates information that can be used to prevent risks and intervene early. The system's operation relies on the timely gathering and dissemination of information about food products and feeds. Mycotoxin contamination of food results in a significant number of reports (about 20% in the European RASFF network) (Pigłowski et al., 2019).

In developing countries, the consumption of contaminated food significantly contributes to avoidable illnesses and deaths in both humans and animals (World Health Organization et al., 2021). Food products are susceptible to contamination at origin and as they move along the value chain, exposing consumers to health risks; this is compounded by rapidly changing production practices, a lack of human and institutional capacity, poor government oversight, and limited public health awareness (BLGG Consortium et al., 2023; Trench et al., 2011). Food and environmental samples can be contaminated by chemicals as well as biological agents. One of the most prevalent roles in detecting chemical contaminants is the examination of chemical leftovers. Researchers have created rapid techniques that can be used in procedures for simultaneously determining certain groups of substances, such as different types of plant protection agents and veterinary medications. Currently, despite numerous options for monitoring and preventing food contamination, there are hazards that we cannot entirely eliminate. Secondary metabolites of mold fungi, known as mycotoxins, are one source of such hazards. The name comes from the Greek word *mycos* (fungi) and the Latin *toxicum* (poison). Many mycotoxins have been identified, but the most essential for food safety include aflatoxins, deoxynivalenol, fumonisins, zearalenone, and ochratoxin A. These fundamental mycotoxin forms can be transformed into metabolites with altered chemical structures and distinct physical, chemical, and biological characteristics. Modified forms may occur in raw materials used for food production and during technological procedures (Humpf et al., 2019).

Mycotoxins are also classified as chemical pollutants because their appearance suggests biological pollution and the existence of toxicogenic and frequently pathogenic fungi. Humans become internally exposed to mycotoxins when they consume infected foods such as peanuts, pecans, cereals, beans, or oilseeds. Certain mycotoxins occur as emerging pollutants in surface waters; therefore, modest exposure may become apparent through drinking water. Animals that consume contaminated feed may be indirectly exposed, which can affect products such as milk, eggs, and offal. Given the most important mould species and their toxins, certain economic crops such as cereals (particularly corn), hot peppers, fruits (such as apples and grapes), and their derivatives may become afflicted. The Rapid Alert System for Food and Feed (RASFF), which operates in EU member states, has identified specific nuts (such as pistachios and peanuts), coffee and cocoa beans, and spices in imported products as the principal causes of mycotoxin exposure. Mycotoxins are primarily produced by filamentous fungi, including

Aspergillus, *Penicillium*, *Alternaria*, and *Fusarium* species. *Claviceps* and *Stachybotrys* species also produce substantial amounts of mycotoxin. Mycotoxins are small hazardous secondary metabolites produced by certain fungal species. They form a group of chemicals that possess different chemical structures (Santos Pereira et al., 2019). Mycotoxins are small organic molecules synthesised as secondary metabolites by certain fungal species that may contaminate various agricultural commodities such as cereals, corn, nuts, spices, soybeans, and coffee beans, among others. Aflatoxins (AFB1, B2, G1 and G2), ochratoxin A (OTA), citrinin, patulin, trichothecenes (mainly deoxynivalenol (DON), T2-toxin (T2) and HT2-toxin (HT2)), fumonisins (FB1, FB2 and FB3) and zearalenone (ZEA) are the most frequently occurring mycotoxins present in cereal crops (Malachová et al., 2018). Over 300 structurally distinct mycotoxins have already been identified, including a class known as minor mycotoxins, which includes fusaproliferin, beauvericin, and enniatins. Beauvericin and enniatin have a wide range of biological actions. Rather than assisting the fungus's growth and regular metabolism, many mycotoxins are secondary metabolites that compete with other species or are implicated in conditions (Pitt et al., 2000; Reverberi et al., 2010). Among these mycotoxins, AFs have gained the greatest attention due to their extreme toxicity. The International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has categorised aflatoxin B1 (AFB1) as a Group 1 agent (potent human carcinogen). It has long been associated with liver cancer, and more recent studies have revealed its deleterious influence on nutritional outcomes and immune repressive effects (Shephard et al., 2008). Medical conditions such as nephropathy, hepatic disorders, haemorrhagic syndromes, immune system dysfunctions, neurological disorders, and different types of cancer can all be brought on by mycotoxins (Armendáriz et al., 2014). These hazardous substances can enter the human body by touch, ingestion, or inhalation (Manteca et al., 2001). According to published data, over 500 million individuals in underdeveloped nations bear the burden of mycotoxins (Agriopoulou et al., 2020). Agricultural technologies for assessing crop health include polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), gas chromatography-mass spectrometry (GC-MS), and flow cytometry (FCM). These methods let us identify a variety of plant diseases, nutrient deficits, weather sensitivity, and stresses. Currently, variations in chemical indicator levels are utilised to assess plant stress. However, existing crop health analysis approaches are labour-intensive, time-consuming, and intended for laboratory settings. Improvements in biosensors look to be critical for a better understanding of agricultural processes (Kundu et al., 2019).

Mycotoxins cannot be completely eradicated because they are a naturally occurring food contaminant that develops in dietary items (Chatterjee et al., 2023). Due to their toxicity, maximum allowable concentrations of mycotoxins are controlled globally, and monitoring their occurrence in diverse commodities is necessary to ensure food safety and consumer protection (Anfossi et al., 2016). As a result, numerous detection systems have been developed to identify even trace levels of toxins in food. Established approaches such as chromatography techniques, including high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-FLD), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and gas chromatography (GC), have been widely used and have high specificity and sensitivity (Selvaraj et al., 2015). Gas chromatography separates the parts of a mixture based on how they

move between the stationary and mobile phases. It also separates volatile chemicals by using a high-boiling liquid that is stuck to a solid as the stationary phase and a gas, like helium, as the mobile phase. The idea is that the different parts are spread out over these phases and interact with each other in different ways based on their structures and properties. This allows the column to hold things for varying durations of time, resulting in separation (Marriott et al., 2002). Traditional processes, on the other hand, are expensive, need a lot of equipment, and require skilled operators; they also lack the speed of cutting-edge approaches (Orooji et al., 2021; Zhang et al., 2021). The immune-chemical approach known as enzyme-linked immunosorbent assay (ELISA) is commonly used to detect mycotoxin metabolites. It has a high sample throughput and great sensitivity and specificity (Krska et al., 2008).

Since it requires a lot of labour and is slower than newer techniques of finding things, point-of-care testing procedures have sparked widespread concern in recent years. They have advantages over central lab procedures, such as faster processing, fewer samples, and ease of use (Mansour et al., 2024; Sohrab et al., 2022). Traditional detection methods are expensive and can sometimes produce false positives or negatives; thus, the search for faster, cheaper, and more accurate approaches continues. Recent breakthroughs in detection technologies, such as microfluidics, lab-on-a-chip systems, smart nanospectroscopy, and sensor technologies, provide potential alternatives for rapid and cost-effective mycotoxin detection. As one of the POINT OF CARE TESTING methodologies and immunochemical-based techniques, Biosensors offer an alternate technique to performing tasks than typically utilised devices (Sohrabi et al., 2021). It is a detecting device that employs an electrical transducer to convert biological reactions from a receptor into an electrical signal that can be recognised, coupled to, and detected by an electrical sensor (Mansour et al., 2024; Sohrabi et al., 2023; Su et al., 2011). The primary premise of biosensor detection is the coupling of a bioreceptor (a biological recognition element) and a transducer (a sensing element), which produces a quantifiable signal proportional to analyte concentration. Different types of biosensors have been discovered based on the bioreceptor type (e.g., enzymes, antibodies, microbes, etc.), yet the significance is usually based on the interaction with analytes (i.e., the need to be highly specific) (Ma et al., 2022).

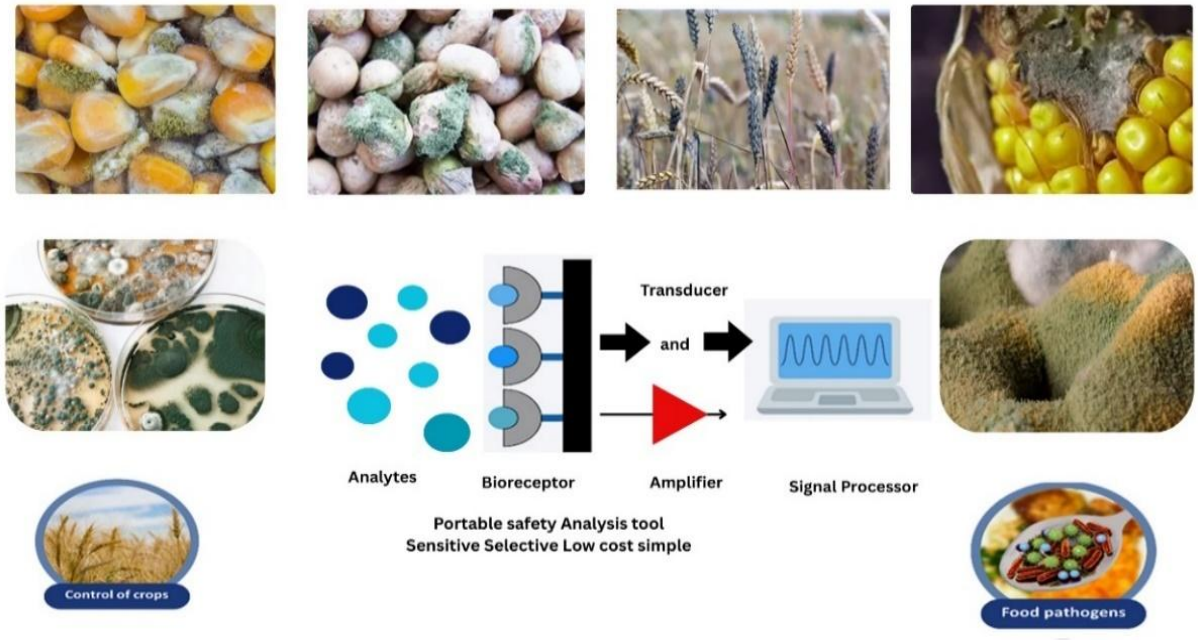

Figure 1: Integrated Mycotoxin Detection System from Bioreceptor to Signal Processing (Ma et al. 2022)

Table 1: List of Mycotoxin, their origin and toxicity.

Mycotoxin	Structure	Main fungal species	Commodities Affected	Toxic Effects
Aflatoxins: B1,B2,G1, G2,M1*	AFB1	*ASP.Flavus,Asp.parasiticus*	Nuts, spices, grains such as maize, rice, wheat, milk and milk products, etc.	Carcinogenic, teratogenic, mutagenic, immunosuppressive (Rushing, B. R et.al.2019)
DON	DON	*F.graminearum,*	Cereals, cereal products	Diarrhea,vomiting,anorexia,immune dysregulation(Liu, D.et.al ,2020)
		F culmorum, F.cerealis		
ZEN	trans-ZEN	*F.graminearum(Gibberella zeae), F.culmorum, F.cerealis, F.equiseti, F.crookwellense,*	Cereals, cereal products, maize, rice, beer, etc.	Hyperoestrogenic, hepatotoxic, haematotoxic, immunotoxic, genotoxic(Kuiper-Goodman, T et.al 1987)
	cis-ZEN	*F.semitectum*		

OTA	OTA	*Asp. ochraceus, Asp. niger,* *Asp. carbonarius,* *Asp. terreus,* *P. verrucosum,* *P. nordicum*	Cereals, wine, coffee, cocoa, beans, dried fruits, nuts, spices, cheese, etc.	Nephrotoxic, hepatotoxic, neurotoxic, teratogenic, immunotoxic(Tao, Y.et.al 2018)
Fumonisins: **FB_1, FB_2, FB_3**	FB1	*F. verticillioides, F. proliferatum*	Mainly maize and maize-based products, sorghum, and asparagus	Carcinogenic, cytotoxic, nephrotoxic, hepatotoxic(Ekwomadu, T. I.et.al 2021; Li, Q et.al2021)
T-2/HT-2 toxin	T-2 TOXIN HT-2 toxin	*F. langsethiae, F. poae, F. sporotrichioides*	Wheat, rye, maize, soybeans	Growth retardation, myelotoxic, hemotoxic, necrotic lesions on contact areas. (Nathanail, A. et al. 2015)
PAT		*P. expansum*	Fruits and vegetables	Nausea, vomiting, and other gastrointestinal intestinal symptoms, kidney damage(Speijers, G. Jet.al 1988)
CIT		*P. citrinum,* *P. camemberti,* *Asp. terreus,* *Asp. Niveus*	Fermented maize, cheese, corn, wheat, barley, red yeast rice, apples, brewed beer, cereal products	Nephrotoxic, may cause liver and kidney diseases, nervous system damage (de Oliveira Filho, J. W. G., et al. 2017)

Aflatoxin b1

Overview of AFB1 and its toxicity

Mycotoxins, a class of secondary metabolites produced by filamentous fungi, contaminate approximately 25% of global agricultural goods at various stages of their economic life cycle: growth, harvest, storage, and processing (Al-Taher et.al 2013) They are a vast group of mycotoxins, but the most predominant include nivalenol, 3-acetyl-deoxynivalenol, 15-actyl-deoxynivalenol, ochratoxin (OTA), aflatoxin B1 (AFB1), and fumonisin B1(FB1) (Pereira et.al 2014; Li et.al 2009). AFB1 is likely the most well-known and certainly the most hazardous member of its class. It is known to bind to cell DNA and raise the risk of liver cancer, even if only trace amounts are present. Considering this, coupled with its mutagenic, teratogenic, immunosuppressive, and carcinogenic method of action, AFB1 has been included by the International Agency for Research on Cancer as the number one group of carcinogens (Pereira et.al, 2014; Li et.al, 2009; Shim et.al2014) Without a doubt, there is a need to keep AFB1 levels under control and contamination to a minimum. As a result, many municipal food and health agencies have established limits on AFB1 levels in various food products. Cereals and cereal-derived products, which pose the greatest risk of contamination, often have limits ranging from 0.05 to 20 ng/mL (Babu et al., 2011). However, in some countries, such as the United States, the Food and Drug Administration (FDA) can set limits on corn and peanuts (Hu et al., 2015). To maintain control over AFB1 levels, a continual and comprehensive analysis of its content in food products is required. Traditional methods, such as high-performance liquid chromatography, mass spectrometry, or a combination of the two, are dependable with high sensitivity and selectivity, but they are costly, time-consuming, and unable to analyse a large number of samples in a variety of environments (Abia et al., 2013; Herzallah et al., 2009; Tokuşoğlu et al., 2005; Yazdanpanah et al., 2013; Shim et al., 2007).

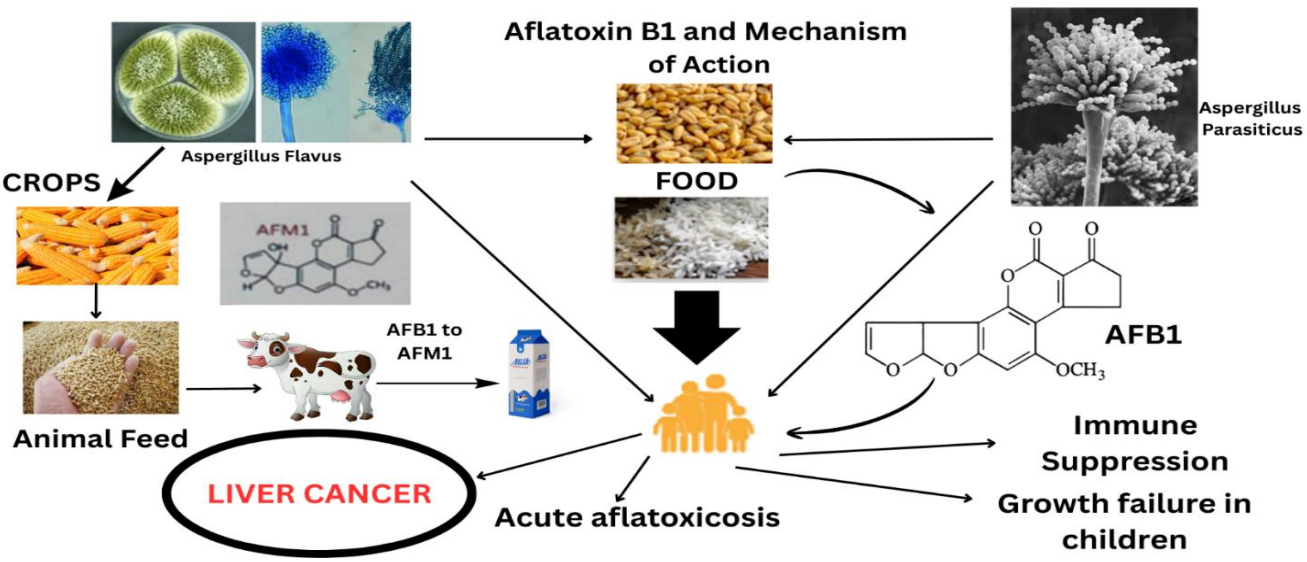

Figure 2: Aflatoxin B1 exposure pathways (Hamid, A. et al.2013) (Li et.al 2022) (Smith et al. 2020)

Aflatoxins, among the several mycotoxins discovered thus far, have emerged as a source of intense scientific interest. This is due to their high toxicity and carcinogenic potential, with several aflatoxin subtypes formally classified as Group 1 carcinogens by the International Agency for Research on Cancer (International Agency for Research on Cancer et al. 2003). These compounds are metabolites from the fungus species Aspergillus flavus and Aspergillus parasiticus, which are commonly found in food and feed, particularly peanuts, corn, and rice. The tropical and subtropical climates promote the growth of both fungi and poisons because they give the ideal temperature and humidity. However, their prevalence in food is not simply driven by geographical location, but may also occur in reaction to insufficient storage, transit, or agricultural processing conditions. (Bennett et al 2003; Pitt et.al 2014)

When consumed in large quantities, aflatoxins can trigger acute toxic responses, resulting in a disease known as aflatoxicosis. Aflatoxicosis can be lethal, especially because it causes hepatic failure. Furthermore, aflatoxins can have chronic harmful consequences, manifesting as carcinogenicity in numerous organs, with a predisposition for the liver and kidneys. (Li et.al 2022)

The epoxidation of AFB1 by cytochrome P450 enzymes, particularly CYP3A4, results in the production of 8,9-epoxy-AFB1. Only the 8,9-exo-epoxy-AFB1 isomer is mutagenic, creating adducts with DNA or proteins, whereas the endo isomer is not harmful. Because of interindividual variations in the expression of cytochrome P450 enzymes, certain persons may be more vulnerable to the creation of the poisonous metabolite, increasing the chance of developing liver cancer (Smith et al. 2020).

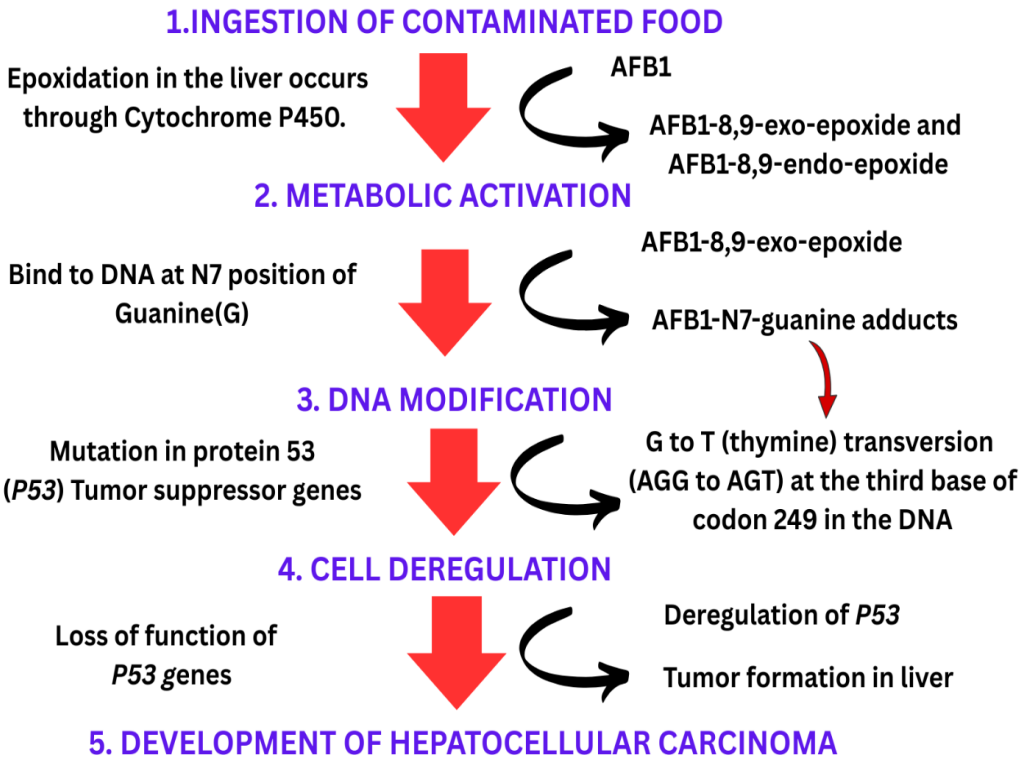

Figure 3: Mechanism of Aflatoxin B1-induced hepatocellular carcinoma (Smith et al. 2020).

As a result, the presence of AFB1 in food has been identified as a severe food safety problem, prompting regulators to establish certain maximum amounts. Permitted standards for AFB1 levels vary across different areas and nations throughout the world. The maximum permitted for most consumer items in the European Union is 2 ng/mL, 10 ng/mL in Japan and Korea, and 20 ng/mL in the United States (European Commission et.al 2006; Tokyo Metropolitan Government et al. 2023; U.S. Food and Drug Administration et al. 2011)

Lateral Flow Biosensor

The IUPAC nomenclature defines a biosensor as a device that uses precise biochemical reactions mediated by isolated immune systems, enzymes, organelles, tissues, or entire cells to detect chemical compounds (analytes), typically through optical, electrical, and thermal signals. A bioreceptor, a transducer, a micro-electronics/electronic system/an electronic assembly, and a display/readout unit are the four standard parts of a biosensor. Biosensors are widely used for detecting allergens in food, toxins in water, chronic illness control, pregnancy tests, and other diagnostic applications. Biosensors are expected to become more prevalent in our daily lives, necessitating the development of user-friendly technology. Although biosensor technology has advanced, it still falls short of the convenience of devices like glucose biosensors and pregnancy tests, which are widely available in pharmacies worldwide. Developing low-cost and effective sensors that detect analytes, including DNA, proteins, and cells, in real-world circumstances remains a challenge (Clark et al., 1953). Researchers could develop paper-based biosensors, which are also known as Lateral Flow Biosensors, similar to immunochromatographic assays (IAs) to separate analytes based on interactions between antigen, antibody, enzyme, substrate, or receptor. Paper is a cost-effective and efficient medium for biosensing technology due to its simplicity, abundance, and ease of manufacturing (Lou et al., 1993; Cho and Paek, 2001; Lönnberg and Carlsson, 2001; Ho and Wauchope, 2002; Shyu et al., 2002). To fulfil cost/efficient requirements in biosensing technology, Paper is used since it is a simple, cheap, abundant, and easy-to-manufacture material (Costa et al.2014)

Introduction of Biosensor

A biosensor is a device that detects biological or chemical reactions by producing signals proportional to the concentration of an analyte in the reaction. Biosensors are used in disease monitoring, drug development, and the detection of contaminants, disease-causing microorganisms, and disease markers in bodily fluids (blood, urine, saliva, and sweat). It consists of the following components.

Analyte: A material of interest that requires detection. For example, glucose is an analyte in a biosensor that detects glucose. A bioreceptor is a molecule that precisely detects an analyte. Bioreceptors include enzymes, cells, aptamers, DNA, and antibodies. Bio-recognition refers to the process of generating signals (such as light, heat, pH, charge, or mass change) as a result of the bioreceptor's interaction with the analyte.

Transducer: A transducer is an element that transforms one type of energy into another. In a biosensor, the transducer converts the bio-recognition event into a quantifiable signal. This process of energy conversion is referred to as signalisation. Most transducers provide optical or electrical signals, which are typically proportional to the number of analyte-bioreceptor

interactions.

Electronics: This component of a biosensor processes the transduced signal and prepares it for display. It is made up of complicated electrical circuitry that provides signal conditioning functions such as amplification and signal conversion from analogue to digital format. The biosensor's display device will then quantify the processed signals.

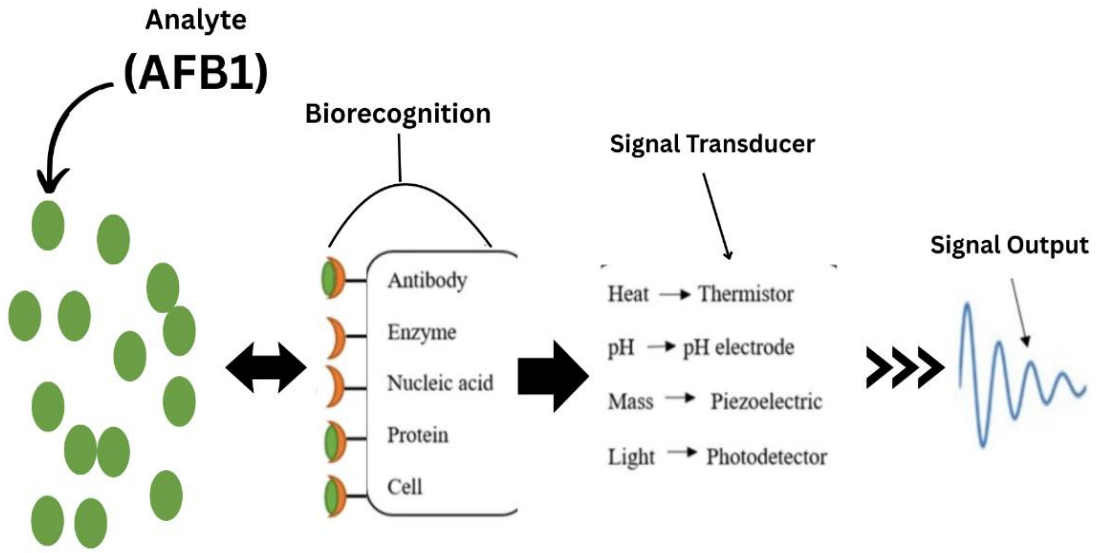

Figure 4: Fundamental components and operation of a Biosensor (Cremer et al. 1906) (Gupta et al., 2019) (Naresh et al.2021; Bhattarai et.al 2020)

History of Biosensor

Biosensors have been around since 1906, when M. Cremer (Cremer et al. 1906) proved that the concentration of an acid in a liquid is proportional to the electric potential that exists between fluid components on opposite sides of a glass membrane. Søren Peder Lauritz Sørensen proposed the notion of pH (hydrogen ion concentration) in 1909, and W.S. Hughes developed an electrode for measuring pH in 1922(Hughes et al. 1922). Griffin and Nelson (Griffin et al., 1916; Nelson et al., 1916) were the first to show that the enzyme invertase could be immobilised on aluminium hydroxide and charcoal between 1909 and 1922. Leland C. Clark, Jr. developed the first 'real' biosensor for oxygen detection in 1956. He is renowned as the 'father of biosensors', and his development of the oxygen electrode retains his name: the 'Clark electrode'(Heineman et al. 2006). Leland Clark demonstrated an amperometric enzyme electrode for the detection of glucose in 1962. Guilbault and Montalvo, Jr. discovered the first potentiometric biosensor to detect urea in 1969(Guilbault et al. 1969). The first commercial biosensor was developed by Yellow Spring Instruments (YSI) in 1975. Table 2 shows the historical overview of biosensors in the period 1970–1992. Remarkable progress has been achieved in the field of biosensors since the development of the i-STAT sensor. Currently, this field is a multidisciplinary area of research that bridges the principles of basic sciences (physics, chemistry, and biology) with fundamentals of micro/nano-technology, electronics, and applied medicine. Lateral flow biosensors, or LFBs, were first developed in the early 1980s

(Glad et al. 1981). Since then, LFBs have been used as commercial products for low-cost, simple-to-use, rapid tests for point-of-care screening of infectious diseases, drugs of abuse, and pregnancy (Glynou et al. 2003).

Table 2. Important cornerstones in the development of biosensors during the period 1970–1992.

1970	Discovery of ion-sensitive field-effect transistor (ISFET) by Bergveld (Bergveld et al. 1970)
1975	Fibre-optic biosensor for carbon dioxide and oxygen detection by Lubbers and Opitz (Vestergaard et al. 2015)
1975	First commercial biosensor for glucose detection by YSI (Yoo et al. 2010)
1975	First microbe-based immunosensor by Suzuki et al.*(Suzuki et al. 1975)*
1982	Fibre-optic biosensor for glucose detection by Schultz (Schultz et al. 1982)
1983	Surface plasmon resonance (SPR) immunosensor by Liedberg (Liedberg et al., 1983)
1984	First mediated amperometric biosensor: ferrocene used with glucose oxidase for glucose detection (Cass et al. 1984)
1990	SPR-based biosensor by Pharmacia Biacore (Vestergaard et al., 2015)
1992	Handheld blood biosensor by i-STAT (Vestergaard et al., 2015)

Characteristics of a biosensor

Every biosensor has specific static and dynamic characteristics. The biosensor's performance reflects the optimum of these qualities.

Selectivity

Selectivity refers to a bioreceptor's capacity to identify a certain analyte in a sample that contains various admixtures and impurities. It is the most essential element of the biosensor. The interaction of an antigen and an antibody is the best illustration of selectivity. Antibodies often function as bioreceptors and are immobilised on the transducer's surface. A solution containing the antigen (often a salt-containing buffer) is then introduced into the transducer, where antibodies interact only with the antigen. Selectivity is the most important factor to consider when selecting bioreceptors for a biosensor.

Reproducibility

The capacity of the biosensor to produce the same results for a replicated experimental setup is known as reproducibility. The precision and accuracy of a biosensor's electronics and transducer define its reproducibility. Accuracy shows the sensor's ability to provide a mean value that is close to the true value when a sample is measured more than once, while precision is the sensor's ability to produce consistent results each time a sample is measured. Reproducible signals give the conclusion drawn about a biosensor's response great robustness and dependability.

Stability

The stability of the biosensing system refers to its level of vulnerability to environmental disturbances. The output signals of a biosensor that is being measured may drift as a result of these disruptions. This may result in a measurement error in the concentration and impact the biosensor's accuracy and precision. When a biosensor is used in situations requiring lengthy incubation periods or ongoing monitoring, stability is the most important characteristic. The stability of a biosensor may be impacted by temperature-sensitive transducers and electronic responses. Therefore, to guarantee a steady response from the sensor, proper electronics tuning is needed. The degree to which the analyte binds to the bioreceptor, or the bioreceptor's affinity, is another element that may affect stability. To strengthen a biosensor's stability, high-affinity bioreceptors promote the analyte's covalent or strong electrostatic bonding. The bioreceptor's gradual deterioration is another element that influences a measurement's stability.

Sensitivity

A biosensor's limit of detection (LOD) or sensitivity is determined by the smallest quantity of analyte that it can detect. Analyte concentrations as low as ng/ml or even fg/ml must be detected by a biosensor in a variety of medical and environmental monitoring applications to verify the existence of analyte traces in a sample. For example, doctors recommend biopsy tests for prostate cancer when the PSA concentration in blood is 4 ng/ml. Sensitivity is therefore regarded as a crucial biosensor characteristic.

The concept of linearity

The characteristic known as linearity, which can be expressed mathematically as y=mc, where c is the analyte concentration, y is the output signal, and m is the biosensor's sensitivity, indicates how accurately the measured response (for a series of measurements with varying analyte concentrations) to a straight line. The resolution of the biosensor and the range of analyte concentrations being tested are related to the biosensor's linearity. The smallest change in analyte concentration necessary to alter the biosensor's response is known as the resolution of the biosensor. The range of analyte concentrations for which the biosensor response varies linearly with the concentration is known as the linear range, which is associated with linearity.

Fundamentals of Biosensors: Essential Components and Working Principle

Essential Components

The bioreceptor, a crucial component painstakingly designed to identify and bind with the target analyte, is at the core of biosensors. Using a variety of bioreceptor arrays, each with unique benefits, this component essentially determines the biosensor's specificity and sensitivity (Huang et al. 2021). The use of immune system-derived antibodies in biosensors is demonstrated by their remarkable specificity and ability to be customised for different disease markers (Shamsuddin et al., 2021). As biological catalysts, enzymes change their activity when they bind to the analyte and provide a detectable signal. This is especially useful in biosensors that are intended to identify particular enzymatic reactions (Kucherenko et al. 2019). Short

DNA or RNA sequences known as aptamers exhibit strong target affinity and adaptability, making them useful in a variety of biosensor applications (Wang et al. 2022). Furthermore, cells—whether in their entirety or in their constituent organelles or membranes offer a dynamic approach to biosensor design, changing their electrical characteristics or signalling activity in response to analyte (Gupta et al., 2019). This complex interaction of bioreceptors highlights the complex and varied nature of biosensor functionality. The chemical information associated with the analyte (present in various biological fluids such as sweat, blood, saliva, interstitial fluid, tears, breath, and human volatiles) is transformed to a readable output via sensing/recognition and transduction. Specificity and selectivity are crucial aspects of biorecognition elements (BERs), also known as bio-receptors, that identify the target analyte (Ramesh et, al2022; Soleymani et al., 2017). Examples of various biorecognition elements are (i) enzymes; (ii) antibodies;(iii) nucleic acids; (iv) molecularly imprinted polymers (MIPs); (v) cells; (vi)aptamers; (vii) DNA, etc. The examples (i–iii) belong to the most widely used bioreceptors. The biorecognition elements immobilize on the biosensor sur-face, allowing the interaction of the target analyte with the immobilize bio-receptors, which will be conveyed to the transducer that transforms the recognition event into a readable output which can be further analysed and amplified(Naresh et al.2021; Bhattarai et.al 2020).The transducing systems can be optical, electrochemical, piezoelectric, ion-sensitive, thermometric, magnetic or acoustic one(Monošík et.al 2012)Bio-sensors may comprise various types of display units such as a computer, liquid crystal display (LCD) or a printer, which provide a pictographic demonstration of the measured signal. According to the needs of the users, the format for the output signals can be different; for instance, the outcome can be in a tabular form, numeric, an image, or graphics.

Working principle

Traditional methods, such as membrane encapsulation, physical adsorption, covalent or noncovalent binding, etc., are employed to immobilise a bioreceptor on a suitable substrate, making it the sensor's principal component (Malhotra et al., 2017). When the sample's analyte attaches to the bioreceptor on the sensing platform, a biomolecular interaction occurs, resulting in an optical, electrical, or chemical response. During the interaction, a new product is occasionally generated, as well as byproducts such as ions, gases, heat, light, and so on. The transducer's surface responses are then converted into electrical or optical signals that may be read using an amplifier. The differential gap between the sample and reference signals is often enhanced during signal processing. The microprocessor unit receives the digital signal, processes it, calibrates it, and displays the results in the appropriate units. A biosensor's performance is dependent on its sensitivity, selectivity, and stability. The biosensor has what is called a "working range, or a range of concentrations of the bio-analyte in which it is effective. Graphene's low noise and excellent electrical conductivity enable its exceptional sensitivity (Yu X et al. 2017). Having the biosensor correctly detect the biomarker molecule is a critical stage in the process. Various proteins or antigens may be present in the target blood sample. Even in this scenario, the biosensor should only react to the target biomolecule, not any other chemicals. The lock-and-key mechanism revealed by antigen-antibody interaction allows for increased and enhanced sensitivity (AI Qaraghuli et al., 2020). Jokerst and his colleagues developed a semiconductor QD-based biosensor capable of detecting all three

cancer biomarkers: carcinoembryonic antigen (CEA), CA 125, and Her-2/Neu. They discovered that it was far more sensitive than the traditional ELISA (Enzyme-based Immunosorbent Assay) test (Jokerst et al. 2009). Any machine that does quantitative analysis must have high measurement accuracy. The analyte concentration had a greater effect on the signal strength. Maintaining a constant signal is critical for getting reliable results. A biosensor must also have a short response time to be used for real-time monitoring purposes. A submicron-sized glucose sensor implanted beneath the skin has been shown to produce findings in less than a millisecond (Kim et al. 2022). The biosensor's endurance is determined by various factors, including the accuracy with which the transducer electronics are calibrated, the bioreceptor's affinity, and the bioreceptor's degradation time or rate, among others (Demir et al. 2024).

Signal transduction and element biorecognition are the fundamentals of how biosensors operate. Any biological material, such as an enzyme, antibody, hormone, nucleic acid, organelle, or entire cell, can be employed as a sensor or detector in a device. However, a particular deactivated enzyme is typically the desired bio-receptor. The transducer is positioned close to the deactivated enzyme. The tested analyte binds to the particular enzyme (bio-receptor) and alters the enzyme's biochemical characteristics. Using an electroenzymatic method, the change in turn produces an electronic response. The chemical process of using a transducer to convert enzymes into corresponding electrical signals is known as an electroenzymatic process. The electrical signal produced by the transducer now directly represents the biological material (in this case, the analyte and enzyme) being measured. To properly analyse and represent the electrical signal, it is typically transformed into a physical display.

Lateral flow assay is an ancient method of diagnosis that has been used since the 1940s. To ensure that the analyte it contains can bind to the conjugate capture reagents and the membrane, the sample (which contains an analyte) is placed onto the sample pad. Before migrating into the detection zone with the conjugated antibody, the treated sample passes through the conjugation pad, which has antibodies that are specific to the target analyte and have been attached primarily to gold nanoparticles (fluorescent particles). This detecting zone (mostly antibodies or antigens) is referred to as a nitrocellulose membrane, a porous membrane typically made of nitrocellulose containing immobilised biological components. The nitrocellulose membrane's function is to bind to the conjugated antibody and participate in interactions between the analyte and the antigen (Koczula et al., 2016). It provides a platform for both reactions during the analysis. In order to permanently bind the antibodies to the nitrocellulose membrane, strong nitrate ester dipoles and peptide bonds in the antibodies interact to create an electrostatic interaction, which is subsequently dried at room temperature. Other substances, including polyvinyl alcohol, block the membrane's free active sites. A reaction on the test line indicates the identification of the sample analyte, whereas a response on the control line verifies appropriate liquid flow across the strip since the membrane has two distinct types of lines (test and control lines). A single line on the pad indicates the test's positive and negative results after the incubation period. These lines of different intensities represent the test's read-out, which can be viewed visually or with a specialised reader. When the residual

sample finally reaches the absorbent pad, which has a huge bed volume, it can finish the sample flow (Hasan et al., 2022).

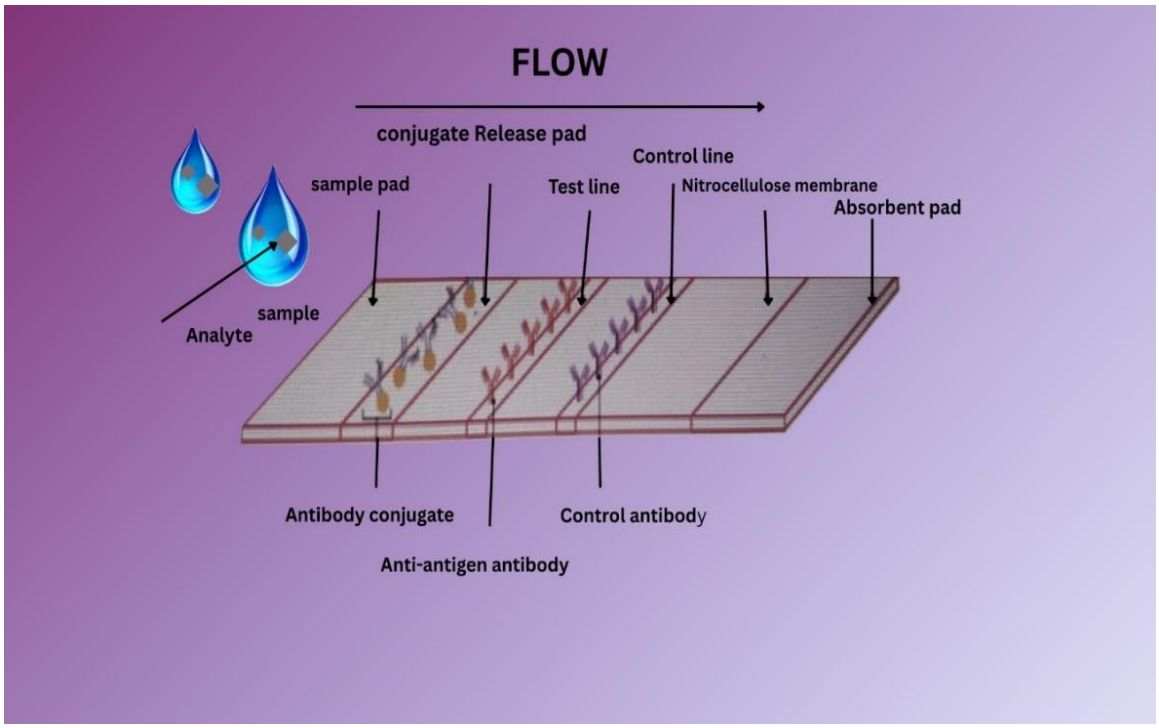

Figure 5: Operational principle of Lateral Flow Assay (Koczula et al., 2016)

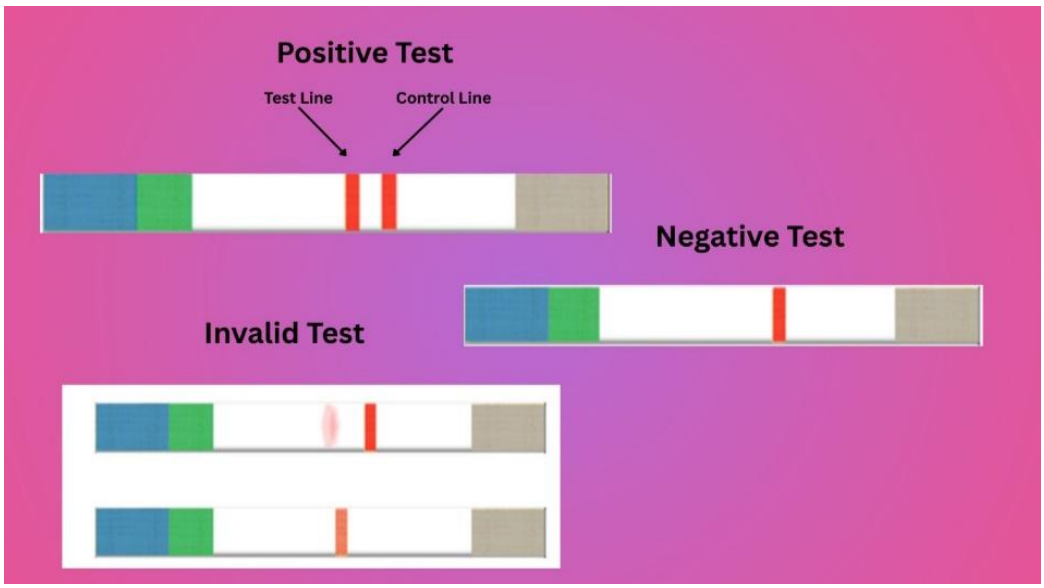

Figure 6: Test results of LFA (Hasan et al., 2022).

Types of Biosensors utilized for Aflatoxin B1 detection

Biosensors are categorised into two groups, either according to the transduction technique used or the biological element used in the analysis. DNA, enzymes, antibodies, microorganisms, tissues, cell receptors, and so on are some of the frequently used biological elements, also known as bio-recognition elements.
The type of transduction employed in the sensor, or the type of physicochemical that results from the sensing event, is the basis for the next and most widely used classification of biosensors. Furthermore, transduction-based biosensors can be categorised into three main groups. They are: Mass-based Biosensors, Optical-based Biosensors, and Electrochemical Biosensors

Mass-based, electrochemical, and optical biosensors are the three types of biosensors that fall under the category of transduction techniques. Magnetoelastic, piezoelectric, and quartz crystal microbalance (QCM) biosensors are examples of mass-based biosensors that detect variations in mechanical or mass characteristics. Amperometric, potentiometric, conductometric, and impedimetric types of electrochemical biosensors are further subdivided into those that translate biochemical interactions into electrical signals. Surface plasmon resonance (SPR), optical fiber-based, and surface-enhanced Raman spectroscopy (SERS) biosensors are examples of optical biosensors that make use of light-based phenomena. Depending on the biological material—such as antibodies, DNA, enzymes, or phages—biosensors can be categorised according to biorecognition elements. Certain biomolecules can be detected thanks to these bioreceptors' selective binding to their target analytes. MEMS-based biosensors use cutting-edge miniaturisation technology to increase sensitivity and precision. Finally, the classification includes biosensors depending on substrate type, such as paper and electrospun materials. These diversified biosensors have several advantages, including expanded surface area for greater sensitivity, biocompatibility, and the ability to develop flexible, wearable biosensors. This classification scheme reflects the wide range of biosensor technologies available for healthcare applications, each designed to meet specific monitoring or diagnostic needs. Biomolecules contain enzymes, cells, antibodies, DNA, and various substances. The signal transduction mechanism is determined by the physicochemical interactions between the biorecognition component and the target molecule in the sample.

Numerous biosensors based on nanomaterials are used to examine and track food contaminated with AFB1. Various transducers that offer different types of analytical data, including current, potential, conductance, impedance, absorbance, and fluorescence, have been used to detect AFB1. The transducer converts the analyte-receptor interaction rate and occurrence into a quantifiable physical phenomenon. Biosensors that are used for the detection of AFB1 have been categorised based on the transducer used, such as electrochemical, optical, or piezoelectric biosensors (Odoemelam et al.2009; Jia et al. 2020).

Electrochemical biosensors for AFB1 Detection

Electrochemical biosensors have sparked particular interest in the assessment of food quality, owing to their small size, low cost, speed, sensitivity, and unique functionality. Electrochemical biosensors create analytical signals such as current, voltage, impedance, or conductance when AFB1 is detected in foods such as maize flour (Yadav et al., 2021; Durán

et al., 2013). These electrochemical biosensors include immunosensors, conductometric, potentiometric, impedimetric, and amperometric biosensors (Shi et al. 2020). Two or three electrodes can be used to create amperometric biosensors; the three-electrode method has shown better analytical performance for AFB1 detection. The three-electrode system consists of the reference, auxiliary, and functional electrodes (Chuan et al., 2011; Pundir et al.2019). By expanding the surface area, the extra auxiliary electrode enhances the electron kinetics when AFB1 is found in food. Whereas electric potential moves between functional and reference electrodes, electric current moves between functional and auxiliary electrodes (Hui et.al, 2020; Pundir et. al, 2019). In electrochemical biosensors, biological molecules are coated on a probing surface. The sensing molecules are maintained in place using a non-interfering membrane. The detecting molecules then respond properly to the identified substance, producing an electrical signal proportionate to the quantity measured. Electrochemical biosensors can use a variety of transducers, including potentiometric, amperometric, and impedimetric, to turn chemical information into a measurable electrical signal. In contrast, the two-electrode system does not have a counter electrode or auxiliary electrode and fails at high temperatures (Sharma et al. 2020). Consequently, amperometric biosensors with two electrodes cannot be used to assess the quality of food samples. Amperometric biosensors measure changes in current with remarkable repeatability, consistency, sensitivity, and selectivity. The amount of AFB1 present in the sample determines how much current changes while keeping the potential constant. However, biosensors can occasionally yield erroneous results due to cross-reactivity, and their operation necessitates costly oligonucleotide segment preparation, specialized equipment, and personnel (Chuan et al. 2011)

Potentiometric biosensors detect a specific analyte, such as AFB1, by measuring the voltage between functional and reference electrodes while maintaining a constant current. Potentiometric biosensors are easy to use and sensitive, with stability and sensitivity determined by several factors, including pH, temperature, immobilization matrix, and immunological cross-reaction (Agriopoulou et al., 2020; Li et, al2016; Eivazzadeh-Keihan et al.2017)

Conductometric biosensors detect AFB1 by measuring the electrical conductivity of an analyte between electrodes while understanding the enzymatic processes that alter the ionic strength of the reaction mixture. Variations in conductivity generated by this shift in the reaction system's ionic concentration serve as an analytical signal to identify the ionic concentration of the sample. Conductometric biosensors are generally economical and sensitive; however, they can produce false positives due to their poor specificity and accuracy (Jaffrezic-Renault et.al, 2008; Grieshaber et.al, 2008; Liu et.al, 2006).

