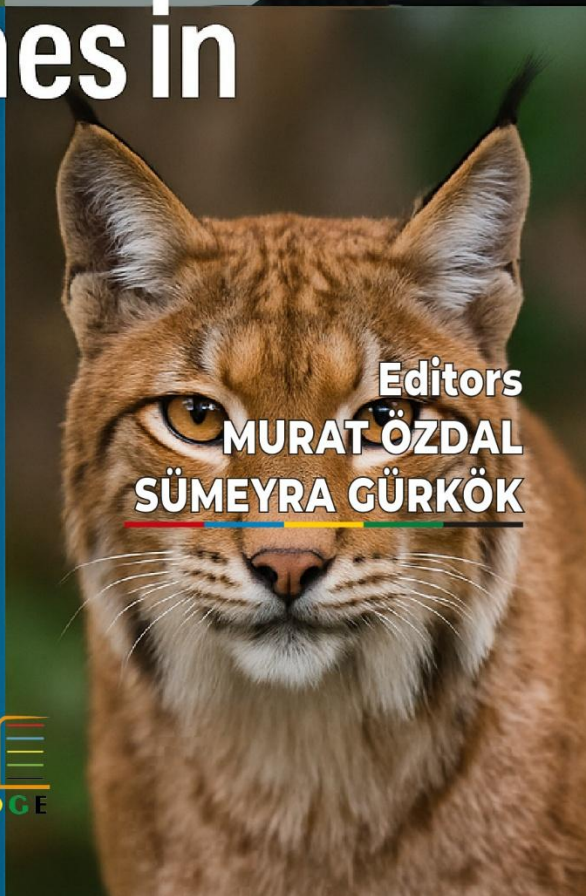
Multidisciplinary Approaches in Biology



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İÇİNDEKİLER

PREFACE

Biology is a continuously evolving science that seeks to understand the complexity of living systems. In recent decades, the integration of various biological disciplines has become increasingly essential to address global challenges in health, environment, and biotechnology. The rapid advances in molecular biology, microbiology, genetics, and environmental sciences have highlighted the importance of multidisciplinary approaches in understanding life and its interactions with the surrounding environment.

This book, *Multidisciplinary Approaches in Biology*, aims to bring together current research perspectives and methodologies from diverse subfields of biology. The chapters encompass a wide range of topics, including microbial biotechnology, metagenomics, bio-based materials, environmental biology, plant sciences, and epigenetic mechanisms. Each contribution reflects the interdisciplinary nature of modern biological research and presents novel insights into both fundamental and applied biological systems.

We would like to express our sincere gratitude to all the authors for their valuable scientific contributions, dedication, and collaboration throughout the preparation of this book. Their expertise and efforts have enriched this collection and made it a valuable resource for the biological sciences community. We also extend our appreciation to the Department of Biology, Faculty of Science, Atatürk University, for their continuous support, and to the

publishing team for their cooperation in bringing this work to completion.

It is our hope that this book will serve as a useful reference for researchers, academicians, and students who seek to explore the dynamic and interdisciplinary nature of contemporary biology.

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2025

EVALUATING SANGER AND NEXT-GENERATION SEQUENCING TECHNIQUES IN MICROBIAL METAGENOMIC APPLICATIONS

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Introduction

Metagenomic approaches have added approximately an innovative transformation in cutting-edge microbiology and environmental genomics with the aid of using allowing the exploration of microbial environment diversity, useful potential, and dynamic structure. These strategies offer get admission to to the genetic make-up of unculturable microorganisms with the aid of using permitting the direct isolation and evaluation of DNA from environmental samples. The effectiveness of metagenomic analyses is mostly formed with the aid of using the potential and accuracy of the sequencing technology employed (Aksu & Karadayı, 2024; Aliyu et al., 2017; Fakıoğlu et al., 2025).

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The Sanger sequencing approach, evolved in 1977 via way of means of Frederick Sanger and his colleagues, turned into lengthy seemed because the gold age in DNA sequencing technology. Based at the precept of dideoxynucleotide chain termination, this approach fashioned the inspiration of genetic studies because of its excessive accuracy and turned into actively utilized in large-scale tasks which includes the Human Genome Project. However, its restrained study period and occasional throughput have rendered it insufficient for studying excessive-extent metagenomic facts (Grada & Weinbrecht, 2013).

Next-Generation Sequencing (NGS) technology, which emerged withinside the early 2000s, revolutionized molecular biology via way of means of overcoming the constraints of traditional methods. This technology permits the parallel sequencing of good sized quantities of facts in a shorter time and at a decrease cost.

Commercial structures which incorporates Roche 454, Illumina, and Ion Torrent have carried out a pioneering role in this variation and function all of sudden acquired splendid adoption. Today, especially in metagenomic studies, NGS systems provide researchers with the opportunity to investigate microbial range in extra depth and detail (Gülşahin, 2024; Goodwin et al., 2016; Rakhalaru et al., 2025).

Sanger and NGS technologies differ significantly in terms of their application protocols, data generation capabilities, error profiles, and analysis requirements. These differences directly impact not only experimental design but also the bioinformatics tools and strategies to be used. The sheer volume of data generated by NGS technologies, in particular, has rendered traditional analysis approaches inadequate, making the use of high-performance bioinformatics analyses essential for processes such as data cleaning,

alignment, annotation, and statistical interpretation (Shendure & Ji, 2008).

As sequencing technologies have advanced, the software tools, databases, and algorithms used to interpret metagenomic data have also evolved rapidly. Thus, metagenomic analyses are no longer just a laboratory process but a multidisciplinary field requiring intensive computational and data processing.

The Evolution of Metagenomic Approaches

Although microorganisms are among the most widespread and diverse life forms in nature, only a small fraction can be isolated and identified using classical microbiological techniques. Studies show that over 99% of microorganisms in environmental environments cannot be cultivated under laboratory conditions (Amann et al., 1995). This has necessitated the development of culture-independent methods to fully understand microbial diversity.

Metagenomics, which emerged in line with this requirement, was first introduced in 1998 by Jo It was introduced to the literature by Handelsman and colleagues. The term metagenomics refers to the assembly of all microbial genomes isolated from a given environmental sample. This approach surpasses classical methods by allowing the analysis of microorganisms' genomes directly from DNA isolated from the environment (Vieites et al., 2008). This new, culture-independent paradigm offers the opportunity to study not only microbial diversity but also functional gene repertoires that change depending on environmental conditions (Handelsman et al., 1998).

Early metagenomic studies focused largely on deciphering the genetic structure of complex microbial communities, such as soil, marine, and human gut microbiota. Craig, in particular, The Sargasso Sea project, led by Venter in 2004, revolutionized

metagenomics with the discovery of thousands of new genetic sequences and potentially new species (Venter et al., 2004). Such studies have contributed not only to the understanding of taxonomic composition but also to environmental adaptation, metabolic potential, and microbial interactions.

Metagenomic evaluation has developed through the years round principal tactics: useful metagenomics and array-primarily based totally metagenomics. Functional metagenomics lets in genetic screening for precise phenotypes through building expression libraries of environmental DNA, at the same time as array-primarily based totally metagenomics pursuits to decide genetic shape via direct DNA sequencing. These tactics may be used complementarily, relying at the studies query and pattern type (Neelakanta & Sultana, 2013).

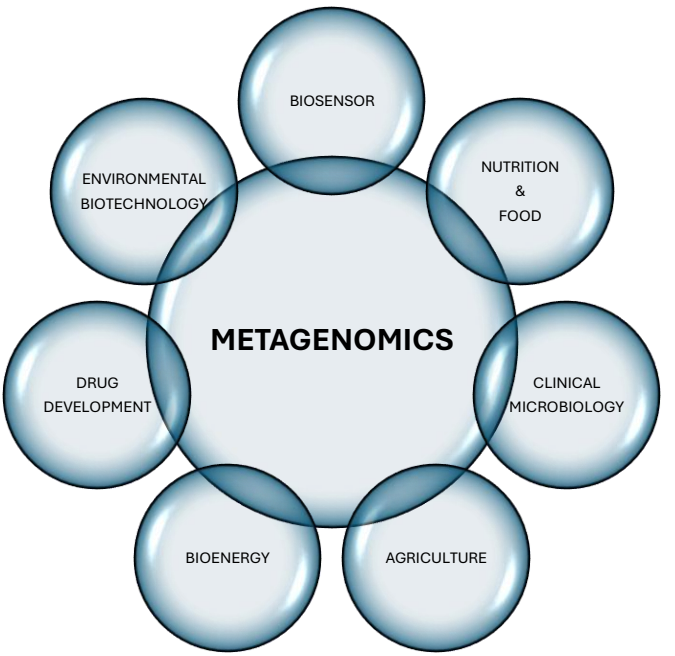
Technological improvements have extensively stepped forward each the feasibility and determination of metagenomic tactics. Initially completed the usage of Sanger sequencing, early analyses had been constrained through excessive charges and coffee facts throughput. However, the arrival of Next-Generation Sequencing (NGS) technology withinside the mid-2000s enabled metagenomic research to be carried out at plenty extra intensity and coverage. This has facilitated the detection of uncommon microbial species and allowed for extra dependable network-degree useful analyses (Alves et al., 2018; Bragg & Tyson, 2014).

Today, metagenomic analyses are completed the usage of numerous strategies, which include shotgun metagenomics and amplicon-primarily based totally sequencing (16S rRNA gene sequencing). Shotgun metagenomics includes random fragmentation and sequencing of all microbial DNA in a pattern, offering each taxonomic and useful insights on the network degree. Amplicon sequencing, on the alternative hand, goals unique gene areas and is

on the whole used for species-degree classification. Each technique has its personal blessings and obstacles and is chosen primarily based totally at the unique studies objectives (Quince et al., 2017).

Metagenomic strategies at the moment are extensively implemented now no longer handiest in primary sciences however additionally in various fields which include medical microbiology, agriculture, environmental biotechnology, meals safety, biosensor, bioenergy and commercial microbiology. In particular, research investigating the effect of the human microbiota on fitness have located metagenomic procedures as a key issue of translational medicine.

Figure 1. *Schematic representation of the application areas of metagenomics.*



The Sanger Sequencing Method and Its Applications

Developed in 1977 through Frederick Sanger and colleagues, the Sanger sequencing approach is taken into consideration one of the foundational strategies of DNA sequencing in molecular biology. This approach is primarily based totally at the precept of dideoxynucleotide chain termination and permits the era of long, high-accuracy series reads. Sanger sequencing changed into seemed because the gold popular in large-scale genetic studies, consisting of the Human Genome Project (Sanger et al., 1977).

Technically, the Sanger method relies on the random incorporation of dideoxynucleotides (ddNTPs) during the synthesis of a new DNA strand by DNA polymerase. Each dideoxynucleotide corresponds to a specific base and halts strand elongation upon incorporation, resulting in DNA fragments of varying lengths. These fragments are then separated by size using gel or capillary electrophoresis, allowing for the determination of the nucleotide sequence.

Among its advantages are high sensitivity, relatively long read lengths (typically 700–1000 base pairs), and a low error rate. However, the method is time-consuming and costly, making it impractical for sequencing large volumes of complex samples. As a result, Sanger sequencing is now predominantly used for targeted sequencing, mutation detection, and validation purposes (Heather & Chain, 2016).

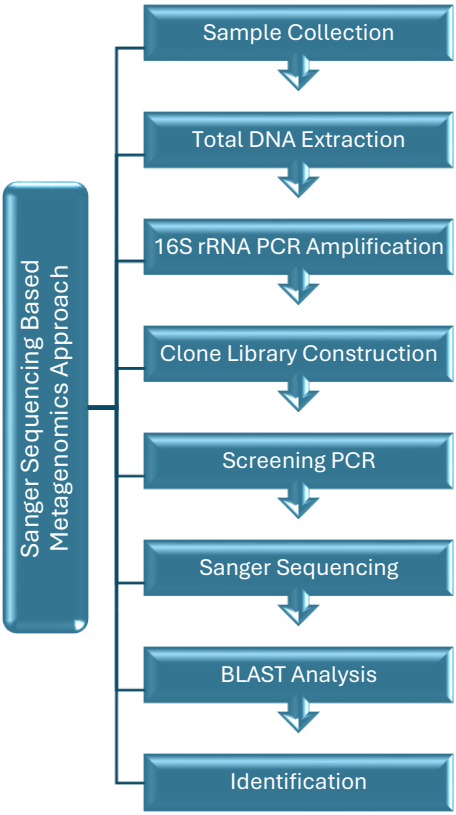
Although the role of Sanger sequencing in metagenomic applications has been limited, its historical significance is substantial. The first metagenomic analyses were performed using this method, contributing greatly to the early discoveries of microbial diversity. Nonetheless, the complexity and volume of data inherent in metagenomic samples necessitated the development of

faster and more scalable next-generation sequencing technologies (Handelsman, 2004).

To better understand the applicability of the Sanger sequencing method, it is useful to outline the general workflow typically employed in such analyses. The process begins with the collection of environmental or clinical samples, followed by total DNA extraction to isolate the genetic material of microbial communities. For bacterial network profiling, PCR amplification of the 16S rRNA gene is generally performed. The ensuing PCR merchandise are used to assemble a clone library, in which DNA fragments are inserted into vectors and converted into capable host cells for propagation.

Selection PCR is then performed on samples selected from positive colonies to confirm the presence of the desired inserts. Following this step, Sanger sequencing is performed on the confirmed samples. The sequences obtained are compared with references in genetic databases and BLAST (Basic Local Alignment Search Tool). This process continues to be a valid method, especially for the detailed analysis of low-complexity microbial communities.

Figure 2. *Stages Applied in the Sanger Sequencing Method.*



Next Generation Sequencing (NGS) Technologies and Metagenomics Applications

Next-Generation Sequencing) was developed in the early 2000s. Sequencing (NGS) technologies have brought groundbreaking innovations to the field of metagenomics. Thanks to its parallel sequencing capacity, NGS enables high data production by sequencing millions of DNA fragments simultaneously in a very short time. These technologies have significantly increased the depth and scope of metagenomics studies and enabled much more detailed analysis of microbial ecosystems (Mardis, 2008).

NGS platforms include Roche 454, Illumina, Ion Various systems exist, such as Torrent and PacBio, each platform has different advantages and limitations. For example, the Illumina system provides high accuracy and high throughput, while PacBio offers long read lengths, making it preferred for deciphering complex genome structures (Goodwin, 2016).

NGS in metagenomic applications, especially shotgun It is extensively used in metagenomics and amplicon- based sequencing methods. Shotgun While metagenomics allows sequencing the genome of the entire microbial community by random fragmentation, amplicon sequencing allows taxonomic classification at the species level by specifically targeting gene regions such as 16S rRNA.

The proliferation of NGS technologies has led to the development of advanced bioinformatics approaches for processing and analyzing metagenomic data. Consequently, larger datasets, failure repair, sequence alignment, and functionalization have contributed to the advancement of the fundamental building blocks of metagenomic analysis.

Next-Generation Sequencing (NGS) demonstrates that individual analyses follow a specific chronological order. The firs step in this process begins with sampling clinic or single-piece samples. Total DNA is isolated from the collected samples, thus obtaining genetic material belonging to the surrounding microbial community.

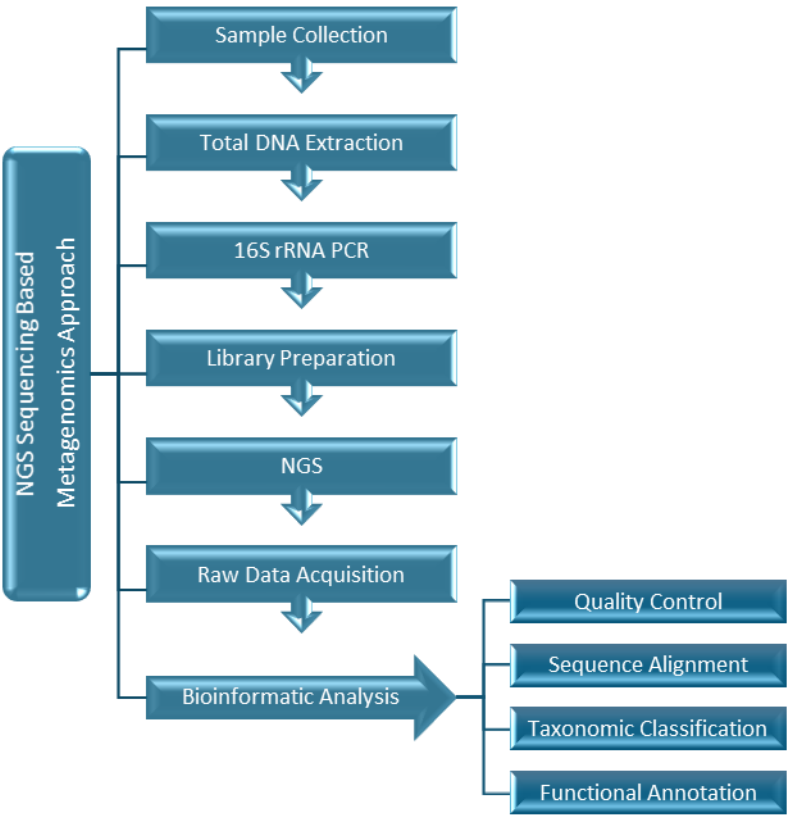
PCR amplification of the 16S rRNA gene, a cammon method for determining microbial diversity, is also applied. The amplification products are used to prepare components suitable for the selected NGS platform, leading to the sequencing phase.

The raw data generated after sequencing is analyzed using various bioinformatics tools. This analysis process includes key

steps such as quality control, sequence alignment, taxonomic representation, and functional annotation.

This systematic approach allows for better identification of microbial particles and a detailed understanding of microbial diversity in biological samples.

Figure 3. *Stages Applied in Next-Generation Sequencing (NGS) Methods.*



Fundamentals of Bioinformatics Analysis and Metagenomic Data Processing

To transform the large-scale data obtained from metagenomic analyses into meaningful biological insights, a comprehensive and

careful bioinformatics process is necessary. The first step in this process is quality control of the raw data. During the quality control phase, low-quality or short sequences are eliminated, adapter sequences are identified, and potential error sources are corrected.

Following quality control, the filtered sequences are aligned to appropriate reference genomes or comprehensive databases. These databases generally contain up-to-date and summarized genome data and are crucial for the accuracy of the analyses.

Following this, the resulting sequences are subjected to taxonomic classification. During this classification process, analyses are conducted specifically for target gene regions, such as 16S rRNA. These analyses reveal the microbial diversity in the sample and allow organisms to be classified based on genome-wide similarities. This provides detailed information about the structural composition of the community.

In the final stage, the genetic sequences are functionally analyzed. This analysis is accomplished through the use of various functional databases designed to predict the biological roles of genes, potential metabolic pathways, enzyme functions, and gene distribution (Bolger et al., 2014).

Today, commonly used tools for analyzing metagenomic data include QIIME, Mothur, MEGAN, MetaPhlAn, and HUMAnN. These software programs largely automate the analysis process, allowing researchers to obtain faster and more reliable results. Table 1 provides a summary comparison of the key features and frequency of use of these tools (Caporaso et al., 2010; Schloss et al., 2009; Huson et al., 2016).

Consequently, the interpretation of metagenomic data relies on robust and accurate bioinformatics approaches, along with high-quality sequencing data, serving as a critical bridge to understanding microbial ecosystems.

Table 1. *Commonly used bioinformatics tools in metagenomic data analysis.*

Software	Main Function	Area of Use	Advantages	Disadvantages
QIIME 2	Taxonomic analysis and diversity calculation	16S rRNA amplicon sequencing	Large user community, easy to use	Processing time can be long for large data sets
Mothur	16S rRNA -based microbial community analysis	Amplicon sequencing	Comprehensive package with many tools	The learning curve can be a bit steep
MEGAN	Taxonomic and functional analysis of metagenomic data	Shotgun metagenomics	Both taxonomic and functional analysis	High memory usage
KRAKEN 2	Sequence-based taxonomic classification	Used in taxonomic analysis	Very fast classification, sensitive and high accuracy rate	High RAM requirement and only taxonomic classification
MetaPhlAn	Microbial community profiling	Shotgun metagenomics	Fast and accurate classification	Focuses only on certain marker genes
HUMAN	Functional annotation and metabolic pathway analysis	Shotgun metagenomics	Provides detailed functional analysis	Can be complicated to set up and use

Figure 4. *The user interface of QIIME 2 (https://qiime2.org/)*

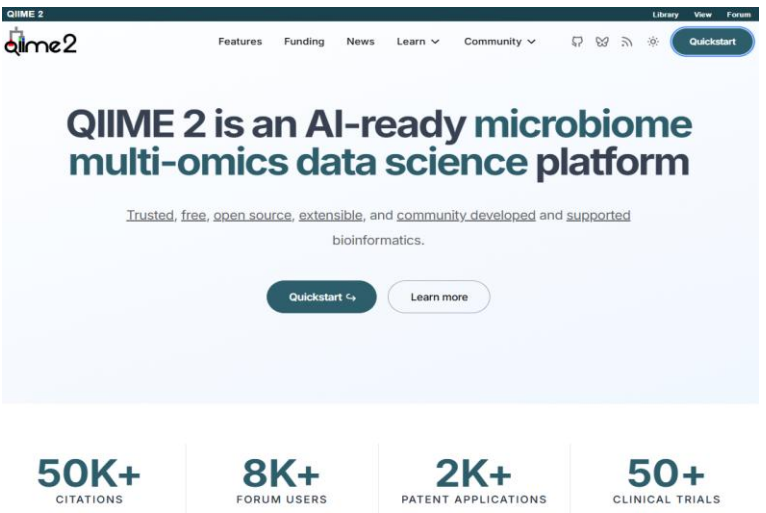


Figure 5. *The user interface of mothur (https://mothur.org/)*

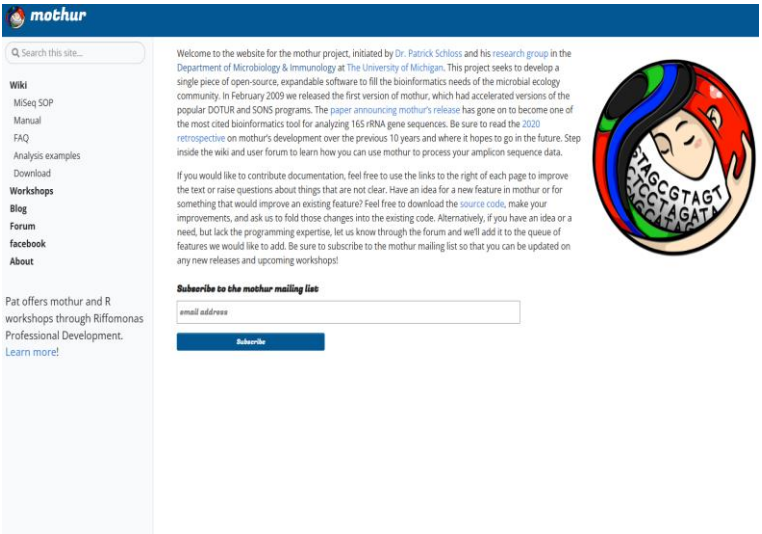


Figure 6. *The user interface of MEGAN (https://uni tuebingen.de/impressum/?in2cookieHideOptIn)*