Immunosensors detect target antigens by electrochemical immunoreactions with specific antibodies. The transducing element immobilises specific antibodies that detect AFB1 in food samples. Immunosensors are repeatable, sensitive, specific, and reliable, but labour-intensive since the immobilised antibodies attach to non-targeted molecules (Sharma et al., 2020; Wu et al., 2020; Zhang et al., 2016). In impedimetric biosensors, the biorecognition element binds to the target analyte (AFB1), resulting in an electrochemical response of altered

capacitance and electrical conductance. This change is recorded as the impedance change caused by the interaction between the analyte and the biorecognition element immobilised at the working electrode. Impedimetric biosensors provide a rapid response time without harmonic distortions; however, they are more expensive and less precise.

Piezoelectric Biosensor for Aflatoxin B1 Detection

In these biosensors, the surface is coated with antibodies that bind to the complementary antigen found in the sample solution. This causes an increase in mass, which lowers their vibrational frequency; this alteration/change is used to calculate the amount of antigen present in the sample solution. The recording and measurement of affinity contacts underpins the operation of piezoelectric biosensing devices. Piezoelectric crystals serve as the sensing element in these biosensors, measuring the changed oscillations caused by mass changes when subjected to an electrical field. Quartz crystal microbalance (QCM) serves as the transducing element in these biosensors. QCM measures the mass per unit area deviation produced by a frequency change in the quartz crystal resonator. This biosensor uses thiolated β-cyclodextrin to immobilise phenoxy-derived dextran on the Au surface of QCM. Concanavalin A, which accumulates on the QCM probe as a result of the dextran-Con A interaction, binds to AFB1 and bovine serum albumin. Nanoliposomes filled with glucose and labelled with monoclonal antibodies specific for AFB1 enhance the strength of the QCM signal. The sample containing AFB1 is introduced to bind with the AFB1-targeted antibody, which connects with the AFB1-BSA on the QCM probe. As a result of the antigen-antibody interaction, the amount of conjugated nanoliposomes on the QCM surface decreases, indicating an increase in AFB1 in the sample (Eivazzadeh-Keihan et al., 2017; Tang et al., 2020). Piezoelectric biosensors for AFB1 detection have shown outstanding sensitivity, independence from radiation and markers, cost-effectiveness, recyclable nature, specificity, repeatability, dependability, and ease of on-site testing. However, these biosensors are less stable at higher temperatures and provide a substantially greater background signal than the analytical signal (Agriopoulou et al. 2020).

Optical biosensors for AFB1 detection

This biosensor measures both catalytic and affinity processes. The biosensor measures the change in fluorescence caused by the products created during the catalytic processes. In other words, biosensors detect the change in the intrinsic optical characteristics of the biosensor surface caused by the addition of dielectric molecules such as protein. The most modern luminescence biosensor detects bacteria in food or clinical samples using the luciferase enzyme. In the presence of O2, luciferase absorbs ATP produced during bacterial lysis to produce light, which is recognized and quantified by a biosensor.

Optical biosensors use fluorescence, phase shift, absorbance, reflectance, and other qualities to evaluate AFB1(Goud et, al 2020)**.** An optical biosensor consists of three components: processing units, signal transducing units, and sensing units. The detection and sensing processes rely on molecular identification and interaction. Biomolecules, such as enzymes and nucleic acids, may precisely recognize other biological entities via affinity binding and catalysis. Molecular recognition in the affinity process is based on both specificity and affinity, resulting in the formation of a stable complex (Soldatkin et.al, 2013; Khansili et.al, 2021). A transducing device converts the recognition from a living being into a recognizable

optical signal (Chen et.al 2020). AFB1 has been detected in a range of food samples utilizing several nanomaterial-based optical biosensors, including lateral flow test strips (LFTS), surface Plasmon resonance (SPR), colorimetric Aptasensor, surface-enhanced Raman scattering (SERS), and fluorescence Aptasensor. A fluorescence biosensor detects the target analyte (AFB1) by measuring fluorescence and absorbance. Light absorption is evaluated using the difference in light intensity between the reference and the sample under inquiry. It is feasible to measure fluorescence intensity directly without comparing it to a reference beam. The performance of a fluorescence biosensor is also evaluated using emission intensity, wavelength, fluorescence anisotropy, and fluorescence duration measurements. Fluorescence Biosensors have outstanding AFB1 detection sensitivity and selectivity, but are susceptible to interferences and autofluorescence. (Odoemelam et.al, 2009; Jaffrezic-Renault et.al, 2008; Schäferling et.al 2006)

SERS is a spectroscopic method that uses Raman spectra and metallic nanoparticles. Important structural information about any analyte can be obtained using this molecular, non-invasive, and non-destructive analytical technique. Because SERS uses nanomaterials to significantly amplify Raman signals, it is an investigative tool for tracking food samples. SERS analysis is sensitive, specific, marker-free, and dependable, and it can detect multiple analytes simultaneously. However, because nano probes are only used once and are especially prone to precipitation, this process is laborious and less reproducible (Li et al. 2017).

Point-of-Care (POC) devices based on nanomaterials for AFB1 detection

The development of point-of-care (POC) devices based on nanomaterials for detecting and assessing food toxins to provide real-time patient care is increasing. POCs offer a useful platform for the prompt, accurate, and on-site detection of AFB1 in the food industry (Choi et al., 2019). POC device fabrication is dependent on the following: a bioreceptor that is expected to be highly selective, a transducer that is adaptable, dependable, sensitive, and reasonably priced; a signal processor that is compact, portable, and with low power consumption, and a detector that must be a user-friendly instrument. The POC device should show that sample analysis is fully automated on a single and integrated platform, enabling end users to conduct tests by creating screen-printed electrodes, shrinking potentiostats, and using smartphones for signal reading (Mohammadniaei et al., 2019). These devices eliminate the need for complex instrumentation, are affordable, quick, and portable, and consequently, it is beneficial for on-site analyte detection. However, due to their hydrophobicity, POCs have poor sensitivity and the reaction buffer is not sufficiently contained in the working zone. A remarkable platform for AFB1 research in numerous food product categories has been made possible by the integration of electrochemical and optical biosensing technology with POC devices (Goud et al., 2020).

Electrochemical POC devices have demonstrated extraordinary analytical capabilities over op tical biosensors,
which are associated with high instrument costs, low reproducibility, sensitivity, and selectivi ty.

Application and Impact of Biosensor

Biosensors have numerous applications aimed at improving people's quality of life. This category includes its applications in environmental monitoring, disease detection, food safety, defence, drug discovery, and many more. One of the most common applications of biosensors is to detect biomolecules that are either disease markers or therapeutic targets. Medical diagnostics: Biosensors identify diseases by measuring blood glucose, cholesterol, and other biomarkers.
Environmental Monitoring: They can help monitor environmental conditions by identifying contaminants in soil and water.
Food Safety: Biosensors are used to detect illnesses, toxins, and allergies in foods. Pharmaceuticals: By tracking biological interactions and evaluating the effectiveness of novel compounds, they aid in the creation of pharmaceuticals. Bioprocessing: Biosensors are used in industrial biotechnology to monitor fermentation processes and enhance microbial growth conditions. Wearable technology uses biosensors to monitor heart rate, hydration, and fitness in real time. Agriculture: Precision agricultural methods are made possible by their assistance in tracking crop conditions, plant diseases, and soil health.

Biodefense: Biosensors are utilised in security and defence applications to detect diseases and biological threats. Looking at the never-ending research on biosensors during the previous few decades, it is clear that they are appealing not only in academia but also in industry. Biosensor technology takes advantage of the unique characteristics of a biological recognition event on a transducing device. In such cases, the analyte-bioreceptor interaction is translated into a user-friendly output. This strategy not only takes advantage of the molecular binding event, but also brings together researchers from other fields of science and engineering to share their expertise. Similar techniques have had a significant impact on biosensor research in its early stages. Furthermore, it has opened up new avenues for scientific study, with a focus on the development of technology that will help a variety of fields, including healthcare. Working in an interdisciplinary field allows you to think outside the box and collaborate with a variety of professions, with each thought contributing to the creation of something meaningful. A basic example is a pregnancy test biosensor, in which biology researchers focused on the biological aspects and collaborated with engineers to work on the system's electronics for the readout. Finally, management personnel are responsible for transferring laboratory research to clients around the world.

Biosensor research is not limited to a certain domain, as evidenced by the recent surge in market availability. Recently, there has been a gradual increase in the number of biosensor-based start-up companies around the world, which is having a significant impact on the healthcare industry. In general, biosensors have found a place in modern society because they strive to improve people's quality of life in a variety of fields such as homeland defence and security, agriculture, food safety, the environment, medicine, and pharmaceuticals.

Challenges and Future Trends

The development of biosensors has been going on for about 50 years, and during the past ten years, academic research in this area has advanced significantly. However, only a small

number of biosensors have achieved worldwide commercial success at the retail level, aside from electrochemical glucose biosensors and lateral flow pregnancy tests. There are a number of reasons for this, including the challenges faced by industry in converting scholarly research into commercially viable prototypes, intricate regulatory concerns in clinical applications, and the challenges of recruiting researchers with experience in biosensor technology or bringing together researchers from various scientific and engineering fields. Another issue is that academic research is driven by peer-reviewed science proposals, as well as funding agencies and politics, which can be fraught with conflicts of interest. A jury of academics frequently determines the priorities of funding bodies, while legislators demand substantial justifications for the funding they accept. If a subject can be made to appear fancy and appealing, it stands a better chance of success. In this regard, biosensor technology has a specific advantage that has been effectively marketed as a priority. Biosensors should be designed to be practical are usable. Given the success of commercial glucose sensors, biosensor research is undoubtedly valuable for the industry's long-term viability. However, it takes a long time to develop a commercially viable technology based on a proof of concept shown in academia. This also entails a variety of dangers that industries are unwilling to accept. Therefore, unresolved mandatory issues about the manufacturing of a Commercial biosensor exist, including: determining which market is interested in a biosensor for a particular target analyte. It defines benefits over current techniques for that analyte's analysis, testing the biosensor's functionality both during operation and after storage. For any realistic commercial application, a biosensor must respond at least after six months of storage. Each biosensor component's stability, cost, and manufacturing ease, risks and moral considerations related to using the created biosensor.

The good news about biosensing technology is that the above-mentioned barriers are being rapidly overcome. Translational research has received significant investment globally, particularly for healthcare applications. This brings industry and academia closer together in order to develop economically viable goods. On the other side, there has been a significant improvement in how scientists collaborate across boundaries. Engineers and physical scientists today have a far better knowledge of basic biomolecular processes, whereas biochemists and molecular biologists are more aware of the capabilities of various technologies. The collaboration of professionals from several disciplines from the start of biosensing development projects is a very appealing offer that will undoubtedly result in advanced and new products on the market.

Despite the need for traceability in the complicated food supply chain, not all food firms have the economic value or scale to invest in. Many studies claim that biosensors are cost-effective; nonetheless, the cost of manufacturing biosensors must be reduced because commercial applications are still scarce. Currently, self-powered biosensors based on biofuel cells have garnered tremendous interest because they might improve the cost-efficiency of biosensors to a degree while being user-friendly and highly appropriate for downsizing, portability, and wearability.

Recent advances in aflatoxin B1 (AFB1) detection employing lateral flow biosensor (LFB) devices point to important future developments aimed at increasing sensitivity and user adaptability. Innovations include the incorporation of luminous reporters such as nanobody-

nano luciferase fusions and fluorescent nanobody-GFP constructions, which achieve lower detection limits suitable for real-food matrices (e.g., almond or oat milk). Concurrently, nanomaterial upgrades using metal nanoparticles, quantum dots, and carbon-based materials increase signal responsiveness and assay reliability. The creation of novel bioreceptors, particularly nanobodies and aptamers, enables increased selectivity, stability, and cost-effectiveness, making them more accessible in low-resource settings. Furthermore, multiplex and dual-target detection technologies are emerging, allowing for the simultaneous identification of AFB1 and other mycotoxins or genetic markers, resulting in comprehensive food safety monitoring. To improve field deployment, LFB devices are increasingly being built to be user-friendly, portable, quick (less than 30 minutes), and compatible with smartphone-based quantitative readouts. Furthermore, attempts to improve shelf life and assay robustness across a variety of environmental settings aim to increase device utility worldwide. In the future, automation and multiplexing are expected to support high-throughput, on-site detection systems, hence increasing food safety surveillance. These technical trajectories promise to make AFB1 biosensing more sensitive, feasible, and widely operable, especially in locations with inadequate laboratory infrastructure.

Despite the importance of a traceability system within the complicated food supply chain, not all food firms have the economic value or scale to invest in. Many studies claim that biosensors are cost-effective, but the cost of manufacturing biosensors must still be reduced because commercial applications are still missing. Currently, self-powered biosensors based on biofuel cells have garnered tremendous interest because they might advance the cost-efficiency of biosensors to another extent while being user-friendly and highly appropriate for miniaturisation, portability, and wearability (Hao et al.,2020). Lack of uniformity in systems, coordination, and allocation of costs and benefits for research and development, as well as differences in globalization pace, climate, geographical location, and natural resources among countries, have posed additional challenges to the efficient application of biosensors in the food system have successfully created a highly heat and storage stable electrochemical biosensor to aid in the fast detection of pesticides in fruits and vegetables in a variety of environments. The development of a biosensor with a 40-day shelf life has contributed to advancements in food safety and analysis technology (Wan et al., 2022; Zheng et al., 2020). As the food system has become more complicated, there is an urgent need for a more integrated food detection system. Biosensors paired with developing technologies such as smartphones, 3D printing, IoT, AI, and blockchain have the potential to propel biosensors to new levels of improvement. For example, combining a biosensor with a smartphone could dramatically enhance detection accuracy and reduce detection time due to automation and cloud data storage from the smartphone. With the current globalisation, in which practically everyone uses a smartphone, smartphone-assisted biosensors have huge promise for onsite detection of food contamination. For example, (Abdelbasset et al. 2023) explained how smartphone-based Aptasensors provide a semi-automated user interface that can be used by an inexperienced person, as well as rapid and wireless data transferability. It might then serve as a foundation for on-site, portable, and simple monitoring in a smart food traceability system. Sensor arrays can increase the specificity of biosensors by accurately identifying very similar and diverse analytes in mixtures for fingerprint identification. This can then be used to detect food adulteration. Finally, biosensor

and IoT technologies could take the lead in wireless transmission technology (Zhang et al., 2022; Xie et al., 2021). Nanotechnology, artificial intelligence (AI), and machine learning (ML) advancements make accurate food quality monitoring possible, improving efficiency, reducing hazards, and assuring regulatory compliance. To maintain data privacy and security when integrating IoT and cloud technologies into biosensor systems, solutions such as end-to-end encryption, secure authentication mechanisms, and compliance with data protection standards like GDPR are advocated. These safeguards secure sensitive information and ensure the confidentiality and integrity of biosensor data. One such example is Intel's safe Device Onboard (SDO) technology, which automates and secures the onboarding of IoT devices to cloud platforms, enabling safe communication and administration throughout the device lifespan (Moulahoum et.al 2024; Chhetri et.al 2024; Jaime et.al 2023; Malik et.al 2021) Furthermore, AI-powered security solutions, such as those from Palo Alto Networks, can detect and respond to anomalous activity in real time, protecting against potential breaches. These innovations redefine food quality management and safety, ushering in a new era of individualised nutrition, autonomous monitoring, and worldwide collaboration, and representing a transformational paradigm shift in the food sector (Patel et al, 2024).

Case Study

In this work, we developed an electrochemical immunosensor based on chitosan graphene nanosheets (CS-GNs) to measure the amount of AFB1 in corn samples. The CS-GNs nanocomposites demonstrated great electrochemical activity, a large specific surface area, and outstanding biocompatibility. These characteristics increase the rate of electron transfer and help immobilise antibodies. SEM was used to characterise the surface of the resulting CS-GNs nanocomposites. The DPV approach served as the basis for optimising antibody concentration, pH, antibody incubation time, and immunoreaction time. Under ideal circumstances, the change in DPV peak currents before and after immunisation was linear over the 0.05–25 ng/mL AFB1 concentration range, with a 0.021 ng/mL (S/N=3) detection limit. For determining the amount of AFB1 in maize samples, the developed immunosensor showed good repeatability, stability, and specificity. Furthermore, the recovery of AFB1 detection in corn samples varied between 97.3% and 101.4%. The findings demonstrate that the created AFB1 immunosensor has the benefits of high selectivity, sensitivity, and ease of use. Because of this, it is a useful tool for identifying and analysing other biomolecules.

The modified Hummers process was used by Srivastava et al. to create graphene oxide (GO). Graphite powder was first pre-oxidised by treating it with a solution of H2SO4, K2S2O8, and P2O5 for four hours at 80 °C. GO was then obtained by stirring it in H2SO4/H3PO4, adding KMnO4, and stirring the mixture for 15 hours at 50 °C. After that, the produced GO was used to create electrochemical immunosensors that could identify AFB1(Srivastava et al.,2016). The modified Hummers method was also used by Bhardwaj et al. to synthesise GO, which was then hydrothermally treated at 200 °C for 10 hours to produce GQDs. To create electrochemical immunosensors for AFB1 detection, the GQDs were synthesised (Bhardwaj et al.,2018). By using a simpler synthesis of CS-GNs nanocomposites, the researchers were able to improve Aflatoxin B1 (AFB1) detection. They used chitosan (CS) as a surfactant for graphene to improve material dispersion, and they used chitosan to immobilise AFB1

antibodies to improve biocompatibility. The technique was used to identify AFB1 in corn samples after graphene nanosheets and chitosan were combined to increase sensitivity and produce a low detection limit of 0.021 ng/ml.

Conclusion

Biosensors are a disruptive technology with enormous potential for improving food safety and quality monitoring. Their capacity to detect pollutants quickly, accurately, and on-site gives them a major edge over conventional analytical approaches. Although biosensors provide diverse platforms for detecting a wide range of pollutants and analytes, their implementation faces hurdles such as sensitivity and specificity limitations, matrix interference, and regulatory requirements. However, current research and development activities, as well as recent developments in nanotechnology, multiplexed detection, and IoT integration, present great potential to overcome these hurdles and transform food safety standards. By addressing these opportunities and problems, biosensors have the ability to improve food safety, quality assurance, and regulatory compliance efforts, ensuring the global food supply chain's integrity and safety for many years. Finally, the review thoroughly investigated the development and implementation of highly specific lateral flow biosensors for aflatoxin B1 detection in food crops, emphasising their potential to improve food safety and quality monitoring. The incorporation of nanomaterials, luminous reporters, and novel bioreceptors has increased the sensitivity and usability of these biosensors, making them more accessible in low-resource situations. The introduction of multiplex and dual-target detection technologies, as well as the development of user-friendly, portable, and smartphone-based quantitative readouts, highlights biosensors' potential for comprehensive food safety monitoring. As the food system gets more complicated, there is a pressing need for a more integrated food detection system, and biosensors combined with emerging technologies such as smartphones, 3D printing, IoT, AI, and blockchain show enormous potential for propelling biosensors to new heights of performance. Finally, widespread adoption of biosensors in the food industry has the potential to redefine food quality management and safety, ushering in a new era of personalised nutrition, autonomous monitoring, and global collaboration, as well as a paradigm shift in the food sector.

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Chapter 3

Behavioural Adaptation of Animals Under Pathogenic Exposure: Mechanisms, Evolutionary Implications, and Ecological Consequences

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Abstract

The evolutionary arms race between pathogenic microorganisms and their metazoan hosts has precipitated the development of sophisticated behavioural adaptations that constitute a remarkable component of the biological immune system across diverse animal taxa. The present review comprehensively examines the current state of knowledge pertaining to pathogen-induced behavioural modifications in animals, with particular emphasis on the mechanistic foundations, evolutionary trajectories, and ecological ramifications of these adaptive responses. Various categories of behavioural adaptation to pathogenic exposure have been documented in the literature, encompassing individual-level prophylactic behaviours, pathogen avoidance strategies, self-medication practices, and complex social immunity mechanisms that function at the population level. These adaptations manifest through diverse sensory modalities including olfactory discrimination of infected conspecifics, behavioural fever responses in ectothermic vertebrates, spatial segregation from contaminated environments, and sophisticated allogrooming protocols in social species. Mechanistic investigations have revealed that pathogen-driven behavioural responses are mediated through conserved neuroimmune pathways involving toll-like receptor signalling, cytokine cascades, and neuromodulatory systems that integrate immunological status with motor output. The evolutionary implications of these adaptations extend beyond immediate survival benefits, influencing mate choice, social structure, and population dynamics in ways that may ultimately affect speciation processes and community assembly patterns. However, the precise genetic architecture underlying behavioural immunity, the trade-offs between pathogen avoidance and other fitness-enhancing activities, and the capacity of these systems to respond to novel

pathogenic threats in anthropogenically modified environments remain inadequately characterised. The present review identifies critical knowledge gaps and emphasises the urgent need for integrative research frameworks that incorporate genomic, neurobiological, and ecological approaches to advance our understanding of this fundamental aspect of host-pathogen coevolution.

Keywords: behavioural immunity, pathogen avoidance, social immunity, host-pathogen coevolution, adaptive behaviour

Introduction

The evolutionary context of pathogen-driven behavioural adaptation

The perpetual evolutionary struggle between pathogenic microorganisms and their multicellular hosts represents one of the most fundamental and pervasive selective pressures operating across the entire spectrum of animal life (Anderson & May, 1991). Pathogenic organisms, encompassing bacteria, viruses, fungi, protozoa, and metazoan parasites, exert profound effects on host fitness through mechanisms of virulence that may result in reduced reproductive capacity, compromised physiological function, and ultimately mortality. In response to this omnipresent threat, natural selection has favoured the evolution of diverse defensive strategies that extend far beyond the conventional physiological immune system to encompass a remarkable array of behavioural adaptations that function as a primary line of defence against pathogenic encounter and subsequent infection (Hart, 1990; Curtis, 2014).

Behavioural immunity, conceptualised as a coordinated suite of cognitive and motor responses that function to detect, avoid, and mitigate pathogenic threats prior to infection, has emerged as a critical component of host defence across virtually all animal taxa (Schaller & Park, 2011). Unlike the physiological immune system, which responds to pathogenic challenges after host invasion has occurred, behavioural immunity operates proactively to prevent initial contact with infectious agents, thereby circumventing the energetic costs and fitness consequences associated with mounting an active immune response (Loehle, 1995). The evolutionary advantages conferred by such preemptive defensive strategies are substantial, particularly given that successful pathogen clearance following infection is never guaranteed and often requires significant metabolic investment that may compromise other fitness-enhancing activities.

Scope and objectives of the present review

The present synthesis aims to provide a comprehensive examination of behavioural adaptations to pathogenic exposure across the animal kingdom, with particular focus on the mechanistic underpinnings, evolutionary origins, and ecological consequences of these phenomena. We begin with an analysis of individual-level behavioural responses, including pathogen detection and avoidance mechanisms, prophylactic behaviours, and self-medication practices. Subsequently, we examine social-level adaptations, encompassing collective immunity strategies, quarantine behaviours, and cooperative health maintenance activities. We further explore the neurobiological and genetic foundations of behavioural immunity, followed by consideration of the evolutionary implications and ecological ramifications of pathogen-driven behavioural evolution. Finally, we identify critical research priorities and methodological approaches that will advance our understanding of this rapidly evolving field.

Literature search strategy and analytical framework

Search methodology

A systematic literature search was conducted across multiple electronic databases, namely Web of Science, PubMed/MEDLINE, Scopus, and Google Scholar, utilising Boolean combinations of the following search terms: ("behavioural immunity" OR "behavioral immunity" OR "pathogen avoidance" OR "disease avoidance") AND ("animal behaviour" OR "animal behavior" OR "host-pathogen" OR "parasite avoidance") AND ("adaptive behaviour" OR "evolutionary adaptation" OR "social immunity" OR "self-medication"). The search encompassed publications from 1980 to 2025 and was not restricted by taxonomic group or geographical region. Additional references were identified through examination of citation networks and consultation of relevant review articles.

Inclusion and exclusion criteria

Studies were included if they satisfied the following criteria: (i) reported original empirical data on behavioural responses to pathogenic threats or infected conspecifics; (ii) demonstrated adaptive significance of the behaviours through fitness measurements or comparative analysis; (iii) employed appropriate controls for non-pathogenic factors that might influence behaviour; and (iv) were published in peer-reviewed journals or authoritative institutional repositories. Studies were excluded if they focused exclusively on physiological immune responses without behavioural components, if the pathogenic stimuli were not

biologically relevant, or if the methodology did not permit clear attribution of observed behaviours to pathogenic exposure.

Individual-level behavioural adaptations to pathogenic exposure

Pathogen detection and discrimination mechanisms

The capacity for accurate detection and discrimination of pathogenic threats represents a fundamental prerequisite for effective behavioural immunity, and natural selection has favoured the evolution of sophisticated sensory mechanisms that enable animals to identify and respond appropriately to disease-related cues in their environment (Kavaliers & Choleris, 2001). Olfactory detection systems have emerged as particularly important modalities for pathogen recognition, as many infectious agents produce distinctive volatile compounds that can be detected at considerable distances from the source. Experimental investigations have demonstrated that diverse taxa, ranging from arthropods to mammals, possess the ability to discriminate between the odour signatures of healthy and infected individuals, often exhibiting pronounced avoidance responses to scents associated with diseased conspecifics (Kavaliers et al., 2005).

Visual cues also provide critical information regarding the health status of potential social partners, with many species demonstrating remarkable sensitivity to subtle changes in appearance, posture, or activity patterns that may indicate pathogenic infection. Caribbean spiny lobsters (*Panulirus argus*) have been shown to exhibit complete avoidance of shelters previously occupied by individuals infected with the lethal virus PaV1, demonstrating sophisticated discrimination capabilities that persist even in the absence of visual contact with infected individuals (Behringer et al., 2006). Similarly, house finches (*Carpodacus mexicanus*) avoid feeding in proximity to conspecifics displaying the characteristic conjunctival swelling associated with mycoplasmal infection, indicating that visual assessment of disease symptoms can override normal gregarious feeding tendencies (Bouwman & Hawley, 2010).

Prophylactic behaviours and self-maintenance activities

Prophylactic behaviours encompass a diverse array of self-directed activities that function to reduce pathogen load or prevent infection in the absence of immediate pathogenic threats (Hart, 1997). Self-grooming represents perhaps the most ubiquitous of these behaviours, serving multiple functions including the mechanical removal of potential pathogens from body surfaces, the distribution of antimicrobial secretions, and the maintenance of integumentary integrity that constitutes the primary barrier against pathogenic invasion. The frequency and

intensity of grooming behaviours have been shown to correlate positively with pathogenic pressure in numerous species, with individuals in pathogen-rich environments exhibiting significantly elevated grooming rates compared to those in pathogen-poor conditions (Zuk & Stoehr, 2002).

Self-medication behaviours, involving the targeted consumption of substances possessing antimicrobial, antiparasitic, or other therapeutic properties, have been documented across a remarkable diversity of animal taxa (Clayton & Wolfe, 1993; de Roode et al., 2013). Chimpanzees (*Pan troglodytes*) selectively consume the bitter pith of *Vernonia amygdalina* trees when suffering from intestinal parasitic infections, with the active compounds in this plant material demonstrating significant anthelmintic properties under controlled experimental conditions (Huffman & Seifu, 1989). Similarly, monarch butterflies (*Danaus plexippus*) infected with the protozoan parasite *Ophryocystis elektroscirrha* exhibit preferential oviposition on milkweed species containing elevated concentrations of cardenolides, toxic compounds that significantly reduce parasite loads in developing larvae (Lefèvre et al., 2010).

Behavioural fever and thermoregulatory responses

Behavioural fever, defined as the active selection of elevated environmental temperatures following pathogenic infection, represents a sophisticated thermoregulatory adaptation that has been documented across a broad spectrum of ectothermic vertebrates and invertebrates (Kluger et al., 1975; Watson et al., 1993). This behavioural response serves to enhance immune function through temperature-dependent activation of cellular defence mechanisms while simultaneously creating thermal conditions that may be suboptimal for pathogen replication and survival. Desert iguanas (*Dipsosaurus dorsalis*) infected with *Aeromonas hydrophila* bacteria demonstrate pronounced behavioural fever, actively seeking basking sites that elevate body temperature by 2-4°C above normal thermoregulatory preferences, with this temperature elevation significantly improving survival rates compared to individuals prevented from exhibiting behavioural fever (Kluger et al., 1975).

Social-level behavioural adaptations and collective immunity

Social immunity mechanisms in eusocial insects

Social insects have evolved perhaps the most sophisticated collective immunity systems documented in the animal kingdom, employing coordinated behavioural strategies that function to protect entire colonies from pathogenic threats (Cremer et al., 2007). These social immunity mechanisms operate through multiple complementary pathways, including enhanced pathogen

detection through collective surveillance, rapid isolation and removal of infected individuals, prophylactic nest hygiene behaviours, and active modification of the chemical environment to create conditions inhospitable to pathogenic growth. Honeybee colonies (*Apis mellifera*) exemplify the remarkable effectiveness of social immunity, with workers exhibiting hygienic behaviour involving the detection and removal of diseased larvae from brood cells, thereby preventing the spread of pathogens such as *Paenibacillus larvae* (the causative agent of American foulbrood) throughout the colony (Spivak & Gilliam, 1998).

Termite colonies employ sophisticated architectural and behavioural strategies to maintain pathogen-free environments within their nest structures (Korb & Aanen, 2003). *Macrotermes* species actively cultivate fungal gardens that serve dual functions as food sources and biological control agents, with the fungi producing antimicrobial compounds that suppress the growth of competing pathogenic microorganisms within the nest environment. Workers continuously monitor the health of fungal cultures and rapidly remove contaminated substrate, effectively preventing the establishment of pathogenic fungi that could devastate both the cultivated fungus and the termite colony itself.

Quarantine and isolation behaviours in vertebrate societies

Social vertebrates have evolved diverse strategies for managing diseased individuals within group contexts, balancing the benefits of sociality against the increased transmission risks associated with group living (Loehle, 1995). Many species exhibit sophisticated quarantine behaviours involving the active exclusion or self-imposed isolation of infected individuals, effectively reducing disease transmission rates within social groups while maintaining overall group cohesion. Caribbean spiny lobsters demonstrate remarkable collective quarantine behaviour, with entire groups vacating shelters contaminated by virus-infected individuals, even when alternative shelter sites are limited and predation risks are elevated (Behringer et al., 2006).

Primate societies exhibit complex responses to illness that incorporate both exclusion of sick individuals and enhanced care behaviours directed toward group members showing signs of disease (Nunn & Altizer, 2006). Chimpanzee communities have been observed to systematically avoid and exclude individuals displaying symptoms of respiratory illness, while simultaneously maintaining surveillance behaviours that enable rapid detection of disease recovery and subsequent reintegration into normal social activities. These sophisticated social

responses suggest that group-living primates possess detailed cognitive representations of disease states and their associated transmission risks.

Allogrooming and cooperative health maintenance

Allogrooming, encompassing the grooming of one individual by another, serves crucial health maintenance functions in numerous social species, extending the protective benefits of self-grooming to include body regions that cannot be effectively accessed through individual grooming efforts (Hart, 1992). This cooperative behaviour provides mutual benefits through the removal of ectoparasites and potential pathogens while simultaneously strengthening social bonds that may be critical for accessing other fitness-enhancing resources. Primate allogrooming networks have been shown to correlate significantly with health outcomes, with individuals possessing more extensive grooming partnerships exhibiting lower ectoparasite loads and reduced incidence of skin infections (Tanaka & Takefushi, 1993).

Table 1: Examples of social immunity mechanisms documented across animal taxa.

Taxonomic Group	Species Example	Immunity Mechanism	Documented Effects
Hymenoptera	*Apis mellifera*	Hygienic behavior; diseased larvae removal	95% reduction in American foulbrood incidence
Isoptera	*Macrotermes bellicosus*	Antimicrobial substrate cultivation	Complete suppression of pathogenic fungi
Decapoda	*Panulirus argus*	Group shelter abandonment	100% avoidance of PaV1-contaminated shelters
Primates	*Pan troglodytes*	Allogrooming; social exclusion	Reduced ectoparasite loads; disease transmission control

Mechanistic foundations of behavioural immunity

Neuroimmune integration and signalling pathways

The integration of immunological status with behavioural output requires sophisticated communication between the immune system and the central nervous system, mediated through conserved molecular pathways that have been maintained across diverse animal taxa (Dantzer et al., 2008). Toll-like receptors (TLRs), originally characterised for their role in pathogen recognition within the innate immune system, have been demonstrated to function as critical mediators of pathogen-induced behavioural changes, with TLR activation leading to the

production of pro-inflammatory cytokines that directly influence neural circuits controlling avoidance behaviours and social interactions (Medzhitov & Janeway, 2000).

Cytokine signalling, particularly through the pro-inflammatory mediators interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and interleukin-6 (IL-6), has been shown to modulate the activity of brain regions involved in social cognition and behavioural decision-making (Kelley et al., 2003). Experimental manipulation of cytokine levels in laboratory animals consistently produces dose-dependent changes in pathogen-related avoidance behaviours, social exploration, and grooming activities, demonstrating direct causal relationships between immune activation and behavioural modification. Furthermore, the expression patterns of cytokine receptors within specific brain regions correspond closely to the neuroanatomical substrates of pathogen-avoidance behaviours, providing mechanistic support for the neuroimmune integration hypothesis.

Genetic architecture and heritability of behavioural immunity traits

Recent advances in quantitative genetics and genomics have begun to elucidate the genetic foundations underlying behavioural immunity, revealing that many pathogen-avoidance behaviours are characterised by moderate to high heritability estimates that facilitate rapid evolutionary responses to changing pathogenic pressures (Poulin, 2007). Quantitative trait locus (QTL) mapping studies in laboratory populations have identified discrete genomic regions associated with variation in grooming frequency, pathogen-avoidance behaviour, and social immunity traits, with many of these QTL colocalising with genes involved in immune function, sensory perception, and neuromodulation.

The hygienic behaviour of honeybees, involving the recognition and removal of diseased larvae, exemplifies the relatively simple genetic architecture that may underlie complex social immunity behaviours (Rothenbuhler, 1964). This adaptive behaviour is controlled by two main genetic loci: one governing the ability to detect diseased brood and another controlling the motor behaviour required for cell uncapping and larval removal. The modular genetic structure of this system enables rapid evolutionary modification of specific components while maintaining overall functional integrity, potentially explaining the remarkable effectiveness and widespread distribution of hygienic behaviour across honeybee populations subjected to pathogenic pressure.

Evolutionary implications and coevolutionary dynamics

Host-pathogen arms races and counter-adaptations

The evolution of behavioural defences against pathogens is inherently embedded within an ongoing coevolutionary arms race between hosts and parasites, with each adaptive innovation by the host generating corresponding selection pressure for pathogenic counter-adaptations that circumvent or exploit these defensive mechanisms (Dawkins & Krebs, 1979). Pathogenic organisms have evolved remarkable strategies for manipulating host behaviour to facilitate their own transmission, representing sophisticated evolutionary responses to behavioural immunity systems (Poulin, 1995). The fungal pathogen *Ophiocordyceps unilateralis* exemplifies such manipulation, precisely controlling the behaviour of infected ants to ensure optimal positioning for spore dispersal while effectively bypassing the host's normal pathogen-avoidance mechanisms (Andersen et al., 2009).

The evolutionary dynamics of these host-pathogen interactions are further complicated by the fact that behavioural immunity systems themselves may create novel selective environments that favour the evolution of alternative pathogenic strategies, including increased virulence, altered transmission modes, or the exploitation of behavioural immunity responses for pathogenic benefit. Mathematical models of coevolutionary dynamics predict that the arms race between behavioural immunity and pathogenic manipulation should result in increasingly sophisticated adaptations on both sides, with the relative evolutionary rates determining the temporary advantage held by either host or pathogen at any given time (Thompson, 2005).

Trade-offs and constraints in behavioural immunity evolution

The evolution of behavioural immunity is constrained by fundamental trade-offs between pathogen avoidance and other fitness-enhancing activities, including foraging, mating, and social interaction (Boots & Bowers, 1999). These trade-offs arise because resources allocated to pathogen-avoidance behaviours are unavailable for alternative activities, and because excessive pathogen avoidance may result in missed opportunities for beneficial social or ecological interactions. The optimal level of behavioural immunity for any given individual or population therefore represents an evolutionary compromise that balances the benefits of reduced pathogen exposure against the costs of foregone activities.

Empirical studies have documented numerous examples of such trade-offs in natural populations. House finches exhibiting pronounced avoidance of diseased conspecifics experience reduced foraging efficiency and altered social structure that may compromise

reproductive success (Hawley et al., 2007). Similarly, Caribbean spiny lobsters that avoid virus-contaminated shelters face increased predation risks and reduced growth rates due to the energetic costs of shelter-seeking behaviour and reduced feeding opportunities (Behringer et al., 2006). These empirical findings support theoretical predictions that behavioural immunity should be optimised rather than maximised, with the degree of pathogen avoidance reflecting the specific ecological and social context within which each species has evolved.

Ecological consequences of pathogen-driven behavioural evolution

Population-level effects and demographic implications

Behavioural responses to pathogenic threats can exert profound effects on population dynamics and demographic structure, with the potential to influence population stability, growth rates, and extinction probabilities under certain circumstances (Hudson et al., 2002). Pathogen-induced avoidance behaviours may reduce effective population density by preventing normal social aggregation, thereby altering competitive interactions, mate choice dynamics, and the efficiency of collective behaviours such as predator detection and resource exploitation. These demographic consequences can propagate through multiple generations, potentially influencing the evolutionary trajectory of populations in ways that extend far beyond the immediate effects of pathogenic mortality.

Spatial segregation of infected and healthy individuals, mediated through behavioural avoidance mechanisms, can result in the formation of distinct population subunits with limited gene flow between them (Real & Biek, 2007). This spatial structure may facilitate local adaptation to specific pathogenic strains while simultaneously reducing the overall genetic diversity within subpopulations. Mathematical models incorporating both pathogen dynamics and behavioural avoidance predict that moderate levels of avoidance behaviour can stabilise population dynamics by reducing disease transmission, while extreme avoidance may lead to population fragmentation and increased extinction risk due to demographic stochasticity in small, isolated subpopulations.

Community-level effects and interspecific interactions

The behavioural responses of animals to pathogenic threats can cascade through ecological communities, affecting interspecific interactions and community structure in complex and often unpredictable ways (Hatcher et al., 2006). When species alter their habitat use, foraging behaviour, or activity patterns in response to pathogenic exposure, these changes can modify predator-prey dynamics, competitive relationships, and mutualistic interactions in

ways that may ultimately affect community composition and ecosystem functioning. Pathogen-induced changes in herbivore behaviour can alter plant community structure through modified grazing patterns, while behavioural changes in pollinators may affect plant reproductive success and gene flow patterns.

Host-specific pathogens may indirectly benefit competing species by inducing behavioural changes that reduce the competitive ability of infected hosts (Hatcher & Dunn, 2011). For example, if infected individuals reduce their foraging activity or avoid optimal habitat patches due to pathogenic exposure, uninfected competitors may gain access to previously monopolised resources. These indirect pathogenic effects, mediated through behavioural modifications rather than direct mortality, may play important roles in structuring ecological communities and maintaining species diversity in systems where competitive exclusion would otherwise eliminate inferior competitors.

Conservation and applied implications

Wildlife disease management and conservation strategies

Understanding the behavioural dimensions of host-pathogen interactions provides crucial insights for wildlife conservation and disease management strategies, particularly in the context of emerging infectious diseases and anthropogenic habitat modification (Daszak et al., 2000). Conservation programmes that account for natural behavioural immunity mechanisms are likely to be more effective than those that focus exclusively on pathogen control or habitat provision without consideration of the behavioural requirements for effective disease avoidance. Habitat connectivity, for example, may be essential for enabling pathogen-avoidance behaviours that require spatial movement away from contaminated areas, while habitat fragmentation may force animals into contact with pathogens they would normally avoid through behavioural mechanisms.

Captive breeding and reintroduction programmes must consider the potential loss of behavioural immunity traits in populations maintained under pathogen-free conditions for extended periods (Woodworth et al., 2002). Animals raised without exposure to relevant pathogens may fail to develop normal pathogen-recognition abilities or appropriate avoidance responses, making them highly vulnerable to disease outbreaks following release into natural environments. Conservation protocols that incorporate controlled exposure to non-lethal pathogenic cues during captive breeding may help maintain these crucial behavioural adaptations while minimising direct health risks to captive populations.

Climate change and novel pathogenic challenges

Anthropogenic climate change is fundamentally altering pathogen distributions and creating novel host-pathogen interactions that may challenge existing behavioural immunity systems (Harvell et al., 2002). Rising temperatures, changing precipitation patterns, and altered seasonality may expose animal populations to pathogenic species for which they have not evolved appropriate behavioural defences, while simultaneously rendering existing behavioural adaptations ineffective or maladaptive under new environmental conditions. Species with rigid, genetically determined behavioural responses may be particularly vulnerable to these novel pathogenic challenges, whereas those with flexible, learning-based immunity systems may be better positioned to adapt to changing pathogenic landscapes.

The capacity for rapid behavioural adaptation to novel pathogens may therefore become an increasingly important determinant of species survival and conservation success in the coming decades. Research priorities should include identification of the genetic and neurobiological mechanisms underlying behavioural flexibility in pathogen recognition and avoidance, assessment of the speed with which new behavioural immunity traits can evolve or be acquired through learning, and development of predictive models that integrate pathogen ecology with behavioural adaptation to forecast species vulnerability under various climate change scenarios.

Future research directions and methodological advances

Integrative approaches and technological innovations

Future progress in understanding behavioural immunity will require the integration of multiple disciplinary approaches, combining insights from evolutionary biology, immunology, neuroscience, and ecology to develop comprehensive models of pathogen-driven behavioural adaptation (Adamo, 2006). Technological advances in animal tracking, physiological monitoring, and genetic analysis are creating unprecedented opportunities to study these phenomena in natural populations under realistic ecological conditions. GPS tracking and accelerometry can provide detailed information about movement patterns and behavioural changes associated with pathogen exposure, while simultaneous monitoring of immunological and stress indicators can elucidate the physiological mechanisms underlying behavioural modifications.

Genomic approaches, including genome-wide association studies (GWAS) and quantitative trait locus mapping, promise to identify the specific genetic variants underlying

variation in behavioural immunity traits, enabling more precise predictions about evolutionary responses to changing pathogenic pressures. Transcriptomic and proteomic analyses can reveal the molecular pathways activated during pathogen-induced behavioural changes, providing mechanistic insights that may be applicable across diverse taxa. Machine learning approaches applied to large behavioural datasets may reveal previously unrecognised patterns in pathogen-avoidance behaviour and identify subtle behavioural changes that precede overt disease symptoms.

Experimental approaches and model systems

The development of standardised experimental protocols for assessing behavioural immunity traits will be crucial for enabling comparative studies across species and ecosystems (Curtis, 2014). Laboratory-based choice experiments, involving the presentation of pathogen-associated cues and measurement of avoidance responses, provide controlled conditions for quantifying individual and population-level variation in pathogen recognition abilities. Field experiments manipulating pathogen exposure or environmental conditions can test the ecological relevance of laboratory findings and assess the costs and benefits of behavioural immunity under natural conditions.

Model systems that combine tractable genetics with well-characterised pathogen interactions, such as *Drosophila melanogaster* with bacterial or fungal pathogens, offer opportunities for detailed mechanistic studies that may reveal fundamental principles applicable to more complex systems. Social insects, particularly honeybees and ants, provide excellent models for investigating the evolution and mechanisms of collective immunity, while vertebrate systems enable exploration of the neurobiological foundations of pathogen-avoidance behaviour. Comparative studies across these diverse model systems will be essential for identifying universal principles versus taxon-specific adaptations in behavioural immunity.

Conclusion

The study of behavioural adaptations to pathogenic exposure reveals the remarkable sophistication of animal responses to disease threats, encompassing individual-level avoidance strategies, sophisticated social immunity mechanisms, and complex neurobiological systems that integrate immunological status with behavioural output. These adaptations represent millions of years of evolutionary refinement in response to one of the most persistent and powerful selective forces operating in natural systems. The evidence reviewed here demonstrates that behavioural immunity functions as a crucial first line of defence against

pathogenic threats, often preventing infections that would otherwise impose substantial fitness costs on affected individuals and populations.

The evolutionary and ecological implications of pathogen-driven behavioural adaptation extend far beyond the immediate benefits of reduced disease transmission, influencing fundamental aspects of animal societies, population dynamics, and community structure in ways that may ultimately affect speciation processes and ecosystem functioning. The ongoing coevolutionary arms race between hosts and pathogens continues to drive innovation in both behavioural immunity systems and pathogenic manipulation strategies, creating a dynamic evolutionary landscape that shapes the diversity of life on Earth.

As we face an era of emerging infectious diseases, rapid environmental change, and novel host-pathogen interactions, understanding the mechanisms and limitations of behavioural immunity becomes increasingly critical for predicting and managing wildlife responses to pathogenic threats. The integration of genomic, neurobiological, and ecological approaches promises to reveal new insights into the evolution and function of these fascinating adaptations, while technological advances in tracking and monitoring will enable unprecedented studies of behavioural immunity in natural populations. Continued research in this field will not only advance our fundamental understanding of host-pathogen coevolution but also provide crucial insights for wildlife conservation and disease management in an increasingly complex and rapidly changing world.

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Chapter 4

Non-Steroidal Anti-Inflammatory Drugs and Their Adverse Effects:

A Comprehensive Review of Clinical Pharmacology

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most globally prescribed pharmacological agents, valued for their analgesic, antipyretic, and anti-inflammatory properties. Their principal mechanism entails inhibition of cyclooxygenase (COX) enzymes—COX-1, which sustains homeostatic prostaglandin synthesis, and COX-2, which is induced at sites of inflammation. Beyond classical COX-dependent pathways, emerging evidence points to COX-independent mechanisms, including modulation of L-selectin-mediated leukocyte adhesion and activation of NADPH-oxidase pathways. While COX-2 selective inhibitors (coxibs) were developed to mitigate gastrointestinal toxicity, they concurrently heightened cardiovascular risk. Pharmacokinetic diversity—in absorption, protein binding, and hepatic biotransformation—further complicates the safety-efficacy balance. This review comprehensively examines NSAID classification, mechanisms of action, clinical indications, adverse effect profiles across organ systems, and emerging therapeutic innovations including nitric oxide-releasing NSAIDs and hydrogen sulfide-releasing conjugates, with the aim of providing clinicians and researchers with an updated framework for rational NSAID therapy.