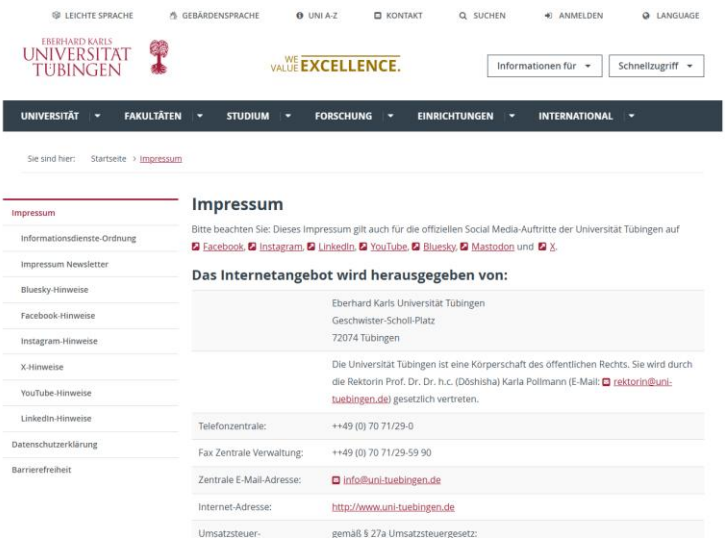


Figure 7. *The user interface of Kraken 2*

(https://ccb.jhu.edu/software/kraken2/)

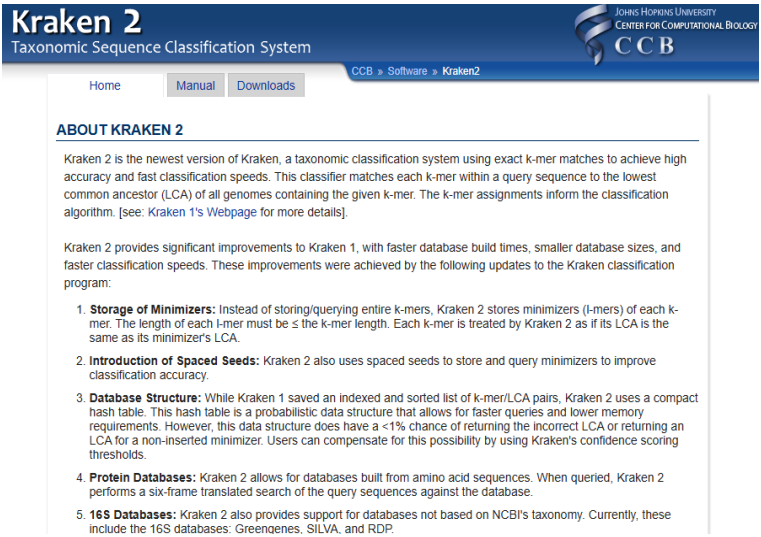


Figure 8. *The user interface of MetaPhlAn*

(https://huttenhower.sph.harvard.edu/metaphlan/)

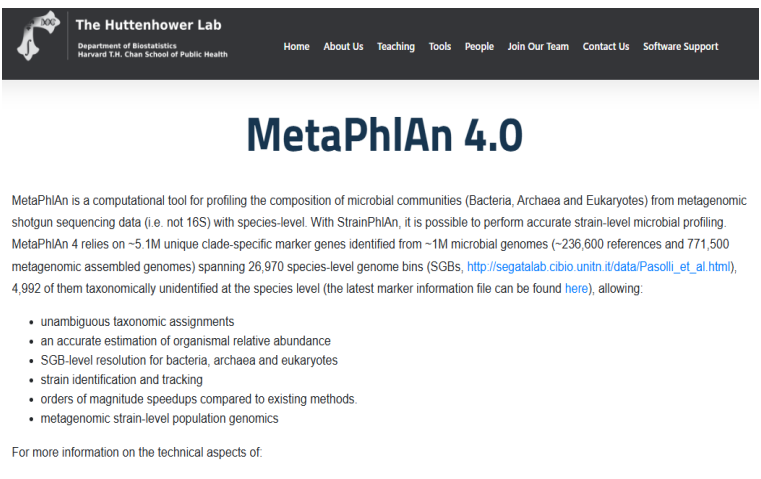
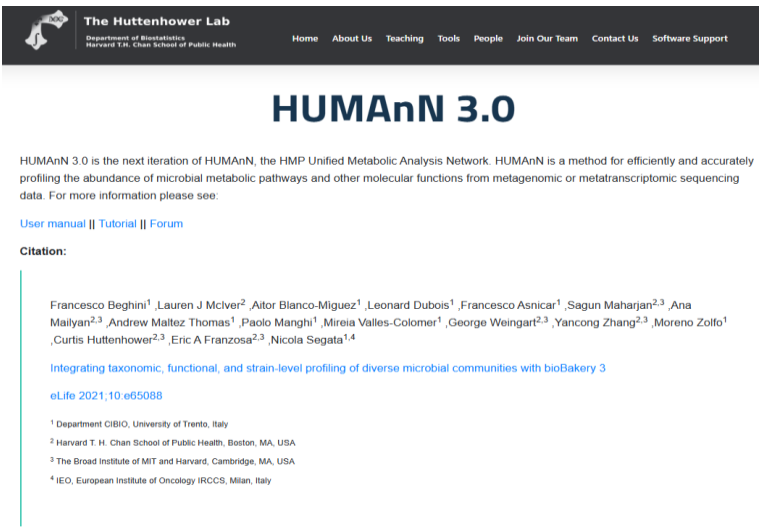


Figure 9. *The user interface of HUMAnN*

(https://huttenhower.sph.harvard.edu/humann)



Comparative Analysis: Sanger Sequencing and Next Generation Sequencing (NGS)

The Sanger sequencing and Next-Generation Sequencing (NGS) strategies utilized in metagenomic analyses mirror one-of-a-kind dimensions of technological improvements and studies desires. Both strategies showcase wonderful benefits and boundaries in phrases of utility regions and information manufacturing capacities (Metzker, 2010).

Developed in 1977, Sanger sequencing has lengthy been appeared because the gold popular in molecular biology because of its excessive accuracy and lengthy study lengths (700–one thousand base pairs). It is primarily based totally on sequencing person DNA molecules and, because of its low throughput, isn't always sensible for large-scale research. This technique is in the main hired for focused sequencing, mutation detection, and validation purposes.

Moreover, Sanger sequencing has appreciably contributed to the unique exam of complicated genome structures (Heather & Chain, 2016).

In contrast, NGS technology, rising withinside the 2000s, have improved the scope of metagenomic research thru excessive parallelism and speedy information manufacturing. NGS systems allow the simultaneous sequencing of hundreds of thousands of brief DNA fragments, taking into consideration unique and excessive-decision analyses of environmental and microbial communities. Although study lengths are commonly shorter than the ones of Sanger sequencing (100–three hundred base pairs), the excessive information throughput and decrease fees make NGS the favored desire for reading complicated and heterogeneous samples (Mardis, 2008).

The blunders profiles of the 2 strategies additionally differ; Sanger sequencing is characterised via way of means of a completely low blunders rate, while NGS technology are greater at risk of base-calling mistakes and sequencing artifacts. This necessitates cautious processing of NGS information the use of bioinformatic tools.

Sanger and NGS technology are frequently used complementarily today. Sanger sequencing is favored for centered analyses requiring excessive accuracy, at the same time as NGS systems are extra appropriate for research concerning a huge wide variety of samples and wide genomic regions. Accordingly, it's miles critical for researchers to genuinely outline their studies targets whilst figuring out the sequencing approach to use. Table 2 below summarizes the key advantages and limitations of both technologies in a comparative manner.

Table 2. *Advantages and disadvantages of Sanger and NGS sequencing methods.*

	Sanger Sequencing	**Next Generation Sequencing (NGS)**
Advantages	High accuracy	Very high data production speed
	Long read lengths (700-1000 bp)	Lower cost
	Effective in target-oriented, specific analyses	Wide coverage with parallel multiplexing
Disadvantages	Low throughput and slow processing time	Shorter read lengths (100-300 bp)
	High cost	Relatively higher error rates
	Limited use in large and complex samples	Requires complex bioinformatics analysis

Conclusion and Future Perspective

Next-generation sequencing (NGS) generation has grown to be a huge milestone withinside the improvement of metagenomic studies. In current years, those strategies have grown to be extra cost-powerful and quicker to generate information, permitting extra distinctive research of microbial diversity. NGS lets in complete statistics to be acquired from environmental samples that have been formerly tough to genetically analyze. This affords scientists with the possibility to higher recognize how ecosystems function (Ayling et al., 2020).

However, third-era sequencing technology also are starting to make huge contributions to metagenomic studies. Longer examine instances and the capacity of a few structures to carry out real-time evaluation are commencing new views for researchers. Artificial intelligence-primarily based totally bioinformatics strategies are more and more getting used and developed. These strategies, in particular, have the cappotential to allow quicker, extra accurate, and

extra complete evaluation of large-scale metagenomic information (Amarasinghe et al., 2020).

Sanger sequencing, taken into consideration one of the classical strategies, stays broadly used because of its excessive accuracy and long-examine capability, specially whilst distinctive research of precise genetic areas is required, notwithstanding advances in generation. Currently, Sanger sequencing is regularly used along side NGS strategies and allows boom the reliability of metagenomic research (Kircher & Kelso, 2010).

Overall, non-stop advances in sequencing generation are broadening our knowledge of microbial ecosystems and commencing up new studies regions in numerous fields, from biotechnology to environmental science. The scope of metagenomic studies is predicted to retain to amplify withinside the future, and the accuracy of the ensuing information is predicted to boom, mainly with the contribution of analytical equipment that assist synthetic intelligence. In parallel with those developments, hobby in metagenomics maintains to grow.

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CURRENT APPROACHES IN PHARMACEUTICAL MICROBIOLOGY

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Introduction

Definition and scope of pharmaceutical microbiology

Pharmaceutical microbiology is an applied science that examines the effects of microorganisms on the production processes, quality control, stability, and safety of medicines. This discipline analyses the roles of both pathogenic and beneficial microorganisms in medicine production and application, while also covering fundamental processes such as the prevention, detection, and control of microbial contamination. Pharmaceutical microbiology is an indispensable quality assurance element at every stage of the pharmaceutical industry, particularly in the production of sterile products (Haider et al., 2024). In addition to traditional microbiological testing methods, pharmaceutical microbiology has

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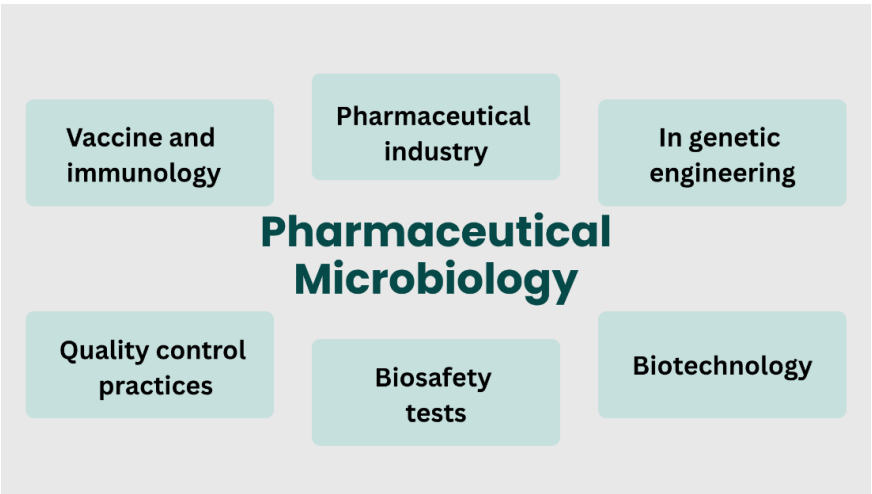
gained more sensitive and specific measurement methods thanks to rapid microbiological methods, molecular diagnostic techniques, and genetic-based analyses developed in recent years. These developments play a critical role in ensuring the microbiological safety of new-generation pharmaceutical products, particularly biotechnological products, live cell therapies, and gene therapies (Chauhan & Jindal, 2020). With advancing technologies, pharmaceutical microbiology has moved beyond traditional quality control applications and has become an interdisciplinary field that integrates bioinformatics, artificial intelligence, and advanced molecular biology techniques. In this respect, it is positioned as a strategic area of expertise in both academic and industrial fields (Kapoor et al., 2020).

Figure.1. Scope of Pharmaceutical Microbiology



Source: Canva (2025) (Designed on 07/10/2025 at https://www.canva.com.)

The place and importance of pharmaceutical microbiology within pharmaceutical sciences

Pharmaceutical microbiology, as one of the fundamental disciplines of pharmaceutical sciences, plays a critical role in

ensuring the microbiological quality of medicines, eliminating contamination from production processes, and controlling microbial-related risks. Pharmaceutical microbiology works in an integrated manner with other disciplines such as pharmaceutical chemistry, pharmacology, pharmaceutical technology, and biotechnology, and is particularly important for the safe production of sterile and biological products (Gilmore & Denyer, 2023). The primary function of pharmaceutical microbiology is to control microorganisms that directly affect product quality. Within this framework, microbiological testing methods (sterility tests, microbial load determinations, pathogen screenings, etc.) are used to verify that medicines comply with pharmacopoeia standards. In this respect, pharmaceutical microbiology is not only part of the quality control process; it also plays an active role in product development, production, storage and distribution stages (Sandle, 2015). In modern pharmaceutical applications, particularly in the production of biological products, vaccines, recombinant proteins, cellular therapies and live microbial products, microbiological control is as vital as the efficacy and reliability of the product (Gunar & Builova, 2016). Pharmaceutical microbiology also ensures the monitoring of the microbiological quality of production environments within the scope of Good Manufacturing Practices (GMP). Practices such as clean room classifications, environmental monitoring protocols, and personnel hygiene fall within the scope of this discipline. Preventing microbial contamination not only ensures product safety but also prevents economic losses and protects patient health (Eissa, 2017). In recent years, advances in molecular microbiology, rapid diagnostic technologies, and artificial intelligence-based quality control systems have increased the impact of pharmaceutical microbiology, transforming this discipline from an area where only classical tests are performed into a multidisciplinary field of expertise. In this context, the role played by pharmaceutical

microbiology throughout the life cycle of pharmaceutical products has deepened with its increasing integration with other scientific disciplines (Nemati et al., 2016). In conclusion, pharmaceutical microbiology is a fundamental scientific discipline that ensures both the safety of products offered to patients and the sustainability of production processes; it occupies a central position within the pharmaceutical sciences. Current developments such as antibiotic resistance, microbiome research, and the rise of biotechnological products further emphasize the importance of this discipline (Tahmasebi et al., 2025).

The relationship between drug safety and microbiological quality

Drug safety is a multifaceted concept that encompasses not only the therapeutic efficacy of pharmaceutical products but also their formulation, manufacture, storage, and administration in a manner that does not harm the patient. In ensuring this safety, microbiological quality emerges as a critical parameter. Microbial contamination poses serious risks, particularly for sterile products, liquid formulations, biotechnological medicines, and topical preparations, and can directly threaten both the clinical efficacy of the product and patient health (Hashim & Celiksoy, 2025). Microbiological quality refers to the requirement that pharmaceutical products contain microorganisms within specified microbial load limits or be completely free of microorganisms. These requirements are defined by pharmacopoeia standards. Absolute sterility is essential for sterile products; in non-sterile products, microbial presence is permitted within certain limits. However, it is important that contaminant microorganisms are non-pathogenic and do not compromise the physicochemical stability of the product (Luis Jimenez, 2004). Microbiological quality deficiencies may lead to systemic infections, immune response disorders, toxin formation, or biological inactivation of the active

ingredient. For example, in recent years, some eye drop products recalled from the market by the US Food and Drug Administration (FDA) were found to be contaminated with *Burkholderia cepacia* and *Pseudomonas aeruginosa*, which caused serious adverse events, including blindness. Such incidents clearly demonstrate the decisive role of microbiological control in drug safety (Tavares et al., 2020). From a drug safety perspective, not only the final product but also the production environment, personnel hygiene, water systems and packaging materials should be assessed as sources of microbiological risk. In accordance with GMP (Good Manufacturing Practice) principles, regular environmental monitoring should be carried out in these areas and contamination risks should be minimized (Sandle, 2019).

Historical background and scientific development

The transition from antiquity to modern pharmaceutical microbiology

Pharmaceutical microbiology, although a relatively young scientific discipline in the modern sense, has origins that stretch back to ancient times in human history. Although the existence of microorganisms was unknown in ancient times, the infectious nature of infections was intuitively recognized, and empirical practices based on this were developed. The Ebers Papyrus and the Hippocratic Corpus, dating back to around 1500 BC, document the use of antiseptic herbal oils and liquids to prevent wound infections. These practices were shaped by observational experience rather than microbiological knowledge (Abdel-Razek et al., 2020). Throughout the Middle Ages, diseases were generally interpreted through metaphysical and theological explanations, and the existence of microbial agents was denied. The prevailing view of the period, the miasma theory, argued that diseases spread through foul odours (Karamanou et al., 2012). The scientific foundations of

pharmaceutical microbiology were laid in the 17th century when Antonie van Leeuwenhoek observed microscopic organisms, which he called ‘animalcules,’ in water droplets and human saliva using simple microscopes. However, the relationship between microorganisms and disease was only scientifically established in the 19th century (Robertson, 2022). In this context, Louis Pasteur and Robert Koch are recognized as the founding figures of modern microbiology and pharmaceutical microbiology. Pasteur's work on fermentation, pasteurization, and the ability of living organisms to cause disease through transmission; and Koch's development of pure culture techniques and his definition of the causative-disease relationship through Koch's postulates, directly formed the basis of pharmaceutical microbiology. This period also saw the emergence of the concepts of sterilization and asepsis (Blevins & Bronze, 2010; Cavaillon & Legout, 2022). The discovery of antimicrobial drugs in the early 20th century, with Alexander Fleming's penicillin derived from Penicillium notatum, marked a revolutionary era in pharmaceutical microbiology (Bennett & Chung, 2001). This development has accelerated not only the treatment of infectious diseases but also the advancement of quality standards that prioritise the microbiological safety of medicines. From the second half of the twentieth century onwards, microbiological contamination control gained a systematic structure alongside GMP principles in pharmaceutical production processes (Chhabra et al., 2024). Today, pharmaceutical microbiology has evolved into an interdisciplinary field equipped with multidimensional tools such as molecular diagnostic techniques, rapid microbiological methods (RMM), genetic typing, and artificial intelligence-supported contamination modelling, in addition to classical culture methods. This transformation is the result of an evolution spanning from ancient intuitive approaches to modern molecular control mechanisms (Surrette et al., 2018).

The historical evolution of pharmacopoeia standards

Pharmacopoeia standards are official references established to ensure the quality, safety and efficacy of medicines. The integration of standards relating to microbiological quality into pharmacopoeias became possible after the scientific acceptance of the theory of microbial disease. In particular, the work of Pasteur and Koch in the 19th century pioneered the development of the concepts of sterilization and asepsis; these developments led to the inclusion of sterility tests in pharmacopoeias such as the USP and BP in the early 20th century (Surrette et al., 2018). Over time, microbial load limits have been established for medicines other than sterile products, and microbiological limit tests and pathogen screenings have been standardized. Since the 2000s, regulation has been harmonized between the USP, Ph. Eur. and Japanese Pharmacopoeia, with the aim of achieving global consistency in testing. Today, pharmacopoeias also incorporate modern approaches such as rapid microbiological methods (RMM), molecular tests, and microbiological risk management. Thus, pharmacopoeia standards have become not only test specifications but also the cornerstones of quality assurance systems (Sutton, 2006).

Basic microbiological methods and techniques

The methods used to ensure the microbiological quality of pharmaceutical products are of critical importance in guaranteeing both product safety and compliance with regulatory requirements (Cengiz & Yapar, 2020). These methods range from classical culture techniques to molecular-level analyses.

Conventional cultural methods

Culture methods are traditional microbiological tests that enable the controlled growth and identification of microorganisms. In these methods, microorganisms are inoculated into growth-

favorable media (agar or liquid) and incubated at the appropriate time and temperature. The purpose of these tests is to determine whether the product carries microbial contamination and to identify the type of contaminant. Although culture methods are low-cost, easy to perform, and provide quantitative data, they may be insufficient for detecting microorganisms that cannot be cultured. Therefore, in some cases, they may need to be supported by molecular methods (Kocagöz, 2013).

Sterility tests

Sterility tests are a microbiological analysis applied to pharmaceutical forms that must be sterile, particularly parenteral, ophthalmic, implant and certain irrigation products. These tests are performed to verify that the product does not contain live microorganisms and are detailed in pharmacopeia monographs such as the USP and European Pharmacopoeia. There are two primary sterility test methods:

1. Membrane Filtration Method: This method is preferred especially for products containing bulky liquids and antibacterial substances. The product is passed through a sterile membrane filter; the filter is transferred to a suitable culture medium and incubated.
2. Direct Inoculation Method: The product is inoculated directly into a sterile culture medium and incubated for 14 days.

Although sterility tests play a decisive role in the release of production batches, a ‘negative result’ cannot be interpreted as ‘absolute sterility’. The sensitivity of the test depends on the risk of contamination of the medium and the validation of the method (Öztürk & Yıldız, 2016; Sutton, 2011).

Microbial load determinations

Microbial load determinations are performed to determine the number of aerobic mesophilic microorganisms present in non-sterile pharmaceutical products (tablets, syrups, ointments, etc.). These tests are mandatory to demonstrate compliance with pharmacopoeial limits. The method is conducted in accordance with the USP and European Pharmacopoeias (Dao et al., 2018). In the method, samples are diluted appropriately and:

- Pour plate
- Spread plate
- One of the membrane filtration methods is applied to solid media.

Incubation periods and temperatures:

- For bacteria: 3–5 days at 30–35 °C
- For yeast/mould: 5–7 days at 20–25 °C

Culture media:

- Soybean Casein Digest Agar: Bacteria
- Sabouraud Dextrose Agar: Yeasts and moulds

Microbial load limits vary according to the product form and route of administration, as specified in the pharmacopoeia. As the product matrix may inhibit the test system in these tests, suitability tests must be performed as part of validation (Charnock, 2004).