Keywords: Non-steroidal anti-inflammatory drugs, Cyclooxygenase inhibition, COX-1 & COX-2, Prostaglandins, Clinical pharmacology, Nitric oxide-releasing NSAIDs, Adverse drug reactions

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) constitute one of the most widely used classes of therapeutic agents worldwide. Globally, over 30 million people consume NSAIDs daily, and prescriptions for these agents account for a significant proportion of outpatient pharmacotherapy across diverse specialties including rheumatology, orthopedics, internal medicine, and primary care (Vane & Botting, 1998). Their therapeutic utility spans acute and chronic pain syndromes, febrile illnesses, and inflammatory disorders of variable etiology.

The modern history of NSAIDs traces back to the mid-twentieth century, with the synthesis of indomethacin in 1963, though the pharmacological rationale crystallized with John Vane's landmark discovery in 1971 that aspirin-like drugs exert their actions by inhibiting prostaglandin biosynthesis—work that earned him the Nobel Prize in Physiology or Medicine in 1982 (Vane, 1971). Subsequent elucidation of the dual cyclooxygenase isoforms, COX-1 and COX-2, in the early 1990s fundamentally reframed understanding of NSAID action and adverse effects, providing the rationale for the development of selective COX-2 inhibitors (Needleman & Isakson, 1997).

Despite decades of use, NSAIDs remain associated with a substantial and clinically significant adverse effect burden. Gastrointestinal complications—ranging from dyspepsia to life-threatening peptic ulcer hemorrhage—represent the most prevalent harms, while cardiovascular, renal, and hepatic toxicities add further complexity to risk-benefit calculations (Lanas & Hunt, 2006). The selective COX-2 inhibitors (coxibs), although offering gastrointestinal advantages, were implicated in increased thrombotic cardiovascular events, leading to the withdrawal of rofecoxib and valdecoxib from the global market (Bresalier et al., 2005).

This review presents a comprehensive appraisal of NSAID pharmacology, encompassing classification, mechanisms of COX-dependent and COX-independent action, pharmacokinetic profiles, clinical applications, multi-system adverse effects, drug interactions, special population considerations, and cutting-edge developments in safer NSAID analogs.

Classification of NSAIDs

NSAIDs constitute a structurally heterogeneous group of compounds united by their capacity to inhibit COX enzymes. They are conventionally classified by chemical structure, COX selectivity, and pharmacokinetic profile.

Chemical Classification

The major chemical classes include: salicylates—the prototypical class, including aspirin and diflunisal; propionic acid derivatives (profens)—such as ibuprofen, naproxen, ketoprofen, and flurbiprofen; acetic acid derivatives—including indomethacin, diclofenac, sulindac, and ketorolac; enolic acid derivatives (oxicams)—such as piroxicam, meloxicam, and tenoxicam; anthranilic acid derivatives (fenamates)—including mefenamic acid and flufenamic acid; and selective COX-2 inhibitors (coxibs)—celecoxib, etoricoxib, and parecoxib (Warner & Mitchell, 2004).

COX Selectivity Classification

Based on relative inhibitory potency at COX-1 versus COX-2, NSAIDs are stratified into non-selective COX inhibitors (e.g., aspirin, ibuprofen, naproxen, indomethacin); preferential COX-2 inhibitors (e.g., meloxicam, nimesulide, diclofenac at low doses); and highly selective COX-2 inhibitors or coxibs (e.g., celecoxib, etoricoxib) (FitzGerald & Patrono, 2001). This selectivity ratio profoundly influences both the therapeutic and adverse effect profiles and forms the cornerstone of rational NSAID prescribing.

Mechanisms of Action

COX-Dependent Mechanisms

The cyclooxygenase enzymes, COX-1 and COX-2, catalyze the committed step in prostanoid biosynthesis: the bis-dioxygenation of arachidonic acid to form the unstable endoperoxide intermediate prostaglandin G_2 (PGG_2), followed by its reduction to prostaglandin H_2 (PGH_2). Downstream tissue-specific synthases then convert PGH_2 into prostaglandins, thromboxanes, and prostacyclin, each mediating distinct physiological and pathological functions (Smith et al., 2000).

COX-1 is constitutively expressed in most tissues, including gastric mucosa, platelets, vascular endothelium, and renal medulla, where it generates prostaglandins critical for gastric cytoprotection, platelet aggregation via thromboxane A_2, and renal blood flow regulation. COX-2, in contrast, is inducible by cytokines, growth factors, tumor promoters, and shear stress; its expression is markedly upregulated at inflammatory foci, producing prostanoids that mediate hyperalgesia, vasodilation, and edema (Vane et al., 1998). Both isoforms share approximately 60% amino acid sequence homology, yet their substrate-binding channel

geometry differs sufficiently that selective inhibitors have been engineered to preferentially occupy the COX-2 active site (Kurumbail et al., 1996).

Aspirin irreversibly acetylates a serine residue within the active site of both isoforms, permanently inactivating them. Because platelets lack nuclei and cannot synthesize new COX-1, aspirin's antiplatelet effect persists for the platelet's lifespan of approximately 7–10 days—the basis of its use in cardiovascular prophylaxis. Other NSAIDs competitively and reversibly inhibit COX active sites, with their duration of action governed by pharmacokinetic parameters rather than irreversible binding (Fuster et al., 1993).

COX-Independent Mechanisms

Growing evidence highlights pharmacological actions of NSAIDs that are not attributable to COX inhibition. Several NSAIDs, particularly at high concentrations, inhibit neutrophil activation and L-selectin shedding from leukocyte surfaces, thereby impairing leukocyte rolling and extravasation at sites of inflammation (Diaz et al., 2000). Additionally, sulindac sulfone and certain NSAIDs activate peroxisome proliferator-activated receptor gamma (PPARγ), inducing apoptosis in cancer cell lines independently of prostaglandin synthesis—a mechanism relevant to the chemopreventive applications of aspirin and other NSAIDs in colorectal neoplasia (Piazza et al., 1995).

NSAIDs have also been shown to modulate NF-κB signaling—a transcription factor central to the expression of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6—and to activate NADPH-oxidase pathways in phagocytes. Furthermore, indomethacin and ibuprofen interfere with mitochondrial electron transport at high concentrations, contributing to cytotoxic effects observed in experimental models (Lecomte et al., 1994; Rao et al., 2003).

Pharmacokinetics and Pharmacodynamics

Absorption and Bioavailability

NSAIDs are generally well absorbed after oral administration, with bioavailabilities ranging from 80–99% for most agents. Being weak acids (pKa 3–5), they undergo pH-dependent dissolution; gastric acidity promotes their non-ionized, lipid-soluble form, facilitating passive absorption across the gastric epithelium. Enteric-coated and extended-release formulations delay absorption, reducing peak plasma concentrations and associated gastrointestinal irritation, though they do not eliminate systemic prostaglandin-mediated gastric toxicity (Davies & Anderson, 1997).

Distribution and Protein Binding

NSAIDs are extensively bound to plasma proteins, particularly albumin, with binding exceeding 90–99% for most agents. This high protein binding limits volume of distribution and reduces free drug available for tissue penetration; however, it also predisposes to competitive displacement interactions with anticoagulants such as warfarin, sulfonylureas, and phenytoin—elevating free drug concentrations and augmenting toxicity risk. Synovial fluid concentrations often approach plasma levels for naproxen and piroxicam, accounting for their prolonged anti-arthritic efficacy (Brune & Patrignani, 2015).

Metabolism and Elimination

Hepatic biotransformation via cytochrome P450 enzymes (primarily CYP2C9) constitutes the principal route of metabolism, with subsequent glucuronide conjugation generating polar metabolites excreted in urine and bile. Plasma half-lives vary substantially: from 0.25 hours for aspirin to 2–4 hours for ibuprofen, 12–17 hours for naproxen, and 38–50 hours for piroxicam—differences directly influencing dosing intervals and accumulation risk in renally impaired patients (Brune & Patrignani, 2015).

Clinical Applications

Musculoskeletal and Rheumatological Disorders

NSAIDs remain first-line pharmacotherapy for osteoarthritis, offering clinically meaningful pain reduction superior to acetaminophen in meta-analytic comparisons (da Costa et al., 2017). In rheumatoid arthritis (RA), NSAIDs provide symptomatic relief of joint pain, stiffness, and swelling, though they do not modify disease progression or arrest joint destruction; they are typically used adjunctively with disease-modifying antirheumatic drugs (DMARDs). For ankylosing spondylitis, NSAIDs represent the cornerstone of pharmacotherapy, with continuous NSAID use demonstrating retardation of radiographic spinal progression in observational studies (Poddubnyy et al., 2012).

In acute gout, high-dose NSAIDs such as indomethacin 50 mg three times daily or naproxen 500 mg twice daily are equivalent to colchicine and systemic corticosteroids for resolution of acute attacks, with clinical benefit apparent within 24 hours of initiation (Terkeltaub et al., 2010). NSAIDs are also employed in reactive arthritis, psoriatic arthritis, and crystal-induced arthropathies.

Pain Management and Other Indications

NSAIDs are integral to multimodal analgesic regimens for post-operative pain, dental pain, dysmenorrhea, headache, and renal colic. Intravenous formulations (ketorolac, ibuprofen lysine) enable their use in hospitalized patients unable to take oral medications. In oncology, NSAIDs—particularly aspirin—have attracted interest as chemopreventive agents for colorectal, breast, and other adenocarcinomas, with epidemiological and randomized trial data supporting risk reduction with regular use (Chan et al., 2007). Aspirin at low doses of 75–325 mg/day is a cornerstone of antiplatelet therapy in secondary cardiovascular prevention.

Adverse Effects

Gastrointestinal Toxicity

Gastrointestinal (GI) toxicity represents the most clinically prevalent adverse effect class and the primary driver of NSAID-related morbidity and mortality. The spectrum ranges from dyspepsia and heartburn in 10–60% of NSAID users to erosive gastropathy, peptic ulcer disease, and potentially fatal upper GI hemorrhage or perforation (Lanas & Hunt, 2006). The pathogenesis is dual: local mucosal irritation from the acidic drug molecule and—more importantly—systemic suppression of COX-1-derived prostaglandins, which normally stimulate mucus and bicarbonate secretion, maintain mucosal blood flow, and promote epithelial restitution.

Epidemiological data indicate a 3- to 5-fold increase in peptic ulcer risk among regular NSAID users, with the annual incidence of clinically significant GI events estimated at 1–4% (Silverstein et al., 1995). Risk factors include age over 65 years, prior peptic ulcer disease, concomitant anticoagulant or corticosteroid use, high NSAID doses, and Helicobacter pylori co-infection. Gastroprotective strategies—proton pump inhibitors (PPIs), misoprostol, or H_2-receptor antagonists—significantly reduce ulcer incidence and should be co-prescribed in high-risk patients. The lower GI tract is also susceptible; NSAID-enteropathy, manifesting as intestinal inflammation, strictures, and protein-losing enteropathy, is increasingly recognized (Bjarnason & Takeuchi, 2009).

Cardiovascular Toxicity

The cardiovascular risk of NSAIDs was thrust into prominence following the withdrawal of rofecoxib in 2004, after the APPROVe trial demonstrated a 2-fold increase in myocardial infarction and stroke risk with long-term use (Bresalier et al., 2005). Selective COX-2 inhibitors shift the thromboxane A_2-prostacyclin balance toward a pro-thrombotic state:

suppression of endothelial COX-2-derived prostacyclin (a potent vasodilator and platelet aggregation inhibitor) while sparing platelet COX-1-derived thromboxane A_2 (a vasoconstrictor and platelet activator) (FitzGerald & Patrono, 2001).

Importantly, subsequent meta-analyses established that this cardiovascular risk is not confined to coxibs. Diclofenac and high-dose ibuprofen confer cardiovascular risk comparable to coxibs, whereas naproxen appears relatively cardiovascular-neutral, though not devoid of risk (Coxib and Traditional NSAID Trialists' Collaboration, 2013). NSAIDs also increase blood pressure by approximately 3–5 mmHg, antagonize the effects of antihypertensive agents, and may precipitate acute decompensation in patients with heart failure by promoting sodium and fluid retention.

Renal Toxicity

The kidneys depend on locally produced prostaglandins—primarily PGE_2 and prostacyclin—to maintain glomerular filtration rate (GFR) and tubular function under conditions of hemodynamic stress, such as volume depletion, heart failure, and chronic kidney disease. NSAID-induced inhibition of these renal prostaglandins can precipitate acute kidney injury (AKI), particularly in vulnerable patients, manifesting as a decline in GFR within days of NSAID initiation. Reversible AKI occurs in approximately 1–5% of NSAID users (Whelton, 1999).

Additional renal manifestations include hyperkalemia via suppression of renin release and aldosterone-dependent potassium secretion, hyponatremia, and interstitial nephritis—the latter being an idiosyncratic reaction characterized by eosinophilic infiltration and sometimes nephrotic-range proteinuria, particularly associated with fenoprofen. Analgesic nephropathy, a syndrome of chronic renal insufficiency caused by prolonged combination analgesic use, remains a cautionary example of cumulative NSAID nephrotoxicity (Eknoyan, 1999).

Hepatic Toxicity

Hepatotoxicity from NSAIDs is relatively uncommon but may be severe. Diclofenac is the most frequently implicated agent, with an estimated incidence of clinically apparent hepatotoxicity of approximately 1 per 10,000–100,000 patient-years; cases range from asymptomatic transaminase elevations to fulminant hepatic failure (Banks et al., 1995). Sulindac has been associated with cholestatic hepatitis, and nimesulide's hepatic risk profile led to its restriction or withdrawal in several European countries. Mechanisms include reactive metabolite formation, immune-mediated hepatocyte injury, and mitochondrial dysfunction.

Other Adverse Effects

NSAIDs have been associated with platelet dysfunction, prolonged bleeding time, and increased surgical bleeding risk. Aspirin irreversibly inhibits platelet aggregation and is typically withheld 7–10 days preoperatively. Respiratory hypersensitivity—aspirin-exacerbated respiratory disease (AERD), comprising the triad of asthma, nasal polyposis, and NSAID hypersensitivity—affects approximately 10% of asthmatics and involves shunting of arachidonic acid metabolism toward leukotriene production following COX inhibition (Stevenson & Simon, 2001). Central nervous system effects including headache, dizziness, and cognitive disturbance are reported, particularly with indomethacin. Skin reactions range from urticaria to Stevens-Johnson syndrome, the latter being a rare but potentially fatal complication.

Drug Interactions and Special Populations

Significant Drug Interactions

NSAIDs participate in numerous clinically significant drug interactions. Their displacement of warfarin from albumin binding sites and concurrent antiplatelet effects markedly elevate hemorrhagic risk; INR monitoring is mandatory upon initiation or cessation of NSAIDs in anticoagulated patients. NSAIDs reduce the natriuretic efficacy of loop and thiazide diuretics and blunt the antihypertensive actions of ACE inhibitors and angiotensin receptor blockers (ARBs). The combination of NSAIDs, ACE inhibitors or ARBs, and diuretics—collectively termed the “triple whammy”—is associated with a substantially elevated risk of AKI (Thomas et al., 2000).

Concurrent use of corticosteroids amplifies NSAID-related GI ulcer risk 2- to 4-fold. NSAIDs reduce renal lithium clearance, necessitating lithium dose reduction and serum level monitoring. High-dose aspirin or NSAIDs may displace methotrexate from protein-binding sites and decrease its renal tubular secretion, potentially elevating methotrexate to toxic concentrations—a potentially fatal interaction requiring vigilance (Aherne et al., 1978).

Special Populations

In the elderly, pharmacokinetic alterations including decreased albumin, reduced renal and hepatic clearance, and increased body fat, combined with pharmacodynamic sensitization, amplify all adverse effects. Geriatric guidelines recommend avoiding high-dose, long-duration NSAID use in patients over 65 years of age (American Geriatrics Society Beers Criteria). In pregnancy, NSAIDs are contraindicated beyond 20 weeks of gestation due to risk of fetal renal

dysfunction, oligohydramnios, and, particularly after 30 weeks, premature constriction of the ductus arteriosus (US FDA, 2020). In pediatric populations, ibuprofen is safe and effective, whereas aspirin is contraindicated due to the risk of Reye's syndrome following viral illness.

Emerging Therapeutic Strategies

Nitric Oxide-Releasing NSAIDs

Nitric oxide-releasing NSAIDs (NO-NSAIDs) represent a class of hybrid molecules engineered to couple conventional NSAIDs with nitric oxide-donating moieties. Nitric oxide, a gasotransmitter produced by endothelial nitric oxide synthase, exerts cytoprotective effects on gastric mucosa by stimulating mucus secretion, increasing mucosal blood flow, inhibiting leukocyte adhesion, and promoting epithelial repair. Preclinical studies consistently demonstrate that NO-NSAIDs retain equianalgesic and anti-inflammatory potency relative to their parent compounds while producing markedly fewer GI erosions (Wallace et al., 1994). Clinical development of naproxcinod (NO-naproxen) demonstrated blood-pressure-neutral anti-arthritic efficacy in phase II trials, though regulatory approval was not obtained.

Hydrogen Sulfide-Releasing NSAIDs

Analogous to NO-NSAIDs, hydrogen sulfide-releasing NSAID conjugates (HS-NSAIDs)—such as ATB-346—exploit the cytoprotective and anti-inflammatory properties of hydrogen sulfide to counteract NSAID-associated gastric injury. In rodent models, ATB-346 virtually eliminated gastric damage while maintaining anti-inflammatory activity, attributed to combined COX inhibition and hydrogen sulfide-mediated gastric protection and leukocyte adhesion reduction (Fiorucci et al., 2011).

Dual COX/5-LOX Inhibitors and Other Advances

Dual inhibitors of COX and 5-lipoxygenase (5-LOX)—such as licofelone—aim to prevent the arachidonic acid shunting toward pro-inflammatory leukotrienes that accompanies COX inhibition, potentially providing superior anti-inflammatory efficacy with reduced GI and cardiovascular risk. Emerging microbiome research indicates that NSAIDs alter gut microbial composition, increasing Gram-negative bacteria and decreasing beneficial Lactobacillus species, which may contribute to enteropathy (Sigthorsson et al., 1998). Advances in pharmacogenomics—incorporating CYP2C9 genotyping, COX-2 polymorphism analysis, and platelet function testing—may further refine NSAID selection and dosing to optimize individual risk-benefit outcomes.

Conclusion

NSAIDs remain indispensable agents in the management of pain, fever, and inflammatory diseases across the full spectrum of clinical practice. Their well-characterized mechanisms of COX inhibition underpin both their therapeutic value and adverse effect profiles. While COX-2 selectivity reduced gastrointestinal complications, it illuminated the profound cardiovascular implications of prostanoid imbalance. The recognition that COX-independent pathways further modulate NSAID bioactivity has enriched the mechanistic understanding of these ubiquitous drugs.

The rational prescription of NSAIDs demands a personalized risk assessment integrating gastrointestinal, cardiovascular, renal, and hepatic risk factors, with appropriate co-prescription of gastroprotective agents, careful monitoring of renal function and blood pressure, and vigilant attention to drug interactions. Selective restriction in high-risk populations—the elderly, pregnant women, and those with established organ disease—remains imperative.

Innovative NSAID derivatives—particularly NO-NSAIDs and HS-NSAIDs—offer promising prospects for retaining efficacy while substantially reducing organ toxicity. As translational pharmacology advances, integrating genomic, metabolomic, and microbiome insights with clinical trial data will be essential to realizing the vision of precision NSAID therapy, maximizing therapeutic benefit while minimizing the substantial morbidity currently attributable to these widely used agents.

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Chapter 5

Pesticide Exposure, MicroRNA Dysregulation and Behavioural Pattern Changes in Children: Implications for Mood, Cognitive Function and Neurodevelopment

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Abstract

Environmental exposure to pesticide residues has become a significant concern for public health, particularly in relation to children's neurological development and behavioural health. Children are more susceptible to environmental toxicants due to their developing physiological systems, immature detoxification pathways, and increased dietary intake relative to body weight. Pesticides commonly used in agriculture can persist in food products, water bodies, and surrounding environments, thereby increasing the likelihood of exposure through diet and environmental contact.

Recent research has highlighted the importance of epigenetic mechanisms in mediating the biological effects of environmental toxicants. Among these mechanisms, microRNAs (miRNAs) have emerged as crucial regulators of gene expression involved in neuronal development, synaptic plasticity, and behavioural regulation. MicroRNAs are small non-coding RNA molecules that modulate gene expression at the post-transcriptional level and play an essential role in brain development and neurobehavioral processes.

Evidence from both experimental and epidemiological studies indicates that pesticide exposure may alter miRNA expression patterns, leading to disruptions in neural signalling pathways and behavioural outcomes. These alterations may manifest as mood instability, attention deficits, anxiety-related symptoms, and cognitive impairment in children. In addition to dietary intake, environmental exposure through recreational activities such as swimming in contaminated water bodies near agricultural areas may further contribute to pesticide exposure.

Understanding the interaction between pesticide exposure, epigenetic regulation, and behavioural outcomes is therefore essential for developing preventive strategies and improving child health outcomes. This review summarizes current knowledge on dietary pesticide

exposure, microRNA dysregulation, and behavioural pattern changes in children, with particular emphasis on mood-related effects and neurodevelopmental implications.

Keywords: Pesticide exposure; MicroRNA; Child behaviour; Mood disorders; Neurodevelopment; Environmental toxicology; Epigenetics

Introduction

Children's health and neurological development are strongly influenced by environmental factors encountered during early life stages. Among these factors, exposure to environmental contaminants such as pesticides has received increasing scientific attention due to its potential association with neurodevelopmental disorders and behavioural abnormalities. Pesticides are extensively used worldwide in modern agriculture to control pests and enhance crop productivity; however, their widespread application has resulted in persistent residues in food, soil, and water ecosystems (Aktar et al., 2009). Consequently, children may be exposed to these chemical compounds through multiple pathways, including dietary consumption of contaminated fruits and vegetables, drinking water, and environmental contact during outdoor activities.

Children are particularly vulnerable to pesticide exposure because their physiological systems are still developing and their metabolic detoxification processes are not fully matured. In addition, children consume more food and water relative to their body weight compared with adults, which may increase their intake of pesticide residues (Landrigan et al., 2018). Early-life exposure to environmental toxicants has been associated with disruptions in neurodevelopmental processes, which may ultimately lead to cognitive deficits, behavioural abnormalities, and emotional disturbances later in life.

Recent advances in molecular biology have revealed that environmental toxicants may exert their biological effects through epigenetic mechanisms that regulate gene expression without altering the DNA sequence. Among these mechanisms, microRNAs (miRNAs) have emerged as key regulators of gene expression involved in neuronal development and brain function. MicroRNAs are small non-coding RNA molecules, typically consisting of approximately 20–24 nucleotides, that regulate gene expression by binding to messenger RNA (mRNA) targets and inhibiting protein synthesis (Bartel, 2009). These molecules play critical roles in brain development, neuronal differentiation, and synaptic plasticity, making them essential regulators of cognitive and behavioural processes.

Evidence from recent studies suggests that exposure to environmental chemicals, including pesticides, may disrupt microRNA expression patterns and thereby influence neurological development. For instance, organophosphate pesticides such as chlorpyrifos have been shown to alter microRNA signaling pathways associated with neuronal growth and synaptic development (Slotkin & Seidler, 2012). Similarly, pyrethroid pesticides have been reported to induce oxidative stress and epigenetic alterations that may affect neuronal communication and behavioural responses (Kim et al., 2017). Such disruptions may contribute to behavioural pattern changes in children, including attention deficits, mood disturbances, learning difficulties, and impaired cognitive performance.

Several epidemiological studies conducted in agricultural communities have reported associations between pesticide exposure and behavioural problems among children. Research has indicated increased risks of attention deficit hyperactivity disorder (ADHD), anxiety-related symptoms, and reduced cognitive performance among children with higher levels of pesticide exposure (Bouchard et al., 2011). Mood-related symptoms such as irritability, emotional instability, and reduced concentration have also been observed in populations exposed to environmental pesticides.

In addition to dietary exposure, environmental exposure pathways such as recreational activities may also contribute to pesticide intake. Children who swim or play in water bodies located near agricultural areas may be exposed to pesticide residues present in surface water and sediment. These exposures may occur through dermal absorption or accidental ingestion of contaminated water (Aktar et al., 2009). Although such exposure routes are less frequently studied, they may represent an additional risk factor for pesticide-related health effects in children.

Understanding the complex interactions between pesticide exposure, epigenetic regulation, and behavioural outcomes is therefore critical for protecting children’s health. The emerging field of environmental epigenetics has provided new insights into how environmental toxicants may influence gene regulation and neurological development. However, despite growing evidence, the molecular pathways linking pesticide exposure to behavioural changes in children remain incompletely understood.

This review aims to examine current scientific evidence regarding the relationship between pesticide exposure, microRNA dysregulation, and behavioural pattern changes in children. Particular emphasis is placed on mood-related effects, cognitive function, and

neurodevelopmental outcomes. By integrating findings from environmental toxicology, molecular biology, and epidemiological research, this review seeks to provide a comprehensive understanding of how pesticide exposure may influence child health through epigenetic mechanisms.

Background

Environmental exposure to pesticides has become a major concern in global public health due to the increasing use of agrochemicals in modern agriculture. Pesticides are chemical compounds designed to control pests, weeds, and plant diseases; however, their widespread application has resulted in the contamination of food products, soil ecosystems, and water resources. As a result, humans are frequently exposed to pesticide residues through dietary intake, environmental contact, and occupational exposure (Aktar et al., 2009). Among different population groups, children represent one of the most vulnerable populations because of their developmental stage, behavioural patterns, and physiological characteristics.

Children have a higher risk of pesticide exposure compared with adults due to several biological and environmental factors. First, children consume larger quantities of food and water relative to their body weight, which increases their exposure to pesticide residues present in agricultural products such as fruits, vegetables, and cereals. Second, their metabolic detoxification systems, including hepatic enzyme pathways, are not fully developed during early life stages. This reduced detoxification capacity may enhance the accumulation of toxic chemicals within the body (Landrigan et al., 2018). Furthermore, behavioural patterns such as hand-to-mouth activities, playing on the ground, and outdoor recreational activities may increase the likelihood of contact with contaminated soil and water.

Pesticides include several major classes of chemical compounds, including organophosphates, carbamates, organochlorines, and pyrethroids. These chemicals are known to exert neurotoxic effects by interfering with neurotransmitter systems, oxidative stress pathways, and neuronal signaling mechanisms (Kim et al., 2017). For instance, organophosphate pesticides inhibit acetylcholinesterase activity, leading to excessive accumulation of acetylcholine in synaptic junctions, which may disrupt neuronal communication and brain development. Prolonged exposure to such compounds during early developmental stages may contribute to cognitive deficits, behavioural disorders, and mood alterations in children.

In recent years, advances in molecular biology have revealed that environmental chemicals may influence gene expression through epigenetic modifications. Epigenetics refers to heritable changes in gene expression that occur without altering the underlying DNA sequence. These modifications include DNA methylation, histone modifications, and microRNA-mediated gene regulation. Among these mechanisms, microRNAs (miRNAs) have emerged as key regulators of gene expression involved in brain development, neuronal differentiation, and synaptic plasticity (Bartel, 2009).

MicroRNAs are small non-coding RNA molecules typically composed of 20–24 nucleotides that regulate gene expression at the post-transcriptional level. They function by binding to complementary sequences on messenger RNA (mRNA), leading to the degradation of target mRNA or inhibition of protein translation. Through this regulatory mechanism, microRNAs play essential roles in cellular development, immune responses, and neural signaling pathways (Bicker & Schratt, 2014). Importantly, many microRNAs are highly expressed in the central nervous system and are involved in processes such as neurogenesis, synaptic development, and neuronal plasticity.

Emerging evidence indicates that environmental toxicants, including pesticides, can alter microRNA expression profiles. Such alterations may disrupt gene regulatory networks involved in brain development and behavioural regulation. Experimental studies have demonstrated that exposure to certain pesticides may modify the expression of microRNAs associated with oxidative stress responses, neuronal apoptosis, and inflammatory signaling pathways (Slotkin & Seidler, 2012). These molecular changes may contribute to long-term neurodevelopmental effects and behavioural abnormalities.

Behavioural pattern changes associated with pesticide exposure have been documented in several studies focusing on child populations living in agricultural areas. These behavioural effects may include symptoms such as hyperactivity, mood swings, reduced attention span, learning difficulties, and emotional instability. In addition, some studies have suggested that pesticide exposure may contribute to anxiety-related behaviours and social interaction difficulties among children (Bouchard et al., 2011). These behavioural changes may affect educational performance, social development, and overall quality of life.

Environmental exposure through recreational activities also represents a potential pathway for pesticide intake in children. Water bodies located near agricultural areas may contain pesticide residues due to runoff from farmland and irrigation systems. Children who

participate in recreational activities such as swimming or playing in contaminated water may experience dermal and accidental oral exposure to these chemicals. Although this pathway is less frequently studied compared with dietary exposure, it may contribute to cumulative pesticide exposure during early life stages (Aktar et al., 2009).

Overall, the background evidence suggests that pesticide exposure represents a complex environmental risk factor capable of influencing neurological development through multiple biological pathways. Understanding the interaction between pesticide exposure, epigenetic regulation, and behavioural outcomes is therefore essential for evaluating potential health risks and developing strategies to protect vulnerable populations such as children.

Literature Review

A growing body of scientific literature has investigated the potential relationship between pesticide exposure and neurodevelopmental outcomes in children. Numerous epidemiological and experimental studies have examined how environmental toxicants influence cognitive development, behavioural patterns, and emotional health. These studies collectively suggest that pesticide exposure may contribute to a range of neurobehavioral alterations through both direct neurotoxic effects and epigenetic mechanisms.

One of the most extensively studied classes of pesticides in relation to neurodevelopmental effects is organophosphate pesticides. Organophosphates are widely used agricultural insecticides that function by inhibiting the enzyme acetylcholinesterase, leading to excessive stimulation of cholinergic neurotransmission. Research conducted in agricultural communities has demonstrated significant associations between prenatal or early-life exposure to organophosphates and cognitive deficits in children (Bouchard et al., 2011). In particular, higher levels of organophosphate metabolites in urine samples have been linked with reduced intelligence quotient (IQ) scores, impaired working memory, and attention deficits in school-aged children.

Several longitudinal studies have further supported these findings. For example, a cohort study conducted in the United States reported that children exposed to higher levels of organophosphate pesticides during prenatal development exhibited lower cognitive performance and increased behavioural problems compared with children with lower exposure levels (Rauh et al., 2011). These behavioural problems included symptoms related to attention deficit hyperactivity disorder (ADHD), impulsivity, and difficulties in emotional regulation.

In addition to organophosphates, other pesticide classes such as pyrethroids and carbamates have also been associated with neurobehavioral outcomes. Pyrethroid pesticides are commonly used in residential pest control and agriculture. Although they are generally considered less toxic than organophosphates, recent studies suggest that chronic exposure to pyrethroids may affect neurological development by inducing oxidative stress and altering neuronal signaling pathways (Kim et al., 2017). Such molecular disruptions may contribute to behavioural changes including anxiety-like behaviour, reduced learning ability, and mood disturbances.

Recent research has also emphasized the importance of epigenetic mechanisms in mediating pesticide-related health effects. MicroRNAs have emerged as particularly important regulators of gene expression in the nervous system. Studies have shown that environmental toxicants can modify microRNA expression patterns, thereby influencing cellular processes involved in brain development and neuronal communication (Bicker & Schratt, 2014). For example, experimental research has demonstrated that exposure to certain pesticides may alter the expression of microRNAs associated with oxidative stress regulation and neuronal apoptosis.

Animal model studies provide additional evidence supporting the role of microRNA dysregulation in pesticide-induced neurotoxicity. Laboratory experiments involving rodents exposed to organophosphate pesticides have reported significant changes in microRNA expression within brain tissues. These alterations were associated with impaired neuronal differentiation and synaptic development (Slotkin & Seidler, 2012). Such findings suggest that microRNA-mediated gene regulation may represent a key pathway through which pesticide exposure affects neurological development.

Behavioural studies have also investigated the psychological and emotional consequences of pesticide exposure in children. Research conducted in agricultural regions of North America and Latin America has reported increased prevalence of behavioural disorders among children exposed to pesticide residues. These behavioural disturbances include hyperactivity, mood instability, decreased attention span, and difficulties in social interaction (Grandjean & Landrigan, 2014). Some studies have also reported associations between pesticide exposure and symptoms related to depression and anxiety.

In addition to dietary exposure, environmental exposure through water contamination has been identified as another potential pathway. Agricultural runoff often carries pesticide residues into nearby rivers, lakes, and irrigation canals. Children who swim or play in these

water bodies may be exposed to pesticide residues through dermal absorption or accidental ingestion of contaminated water. Although limited research has examined this exposure pathway specifically, existing studies suggest that environmental contamination of water systems may contribute to cumulative pesticide exposure (Aktar et al., 2009).

Despite increasing evidence linking pesticide exposure with neurodevelopmental effects, several gaps remain in current research. Many studies focus primarily on individual pesticide compounds, whereas real-world exposures often involve mixtures of multiple chemicals. Furthermore, the molecular pathways connecting pesticide exposure with behavioural outcomes are still not fully understood. Future research integrating environmental monitoring, molecular biology, and epidemiological analysis will be essential for developing a more comprehensive understanding of pesticide-related health risks in children.

Overall, the literature indicates that pesticide exposure may significantly influence neurodevelopmental processes and behavioural patterns in children. Epigenetic mechanisms such as microRNA dysregulation appear to play an important role in mediating these effects. Continued research in this area is necessary to identify specific molecular pathways, evaluate long-term health consequences, and develop effective strategies for reducing pesticide exposure in vulnerable populations.

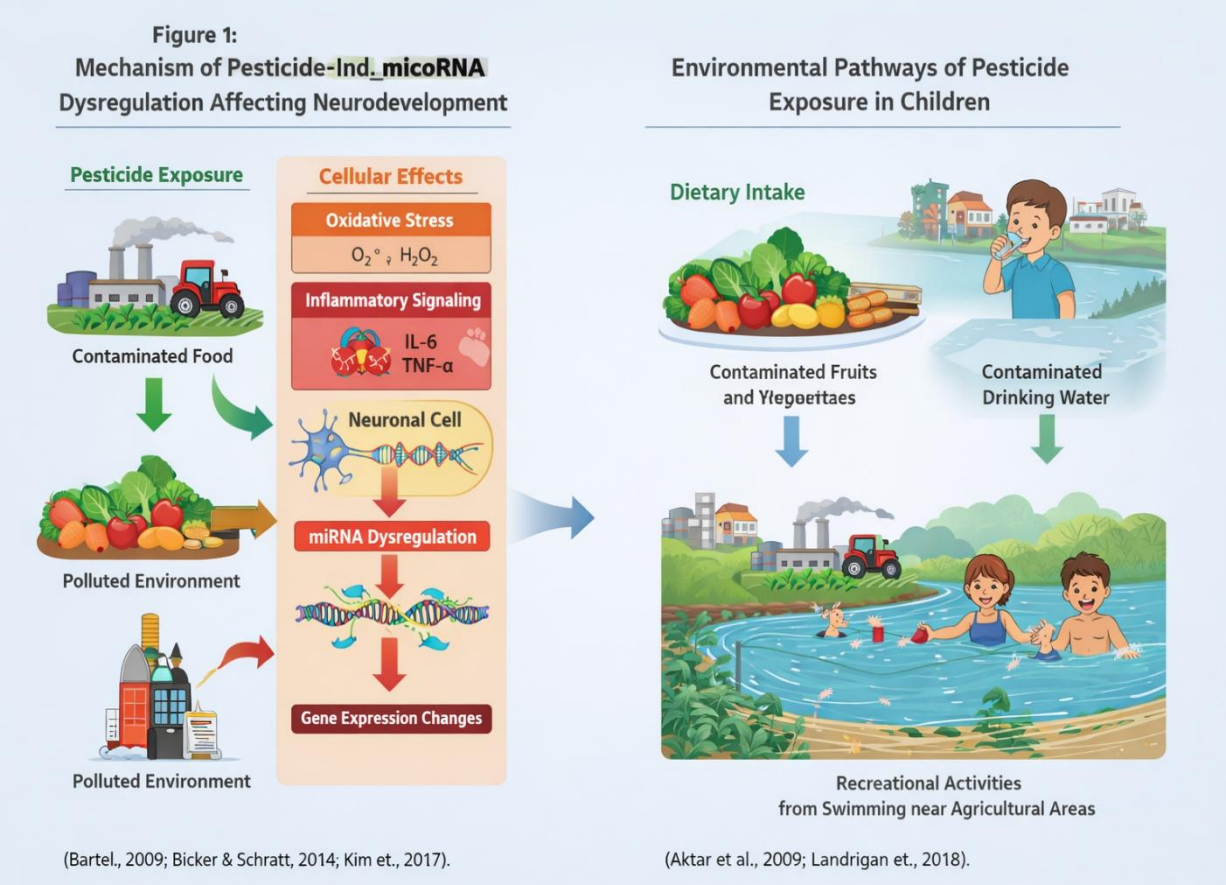

Figure 1. Mechanism of pesticide-induced microRNA dysregulation affecting neurodevelopment in children. This schematic illustrates how pesticide exposure through food or environmental sources induces oxidative stress and inflammatory signaling, leading to altered microRNA expression in neuronal cells. Dysregulation of microRNAs may influence gene expression associated with synaptic development, neuronal signaling pathways, and behavioural regulation. Adapted from previous studies examining environmental toxicant–induced epigenetic changes (Bartel, 2009; Bicker & Schratt, 2014; Kim et al., 2017).

Figure 2. Environmental pathways of pesticide exposure in children. Major exposure pathways include dietary intake of contaminated fruits and vegetables, ingestion of contaminated drinking water, and environmental contact through recreational activities such as swimming in pesticide-contaminated water bodies near agricultural areas. These combined exposure routes may contribute to cumulative pesticide intake and potential neurobehavioral health effects in children (Aktar et al., 2009; Landrigan et al., 2018).

Pesticide-Induced MicroRNA Dysregulation Mechanisms

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression by binding to complementary sequences on messenger RNA (mRNA), thereby inhibiting translation or promoting mRNA degradation. These molecules play critical roles in cellular differentiation, metabolic regulation, immune responses, and neuronal development. In the central nervous system, miRNAs regulate processes such as synaptic plasticity, neuronal survival, and neural circuit formation, making them essential for normal cognitive and behavioural development (Bartel, 2009).

Increasing evidence suggests that environmental toxicants, including pesticides, may influence the expression and activity of microRNAs, thereby disrupting gene regulatory networks. Pesticides may alter miRNA expression through several biological mechanisms, including oxidative stress induction, mitochondrial dysfunction, inflammation, and disruption of signaling pathways involved in neuronal development. These mechanisms may ultimately lead to altered gene expression patterns that affect brain structure and function.

One of the primary pathways through which pesticides influence miRNA expression is oxidative stress. Many pesticides generate reactive oxygen species (ROS) during metabolic breakdown, which can damage cellular components such as lipids, proteins, and DNA. Oxidative stress has been shown to influence epigenetic regulatory mechanisms, including miRNA expression patterns (Kim et al., 2017). Altered miRNA expression in response to

oxidative stress may affect genes involved in neuronal signaling, apoptosis, and neuroinflammation.

Organophosphate pesticides are among the most widely studied compounds associated with miRNA dysregulation. These pesticides inhibit acetylcholinesterase activity, leading to accumulation of acetylcholine and overstimulation of cholinergic synapses. Experimental studies have shown that exposure to organophosphates such as chlorpyrifos can alter the expression of multiple miRNAs involved in neuronal differentiation and synaptic plasticity (Slotkin & Seidler, 2012). These changes may disrupt neurodevelopmental processes and contribute to long-term behavioural and cognitive impairments.

Another mechanism through which pesticides influence miRNA regulation is through inflammatory signaling pathways. Chronic exposure to environmental toxicants may activate immune responses within neural tissues, leading to the release of inflammatory cytokines. Neuroinflammation has been associated with changes in the expression of specific miRNAs that regulate immune and neuronal pathways. Dysregulation of these miRNAs may affect neuronal survival and communication, potentially contributing to neurodevelopmental disorders (Bicker & Schratt, 2014).

Mitochondrial dysfunction also plays a significant role in pesticide-induced epigenetic alterations. Certain pesticides interfere with mitochondrial respiration and energy production, leading to increased oxidative stress and cellular damage. Mitochondrial impairment has been linked to altered expression of miRNAs involved in metabolic regulation and neuronal signaling. These changes may impair neuronal growth and synaptic formation during early brain development.

Animal studies have provided important insights into the relationship between pesticide exposure and miRNA dysregulation. Laboratory experiments involving rodent models exposed to pesticides have demonstrated significant changes in miRNA expression within brain tissues. These alterations have been associated with impaired neuronal differentiation, synaptic development, and behavioural abnormalities (Slotkin & Seidler, 2012). Such findings suggest that miRNA-mediated gene regulation may represent a key molecular pathway linking pesticide exposure to neurodevelopmental outcomes.

Furthermore, environmental exposure to pesticide mixtures may produce cumulative effects on miRNA expression. In real-world conditions, individuals are often exposed to multiple pesticide compounds simultaneously rather than a single chemical. These combined

exposures may influence several gene regulatory pathways, resulting in complex epigenetic responses that affect neurological development. Understanding these interactions remains a major challenge in environmental toxicology research.

Overall, pesticide-induced microRNA dysregulation represents an important molecular mechanism through which environmental toxicants may influence brain development and behavioural outcomes in children. Continued research is necessary to identify specific miRNA targets affected by pesticide exposure and to understand how these alterations contribute to neurodevelopmental disorders.

Behavioural and Mood Changes in Children

Behavioural and emotional development during childhood is a complex process influenced by genetic, environmental, and social factors. Increasing evidence suggests that environmental toxicants such as pesticides may contribute to behavioural disturbances and mood alterations in children. These behavioural changes may arise from disruptions in neural development, neurotransmitter signaling, and epigenetic regulatory mechanisms.

One of the most frequently reported behavioural outcomes associated with pesticide exposure is attention deficit hyperactivity disorder (ADHD). ADHD is characterized by symptoms such as hyperactivity, impulsivity, and difficulty maintaining attention. Epidemiological studies have reported associations between organophosphate pesticide exposure and increased prevalence of ADHD-related symptoms among children. For example, a population-based study found that children with higher urinary concentrations of organophosphate metabolites exhibited significantly greater risks of attention deficits and hyperactivity (Bouchard et al., 2011).

Mood disturbances are another behavioural outcome that may be associated with pesticide exposure. Mood regulation involves complex neural networks within the brain, including pathways associated with dopamine, serotonin, and other neurotransmitters. Disruption of these pathways during early development may lead to emotional instability, irritability, and anxiety. Research has indicated that exposure to certain pesticides may affect neurotransmitter balance and neural signaling pathways involved in emotional regulation (Kim et al., 2017).

Cognitive impairment and learning difficulties have also been reported among children exposed to environmental pesticides. Cognitive functions such as memory, language development, and problem-solving ability depend on proper neuronal development and

synaptic connectivity. Exposure to neurotoxic chemicals during critical developmental periods may disrupt these processes, resulting in reduced academic performance and learning capacity (Grandjean & Landrigan, 2014). Several cohort studies have reported reduced intelligence quotient (IQ) scores among children with higher prenatal pesticide exposure.

Anxiety-related behaviours and social interaction difficulties have also been observed in populations exposed to pesticides. Children exposed to environmental toxicants may exhibit symptoms such as increased nervousness, reduced social engagement, and difficulties adapting to social environments. These behavioural changes may influence interpersonal relationships and emotional well-being throughout childhood and adolescence.

Environmental exposure through recreational activities may also contribute to pesticide-related behavioural effects. In agricultural regions, pesticide residues may contaminate nearby rivers, lakes, and irrigation canals through runoff. Children who participate in recreational activities such as swimming in these water bodies may experience dermal exposure or accidental ingestion of contaminated water. Although research on this exposure pathway remains limited, environmental monitoring studies suggest that pesticide contamination of surface water may represent an additional source of exposure for children (Aktar et al., 2009).

The behavioural outcomes associated with pesticide exposure are likely influenced by multiple interacting factors, including genetic susceptibility, nutritional status, and socioeconomic conditions. Furthermore, the timing and duration of exposure may play a crucial role in determining the severity of behavioural effects. Early-life exposure during critical stages of brain development may produce more significant and long-lasting consequences compared with exposure occurring later in life.

Recent research has highlighted the potential role of epigenetic mechanisms, particularly microRNA dysregulation, in mediating these behavioural effects. Altered miRNA expression may influence genes involved in neuronal signalling, synaptic plasticity, and neurotransmitter regulation. Such changes may contribute to behavioural abnormalities such as mood disturbances, attention deficits, and cognitive impairment.

Overall, the available evidence suggests that pesticide exposure represents a significant environmental risk factor for behavioural and mood changes in children. Although many studies have demonstrated associations between pesticide exposure and neurobehavioral

outcomes, further research is needed to clarify the underlying molecular mechanisms and to determine safe exposure levels for vulnerable populations.

Environmental Exposure Pathways (Food and Swimming Water)

Environmental exposure to pesticides occurs through multiple pathways, among which dietary intake and environmental contact with contaminated water represent two of the most significant routes for children. Due to their physiological characteristics, behavioural patterns, and developmental stage, children are more susceptible to environmental chemical exposure than adults. Understanding these exposure pathways is essential for evaluating the potential health risks associated with pesticide residues and their influence on neurological development and behavioural outcomes.

Dietary intake is considered the primary route of pesticide exposure in children. Modern agricultural practices rely heavily on pesticide application to increase crop yield and prevent damage caused by pests and plant diseases. As a result, residues of pesticides may remain on fruits, vegetables, grains, and other food products even after harvesting and processing. Several monitoring studies have reported detectable levels of pesticide residues in commonly consumed food items, raising concerns about long-term dietary exposure in human populations (Aktar et al., 2009).

Children often consume higher amounts of fruits and vegetables relative to their body weight compared with adults, which may increase their exposure to pesticide residues. Furthermore, children's detoxification systems, including hepatic enzyme activity responsible for metabolizing xenobiotics, are not fully developed during early life stages. This reduced metabolic capacity may increase the retention of pesticide compounds within the body and enhance their toxic effects (Landrigan et al., 2018). Prenatal exposure through maternal dietary intake may also influence fetal development, as certain pesticides are capable of crossing the placental barrier and affecting the developing nervous system.

In addition to dietary intake, drinking water can also serve as a source of pesticide exposure. Agricultural runoff, irrigation drainage, and improper disposal of pesticide-containing waste may contaminate groundwater and surface water systems. Residues of pesticides have been detected in rivers, lakes, and groundwater sources located near agricultural regions, potentially exposing nearby communities to environmental contaminants (Kim et al., 2017). Chronic consumption of contaminated water may contribute to cumulative pesticide exposure over time.

Recreational activities represent another environmental exposure pathway that may affect children living in agricultural regions. Children frequently engage in outdoor activities such as swimming, playing, or bathing in local water bodies, including rivers, ponds, and irrigation canals. In areas where agricultural runoff enters water systems, these water bodies may contain detectable levels of pesticide residues. Dermal contact with contaminated water and accidental ingestion during swimming may contribute to pesticide intake (Aktar et al., 2009).