Antibiogram and microbial susceptibility tests

Antibiograms and microbial susceptibility tests are fundamental pharmaceutical microbiology techniques that enable the quantitative or semi-quantitative determination of microorganisms' in vitro susceptibility levels to antimicrobial agents.

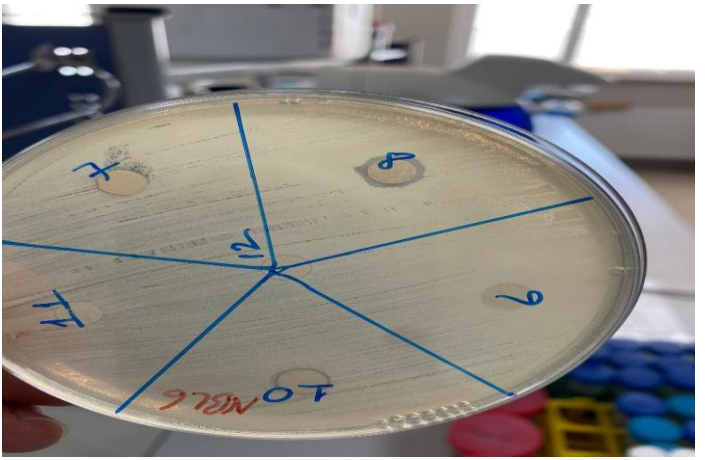
These tests play a critical role not only in guiding treatment in the clinical setting, but also in the development of pharmaceutical products, control of microbial contamination, and determination of protective efficacy (Gajic et al., 2022). Microbial susceptibility tests are used to determine the minimum inhibitory concentration (MIC) and/or minimum bactericidal concentration (MBC) of antibacterial, antifungal, and antiviral agents against specific microorganisms. While demonstrating the efficacy of antibiotics, they are indispensable in characterizing contaminants encountered in pharmaceutical production environments, testing the efficacy of antimicrobial preservatives used, and particularly in monitoring antimicrobial resistance (AMR) processes. These tests enable:

- Preclinical evaluation of new antimicrobial agents can be performed.
- The efficacy of antimicrobial preservatives in pharmaceutical products can be verified.
- The antibiotic resistance profiles of contaminant microorganisms isolated from the production environment can be determined (van Belkum et al., 2020).

3. Disk Diffusion Method (Kirby–Bauer Test)

It is the most commonly used sensitivity test. A standardized bacterial suspension (0.5 McFarland standard) is spread onto the surface of Mueller-Hinton agar. Antibiotic-containing discs are placed on this surface and left to incubate for 16–18 hours. The effect of antibiotics on microorganisms is evaluated by measuring the diameter of the inhibition zones formed. These measurements are interpreted according to CLSI or EUCAST standards (Jorgensen & Turnidge, 2015; Liu et al., 2023).

Figure.2. Disk Diffusion Method



Source: Photographs were taken during experiments conducted in the molecular biology laboratory of Atatürk University, Faculty of Science, Department of Biology.

4. Dilution Methods (Micro and Macro Dilution)

Different concentrations of the antibiotic are incubated with the microorganism in liquid medium to determine the Minimum Inhibitory Concentration (MIC) value. This method is considered the gold standard, particularly in research and product development studies (Vanegas et al., 2021).

Microdilution method: 96-well microplates are used.

Macrodilution method: This is a more traditional method performed using tubes.

MIC: The minimum antimicrobial concentration that inhibits the growth of microorganisms.

MBC: The minimum antimicrobial concentration that kills 99.9% of microorganisms.

5. E-test (Epsilometer Test)

Special strips containing varying concentrations of an antibiotic are placed on agar. An inhibition zone forms along the strip, and the MIC value is read directly on the strip. This method combines the advantages of the disc diffusion and dilution methods (Michael et al., 2021).

6. Automatic Systems

Automated susceptibility testing devices such as VITEK 2®, Phoenix® or MicroScan® are also widely used to increase laboratory efficiency. These systems can provide both identification and susceptibility testing in a short time. However, they have limitations such as cost and calibration requirements (Khan et al., 2021).

Antibiograms and microbial susceptibility tests are indispensable methods used not only to provide clinical treatment guidance but also for multifaceted purposes such as the development of pharmaceutical products, monitoring contamination risks, and determining the efficacy of antimicrobial preservatives. Given the increasing threat of antimicrobial resistance, the correct application of these tests and their execution in accordance with continuously updated protocols is of strategic importance for drug safety (Wenzler et al., 2023).

Microbiological endotoxin tests

Microbiological endotoxin tests are performed to assess whether pharmaceutical products administered directly into the body, such as parenteral drugs, implants, biological products, and haemodialysis solutions, are safe from endotoxin contamination caused by lipopolysaccharide (LPS) found in the cell walls of Gram-negative bacteria. These tests are complementary to sterility tests and play a critical role, particularly in preventing pyrogenic reactions (Spoladore et al., 2021).

Endotoxins are molecules, particularly those with a Lipooligosaccharide (LOS) and Lipopolysaccharide (LPS) structure. These molecules can trigger a powerful pro-inflammatory response via Toll-like receptor 4 (TLR4) in the immune system, leading to fever, shock, and fatal systemic inflammatory syndromes. The toxic effects of endotoxins can occur even in the absence of live bacteria, meaning that even a sterile product can be pyrogenic. For this reason, endotoxin testing is a critical quality indicator in pharmaceutical quality control systems (Dubczak et al., 2021).

7. LAL (Limulus Amebocyte Lysate) Test

The LAL test is a biologically based test that detects the presence of endotoxins using lysate obtained from the amebocytes of the North American horseshoe crab (Limulus polyphemus). Endotoxins activate a zymogen enzyme called factor C within the LAL. This activation ultimately activates coagulation factors, resulting in gel formation, turbidity, or chromogenic change (Su et al., 2015).

Advantages:

- High sensitivity
- Results in a relatively short time
- Compliance with clinical and pharmaceutical regulations

Limitations:

- The LAL reagent is of animal origin (ethical and sustainability concerns)
- Certain pharmaceutical components may affect the test result by masking or enhancing it.
- Interference and validation are mandatory for each product prior to testing.

8. Alternative Methods: rFC (Recombinant Factor C) Test

Today, the rFC (recombinant Factor C) test, developed using recombinant DNA technology for reasons of animal welfare and sustainability, is emerging as an ethical alternative to the LAL test. In this method, factor C protein is produced recombinantly without the need for Limulus, and results in fluorescence or chromogenic signal production in the presence of endotoxins.

Features of the rFC Test:

- Does not require the use of animals (ethical superiority)
- High specificity and accuracy
- Low variation, high reproducibility

Microbiological endotoxin tests are among the essential analyses for the safety of pharmaceutical products. Although the LAL test has traditionally been widely used, recombinant methods offer more advantageous options in terms of both ethics and sustainability. In the field of pharmaceutical microbiology, the validation of these tests and their execution in accordance with regulations is mandatory for both patient safety and product quality (Piehler et al., 2020).

Current Technological Approaches and Innovations

Pharmaceutical microbiology has been reshaped in recent years through the application of advanced technologies in order to overcome the limitations of classical methods and to evaluate microbial safety more rapidly, accurately and specifically (Nemati et al., 2016). The integration of these technologies into pharmaceutical microbiology and their practical implications are currently utilized in numerous fields.

Rapid microbiological methods (RMM)

Although classical methods used in pharmaceutical microbiology are highly reliable, they have limitations such as long incubation times, low sensitivity, and susceptibility to human error. These constraints particularly prolong the product release process and lead to delayed intervention in contamination situations. Therefore, in recent years, rapid microbiological methods (RMM) have played an important role in the modernization of industrial production processes. RMM are technological methods that enable microbial contamination to be detected in a shorter time, with higher sensitivity and specificity, compared to classical culture techniques. These methods are widely used in pharmaceutical product release, environmental monitoring, water quality control, personnel hygiene monitoring, and biological product validation (Luis Jimenez, 2001). These systems are based on different technological approaches that enable the direct or indirect detection of microorganisms.

Optical and imaging-based systems enable digital detection based on the physical properties of microorganisms. For example, the bioMerieux ScanRDI® system can detect fluorescently stained microorganisms within a few hours using laser scanning, while flow cytometry analyses live cells based on size, granularity, and morphology by staining them. Furthermore, techniques such as FISH (fluorescence in situ hybridization) can be used to achieve hybridization at the cellular level using fluorescent probes targeting specific genetic sequences. Another approach, bioluminescence systems, detects live cells by measuring the presence of intracellular ATP. ATP is a universal energy molecule found in all living cells and can be easily measured by emitting light through the luciferin–luciferase reaction. With systems such as Clean-Trace™, hygiene analyses, particularly for surface contamination, can be performed in a matter of minutes. Metabolic activity-based systems determine the viability of microorganisms indirectly through metabolic by-

products rather than directly through growth. In these systems, the presence of living cells is detected by measuring the oxygen consumption, carbon dioxide production, or pH changes of microorganisms. Impedance measurement techniques record changes in the electrical conductivity of the environment, while in colorimetric systems, colour changes resulting from metabolic activity are observed (Brito et al., 2021). Molecular diagnostic-based systems, which are among the most advanced versions of RMM technologies, are based on nucleic acid amplification. PCR and qPCR enable highly sensitive detection by amplifying genetic sequences specific to contaminant DNA. qPCR systems allow not only detection but also quantitative analysis. New-generation isothermal techniques such as LAMP (Loop-mediated Isothermal Amplification) enable DNA amplification without temperature control, increasing their field usability. Furthermore, DNA microarray and biosensor systems enable the simultaneous detection of multiple target microorganisms. Such molecular systems offer culture-independent approaches, making them particularly advantageous for detecting microorganisms that are difficult to culture (Hosseini et al., 2023; Xing et al., 2022).

Pharmaceutical applications of rapid microbiological methods;

- Product Release: RMM systems are preferred for rapid release, particularly in biological products with a short shelf life.
- Cleanroom Monitoring: Real-time monitoring of airborne microbial load can be achieved in GMP Class A/B areas.
- Process Validation: Instant feedback can be obtained for the control of filtration, aseptic filling, and sterilization steps.

- Microbiological Quality of Water: Water quality can be monitored in WFI (Water for Injection) and PW (Purified Water) systems using online RMM systems (Easter, 2003; Luis Jimenez, 2001).

The advantages of these methods include time savings (results within hours), process monitoring with real-time data, minimization of operator errors, and reduction of product recall risk. The disadvantages include high system cost, interaction with certain products (e.g., dye or viscosity), complex validation processes, and the need for specific adaptation for each pharmaceutical form (Law et al., 2015).

PCR, qPCR and genetic identification techniques

In pharmaceutical microbiology, considering the limitations of classical culture methods (e.g. long incubation times, inability to detect non-culturable microorganisms), molecular diagnostic techniques have initiated a significant transformation, particularly since the 2000s. In this context, polymerase chain reaction (PCR) and its advanced versions, qPCR and multiplex PCR, have become indispensable for the rapid and specific detection of microorganisms at the genetic level (Serapide et al., 2025). PCR is an amplification technique that enables the in vitro replication of a target DNA region into millions of copies. This process, carried out with the help of the enzymatically active Taq DNA polymerase, consists of three basic steps: denaturation, primer annealing, and extension. This cycle is repeated 30–40 times to achieve amplification (Khehra et al., 2023). In pharmaceutical microbiology, PCR is widely used in areas such as the detection of microbial contaminants at the species level, the identification of clinically significant resistance genes, the confirmation of difficult-to-identify agents such as *Mycoplasma, Bacillus*, and *Pseudomonas* in biological products, and the monitoring of environmental microbial load in water systems

(Jimenez et al., 1999; Ragheb et al., 2012). qPCR is a technique that, in addition to conventional PCR, provides quantitative data by simultaneously monitoring the amplification process. In this method, probes that produce a fluorescent signal during DNA amplification (e.g., SYBR Green, TaqMan) are used. The resulting curves are analyzed based on the cycle number at which DNA is first detected (Ct value) to determine the microbial density in the sample. In pharmaceutical applications, qPCR is valuable for the rapid and accurate numerical determination of microbial load, rapid contamination screening, genetic purity analyses in biological products, and real-time microbial monitoring integrated with RMM systems (Schröder et al., 2023). Multiplex PCR enables the simultaneous amplification of multiple target genes within the same reaction tube. This method is particularly useful when screening for numerous microorganisms or genetic markers at the same time. In pharmaceutical production environments, it is used to simultaneously detect both bacterial and fungal contaminants, examine resistance genes and virulence factors together, and distinguish product-related biological impurities (Farajnia et al., 2009).

Next-generation sequencing (NGS) applications

Next-generation sequencing (NGS) is an advanced genomic analysis technology that enables the high-throughput, parallel and rapid reading of DNA or RNA sequences. Compared to conventional Sanger sequencing, NGS allows the simultaneous analysis of millions of genetic fragments in a much shorter time. The basic principle involves fragmenting all genetic material in the sample, labelling it with adapter sequences, then subjecting it to amplification and sequencing cycles, followed by bioinformatic analysis of the data (Wensel et al., 2022). Contamination control and microbial quality management in pharmaceutical production environments are critical for product safety. Due to the limitations of

traditional culture methods (e.g., detection of only cultivable organisms), culture-independent techniques such as NGS provide a higher level of sensitivity and coverage. NGS is used in pharmaceutical microbiology for purposes such as fully profiling microbial diversity (microbiome), detecting unknown or non-cultivable contaminants, genetically supporting environmental monitoring programmes, phylogenetically tracking the source of contamination, and analysing microbial resistance at the genomic level (Mandlik et al., 2024).

Biosensors and microfluidic systems

Biosensors are analytical devices that integrate a biological recognition element (e.g., enzyme, antibody, DNA probe) with a physical transducer component. In microbiological applications, these systems are widely used to detect the presence of pathogenic microorganisms in a specific and rapid manner. Protecting pharmaceutical products from microbial contamination is of vital importance, particularly in sterile products. Therefore, biosensors offer an important innovation in the field of drug production for both environmental monitoring and product-based testing (Qian et al., 2021). Biosensor technology is generally classified into the following subtypes:

- Electrochemical biosensors: These indirectly detect the presence of microorganisms by measuring microbial metabolites. For example, they generate electrical signals via protons or electrons released through microbial respiration.
- Optical biosensors: Detect biomolecular interactions in real time using fibre optic systems or surface plasmon resonance (SPR) technology.

- Piezoelectric biosensors: Analyses mass changes resulting from molecular binding, such as antigen-antibody interactions, through vibration frequency.

These systems provide results much faster than conventional culture-based methods and can detect even low levels of microorganism presence with high sensitivity (Naresh & Lee, 2021).

Microfluidic systems are microdevices, typically made from materials such as glass, silicone or polydimethylsiloxane (PDMS), that enable the direction of fluids in sub-millimetre channels. These systems are designed for the analysis of small-volume samples and form the basis of lab-on-a-chip technology (Al-wdan et al., 2023). From a pharmaceutical microbiology perspective, microfluidic systems reduce sample volume (enabling work at the microlitre level), shorten reaction times, offer automation and parallel analysis capabilities, and provide real-time monitoring and quantitative assessment. Thanks to the sensor technologies integrated into these systems, multiple microbial parameters can be analyzed simultaneously. For example, microfluidic PCR systems provide high accuracy in pathogen detection by integrating DNA amplification (Li et al., 2019). Biosensors and microfluidic systems are technologies that enable rapid, sensitive, and specific analyses in pharmaceutical microbiology. While they do not entirely replace traditional testing methods, they serve as innovative complementary tools, particularly in critical areas such as contamination detection, environmental monitoring, and process validation. The integration of these systems with evolving nanotechnology and artificial intelligence-supported analysis platforms will enable even more sensitive and versatile microbiological assessments in the future (Chen et al., 2023).

Artificial intelligence-supported contamination prediction and risk assessment

Artificial intelligence is a powerful technology with the potential to optimize quality assurance processes in the pharmaceutical industry. In particular, the risk of contamination in sterile and semi-sterile production environments is a critical factor that directly affects product safety. At this point, artificial intelligence algorithms enable risk assessment through a predictive and data-driven approach that goes beyond traditional quality control techniques (Huanbutta et al., 2024). Some current application areas of artificial intelligence include process monitoring and anomaly detection, contamination source identification, risk-based decision support systems, and numerous other R&D processes (Suriyaamporn et al., 2024).

Advantages:

- Reduces response times and intervention times.
- Increases detection accuracy.
- Enables pattern recognition from large data sets.

Disadvantages:

- Requires high-quality, labelled data.
- Requires model transparency for regulatory compliance.
- May contain algorithmic bias and false negative/positive rates (Burns et al., 2023).

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ISOLATION AND IDENTIFICATION OF PROBIOTIC BACTERIA

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Introduction

Probiotics are live microorganisms, usually bacteria, that confer health benefits on the host when consumed in appropriate amounts (Al-Dhabi et al., 2020; Zhang et al., 2020). These beneficial microbes can often be consumed directly through fermented foods or supplements, such as yogurt, kefir, pickles, pickled olives, fermented sausage, pastrami, and kimchi. Commercially available probiotic products (capsules or sachets) often contain bacteria primarily from the Lactobacillus and *Bifidobacterium* genera (Salim et al., 2020; Selvaraj and Gurumurthy, 2023; Ozdal, 2024).

Lactic acid bacteria (LAB) represent an important group of probiotics widely used in commercial fermented foods. They are Generally Recognized as Safe (GRAS), possessing characteristics

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that make them ideal for this consumption (Zhang et al., 2020; Khusro et al., 2021). LAB, particularly *Lactobacillus* species, are extensively used as primary or starter cultures in dairy production. In recent years, probiotics have gained significant importance due to their ability to regulate the host's digestive system against pathogens. Consequently, probiotics have been investigated in recent years as bioactive preservatives in foods and as alternatives to antibiotics in clinical treatments (Lashani et al., 2020; Rashed et al., 2022).

Probiotic consumption has been linked to improvements in a variety of health conditions, including irritable bowel syndrome, diarrhea, constipation, hypertension, diabetes, Parkinson's disease, Alzheimer's disease, cardiovascular disease, obsessive-compulsive and depressive disorders, and learning disabilities. However, for a strain to be functionally effective, it must possess several key beneficial qualities. There are beneficial probiotic bacteria that adapt to these undesirable gastrointestinal conditions and protect the host (A'inurrofiqin et al., 2022). Furthermore, by utilizing the high exopolysaccharide (EPS) production characteristic of commercial probiotic strains and the ability to digest fibrous foods, researchers can produce functional fermented foods that offer greater health benefits than traditional food products, leading to a continuous search for new LAB strains with superior probiotic properties. (Selvaraj and Gurumurthy, 2023). Recently, probiotic supplements have received much attention as a result of reducing reliance on antibiotic use in infectious diseases. This is a critical development because drug resistance, which is rapidly emerging in pathogens, frequently undermines the effectiveness of conventional antibiotic treatments. (Das et al., 2022; M'Hamed et al., 2022). For people with recurrent infections, antibiotic resistance transforms infection from a routine risk to a source of clinical concern. Untreated or resistant infections can rapidly worsen, leading to significant complications and the need for hospitalization. As a result, probiotics have become

an attractive and preventive potential to prevent or delay the development of multidrug-resistant (MDR) bacteria. (Lim and Im, 2009;)

Probiotics have remarkable results in combating infectious diseases through multifaceted mechanisms such as protecting and repairing the intestinal epithelial barrier and inhibiting pathogens (Kirtzalidou et al., 2011). Furthermore, the understanding of the functional capabilities of these microbes is advancing; metabolic pathway analysis of probiotic bacteria, as proposed by Le and Yang (2022) and Le et al. (2023), has the potential to effectively elucidate their metabolic status and provide important advice for the industrial development of advanced functional fermented foods, including milk. In recent studies, increasing focus has been placed on millet and its derived beverages as highly ideal raw materials for the production of emerging probiotic products. (Desrouilleres et al., 2020). Grain-based substrates are highly effective in promoting the growth and survival of probiotic bacteria. These substrates are also rich in nutrients, including essential dietary fiber, protein, carbohydrates, vitamins B and E, iron, trace minerals, and fiber. This reinforces the importance of whole grains as a healthy and beneficial food choice. In particular, grains such as millet and pearl millet have been shown to work effectively with various probiotic strains (Sachdev et al., 2023). Their high fiber content acts as prebiotics and provides nutrients for probiotic cultures. Their matrices provide a protective environment that protects probiotic cells from the harsh conditions of the digestive tract, thus making them ideal substrates for the manufacture of superior probiotic products (Desrouilleres et al., 2020). (Bembem and Agrahar-Murugkar, 2020).

Probiotic Delivery Systems and Functional Characterization

Probiotics can be delivered to consumers through various delivery systems, most notably probiotic-enriched functional foods

and nutraceutical formulations (Table 1). In the realm of functional foods, probiotic strains can easily be included in the fermentation. This integration is perfectly illustrated by how probiotic beer is produced. (Camelo-Silva et al., 2024), . In addition to food-based delivery, probiotics are widely available as dietary supplements or nutraceutical capsules, which may contain either single or multi-strain formulations. The biological activity of these probiotics is highly species- and strain-specific, influencing their overall functionality and clinical efficacy. Comparative analyses between single-strain and multi-strain probiotics have been shown in several meta-analysis studies (McFarland, 2021), yielding inconclusive findings. Multi-strain probiotic formulations are often assumed to offer a broader range of health benefits.

Before proceeding to in vivo evaluations or clinical trials, it is essential to conduct a comprehensive investigation into the safety and health-promoting properties of probiotic candidates. In vitro evaluations have focused on evaluating the performance of probiotics, such as tolerance to acidic pH, bile salts, and digestive enzymes that can adhere to intestinal epithelial cells and resist antibiotics. These micro-level parameters are no longer considered sufficient on their own.

The application of probiotic genomics has advanced significantly in recent years in the characterization of probiotic microorganisms, which has enabled the identification of genes associated with probiotic traits, including genes associated with virulence, drug resistance, bioactive amine production, and other technologically significant traits (Da Silva et al., 2023). Gene identification alone is not sufficient and is not the ultimate goal; current research also focuses on evaluating the expression levels and regulatory conditions of these genes in different physiological contexts (Isenring et al., 2021).

To use probiotics as therapeutic agents in the prevention and treatment of diseases, it is essential to have a deep understanding of probiotic-host interactions. Such interactions mainly occur by regulating the intestinal microbiota, enhancing the integrity of the intestinal barrier, balancing the immune system, and stimulating the production of metabolic products, especially short-chain fatty acids (SCFAs) (Kuesi et al., 2021). Multi-omics (probiogenomics) has been instrumental in providing a more comprehensive understanding of these complex host-microbe interactions. For example, metagenomics facilitates the determination of the role of probiotics on gut microbial content and function (Koji et al., 2023). Studies that have been initiated to analyze host gene expression profiles after probiotic administration are helping to better understand how probiotics affect host cellular pathways and physiological responses (Suzuki et al., 2022). Complementing these methods, the analysis of compounds produced during probiotic metabolism provides a challenging perspective on the biochemical interactions between probiotics and the host (Yang et al., 2022).