Although dermal exposure is generally considered less significant than dietary exposure, repeated contact with contaminated water during recreational activities may lead to cumulative chemical absorption. Certain pesticide compounds are capable of penetrating the skin barrier, particularly when exposure occurs over extended periods or when skin surfaces are damaged. Additionally, inhalation of aerosolized pesticide particles near agricultural spraying areas may further contribute to environmental exposure.

Environmental monitoring studies have reported varying levels of pesticide contamination in surface water systems across agricultural regions worldwide. These contaminants may include organophosphate, carbamate, and pyrethroid pesticides, depending on local agricultural practices. The presence of such chemicals in water ecosystems not only affects aquatic organisms but may also pose potential health risks to nearby human populations.

Children living in rural and agricultural communities may therefore experience multiple exposure pathways simultaneously, including dietary intake, drinking water contamination, and environmental contact during recreational activities. These combined exposures may lead to cumulative pesticide exposure, which may increase the likelihood of adverse health effects, including neurodevelopmental disorders and behavioural abnormalities.

Understanding environmental exposure pathways is essential for identifying potential sources of pesticide contamination and developing effective strategies to reduce exposure risks. Improved agricultural practices, enhanced food monitoring programs, and environmental regulations aimed at reducing pesticide runoff may help minimize exposure in vulnerable populations such as children.

Public Health Implications

The increasing evidence linking pesticide exposure to neurodevelopmental and behavioural outcomes in children has important implications for public health policy and environmental regulation. Protecting children's health from environmental toxicants requires a

comprehensive understanding of exposure pathways, biological mechanisms, and long-term health consequences associated with pesticide residues.

One of the most significant public health concerns associated with pesticide exposure is the potential impact on neurological development. The developing brain is highly sensitive to environmental chemicals, particularly during prenatal and early childhood stages when critical processes such as neuronal differentiation, synaptic formation, and neural circuit development occur. Disruption of these processes by environmental toxicants may lead to long-lasting cognitive and behavioural impairments (Grandjean & Landrigan, 2014). Consequently, reducing pesticide exposure during early life stages should be considered a priority in public health strategies.

Table 1. Summary of selected studies linking pesticide exposure with behavioural and neurodevelopmental outcomes in children.

Study	**Population**	**Exposure Type**	**Key Findings**
Bouchard et al., 2011	Children (USA)	Organophosphate pesticides	Higher exposure associated with increased ADHD symptoms
Eskenazi et al., 2007	Agricultural community children	Prenatal pesticide exposure	Lower cognitive performance and developmental delays
Engel et al., 2011	Birth cohort	Prenatal organophosphate exposure	Behavioral problems and reduced attention span
Rauh et al., 2011	Urban children	Chlorpyrifos exposure	Reduced IQ and working memory
Marks et al., 2010	School-age children	Organophosphate metabolites	Increased attention and learning difficulties
Kim et al., 2017	Experimental studies	Pyrethroid pesticides	Oxidative stress and neuronal toxicity

Grandjean & Landrigan, 2014	Global review	Environmental neurotoxicants	Early exposure linked with neurodevelopmental disorders
Lu et al., 2006	Dietary exposure study	Pesticide residues in food	Organic diet reduced pesticide metabolites in children

Future Research Directions

Although significant progress has been made in understanding the potential relationship between pesticide exposure and neurodevelopmental outcomes in children, several important research gaps remain. Addressing these gaps will be essential for improving risk assessment, developing preventive strategies, and protecting vulnerable populations from environmental toxicants. Future research should focus on integrating epidemiological, molecular, and environmental studies to provide a comprehensive understanding of how pesticide exposure influences neurological development and behavioural patterns in children.

One important area for future investigation is the identification of specific microRNAs involved in pesticide-induced neurotoxicity. While several studies have demonstrated that pesticide exposure can alter miRNA expression patterns, the precise regulatory pathways linking these molecular changes to behavioural outcomes are not fully understood. Advanced genomic and transcriptomic techniques may help identify miRNA signatures associated with pesticide exposure and neurodevelopmental disorders. These molecular markers could potentially serve as early indicators of pesticide-induced neurological damage.

Another important research direction involves the investigation of combined exposures to multiple environmental toxicants. In real-world conditions, children are rarely exposed to a single pesticide compound. Instead, they are typically exposed to complex mixtures of pesticides and other environmental chemicals. These combined exposures may interact synergistically or antagonistically, producing biological effects that differ from those caused by individual compounds. Future studies should therefore focus on evaluating the cumulative effects of multiple pesticide exposures and their combined influence on gene regulation and behavioural development.

Longitudinal cohort studies are also needed to better understand the long-term health consequences of early-life pesticide exposure. Many existing studies examine short-term behavioural outcomes, but the long-term neurological and psychological impacts of pesticide exposure during childhood remain less well understood. Long-term follow-up studies that track children from prenatal stages through adolescence and adulthood may provide valuable insights into how early environmental exposures influence lifelong health and cognitive performance.

Environmental monitoring and exposure assessment techniques should also be improved to obtain more accurate estimates of pesticide exposure levels. Advanced analytical technologies such as high-resolution mass spectrometry and biomonitoring approaches may help detect pesticide residues and their metabolites in biological samples with greater sensitivity and accuracy. Such methods could enhance the reliability of epidemiological studies investigating the relationship between pesticide exposure and health outcomes.

In addition, research focusing on environmental exposure pathways, including dietary intake and recreational activities such as swimming in contaminated water bodies, remains limited. More comprehensive environmental studies are needed to quantify pesticide concentrations in food products, drinking water, and recreational water systems in agricultural regions. These studies could help identify critical exposure sources and guide the development of targeted public health interventions.

Another promising area of research involves the development of preventive and therapeutic strategies aimed at mitigating pesticide-induced neurotoxicity. Nutritional interventions, antioxidant therapies, and lifestyle modifications may help reduce oxidative stress and protect neural tissues from environmental toxicants. However, further research is required to determine the effectiveness of such strategies in reducing pesticide-related health risks.

Finally, interdisciplinary collaboration among environmental scientists, toxicologists, epidemiologists, and public health professionals will be essential for addressing the complex challenges associated with pesticide exposure and child health. Integrating knowledge from multiple scientific disciplines may facilitate the development of comprehensive strategies for reducing environmental exposure and protecting children's neurological development.

Conclusions

Environmental exposure to pesticides represents a significant public health concern, particularly for children whose physiological and neurological systems are still undergoing

critical stages of development. The widespread use of pesticides in modern agriculture has increased the likelihood of exposure through multiple pathways, including dietary intake, contaminated water sources, and environmental contact during outdoor recreational activities. These exposure pathways may contribute to cumulative pesticide intake in children, potentially affecting their neurological development and behavioural health.

Recent advances in environmental toxicology and molecular biology have provided new insights into the biological mechanisms underlying pesticide-induced health effects. Among these mechanisms, epigenetic regulation—particularly microRNA-mediated gene expression—has emerged as an important pathway through which environmental toxicants may influence neuronal development. MicroRNAs play crucial roles in regulating gene networks involved in synaptic formation, neuronal differentiation, and neural signaling processes. Disruption of these regulatory pathways by pesticide exposure may therefore lead to long-term neurodevelopmental consequences.

Evidence from experimental and epidemiological studies suggests that pesticide exposure may be associated with various behavioural and cognitive outcomes in children. These outcomes include attention deficits, hyperactivity, mood instability, learning difficulties, and anxiety-related behaviours. Such behavioural changes may affect academic performance, social interactions, and emotional well-being throughout childhood and adolescence. Although the exact molecular mechanisms remain incompletely understood, growing evidence indicates that pesticide-induced microRNA dysregulation may contribute to these neurobehavioral effects.

Environmental exposure pathways also play an important role in determining the overall risk of pesticide-related health effects. Dietary intake of contaminated food products remains the primary exposure route for most populations, while environmental contact through drinking water or recreational activities may contribute additional exposure. Children living in agricultural communities may therefore experience multiple exposure pathways simultaneously, increasing their vulnerability to pesticide-related health risks.

The findings discussed in this review highlight the need for improved environmental monitoring, stronger food safety regulations, and greater public awareness regarding pesticide exposure. Implementing sustainable agricultural practices, reducing pesticide application, and improving water quality management may help minimize environmental contamination and protect vulnerable populations. Public health strategies aimed at educating communities about

safe food handling practices and environmental protection may also contribute to reducing pesticide exposure.

In conclusion, pesticide exposure represents a complex environmental health issue with significant implications for children's neurological development and behavioural outcomes. Continued research integrating molecular biology, environmental monitoring, and epidemiological analysis will be essential for clarifying the mechanisms underlying pesticide-related health effects. Such knowledge will support the development of effective prevention strategies and public health policies aimed at safeguarding children's health and promoting sustainable environmental management.

Food safety monitoring represents an essential component of pesticide exposure prevention. Regulatory agencies in many countries have established maximum residue limits (MRLs) for pesticide concentrations in food products. However, continuous monitoring is necessary to ensure that these limits are maintained and that consumers are protected from excessive exposure. Strengthening food safety regulations and encouraging the adoption of integrated pest management strategies may help reduce pesticide residues in agricultural products.

Public awareness and education also play an important role in minimizing pesticide exposure. Educating parents, farmers, and communities about safe pesticide use, proper washing of fruits and vegetables, and environmental protection practices can significantly reduce the risk of exposure. For example, washing and peeling fruits and vegetables may help remove surface pesticide residues, thereby reducing dietary intake.

Environmental management strategies are equally important for reducing pesticide contamination in water systems. Agricultural runoff is one of the primary sources of pesticide pollution in rivers, lakes, and groundwater. Implementing sustainable farming practices such as buffer zones, reduced pesticide application, and improved irrigation management can help minimize the transport of pesticides into aquatic environments.

Public health interventions should also focus on monitoring vulnerable populations, particularly children living in agricultural regions where pesticide exposure levels may be higher. Biomonitoring studies that measure pesticide metabolites in biological samples such as urine or blood can provide valuable insights into exposure levels and potential health risks. Such data may help policymakers design targeted interventions aimed at protecting high-risk communities.

Another important public health consideration is the potential role of epigenetic mechanisms, including microRNA dysregulation, in mediating pesticide-related health effects. Understanding these molecular pathways may provide new opportunities for early detection of pesticide-induced health risks. Biomarkers based on epigenetic changes may eventually be used to identify individuals who are more susceptible to environmental toxicants.

International organizations and public health agencies have increasingly recognized the need to regulate pesticide use and protect vulnerable populations from environmental chemical exposure. Collaborative efforts involving governments, researchers, and agricultural industries are essential for developing effective strategies that balance agricultural productivity with human health protection.

In summary, pesticide exposure represents a complex environmental health challenge with significant implications for child development and public health. Reducing exposure through improved agricultural practices, strengthened food safety regulations, and public education programs is essential for protecting children's health and promoting sustainable environmental management.

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Chapter 6

Quorum Sensing: Molecular Mechanisms and Therapeutic Implications in Bacterial Communication

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Abstract

Quorum sensing represents a sophisticated cell-to-cell communication mechanism whereby bacteria coordinate collective behaviors in response to population density through production, release, and detection of signaling molecules called autoinducers. This review examines the molecular architecture of quorum sensing systems, including the canonical LuxI/LuxR paradigm in Gram-negative bacteria and oligopeptide-based systems in Gram-positive organisms. We critically evaluate the role of quorum sensing in regulating virulence factor expression, biofilm formation, bioluminescence, and secondary metabolite production across diverse bacterial species. Recent advances in understanding interspecies and interkingdom signaling networks have revealed quorum sensing as a central regulatory hub influencing microbial ecology and pathogenesis. Furthermore, we discuss the therapeutic potential of quorum quenching strategies, including small molecule inhibitors and enzymatic degradation of autoinducers, as novel anti-virulence approaches to combat antibiotic-resistant infections. The integration of synthetic biology and systems-level analyses promises to unlock new dimensions in manipulating bacterial communication networks for biotechnological and clinical applications. This review synthesizes current knowledge on molecular mechanisms, ecological implications, and therapeutic applications of quorum sensing, highlighting future research directions emphasizing the ecological context of quorum sensing and its exploitation in precision medicine for developing next-generation antimicrobial strategies that circumvent traditional resistance mechanisms.

Keywords: Anti-virulence therapy, Autoinducers, Bacterial communication, Biofilm formation, Quorum sensing

Introduction

Bacteria were long perceived as unicellular organisms operating independently, governed solely by intrinsic genetic programs and environmental stimuli. This paradigm shifted dramatically with the discovery of quorum sensing, a sophisticated mechanism enabling bacteria to monitor population density and coordinate group behaviors through chemical communication. Quorum sensing allows bacterial populations to function as multicellular entities, executing complex tasks that would be impossible or inefficient for individual cells. These coordinated activities include bioluminescence production, biofilm formation, virulence factor expression, antibiotic synthesis, sporulation, and conjugation (Waters & Bassler, 2005).

The ecological and medical significance of quorum sensing cannot be overstated. In natural environments, quorum sensing mediates critical interactions within microbial communities, influencing nutrient cycling, symbiotic relationships, and competitive dynamics. In clinical contexts, quorum sensing controls expression of virulence determinants in pathogenic bacteria, making it a central factor in infectious disease progression. The alarming rise of antibiotic resistance has intensified interest in quorum sensing as a therapeutic target, as disrupting bacterial communication offers an alternative strategy that may impose less selective pressure for resistance evolution compared to conventional antibiotics (Defoirdt, 2018).

This review provides a comprehensive examination of quorum sensing mechanisms, their regulation of bacterial physiology and pathogenesis, and their potential exploitation for therapeutic and biotechnological applications. We synthesize current understanding of the molecular machinery underlying bacterial communication, analyze its role in diverse biological processes, and evaluate emerging strategies for manipulating these systems to combat infectious diseases and enhance industrial fermentation processes.

Molecular Architecture of Quorum Sensing Systems

The LuxI/LuxR Paradigm in Gram-Negative Bacteria

The LuxI/LuxR system of the bioluminescent marine bacterium Vibrio fischeri represents the archetypal quorum sensing mechanism in Gram-negative bacteria. In this system, LuxI functions as an autoinducer synthase that catalyzes production of N-acyl homoserine lactones (AHLs), the primary signaling molecules in Gram-negative quorum sensing. These AHLs consist of a homoserine lactone ring linked to an acyl chain of variable length and substitution, which determines signal specificity (Whitehead et al., 2001).

AHLs are small, membrane-permeable molecules that diffuse freely across bacterial cell membranes, accumulating both intracellularly and in the surrounding environment. At low cell densities, AHL concentrations remain below threshold levels. As bacterial populations grow, AHL concentrations increase proportionally until reaching a critical threshold that correlates with high cell density. At this point, AHLs bind to cognate LuxR-type transcriptional regulators, inducing conformational changes that enable DNA binding and transcriptional activation or repression of target genes (Fuqua et al., 1994).

The LuxI/LuxR circuit creates a positive feedback loop, as activated LuxR typically upregulates luxI expression, leading to exponential accumulation of autoinducer and rapid synchronization of population-wide gene expression changes. This elegant regulatory architecture enables bacteria to sense population density with remarkable precision and coordinate responses only when sufficient cell numbers are present to make collective action effective.

Oligopeptide-Based Systems in Gram-Positive Bacteria

Gram-positive bacteria employ mechanistically distinct quorum sensing systems based on oligopeptide autoinducers rather than AHLs. These autoinducing peptides (AIPs) are ribosomally synthesized as precursor peptides that undergo post-translational modification and proteolytic processing before secretion via dedicated ABC transporters (Kleerebezem et al., 1997). Unlike the freely diffusible AHLs of Gram-negative bacteria, oligopeptide signals cannot cross membranes passively and must be actively exported.

Detection of extracellular AIPs occurs through two-component signal transduction systems comprising a membrane-bound histidine kinase receptor and a cytoplasmic response regulator. When AIP concentrations reach threshold levels, binding to the histidine kinase triggers autophosphorylation and subsequent phosphotransfer to the response regulator, which then modulates transcription of target genes. This system is exemplified by the Agr system of Staphylococcus aureus, which regulates virulence factor production and biofilm formation (Novick & Geisinger, 2008).

The diversity of oligopeptide sequences and processing mechanisms provides Gram-positive bacteria with extensive potential for signal specificity and discrimination between self and non-self-signals. Multiple quorum sensing systems often coexist within single bacterial species, creating complex regulatory networks that integrate diverse environmental and physiological signals.

LuxS and AI-2: A Universal Interspecies Signal

The discovery of AI-2, produced by the LuxS enzyme, revealed a communication system shared across both Gram-positive and Gram-negative bacteria. LuxS catalyzes synthesis of 4,5-dihydroxy-2,3-pentanedione (DPD), which spontaneously cyclizes to form various furanosyl borate diester derivatives collectively termed AI-2. Unlike species-specific AHLs and AIPs, AI-2 appears to function as a universal signal enabling interspecies communication within mixed microbial communities (Vendeville et al., 2005).

The widespread distribution of luxS genes suggests that AI-2 signaling plays important roles in coordinating multispecies behaviors in complex microbial ecosystems. However, the precise functions of AI-2 signaling remain subjects of active investigation, as LuxS also participates in the activated methyl cycle, making it difficult to distinguish quorum sensing functions from metabolic roles.

Physiological Processes Regulated by Quorum Sensing

Virulence Factor Expression and Pathogenesis

Quorum sensing plays central roles in bacterial pathogenesis by coordinating expression of virulence factors in response to population density. This strategy enables pathogens to establish infections while avoiding premature immune detection. Only after reaching sufficient numbers do bacteria activate production of toxins, degradative enzymes, and other virulence determinants that damage host tissues and suppress immune responses (Rutherford & Bassler, 2012).

In Pseudomonas aeruginosa, a major opportunistic pathogen, multiple interconnected quorum sensing systems regulate production of elastase, pyocyanin, rhamnolipids, and numerous other virulence factors. Quorum sensing mutants show dramatically attenuated virulence in animal infection models, demonstrating the critical importance of coordinated gene expression in pathogenesis. Similar patterns occur in diverse pathogens including Vibrio cholerae, Burkholderia cepacia, and Staphylococcus aureus, establishing quorum sensing as a common virulence strategy (Williams & Cámara, 2009).

Biofilm Formation and Architecture

Biofilms are structured microbial communities encased in self-produced extracellular polymeric matrices that adhere to surfaces. Quorum sensing regulates multiple stages of biofilm development, including initial attachment, maturation, and dispersal. In many bacteria,

quorum sensing activation promotes biofilm formation through upregulation of genes encoding matrix components such as polysaccharides, proteins, and extracellular DNA (Davies et al., 1998).

The relationship between quorum sensing and biofilm formation is complex and species-dependent. In P. aeruginosa, quorum sensing positively regulates biofilm maturation and structural differentiation, while in some other species, quorum sensing triggers biofilm dispersal, releasing cells to colonize new environments. This regulatory versatility reflects the adaptive value of coordinating biofilm behaviors with population density.

Biofilms pose serious challenges in clinical settings, as they exhibit enhanced resistance to antibiotics and immune clearance. Medical device-associated infections, chronic wounds, and persistent respiratory infections in cystic fibrosis patients all involve biofilm-embedded bacteria regulated by quorum sensing, making disruption of these communication systems an attractive therapeutic strategy.

Secondary Metabolite Production

Many bacteria produce secondary metabolites including antibiotics, siderophores, and signaling molecules whose synthesis is regulated by quorum sensing. This coordination ensures that metabolically expensive secondary metabolite production occurs only when bacterial populations are sufficiently large to make these compounds effective. For example, the antibiotic carbapenem in Erwinia carotovora and phenazines in P. aeruginosa are quorum sensing-controlled (Whitehead et al., 2001).

Industrial fermentation processes increasingly exploit quorum sensing regulation to optimize production of commercially valuable secondary metabolites. Understanding the regulatory networks controlling biosynthetic pathways enables rational engineering of bacterial strains with enhanced productivity through manipulation of quorum sensing circuits.

Quorum Quenching: Therapeutic Strategies

Small Molecule Inhibitors

Quorum quenching encompasses diverse strategies for disrupting bacterial communication, including competitive inhibition of signal-receptor binding, interference with signal biosynthesis, and enzymatic degradation of autoinducers. Small molecule inhibitors that structurally mimic autoinducers but lack agonist activity can competitively block receptor binding, preventing activation of quorum sensing-regulated genes (Rasmussen & Givskov,

2006).

Synthetic analogues of AHLs with modified acyl chains or lactone rings have been developed as quorum sensing antagonists. Additionally, natural compounds from plants, algae, and other organisms exhibit anti-quorum sensing activity. Halogenated furanones from the marine alga Delisea pulchra, for instance, interfere with AHL-mediated quorum sensing and reduce biofilm formation in multiple bacterial species. These compounds represent promising leads for development of anti-virulence therapeutics (Givskov et al., 1996).

Enzymatic Degradation of Autoinducers

Numerous organisms produce enzymes that degrade bacterial autoinducers, a phenomenon termed quorum quenching. AHL-lactonases and AHL-acylases hydrolyze AHL signals, while various peptidases can degrade oligopeptide autoinducers. These enzymes may have evolved as defense mechanisms, allowing host organisms to disrupt pathogen communication (Dong et al., 2007).

Therapeutic applications of quorum quenching enzymes include their use as antimicrobial agents or in coating medical devices to prevent biofilm formation. Encapsulation of quorum quenching enzymes in controlled-release formulations or expression in probiotic bacteria represents innovative delivery strategies currently under investigation.

Advantages and Challenges

Quorum quenching offers several theoretical advantages over traditional antibiotics. By targeting virulence regulation rather than bacterial viability, these approaches may impose reduced selective pressure for resistance evolution. Furthermore, anti-virulence strategies could preserve beneficial commensal bacteria while specifically attenuating pathogenic behaviors. However, challenges remain, including the need for broad-spectrum inhibitors effective against multiple quorum sensing systems, potential compensatory mechanisms in bacteria, and unknown long-term ecological consequences (Defoirdt, 2018).

Synthetic Biology and Biotechnological Applications

Synthetic biology approaches enable rational engineering of quorum sensing circuits for diverse applications. Modified quorum sensing systems have been designed to control industrial fermentation processes, coordinate division of labor in engineered bacterial consortia, and create biosensors for detecting pathogens or environmental contaminants. The modular nature of quorum sensing components facilitates their integration into synthetic

genetic circuits with predictable behaviors (Brenner et al., 2008).

In biomanufacturing, quorum sensing-controlled expression systems offer advantages for optimizing production timing and reducing metabolic burden on host cells. By delaying expression of recombinant proteins or secondary metabolites until high cell densities are achieved, these systems maximize product yields while maintaining cell viability during growth phases.

Future Directions and Conclusions

Understanding quorum sensing has revolutionized our conception of bacterial behavior, revealing sophisticated social interactions previously unrecognized in prokaryotes. Future research directions include elucidating the full complexity of quorum sensing networks within natural microbial communities, developing next-generation anti-virulence therapeutics, and exploiting quorum sensing for enhanced biotechnological processes. Systems biology approaches integrating genomics, transcriptomics, and metabolomics will be essential for comprehensive understanding of these regulatory networks. The ecological context of quorum sensing, including its roles in interspecies interactions and evolution of cooperation, represents an exciting frontier with implications extending beyond microbiology to fundamental questions in evolutionary biology and ecology. As antibiotic resistance continues threatening global health, quorum sensing-based therapeutics offer hope for innovative treatment strategies that work in harmony with, rather than against, natural bacterial ecology.

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Chapter 7

The Dark Side of Clenbuterol: A Review of Performance Enhancing Drugs in Sports

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Abstract

Originally created as a bronchodilator for veterinary usage, Clenbuterol is a long-acting β_2-adrenergic agonist. However, its off-label misuse as a performance-enhancing drug in bodybuilding and sports has drawn a lot of attention. It has potential for boosting lean muscle mass and decreasing body fat because of its anabolic and lipolytic qualities, which are mainly mediated by β_2-adrenergic receptor activation and downstream signalling pathways including PI3K/Akt/mTOR. Clenbuterol enhances resting metabolic rate, fat oxidation, and muscle protein synthesis, according to data from human, animal, and case-report studies; however, these advantages come with serious metabolic and cardiovascular hazards. At very low doses, reported side effects range from tremors, hypokalaemia, and tachycardia to serious consequences such myocardial infarction, arrhythmias, myocarditis, and rhabdomyolysis. Overall, there are significant health concerns associated with the unsupervised use of clenbuterol for performance or aesthetic improvement, even while it has limited therapeutic potential in certain clinical situations, such as muscular wasting disorders. Increased awareness, stricter regulation, improved food monitoring, and education are essential to mitigate abuse and protect athlete health.

Keywords: Anabolic effects, β_2-adrenergic agonist. Cardiovascular toxicity, Clenbuterol, Performance enhancing drug

Introduction

Long-acting β2-adrenergic agonist clenbuterol was first created in 1977 as a bronchodilator to treat asthma, but because of its strong anabolic and lipolytic properties, it has gained attention for off-label use in humans (Jessen, S.et.al.2020) It is still often abused in the bodybuilding, weight reduction, and performance improvement industries despite not being authorised for human use in the USA and many European nations (Kintz, P.et.al 2019). We can understand it through mechanisms involving the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) axis, clenbuterol elevates the resting metabolic rate and fat oxidation in skeletal muscle by activating β2-adrenergic receptors.(Jessen, S et.al.2020) Similarly another anabolic androgen steroid oxandrolone, combined with a high-protein diet (2 g/kg/day) helps in faster rate of weight gain and restoration of muscle function in the recovery phase (Demling, R. H., & DeSanti, L. et.al 2003).Shortcuts to dieting have been advocated as society has placed more emphasis on physical beauty. As a result, more people are using "image- and performance-enhancing drugs," such as β2 agonists like clenbuterol (Milano, G et.al.2018) Additionally, clenbuterol can increase muscle mass and promote weight loss by acting on adipocytes' β3-adrenergic receptors. One significant issue is with bodybuilders, amateur athletes, and elite athletes who want to lose body fat while improving their physical appearance and performance (Milano, G et.al 2018). Apart from this deliberate consumption of clenbuterol, there have been cases of pills that were promoted as fat burners but contained trace levels of clenbuterol and were labelled as thermogenic goods or metabolism boosters. Numerous nations' health authorities and the Food and Drug Administration (FDA) have warned that clenbuterol can be present in herbal mixes, and dietary items like "fat burners" marketed under names like ClenXDV, Clen 40. (Tucker, J et.al 2018) Consuming tainted meat (animals that have given those steriod to develop lean meat) has recently been identified as another source of unintentional doping. Because of this, athletes who eat such tainted meat can unintentionally consume traces of clenbuterol, which could result in an adverse analytical finding (AAF), which is a positive anti-doping test result that shows a prohibited substance was present in their biological sample (Geyer, H. et.al 2014). Despite having a therapeutic effect it's higher dose has a significant risk of causing sympathomimetic adverse effects, including tachycardia, tremors, hypokalaemia, and cardiac arrhythmias, which have been proven to be human toxicity(Duabert et.al.2007).World Anti-Doping Agency (WADA)-accredited labs perform the testing for the presence of such drugs , and the athlete must even provide proof if they had accidental

consumption(Geyer, H et.al.2004).Differentiating between unintentional consumption and intentional ingestion of clenbuterol or other performance enhancing drugs remains challenging(Walpurgis, K et.al.2020)(Geyer, H et.al.2014)Because of its pharmacodynamic properties, clenbuterol has also emerged as a potential treatment option for reducing muscle atrophy in clinical settings where conventional anabolic drugs frequently have poor results, such as spinal cord injury(Otzel, D et.al.2021) Growing instances of abuse and contamination have sparked worries about their safety profiles and laws around the world(Brett, J et.al2014)The ambition to obtain a competitive advantage, his attempt for the ideal physique, and the pressure to live up to social beauty standards are some of the reasons why bodybuilders take such performance-enhancing drugs like clenbuterol. Furthermore, some sportsmen might believe that in order to stay up with their peers, they must use such performance enhancing drugs (Tavares, A et.al.2020)

Pharmacodynamics of Clenbuterol

Sportsmen are interested in two different impacts that clenbuterol produces. It accelerates the deposition of muscle proteins and encourages lipolysis. Both of these effects are believed to be independent of each other. Despite not causing hyperplasia, clenbuterol causes skeletal and cardiac muscle to enlarge (Jessen, S., Solheim, et.al 2020). It is believed that the balance between protein synthesis and breakdown determines how well muscle grows. Clenbuterol suppresses both synthesis and breakdown, with the latter appearing to be more suppressed; which results in increased synthesis of muscle proteins (Hostrup, M.et.al.2025) Plasma levels of insulin, growth hormone, triiodothyronine, and testosterone have not shown any noticeable alterations in clenbuterol-treated participants' endocrine tests. While chronic administration of clenbuterol did not enhance muscle blood flow, acute dosing did (Soppa, G. K. R.et.al 2005).The growth-promoting impact is temporary and appears to fade with continued therapy(.Navegantes, L. C.et.al 2003) Beta 2 adrenergic drugs have been used in human participants in two investigations. One study did note that when clenbuterol was inhaled before testing, human subjects' peak power production, as measured on a bicycle ergometer, increased significantly. A second study examined how skeletal muscle function was affected following 14 days and 21 days treatment with either a placebo or a slow-release form of salbutamol (Hostrup, M.et.al 2024) After taking salbutamol for 21 days, the strength of the quadriceps and hamstrings rose, while the placebo group showed no improvements. The grip strength, however, did not significantly change. According to these findings, voluntary muscular strength is increased by short-term salbutamol administration; however, the strength of this

impact differs according on the muscle group. According to this study, B-adrenoreceptor agonists may have therapeutic value in modifying human skeletal muscle function. (Hostrup, M.et.al 2018).

Anabolic effects and Lipolysis

Consuming clenbuterol has a major impact on a number of metabolic markers in young, healthy males. It improves fat oxidation by 39% and raises the resting metabolic rate by 21%. At the molecular level, clenbuterol increases the phosphorylation of protein kinase A (PKA) substrates by 35% and mTOR at Ser2448 by 121%, suggesting improved adrenergic and anabolic signalling. The amount of glycogen in skeletal muscle does not vary in spite of these molecular and metabolic changes. Additionally, clenbuterol significantly raises the plasma concentrations of a number of hormones and substrates, such as lactate (87%), insulin (105%), glucose (25%), and free fatty acids (129%), indicating extensive systemic metabolic activity. (Jessen et.al.2020)

By promoting Akt/forkhead box O3 (FoxO3) phosphorylation, clenbuterol increases cyclic adenosine monophosphate (cAMP) levels and inhibits the expression of atrogin 1 and muscle RING-finger protein-1 (MuRF1) that is increased by fasting. By promoting protein anabolism and suppressing cathepsin L and the ubiquitin ligase-dependent proteolytic pathway, it also controls the process of muscle atrophy (Dutt, V et.al.2015). According to these findings, clenbuterol short-term increases fat oxidation and resting metabolic rate while also triggering anabolic pathways that boost muscle protein synthesis, including protein kinase A (PKA) and mechanistic target of rapamycin (mTOR). Its extensive use as a performance-enhancing drug is a result of these combined effects. But in the long run, the notable spikes in insulin, lactate, and plasma glucose levels raise questions about possible metabolic strain. Long-term or high-dose use can raise the risk of insulin resistance, glycaemic dysregulation, and cardiovascular disease, underscoring the risks of abuse or long-term exposure.

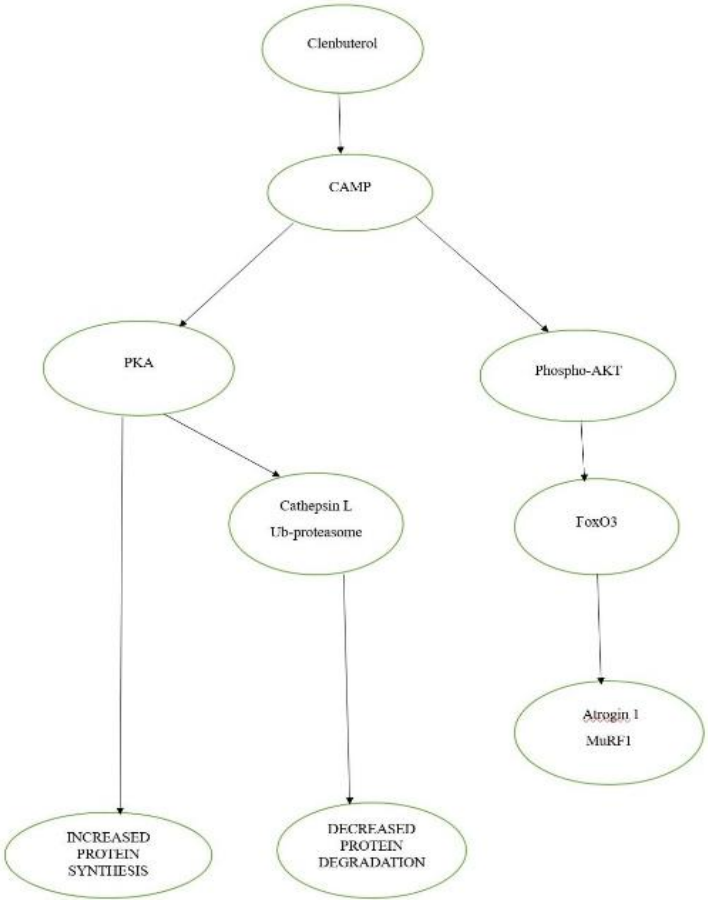

Figure 1: Action of clenbuterol (both anabolic and catabolic) (Dutt, V et.al.2015)

Because clenbuterol has a high oral bioavailability, ingestion is typically the method of misuse. For athletes, a typical "anabolic" dose of clenbuterol ranges from 60 to 100 µg per day for 6 to 12 weeks (Kennedy, M. C et.al.2000). Much higher dosages of clenbuterol (e.g., 1.575 µg daily for 14 months) were used alongside with anabolic steroids, according to a case repor(Liu, J.-D et.al 2019)

Peak plasma clenbuterol levels (i.e., around 0.3 ng/mL) occurred two to three hours following the consumption of a single 20 µg clenbuterol dose in a study including nine healthy male volunteers (Couet, W.et.al.1989) A single dose has a comparatively high bioavailability (about 70–80%). When clenbuterol is inhaled, its systemic absorption is significantly lower than when it is taken orally. According to in vitro research, clenbuterol is 50–98% protein bound, the volume of distributionis also relatively very large (Yamamoto, I.et.al.1985). After clenbuterol is metabolised in the liver, there is evidence of several metabolites, but their structures are still unknown. The primary metabolite is most likely a 4-mono-nitro molecule, and dechlorination produces a secondary metabolite. (Brambilla, G.et.al.2007). About 30% of

the absorbed dose of clenbuterol in volunteer trials is excreted by the kidneys as unprocessed clenbuterol (Witkowska-Piłaszewicz, O.et.al 2021)The remaining clenbuterol elimination is due to biliary and renal excretion of conjugated metabolites, primarily sulphates. Clenbuterol has a plasma elimination half-life of around 25–39 hours.(.Meza-Márquez, O. G.et.al 2012). The plasma concentration remained between 0.2 and 0.3 ng/mL with twice-daily 20 μg doses and between 0.5 and 0.6 ng/mL with twice-daily 40 μg doses after repeated treatment, which resulted in a steady-state concentration by the fourth day. About 20% of the clenbuterol that was administered was eliminated through the urine in less than 72 hours, mostly in the form of glucuronide and sulfate conjugates or in its intact form. Soma, L. R.,et.al 2004)

Adverse side effects of clenbuterol abuse

Clenbuterol is a strong sympathetic stimulant and a β2-adrenergic agonist. Because of its anabolic and lipolytic properties, it is occasionally abused, but it can have serious, multisystemic side effects. (Gauthier J.et.al.2001) (Spiller, H. A.et.al.2013).

Clenbuterol is commonly abused in addition with other performance-enhancing drugs, which may increase its toxicity to the heart and nervous system, according to a descriptive study on drug usage (Gauthier J.et.al.2001) Finding the main cause of adverse effects is made more difficult by this multiple medications.

However, numerous case studies have shown that clenbuterol alone can cause cardiovascular toxicity, such as myocardial infarction (MI) and arrhythmias, underlining the drug's potential for harm even when used alone. (Barry, A. R.et.al.2013;Moriarty, N.et.al.2020, Quinley, K. E.et.al.2016)

Psychological and social drivers inducing unsupervised use

Bodybuilders exhibit mental health issues, behavioural addiction, obsessive-compulsive disorder, and muscle dysmorphia, also known as bigorexia, a disorder related to body image (Coquet, R et.al.2018) Seeking a "extraordinary self": The desire for an idealised, "extraordinary" self is one of the main drivers behind uncontrolled clenbuterol use. Since bodybuilders are high-achieving athletes, they frequently view clenbuterol as a way to quickly achieve a competitive physique (Macho, J et.al.2021). Conflict arises between the "extraordinary" and "ordinary" selves as a result of this desire to change physically while retaining a sense of normalcy outside of the gym. Managing daily life and athletic goals: Athletes may justify clenbuterol use as a desired strategy to maintain their personal and athletic

objectives. (Macho, J et.al.2021). Feelings of dissatisfaction with oneself lead to the usage of anabolic steroids and supplements. As expected, doping is very common among competitive bodybuilders (77.8%) (Kennedy M. C. et al 2000). Users of androgenic anabolic steroids may be fascinated by how drugs affect human physiology, driven by a desire to look and feel good, to enjoy life to the fullest, even if it means taking chances, and to "prepare for and perform at competition."(Coquet, R et.al.2018). People frequently believe that using performance enhancers is necessary to preserve their competitive edge and social functioning, as explained in the context of other appearance and performance-enhancing drugs (APEDs). Their investment in and out of the gym is more a reflection of their performance, knowledge, and way of life. A growing number of bodybuilders and athletes are using clenbuterol without the guidance of a physician in an attempt to improve their performance and physical appearance. Originally created as a bronchodilator, clenbuterol has become well-liked in the bodybuilding scene because of its capacity to increase lean muscle mass and decrease fat. Because of its apparent efficacy and quick results, athletes continue to use clenbuterol despite the possibility of serious side effects (Macho, J et.al.2021).

Below is a brief description about other performance enhancing drugs:

a) Anabolic androgenic steroids (AAS)

With both anabolic ("muscle building") and androgenic ("masculinizing") qualities, the anabolic-androgenic steroids (AASs) family of hormones consists of the natural male hormone testosterone and its several synthetic derivatives (Kanayama, G.et.al.2008)(Kanayama, G et.al.2010).When used in supraphysiologic dosages, AAS enables users to improve their athletic performance and muscle strength, usually well beyond what is possible through natural means.(Hassan, A.et.al.2013)(Kanayama, G.et.al.2008) The vast majority of illegal AAS users are not professional athletes; in fact, many are just people looking to gain muscle strength.(Kanayama, G.et.al.2008)According to a recent meta-analysis, the lifetime prevalence of AAS misuse is 18.4% among recreational athletes and 6.4% among men globally(Sagoe, D.et.al.2015).Bodybuilders, weightlifters, and other athletes frequently abuse AS in an attempt to boost their lean mass, muscle growth, and performance.(Aparicio, V.et.al.2013)(Frati, P.et.al.2015)One drawback of human research is that data regarding AAS use is typically self-reported, making it difficult to determine the precise dosage. Additionally, AASs are used in combination with other medications or substances (referred to as "steroid-accessory drugs"), such as growth hormone, ephedrine, and

diuretics, for which it is challenging to distinguish between their harmful effects. (Aparicio, V.et.al.2013)(Frati, P.et.al.2015)

AASs are actually used to treat conditions such delayed male puberty, aplastic anemia, hypogonadism, malnourishment, and testosterone insufficiency (Hassan, A.et.al.2013) with the use of AASs insulin sensitivity is minimized (Rasmussen, J. et.al.2017)

b) Stimulants

Among the stimulants are cocaine, fenfluramine, pemoline, selegiline, sibutramine, strychnine, amphetamine, D-methamphetamine, methylphenidate, ephedrine, pseudoephedrine, caffeine, dimethylamylamine, and modafinil. Stimulants can reduce tiredness, increase anaerobic performance, improve reacting speed, increase alertness, improve endurance, and lead to weight loss, according to researchresearch(Eichner E. R.et.al2008) Interestingly, WADA permits the use of coffee but prohibits stimulants as a class. A wide range of stimulants and other chemicals, such as caffeine, the amino acids taurine and L-carnitine, glucuronolactone, ginkgo biloba, ginseng, and others, are now frequently included in energy drinks (Higgins, J. P.et.al.2010).Each can or bottle may contain up to 500 milligrammes of caffeine. It's unknown if the additional components in energy drinks have any possible performance advantages. Taurine, for instance, may increase exercise capacity by reducing DNA damage caused by exercise, although the levels present in common beverages are most likely far below what is required to have a performance-enhancing effect.(Higgins, J. P.et.al.2010) potential side effects of using stimulants includes - Hypertension, tachycardia, myocardial infarction, stroke, heat stroke, weight loss, rhabdomyolysis, headache, nausea, tremor, insomnia, anxiety/panic attacks, agitation, aggression, psychosis(Baron, D. A.et.al.2013)

c) Growth hormone and growth factors

WADA also prohibits growth hormone and growth factors. Recombinant human growth hormone has been shown in studies to reduce fat tissue and increase muscular mass. Male recreational athletes were randomly selected to receive growth hormone subcutaneously at a dose of 2 mg per day, testosterone intramuscularly at a dose of 250 mg per week, a combination of the two treatments, or a placebo in one sample study (Meinhardt, U et.al.2010) Recreational female athletes were randomly assigned to receive either a placebo or 2 mg of growth hormone each day. Growth hormone significantly lowered fat mass, increased lean body mass, and enhanced sprint capacity in both males and females (but did not affect strength, power, or endurance).

When testosterone and growth hormone were given to males all at once, the boost in sprint ability was considerably more pronounced.

Growth factors include insulin and insulin-like growth factor. They have not been tested in athletes, but it is assumed that they have effects akin to those of growth hormone. These drugs are used by athletes due to their perceived ability to increase muscle mass. (Holt, R. I.et.al.2008)

Common side-effects of human growth hormone includes insulin resistance, hyperglycemia, diabetes mellitus, cardiomegaly, hastened epiphyseal closure in adolescents, myopathy, hypertension, edema, carpal tunnel syndrome (Baron, D. A.et.al.2013)

Evolution of Performance enhancing drugs over time

It's important to note that users may switch between these kinds during their sports careers. While more seasoned athletes frequently adopt a more measured and health-conscious viewpoint as they recognise long-term health dangers, early-stage users may choose to take risks (Macho, J.et.al.2021).

Influence of social factors

Unsupervised use is greatly impacted by the normalization of clenbuterol use in bodybuilding communities. When athletes observe their peers doing drugs with no obvious repercussions right away, they could think the risks are low or exaggerated and in this way they get trapped in the vicious cycle of abuse (Macho, J.et.al.2021)

Cultural norms and media influence: Clenbuterol is frequently portrayed on social media and in online fitness communities as a safe and efficient way to achieve the ideal body. Scientific information showing the health dangers of unsupervised usage may be disguised by this constructive interpretation (Petróczi, A.et.al.2008)

Uncertainty in risk perception: People frequently believe they know enough to control the risks connected to clenbuterol. Like doing PCT (post cycle therapy) so that ut get flushes out of the body system but nevertheless, this feeling of control may cause one to dangerously underestimate possible negative consequences, such as neurological and cardiovascular issues. (Macho, J.et.al.2021)

Effects of clenbuterol on animals during clinical trials

Rats given 0.003–3 mmol/kg of clenbuterol for 14 days had cardiac and skeletal muscle necrosis, possibly as a result of decreased oxygen delivery and increased muscle fatigability (Burniston, J. G.et.al. 2002). Long-term clenbuterol treatment horses raised the likelihood of aortic root rupture in equine investigations by increasing the heart rate (HR), right ventricular pressure (RVP), and aortic root diameter during maximal exercise. Furthermore, it proved that cardiac and skeletal myocytes had elevated drug concentrations (4–12 times) (Whitmore, J et.al 2021). In rats, clenbuterol changed slow-twitch (aerobic) fibers into fast-twitch (anaerobic) fibers, which could be bad for endurance. Because of this change, muscles are more adapted for brief bursts of activity as opposed to long-term endurance (Polla,B.et.al 2001, (Roberts, M. D.et.al:2023)

Clenbuterol usage was linked to lower aerobic performance in animal experiments (e.g., horses). This is probably because changes in muscle composition and increased muscular mass are not ideal for endurance exercises (Kearns, C. F.et.al2002). Clenbuterol raises a horse's fat-free mass (FFM) whether or not they are active. Compared to the four weeks required in the clenbuterol-plus-exercise group, the increase in the clenbuterol-only group occurred more quickly, reaching substantial after two weeks. This implies that exercise and clenbuterol may work in concert to reduce body fat but against FFM increase (Kearns, C. F.et.al 2001).Giving horses clenbuterol significantly raised their adiponectin and lowered their leptin levels. These alterations are associated with a decrease in body fat, because both adipokines are linked to fat mass. These changes show that clenbuterol influences the hormonal control of appetite and energy balance in addition to reducing fat.Kearns (C. F.et.al.2006).

Case report in Humans

Adults without asthma were never the subject of any human research. This medication is prohibited for human consumption by the Food Safety and Inspection Service (FSIS) and the FDA. Due to significant health risks associated with its detrimental effects on the cardiovascular system, including elevated heart rate, hypertension, and arrhythmias, which can be harmful for people without respiratory disorders like asthma, clenbuterol has not been investigated in adults who are not asthmatic. Animals use it to improve their muscle growth and performance. This medication for muscle growth and performance in food and animals was prohibited by the FDA and FSIS.