Table 1. Analytical approach used in describing probiotics and a look at probiotic drug delivery systems

Category	**Description /Application**	**Examples**	**References**
Food-based delivery systems	Incorporation of probiotics into functional foods via fermentation or as added ingredients	Probiotic beer; probiotic chocolate	Camelo-Silva et al. (2024); Díaz et al. (2023)
Nutraceutical and supplement delivery	Encapsulation of probiotics in capsules or powders as dietary supplements	Single-strain and multi-strain probiotic formulations	McFarland (2021)
Traditional *in vitro* characterization	Evaluation of survival and functionality under simulated gastrointestinal conditions	pH tolerance, bile salt resistance, digestive enzyme tolerance, and antibiotic resistance	Chen et al. (2022)
Gene expression studies	Investigation of probiotic gene activity and regulation	Transcriptomic analysis under stress or host-simulated conditions	Isenring et al. (2021)
Host–probiotic interaction studies	Exploration of the physiological outcome of probiotics on host cells and microbial founder	*In vitro* cell line models, animal models, and clinical studies	Kiousi et al. (2021)
Multi-omics and systems biology	Integrated use of metagenomics, transcriptomics, and metabolomics	Metagenomic gut microbiota profiling; metabolomic analysis of SCFAs and bioactives	Kwoji et al. (2023); Suzuki et al. (2022)

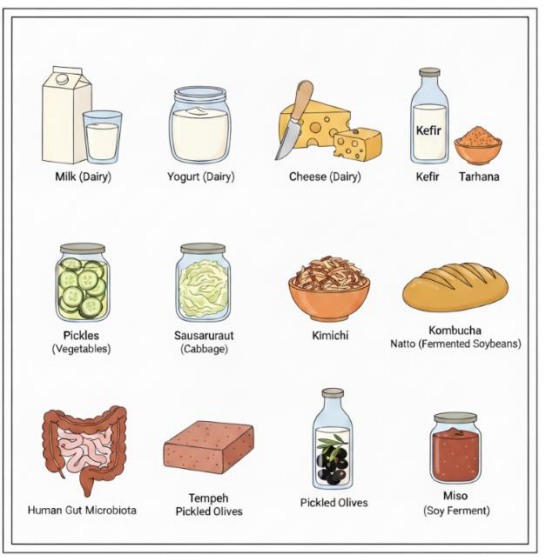
Probiotic Sources and Classification

Probiotic bacteria are mainly isolated from the human gut, breast milk and fermented foods/beverages (Figure 1). However, commercialization of probiotic bacteria from fermented foods has not yet progressed. Regulatory frameworks and laws vary across countries and regions, however most impose strict safety standards on traditional and non-traditional probiotics (Ozdal, 2024). For example, products that have health and beneficial effects or disease reduction are often required to provide substantial evidence of efficacy and safety (Spacova et al., 2023). Fermented products, both animal and plant, serve as a good source of probiotic microbes (Hill et al., 2014; Ozdal, 2024). The dominant sources for probiotic isolation include fermented meats, dairy products, fish, vegetables and sourdough. However, given its rich microbial habitat, the human gastrointestinal tract remains the oldest and most productive source for probiotic strains. Numerous genera and strains of *Lactobacillus* and *Bifidobacterium* have been successfully isolated from the healthy human intestine (Wall et al., 2008).

Human breast milk is another interesting source that provides both safety and diversity of probiotics (D'Alessandro et al., 2022). Breast milk harbors a wide range of probiotic bacteria, including bacterial genera such as *Lactobacillus, Staphylococcus, Streptococcus, Lactococcus, Micrococcus, Enterococcus, and Bifidobacterium*, each of which shows promising probiotic potential (Dombrowska-Pali et al., 2024). Probiotics can be classified into traditional (or first-generation) and next-generation probiotics based on their history of safe use. Traditional probiotics have a long history of safety and beneficial effects on host health (Luo et al., 2019; Binda et al., 2020). These microorganisms generally have a Qualified Safety (QPS) designation by the European Food Safety Authority (EFSA) and are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA) (Lin et al., 2019). Species

widely used in the production of functional foods include *Bifidobacterium, Lactobacillus, Saccharomyces, Bacillus, Escherichia coli, Enterococcus,* and *Vissella* (Tsai et al., 2019). While, groups such as diverse species belonging to the phyla Bacteroidetes, Actinobacteria, Verrucomicrobia and Firmicutes are new generation probiotics (NGPs) that have emerged from next-generation sequencing (NGS) and functional genomics research, which has enabled precise molecular identification and functional characterization of novel bacterial isolates (Chang et al., 2019). Unlike traditional probiotics, next-generation probiotics are primarily designed as Next-Generation Probiotics agents somewhat similar to conventional food supplements. Furthermore, this category also includes bioengineered probiotic strains, where targeted genetic modifications enhance therapeutic potential and techno-functional properties. For instance, *Escherichia coli* strains have been extensively studied as bioengineered probiotics, some progressing to clinical trial phases in recent gene-editing research (Ma et al., 2022).

Figure 1. Some of the isolation sources of probiotic bacteria



Isolation of Probiotic Bacteria

Probiotics can be isolated from various sources, including both traditional and non-traditional sources (Sornplang and Piyadeatsoontorn, 2016). Non-traditional microorganism sources can be used to isolate potential probiotics from various origins, including non-gut sources and non-dairy fermented food products (Ramirez-Chavarin et al., 2013; Sornplang and Piyadeatsoontorn, 2016, Ozdal, 2024). It is generally assumed that consuming fermented products rich in lactic acid bacteria (LAB), such as yogurt, kefir, pickles, tempeh, and kombucha, provides health benefits (Jan, 2024). The development of food fermentation and preservation methods has helped domesticate some of these bacterial species (Douglas and Klaenhammer, 2010). MRS (De Man Rogosa) medium/agar and TSA are used for the isolation of probiotic bacteria from collected samples. The isolation process is carried out by incubation at 30 °C for 24-72 hours in an anaerobic jar.

Determination of the Probiotic Properties of the Isolated Bacteria

Various tests are used to demonstrate the probiotic properties of isolated bacteria. This evaluation reflects their ability to tolerate gastrointestinal conditions, inhibit pathogenic microorganisms, and adhere to intestinal epithelial cells. The results provide a detailed description of their potential as probiotic strains. The pure isolates obtained can be tested for Gram staining, motility, and catalase production by adding a drop of hydrogen peroxide to an isolated colony on a microscope slide. Gram-positive and catalase-negative isolates are selected for further analyses, such as carbohydrate fermentation test, acid tolerance, hemolysis test, bile tolerance, autoaggregation, cell surface hydrophobicity, ability to survive at different temperatures, phenol, NaCl concentrations, antibiotic susceptibility, and antimicrobial activity. All strains can be stored at

−20 °C as 50% glycerol stock for long-term storage (Amer et al., 2013; Bazireh et al., 2020; Divyashree et al., 2024; Kouadio et al., 2024; Madushanka et al., 2025). Gram (+) *Staphylococcus aureus*, *Bacillus subtilis*, Gram (-) *Escherichia coli,* and *Pseudomonas aeruginosa* can be used in antimicrobial activity tests.

The identification of probiotic bacteria is performed using Gram staining, colony morphology, motility, gas production, oxidase and catalase tests, as well as 16S rDNA analysis.

Assessment of Acid Tolerance

To prove that a bacterial strain is an effective probiotic, one of the most difficult obstacles to overcome is the highly acidic environment of the human stomach. This biological barrier has evolved to protect the body from potent pathogens, but it also significantly affects the survival of beneficial bacteria on their way to the intestines (Fuller, 1989). For this reason, assessing the acid tolerance of isolated strains is an important step in evaluating their probiotic potential (Chartreis et al., 1998). MRS culture medium is adjusted using 1N HCl and 1N NaOH at pH levels of 1, 2, 3 and 4, and freshly prepared bacterial cultures are added to the corresponding MRS tubes. The tubes are incubated for 48 hours at 37°C, and the turbidity of the culture medium is checked after 24–48 hours (Chen et al., 2022).

NaCl Tolerance Test

Probiotic strains should also be resistant to various physical stress conditions. These physical stresses are exposure to varying degrees of osmotic pressure. The NaCl tolerance test indicates the ability of isolates to survive and grow at high salt concentrations and provides insight into their capacity to adapt to osmotic stress (Yadavand Shukla, 2017). The NaCl tolerance of isolated bacterial cultures is assessed using MRS culture media containing 2%, 4%,

6% and 8% NaCl. Freshly prepared cultures are incubated for 48 hours at 37 °C and the turbidity of the medium is assessed after 24 and 48 hours (Chen et al., 2022).

Bile Tolerance Test

Bile tolerance testing is performed to assess the ability of candidate strains to survive and proliferate in the presence of bile salts. Another important chemical challenge in the gastrointestinal tract is bile salts, which probiotic strains must be able to withstand after overcoming the highly acidic environment of the stomach (Shah, 2001). Bile resistance is a key determinant of the capacity of a strain to colonize the intestine and exert its beneficial effects (Leung and Shah, 2005). The bile salt tolerance of isolated samples is assessed using 10 ml of MRS medium containing 0.5%, 1.0%, 1.5% and 2% bile salts. The culture is incubated at 37°C and samples are taken after 24 hours (Chen et al., 2022).

Hemolytic Activity

On blood agar plates prepared with 5% sheep blood, the hemolytic activity of the isolates is examined and evaluated after incubation at 37 ° C for 48 hours. And they are classified based on the degree of lysis of red blood cells around the colonies, with clear areas around the colonies known as beta-hemolysis, green areas as alpha-hemolysis, and the absence of any area as gamma-hemolysis. Which are usually non-hemolytic (gamma-hemolysis) probiotic strains (Wei et al., 2022).

Pancreatic Fluid Tolerance Test

The selected probiotic strains must also tolerate the highly enzymatic environment of the terminal small intestine, which is influenced by pancreatic secretions (Jacobse et al., 1999). This test assesses the ability of the strains to resist the lytic activity of pancreatic enzymes and, consequently, to determine their survival

rate during their progression to colonization sites in the terminal ileum and colon (Gueimonde and Salminen, 2006). An important factor in the selection of probiotics is tolerance to simulated pancreatic fluid, since strong enzymatic components - such as trypsin and chymotrypsin - can disrupt bacterial cell walls and membranes (Havenaar and Huis in 't Veld, 1992). The ability to resist under these conditions suggests that this strain has effective defense mechanisms that enable it to cross the entire small intestine and reach the sites of colonization in sufficient numbers to exert its beneficial effects (Chantawannakul et al., 2018). With this method, after incubation at 37 °C for 24 h, 30 µL of each bacterial culture grown in MRS medium is transferred to microtiter plates containing 270 µL of test medium consisting of 150 mM $NaHCO_3$ and 1.9 mg/mL pancreatin adjusted to pH 8. The cultures are incubated at 37 °C with shaking for 3 and 6 h. The survival rate of the strains is determined by culturing samples on MRS agar at times 0, 3, and 6 h (Wei et al., 2022).