There was no overall enhancement in walking or respiratory function, although there was a slight improvement in handgrip and blood creatine kinase levels in a randomized clinical study of clenbuterol in spinal and bulbar muscular atrophy (The therapy group also had an increase in heart rate and tremors(Querin, G.et.al.2013).Though safety issues prohibit its use in human studies, clenbuterol has demonstrated promise in animal models of cancer cachexia management in preventing muscle loss(Mantovani, G.et.al.2001) Although it may be useful in reducing cachexia-induced muscle loss in animal models, its use in human research is prohibited due to possible adverse effects and significant cardiovascular risks(Gorjao, R.et.al.2019). In a study on clenbuterol's effects on skeletal muscle gene expression, estimated measurements revealed that clenbuterol can raise daily energy expenditure from roughly 2000 to 2400 kcal (Jessen, S.et.al.2020) However, this assumption assumes a steady ~20% increase over 24 hours, which would not accurately reflect the pharmacodynamics because clenbuterol's impact probably reduces during that time. G-protein-coupled receptors called β2-adrenoceptors (β2-AR) are widely distributed throughout skeletal muscles. Since β2-AR signalling is essential for controlling skeletal muscle development and proliferation, it is a desirable therapeutic target for the treatment of skeletal muscle wasting diseases. The potential of a number of β2-adrenoceptor agonists, such as clenbuterol and formoterol, to reduce skeletal muscle atrophy by altering this signalling pathway is presently being studied(Dutt, V.et.al.2015)In order to examine overall results, clinical trials combining clenbuterol with other adrenergic agonists (such phenylpropanolamine, norepinephrine, and midodrine) were carried out in a study on the use of adrenergic agonists in the treatment of urine incontinence. According to the available data, adrenergic agonists (as a class) have limited advantages in reducing subjective symptoms like incontinence episodes and pad use, but they do not result in complete continence. Sleeplessness, restlessness, and vasomotor symptoms were among the side effects that 28% of participants on adrenergic medications experienced, however they were usually mild and few resulted in dropouts. However, it is important to use caution when interpreting the results' strength and applicability because each study included an average of just 42 people (Alhasso, A.et.al.2005)Beyond its anabolic and thermogenic properties, clenbuterol has new therapeutic potential for the treatment of neuropathic pain. It works in a similar way to antidepressants, which also need to be taken continuously to effectively treat neuropathic pain. Regardless of the delivery mechanism (transdermal gel, implant, or inhalation), pain control remained consistent (Barrot, M.et.al.2009). Numerous incidents demonstrating the adverse effects of clenbuterol on patients have been documented. According to a case study, a young guy in his 20s who frequently uses clenbuterol for bodybuilding

complained of severe chest pain. Later, it was determined to be MI with grade 4 thrombus in the proximal left anterior descending artery, high serum troponin levels, and ST-segment elevation on the ECG(Aggarwal, A.et.al.2025)A case report showed a patient who complained of chest pain for three days after taking 40 μg/day of low-dose clenbuterol for a week (Moriarty, N.et.al.2020).Elevated troponin levels and ST depression were seen on the ECG. A type II MI preliminary tentative diagnosis was taken into consideration. Myocarditis was confirmed by cardiac MRI, which revealed cardiac oedema and delayed epicardial enhancement. Even ordinary or low doses of clenbuterol (40 μg/day) can result in significant adverse cardiac events, as this example demonstrated.

Legal and ethical concern of Clembuterol as a drug

In both professional and amateur sports, the use of clenbuterol as a performance-enhancing substance presents serious moral and legal issues. It also has greater consequences for credibility in competitive athletics. The World Anti-Doping Agency (WADA) has classed clenbuterol as a prohibited substance because to its anabolic and performance-enhancing qualities, which include rapid fat metabolism, increased lean muscle mass, and improved aerobic capacity. In a similar vein, the US Food and Drug Administration (FDA) lists clenbuterol as an unapproved drug for human use outside of certain veterinary purposes and forbids its use in animals raised for food.

Using liquid chromatography-mass spectrometry techniques, clenbuterol can be found in urine at amounts of a few pg/mL during normal sports drug testing (Walpurgis, Ket.al.2020). A WADA-accredited laboratory may record low amounts of clenbuterol (urine concentrations of less than 5 ng/mL) in an athlete's sample as an unusual result rather than an automatic violation, according to a new amendment that went into effect on June 1, 2019. In these cases, more research is carried out to determine whether meat contamination is a likely cause. On the other hand, concentrations of ≥5 ng/mL are subject to the usual anti-doping adjudication process, and the athlete's background and circumstances will determine if they result in any penalties

Conclusion

Originally developed for veterinary usage, clenbuterol is now abused as a performance-enhancing medication. It is infamous for being used off-label in both human and animal situations, especially by fitness-minded people. Due to a variety of societal and personal factors, its popularity endures despite governmental restrictions in many nations. They include

the desire to enhance performance or body image, peer pressure, and physical beauty concerns. Unmonitored use can have serious consequences, particularly for the health of the heart and muscles. More thorough food monitoring, more public awareness, and a more distinct line between unintentional and deliberate exposure in performance settings are all need to address this problem. It is essential to foster a culture of safety and responsible performance enhancement. Further research into safer substitutes might provide answers in the future.

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Chapter 8

Decoding the Dark Genome: Emerging Roles of Long Noncoding RNAs(lncRNAs) in Gene Regulation and Disease.

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Abstract

Long noncoding RNAs (lncRNAs), once dismissed as transcriptional noise within the so-called 'Dark genome' (uncharacterized noncoding regions), are now recognized as pivotal regulators of gene expression, particularly at the epigenetic level. Typically exceeding 200 nucleotides in length, lncRNAs often possess multiple functional domain within single molecule, enabling them to exhibit multifunctionality (eg. Scaffold + sponge). lncRNAs lack protein coding potential due to the absence of canonical open reading frames. Their functional versatility is largely attributed to their ability to form intricate secondary and tertiary structure, enabling them to bind with various molecular targets. They are predominantly transcribed by RNA polymerase II from diverse genomic regions including intergenic, antisence, and enhancer-associated loci. Various lncRNAs have been found that regulate gene expression through multiple mechanisms,including epigenetic modulation, chromatin remodeling by recruiting histone and DNA-modifying enzymes, thereby altering chromatin accessibility, also influence transcriptional activation or repression, and control post - transcriptional processes. Dysregulation of lncRNAs expression is increasingly recognized as a hallmark of various pathological conditions. Notable, liquid biopsy samples, including plasma, saliva, and urine, often reveal diseases - specific lncRNAs expression profiles that distinguish patients from healthy individuals. Emerging evidence implicates lncRNAs in the modulation of key molecular pathways across a wide spectrum of diseases, including autoimmune disorders, neurodegenerative diseases, cardiovascular conditions, and numerous cancers. Decoding the potential of lncRNAs as non-invasive biomarkers and therapeutic targets offers new avenues for diseases diagnosis, prognosis, and precision medicine.

Keywords: Chromatin remodeling, Diseases, Epigenetic, Long noncoding RNAs (lncRNAs), Non-invasive biomarkers, Precision medicine, Therapeutic targets.

Introduction

Long non-coding RNAs (lncRNAs) have become recognized as an essential and highly dynamic component of the eukaryotic transcriptome, fundamentally expanding the classical protein-centric paradigm of gene regulation. Defined as RNA transcripts longer than 200 nucleotides with little or no protein-coding potential, lncRNAs are predominantly transcribed by RNA polymerase II and often undergo capping, splicing, and polyadenylation similar to mRNAs. LncRNAs originate from a broad spectrum of genomic regions, including intergenic spaces, introns, antisense strands, enhancer elements, and bidirectional promoters, reflecting their extensive transcriptional heterogeneity and regulatory complexity. In contrast to protein-coding genes, the functional roles of lncRNAs are primarily governed by their structural conformations and interaction capacities rather than by sequence conservation. Through intricate secondary and tertiary structures, lncRNAs function as molecular scaffolds, guides, decoys, and signalling mediators, facilitating interactions with DNA, RNA, and proteins (Wang & Chang,2011). LncRNAs can epigenetically regulate gene expression through chemical modifications of DNA and histones without altering the underlying sequence. These changes, influenced by internal and environmental factors, affect development, growth, reproduction, aging, and susceptibility to diseases (Esteller, 2011). Importantly, dysregulation of lncRNA expression and function has been increasingly recognized as a hallmark of numerous human diseases. Aberrant lncRNA networks contribute to the pathogenesis of neurodegenerative disorders, cardiovascular diseases, autoimmune conditions, and cancer by modulating key pathways involved in inflammation, apoptosis, oxidative stress, immune activation, and cell proliferation (Wapinski & Chang, 2011).Notably, many lncRNAs exhibit tissue- and disease-specific expression patterns and are detectable in biofluids such as blood, cerebrospinal fluid (CSF), saliva, and urine, underscoring their promise as minimally invasive biomarkers (Chen et al., 2019).

In parallel, advances in RNA-based technologies have highlighted lncRNAs as attractive therapeutic targets. Strategies aimed at silencing pathogenic lncRNAs or restoring protective ones using antisense oligonucleotides (ASOs), RNA interference (RNAi), or genome-editing approaches are gaining momentum (Wahlestedt, 2013). Collectively, these insights place lncRNAs at the intersection of epigenetic regulation, disease pathogenesis, and precision medicine. This review focuses on the biogenesis, mechanisms of action, and pathological roles of lncRNAs across major human diseases, emphasizing their emerging potential as diagnostic biomarkers and therapeutic targets.

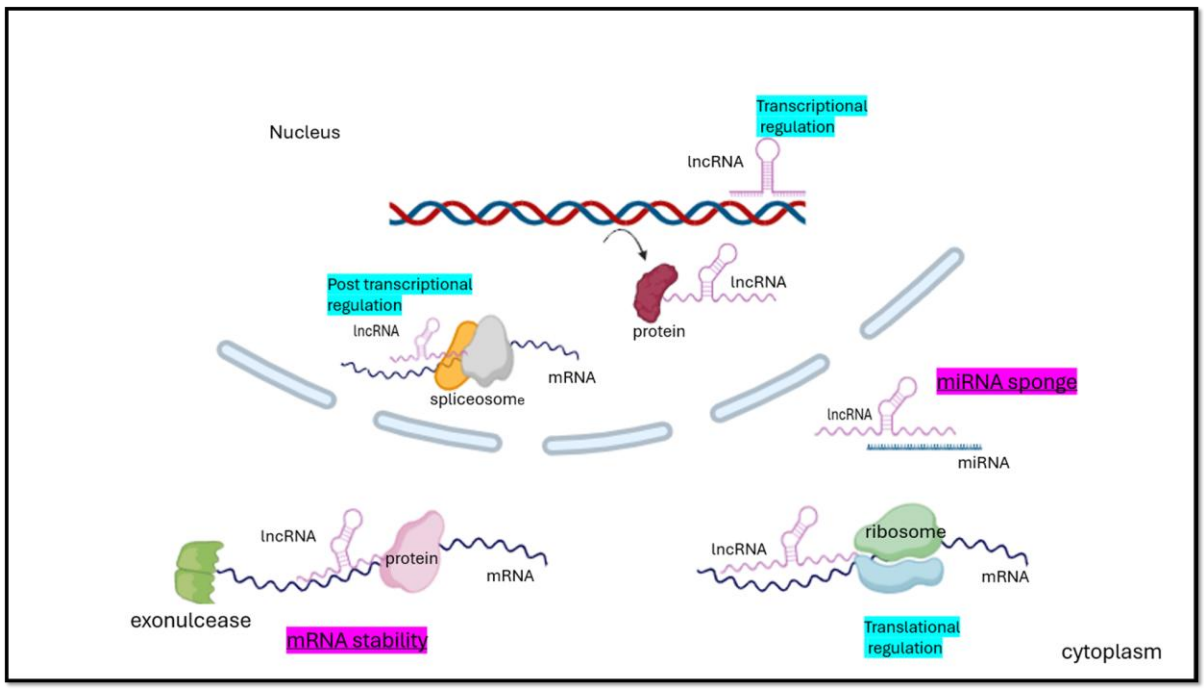

Figure 1: Multilevel Gene Regulatory Functions of Long Non-Coding RNAs in Nuclear and Cytoplasmic Compartments - Long non-coding RNAs (lncRNAs) regulate gene expression across nuclear and cytoplasmic compartments by modulating chromatin architecture, transcriptional activity, RNA processing, microRNA function, mRNA stability, and translation, thereby orchestrating multilayered regulatory networks essential for cellular homeostasis and functional adaptability

Biogenesis of lncRNAs

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that are synthesized primarily by RNA polymerase II (Pol II). Similar to mRNAs, most lncRNAs acquire a 5′ methyl-guanosine cap, undergo splicing, and receive a 3′ poly(A) tail, although they do not encode proteins (Schmitz et al., 2016). Based on their genomic location and transcriptional orientation relative to protein-coding genes, lncRNAs are broadly classified into five categories: sense, antisense, bidirectional, intronic, and intergenic. Sense and antisense lncRNAs overlap with protein-coding genes on the same or opposite strands, respectively. Bidirectional lncRNAs are transcribed from promoters located within 1 kb of a coding gene but in the opposite direction. Intronic lncRNAs derive from long introns, and intergenic lncRNAs (lincRNAs) arise from noncoding regions between genes (Ma et al., 2013). While the

biogenesis of most lncRNAs resembles that of mRNAs, several undergo noncanonical processing pathways. For example, RNase P cleaves specific lncRNAs such as MALAT1 and NEAT1-2 (also known as Menβ) at their 3′ tRNA-like structures to generate mature, stable ends (Wilusz et al., 2012). Some lncRNAs are stabilized by small nucleolar RNA-protein complexes (snoRNPs) that cap their ends, protecting them from exonuclease degradation.

lncRNA expression is finely controlled by epigenetic factors. Histone modifications such as H3K56 acetylation and chromatin remodelers like the SWI/SNF complex can stimulate antisense lncRNA transcription, whereas the Chromatin Assembly Factor 1 (CAF-1) complex often acts as a repressor (Maruyama & Suzuki, 2012). DNA methylation also plays a critical role; for instance, methylation at the paternal Air locus modulates its expression and influences the activity of nearby genes. The degradation of lncRNAs is a regulated process, mediated by nuclear exosomes via complexes like Nrd1-Nab3-Sen1 and the TRAMP complex, or by the 5'-3' exoribonuclease Xrn1 in the cytoplasm, with proteins like UPF1 providing protection from decay (Skourti-Stathaki et al., 2011). Although many regulatory factors have been identified, the full spectrum of mechanisms governing lncRNA biogenesis remains an active area of investigation.

Functions of lncRNAs

Long non-coding RNAs regulate gene expression through diverse mechanisms that rely on their ability to form complex structures and interact with DNA, RNA, and proteins. These functions can be categorized into several archetypes: signals, decoys, scaffolds, guides, and translational regulators (e.g., SINEUPs) (Wang & Chang, 2011). Signal lncRNAs act as molecular indicators of specific cellular states or signaling pathway activation. Their expression is often tightly regulated and transient, serving as a readout of biological events. The canonical example is Xist, whose expression on one of the two X chromosomes in females triggers X-chromosome inactivation. Xist coats the chromosome in cis, recruiting chromatin-modifying complexes like Polycomb Repressive Complex 2 (PRC2) to induce heterochromatin formation and silence gene expression across the entire chromosome (Penny et al., 1996). Decoy lncRNAs function as molecular sponges that sequester regulatory molecules, thereby liberating other targets. They can bind and titrate transcription factors,RNA-binding proteins (RBPs), or, most commonly, microRNAs (miRNAs). For example, the lncRNA NKX2-1-AS1 enhances SERPINE1 expression by sponging miR-145-5p. This derepresses the target of miR-145-5p, activating VEGFR-2 signaling and promoting angiogenesis, which has been implicated

in gastric cancer progression (Jiang et al.,2019). Scaffold lncRNAs serve as platforms to assemble multiple proteins into functional ribonucleoprotein (RNP) complexes. By bringing together enzymes and their substrates or different components of a regulatory pathway, they facilitate chromatin modification and transcriptional regulation.

A well-documented example is HOXA11-AS, which scaffolds PRC2, Lysine-Specific Demethylase 1 (LSD1), and DNA Methyltransferase 1 (DNMT1). This complex coordinately represses the expression of target genes like PRSS8 and KLF2, thereby enhancing gastric cancer cell proliferation and invasion (Sun et al., 2016). Guide lncRNAs bind to proteins and direct the resulting RNP complex to specific genomic loci to modulate gene expression. The lncRNA HOTTIP, transcribed from the HOXAlocus, interacts with the WDR5 protein and guides the WDR5/MLL complex to the 5' end of the HOXA gene cluster. This action promotes H3K4 trimethylation and activates the transcription of neighboring genes, playing a critical role in developmental patterning (Wang et al., 2011). SINEUPs represent a distinct class of natural and synthetic antisense lncRNAs that enhance the translation of target mRNAs without altering mRNA levels. Natural SINEUPs, like AS-Uchl1, contain an inverted SINEB2 element that interacts with the ribosome and promotes the translation of its sense target, Uchl1 mRNA (Carrieri et al., 2012). Synthetic SINEUPs have been engineered to specifically boost the production of desired proteins, demonstrating therapeutic potential for haploinsufficiency disorders or other conditions caused by insufficient protein expression (Zucchelli et al., 2015).

Role of Long Non-coding RNAs in Disease

Neurodegenerative disease

Neurodegenerative diseases are a major cause of morbidity in the elderly, involving the progressive loss of neurons in specific brain regions. While clinically distinct, these diseases share underlying mechanisms such as protein misfolding, neuroinflammation, and oxidative stress. An estimated 40% of lncRNAs are expressed in brain tissue, where they serve as critical modulators of neuronal function and survival (Qureshi & Mehler, 2012). Their dysregulation is increasingly implicated in the onset and progression of Alzheimer's and Parkinson's diseases.

Alzheimer's Disease (AD)

In Alzheimer's disease (AD), lncRNAs contribute to the two main pathological hallmarks: amyloid-β (Aβ) plaques and neurofibrillary tangles (NFTs) of hyperphosphorylated

Tau. The brain-specific lncRNA BACE1-AS is upregulated in AD patients. It contains a sequence complementary to the BACE1 mRNA, which encodes the β-secretase enzyme responsible for the initial cleavage of amyloid precursor protein (APP). By forming an RNA duplex, BACE1-AS stabilizes the BACE1 mRNA and enhances its translation, leading to increased Aβ production and plaque formation (Faghihi et al., 2008). Inhibiting BACE1-AS has been shown to reduce Aβ levels in cellular models, highlighting it as a therapeutic target (Modarresi et al., 2012). The lncRNA MAPT-AS1 is transcribed antisense to the MAPT gene, which encodes the Tau protein. Dysregulation of MAPT-AS1 has been linked to altered splicing and expression of Tau, promoting its hyperphosphorylation and subsequent aggregation into neurofibrillary tangles (NFTs) (Zhu et al., 2020).

lncRNAs NEAT1 and MALAT1, are involved in neuroinflammation, neuronal death, a key driver of AD progression. NEAT1 is a scaffold for paraspeckles and its expression is elevated in AD brains, where it modulates the expression of inflammatory genes in microglia (Cai et al., 2021). MALAT1 also regulates microglial activation and neuronal apoptosis, and its increased levels in plasma and CSF of AD patients correlate with cognitive decline (Ma et al., 2019). As biomarkers, elevated BACE1-AS, MAPT-AS1, MALAT1, and NEAT1 in plasma or cerebrospinal fluid correlate with disease severity, while changes in their levels predict progression and treatment response, highlighting their diagnostic and therapeutic potential in AD.

Parkinson's Disease (PD)

In Parkinson's disease (PD), which is characterized by the loss of dopaminergic neurons and the presence of α-synuclein aggregates (Lewy bodies), lncRNAs regulate key pathological pathways.

Antisense lncRNA HOTAIR is upregulated in cellular models of PD. It acts as a miRNA sponge for miR-126, leading to increased expression of its target, RAB3IP, which promotes neuronal apoptosis and oxidative stress (Ye et al., 2018). The antisense lncRNA AS Uchl1 is a natural SINEUP that enhances the translation of Ubiquitin carboxy-terminal hydrolase L1 (UCHL1), an enzyme critical for protein degradation. Dysregulation of AS Uchl1 impairs the ubiquitin-proteasome system, contributing to α-synuclein accumulation and Lewy body formation (Carrieri et al., 2012).

lncRNA NEAT1 Induces microglial activation and inflammation, exacerbating neuronal injury. (Lo et al., 2021). The lncRNA SNHG1 is also upregulated in PD models,

where it activates the NF-κB signaling pathway, increasing the production of pro-inflammatory cytokines like TNF-α and IL-1β and accelerating dopaminergic neuron degeneration (Cai et al., 2020). Circulating levels of these lncRNAs are being explored as potential biomarkers for early PD diagnosis, disease monitoring, and therapeutic evaluation.

Cardiovascular Diseases (CVDs)

In cardiovascular diseases (CVDs), lncRNAs are key regulators of cardiac development, remodeling, and pathology. They influence atherosclerosis, myocardial infarction (MI), and heart failure by modulating apoptosis, inflammation, fibrosis, and angiogenesis.

Antisense lncRNAs ANRIL in the INK4 Locus (ANRIL) is strongly associated with atherosclerosis. It is transcribed from the CDKN2A/B locus and represses the expression of tumor suppressor genes p15/INK4b and p16/INK4a by recruiting PRC1 and PRC2. This promotes vascular smooth muscle cell proliferation and inflammation, contributing to plaque formation (Congrains et al., 2013). Myocardial Infarction Associated Transcript (MIAT) was one of the first lncRNAs linked to MI risk. MIAT acts as a sponge for several miRNAs, including miR-150-5p. By sequestering miR-150-5p, it upregulates its target genes involved in endothelial dysfunction and fibrosis, exacerbating cardiac injury post-MI (Qu et al., 2017).

Conversely, some lncRNAs MALAT1 & CAREL are protective. MALAT1 promotes angiogenesis and protects endothelial cells from apoptosis under hypoxic conditions (Michalik et al., 2014). Cardiac-apoptosis-related lncRNA (CAREL) prevents cardiomyocyte apoptosis by sponging miR-128a, thereby enhancing the expression of the anti-apoptotic protein BCL2L11 (Li et al., 2021). These examples highlight the therapeutic potential of modulating lncRNA levels to restore cardiac function.

Autoimmune disorders

In autoimmune disorders, multiple lncRNAs play crucial roles in regulating immune responses, inflammation, and tissue damage by acting via miRNA sponging or transcriptional modulation. Targeting these lncRNAs offers therapeutic potential, while their altered expression serves as diagnostic and prognostic biomarkers in diseases such as rheumatoid arthritis and multiple sclerosis.

Rheumatoid Arthritis (RA)

RA is a chronic inflammatory disorder affecting synovial joints. Several lncRNAs contribute to its pathogenesis by modulating inflammation and synovial cell proliferation. In RA, HOTAIR is highly expressed in synovial tissues. It acts as a sponge for miR-138, which targets the V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB). By sequestering miR-138, HOTAIR increases MAFB expression, which in turn promotes the production of inflammatory cytokines like IL-6 and

MMP-13, leading to synovial inflammation and cartilage degradation (Li et al., 2017). Growth Arrest-Specific 5 (GAS5) is generally considered a tumor suppressor, but in RA, its role is complex. It is downregulated in RA synovial fibroblasts and acts as a sponge for miR-222, which promotes apoptosis. Low GAS5 levels contribute to the excessive proliferation and resistance to apoptosis of these cells, a hallmark of RA pathology (Zheng et al., 2018).

TNFα and HNRP-L Related Immunoregulatory LincRNA (THRIL) regulates the expression of TNF-α, a key cytokine in RA. THRIL forms a complex with the protein hnRNP-L, which binds to the TNFα promoter and enhances its transcription. High levels of THRIL in immune cells contribute to the chronic inflammation seen in RA (Li et al., 2014).

Multiple Sclerosis (MS)

MS is a chronic autoimmune disease of the central nervous system characterized by demyelination and neurodegeneration. lncRNAs are implicated in regulating both immune-mediated damage and neuroprotective responses. NEAT1 and MALAT1 are involved in the neuroinflammatory processes of MS. They regulate cytokine production in T-cells and microglia, and their elevated expression in the blood and CSF of MS patients correlates with disease activity and relapse rates (Einali et al., 2019). Silencing NEAT1 has been shown to ameliorate disease symptoms in animal models of MS by reducing T-cell infiltration into the CNS.

In MS, GAS5 is upregulated in active lesions and contributes to the apoptosis of oligodendrocytes, the cells responsible for myelination. It can also modulate T-cell differentiation, favoring pro-inflammatory Th1 and Th17 lineages, thus exacerbating the autoimmune attack (Sun et al., 2017). Taurine Upregulated Gene 1 (TUG1) appears to have a neuroprotective role. It is downregulated in MS lesions and its overexpression has been shown

to protect neurons from inflammatory damage and promote oligodendrocyte precursor cell differentiation, suggesting it could be a target for remyelination therapies (Kefas et al., 2019).

Cancer

In cancer, lncRNAs can act as either oncogenes or tumor suppressors, influencing all major hallmarks of cancer, including proliferation, metastasis, angiogenesis, and therapy resistance.Most studied explains oncogenic lncRNAs, HOTAIR is overexpressed in numerous cancers, including breast, colon, and liver cancer. It acts as both a scaffold and a guide, binding to PRC2 and guiding it to hundreds of target genes to induce H3K27 methylation and transcriptional repression of tumor suppressors (Gupta et al., 2010). Its high expression is often associated with poor prognosis and metastasis. Initially identified in lung cancer, MALAT1 is overexpressed in many malignancies. It regulates alternative splicing of pre-mRNAs and also acts as a miRNA sponge, promoting cell proliferation, migration, and invasion (Gutschner et al., 2013). Plasmacytoma Variant Translocation 1 (PVT1) is co-amplified with the MYC oncogene in many cancers. PVT1 stabilizes the MYC protein by protecting it from degradation and also acts as a sponge for multiple tumor-suppressive miRNAs, thereby promoting tumor growth (Tseng et al., 2014).

Tumor-Suppressive lncRNAs Maternally Expressed Gene 3 (MEG3) is downregulated or lost in a wide range of cancers. It can induce cell cycle arrest and apoptosis by activating the p53 pathway. MEG3 acts as a scaffold to bring p53 and other regulatory proteins together, enhancing p53's transcriptional activity (Zhou et al., 2012). GAS5 can arrest cell growth. It is downregulated in many cancers and functions by sponging oncogenic miRNAs (e.g., miR-21) and acting as a decoy for the glucocorticoid receptor, preventing it from binding its DNA response elements and activating pro-survival genes (Mourtada-Maarabouni et al.,2009). NF-κB Interacting LncRNA (NKILA) suppresses cancer metastasis by inhibiting NF-κB signaling. It directly binds to the NF-κB complex in the cytoplasm, masking its phosphorylation sites and preventing its nuclear translocation and subsequent activation of pro-metastatic genes (Liu et al., 2015). Targeting these lncRNAs offers novel therapeutic avenues. Oncogenic lncRNAs can be silenced with ASOs or siRNAs, while tumor-suppressive lncRNAs can be restored using gene delivery systems.

Conclusion

Long non-coding RNAs have emerged as central regulators of gene expression, substantially expanding our understanding of epigenetic and transcriptional control beyond protein-coding genes. Their diverse genomic origins, complex biogenesis, and capacity to form intricate secondary and tertiary structures enable dynamic interactions with DNA, RNA, and proteins. Through mechanisms such as signaling, scaffolding, guiding, and decoy activity, lncRNAs influence chromatin remodeling, transcription, and post-transcriptional regulation, thereby supporting cellular homeostasis, development, and environmental adaptation. The profound impact of lncRNA dysregulation across a wide spectrum of human diseases—from neurodegeneration and cardiovascular disorders to autoimmunity and cancer—highlights their critical role in pathogenesis.

Furthermore, advances in RNA-based therapeutic technologies have accelerated the translation of lncRNA research into clinical applications. Targeting pathogenic lncRNAs or restoring protective ones using antisense oligonucleotides, RNA interference, or genome-editing approaches represents a novel and precise therapeutic strategy. The tissue-specific expression and stability of lncRNAs in accessible biofluids also position them as highly promising biomarkers for early diagnosis, prognosis, and monitoring treatment response. Despite significant progress, challenges remain, including the incomplete functional annotation of the vast lncRNA transcriptome, their often context-dependent effects, and the need for safe and efficient delivery systems for lncRNA-targeted therapies. Overall, lncRNAs represent a critical regulatory layer linking epigenetic control to disease mechanisms and precision medicine. Continued integration of high-throughput genomics, mechanistic investigations, and rigorous clinical validation will be essential to unlock their full potential as reliable biomarkers and transformative therapeutic targets, paving the way for more personalized and effective disease management strategies.

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Chapter 9

Decoding Evolutionary Transitions: From Phylogenetic Trees to Trait Architectures

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Abstract

Phylogenetic trees constructed from molecular and morphological data provide a critical framework for reconstructing trait evolution and ancestral states. Methods including parsimony, maximum likelihood (ML), and Bayesian inference—implemented in tools such as BEAST and MrBayes—enable time-calibrated trait modeling. Statistical approaches including Pagel's λ, phylogenetic independent contrasts (PIC), and likelihood ratio tests facilitate detection of convergent and divergent evolutionary signals. Phylogenetic Generalized Least Squares (PGLS) and Ornstein–Uhlenbeck (OU) models quantify trait–environment relationships, elucidating how ecological gradients shape traits such as drought resistance, metabolic rate, and reproductive strategy. Integration of next-generation sequencing, genome-wide association studies (GWAS), transcriptomics, epigenomics, machine learning, and 3D morphometrics has substantially improved trait inference resolution. Persistent challenges—including homoplasy, horizontal gene transfer (HGT), incomplete lineage sorting (ILS), and sampling bias—underscore the need for rigorous methodology. Nonetheless, trait–tree synthesis enables predictive modeling of species resilience, informs conservation prioritization, and advances understanding of functional biology and evolutionary distinctiveness.

Keywords: phylogenetic inference, trait evolution, comparative methods, convergent evolution, omics integration, conservation biology

Introduction

Tracing how species traits evolve over time represents a central challenge in evolutionary biology. Phylogenetic trees—branching diagrams derived from genetic or morphological data—provide the structural scaffold for this endeavor, enabling reconstruction of ancestral states and identification of major evolutionary transitions, such as the origin of avian flight or the evolution of plant drought resistance (Revell, 2017). These trees differentiate traits inherited from common ancestors from those arising through convergent or divergent evolutionary processes (Felsenstein, 2019). Statistical methods such as parsimony, ML, and Bayesian inference are routinely applied, with software platforms including BEAST and MrBayes enabling time-calibrated phylogenies that account for the temporal sequence of evolutionary events (Drummond & Bouckaert, 2015).

The integration of advanced genomics, computational tools, and ecological data is rapidly transforming this field. However, challenges including homoplasy, ILS, HGT, and sampling bias continue to complicate accurate inference (Maddison & FitzJohn, 2015). This review synthesizes current methods, computational advances, and applications of phylogenetic trait analysis, with particular emphasis on their utility for predictive ecology and conservation.

Phylogenetic Methods for Trait Inference

Parsimony-based methods minimize evolutionary changes but can oversimplify complex patterns, particularly for traits with multiple independent origins. ML and Bayesian inference (BI) methods provide superior frameworks for managing uncertainty and accommodating complex evolutionary models (Young et al., 2020). Bayesian approaches—implemented in BayesTraits, MrBayes, and BEAST—incorporate uncertainties in both the phylogenetic model and evolutionary process, producing time-calibrated phylogenies critical for understanding the temporal origins of traits (Herbst et al., 2017; Goolsby, 2017).

Scalability remains a practical concern. BEAST 2 with BEAGLE libraries employs parallelized algorithms suitable for large datasets, while Hamiltonian Monte Carlo (HMC) sampling has further enhanced the speed and accuracy of trait inferences (Baele et al., 2021). For continuous traits (e.g., body size, metabolic rate), rerooting-free ML algorithms permit rapid ancestral state reconstruction in linear time (Goolsby, 2017). Comparative analyses indicate that Bayesian methods—particularly MrBayes and RevBayes—generally outperform parsimony and ML approaches under incomplete datasets or complex morphological data

(Höhna et al., 2020). A key limitation is trait lability: under high lability or elevated extinction rates, ancestral state reconstruction accuracy declines substantially (Felsenstein, 2020).

Convergent and Divergent Evolutionary Signals

Convergent evolution—the independent acquisition of similar traits in distantly related lineages in response to analogous environmental pressures—and divergent evolution—the development of distinct traits among closely related species adapting to different niches—represent fundamental macroevolutionary phenomena. PIC and likelihood ratio tests account for the non-independence of species data arising from shared ancestry. Pagel's λ quantifies phylogenetic signal in traits, with high values indicating strong phylogenetic constraint and low values suggesting convergence (Blomberg et al., 2003).

Repeated convergence in body morphology and social behavior has been documented in vertebrates in response to predation and drought stimuli, extending convergence beyond isolated traits to encompass broader biological dimensions (Arbuckle et al., 2014). Genomic analyses reveal that phenotypically convergent species may achieve similar outcomes via common or divergent genetic pathways (Stern, 2013), while divergent traits can be traced to specific molecular variants driving adaptive separation among closely related taxa (Zhang & Kumar, 2021). Ecologically, convergent traits signal strong directional selection, whereas divergent traits reflect niche diversification and contribute to ecosystem complexity (Losos & Mahler, 2010).

Trait Ecology: Environmental Correlates and Adaptive Landscapes

Environmental gradients strongly shape trait evolution across lineages. PGLS corrects for shared evolutionary history while quantifying trait–environment associations, improving the accuracy of comparative analyses (Mazel et al., 2016). OU models track trait evolution toward new adaptive optima in response to changing ecological pressures and are best evaluated via ML or Bayesian approaches (Cooper et al., 2015). Forest plant studies illustrate these principles: leaf architecture responds to light and nutrient gradients, wood density varies with water availability and topography, and seed size predicts range size modulated by dispersal mechanism (Ávila-Lovera et al., 2023).

Phylogenetic niche conservatism—the tendency of related species to retain ancestral ecological traits—may constrain adaptation to novel environments and is detectable as elevated phylogenetic signal (Wiens et al., 2010). Bayesian extensions of PGLS further accommodate uncertainties in phylogenetic topology, evolutionary models, and measurement error,

enhancing inference reliability (Cavender-Bares et al., 2019). Caution is warranted with OU models under limited sample sizes, as parameter estimates—particularly for the selection strength α—exhibit substantial bias, necessitating simulation-based validation prior to interpretation.

Computational and Genomic Advances

Next-generation sequencing and multi-omics methodologies have substantially expanded the capacity to resolve trait architectures within phylogenetic frameworks. Transcriptomic data from RNA sequencing links gene expression profiles to phenotypic variation across species, enabling examination of gene expression plasticity in non-model organisms exposed to environmental gradients. GWAS identifies genomic loci associated with ecologically relevant traits such as drought tolerance and metabolic efficiency; when integrated with species-level phylogenies, GWAS distinguishes whether trait similarities reflect shared ancestry or independent evolution (Josephs et al., 2017).

Epigenomic data—particularly DNA methylation—documents heritable gene regulatory changes not encoded in the primary sequence, offering a mechanism for rapid phenotypic response to environmental change (Zhang et al., 2020). Machine learning and deep learning models applied to large genomic and morphological datasets can classify species by genetic markers, detect macroevolutionary patterns, and predict trait trajectories with higher accuracy than classical approaches (Bishop et al., 2022). Three-dimensional morphometrics combined with high-resolution imaging further enables fine-grained quantification of structural traits across lineages, facilitating detection of subtle evolutionary change (Navarro et al., 2020).

Methodological Limitations

Despite methodological progress, several challenges limit the reliability of phylogenetic trait inference. ILS—where ancestral polymorphisms persist across speciation events—causes gene tree–species tree discordance. In primates, ILS affects up to 64% of the genome, complicating trait reconstruction (Zhang et al., 2023); in marsupials, ILS-driven hemiplasy generates spurious signals of morphological convergence (Cell, 2022). Homoplasy—the recurrent, independent evolution of similar traits—obscures true phylogenetic relationships, particularly in ancient lineages, even when extensive genetic data are available (PMC, 2006). HGT introduces non-vertical inheritance in microbial and some plant systems, creating apparent genomic similarity between unrelated taxa; rigorous detection using high-quality reference genomes is required to avoid incorrect topological inferences

(Wickell, 2020). Uneven taxon sampling generates systematic biases, causing distantly related taxa to appear more closely related than they are. Collectively, these issues demand careful data curation, model selection, multi-locus approaches, and cross-validation strategies to ensure robust trait–tree integration.

Applications in Predictive Ecology and Conservation

Phylogenetic trait-based frameworks provide powerful tools for conservation prioritization. Evolutionary distinctiveness (ED) metrics identify species with irreplaceable evolutionary heritage, while the EDGE (Evolutionarily Distinct and Globally Endangered) framework integrates ED with extinction risk to direct conservation investment toward evolutionarily significant threatened species (Gumbs et al., 2023). Species Distribution Models (SDMs) forecast range shifts under climate change, habitat degradation, or invasion pressure by modeling trait–environment relationships across geographic space (Elith et al., 2009). Functional diversity metrics—including functional redundancy and response diversity—derived from phylogenetically informed trait data serve as indicators of ecosystem resilience (Pillar et al., 2013). Community-Weighted Mean (CWM) indices track human-driven shifts in functional trait composition, providing early warning signals of ecosystem state change before species-level responses become apparent (Carreño-Rocabado et al., 2012).

Future Directions

The field is entering a data-intensive phase characterized by integration of AI, high-throughput data, and real-time environmental monitoring. Deep learning platforms such as Phantom of the Elytra extract morphological traits from high-resolution images at speed and accuracy exceeding manual methods (Hunt & Pedersen, 2025), while Phylo-NN simultaneously identifies evolutionary signals from phylogenetic and image data (Elhamod et al., 2023). PhyloG2P frameworks detect convergent evolution and trait associations by tracking recurrent amino acid substitutions and rate shifts across lineages, with future iterations expected to incorporate epigenetic, ecological, and population-scale data (Macdonald et al., 2025).

The transition toward network-based models capable of representing reticulate evolution—including hybridization, introgression, and HGT—addresses a critical limitation of strictly bifurcating tree models, particularly for microbial and plant systems (Elsevier, 2024). Environmental DNA (eDNA), remote sensing, and citizen science are enabling real-time, landscape-scale trait monitoring, which AI-based systems can integrate to predict trait

evolution under changing conditions while accounting for phylogenetic conservatism and trait collinearity (Álvarez-Carretero et al., 2022; Ao et al., 2023). These converging advances position trait–tree synthesis as an increasingly predictive discipline, with broad applications in functional biology, evolutionary theory, and applied conservation.

Conclusion

The integration of phylogenetic trees with trait architectures represents a methodological and conceptual advance that moves evolutionary biology beyond lineage topology toward mechanistic understanding of how and why traits emerge, persist, or are lost. By combining probabilistic phylogenetic inference with multi-omics data, ecological gradients, and AI-driven analytics, researchers can now decode evolutionary transitions at unprecedented resolution. Addressing persistent methodological challenges—ILS, homoplasy, HGT, and sampling bias—through rigorous, multi-evidence approaches remains essential. The synthesis of trees and traits not only illuminates evolutionary history but also provides predictive tools of direct relevance to conservation planning, ecosystem management, and understanding life's adaptive responses to global change.

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Chapter 10

Microbial Traits in Context: The Shift from Descriptive Profiling to Integrative Soil Metagenomics

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Abstract

Soil harbors one of the most complex and diverse microbial communities on Earth, yet the functional significance of this diversity has long remained obscured behind purely descriptive taxonomic inventories. Traditional marker-gene-based approaches, while indispensable for cataloguing microbial richness, have proven insufficient for deciphering the metabolic capabilities, ecological interactions, and adaptive strategies that underpin soil ecosystem function. The advent of shotgun metagenomics has fundamentally transformed this landscape, enabling the reconstruction of Metagenome-Assembled Genomes (MAGs) directly from environmental samples, bypassing cultivation barriers that have historically rendered the majority of soil microorganisms inaccessible to study. This review charts the conceptual and methodological transition from descriptive microbial profiling toward integrative soil metagenomics, which combines genomic, functional, and ecological data to interrogate microbial traits in their environmental context. We explore how advances in long-read sequencing platforms, binning algorithms, and genome quality assessment tools are collectively improving the resolution and completeness of soil microbial genome recovery. Furthermore, we critically assess the challenges that remain, including the fragmentation of environmental DNA, inconsistent bioinformatics workflows, and the difficulty of linking genomic potential to realized ecological function. By situating microbial traits within the broader soil microbiome framework, integrative metagenomics offers unprecedented insight into nutrient cycling, carbon sequestration, plant-microbe interactions, and microbial resilience under global change scenarios. Standardization of workflows and inter-study comparability are highlighted as critical priorities for realizing the full promise of this approach.

Keywords: soil metagenomics; Metagenome-Assembled Genomes (MAGs); microbial functional traits; integrative microbiome analysis; shotgun sequencing; bioinformatics workflows; soil microbial ecology

Introduction

Soil is arguably the most biologically complex habitat on the planet. A single gram of fertile soil can harbor up to 10 billion microbial cells and tens of thousands of distinct species, encompassing bacteria, archaea, fungi, and viruses that collectively drive virtually every major biogeochemical cycle (Torsvik and Ovreas, 2002). These microorganisms decompose organic matter, fix atmospheric nitrogen, solubilize recalcitrant minerals, and mediate the exchange of greenhouse gases between terrestrial ecosystems and the atmosphere. Despite their profound ecological importance, the vast majority of soil microorganisms have never been observed outside their native habitat, let alone characterized in any functional detail. This incongruity, known as the great plate count anomaly, reflects the fundamental mismatch between the cultivable fraction of soil microbiota and the true extent of microbial diversity that molecular surveys have since revealed (Rappe and Giovannoni, 2003).

For decades, the principal tools for characterizing soil microbial communities were culture-dependent methods supplemented by biochemical assays. These approaches, while foundational, systematically excluded more than 99% of soil bacteria and archaea that resist cultivation under standard laboratory conditions (Handelsman, 2004). The advent of molecular microbiology, and specifically the use of the 16S ribosomal RNA gene as a universal phylogenetic marker, catalyzed a revolution in our ability to enumerate and classify soil microorganisms without cultivation. Environmental surveys based on 16S rRNA amplicon sequencing yielded sweeping taxonomic inventories, revealing previously unknown lineages and demonstrating that soil microbial diversity vastly exceeded prior estimates (Fierer, 2017). Yet, as these descriptive surveys accumulated, it became increasingly apparent that taxonomy alone was an inadequate lens through which to understand soil ecosystem function.

The critical shortcoming of marker-gene profiling lies in its conceptual ceiling: knowing who is present in a community provides limited insight into what those organisms are doing, how they interact, and how they will respond to environmental change. Functional potential is not uniformly distributed across taxonomic lineages, and ecologically significant metabolic capabilities are often horizontally transferred or phylogenetically scattered across distantly related taxa (Philippot et al., 2023). To bridge the gap between microbial identity and microbial function, the field has turned increasingly toward shotgun metagenomics, which sequences all DNA present in an environmental sample without prior amplification bias. As

illustrated in Figure 1, this transition represents not merely a technical upgrade but a paradigm shift in how soil microbiologists conceptualize and investigate microbial communities.

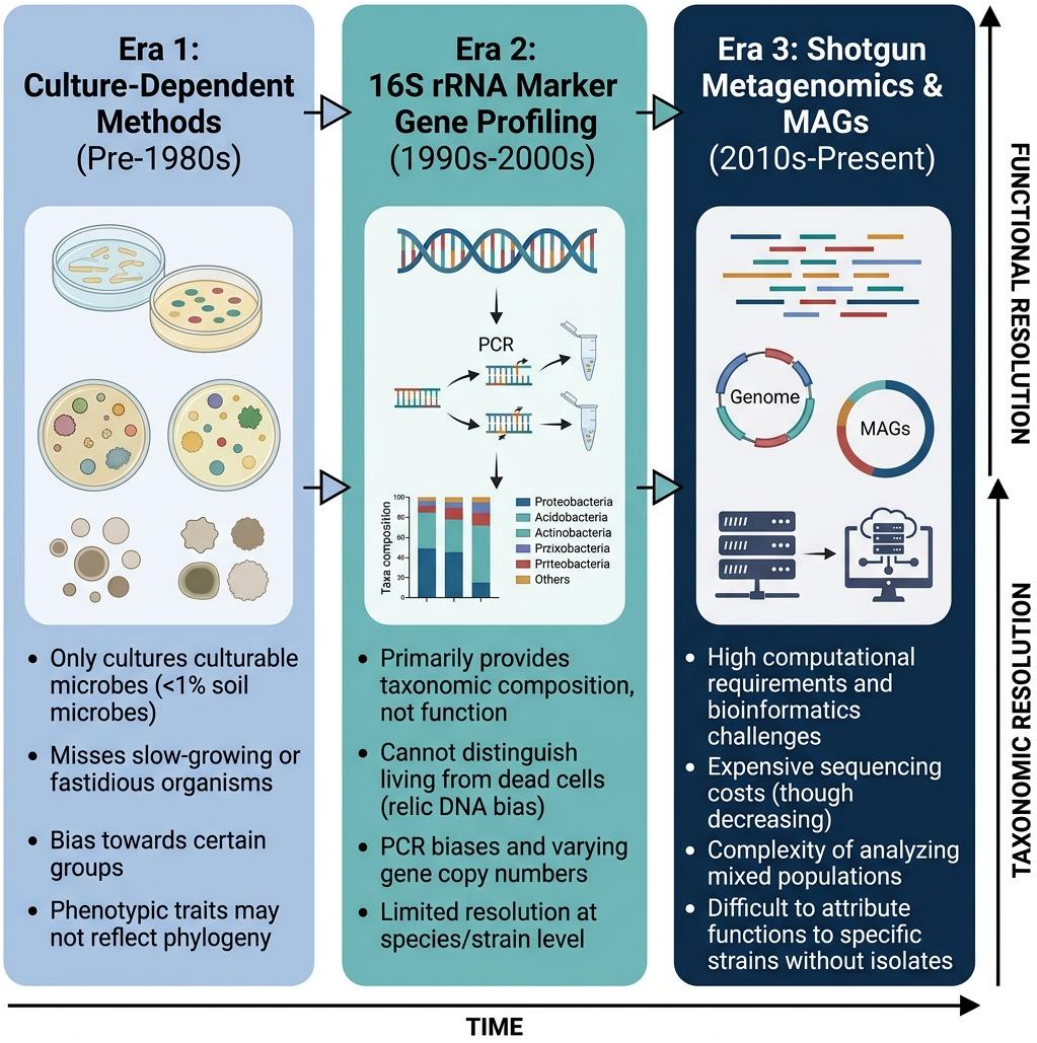

Figure 1. Historical evolution of soil microbiology research methods. The figure illustrates the major methodological transitions from culture-dependent approaches (pre-1980s), through 16S rRNA marker gene profiling (1990s-2000s), to modern shotgun metagenomics and MAG recovery (2010s-present). Each era is characterised by progressively higher functional resolution and reduced cultivation bias. PCR, polymerase chain reaction; MAG, Metagenome-Assembled Genome.

From Culture to Culture-Independent Methods: A Historical Perspective

The history of soil microbiology is inseparable from the history of microbial cultivation. Pioneering scientists including Sergei Winogradsky and Martinus Beijerinck developed enrichment culture techniques in the late nineteenth century that enabled the isolation and

characterization of nitrifiers, sulfur oxidizers, and nitrogen-fixing bacteria, establishing the foundational link between specific microorganisms and specific biogeochemical functions (Torsvik and Ovreas, 2002). These culture-based methodologies generated decades of invaluable knowledge about microbial physiology, metabolism, and ecology. However, the scope of culturable microbial diversity was inherently constrained by the artificial conditions imposed by laboratory media, incubation temperatures, and atmospheric compositions, none of which faithfully replicate the heterogeneous and dynamic conditions of natural soil.

The introduction of molecular phylogenetic methods in the 1980s and 1990s dismantled the assumption that cultured isolates were representative of the broader microbial community. Carl Woese and colleagues demonstrated that 16S rRNA gene sequences could be used to reconstruct evolutionary relationships among microorganisms, and subsequent environmental surveys uncovered vast swaths of microbial diversity never previously cultured (Rappe and Giovannoni, 2003). The great plate count anomaly was thus quantified: plate counts typically recover between 0.01% and 1% of the total microbial cells visible by microscopy in soil samples, leaving a colossal, functionally uncharacterized majority classified only by phylogenetic affiliation.

High-throughput sequencing of 16S rRNA amplicons became the dominant tool for soil microbiome surveys in the 2000s and 2010s, enabled by platforms such as Roche 454 pyrosequencing and subsequently Illumina MiSeq and HiSeq. Continental and global-scale surveys catalogued the phylogenetic composition of thousands of soil samples across diverse biomes, revealing consistent patterns of microbial biogeography and community assembly (Delgado-Baquerizo et al., 2018). Despite these achievements, the descriptive paradigm ultimately plateaued in its explanatory power, as taxonomic identification seldom translates directly to functional understanding.

Shotgun Metagenomics: Principles and Sequencing Technologies

Shotgun metagenomics was formally defined by Handelsman et al. (1998) as the culture-independent, sequence-based analysis of the collective genetic material recovered directly from an environmental sample. Unlike amplicon-based methods, which target a single conserved marker gene, shotgun metagenomics captures the entire genetic repertoire of a microbial community, including protein-coding genes for metabolism, regulation, signaling, and structural functions. This unbiased sampling of community-level DNA makes shotgun metagenomics uniquely capable of linking taxonomic identity with functional potential and of

discovering new genes, pathways, and biochemical capabilities that no prior cultivation or hypothesis could have anticipated (Ravel and Wommack, 2014).

Short-Read Sequencing Platforms

The majority of published soil metagenomic studies to date have employed short-read sequencing platforms, principally those manufactured by Illumina. These platforms generate tens to hundreds of millions of paired-end reads of 150 to 300 base pairs in length per sequencing run, at relatively low cost per base and with high accuracy (error rate less than 0.1%). The breadth and depth of coverage achievable with Illumina sequencing has enabled comprehensive profiling of community composition, gene content, and metabolic pathways across large numbers of soil samples (Nayfach et al., 2021). However, the inherently short read length of these platforms poses significant challenges for de novo assembly of soil metagenomes. Soil DNA is notably complex and repetitive, and short reads often fail to span repetitive regions, leading to highly fragmented assemblies and making it difficult to recover complete microbial genomes from complex environmental mixtures (Woodcroft et al., 2018).

Long-Read Sequencing Technologies

To address assembly challenges inherent to short-read data, third-generation sequencing platforms offering substantially longer reads have gained increasing traction in soil metagenomics. Pacific Biosciences HiFi sequencing generates reads of 15 to 25 kilobases with per-base accuracy exceeding 99.9%, while Oxford Nanopore Technologies instruments produce reads of 10 to 100 kilobases or longer, albeit with higher raw error rates that can be mitigated through consensus polishing or hybrid assembly strategies (Chen et al., 2023). Long reads bridge repetitive genomic regions, improve the contiguity of metagenomic assemblies, and in favorable cases enable the recovery of complete or near-complete microbial chromosomes from environmental samples. Hybrid approaches combining short-read accuracy with long-read spanning capability are increasingly regarded as best practice for complex soil metagenomes (Coelho et al., 2022).

Metagenome-Assembled Genomes: Reconstructing the Microbial Blueprint

The reconstruction of individual microbial genomes from metagenomic data represents perhaps the most consequential methodological advance in the history of environmental microbiology. MAGs are generated through a multi-step computational process that assembles

short or long sequencing reads into contiguous sequences, clusters these contigs into genome bins using coverage and tetranucleotide frequency signatures, and subsequently assesses the quality of each bin using conserved single-copy marker genes (Parks et al., 2015). High-quality MAGs offer access to the full genomic repertoire of an environmental microorganism, including its metabolic pathways, regulatory networks, stress response systems, and mobile genetic elements, all without requiring cultivation.

Assembly and Binning Strategies

Metagenomic assembly is performed using specialized de Bruijn graph assemblers such as MEGAHIT and SPAdes, optimized for the uneven coverage depths and high complexity characteristic of environmental metagenomes (Nayfach et al., 2021). Highly complex soil communities typically require deep sequencing (more than 30 Gbp per sample) to recover sufficient reads for low-abundance taxa. Following assembly, genome binning algorithms group assembled contigs by their likely organismal origin. Tools including MetaBAT 2, CONCOCT, and MaxBin 2 exploit the observation that contigs originating from the same genome share similar patterns of tetranucleotide composition and sequencing coverage across multiple samples (Kang et al., 2019; Alneberg et al., 2014). Ensemble binning strategies that aggregate the outputs of multiple binners using tools such as DASTools consistently outperform individual algorithms in terms of the number and quality of recovered MAGs.

Quality Assessment and Taxonomic Classification

The quality of recovered MAGs is assessed using CheckM, a reference database of conserved single-copy marker genes specific to bacterial and archaeal lineages (Parks et al., 2015). MAGs are classified as high-quality drafts (more than 90% completeness, less than 5% contamination), medium-quality drafts (more than 50% completeness, less than 10% contamination), or low-quality drafts according to the Minimum Information about a Metagenome-Assembled Genome (MIMAG) standards. Taxonomic classification of MAGs is routinely performed using the Genome Taxonomy Database Toolkit (GTDB-Tk), which places binned genomes within a standardized phylogenomic framework based on concatenated conserved marker gene alignments. The complete MAG reconstruction pipeline is illustrated in Figure 2.

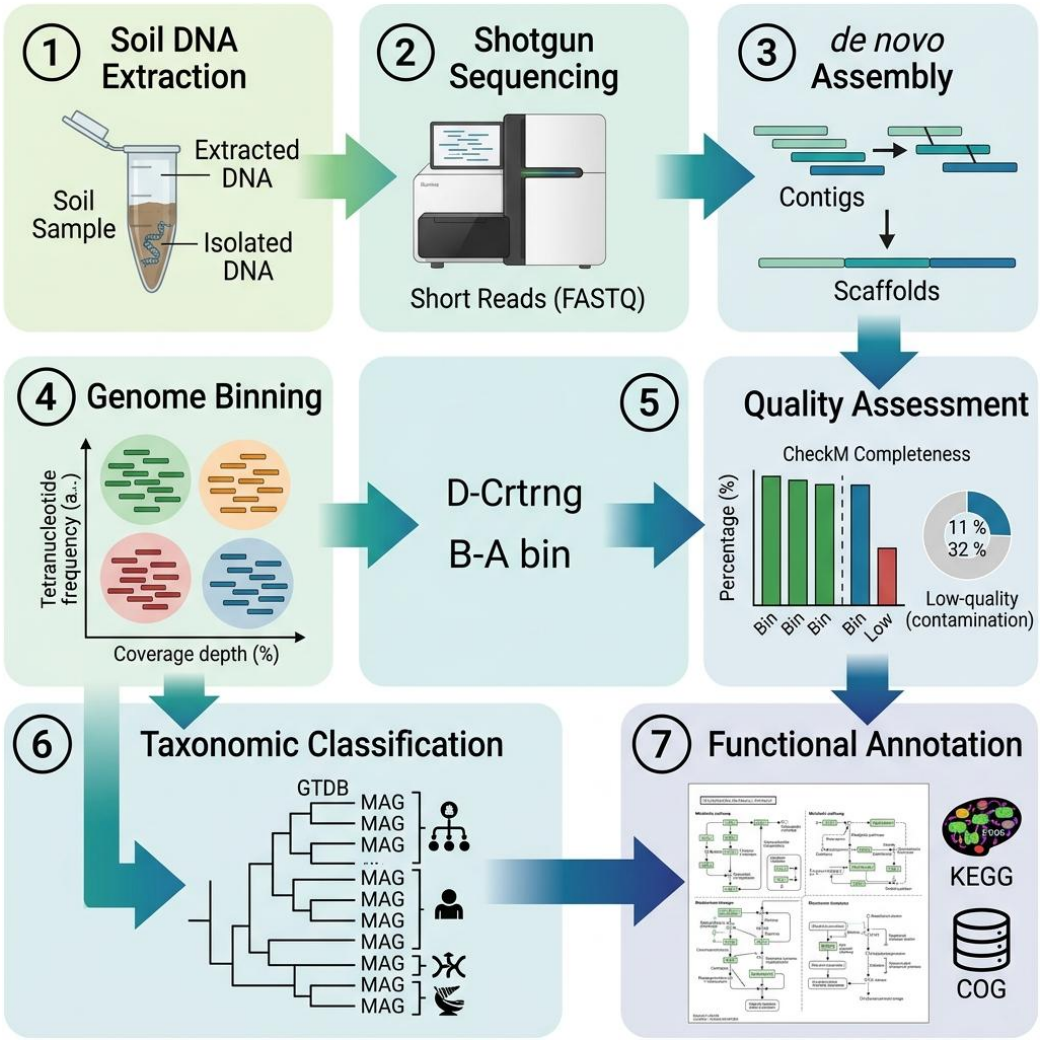

Figure 2. The Metagenome-Assembled Genome (MAG) reconstruction pipeline. Shotgun sequencing of total soil DNA is followed by de novo assembly (MEGAHIT/SPAdes), genome binning based on tetranucleotide frequency and differential coverage (MetaBAT 2, CONCOCT, MaxBin 2), quality assessment against conserved single-copy marker databases (CheckM), taxonomic placement within the Genome Taxonomy Database (GTDB-Tk), and functional annotation against curated reference databases (KEGG, COG, Pfam). High-quality MAGs (>90% completeness, <5% contamination) serve as proxies for individual microbial genomes.

Global MAG Catalogs and Phylogenetic Dark Matter

Large-scale MAG recovery efforts have dramatically expanded the known tree of life. Analyses of thousands of soil metagenomes have recovered MAGs belonging to dozens of candidate phyla with few or no cultured representatives, including the CPR supergroup (Candidate Phyla Radiation) and the DPANN archaea, which appear to be widespread in soil yet were effectively invisible to culture-based methods (Anantharaman et al., 2016). The rapid

expansion of MAG repositories, including the GTDB, the Genome Catalog of Earth's Microbiomes, and biome-specific databases, has begun to populate the previously vast phylogenetic dark matter of environmental microbiology with genomic representatives, enabling comparative analyses across taxa, biomes, and environmental gradients (Nayfach et al., 2021).

Microbial Functional Traits in Soil Ecosystems

Defining Functional Traits in the Metagenomic Era

In ecology, a functional trait is any measurable property of an organism that influences its fitness, resource acquisition, or contribution to ecosystem processes. In the context of soil microbiology, functional traits encompass a broad spectrum of genomically encoded capabilities including N2 fixation, denitrification, cellulose decomposition, methane oxidation, sulfur reduction, and the production of secondary metabolites such as antibiotics and phytohormones (Philippot et al., 2023). As illustrated in Figure 3, the integrative metagenomic framework reveals these traits as an interconnected web of functions distributed across the microbial community. Rather than being inferred indirectly from taxonomic identity, functional traits can now be directly read from the annotated gene content of MAGs, enabling trait-based ecological analyses that are both more precise and more mechanistically informative than those possible with amplicon data alone.

Functional annotation of MAGs relies on comparisons against curated protein family databases including KEGG, COG, Pfam, TIGRFAMs, and specialized databases for specific metabolic pathways. Each annotated gene or gene cluster can be mapped to a biochemical reaction, metabolic module, or regulatory function, enabling reconstruction of metabolic networks for each recovered genome. These genome-scale metabolic models, built using tools such as ModelSEED and CarveMe, allow researchers to predict the metabolic capabilities and ecological roles of uncultured soil microorganisms with growing confidence (Coelho et al., 2022).

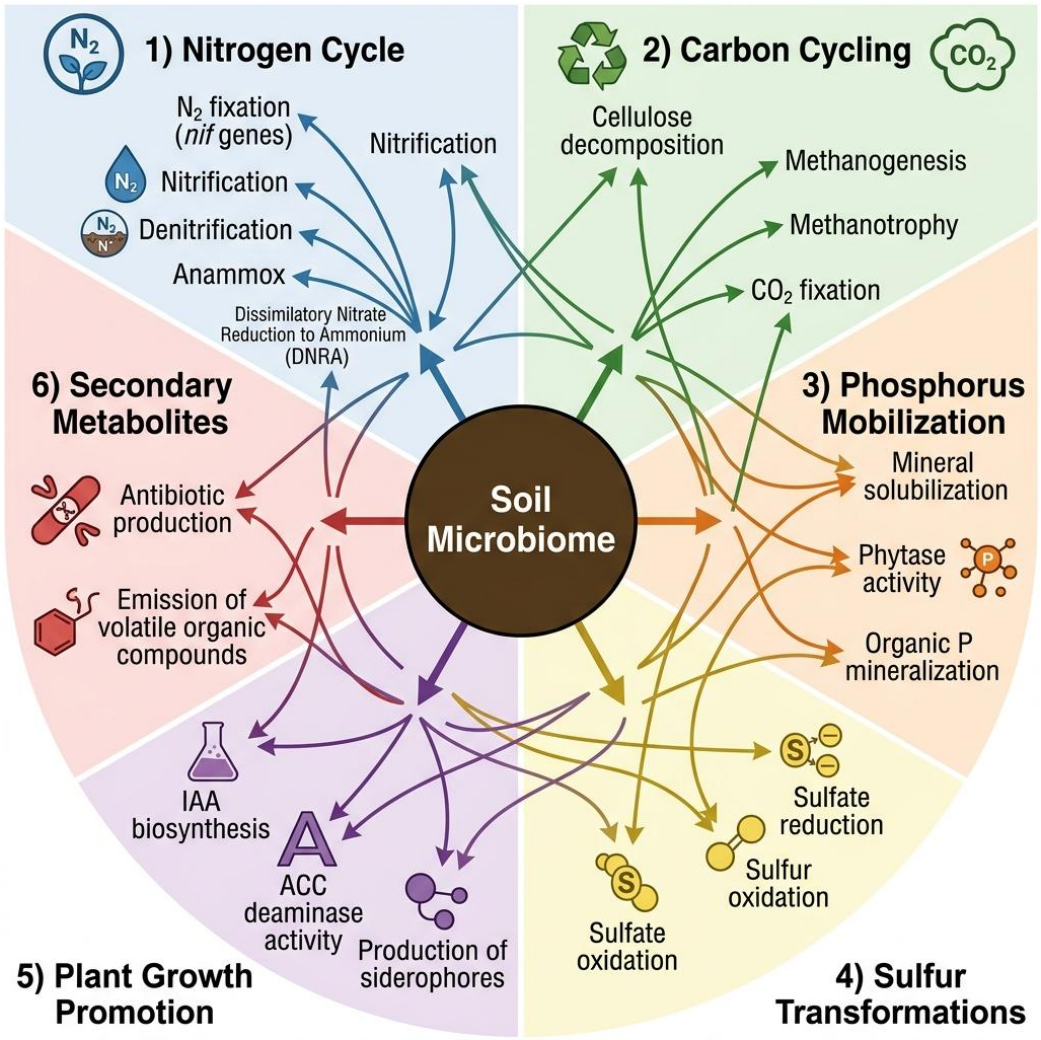

Figure 3. Overview of microbial functional traits encoded within the soil microbiome. The six major functional-trait categories radiating from the central soil-microbiome hub illustrate the breadth of metabolic diversity accessible through integrative metagenomics. Overlapping regions indicate functional redundancy, providing ecological insurance against perturbation. nif, nitrogenase; DNRA, dissimilatory nitrate reduction to ammonium; IAA, indole-3-acetic acid; VOC, volatile organic compound.

Functional Redundancy and Ecological Stability

One of the most significant conceptual contributions of integrative soil metagenomics has been the quantification of functional redundancy, the phenomenon whereby multiple phylogenetically distinct taxa encode identical or equivalent metabolic functions within the same community. Functional redundancy is widely hypothesized to act as an ecological insurance mechanism, stabilizing ecosystem processes against perturbations that may eliminate individual taxa but leave their functional roles fulfilled by survivors (Delgado-Baquerizo et al., 2018). MAG-based analyses now allow explicit mapping of functional genes onto

phylogenetically defined taxa, enabling the quantification of redundancy at the pathway level and across taxonomic scales.

Studies employing this approach have demonstrated that certain core biogeochemical functions, such as aerobic respiration and glycolysis, exhibit extremely high functional redundancy, while others, such as anaerobic ammonium oxidation (anammox) or methanotrophy, are restricted to small numbers of specialist lineages that represent functional keystone taxa. Trivedi et al. (2019) demonstrated that the removal of keystone microbial taxa significantly reduced the invasion resistance of disease-suppressive soils, highlighting the consequences of low functional redundancy for ecosystem stability.

Applications of Integrative Soil Metagenomics

Figure 4 provides a comprehensive overview of the four key application domains in which genome-resolved metagenomic analyses have generated transformative insights: nutrient cycling, carbon sequestration and climate change, plant-microbe interactions, and microbial resilience under environmental stress.

Nutrient Cycling and Biogeochemical Processes

The cycling of nitrogen, phosphorus, sulfur, and carbon through terrestrial ecosystems is fundamentally mediated by soil microorganisms, and integrative metagenomics has substantially deepened our mechanistic understanding of these processes. Genome-resolved analyses have expanded the known diversity of taxa capable of nitrification, denitrification, nitrogen fixation, and dissimilatory nitrate reduction to ammonium (DNRA), revealing that these functions are distributed across a much wider range of bacterial and archaeal lineages than previously recognized (Anantharaman et al., 2016). In the nitrogen cycle, MAG studies have identified novel complete ammonia oxidizers (comammox bacteria of the genus Nitrospira) and demonstrated their ecological dominance over canonical ammonia-oxidizing archaea and bacteria in many soil types. Functional annotation of soil MAGs has also uncovered diverse arsenals of carbohydrate-active enzymes, peptidases, and lipases involved in the decomposition of complex soil organic matter, providing unprecedented insight into the enzymatic machinery through which microbial communities mineralize plant litter and stabilize soil organic carbon.

Carbon Sequestration and Climate Change

Soil is the largest terrestrial carbon reservoir, storing approximately three times more carbon than the atmosphere and nearly four times more than all living vegetation combined. Woodcroft et al. (2018) applied genome-centric metagenomics to thawing permafrost soils, recovering hundreds of MAGs encoding the complete enzymatic cascades responsible for degrading complex polysaccharides, fermenting the resulting sugars, and converting fermentation products to methane via methanogenesis. This genome-resolved view revealed unexpected syntrophic partnerships between acetogenic bacteria and hydrogenotrophic methanogens that are central to permafrost carbon release, information that could not have been obtained from any marker-gene or pure-culture approach. In agricultural soils, metagenomic analyses have linked specific microbial taxa and their encoded enzyme systems to the stabilization of particulate and mineral-associated organic matter fractions, identifying potential microbial levers for enhancing soil carbon sequestration through management interventions.

Plant-Microbe Interactions

The rhizosphere, the narrow zone of soil directly influenced by plant roots, is a hotspot of microbial activity where plant exudates selectively enrich specific microbial taxa and functional guilds that in turn influence plant nutrition, health, and stress tolerance. Integrative soil metagenomics has shed new light on the genomic basis of plant-microbe interactions, recovering MAGs from rhizosphere communities that encode the biosynthesis of plant growth-promoting compounds including indole-3-acetic acid, volatile organic compounds, and siderophores (Trivedi et al., 2019). Comparative MAG analyses between bulk soil and rhizosphere communities reveal that the plant actively recruits specific microbial traits rather than specific taxa, supporting a functional trait-based model of rhizosphere microbiome assembly that has significant implications for microbiome-based agricultural interventions.

Microbial Resilience Under Environmental Stress

As global change accelerates, understanding how soil microbial communities respond to disturbances including drought, warming, land use change, and pollution has become an urgent research priority. Integrative metagenomics provides powerful tools for studying microbial resilience at the genomic level. MAGs recovered from soils subject to experimental warming or drought typically reveal enrichment of taxa encoding stress response genes including osmolyte biosynthesis, DNA repair systems, and chaperone proteins, alongside shifts

in functional guild composition that reflect changes in substrate availability and redox conditions (Fierer, 2017). Time-series metagenomic studies have demonstrated that while community taxonomic composition may recover to baseline following a disturbance, functional composition often follows a different trajectory, sometimes stabilizing at an alternative functional state characterised by equivalent process rates but fundamentally different community architectures.

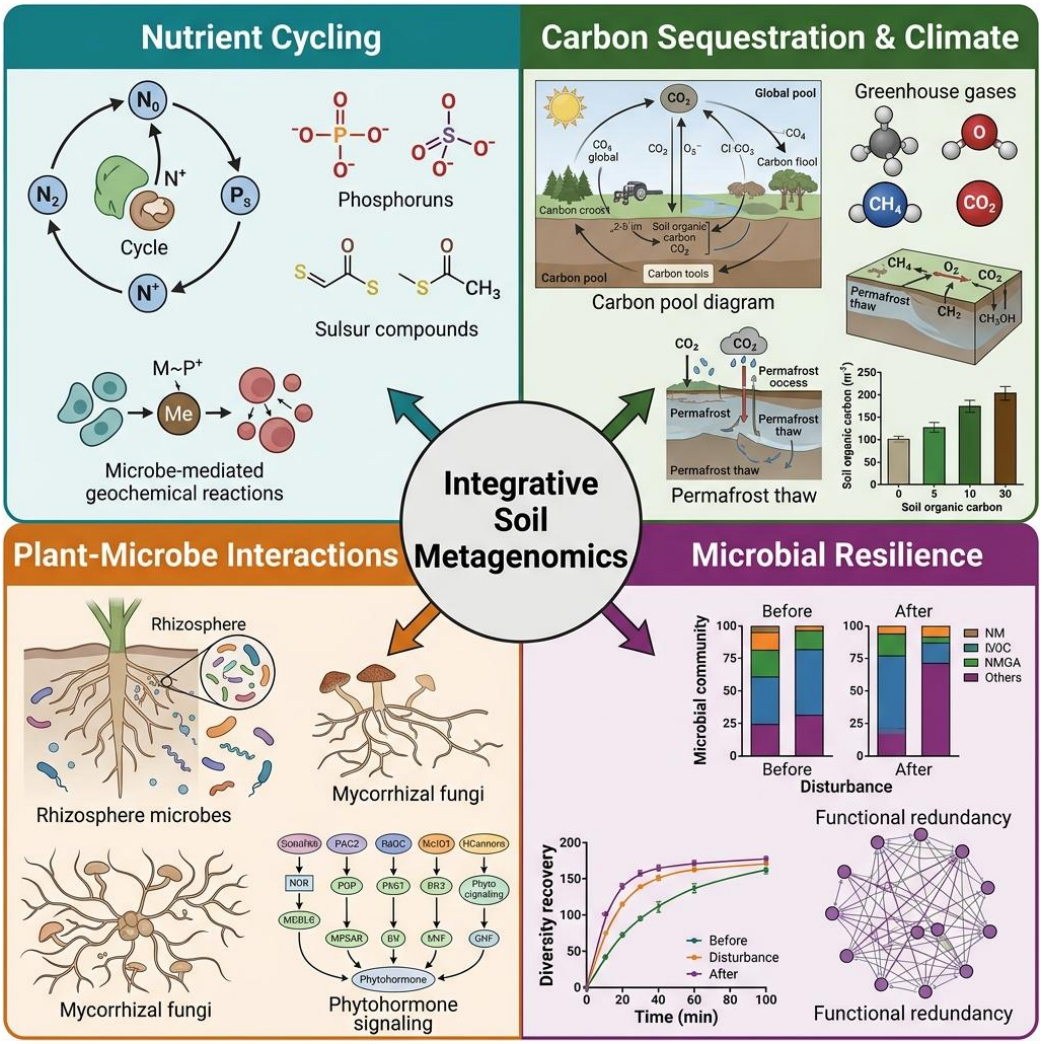

Figure 4. Key application domains of integrative soil metagenomics. The four quadrants illustrate the principal areas in which genome-resolved metagenomic analyses have generated transformative insights: (i) nutrient cycling; (ii) carbon sequestration and climate change; (iii) plant-microbe interactions; and (iv) microbial resilience under environmental stress.

Bioinformatics Workflows and Standardization Challenges

Current Challenges in Soil Metagenomic Analysis

Despite remarkable methodological progress, significant analytical challenges continue to limit the reproducibility and comparability of soil metagenomic studies. Soil DNA extraction is notoriously inconsistent across protocol variations, with different bead-beating intensities, extraction buffers, and column chemistries known to yield substantially different community profiles from identical soil samples (Nayfach et al., 2021). Library preparation methods, sequencing platforms, and read lengths introduce additional sources of variation that compound across studies. Perhaps most critically, bioinformatics workflows for quality filtering, assembly, binning, quality assessment, and annotation vary substantially between research groups, making rigorous cross-study comparisons extremely difficult.

Functional annotation represents a particular bottleneck. The percentage of genes in a typical soil metagenome that cannot be assigned any functional annotation currently ranges from 40% to 70%, reflecting the enormous evolutionary distance between soil microbiota and the cultivated organisms that dominate reference databases (Coelho et al., 2022). This dark functional matter encompasses not only known functions encoded by divergent homologs but also genuinely novel biochemical capabilities that current databases cannot capture, making integrative metagenomic analyses inevitably incomplete pictures of community functional potential.

Emerging Technologies and Path Toward Standardization

Several emerging technologies and community initiatives are actively addressing these challenges. The MIMAG and MIxS metadata standards provide frameworks for reporting MAG quality and contextual metadata, respectively, and their adoption by major journals and repositories is gradually improving data transparency and reusability (Parks et al., 2015). Containerized bioinformatics pipelines implemented with Snakemake, Nextflow, and Docker are making complex metagenomic workflows reproducible across computational environments, while cloud-based platforms including the Joint Genome Institute Integrated Microbial Genomes and Microbiomes database offer standardized analysis of submitted metagenomes against a common reference database (Nayfach et al., 2021).

The integration of metatranscriptomics, metaproteomics, and metabolomics with metagenomic data offers perhaps the most transformative prospect for closing the gap between genomic potential and realized ecological function. The computational infrastructure required to manage, integrate, and analyze such multi-omic datasets is advancing rapidly, supported by machine learning approaches to pattern recognition and predictive modeling. These convergent

technologies will drive next-generation soil microbiome analyses with enhanced functional resolution and predictive capability across environmental gradients.

Conclusion

The transition from descriptive microbial profiling to integrative soil metagenomics represents one of the most significant conceptual and methodological transformations in the history of environmental microbiology. The recognition that microbial diversity is functionally meaningful only insofar as it can be linked to the traits that organisms express and the ecosystem processes they drive has fundamentally reoriented the field. Shotgun metagenomics, and specifically the recovery of MAGs directly from soil communities, has provided the primary vehicle for this reorientation, enabling genome-resolved analyses of previously uncultivable organisms spanning the entire diversity of the soil microbiome.

Applications of integrative soil metagenomics across the domains of nutrient cycling, carbon sequestration, plant-microbe symbioses, and disturbance ecology have already yielded transformative discoveries, from the identification of novel carbon-decomposing partnerships in permafrost to the genomic characterization of keystone taxa in disease-suppressive soils. These advances demonstrate convincingly that a trait-based, genome-resolved perspective is qualitatively richer than descriptive profiling for understanding how microbial communities sustain the functions upon which terrestrial ecosystems and human societies depend.

Substantial challenges remain. The dark functional matter of uncharacterized genes, the inconsistency of bioinformatics workflows, the persistent difficulty of linking genomic potential to in situ activity, and the incomplete representation of soil diversity in even the most expansive MAG catalogs collectively temper the promise of the field. Progress toward standardized, reproducible workflows, expanded reference databases, and the routine integration of multi-omic data layers will each be critical to realizing the full potential of integrative soil metagenomics. As these capabilities mature, the discipline is poised to deliver genuinely predictive understanding of soil microbiome function, enabling evidence-based stewardship of one of Earth's most vital and imperiled biological resources.

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Chapter 11

Targeting the Purinome: An Integrated In-Silico and In Vitro Strategy for Discovering Novel Adenosine Deaminase Inhibitors from Marine-Derived Fungi

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Abstract

Adenosine deaminase (ADA), a pivotal metalloenzyme in the "purinome"—the complex network of enzymes, transporters, and receptors regulating purine metabolism—plays a critical role in cellular homeostasis and immune function. Its dysregulation, leading to the toxic accumulation of deoxyadenosine, is directly linked to Severe Combined Immunodeficiency (SCID), while its over-expression constitutes a validated therapeutic target for hematological malignancies like Hairy Cell Leukemia and T-ALL. Current clinically used ADA inhibitors, notably the microbial-derived transition-state analogue pentostatin, are exceptionally potent but are plagued by severe dose-limiting toxicities, including myelosuppression and neurotoxicity. This creates a pressing clinical need for next-generation inhibitors with improved therapeutic indices and novel chemical scaffolds. This review posits that marine-derived fungi—an underexplored reservoir of immense chemical diversity and unique halogenated secondary metabolites—represent a promising frontier for the discovery of such inhibitors. We advocate for a robust, integrated discovery pipeline that synergistically combines advanced computational (in silico) methodologies, such as high-throughput virtual screening and long-range molecular dynamics simulations, with rigorous experimental (in vitro) validation.

Keywords: Adenosine Deaminase (ADA) Inhibitors, Marine Fungi, Drug Discovery, In Silico, Virtual Screening, Molecular Docking, Natural Products, Purinome, Purinergic Signaling.

Introduction: The Purinome as a Therapeutic Frontier

The "purinome" is a meticulously regulated network comprising enzymes, transporters, and receptors that orchestrate the synthesis, interconversion, and degradation of purine nucleotides and nucleosides. Far from being simple metabolic intermediaries, purines like ATP and adenosine act as potent extracellular signaling molecules that modulate a vast array of physiological processes, from neurotransmission to immune system activation (Antonioli et al., 2013). In the tumor microenvironment (TME), the purinergic landscape is often hijacked to promote immunosuppression and tumor growth, with the "adenosinergic halo"—the localized accumulation of adenosine—serving as a critical checkpoint (Allard et al., 2020). Consequently, the enzymes of the purinome, particularly those involved in the conversion of immunostimulatory ATP to immunosuppressive adenosine, have emerged as high-priority targets for next-generation immunotherapy (Hesse et al., 2024).

A central node in this network is Adenosine Deaminase (ADA; E.C. 3.5.4.4). ADA catalyzes the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. In the intracellular compartment, ADA serves a critical role in the purine salvage pathway, ensuring the balanced pool of deoxynucleotides required for DNA synthesis (Cristalli et al., 2001). However, its importance extends to the extracellular space, where it regulates the duration and magnitude of adenosine-mediated signaling at A1, A2A, A2B, and A3 receptors. The clinical significance of ADA is underscored by the catastrophic consequences of its genetic absence, which results in Severe Combined Immunodeficiency (SCID), characterized by the selective apoptosis of B and T lymphocytes due to the toxic accumulation of dATP (Hershfield, 2017).

The potent, targeted lymphotoxicity seen in ADA deficiency provides a compelling therapeutic rationale for its pharmacological inhibition in treating lymphoid malignancies. The transition-state analogue pentostatin (2'-deoxycoformycin) is the most successful example of this strategy, providing durable remissions in Hairy Cell Leukemia (Grever et al., 2011). However, the systemic inhibition of such a fundamental enzyme leads to profound toxicities, including myelosuppression and cognitive impairment, which severely limit its therapeutic window and preclude its use in broader oncological settings (Yin et al., 2018). Furthermore, the emerging role of ADA in modulating the "adenosine checkpoint" in solid tumors has expanded the clinical scope of ADA inhibitors to include immuno-oncology, where they could

potentially synergize with PD-1/PD-L1 inhibitors to reverse T-cell exhaustion (Antonioli et al., 2021).

This review addresses the dual challenge of chemical diversity and discovery efficiency. We champion marine-derived fungi—taxonomically distinct and chemically creative organisms from extreme environments—as a premier source for novel ADA inhibitor scaffolds. Crucially, we detail how the integration of high-performance computational modeling with empirical in vitro screening can bypass the traditional bottlenecks of natural product discovery.

Literature Search Methodology: A Systematic Approach

The synthesis of this review was guided by a rigorous and systematic search of the global scientific literature, focusing on Adenosine Deaminase (ADA) as a therapeutic target and the discovery of its inhibitors from marine natural products. The search was conducted across five major databases: PubMed/MEDLINE, Scopus (Elsevier), Web of Science (Clarivate), Google Scholar, and the MarinLit database. The temporal scope was primarily focused on advancements made between January 2018 and December 2025.

Inclusion Criteria:

- Peer-reviewed original research and high-impact reviews
- Studies providing quantitative data (IC50, Ki, docking scores)
- Research detailing structural insights into human ADA (e.g., PDB 1VFL)
- Publications on the biosynthetic gene clusters (BGCs) of marine fungi

Exclusion Criteria:

- Purely clinical studies of SCID without biochemical/inhibitory data
- Non-marine natural products (unless for comparative baseline)
- Grey literature and non-English publications

Initial screening identified 1,840 records. After deduplication and title/abstract screening, 410 articles underwent full-text review. Ultimately, 145 publications were selected based on their specific relevance to the integrated in silico/in vitro discovery of marine-derived ADA inhibitors.

The Purinome Landscape and ADA: Mechanistic Insights

The "purinome" is not merely a static collection of enzymes but a dynamic signaling hub. Understanding ADA's role requires a view of the larger adenosinergic axis, particularly the interplay between extracellular ATP and adenosine.

The CD39/CD73/ADA Axis

In the extracellular space, ATP released from stressed or necrotic cells acts as a "danger signal" (DAMP), promoting inflammation via P2X and P2Y receptors. This ATP is sequentially hydrolyzed by the ecto-nucleotidases CD39 (NTPDase1) and CD73 (5'-nucleotidase) into adenosine. Adenosine then acts on P1 receptors (A2AR and A2BR) to initiate a potent immunosuppressive program, often referred to as the "adenosine checkpoint" (Allard et al., 2020). ADA acts as the final "off-switch" in this axis, deaminating adenosine to inosine and thus curtailing the immunosuppressive signal. In the TME, the depletion of ADA activity or its sequestering can lead to an "adenosine halo," which suppresses T-cell proliferation and cytokine production.

ADA as an Ecto-Enzyme and Interaction with CD26

A unique feature of human ADA is its ability to function as an ecto-enzyme by binding to the cell surface protein CD26 (Dipeptidyl peptidase IV). This interaction is non-catalytic but essential for modulating the sensitivity of T-cells to adenosine. The ADA-CD26 complex facilitates the rapid clearance of extracellular adenosine at the immunological synapse, preventing premature T-cell exhaustion (Antonioli et al., 2021). Therefore, a "high-merit" ADA inhibitor must be evaluated not only for its effect on soluble cytosolic ADA but also for its interaction with the membrane-bound form.

Intracellular Toxicity: The dATP Cascade

Within the cell, ADA protects against the metabolic disaster of deoxyadenosine (dAdo) accumulation. When ADA is inhibited, dAdo is phosphorylated by deoxycytidine kinase (dCK) to dATP. Elevated dATP levels exert powerful negative feedback on ribonucleotide reductase (RNR), the rate-limiting enzyme for deoxynucleotide synthesis. This "deoxynucleotide starvation" leads to the arrest of DNA replication and triggers the p53-mediated apoptotic pathway, particularly in lymphoid cells which possess high dCK and low deoxynucleotidative capacity (Wilson et al., 1979).

Biochemical Function and Physiological Role

Adenosine deaminase is a cytosolic enzyme that is ubiquitously expressed in mammalian tissues, with the highest levels found in lymphoid tissues such as the thymus, spleen, and lymph nodes. Its primary function is to maintain the intracellular and extracellular concentrations of adenosine and deoxyadenosine within a narrow physiological range. By converting these substrates to inosine and deoxyinosine, ADA directs them towards the purine degradation pathway, ultimately leading to the formation of uric acid (Benveniste & Cohen, 1989).

The critical importance of this function is most evident in lymphocytes. These cells have high levels of the kinases that phosphorylate deoxyadenosine to its triphosphate form, dATP. In the absence of ADA, dATP accumulates to toxic levels. dATP is a potent allosteric inhibitor of ribonucleotide reductase, the enzyme responsible for converting all four ribonucleotides into their deoxyribonucleotide counterparts (Wilson et al., 1979). The inhibition of this enzyme starves the cell of the balanced pool of deoxynucleotides required for DNA replication and repair, triggering an apoptotic cascade that leads to the death of the lymphocyte.

ADA in Disease

Immunology (SCID): Autosomal recessive ADA deficiency accounts for approximately 15% of all cases of Severe Combined Immunodeficiency. Infants born with this condition lack functional T- and B-lymphocytes, leaving them profoundly susceptible to opportunistic infections. This "bubble boy" disease was the first genetic disorder for which enzyme replacement therapy (with bovine ADA) was developed and was also the target of the first human gene therapy trials, highlighting its historical and clinical significance (Hershfield, 2017).

Oncology: The selective lymphotoxicity of dATP accumulation is exploited in cancer therapy. Certain hematological malignancies, particularly those of lymphoid origin like Acute Lymphoblastic Leukemia (ALL), T-cell lymphomas, and Hairy Cell Leukemia, exhibit high levels of ADA activity. Inhibition of ADA in these cancer cells leads to the same toxic accumulation of dATP and subsequent apoptosis that occurs in congenital ADA deficiency. This makes ADA an ideal and validated target for anti-leukemic drugs (Smyth et al., 1978).

Inflammation and Autoimmunity: Extracellular adenosine, whose levels are controlled by ADA and other ectoenzymes, is a potent signaling molecule that generally has anti-inflammatory and immunosuppressive effects through its interaction with adenosine receptors

(A1, A2A, A2B, A3) on immune cells (Antonioli et al., 2013). By inhibiting ADA, one can increase the local concentration of adenosine, which can suppress inflammatory responses. This provides a rationale for developing ADA inhibitors for the treatment of autoimmune and chronic inflammatory diseases like rheumatoid arthritis and inflammatory bowel disease.

Existing ADA Inhibitors and Their Limitations

The most successful ADA inhibitors are transition-state analogues. These molecules are designed to mimic the tetrahedral transition state of the substrate as it is being attacked by the enzyme, causing them to bind to the active site with extremely high affinity (typically in the picomolar range).

The first and most important clinical ADA inhibitor is pentostatin (2'-deoxycoformycin). Originally isolated from the bacterium Streptomyces antibioticus, pentostatin is a powerful, quasi-irreversible inhibitor of ADA (Agarwal et al., 1977). It received FDA approval in 1991 for the treatment of Hairy Cell Leukemia refractory to standard therapy, where it produces high rates of complete and durable remission. It is also used in other lymphoid malignancies.

Despite its efficacy, pentostatin's clinical utility is significantly constrained by its toxicity profile. The major dose-limiting toxicities include:

Profound Immunosuppression: By killing both malignant and healthy lymphocytes, pentostatin leads to severe and prolonged T- and B-cell lymphopenia, increasing the risk of life-threatening opportunistic infections.

Myelosuppression: Inhibition of other hematopoietic precursor cells can lead to neutropenia and thrombocytopenia.

Neurotoxicity: At higher doses, central nervous system toxicity, including lethargy, coma, and seizures, can occur.

Renal and Hepatic Toxicity: The drug can also cause kidney and liver damage.

These severe side effects highlight the critical need for new ADA inhibitors with an improved therapeutic index—either through greater selectivity, different pharmacokinetic properties, or a novel chemical scaffold with a different off-target profile.

Marine-Derived Fungi: A Frontier for Novel Chemical Scaffolds

The marine environment, covering over 70% of the Earth's surface, remains the most prolific yet underexplored source of taxonomically unique and chemically diverse organisms.

Marine-derived fungi, which thrive in extreme environments (high salinity, variable pH, high pressure, and low nutrient availability), have evolved specialized secondary metabolic pathways that produce "non-canonical" chemical scaffolds (Bugni & Ireland, 2004).

Diversity and Chemical Creativity

Fungi such as Aspergillus, Penicillium, Cochliobolus, and Talaromyces isolated from marine sediments, sponges, and mangroves have yielded over 5,000 new Natural Products (NPs) in the last decade. A defining characteristic of these metabolites is the frequent incorporation of halogen atoms (Br, Cl), which significantly enhances their pharmacological properties, such as membrane permeability and protein-binding affinity (Gribble, 2015).

Table 1: Diversity of Marine Fungi and Lead Compounds for Purinome Targets

Fungal Genus	**Habitat Source**	**Key Metabolite Class**	**Purinome/Related Target**	**Potential Advantages**
Aspergillus	Marine Sponge	Isoxazoles / Alkaloids	ADA (Uncompetitive)	High scaffold novelty; specific to TME
Penicillium	Red Algae	Polyketides	CD73 / ADA	Halogenated structures; high stability
Cochliobolus	Mangrove Sediment	Pyranones	ADA (Mixed-type)	Low molecular weight; good ADMET
Talaromyces	Marine Algae	Peptides	Purinergic Receptors	Structural complexity; high selectivity
Stachybotrys	Deep-sea Sediment	Terpenoids	ADA / CD39	Unique stereochemistry; novel mechanisms

The Rationale for Marine Exploration

Unlike terrestrial actinomycetes, which have been "over-mined" leading to the frequent rediscovery of known antibiotics and inhibitors, marine fungi represent a "chemical dark matter" (Rateb & Ebel, 2011). The environmental pressures of the deep sea force these organisms to produce defensive and competitive molecules that frequently target essential metabolic nodes like the purinome of competitors or hosts. This natural selection has already pre-optimized many marine metabolites for high-affinity binding to conserved eukaryotic enzymes like ADA.

The Integrated Discovery Pipeline: In Silico Meets In Vitro

To efficiently mine the chemical space of marine fungi for ADA inhibitors, a modern, integrated workflow that synergizes computational and experimental approaches is essential. This strategy uses the predictive power of in silico methods to guide and focus the resource-intensive experimental work.

High-Throughput Virtual Screening (HTVS)

This phase uses the high-resolution crystal structure of human ADA (e.g., PDB 1VFL, complexed with transition-state analogues) to screen natural product libraries.

Target Preparation: Human ADA is a monomeric protein with a zinc ion in the active site. Preparation involves removing water molecules, adding polar hydrogens, and establishing the protonation states of active site residues like Asp295 and His238.

Virtual Library Generation: Databases like MarinLit and COCONUT (Collection of Open Natural Products) are filtered for marine fungal metabolites. 3D conformers are generated using OMEGA or OpenBabel.

Molecular Docking: Tools like AutoDock Vina or Glide (Schrödinger) are used to perform rigid and flexible docking. Scoring functions estimate the free energy of binding (ΔG_bind).

Advanced Refinement: Molecular Dynamics and MM-GBSA

Static docking often fails to capture the induced-fit mechanism of the ADA active site. Therefore, top-ranked hits must undergo:

Molecular Dynamics (MD) Simulations: Using force fields like AMBER or GROMOS, the protein-ligand complex is simulated in a periodic water box for 100–500 ns. This evaluates the stability of the binding pose and the role of "bridging" water molecules.

MM-GBSA Calculations: Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) provides a more accurate estimation of binding affinity by averaging energies over multiple MD snapshots, identifying the specific energetic contributions (van der Waals, electrostatic, solvation) of individual residues (Uba et al., 2023).

Table 2: Comparison of Computational Tools for ADA Inhibitor Discovery

Tool / Method	**Purpose**	**Strength**	**Limitation**
AutoDock Vina	Virtual Screening	Open-source; fast; high accuracy	Static protein; simple scoring
Glide (SP/XP)	Precise Docking	Excellent water handling; hydrophobic mapping	Commercial license required
GROMACS / AMBER	MD Simulations	Captures protein flexibility and entropy	Computationally expensive
MM-GBSA	Affinity Prediction	Accurate ΔG estimation; residue-wise decomposition	Sensitive to force field choice
DeepChem (GNN)	AI/ML Prediction	Rapid ADMET / QSAR prediction	Requires large training datasets

AI and Machine Learning Integration

Modern pipelines now incorporate deep learning models (e.g., Graph Neural Networks) to predict the bioactivity of metabolites directly from their SMILES strings. These models can filter out "pan-assay interference compounds" (PAINS) and predict blood-brain barrier (BBB) permeability—a critical factor given pentostatin's neurotoxicity (Schneider, 2018).

Experimental Evidence and Case Studies: Marine Success Stories

The transition from in silico prediction to in vitro reality is best illustrated by several recent success stories where marine fungal metabolites were identified as novel ADA inhibitors.

Case Study 1: Isoxazoles from Aspergillus niger

Zhang et al. (2018) employed a bioassay-guided approach to screen endophytes from marine-adjacent medicinal plants. They isolated 3-(4-nitrophenyl)-5-phenyl isoxazole, a structurally unique alkaloid. Kinetic analysis revealed it to be an uncompetitive inhibitor with an IC50 of 0.347 mM against HepG2-derived ADA. Unlike pentostatin, which binds the transition state, this isoxazole likely binds the enzyme-substrate complex, potentially offering a different selectivity profile.

Case Study 2: Pyranone Derivatives from Cochliobolus sp.

A study of sediment-derived *Cochliobolus sp.* yielded a novel pyranone derivative (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one). This compound demonstrated mixed-type inhibition, suggesting it could bind both the free enzyme and the enzyme-substrate complex (Zhang et al., 2020). Its low molecular weight and high solubility make it a "lead-like" molecule for further medicinal chemistry optimization.

Case Study 3: Phenolic Synergies

Beyond alkaloids, phenolic compounds like Gallic acid and Caffeic acid, frequently found in marine fungal extracts, have been validated through integrated workflows. Uba et al. (2023) demonstrated through MD simulations that these simple phenolics form stable hydrogen bonds with the catalytic water molecule in the ADA active site, with ΔG_bind values reaching -8.5 kcal/mol.

Table 3: Comparative Inhibitory Potency and Binding Affinity of Selected ADA Inhibitors

Compound Name	**Fungal / Source Origin**	**Inhibition Type**	**IC50 / Ki Value**	**ΔG Binding (kcal/mol)**
Pentostatin (PNT)	Streptomyces (Terrestrial)	Transition-state	Ki = 0.9 pM	-12.5 (Exp)
3-(4-nitrophenyl) isoxazole	Aspergillus niger	Uncompetitive	IC50 = 347 µM	-7.2 (Docking)
Kojic Acid Derivative	Cochliobolus sp.	Mixed-type	IC50 = 420 µM	-6.8 (Docking)
Gallic Acid	Marine Phenolics	Competitive	IC50 = 120 µM	-8.5 (MM-GBSA)

Benzimidazole-5	Synthetic / Bio-inspired	Mixed-type	IC50 = 15 µM	-9.1 (MM-GBSA)

Selectivity Studies: The Purinome Challenge

A critical "high-merit" requirement is the assessment of selectivity. Novel marine ADA inhibitors should ideally be tested against related enzymes like adenosine kinase (AK), CD73, and AMP deaminase to ensure that the global purine balance is not catastrophically disrupted, which is a major cause of pentostatin's toxicity.

Clinical and Translational Relevance: From Reef to Relief

The transition of a marine fungal lead into a clinical candidate requires overcoming significant pharmacological and logistical hurdles. For ADA inhibitors, the primary goal is to achieve a superior "therapeutic index" compared to pentostatin.

Addressing the "Supply Problem"

A historical bottleneck in marine natural product discovery is the "supply problem"—the inability to harvest sufficient biomass from the environment for clinical trials. Modern solutions involve:

Genome Mining and Heterologous Expression: By sequencing the genome of the producing fungus, researchers can identify the Biosynthetic Gene Clusters (BGCs) responsible for the active metabolite. These BGCs can be inserted into robust industrial hosts like *Aspergillus niger* or *Pichia pastoris* for large-scale fermentation (Harvey et al., 2015).

Total Synthesis and Semi-Synthesis: Structural simplification of a complex marine scaffold can lead to "analogue-based drug discovery." For instance, the benzimidazole nucleus found in some marine metabolites can be easily synthesized and optimized for improved ADMET properties (Arun et al., 2020).

ADMET Optimization and Safety

The severe neurotoxicity of pentostatin is partly due to its ability to cross the blood-brain barrier (BBB) and inhibit neuronal ADA. Marine-derived scaffolds, particularly those with higher molecular weights or specific polar surface areas (PSA), can be rationally designed or selected to exclude from the central nervous system (CNS), thereby reducing off-target effects. Furthermore, the use of in silico PBPK (Physiologically Based Pharmacokinetic)

models allows for the prediction of tissue distribution before the first in vivo dose is administered.

Current Limitations and Knowledge Gaps

Protein Flexibility: Most high-throughput virtual screens rely on rigid-body docking, which may miss "cryptic pockets" that only open upon ligand binding.

The "Silent" BGC Problem: Many potent ADA inhibitors are only produced by fungi under specific ecological stress (e.g., competition with specific bacteria). Standard laboratory cultures often fail to trigger these biosynthetic pathways.

In Vitro vs. In Vivo Correlation: The efficacy of an ADA inhibitor in a purified enzyme assay does not always translate to cellular models where the "adenosine halo" is regulated by multiple redundant enzymes like AK or ecto-ADA (Antonioli et al., 2021).

Future Directions: The Next Generation of Purinome Modulators

The next decade will see the convergence of several high-impact technologies in the field of purinome drug discovery.

Structural Biology: Cryo-EM and AlphaFold

Advances in Cryo-Electron Microscopy (Cryo-EM) now allow for the visualization of ADA in complex with large, non-purine-like marine metabolites that were previously difficult to crystallize. Furthermore, AI tools like AlphaFold-Multimer are being used to predict the structure of the ADA-CD26 complex, opening the door for the discovery of "protein-protein interaction" (PPI) inhibitors that could selectively modulate extracellular ADA signaling without affecting intracellular metabolism.

Synthetic Biology and CRISPR

CRISPR-Cas9 mediated genome editing is being used to "wake up" silent biosynthetic gene clusters in marine fungi. By overexpressing specific transcription factors or swapping promoters, researchers can force the production of novel metabolites that have never been seen in nature before (Zhang et al., 2022).

Precision Medicine and the Purinome

As our understanding of the "purinome fingerprint" of different cancers grows, ADA inhibitors could be used as part of a precision medicine strategy. Patients with high CD73/ADA

ratios in their tumors may be the ideal candidates for these therapies, potentially identified through non-invasive 18F-labeled adenosine PET imaging (Hesse et al., 2024).

Conclusion

Adenosine deaminase remains a cornerstone of the purinergic signaling network and a highly validated, yet challenging, therapeutic target. The significant clinical limitations of current inhibitors underscore the need for a paradigm shift in drug discovery. Marine-derived fungi offer an unparalleled reservoir of chemical novelty, providing the structural diversity needed to escape the "purine-analogue trap." By integrating the speed of high-performance in silico modeling with the rigor of in vitro enzymatic validation and the power of synthetic biology, we can transform the chemical treasures of our oceans into the next generation of life-saving medicines. This convergent strategy provides a robust roadmap for targeting the purinome, offering hope for more effective and less toxic treatments for patients with hematological malignancies and immune-mediated disorders.

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Chapter 12

Gut Microbiome and Type 2 Diabetes Management: Mechanisms, Interventions, and Therapeutic Implications

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Abstract

The gut microbiome, comprising trillions of microorganisms inhabiting the gastrointestinal tract, has emerged as a pivotal mediator of metabolic homeostasis and a key determinant of susceptibility to Type 2 diabetes mellitus (T2DM). Mounting evidence from epidemiological, experimental, and clinical studies demonstrates that dysbiosis—characterised by reduced microbial diversity, depletion of short-chain fatty acid (SCFA)-producing taxa, and enrichment of pro-inflammatory species—is a consistent hallmark of T2DM pathophysiology. The present review comprehensively examines the current state of knowledge concerning the bidirectional relationship between gut microbiota composition and T2DM, with particular emphasis on mechanistic pathways, therapeutic interventions, and translational implications. Specific mechanisms through which the gut microbiome modulates glycaemic control include SCFA-mediated regulation of insulin secretion and sensitivity, bile acid metabolism, intestinal permeability and endotoxaemia-driven chronic inflammation, and enteroendocrine signalling via incretins and the gut-brain axis. Therapeutic strategies targeting the gut microbiome for T2DM management—encompassing dietary modification, probiotic and prebiotic supplementation, synbiotics, and faecal microbiota transplantation—are critically appraised with respect to their efficacy, mechanisms of action, and clinical feasibility. The review further addresses the modulatory effects of antidiabetic pharmacotherapy on microbiome composition, particularly metformin, and explores the potential for microbiome-based biomarkers to inform precision diabetes management. Critical knowledge gaps pertaining to causality, inter-individual variability, and long-term safety of microbiome-targeted interventions are identified, and an integrative research agenda incorporating metagenomics, metabolomics, and clinical trial design is proposed to advance this rapidly evolving field.

Keywords: gut microbiome, Type 2 diabetes, dysbiosis, short-chain fatty acids, faecal microbiota transplantation, probiotics, metabolic syndrome

Introduction

The metabolic significance of the gut microbiome

The human gastrointestinal tract harbours an extraordinarily diverse community of microorganisms—encompassing bacteria, archaea, fungi, viruses, and protozoa—that collectively constitute the gut microbiome, a dynamic ecosystem of profound metabolic and immunological importance (Turnbaugh et al., 2006). With an estimated 38 trillion microbial cells occupying the intestinal niche and encoding a metagenome approximately 150 times the size of the human genome, the gut microbiome functions as a virtual metabolic organ, performing biochemical transformations that profoundly influence host physiology (Sender et al., 2016). The recognition that this microbial community participates intimately in nutrient metabolism, immune system development, and endocrine signalling has fundamentally transformed our understanding of metabolic disease aetiology and opened new avenues for therapeutic intervention.

Type 2 diabetes mellitus (T2DM) represents one of the most prevalent and rapidly escalating metabolic disorders globally, affecting approximately 537 million adults worldwide in 2021 and projected to reach 783 million by 2045 (IDF, 2021). Characterised by chronic hyperglycaemia resulting from progressive insulin resistance and relative insulin deficiency, T2DM is intimately associated with obesity, dyslipidaemia, and systemic chronic low-grade inflammation, all of which are now recognised to be influenced substantially by gut microbiome composition and function (Qin et al., 2012). The paradigm-shifting demonstration that germ-free mice are resistant to diet-induced obesity and exhibit improved insulin sensitivity compared to conventionally raised counterparts provided foundational evidence that gut microbiota causally contribute to metabolic phenotype, galvanising intensive research into microbiome-T2DM interactions (Bäckhed et al., 2004).

Epidemiological and metagenomic studies have consistently identified characteristic dysbiotic signatures in individuals with T2DM, including reduced abundance of butyrate-producing genera such as Faecalibacterium, Roseburia, and Bifidobacterium, alongside enrichment of opportunistic pathogens including Clostridium, Fusobacterium, and

Ruminococcus gnavus (Qin et al., 2012; Karlsson et al., 2013). These compositional alterations are accompanied by functional deficiencies in SCFA biosynthesis, impaired intestinal barrier integrity, and dysregulated bile acid metabolism, collectively creating a pro-inflammatory milieu that promotes insulin resistance and beta-cell dysfunction. The mechanistic links between these microbial alterations and diabetic pathophysiology have been progressively elucidated through a combination of gnotobiotic animal models, in vitro studies, and increasingly sophisticated human clinical investigations.

Scope and objectives of the present review

The present synthesis aims to provide a comprehensive and critical examination of the role of the gut microbiome in T2DM management, integrating mechanistic insights with therapeutic evidence to inform future research and clinical practice. We begin with a systematic appraisal of gut microbiome compositional and functional alterations in T2DM, followed by an in-depth analysis of the mechanistic pathways linking microbial dysbiosis to metabolic dysfunction. Subsequently, we evaluate the current evidence base for microbiome-targeted therapeutic interventions, including dietary approaches, probiotic and prebiotic supplementation, and faecal microbiota transplantation. We further examine the complex bidirectional interactions between antidiabetic pharmacotherapy and gut microbiota, and consider the emerging potential of microbiome-based biomarkers for personalised diabetes management. The review concludes by identifying critical knowledge gaps and proposing an integrative research framework to advance this rapidly evolving and clinically significant field.

Literature Search Strategy and Analytical Framework

Search methodology

A systematic literature search was conducted across multiple electronic databases, namely PubMed/MEDLINE, Web of Science, Scopus, Embase, and Google Scholar, using Boolean combinations of the following search terms: ("gut microbiome" OR "gut microbiota" OR "intestinal microbiota" OR "intestinal microbiome") AND ("Type 2 diabetes" OR "T2DM" OR "type 2 diabetes mellitus" OR "insulin resistance" OR "glycaemic control") AND ("dysbiosis" OR "probiotics" OR "prebiotics" OR "faecal microbiota transplantation" OR "short-chain fatty acids" OR "bile acids" OR "gut permeability"). The search encompassed publications from 1990 to 2024 without restriction by geographical region, study design, or organism. Additional references were identified through manual examination of citation

networks, systematic review bibliographies, and consultation with subject-specific authoritative sources.

Inclusion and exclusion criteria

Studies were included if they satisfied all of the following criteria: (i) reported original empirical data on associations between gut microbiome composition or function and T2DM outcomes; (ii) employed validated microbiome characterisation methods including 16S rRNA gene sequencing, shotgun metagenomics, or culture-based approaches; (iii) included appropriate control groups or comparative analyses enabling attribution of observed effects to microbial factors; and (iv) were published in peer-reviewed journals or established institutional repositories. Studies were excluded if they focused exclusively on Type 1 diabetes without relevance to T2DM mechanisms, if microbiome characterisation was insufficiently detailed to permit meaningful interpretation, or if the study population comprised fewer than ten participants without adequate statistical justification. Review articles and meta-analyses were included for contextual synthesis but not as primary evidence sources.

Gut Microbiome Composition and Dysbiosis in Type 2 Diabetes

Characteristic microbial signatures in T2DM

Landmark metagenomic studies have delineated a consistent dysbiotic signature associated with T2DM, characterised by reduced overall alpha diversity and specific taxonomic shifts that distinguish diabetic from normoglycaemic individuals across diverse geographical and ethnic populations (Qin et al., 2012; Karlsson et al., 2013). The landmark Chinese cohort study by Qin et al. (2012) identified 47 metagenomic species significantly enriched or depleted in T2DM subjects, establishing that the diabetic gut microbiome is characterised by a moderate degree of dysbiosis with particular depletion of several butyrate-producing bacterial species. These compositional alterations exhibit remarkable consistency across independently conducted studies, suggesting that they reflect fundamental pathophysiological relationships rather than population-specific confounders.

At the phylum level, T2DM is consistently associated with alterations in the Firmicutes/Bacteroidetes ratio, although the direction of this change has been variably reported across studies, potentially reflecting differences in dietary patterns, medication use, and disease severity (Larsen et al., 2010). At the genus and species levels, the most reproducible findings include reduced abundance of Faecalibacterium prausnitzii, Akkermansia muciniphila, Bifidobacterium longum, Roseburia intestinalis, and Eubacterium hallii, alongside increased

representation of Ruminococcus gnavus, Clostridium ramosum, Fusobacterium nucleatum, and various Proteobacteria (Gurung et al., 2020). Faecalibacterium prausnitzii is of particular interest, as it is the most abundant single bacterial species in the healthy human gut and a major producer of butyrate, an SCFA with pleiotropic anti-inflammatory and metabolic regulatory functions.

Functional dysbiosis and metabolic consequences

Beyond compositional alterations, T2DM is characterised by functional dysbiosis involving impairment of key microbial metabolic pathways that influence host glycaemic regulation. Metagenomic functional profiling has revealed consistent enrichment of oxidative stress resistance genes, membrane transport functions, and lipopolysaccharide (LPS) biosynthesis pathways in T2DM microbiomes, alongside deficiencies in butyrate synthesis genes, cofactor and vitamin metabolism, and carbohydrate-active enzyme diversity (Qin et al., 2012). These functional alterations translate into measurable changes in the metabolome, with reduced faecal SCFA concentrations and elevated circulating levels of branched-chain amino acids—produced by gut bacteria—being among the most consistently reported metabolomic features of T2DM (Pedersen et al., 2016).

Reduced production of SCFAs, particularly butyrate, acetate, and propionate, by the dysbiotic T2DM microbiome has cascading consequences for intestinal barrier integrity, immune regulation, and enteroendocrine signalling. Butyrate serves as the primary energy substrate for colonocytes and exerts critical functions in maintaining tight junction protein expression and mucus layer thickness, the disruption of which contributes to increased intestinal permeability and translocation of microbial products into the systemic circulation (Canani et al., 2011). This microbial translocation drives chronic low-grade endotoxaemia that is now recognised as a central mechanism linking gut dysbiosis to systemic insulin resistance, as elegantly demonstrated by Cani et al. (2007) who showed that continuous infusion of LPS at concentrations comparable to those observed postprandially in obese individuals was sufficient to induce insulin resistance and adipose tissue inflammation in mice.

Mechanistic Pathways Linking Gut Microbiome to T2DM Pathophysiology

Short-chain fatty acid signalling and insulin sensitivity

Short-chain fatty acids, produced through microbial fermentation of dietary fibre and resistant starch in the distal intestine, represent the most extensively characterised mechanism through which the gut microbiome influences glycaemic regulation (Sonnenburg & Bäckhed, 2016). Butyrate, propionate, and acetate exert their metabolic effects through multiple complementary mechanisms, including activation of G protein-coupled receptors (GPR41, GPR43, and GPR109a), inhibition of histone deacetylases (HDACs) with consequent epigenetic regulation of gene expression, and direct stimulation of enteroendocrine L-cells to secrete glucagon-like peptide-1 (GLP-1) and peptide YY (PYY)—incretins that potentiate glucose-stimulated insulin secretion and reduce appetite (Kimura et al., 2013).

Propionate, absorbed and transported to the liver via the portal circulation, serves as a gluconeogenic substrate and activates intestinal gluconeogenesis through a gut-brain neural circuit that improves whole-body insulin sensitivity and reduces hepatic glucose production (De Vadder et al., 2014). This finding is particularly significant as it demonstrates a direct mechanistic link between microbial fermentation products and central metabolic regulation, potentially explaining the paradoxical glucose-lowering effects of dietary fibre consumption that exceed what would be predicted from simple caloric restriction. Acetate exerts central effects on appetite regulation through hypothalamic mechanisms and influences hepatic lipogenesis, contributing to the broader metabolic benefits associated with SCFA-producing microbiome configurations.

Intestinal permeability, endotoxaemia, and inflammatory pathways

The integrity of the intestinal epithelial barrier constitutes a critical interface between the gut microbiome and the systemic immune and metabolic systems, and its compromise under conditions of dysbiosis represents a key pathophysiological mechanism in T2DM (Bischoff et al., 2014). Under healthy conditions, tight junction proteins including occludin, claudin-1, and zonula occludens proteins maintain paracellular impermeability that prevents translocation of microbial products including LPS, peptidoglycans, and flagellin into the portal and systemic circulation. Dysbiosis-associated reduction in SCFA availability, decreased mucus-degrading capacity, and direct pathobiont-mediated disruption of tight junction

complexes collectively compromise barrier integrity, resulting in elevated circulating endotoxin levels—a condition termed metabolic endotoxaemia.

Lipopolysaccharide, derived from the outer membrane of gram-negative bacteria, activates innate immune responses through toll-like receptor 4 (TLR4) and its co-receptor CD14, triggering nuclear factor-κB (NF-κB)-mediated transcription of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 in adipose tissue, liver, and skeletal muscle (Cani et al., 2007). These cytokines impair insulin signalling through multiple mechanisms, including serine phosphorylation of insulin receptor substrate-1 (IRS-1) that prevents downstream PI3K-Akt pathway activation essential for glucose transporter GLUT4 translocation to the plasma membrane. The resultant insulin resistance in peripheral tissues, combined with LPS-mediated impairment of pancreatic beta-cell function and survival, creates a self-reinforcing pathological cycle that progressively deteriorates glycaemic control.

Bile acid metabolism and nuclear receptor signalling

Bile acids, synthesised from cholesterol in the liver and subjected to extensive microbial biotransformation in the intestinal lumen, function as critical signalling molecules that regulate glucose and lipid metabolism through their interactions with the nuclear receptor farnesoid X receptor (FXR) and the membrane receptor TGR5 (Wahlström et al., 2016). The gut microbiome, through the action of bile salt hydrolase enzymes and 7α-dehydroxylation reactions, transforms primary bile acids (cholic acid and chenodeoxycholic acid) into secondary bile acids (deoxycholic acid and lithocholic acid) that exhibit distinct receptor affinities and signalling properties. Dysbiosis in T2DM is associated with altered secondary bile acid profiles that impair FXR-mediated suppression of hepatic bile acid synthesis and gluconeogenesis, while simultaneously compromising TGR5-stimulated GLP-1 secretion from enteroendocrine L-cells.

The FXR-fibroblast growth factor 15/19 (FGF15/19) axis, which normally suppresses hepatic glucose production and promotes glycogen synthesis in response to postprandial bile acid signalling, is functionally impaired in T2DM due to microbiome-mediated alterations in bile acid composition (Prawitt et al., 2011). This impairment contributes to excessive hepatic gluconeogenesis and postprandial hyperglycaemia that are characteristic features of early T2DM. The therapeutic relevance of this pathway is underscored by the observation that bariatric surgery, which dramatically alters gut microbiome composition and bile acid profiles, produces metabolic benefits including T2DM remission that exceed what would be predicted

from weight loss alone, suggesting that microbiome-mediated bile acid changes contribute substantially to the metabolic efficacy of these procedures.

Gut-brain axis and neuroendocrine regulation of glycaemia

The bidirectional communication network between the gastrointestinal tract and the central nervous system—the gut-brain axis—serves as a critical conduit through which the gut microbiome influences food intake, energy expenditure, and glycaemic regulation (Cryan et al., 2019). Microbial metabolites and products influence hypothalamic circuits controlling appetite and energy homeostasis through multiple routes including vagal afferent signalling, enteroendocrine hormone release, and systemic circulation of bioactive compounds that cross the blood-brain barrier. Germ-free animals display markedly altered hypothalamic gene expression, impaired serotonin signalling, and dysregulated neurogenesis compared to conventionally colonised counterparts, demonstrating the profound influence of the microbiome on central nervous system function.

The incretin hormones GLP-1 and GLP-2, secreted by intestinal L-cells in response to nutrient exposure and microbial metabolites including SCFAs and secondary bile acids, are of particular therapeutic relevance to T2DM management (Drucker, 2006). GLP-1 potentiates glucose-stimulated insulin secretion, suppresses glucagon release, delays gastric emptying, and promotes satiety, while GLP-2 enhances intestinal barrier integrity and mucosal healing. The gut microbiome modulates L-cell function through multiple mechanisms, and dysbiosis-associated reductions in SCFA and secondary bile acid availability compromise GLP-1 secretory capacity, potentially contributing to the incretin defect that characterises T2DM. Restoration of microbiome function through targeted interventions may therefore enhance endogenous incretin signalling and complement pharmacological GLP-1 receptor agonist therapy.

Therapeutic Interventions Targeting the Gut Microbiome in T2DM

Dietary modification and prebiotic supplementation

Dietary composition exerts the most rapid, potent, and sustained influence on gut microbiome composition and function of any modifiable lifestyle factor, and represents the foundational intervention for microbiome-targeted T2DM management (David et al., 2014). Plant-derived dietary fibre and resistant starch, preferentially fermented by beneficial microbiota including Bifidobacterium and Lactobacillus species, promote SCFA production, enhance microbial diversity, and selectively enrich butyrate-producing taxa that are depleted

in T2DM. Randomised controlled trials have consistently demonstrated that high-fibre dietary interventions improve glycaemic indices, reduce HbA1c levels, and favourably alter microbiome composition in T2DM patients, with the magnitude of glycaemic benefit correlating with the degree of microbiome compositional change (Zhao et al., 2018).

Prebiotic compounds—non-digestible food ingredients that selectively stimulate the growth and/or activity of beneficial microorganisms—include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), arabinoxylan, and resistant starch, each with distinct fermentation profiles and microbiome-modulating properties (Gibson et al., 2017). Meta-analyses of prebiotic supplementation trials in T2DM and prediabetic populations have documented significant reductions in fasting blood glucose (mean reduction 0.82 mmol/L), HbA1c, insulin resistance indices, and circulating inflammatory markers, alongside increases in Bifidobacterium and Lactobacillus abundance and faecal SCFA concentrations. Mediterranean and plant-based dietary patterns, characterised by high fibre, polyphenol, and omega-3 fatty acid content, have emerged as particularly effective for microbiome-mediated metabolic improvement in T2DM.

Probiotic and synbiotic interventions

Probiotics, defined as live microorganisms that confer health benefits when administered in adequate amounts, have been evaluated extensively in T2DM management, with particular focus on Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium lactis, Bifidobacterium longum, and various multi-strain combinations (Hill et al., 2014). Randomised controlled trials and meta-analyses have documented modest but statistically significant improvements in glycaemic control following probiotic supplementation in T2DM patients, including reductions in fasting plasma glucose (mean 0.57 mmol/L), HbA1c (mean 0.54%), fasting insulin, and HOMA-IR, alongside improvements in lipid profiles and inflammatory markers (Samah et al., 2016). Mechanisms underlying probiotic glycaemic benefits include enhancement of intestinal barrier integrity, modulation of bile acid metabolism, stimulation of GLP-1 secretion, and competitive exclusion of pathobiont species.

Next-generation probiotics targeting specific mechanistic pathways in T2DM have attracted considerable attention, with *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* emerging as leading candidates based on their consistent depletion in diabetic microbiomes and compelling mechanistic evidence for metabolic benefits (Plovier et al., 2017). A landmark human clinical trial demonstrated that oral administration of pasteurised

Akkermansia muciniphila to insulin-resistant individuals over three months improved insulin sensitivity, reduced circulating insulin and plasma total cholesterol levels, and attenuated gut barrier dysfunction without any adverse effects, providing the first direct human evidence for next-generation probiotic efficacy in metabolic disease (Depommier et al., 2019). Synbiotic formulations combining probiotics with their preferred prebiotic substrates demonstrate synergistic metabolic benefits exceeding those of either component administered alone.

Faecal microbiota transplantation (FMT)

Faecal microbiota transplantation, involving the transfer of faecal material from healthy donors to recipients with dysbiosis-associated conditions, represents the most radical and potentially transformative microbiome-targeted intervention for T2DM, capable of producing rapid and comprehensive restoration of microbiome composition (Smits et al., 2017). Proof-of-concept was established in a seminal Dutch clinical trial demonstrating that infusion of microbiota from lean donors into obese metabolic syndrome patients significantly improved peripheral insulin sensitivity within six weeks, with the magnitude of improvement correlating with increased abundance of SCFA-producing bacteria including Roseburia intestinalis (Vrieze et al., 2012). Subsequent controlled trials in T2DM populations have reported variable outcomes, with the most consistent benefits observed in patients with the most severe baseline dysbiosis and in those receiving microbiota from carefully selected metabolically healthy, high-fibre-diet donors.

Current limitations of FMT for T2DM management include the variable and often transient nature of microbiome engraftment, the critical importance of donor selection on therapeutic outcomes, potential safety concerns related to pathogen transmission (including the documented transmission of multidrug-resistant organisms in immunocompromised recipients), and the practical challenges of standardisation and regulatory oversight (Wilson et al., 2019). Ongoing research efforts are directed toward identifying the specific microbial consortia responsible for metabolic benefits, developing defined synthetic microbiome preparations that could overcome the variability and safety concerns of donor-derived FMT, and establishing optimal administration routes, dosing frequencies, and co-treatment protocols for maximising durable microbiome engraftment.

Table 1: Summary of gut microbiome-targeted interventions and their documented effects in T2DM management.

Intervention Type	Key Agent/Approach	Primary Mechanism	Documented Glycaemic Effect
Dietary fibre	Inulin, FOS, arabinoxylan, resistant starch	SCFA production; Bifidobacterium enrichment; GLP-1 stimulation	HbA1c reduction ~0.5%; fasting glucose reduction ~0.8 mmol/L
Probiotics	*Lactobacillus, Bifidobacterium, Akkermansia muciniphila*	Barrier restoration; bile acid modulation; anti-inflammatory cytokines	HbA1c reduction ~0.54%; insulin resistance improvement
Synbiotics	Probiotic + matched prebiotic combinations	Synergistic enhancement of engraftment and SCFA yield	Superior glycaemic and lipid outcomes vs. individual components
FMT	Lean/healthy donor faecal infusion (duodenal or colonoscopic)	Comprehensive microbiome reconstitution; SCFA-producer engraftment	Improved insulin sensitivity; variable glycaemic response; donor-dependent

Pharmacological Interactions with Gut Microbiota in T2DM

Metformin and microbiome modulation

Metformin, the most widely prescribed first-line pharmacotherapy for T2DM, exerts significant effects on gut microbiome composition that are now believed to contribute substantially to its glucose-lowering mechanisms beyond its well-characterised inhibition of hepatic gluconeogenesis through AMPK activation (Forslund et al., 2015). Metformin treatment is consistently associated with enrichment of Akkermansia muciniphila, Bifidobacterium species, and several SCFA-producing taxa, alongside reductions in Clostridiales and other potentially pathobiotic bacteria. Critically, a landmark study by Forslund et al. (2015) demonstrated that many of the microbiome differences previously attributed to T2DM per se were in fact driven by metformin treatment, underscoring the importance of carefully accounting for medication use in microbiome-T2DM studies.

Mechanistic investigations have revealed that metformin accumulates at extraordinarily high concentrations in the intestinal lumen—up to 300-fold higher than plasma

concentrations—where it directly modulates bacterial gene expression, inhibits bacterial respiratory chain complex I, and alters bile acid metabolism in ways that parallel its systemic metabolic effects (Duca et al., 2015). Germ-free mouse studies have demonstrated that the glucose-lowering effects of metformin are substantially attenuated in the absence of gut microbiota, and that transplantation of metformin-modified microbiota from treated mice to untreated recipients reproduces a portion of the metabolic benefits, establishing a causal role for microbiome modulation in metformin efficacy. These findings have profound implications for personalised T2DM pharmacotherapy, suggesting that baseline microbiome composition may predict metformin responsiveness.

Other antidiabetic agents and microbiome interactions

Emerging evidence indicates that multiple classes of antidiabetic pharmacotherapy beyond metformin exert significant effects on gut microbiome composition, and that some therapeutic benefits may be mediated in part through microbiome-dependent mechanisms (Wu et al., 2017). GLP-1 receptor agonists, which produce substantial metabolic improvements including weight loss and cardiovascular risk reduction beyond their incretin-mediated effects, are associated with alterations in microbiome composition that include enrichment of Akkermansia muciniphila and reductions in abundance of potentially inflammatory taxa. Alpha-glucosidase inhibitors such as acarbose, which act primarily in the intestinal lumen to delay carbohydrate digestion, produce marked shifts in gut microbiome composition through increased availability of undigested carbohydrates for microbial fermentation, with associated changes in SCFA profiles and bile acid metabolism that may contribute to their glycaemic and cardiovascular benefits.

SGLT-2 inhibitors, which reduce glycaemia by increasing urinary glucose excretion, have been shown to alter gut microbiome composition in ways consistent with reduced carbohydrate availability, including shifts toward saccharolytic fermentation pathways (Lee et al., 2018). These microbiome changes are distinct from those induced by dietary carbohydrate restriction despite similar changes in substrate availability, suggesting drug-specific direct effects on intestinal microbial communities beyond their glycosuric mechanisms. The clinical relevance of these interactions is highlighted by observations that the cardiovascular and renal protective effects of SGLT-2 inhibitors, which substantially exceed what would be predicted from glucose-lowering alone, may involve microbiome-mediated mechanisms that await full characterisation.

Microbiome-Based Biomarkers for Precision Diabetes Management

Predictive biomarkers for T2DM risk stratification

The recognition that specific microbiome compositional and functional signatures precede and predict T2DM development has created opportunities for microbiome-based risk stratification that could enable earlier and more targeted preventive interventions (Allin et al., 2018). Prospective cohort studies have demonstrated that reduced abundance of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and butyrate-producing Firmicutes, combined with elevated Lachnospiraceae incertae sedis and specific metabolic syndrome-associated taxa, characterises microbiome configurations that predict progression from normoglycaemia to prediabetes and T2DM over multi-year follow-up. Integration of microbiome data with conventional clinical risk factors including BMI, family history, and fasting glucose substantially improves predictive accuracy for T2DM risk compared to clinical variables alone.

Metabolomics-based biomarkers derived from gut microbial activity offer complementary predictive value, with elevated plasma concentrations of branched-chain amino acids (valine, leucine, isoleucine), aromatic amino acids, and specific bile acid species demonstrating predictive value for future insulin resistance and T2DM development (Pedersen et al., 2016). The combination of microbiome compositional data with faecal and plasma metabolomics within machine learning frameworks has demonstrated impressive discriminatory capacity for distinguishing T2DM patients from normoglycaemic controls, with some models achieving area under the ROC curve values exceeding 0.90. However, prospective validation of these multivariate biomarker panels in diverse population cohorts remains essential before clinical deployment can be contemplated.

Personalised dietary responses and microbiome configuration

One of the most clinically significant recent discoveries in microbiome-diabetes research is the demonstration that inter-individual variability in postprandial glycaemic responses to identical foods is substantially determined by gut microbiome composition, potentially explaining the limited efficacy of standardised dietary recommendations for T2DM management (Zeevi et al., 2015). The landmark Weizmann Institute study by Zeevi et al. (2015) demonstrated that postprandial glycaemic responses to 46,898 meals consumed by 800 participants were highly variable between individuals even for identical foods, and that microbiome composition was a significant predictor of this variability alongside other personal characteristics. Machine learning algorithms incorporating microbiome data predicted

personalised glycaemic responses with accuracy exceeding that of conventional carbohydrate counting, and microbiome-based personalised dietary recommendations successfully reduced postprandial hyperglycaemia in a subsequent randomised crossover trial.

These findings fundamentally challenge the paradigm of universal dietary guidelines for T2DM management and suggest that microbiome profiling could enable genuinely personalised nutritional interventions that match dietary prescription to individual microbiome configurations for optimal glycaemic outcomes. Ongoing clinical trials are evaluating whether microbiome-guided dietary personalisation produces superior long-term glycaemic control compared to standard dietary advice in T2DM populations, with preliminary results suggesting clinically meaningful advantages. The practical implementation of this approach will require the development of rapid, affordable, and clinically actionable microbiome profiling technologies alongside validated computational tools for dietary recommendation generation.

Future Research Directions and Methodological Advances

Establishing causality and addressing confounding

Despite the compelling evidence for associations between gut microbiome dysbiosis and T2DM, establishing definitive causal relationships remains methodologically challenging due to the pervasive confounding effects of diet, medications, physical activity, and host genetics that simultaneously influence both microbiome composition and glycaemic regulation (Clemente et al., 2012). The development of Mendelian randomisation approaches using genetic instruments for microbiome composition as proxies for lifelong microbiome exposure offers a promising strategy for causal inference that is less susceptible to environmental confounding than conventional observational study designs. Large-scale biobank cohorts with paired genetic, microbiome, metabolomic, and clinical data over extended follow-up periods will be essential for implementing these analytical frameworks.

Gnotobiotic animal models, in which specific human microbiome configurations can be transplanted into germ-free hosts, remain the gold standard for establishing causality between microbiome alterations and metabolic phenotypes, but their translational relevance to human T2DM is constrained by species differences in intestinal physiology, immune system development, and dietary patterns. The development of humanised mouse models with more physiologically relevant intestinal environments, and the increasing availability of organoid systems incorporating both epithelial and microbial components, offer opportunities for mechanistic studies in more translationally relevant systems. Prospective longitudinal cohort

studies with high-resolution time-series microbiome profiling and concurrent metabolomic monitoring will be crucial for establishing the temporal sequence of microbiome alterations in relation to metabolic deterioration in pre-diabetic individuals.

Technological innovations and integrative approaches

The integration of multi-omics data streams—including metagenomics, metatranscriptomics, metaproteomics, and metabolomics—with advanced machine learning and systems biology approaches promises to reveal mechanistic insights into microbiome-T2DM interactions that cannot be obtained from individual data types in isolation (Sonnenburg & Bäckhed, 2016). Network analyses of microbiome-metabolome-host transcriptome interactions have begun to identify key regulatory nodes and functional modules that mediate the influence of microbiome composition on metabolic phenotype, revealing unexpected mechanistic connections and potential therapeutic targets. The development of microfluidic gut-on-a-chip systems that incorporate patient-specific microbiome communities and intestinal epithelial cells offers unprecedented opportunities for personalised mechanistic studies and drug screening in vitro.

Precision microbiome engineering through bacteriophage-mediated selective depletion of specific pathobiont species, CRISPR-based modification of bacterial metabolic pathways, and designer synthetic microbiome consortia targeting specific mechanistic deficiencies represents a transformative future therapeutic frontier that could overcome the limitations of current microbiome-targeted interventions (Zmora et al., 2018). Live biotherapeutic products based on next-generation probiotic candidates including Akkermansia muciniphila and Faecalibacterium prausnitzii are currently advancing through clinical development pipelines, with regulatory frameworks for their evaluation being actively developed by international health authorities. The successful clinical translation of these innovations will require integration of mechanistic insights with rigorous clinical trial evidence and careful consideration of safety, manufacturability, and equitable access.

Conclusion

The gut microbiome has emerged as a fundamental determinant of T2DM susceptibility, pathophysiology, and therapeutic response, operating through multiple interconnected mechanisms including SCFA-mediated insulin sensitisation, bile acid signalling, intestinal barrier regulation, endotoxaemia-driven inflammation, and gut-brain axis modulation. The evidence reviewed here establishes that characteristic dysbiotic signatures in

T2DM are not merely epiphenomenal associations but functional contributors to the metabolic deterioration that defines disease progression, with restoration of microbiome health offering genuine therapeutic potential across multiple intervention modalities.

Dietary modification and prebiotic supplementation represent accessible, safe, and evidence-supported microbiome-targeted interventions for T2DM management that can be integrated into existing clinical guidelines. Probiotic and synbiotic formulations, particularly those incorporating next-generation candidates such as Akkermansia muciniphila, offer promising complementary strategies with an emerging evidence base. Faecal microbiota transplantation, while demonstrating remarkable proof-of-concept results, requires further optimisation of donor selection, engraftment protocols, and safety monitoring before widespread clinical application. The profound influence of standard antidiabetic pharmacotherapy, particularly metformin, on microbiome composition necessitates careful consideration of medication effects in microbiome research and opens new avenues for synergistic combination approaches.

The emerging capacity for microbiome-based personalised dietary prescription represents perhaps the most immediately translatable clinical application, with the potential to substantially improve upon the modest glycaemic outcomes achieved by standardised dietary recommendations. As we advance toward an era of precision diabetes medicine, the gut microbiome will undoubtedly occupy a central position in risk stratification, therapeutic selection, and monitoring of treatment response. Realising this potential will require sustained investment in multi-omics research infrastructure, development of rapid and affordable clinical microbiome profiling platforms, and the conduct of rigorously designed clinical trials that incorporate microbiome outcomes alongside conventional metabolic endpoints. The convergence of mechanistic understanding, technological capability, and clinical evidence positions microbiome-targeted strategies as a transformative frontier in the comprehensive management of Type 2 diabetes mellitus.

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Chapter 13

Impact of Non-Steroidal Anti-Inflammatory Drugs on Gastrointestinal Health: Pathophysiological Mechanisms and Emerging Insights

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed pharmacological agents because of their analgesic, anti-inflammatory, and antipyretic properties. Prolonged use or administration at higher doses is strongly associated with gastrointestinal (GI) complications primarily due to their inhibitory action on both cyclooxygenase (COX)-1 and COX-2 enzymes. This dual inhibition reduces the production of gastroprotective prostaglandins in the gut, leading to loss of mucosal defense, ulcer formation, and bleeding tendencies. Traditional NSAIDs, by targeting both isoforms indiscriminately, have been strongly linked with mucosal injury extending from the stomach to the large intestine. To overcome such complications, several innovative therapeutic strategies have emerged. One of the most widely studied approaches is using COX-2 selective inhibitors, such as Coxibs, which deliver anti-inflammatory effects while reducing suppression of COX-1–mediated protective pathways. Another emerging option is nitric oxide–releasing NSAIDs (NO-NSAIDs), which liberate nitric oxide to improve mucosal circulation and support tissue repair while maintaining therapeutic action. Additionally, dual inhibitors targeting both COX and lipoxygenase (LOX) pathways have shown beneficial effects in reducing inflammation while simultaneously lowering the risks of gastric and cardiovascular side effects linked with standard NSAID use. Despite these developments, none of these alternatives fully eliminates GI risks, particularly in vulnerable patients like the elderly and individuals with prior ulcer history. Future research should prioritise designing drug formulations that maintain therapeutic effectiveness while enhancing safety, thereby advancing the development of next-generation, gastroprotective anti-inflammatory therapies.

Keywords: NSAIDs, Inflammation, COX/LOX Inhibitors, Prostaglandins, Gastrointestinal Injury

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used medications worldwide, prized for their potent analgesic, anti-inflammatory, and antipyretic effects. Their therapeutic benefits are invaluable in managing a wide range of conditions, from acute pain and fever to chronic inflammatory diseases like arthritis. However, the clinical utility of NSAIDs is significantly hampered by their propensity to cause serious gastrointestinal (GI) complications (Lanza et al., 2009). These adverse effects, collectively termed NSAID-induced gastroenteropathy, can range from dyspepsia and mucosal erosions to life-threatening events such as peptic ulceration, bleeding, and perforation (Sostres et al., 2013). The risk of these complications increases substantially with NSAID use, posing a significant burden on public health and healthcare systems (Goldstein and Cryer, 2015).

The primary mechanism underlying both the therapeutic effects and the GI toxicity of NSAIDs is the inhibition of cyclooxygenase (COX) enzymes. These enzymes, existing in two main isoforms (COX-1 and COX-2), are responsible for the synthesis of prostaglandins (PGs). While the inhibition of COX-2 mediates the desired anti-inflammatory effects, the concurrent inhibition of the constitutively expressed COX-1 isoform in the GI tract leads to the depletion of gastroprotective prostaglandins. These prostaglandins are essential for maintaining mucosal integrity through various mechanisms, including stimulating mucus and bicarbonate secretion, maintaining mucosal blood flow, and promoting epithelial cell restitution and proliferation (Takeuchi, 2010). By inhibiting COX-1, traditional NSAIDs deplete these protective PGs, leaving the gastric mucosa vulnerable to injury from endogenous aggressors like gastric acid and pepsin (Sostres et al., 2013). This systemic effect is independent of the route of NSAID administration and is the primary driver of ulcer formation and other upper GI complications.

In response to the high incidence of NSAID-induced GI toxicity, significant research efforts have been directed towards developing safer alternatives. This has led to the creation of COX-2 selective inhibitors, or coxibs, which were designed to spare the gastroprotective functions of COX-1. While coxibs have demonstrated a significant reduction in upper GI complications compared to non-selective NSAIDs, concerns about their cardiovascular safety have emerged, and they do not completely eliminate the risk of GI injury, particularly in the lower GI tract (Chan et al., 2017). This has spurred the development of even more innovative strategies, including nitric oxide-releasing NSAIDs (NO-NSAIDs), dual COX/lipoxygenase

(LOX) inhibitors, and phosphatidylcholine-associated NSAIDs, all aimed at uncoupling the anti-inflammatory efficacy of NSAIDs from their GI toxicity.

This review provides a comprehensive overview of the pathophysiological mechanisms of NSAID-induced GI injury, from the well-established role of prostaglandin inhibition to more recently appreciated factors like mitochondrial dysfunction and gut microbiota dysbiosis. It also examines the clinical spectrum of NSAID gastroenteropathy and critically evaluates the progress and limitations of emerging therapeutic strategies designed to mitigate these risks. By synthesizing the current state of knowledge, this review aims to highlight the ongoing challenges and future directions in the quest for safer and more effective anti-inflammatory therapies.

Pathophysiological mechanisms of NSAID-induced GI injury

The pathogenesis of NSAID-induced gastrointestinal injury is a complex process involving multiple, often interconnected, mechanisms. While the systemic inhibition of prostaglandins is the most well-understood pathway, other factors, including direct topical effects, mitochondrial damage, and alterations of the gut microbiome, play significant roles in initiating and perpetuating mucosal damage.

Inhibition of Cyclooxygenase (COX) and Prostaglandin Depletion

The cornerstone of NSAID-induced GI toxicity is the inhibition of cyclooxygenase (COX) enzymes. COX-1 is constitutively expressed in most tissues, including the gastric mucosa, where it synthesizes prostaglandins (PGs) that are vital for maintaining mucosal defense (Wallace, 2008). These PGs, such as PGE2 and PGI2, exert their gastroprotective effects by stimulating the secretion of mucus and bicarbonate, increasing mucosal blood flow, and promoting epithelial cell restitution and proliferation (Takeuchi, 2010). By inhibiting COX-1, traditional NSAIDs deplete these protective PGs, leaving the gastric mucosa vulnerable to injury from endogenous aggressors like gastric acid and pepsin (Sostres et al., 2013). This systemic effect is independent of the route of NSAID administration and is the primary driver of ulcer formation and other upper GI complications.

Topical Irritant Effects on the GI Mucosa

In addition to their systemic effects, many NSAIDs have a direct topical irritant effect on the GI epithelium. Being weak acids, NSAIDs are non-ionized in the highly acidic environment of the stomach, which allows them to freely diffuse across the lipid membranes of epithelial cells. Once inside the cell, where the pH is neutral, they become ionized and

"trapped," leading to intracellular accumulation and direct cellular injury (Bjarnason et al., 2018). This "ion trapping" mechanism can disrupt cellular integrity, impair mitochondrial function, and uncouple oxidative phosphorylation, ultimately leading to cell death and mucosal erosion (Sostres et al., 2013). This topical damage is particularly relevant in the initial stages of injury and is more pronounced with certain NSAIDs than others.

Mitochondrial Dysfunction and Oxidative Stress

A growing body of evidence suggests that mitochondrial dysfunction is a key event in NSAID-induced gastropathy. NSAIDs can uncouple oxidative phosphorylation in mitochondria, leading to decreased ATP production and increased generation of reactive oxygen species (ROS) (Sostres et al., 2010). This leads to a state of oxidative stress, where the production of damaging ROS overwhelms the cell's antioxidant defenses. Oxidative stress can damage cellular lipids, proteins, and DNA, contributing to mucosal injury and inflammation. The subsequent cellular damage and energy depletion compromise the ability of the mucosal cells to repair and maintain the epithelial barrier, further exacerbating the injury (Takeuchi, 2010).

Role of Gut Microbiota Dysbiosis

The gut microbiota is increasingly recognized as a critical player in NSAID-induced GI injury, particularly in the small intestine (NSAID enteropathy). NSAIDs can alter the composition and function of the gut microbiota, leading to dysbiosis (Cangemi et al., 2016). This imbalance can have several detrimental effects. Firstly, certain bacterial species can metabolize NSAIDs, potentially altering their toxicity and disposition. Secondly, dysbiosis can lead to a reduction in the production of beneficial microbial metabolites, such as short-chain fatty acids, which are important for maintaining gut barrier function (Montalto et al., 2021). Thirdly, NSAIDs increase intestinal permeability, allowing bacteria and their products, such as lipopolysaccharide (LPS), to translocate into the mucosa. This triggers an inflammatory response, mediated by Toll-like receptors, which further contributes to tissue damage (Watanabe et al., 2020). The fact that NSAID-induced enteropathy does not occur in germ-free animals underscores the critical role of the gut microbiota in this process (Wallace et al., 2011).

Emerging therapeutic strategies for GI-sparing NSAIDs

The significant gastrointestinal toxicity of traditional NSAIDs has driven extensive research into developing safer alternatives that retain anti-inflammatory efficacy while minimizing GI damage. Several innovative strategies have emerged, each with its own

mechanistic rationale and clinical potential.

COX-2 Selective Inhibitors (Coxibs)

The development of COX-2 selective inhibitors, or coxibs, was a landmark in the quest for safer NSAIDs. The rationale behind coxibs is to selectively inhibit the COX-2 enzyme, which is upregulated at sites of inflammation, while sparing the COX-1 isoform, which is responsible for producing gastroprotective prostaglandins (Hawkey, 2001). Clinical trials have consistently shown that coxibs, such as celecoxib and etoricoxib, are associated with a significantly lower incidence of upper GI events, including endoscopic ulcers and bleeding, compared to non-selective NSAIDs (Chan et al., 2017; Goldstein & Cryer, 2015). However, the gastrointestinal benefits of coxibs are not absolute. They do not completely eliminate the risk of GI complications, and concerns about their cardiovascular safety have led to the withdrawal of some coxibs from the market (Sostres et al., 2013). Furthermore, the concomitant use of low-dose aspirin for cardiovascular prophylaxis can attenuate the GI-sparing benefits of coxibs (Lanza et al., 2009).

Nitric Oxide-Releasing NSAIDs (NO-NSAIDs)

Nitric oxide (NO)-releasing NSAIDs represent another promising strategy. These drugs consist of a conventional NSAID molecule covalently linked to an NO-releasing moiety. The principle is to leverage the protective effects of nitric oxide on the GI mucosa, which include increasing mucosal blood flow, inhibiting leukocyte adhesion, and promoting ulcer healing (Wallace and Cirino, 1994). By releasing NO at the site of potential injury, NO-NSAIDs aim to counteract the deleterious effects of prostaglandin inhibition. Preclinical studies have shown that NO-NSAIDs retain the anti-inflammatory activity of the parent NSAID while causing significantly less GI damage (Fiorucci et al., 2001). For instance, NO-naproxen has been shown to be as effective as naproxen in reducing inflammation but with a markedly improved GI safety profile (Reuter et al., 1994). Despite promising preclinical data, the clinical development of NO-NSAIDs has been challenging, and none have yet received regulatory approval for widespread use (Wallace, 2006).

Dual COX/LOX Inhibitors

The inhibition of the COX pathway by NSAIDs can lead to a shunting of arachidonic acid metabolism towards the 5-lipoxygenase (5-LOX) pathway, resulting in increased production of pro-inflammatory leukotrienes. These leukotrienes can contribute to GI damage by promoting vasoconstriction and leukocyte infiltration. Dual COX/LOX inhibitors were

developed to block both pathways simultaneously, with the aim of achieving a more potent anti-inflammatory effect and an improved GI safety profile (Raskin, 2003). Licofelone is a well-studied example of a dual COX/LOX inhibitor that has shown promising results in clinical trials, demonstrating anti-inflammatory efficacy comparable to traditional NSAIDs but with a significantly lower incidence of GI adverse events (Martel-Pelletier et al., 2003). However, like other novel anti-inflammatory agents, the development of dual COX/LOX inhibitors has faced challenges, including concerns about hepatotoxicity with some compounds (Celotti & Laufer, 2001).

Other Emerging Strategies

Other innovative approaches are also being explored. Phosphatidylcholine-associated NSAIDs (PC-NSAIDs) are designed to mimic the protective properties of the mucosal barrier by associating the NSAID with a phospholipid, thereby reducing its topical irritant effects (Lichtenberger et al., 2012). Hydrogen sulfide (H2S)-releasing NSAIDs are another class of drugs in development, leveraging the gastroprotective effects of H2S, which include antioxidant and anti-inflammatory actions (Wallace et al., 2010). Furthermore, modulating the gut microbiota through the use of probiotics, prebiotics, or fecal microbiota transplantation is being investigated as a potential strategy to mitigate NSAID-induced enteropathy (Montalto et al., 2021). These emerging strategies highlight the ongoing efforts to develop safer and more effective anti-inflammatory therapies that can overcome the limitations of traditional NSAIDs.

Conclusion and future directions

The development of safer alternatives to traditional NSAIDs remains a significant challenge in modern pharmacology. While our understanding of the pathophysiological mechanisms of NSAID-induced GI injury has advanced significantly, translating this knowledge into clinically successful, gastroprotective anti-inflammatory drugs has proven to be a complex endeavor. COX-2 selective inhibitors, while effective in reducing upper GI toxicity, have raised cardiovascular safety concerns and do not fully protect the lower GI tract. Newer strategies, such as NO-NSAIDs and dual COX/LOX inhibitors, have shown promise in preclinical models but have yet to overcome the hurdles of clinical development.

Future research must continue to explore novel therapeutic targets and drug delivery systems. A deeper understanding of the interplay between NSAIDs, the gut microbiota, and mucosal immunity is crucial for developing targeted interventions for NSAID enteropathy. Personalized medicine approaches, considering an individual's genetic predisposition, risk

factors, and gut microbiome profile, may allow for a more tailored and safer use of NSAIDs. The ultimate goal remains the development of a "super-aspirin"'97an anti-inflammatory agent that offers the full therapeutic benefits of NSAIDs without their debilitating gastrointestinal and cardiovascular side effects. Continued innovation and interdisciplinary research will be key to achieving this goal and improving the safety of one of the world's most widely used classes of drugs.

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Declaration of competing interest

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Chapter 14

Microbial Therapeutics in Oncology: A Comprehensive Review of Bacteria-Based Strategies for Cancer Treatment

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Abstract

Cancer remains one of the most devastating diseases globally, accounting for nearly 10 million deaths in 2022 alone. Conventional treatments including surgery, chemotherapy, and radiotherapy, while effective, are often limited by systemic toxicity, incomplete tumour eradication, and the emergence of drug resistance. A resurgence of interest in microbial-based therapies has positioned bacteria and the broader microbiome as powerful tools in the oncologist's arsenal. This review synthesises current evidence on four major axes of microbial cancer therapy: the use of oncolytic bacteria and genetically engineered bacterial strains to directly target and destroy tumour cells; the well-established role of Bacillus Calmette-Guérin (BCG) as an immunotherapy for non-muscle-invasive bladder cancer; the influence of the gut microbiome on immune checkpoint inhibitor (ICI) efficacy; and the emerging clinical applications of faecal microbiota transplantation (FMT) as an adjunct to cancer immunotherapy. We examine relevant preclinical and clinical data, mechanistic insights into tumour microenvironment modulation, and outstanding translational challenges. Microbial therapeutics represent a compelling frontier in precision oncology, with the potential to enhance immune-mediated tumour killing, overcome immunotherapy resistance, and serve as programmable drug-delivery vehicles tailored to individual tumour biology.

Keywords: Oncolytic bacteria; tumour microenvironment; gut microbiome; immune checkpoint inhibitors; BCG; faecal microbiota transplantation; cancer immunotherapy; synthetic biology

Introduction

Cancer is the second leading cause of death worldwide, with approximately 19.97 million new cases diagnosed in 2022 (Siegel et al., 2023). Despite decades of advances in surgery, radiotherapy, targeted therapy, and immunotherapy, significant clinical challenges persist: systemic toxicity of cytotoxic agents, the immunosuppressive tumour microenvironment (TME), and resistance to existing therapies are obstacles that conventional approaches struggle to overcome entirely.

The therapeutic use of microorganisms in cancer has a longer history than commonly appreciated. The pioneering work of William Coley in the late nineteenth century in which he observed spontaneous tumour regression following streptococcal skin infections in cancer patients laid the foundation for what we now term bacterial immunotherapy (Roe et al., 2023). Coley subsequently developed 'Coley's toxins', a mixture of heat-killed bacteria that induced durable remissions in cases of sarcoma, myeloma, lymphoma, and carcinoma. This pivotal observation preceded modern immunotherapy by nearly a century and demonstrated that the immune system, appropriately activated, could recognise and destroy cancer cells.

In the ensuing decades, research into microbial cancer therapies largely gave way to the rise of chemotherapy. However, one microbial-based treatment did successfully enter clinical practice: intravesical Bacillus Calmette-Guérin (BCG) instillation for non-muscle-invasive bladder cancer (NMIBC), which remains the standard of care more than 30 years after its approval (Wang et al., 2024). The success of BCG, combined with breakthroughs in genomics, synthetic biology, and our understanding of the gut microbiome, has catalysed a renaissance in microbial oncology.

Today, researchers are exploiting multiple microbial modalities. Naturally occurring and genetically engineered bacteria are being developed as oncolytic agents capable of homing selectively to the hypoxic cores of solid tumours regions inaccessible to most conventional drugs (Min et al., 2019). Simultaneously, a wealth of evidence has demonstrated that the composition of the gut microbiome profoundly influences the efficacy of immune checkpoint inhibitors (ICIs), the dominant modality in modern cancer immunotherapy (Zhao et al., 2024). Faecal microbiota transplantation (FMT) has emerged as a clinically feasible method to reshape the gut microbial ecosystem and enhance ICI responses in patients with advanced malignancies (Routy et al., 2023).

This review provides a comprehensive overview of the major strategies by which microbes are being harnessed to treat cancer, drawing on recent preclinical studies, clinical trials, and mechanistic insights published primarily between 2019 and 2025.

Oncolytic Bacteria: Direct Tumour Targeting and Destruction

Tumour-Targeting Mechanisms

A defining biological advantage of oncolytic bacteria over conventional therapeutics is their capacity to selectively accumulate within solid tumours. Several interrelated mechanisms drive this tropism. Tumours characteristically exhibit regions of hypoxia, necrosis, and disordered vasculature that create nutrient-rich niches poorly accessible to the immune system and conventional drugs. Obligate anaerobes such as *Clostridium* and *Bifidobacterium* thrive in these hypoxic zones, while facultative anaerobes like *Salmonella typhimurium* and *Escherichia coli* exploit both the aerobic periphery and the necrotic tumour core (Min et al., 2019). Bacteria are estimated to accumulate at tumour-to-normal-tissue ratios of up to 1,000:1 following systemic administration, providing an inherent degree of tumour specificity (Roe et al., 2023).

Beyond passive accumulation, certain bacterial species actively invade cancer cells. Salmonella employs its type III secretion system to inject effector proteins that disrupt host cell signalling and enable intracellular colonisation (Min et al., 2019). *Listeria monocytogenes*, through the surface protein internalin A, can penetrate tumour cells and the blood-brain barrier, making it especially relevant for central nervous system malignancies. Once inside, these bacteria directly lyse cancer cells, releasing tumour-associated antigens (TAAs) that prime adaptive antitumour immune responses.

A recent landmark study published in Nature Biomedical Engineering demonstrated that a consortium of two naturally occurring tumour-resident bacteria *Proteus mirabilis* (A-gyo) and *Rhodopseudomonas palustris* (UN-gyo) in a precisely defined 3:97 ratio eradicated diverse tumour types in both immuno-competent and immuno-compromised mouse models, including human xenografts, without inducing cytokine release syndrome (Nakashima et al., 2025). Mechanistic analyses revealed that the consortium triggered selective intratumoural thrombosis and vascular collapse, underpinned by cytokine induction, fibrin deposition, and platelet aggregation, culminating in tumour necrosis. This drug-free approach raises intriguing possibilities for patients with compromised immune systems in whom conventional immuno-therapies are less effective.

Key Bacterial Genera in Oncolytic Therapy

Salmonella typhimurium has attracted the most extensive investigation as an oncolytic agent. Attenuated strains lacking aroA/aroD metabolic genes are auxotrophic and incapable of systemic pathogenicity, yet retain robust tumour-colonising activity. Phase I clinical trials with VNP20009, a lipid A-modified attenuated *Salmonella* strain, demonstrated safety and confirmed intratumoural colonisation in patients with metastatic melanoma and renal cell carcinoma, although objective responses were limited, prompting further engineering efforts (Min et al., 2019). More recent work has demonstrated that engineered *Salmonella* expressing human papillomavirus (HPV) antigens elicited potent antitumour immunity in a cervical cancer mouse model (Wang et al., 2024).

Clostridium species, as obligate anaerobes, are particularly suited to colonising the hypoxic and necrotic cores of solid tumours. Clostridium novyi-NT, from which the primary toxin gene (ntx) has been deleted, has shown promising antitumour activity following intratumoral injection of spores, with phase I clinical studies demonstrating extensive tumour destruction via gas pocket formation in lesion biopsies, as confirmed by computed tomography imaging (Min et al., 2019). The first bacterial oncolytic drug in China *Clostridium ghonii* spore freeze-dried powder for injection entered a phase II clinical trial for advanced soft tissue sarcoma (Chen et al., 2023).

Listeria monocytogenes has been extensively engineered as a cancer vaccine platform. The attenuated strain CRS-207, engineered to express the tumour antigen mesothelin, elicited robust mesothelin-specific T cell responses in mouse models and demonstrated tolerability in patients with refractory mesothelioma, ovarian cancer, lung cancer and pancreatic cancer (Wang et al., 2024). CRS-207 has also been evaluated in combination with GVAX (irradiated, GM-CSF-secreting tumour cells) in pancreatic ductal adenocarcinoma, where the combination improved overall survival compared to chemotherapy in a randomised phase II trial.

Bifidobacterium, a probiotic genus, preferentially colonises the necrotic regions of solid tumours and has been investigated both as a direct oncolytic agent and as a carrier for therapeutic payloads. Several studies have shown that Bifidobacterium can enhance the antitumour efficacy of immune checkpoint blockade by modulating the TME and stimulating dendritic cell maturation (Zhao et al., 2024).

Genetically Engineered Bacteria and Synthetic Biology

The integration of synthetic biology has transformed the therapeutic potential of oncolytic bacteria, enabling precise spatial and temporal control of therapeutic payload delivery within the TME. Engineered bacteria can be programmed to produce anticancer molecules in situ including cytokines, toxins, enzymes and checkpoint inhibitor antibodies only upon detecting tumour-derived signals, thereby maximising therapeutic concentrations at the tumour site while minimising systemic toxicity (Chen et al., 2025).

An elegant approach developed by Gurbatri and colleagues employed engineered *E. coli* equipped with a quorum-sensing circuit that triggers synchronised bacterial lysis upon reaching a defined cell density within tumours, releasing a programmed payload including the cytokine IL-12 and the anti-CD47 nanobody. This 'bacterial time bomb' approach achieved complete tumour remission in preclinical colorectal cancer models and initiated systemic immune responses that suppressed distant metastases (Gurbatri et al., 2022, cited in Chen et al., 2025).

Thermally inducible systems have also been developed. Wang et al. engineered non-pathogenic *E. coli* incorporating a thermally sensitive promoter driving expression of the pore-forming toxin cytolysin A (ClyA). At 45°C achievable locally via focused ultrasound or photothermal nanoparticles ClyA is expressed and forms membrane-perforating channels in cancer cells, enabling spatially controlled oncolysis (Roe et al., 2023).

Bacteria are also being developed as living drug-delivery vehicles. In a particularly innovative approach reported by Khanduja et al. in 2024, a Salmonella-based platform was engineered to deliver the genome of the oncolytic virus 'minute virus of mice' into cancer cells (Chen et al., 2025). Following bacterial invasion and lysis inside tumour cells, plasmids encoding the viral genome were released, producing infectious virions that then proceeded to selectively lyse the infected tumour cells effectively creating a self-amplifying oncolytic cycle. Such hybrid bacteria-virus strategies combine the tumour-targeting precision of bacteria with the deep cellular penetration and potent lytic activity of viruses.

Nanomaterial-assisted oncolytic bacteria (NAOB) represent another frontier. Coating bacteria with gold nanoparticles (generating Bac@Au biohybrids), for example, confers photothermal responsiveness, enabling near-infrared laser-triggered bacterial activation and tumour killing in solid tumour models (Li et al., 2024). These nanobiohybrid platforms enhance

tumour penetration, allow real-time imaging guidance, and can be loaded with chemotherapeutic payloads.

Bacillus Calmette-Guérin (BCG) in Bladder Cancer

BCG as the Prototype Microbial Cancer Immunotherapy

Bacillus Calmette-Guérin is a live attenuated strain of Mycobacterium bovis, first developed as a tuberculosis vaccine in 1921. Its antitumour properties were serendipitously discovered in the 1970s, and it was approved by the US Food and Drug Administration in 1990 for intravesical instillation in NMIBC making it the oldest and arguably most successful microbial cancer therapy in clinical use (Kawai et al., cited in Chen et al., 2023). Bladder cancer is common, with approximately 83,190 new cases projected in the United States in 2024, of which roughly 75% are non-muscle-invasive at diagnosis (Ślusarczyk et al., 2024).

Intravesical BCG reduces tumour recurrence and decreases the risk of progression to muscle-invasive disease in patients with high-risk NMIBC. Both urothelial cells and immune cells play crucial roles in BCG's antitumour mechanism (Redelman-Sidi et al., 2014). Following instillation, BCG adheres to fibronectin on the urothelial surface a process mediated by the bacterial fibronectin attachment protein and is internalised by both normal and malignant urothelial cells. Internalised BCG triggers the secretion of proinflammatory cytokines and chemokines including IL-6, IL-8, TNF-α, and GM-CSF, leading to the recruitment and activation of innate and adaptive immune cells, culminating in CD4+ and CD8+ T-cell-mediated tumour killing (Biot et al., cited in Packiam et al., 2024).

Resistance to BCG and Emerging Combination Strategies

Despite its efficacy, BCG fails in 30–50% of patients, with approximately half of failures occurring within six months of initiation (Packiam et al., 2024). Resistance arises through multiple mechanisms: immunosuppression within the bladder TME, downregulation of BCG-binding fibronectin, epigenetic silencing of BCG-responsive genes, and an immunosuppressive microenvironment dominated by regulatory T cells and myeloid-derived suppressor cells (MDSCs). A worldwide supply shortage further complicates its clinical deployment.

The KEYNOTE-057 trial established pembrolizumab, an anti-PD-1 antibody, as the first approved therapy for BCG-unresponsive NMIBC with carcinoma in situ, achieving complete response rates of approximately 40% (Balar et al., cited in Packiam et al., 2024).

More recently, the phase III CREST trial examined the combination of sasanlimab-a novel subcutaneous PD-1 inhibitor-with standard BCG induction and maintenance therapy. Results published in Nature Medicine demonstrated a 32% lower risk of recurrence, progression, or death compared to BCG alone, with three-year event-free survival of 82% in the combination arm versus 75% in the BCG-alone arm (Shore et al., 2025). This trial provides the strongest evidence to date for combining PD-1 checkpoint blockade with BCG in high-risk NMIBC.

Preclinical and early-phase clinical data also support combining BCG with IL-15 superagonists. In a phase I trial, all nine patients treated with BCG plus ALT-803 (an IL-15 superagonist complex) were disease-free at 24 months, with no severe adverse events (Ślusarczyk et al., 2024). The role of the bladder microbiome in modulating BCG response is also an active area of investigation, with studies identifying urinary microbiota diversity as a predictor of treatment outcome; enriching specific members of the Actinomycetota and Pseudomonadota phyla may potentiate BCG efficacy and warrants prospective evaluation (Russo et al., 2024).

The Gut Microbiome and Immune Checkpoint Inhibitor Efficacy

Mechanistic Links Between the Gut Microbiota and ICI Response

Immune checkpoint inhibitors particularly anti-PD-1, anti-PD-L1, and anti-CTLA-4 antibodies have transformed the treatment of advanced malignancies including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), and urothelial carcinoma. Approved for over twenty cancer types, ICIs reinvigorate cytotoxic T-cell responses against tumours by blocking inhibitory immune checkpoints (Hwang et al., 2023). Despite this, primary or acquired resistance affects 50% of melanoma patients and 25-44% of NSCLC patients, representing a major unmet clinical need (Zhao et al., 2024).

A paradigm-shifting body of evidence has established that the composition of the gut microbiota is a key determinant of ICI efficacy. The field was launched in 2015 by two landmark studies demonstrating that specific gut bacteria could enhance anti-CTLA-4 therapy in mice and that antibiotic-induced gut dysbiosis abrogated ICI responses (Vétizou et al., 2015; cited in Hwang et al., 2023). Subsequent mechanistic research has shown that gut bacteria interact with ICI therapy through multiple non-redundant pathways: by programming innate dendritic cell maturation and antigen presentation; by producing metabolites (short-chain fatty acids, inosine, indole derivatives) that modulate T-cell differentiation and function; and by

acting as molecular mimics that activate cross-reactive T cells targeting tumour neoantigens (Zhao et al., 2024).

Specific microbial metabolites have emerged as critical mediators. Roseburia intestinalis-derived butyrate enhances anti-PD-1 efficacy in colorectal cancer by activating cytotoxic CD8+ T cells (Kang et al., 2023). Microbiome-derived inosine modulates checkpoint inhibitor responsiveness by engaging adenosine A2A receptors on T cells, promoting Th1 differentiation and IFN-γ production, thereby boosting antitumour immunity (Mager et al., 2020, cited in Chen et al., 2025). In a 2024 preclinical study, Fusobacterium nucleatum was shown to produce butyrate that downregulates PD-1 expression on CD8+ T cells in colorectal cancer models via epigenetic modification of H3K27ac, alleviating T-cell exhaustion (Jia et al., 2024, cited in Gut Microbiota in Cancer Immunity 2025).

Clinical Evidence: Microbiome Composition and ICI Response

Clinical studies across multiple cancer types have consistently identified differences in gut microbiome composition between ICI responders and non-responders. In patients with NSCLC, *Akkermansia muciniphila*, *Alistipes* spp., *Ruminococcus* spp., and *Eubacterium* spp. were enriched in individuals who responded to anti-PD-1 therapy (Chen et al., 2024, cited in JCI Review 2025). In melanoma patients receiving anti-CTLA-4 therapy, higher baseline abundance of *Faecalibacterium prausnitzii* was associated with improved progression-free survival and reduced colitis risk. In RCC, *Bacteroidetes*-to-*Firmicutes* ratio and *Akkermansia muciniphila* abundance have been linked to ICI benefit (Hwang et al., 2023).

Notably, antibiotic use prior to ICI initiation which causes gut dysbiosis is consistently associated with reduced ICI efficacy and shorter survival across cancer types. In patients with RCC receiving Nivolumab and Cabozantinib, supplementation with CBM588, a bifidogenic live bacterial product containing *Clostridium butyricum*, demonstrated preliminary signals of improved clinical activity of the ICI-based combination in a phase I randomised trial (Dizman et al., 2022, cited in Ciccarese et al., 2024). These findings underscore the gut microbiome as a modifiable determinant of ICI outcomes.

The gut microbiome also influences immune-related adverse events (irAEs) associated with ICIs. Specific microbial compositions have been associated with heightened risk of ICI-colitis, the most clinically significant irAE. Dysbiosis characterised by low *Ruminococcaceae* abundance and high *Bacteroidaceae* abundance predisposes patients to CTLA-4 blockade-induced colitis (Hwang et al., 2023). This bidirectional influence of the microbiome modulating

both antitumour efficacy and immune toxicity positions microbiota profiling as a potentially valuable clinical biomarker for patient stratification and monitoring.

Faecal Microbiota Transplantation in Cancer Treatment

Rationale and Preclinical Evidence

The demonstration that gut microbiome composition determines ICI efficacy immediately raises the therapeutic possibility of reshaping the microbiome in patients with suboptimal microbial profiles. Faecal microbiota transplantation (FMT), in which the entire gut microbial community from a healthy or ICI-responsive donor is transferred to the recipient, represents the most direct approach to this goal. Preclinical models provided early proof-of-concept: transplanting stool from ICI-responding patients into germ-free or antibiotic-pre-treated mice phenocopied the donor response to anti-PD-1 therapy, with increased tumour-infiltrating CD8+ T cells and reduced immunosuppressive regulatory T cells observed in tumour tissue (Chen et al., 2022, cited in Xu et al., 2025).

Mechanistically, donor FMT restores gut microbiota diversity and reprogrammes the gut ecosystem. In mouse models, FMT from anti-PD-1 responders enhanced antitumour immunity through multiple mechanisms: promoting dendritic cell maturation and antigen cross-presentation, enriching tumour-infiltrating lymphocytes (TILs), suppressing myeloid-induced immunosuppression, and activating CD56+CD8+ T cells in the TME (Chen et al., 2022). Critically, FMT from non-responders conferred resistance to ICI therapy when transplanted into recipient mice, demonstrating the bidirectional and causal nature of the microbiome-ICI relationship.

Clinical Trials of FMT Combined with ICIs

Several early-phase clinical trials have now demonstrated the feasibility and encouraging efficacy of FMT combined with ICIs in patients with advanced cancers. A phase I trial (NCT03772899) combined healthy donor FMT with anti-PD-1 in patients with advanced melanoma naive to ICI therapy. Results demonstrated that FMT was safe in the first-line setting, with avatar mouse models confirming increased anti-PD-1 efficacy from healthy donor FMT (Routy et al., 2023).

A seminal study by Kim et al. in 2024 evaluated FMT in 13 patients with unresectable or metastatic solid cancers (gastric, oesophageal and hepatocellular carcinomas) who had progressed on anti-PD-1 therapy. FMT was administered from donors who had experienced

complete or partial responses to anti-PD-1. Remarkably, six of thirteen refractory patients re-responded to immunotherapy following FMT, including a hepatocellular carcinoma patient who, having failed the first FMT, experienced dramatic tumour shrinkage after switching to a different donor for the second FMT (Kim et al., 2024). The investigators identified *Prevotella merdae* Immunoactis as a novel strain associated with enhanced FMT efficacy and *Lactobacillus salivarius* and *Bacteroides plebeius* as detrimental species - findings with direct implications for rationally designed FMT products.

The phase 2 FMT-LUMINate trial, published in Nature Medicine in 2025, evaluated healthy donor FMT plus anti-PD-1 in patients with NSCLC (n=20) or anti-PD-1 plus anti-CTLA-4 in melanoma (n=20) in the first-line setting. Eligible patients received a single oral capsule FMT prior to ICI initiation. Encouraging efficacy was observed in both cohorts, with responses linked to significantly greater loss of baseline bacterial species suggesting that successful microbiome remodelling, rather than a specific donor species, drives clinical benefit (Duttagupta et al., 2025).

The TACITO trial, presented at ESMO 2024, was a randomised phase II study comparing FMT versus placebo in patients with metastatic RCC receiving pembrolizumab plus axitinib. The primary endpoint was met: FMT increased the 1-year progression-free survival rate by 31.7% compared to placebo (66.7% vs 35%). Median PFS was 14.2 months in the FMT group versus 9.2 months in the placebo group, and overall response rate was 52% versus 28%, respectively (Ciccarese et al., 2024). This represents among the most compelling randomised clinical evidence to date for microbiome manipulation as a cancer treatment adjunct.

A meta-analysis of 10 clinical studies involving 164 patients with solid tumours, encompassing data up to September 2024, reported a pooled objective response rate (ORR) of 43% (95% CI: 35–51%) for FMT combined with ICIs (Xu et al., 2025). Subgroup analysis revealed that combined anti-PD-1 plus anti-CTLA-4 therapy yielded a significantly higher ORR (60%) compared to anti-PD-1 monotherapy (37%), suggesting that FMT may be particularly synergistic with dual ICI blockade.

FMT has also demonstrated utility in managing ICI-induced colitis. Wang et al. reported the first successful use of FMT to treat ICI-associated colitis, with patients achieving complete clinical relief, restoration of normal bowel motility, and endoscopic regression of ulcers (Chen et al., 2022). This dual benefit-simultaneously improving ICI efficacy and

managing its most serious gastrointestinal toxicity-positions FMT as a highly attractive clinical tool.

Combination Strategies and Emerging Approaches

The integration of microbial therapies with established modalities-chemotherapy, radiotherapy, adoptive cell therapies and targeted agents has opened a new dimension in precision oncology. Engineered oncolytic bacteria are being combined with CAR-T cells, NK cells, and oncolytic viruses to achieve synergistic antitumour effects (Chen et al., 2025). Bacteria that colonise the immunosuppressive TME can remodel it by secreting pro-inflammatory cytokines and consuming immunosuppressive metabolites, thereby creating a permissive environment for adoptive T-cell therapies to function optimally.

Dietary interventions, prebiotics and probiotics represent simpler though less powerful strategies to modulate the microbiome in support of ICI therapy. Spencer et al. demonstrated that dietary fibre and probiotic supplementation influenced the gut microbiome composition and correlated with improved melanoma immunotherapy response (Spencer et al., 2021, cited in JCI Review 2025). Microbial peptides derived from the microbiota are also being investigated as cancer vaccines. EO2401, a microbial peptide that mimics tumour-associated antigens, activates memory T cells and elicits potent immune responses; in the ROSALIE phase I/II trial in glioblastoma, EO2401 combined with nivolumab and bevacizumab yielded a median overall survival of 14.5 months with an 18-month survival rate of 43.1% (Gut Microbiota and Cancer Immunotherapy 2025).

Bacteriophage therapy the use of viruses that selectively infect bacteria is also gaining traction as a means to modulate the gut microbiome with greater precision than antibiotics or FMT. CRISPR-Cas3-engineered phages can selectively eliminate specific pathogenic bacteria species from the gut microbiome, potentially correcting dysbiosis while sparing beneficial commensals (Gut Microbiota and Cancer Immunotherapy 2025). The integration of phage therapy with cancer immunotherapy remains largely preclinical but represents a compelling future direction.

Challenges and Translational Considerations

Despite the remarkable progress reviewed here, several major challenges must be addressed to realise the full clinical potential of microbial cancer therapies. In the domain of oncolytic bacteria, the primary concerns are biosafety, uncontrolled replication, and systemic

toxicity. Even attenuated strains carry residual pathogenicity risk in immunocompromised patients, and unpredictable bacterial replication within the TME creates uncertainty in dosing and response prediction (Chen et al., 2025). Advances in synthetic biology including auxotrophic strain engineering, kill-switch circuits and exogenously inducible gene expression systems are actively addressing these risks by enabling precise spatiotemporal control over bacterial therapeutic activity.

Manufacturing and regulatory scalability represent additional hurdles. Living bacterial therapeutics require specialised containment, cold-chain logistics, and quality control measures that are considerably more complex than those for small-molecule drugs or monoclonal antibodies. Regulatory frameworks for living biotherapeutic products (LBPs) are still evolving, with agencies including the FDA and EMA developing specific guidance for this product class.

For gut microbiome interventions, the central challenge is the substantial inter-individual variability in microbiome composition and the resulting inconsistency in clinical outcomes. Studies identifying microbiome-ICI response associations have identified different bacteria across cohorts *Akkermansia muciniphila*, *Ruminococcaceae*, *Bifidobacterium*, *Bacteroides thetaiotaomicron* underscoring the complexity of host-microbiome interactions and the absence of a single universal 'responder microbiome' (Zhao et al., 2024). FMT safety is also a concern: in previous clinical trials, patients developed broad-spectrum β-lactamase-producing *E.coli* bacteraemia after FMT, with one fatality and the COVID-19 pandemic highlighted risks of transmitting pathogens through stool (Chen et al., 2022). Rigorous donor screening, standardised FMT preparation and encapsulation technologies are mitigating these risks.

Finally, patient selection and biomarker identification remain critical unresolved issues. Comprehensive characterisation of patients' baseline microbiome, immune landscape and tumour biology will be essential for identifying who will most benefit from a particular microbial intervention. Integration of multi-omic data (metagenomics, metabolomics, immunophenotyping) with machine learning approaches is increasingly being applied to this challenge and promises to enable precision microbial oncology.

Conclusions and Future Directions

The past decade has witnessed a transformation in our understanding of the relationship between microorganisms and cancer. What began with Coley's empirical observations of tumour regression following bacterial infection has evolved into a sophisticated, multi-pronged

field at the intersection of microbiology, synthetic biology, immunology, and oncology. Three interconnected themes emerge from the current evidence base.

First, bacteria can be engineered into precisely targeted, programmable cancer therapies. Their capacity to selectively colonise tumour hypoxia, invade cancer cells, and deliver therapeutic payloads in situ combined with the ability to control their behaviour through synthetic biology circuits makes them uniquely versatile agents that complement the capabilities of small molecules and biologics. The clinical success of BCG in bladder cancer provides proof-of-concept that a living bacterial therapeutic can achieve durable remissions, and the next generation of engineered oncolytic bacteria is positioned to build substantially on this foundation.

Second, the gut microbiome is a central and modifiable determinant of cancer immunotherapy outcomes. The consistent finding across cancer types that microbiome composition predicts ICI response, combined with the bidirectional causal evidence from FMT studies in both mice and humans, establishes the gut microbiota as a tractable therapeutic target. The recent TACITO trial, demonstrating a 32% improvement in progression-free survival with FMT added to ICI-based therapy in RCC, marks a significant milestone towards the integration of microbiome modulation into standard oncological practice.

Third, translational success will require overcoming substantial challenges in safety, manufacturing, and patient stratification. Interdisciplinary collaborations between microbiologists, oncologists, bioengineers, regulatory scientists and bioinformaticians will be essential. Development of synthetic FMT products comprising defined, reproducible consortia of beneficial bacteria rather than whole donor stool may ultimately prove more controllable and scalable than traditional FMT.

Looking ahead, the convergence of single-cell genomics, spatial transcriptomics and artificial intelligence-driven microbiome analysis will enable unprecedented resolution in understanding how specific microbial species, strains, and metabolites interact with individual tumours and immune landscapes. The ultimate vision is a future in which microbial therapies whether delivered as engineered living medicines, microbiome-modulating FMT products, or dietary interventions are personalised to the individual patient's tumour biology and microbiome signature, maximising therapeutic efficacy and minimising harm. This vision is no longer speculative; it is rapidly becoming a clinical reality.

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Chapter 15

The Roles of HSP70 In Microbial Pathogenesis: Molecular Chaperones as Virulence Determinants and Host-Pathogen Interaction Mediators

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Abstract

Heat shock protein 70 (HSP70) represents a highly conserved family of molecular chaperones critical to microbial pathogenesis. Widely distributed across microbial taxa, HSP70 acts dually as a pathogen-derived virulence factor and host cellular target. By retaining essential chaperone functions under stress, pathogen-encoded HSP70 ensures microbial survival in hostile host environments. This review highlights the cytotoxic, immunomodulatory, and invasion-promoting properties of HSP70. Key roles in pathogenesis include facilitating bacterial adhesion, enhancing intracellular survival via stress response coordination, modulating immune responses through Toll-like receptor activation, and promoting antibiotic resistance. Pathogen-derived HSP70 directly interacts with host receptors, disrupts cellular homeostasis, and exploits host chaperone networks in species such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Helicobacter pylori*, and *Candida albicans*. Despite these insights, the tissue-specific roles of HSP70 isoforms during infection and the precise temporal dynamics of HSP70 expression remain poorly understood. This review identifies critical research gaps, emphasizing the need for comprehensive molecular investigations into HSP70-mediated host-pathogen interactions to drive the development of novel therapeutic targets.

Keywords: HSP70, heat shock proteins, microbial pathogenesis, virulence factors, host-pathogen interactions, molecular chaperones

Introduction

Heat shock proteins and the emergence of HSP70 as pathogenicity factors

The discovery of heat shock proteins over five decades ago has fundamentally transformed our understanding of cellular stress responses and protein homeostasis across all domains of life. Among the most extensively studied members of this protein family, heat shock protein 70 (HSP70) has emerged as a master regulator of cellular survival under adverse conditions, with its roles extending far beyond simple stress response to encompass critical functions in microbial pathogenesis. The evolutionary conservation of HSP70 across bacteria, fungi, parasites, and viruses underscores its fundamental importance in maintaining cellular integrity and facilitating adaptation to hostile environments, including the challenging milieu encountered during host-pathogen interactions (Welch, 1992; Lindquist & Craig, 1988).

Microbial pathogens encounter numerous stressors during the infection process, including temperature fluctuations, pH changes, oxidative stress, nutrient limitation, and host immune responses. The ability to rapidly respond to these challenges through HSP70-mediated protein folding assistance, aggregation prevention, and cellular repair mechanisms has been recognized as a crucial determinant of pathogenic success. Moreover, recent investigations have revealed that pathogen-derived HSP70 proteins function not merely as intracellular protective factors, but also as secreted virulence determinants capable of directly manipulating host cellular processes and immune responses (Henderson et al., 2013; Burnie et al., 2006).

Structural and functional characteristics of microbial HSP70 proteins

Microbial HSP70 proteins share the characteristic bipartite architecture observed in their eukaryotic counterparts, consisting of an N-terminal nucleotide-binding domain (NBD) and a C-terminal substrate-binding domain (SBD). The NBD exhibits ATPase activity that drives conformational changes essential for substrate binding and release cycles, while the SBD recognizes hydrophobic amino acid sequences in unfolded or misfolded proteins. This fundamental structure is remarkably conserved across microbial species, reflecting the critical importance of chaperone function in cellular survival (Mayer & Bukau, 2005).

However, microbial HSP70 proteins exhibit unique features that distinguish them from host counterparts and contribute to their pathogenic potential. Many bacterial HSP70 proteins, particularly those encoded by intracellular pathogens, contain specialized signal sequences that facilitate secretion into the host cell cytoplasm or extracellular environment. Additionally,

some microbial HSP70 proteins have evolved specific substrate recognition capabilities that enable interaction with host cellular components, thereby expanding their functional repertoire beyond traditional chaperone activities to include host cell manipulation and immune system modulation (Young et al., 2003; Tsan & Gao, 2009).

Literature search strategy and study selection

Search methodology

A comprehensive literature search was conducted across six major electronic databases, namely PubMed/MEDLINE, Scopus, Web of Science, Embase, Google Scholar, and the Cochrane Library, using Boolean combinations of the following search terms: ("HSP70" OR "heat shock protein 70" OR "DnaK") AND ("microbial pathogenesis" OR "bacterial virulence" OR "fungal pathogenicity" OR "viral replication" OR "host-pathogen interaction") AND ("virulence factors" OR "immune evasion" OR "cellular invasion" OR "antibiotic resistance" OR "stress response"). The search was conducted in January 2025 and encompassed publications from 1990 to 2024 to capture the evolution of understanding in this field.

Inclusion and exclusion criteria

Studies were included in the present review if they satisfied the following criteria: (i) investigated HSP70 proteins from microbial pathogens including bacteria, fungi, parasites, or viruses; (ii) examined the role of HSP70 in pathogenesis, virulence, or host-pathogen interactions; (iii) provided experimental evidence for HSP70 function in microbial survival, immune evasion, or host cell manipulation; and (iv) were published in peer-reviewed journals in the English language. Studies were excluded if they focused solely on HSP70 as a vaccine candidate without mechanistic insights, if they examined only host HSP70 responses without pathogen involvement, or if they represented reviews, conference abstracts, or commentaries without original experimental data.

HSP70 in bacterial pathogenesis

Bacterial HSP70 proteins, commonly designated as DnaK in prokaryotic nomenclature, represent essential components of the bacterial stress response machinery that have been co-opted as virulence factors across diverse pathogenic species. The multifaceted roles of bacterial HSP70 in pathogenesis encompass both intracellular functions that enhance bacterial survival under host-imposed stress conditions and extracellular activities that directly manipulate host

cellular processes. The strategic importance of HSP70 in bacterial pathogenesis is underscored by its conservation among successful pathogens and its upregulation in response to host-derived stress signals.

Table 1: HSP70-mediated virulence mechanisms in bacterial pathogens.

Bacterial Species	HSP70 Mechanism	Pathogenic Role	Clinical Significance
M. tuberculosis	Stress response; Immune evasion	Macrophage survival; Latency	Drug resistance; Chronic infection
L. pneumophila	Host cell invasion; Replication	Intracellular survival	Legionnaires' disease severity
H. pylori	Gastric colonization; Acid resistance	Chronic inflammation	Peptic ulcer; Gastric cancer risk
S. pneumoniae	Complement resistance; Adhesion	Respiratory tract invasion	Pneumonia; Meningitis severity
E. coli	Heat shock response; Biofilm formation	Antibiotic tolerance	UTI persistence; MDR infections

Mycobacterium tuberculosis HSP70: mediating survival in hostile environments

Mycobacterium tuberculosis HSP70, encoded by the dnaK gene, represents one of the most extensively studied bacterial heat shock proteins in the context of pathogenesis. The protein plays crucial roles in enabling *M. tuberculosis* to survive within macrophage phagosomes, where the bacteria encounter multiple stress conditions including acidic pH, reactive oxygen species, and nutrient limitation. Several studies have established that HSP70 expression is dramatically upregulated during intracellular infection, with levels increasing up to 15-fold compared to extracellular growth conditions (Garbe et al., 1993; Ramos et al., 2004).

The protective function of *M. tuberculosis* HSP70 extends beyond simple protein folding assistance to encompass sophisticated mechanisms of immune evasion and drug resistance. The protein has been demonstrated to interact with host cell signaling pathways, particularly those involved in apoptosis regulation and inflammatory responses. Additionally, HSP70 contributes to the development of antibiotic tolerance through its role in maintaining protein homeostasis under drug-induced stress conditions, thereby facilitating the

establishment of persistent infections that characterize tuberculosis pathogenesis (Hingley-Wilson et al., 2003; Young et al., 2003).

Legionella pneumophila HSP70: facilitating intracellular replication

Legionella pneumophila HSP70 exhibits unique characteristics that distinguish it from other bacterial heat shock proteins, particularly in its role as a secreted virulence factor. The protein is actively transported into host cells via the Dot/Icm type IV secretion system, where it performs multiple functions essential for bacterial survival and replication within the Legionella-containing vacuole (LCV). Recent investigations have revealed that *L. pneumophila* HSP70 directly interacts with host cell proteins to subvert normal cellular processes and create a replication-permissive environment (Hickey et al., 2008; Zusman et al., 2003).

The secreted HSP70 of *L. pneumophila* has been shown to modulate host cell trafficking pathways, preventing lysosomal fusion and maintaining the neutral pH of the LCV. Furthermore, the protein contributes to the recruitment of endoplasmic reticulum-derived membranes to the LCV, thereby providing both structural support and metabolic resources necessary for bacterial multiplication. The clinical significance of these mechanisms is underscored by observations that HSP70-deficient *L. pneumophila* strains exhibit markedly reduced virulence in both cell culture and animal models of Legionnaires' disease (Fernandez et al., 2006).

HSP70 in fungal pathogenesis

Fungal pathogens have evolved sophisticated HSP70-mediated mechanisms to survive and proliferate within mammalian hosts, where they encounter numerous environmental stresses including elevated temperature, oxidative stress, and antifungal immune responses. The diversity of fungal HSP70 proteins reflects the complex lifecycle and pathogenic strategies employed by different fungal species, with each having adapted their chaperone systems to meet specific ecological and pathogenic requirements. The strategic importance of HSP70 in fungal virulence is demonstrated by its conservation across pathogenic fungi and its critical role in morphological transitions, drug resistance, and immune evasion.

Candida albicans HSP70: orchestrating morphological transitions and immune evasion

Candida albicans expresses multiple HSP70 isoforms that collectively contribute to its remarkable pathogenic versatility and ability to cause infections ranging from superficial mucosal colonization to life-threatening systemic candidemia. The most prominent of these, HSP70-1 and HSP70-2, exhibit differential expression patterns in response to various environmental stresses and play distinct roles in supporting fungal virulence. HSP70-1 is primarily involved in maintaining cellular homeostasis during thermal stress and pH fluctuations, while HSP70-2 plays a more specialized role in facilitating morphological transitions between yeast and hyphal forms (Perdomo et al., 2006; Sun et al., 2005).

The morphological plasticity of *C. albicans*, mediated in part by HSP70 function, represents a critical virulence mechanism that enables the fungus to adapt to diverse host microenvironments and evade immune recognition. HSP70 proteins facilitate the massive protein remodeling required during yeast-to-hyphae conversion, ensuring proper folding of newly synthesized proteins and preventing aggregation of cellular components during this metabolically intensive process. Moreover, recent studies have demonstrated that *C. albicans* HSP70 proteins can be secreted into the extracellular environment, where they function as immunomodulatory factors capable of suppressing host inflammatory responses and promoting fungal survival (Panaretou et al., 2001).

HSP70 in viral pathogenesis

Viral pathogens, despite their obligate intracellular lifestyle and limited coding capacity, have evolved sophisticated strategies to exploit host HSP70 networks for their replication and survival. Unlike bacteria and fungi that encode their own HSP70 proteins, viruses primarily manipulate host chaperone systems through various mechanisms including transcriptional activation, post-translational modification, and subcellular redistribution. The intimate relationship between viral replication and HSP70 function reflects the fundamental dependence of viral proteins on cellular folding machinery, as well as the strategic advantage gained by modulating host stress responses and immune surveillance mechanisms.

Influenza virus HSP70 exploitation: facilitating viral protein folding and immune evasion

Influenza viruses have developed intricate mechanisms to exploit host HSP70 chaperone networks for multiple aspects of their replication cycle, including viral protein folding, ribonucleoprotein complex assembly, and immune system modulation. The viral

nucleoprotein (NP) and polymerase components require extensive chaperone assistance for proper folding and assembly into functional replication complexes, with HSP70 playing a particularly critical role in preventing aggregation of these highly expressed viral proteins. Studies using temperature-sensitive HSP70 mutants have demonstrated that chaperone function is essential for influenza virus replication, with HSP70 deficiency leading to accumulation of misfolded viral proteins and severe impairment of virus production (Chase et al., 1983; Momose et al., 2001).

Beyond its role in viral protein folding, HSP70 contributes to influenza virus immune evasion through modulation of host antiviral responses. The virus induces HSP70 expression early in infection, which subsequently interferes with type I interferon signaling pathways and reduces the efficiency of antigen presentation to T lymphocytes. This HSP70-mediated immune suppression provides the virus with a temporal window for efficient replication before effective adaptive immune responses can be mounted. The clinical relevance of these mechanisms is highlighted by correlations between HSP70 expression levels and disease severity in influenza patients, suggesting that viral manipulation of chaperone networks contributes significantly to pathogenesis (Hirayama et al., 2004).

Host HSP70 responses and pathogen exploitation

Host cells respond to microbial infection through rapid upregulation of HSP70 expression as part of the cellular stress response, a reaction that initially serves protective functions but can be subsequently exploited by sophisticated pathogens for their own benefit. The dual nature of host HSP70 responses during infection exemplifies the complex evolutionary arms race between pathogens and their hosts, where cellular defense mechanisms are continually co-opted and subverted by microbial invaders. Understanding these intricate host-pathogen interactions involving HSP70 is crucial for developing therapeutic strategies that can enhance protective immunity while minimizing pathogen-beneficial effects.

Toll-like receptor activation and innate immune signaling

Extracellular HSP70, whether released from stressed host cells or secreted by pathogens, functions as a damage-associated molecular pattern (DAMP) capable of activating innate immune responses through toll-like receptor (TLR) recognition. Host-derived HSP70 interacts primarily with TLR2 and TLR4 on immune cells, triggering proinflammatory cytokine production and activation of adaptive immune responses. This mechanism serves an

important protective function by alerting the immune system to cellular damage and pathogen presence. However, several pathogens have evolved strategies to exploit this same pathway for their own advantage, using HSP70-TLR interactions to manipulate immune responses in ways that favor pathogen survival and dissemination (Asea et al., 2002; Vabulas et al., 2002).

The manipulation of TLR signaling by pathogen-derived HSP70 proteins represents a sophisticated immune evasion strategy that can lead to inappropriate inflammatory responses, tissue damage, and chronic infection. Some bacterial HSP70 proteins exhibit modified structures that enable preferential binding to specific TLR subtypes, thereby skewing immune responses away from effective antimicrobial activities toward less harmful inflammatory pathways. This mechanism has been particularly well characterized in mycobacterial infections, where HSP70-mediated TLR activation contributes to the granulomatous inflammatory response that, while attempting to contain the infection, also provides a protective niche for bacterial persistence (Bulut et al., 2005; Henderson et al., 2010).

Therapeutic implications and drug targeting

The central role of HSP70 in microbial pathogenesis has identified these chaperone proteins as attractive targets for novel antimicrobial therapeutic development. Unlike traditional antibiotics that target essential cellular processes, HSP70 inhibitors offer the potential for disrupting multiple aspects of pathogen biology simultaneously, including stress responses, virulence factor expression, and host cell manipulation. The evolutionary conservation of HSP70 structure and function across microbial species suggests that broad-spectrum therapeutic approaches may be feasible, though the challenge of achieving selectivity between pathogen and host chaperones remains a critical consideration in drug development efforts.

Table 2: HSP70-targeting compounds with antimicrobial activity.

Compound	**Target**	**Mechanism**	**Antimicrobial Effect**
VER-155008	ATP binding	ATPase inhibition	Bacterial stress sensitivity
MAL3-101	Allosteric site	Conformational disruption	Fungal growth inhibition
Apoptozole	Co-chaperone	HSP70-HSP40 disruption	Viral replication inhibition

Quercetin	Multiple sites	Expression inhibition	Broad-spectrum activity

Conclusion

It is a well-established fact that microbial pathogens across diverse taxa have evolved sophisticated mechanisms to exploit HSP70 chaperone networks for their survival and pathogenic success. They accomplish this through various biochemical, cellular, and molecular strategies aimed at maintaining proteostasis under stress conditions while simultaneously manipulating host cellular processes. Long-term or severe microbial infections, however, can overwhelm host HSP70 systems and impair fundamental cellular functions, including protein homeostasis, immune surveillance, and tissue integrity. Evidence obtained from numerous laboratory and clinical studies has clearly indicated that HSP70 proteins function as critical determinants of microbial pathogenesis, exerting their effects through an interconnected network of stress response, virulence enhancement, and host manipulation mechanisms.

Changes in HSP70 expression and activity resulting from microbial infection have been demonstrated across multiple pathogen-host systems under both laboratory and clinical conditions, and these alterations correlate directly with disease severity and therapeutic outcomes. Furthermore, the conservation of HSP70-mediated pathogenic mechanisms across bacterial, fungal, and viral pathogens suggests fundamental evolutionary advantages conferred by chaperone exploitation, advantages that may persist even under conditions of antimicrobial therapy and host immune pressure. Similar pathogenic roles of HSP70 proteins, observed across diverse microbial species in various infection models, are likely to be reflected in clinical infections, since the biochemical pathways of protein folding and stress response are highly conserved processes across all domains of life. Additionally, various results obtained from in vitro, in vivo, and clinical studies support this hypothesis.

Reports on HSP70-mediated virulence mechanisms in microbial pathogens reveal that even subtle manipulations of chaperone networks can profoundly impact infection outcomes and therapeutic efficacy. This challenge of HSP70 exploitation in infectious diseases is likely to become increasingly important as antimicrobial resistance continues to emerge and spread globally. Our current understanding of the temporal dynamics of HSP70 expression during infection and the precise molecular mechanisms governing pathogen-host chaperone interactions is still not adequate to fully exploit these systems for therapeutic intervention. An integrated multi-disciplinary approach, combining structural biology, systems biology, and

clinical investigation, is required to advance our understanding of HSP70-mediated pathogenesis and translate these insights into effective therapeutic strategies.

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