Hydrophobicity

The ability of microorganisms to adhere to hydrocarbons was assessed according to the method of Vinderola and Reinheimer (2003) as an indicator of the hydrophobicity of their cell surface. Strain cultures were centrifuged at 12,000 × g for 5 min at 5 °C, washed twice with 50 mM K_2HPO_4 buffer (pH 6.5), and finally resuspended in the same buffer. The cell suspension was adjusted to an optical density of approximately 1.0 at 560 nm. Then, 3 ml of the bacterial suspension was mixed with 0.6 ml of n-hexadecane and vortexed for 120 s. The two phases were separated for 30 min at 37 °C. The aqueous phase is carefully removed and its absorbance is measured at 560 nm. The decrease in absorbance of the aqueous phase serves as a measure of the hydrophobicity of the cell surface (H%), which is calculated using the following formula: $H\% = [(A_0$

$- A)/A_0] \times 100$, where A_0 and A are the absorbance values before and after extraction with n-hexadecane, respectively.

Phenol Tolerance

Finally, the capacity of the probiotic strain to survive and maintain activity in the colon is the major factor. The colon is home to various potentially harmful compounds. Phenolic compounds, which are produced from the breakdown of amino acids such as tyrosine, are of particular importance in this regard (Smith and Williams, 1984). Phenol tolerance testing is a key factor for effective colonization and sustained activity in the colon (Zarath and Palauchino, 2023). According to the method proposed by Tepley (1984), MRS medium containing 0.4% (v/v) phenol is inoculated with 200 µl of active bacterial culture per sample and incubated at 30°C. After 24 h of incubation, the number of viable cells is determined by culturing on MRS agar.

Autoaggregation

Self-adhesion is an important property that indicates the potential of a strain to colonize host tissues, initiate biofilm formation, and maintain long-term survival in the gastrointestinal tract. (Manhar, et al., 2015) Self-aggregation and co-aggregation assays were performed with modifications based on the methods described by Kos et al. (2003) and Tuo et al. (2013). Lactobacillus strains were cultured in MRS medium under an enriched atmosphere of 5% CO_2 at 37°C for 20 h. Bacterial cells were harvested by centrifugation at 5000 × g for 20 min, washed twice with PBS, and resuspended in PBS. The optical density of the suspension at 600 nm (A_{600}) is adjusted to 0.25, which corresponds to approximately 10^7–10^8 CFU/mL, to standardize the bacterial concentration. 4 mL of the suspension is vortexed (initial measurement) and then incubated for 2 hours at 37°C (A_2h).

Conclusion

Isolation and identification of probiotic strains requires a multidisciplinary strategy that encompasses microbiology, immunology, and regulatory frameworks. Each step in turn contributes to their safety and efficacy. From the initial selection of candidate strains to conducting clinical trials. Strain-specific evaluations, the use of standardized methods, and strict adherence to ethical guidelines are the best practices to ensure reproducible and reliable results.

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BACTERIAL OUTER MEMBRANE VESICLES AND THEIR BIOTECHNOLOGICAL APPLICATIONS

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Introduction

The ways in which bacteria interact with their environment extend far beyond direct cell-to-cell contact or the exchange of dissolved molecules. Over the past years, membrane vesicles (MVs) have gained recognition as pivotal components that influence adaptation to environmental changes, survival strategies, communication pathways, and interaction with host organisms. Produced by both Gram-negative and Gram-positive bacteria, these nanosized vesicles typically enclosed within a lipid bilayer are secreted into the extracellular space. They transport a variety of

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biomolecules, including proteins, lipids, nucleic acids, toxins, and signaling substances, thereby contributing to numerous aspects of microbial physiology and the dynamics of microbial ecosystems.

MVs are involved not only in bacterial pathogenicity and immune system interactions but also play essential roles in processes such as antimicrobial resistance, horizontal gene transfer, and biofilm development. Gaining a deeper understanding of how these vesicles form and the molecular cargo they contain opens new avenues for explaining bacterial adaptation to both environmental factors and host systems. In parallel, their potential in biotechnology has recently drawn increasing scientific attention. Especially the outer membrane vesicles (OMVs) released from Gram-negative bacteria are the ones extensively studied. This chapter provides information about the structural and biological properties of OMVs, their mechanisms of formation, their functions, and potential applications.

Bacterial Membrane Vesicles (BMVs)

BMVs are naturally released, non-replicative nanostructures ranging from 20 to 400 nm in diameter. These structures are composed of bilayer lipid membranes (Toyofuku et al., 2019). BMVs carry proteins, lipids, nucleic acids and other molecules which enables the bacteria producing OMVs to interact with their environment (Schwechheimer and Kuehn, 2015). For many years it is thought that BMVs were produced only by Gram-negative bacteria. In 1965, the researchers observed that Gram-negative bacterium *Escherichia coli* grown under lysine limiting conditions secretes high amount of free lipopolysaccharide (LPS) into the extracellular medium (Bishop and Work, 1965). Knox et al. (1966) showed vesicles budding from the bacterial cell membrane using electron microscope which provides one of the earliest evidence of vesicle formation from bacterial cells. Later findings showed that

this process was not restricted to stress conditions. In 1967, *Vibrio cholerae* (Chatterjee and Das, 1967) and, in 1973, *Neisseria meningitidis* (Devoe and Gilchrist, 1973) were both observed to produce outer membrane vesicles (OMVs) under normal growth conditions. These studies showed that OMVs are produced not only under stress conditions, but also under normal growth conditions. In 1989, it was reported that OMVs released by *Neisseria gonorrhoeae* contain lipids, nucleic acids and proteins (Dorward et al., 1989).

It was thought that the BMVs are the by-products of contamination and cell lysis until the early 2000s However, following research demonstrated that these structures are products of active biological processes. This reveals that BMVs are not passive waste but structures of cells involved in bacterial communication, virulence, gene transfer, and environmental adaptation (Kuehn and Kesty, 2005; Schwechheimer and Kuehn, 2015). As mentioned before, for a long time, vesicle production was believed to occur only in Gram-negative bacteria. However, in 2009 it was found that the Gram-positive bacterium *Staphylococcus aureus* was also producing vesicles from the cytoplasmic membrane (CMVs) (Lee et al., 2009). Consequently, *S. aureus* is recognized as the first Gram-positive species confirmed to generate vesicles. Further studies revealed other Gram positive bacteria such as *Bacillus anthracis* (Rivera et al., 2010), *Mycobacteria* (Prados-Rosales et al., 2011), *Listeria monocytogenes* (Lee et al., 2013) and *Streptococcus pneumoniae* (Olaya-Abril et al., 2014) also produces CMV. These findings demonstrated that, despite their thick peptidoglycan layer, Gram-positive bacteria are also capable of actively releasing vesicles. In 2013, the formation of an outer–inner membrane vesicle (O-IMV) from Gram-negative bacterium *Shewanella vesiculosa* was reported for the first time (Prez-Cruz et al., 2013) This O-IMV was a bilayered lipid structure and originating from both the outer and inner membranes of the bacterium. Moreover, it was noted that the

bacterium produces both classical OMVs and O-IMVs; however, the DNA is found in the O-IMVs. Later studies demonstrated that various bacteria can produce O-IMVs and that DNA is generally packaged into this specific type of MV (Hagemann et al., 2014; Devos et al., 2017; Takaki et al., 2020). These findings are important because they demonstrate that BMV can have different structures and be produced by different bacterial groups. However, majorities of the studies on OMVs still focus on OMVs secreted by Gram-negative bacteria. The early discovery of OMVs, their relative ease of isolation, and their well-established roles in pathogenesis are the main reasons for this. Today, OMVs retain their importance for their potential applications in biotechnology and medicine as well as for understanding bacterial biology. Therefore, the following section will examine in detail the structural and biological properties of OMVs, their formation mechanisms, functions, and biotechnological applications.

Outer Membrane Vesicles (OMVs)

Outer membrane vesicles are vesicles that bud from the outer membrane of Gram-negative bacteria. Their size generally range from 20 to 400 nm. They retain the LPS, phospholipids, and outer membrane proteins from the bacterial outer membrane on their surface. In addition, they may also contain peptidoglycan, periplasmic proteins. Rarely nucleic acids from the periplasm can be also found in the structure of OMVs (Magaña et al., 2023).

Although a universal mechanism for OMV formation in Gram-negative bacteria has not yet been defined, several pathways have been proposed. One such mechanism is the weakening of the linkage between the outer membrane and the peptidoglycan layer, which initiates vesicle budding; indeed, in *E. coli*, the absence of the Lpp protein, which provides this linkage, or of the L,D-transpeptidases YcfS, YbiS, and ErfK, has been reported to cause a

marked increase in OMV production compared to wild-type strains (Schwechheimer et al., 2013). Another hypothesis suggests that the accumulation of certain components in the periplasm (particularly misfolded proteins) generates turgor pressure on the outer membrane. The outer membrane then buds outward, leading to vesicle formation. For example, McBroom and Kuehn, (2007) demonstrated that a DegP periplasmic protease/chaperone mutation in *E. coli* resulted in the accumulation of misfolded proteins, which in turn enhanced OMV production. The researchers proposed that the observed hypervesiculation was due to increased periplasmic pressure and was independent of OM-PG linkage (Schwechheimer and Kuehn, 2013; Ojima et al., 2020). According to a third model, specific signaling molecules interact with lipid A and insert into the outer membrane. This causes its expansion relative to the inner membrane and triggering vesicle formation. For instance, the *Pseudomonas* quinolone signal (PQS) can trigger OMV formation once transported to the outer membrane. The interaction of PQS with LPS causes and expansion in the membrane which initiates vesicul formation (Mashburn-Warren et al., 2009; Florez et al., 2017). In another mechanism, endolysin activity causes degradation of the cell wall, which may result in cell lysis. During this process, both outer and inner membrane vesicles may be formed, carrying DNA, proteins, and other cellular contents (Nagakubo et al., 2020; Juodeikis and Carding, 2022).

While the molecular mechanisms underlying OMV biogenesis have remained unclear for a long time, there are recent findings paving the way for understanding the regulatory pathways. Mutations in protein complexes that involved in the cell envelope integrity (such as OmpA, Lpp, and the Tol-Pal system) weaken the outer membrane and the peptidoglycan layer connections, which facilitates vesicle budding (Schwechheimer and Kuehn, 2015). Moreover, environmental stresses (such as antibiotic exposure, iron

limitation, and pH changes) and intracellular signaling pathways (including the σE stress response and quorum sensing) can also influence OMV production (Roier et al., 2016; Toyofuku et al., 2019). In addition, disruption of mechanisms that preserve lipid asymmetry, such as the VacJ/Yrb ABC transporter system, which prevents abnormal phospholipid accumulation in the outer membrane, also enhances spontaneous vesicle formation (Roier et al., 2016). These findings indicate that OMV biogenesis is not a passive process, but rather a complex mechanism dynamically regulated by various genetic and environmental factors.

Functions of OMVs

OMVs are not only carriers; they also regulate intracellular activities and coordinate complex interactions with the environment. The main functions of OMVs in bacterial physiology and interactions within ecosystems are also discussed in this chapter.

Bacterial Survival Against Stress Factors

OMVs are an important component of bacterial survival strategies against variable environmental conditions. Gram-negative bacteria can increase OMV production when exposed to environmental stresses such as pH changes, oxidative stress, temperature shifts, and nutrient limitation. This provides the cell various advantages including protection from harmful factors, removal of toxic compounds, and maintenance of cellular homeostasis. In addition, these vesicles may serve as potential decoys against targeted attacks on bacteria. For example, when *E. coli* is infected by T4 or T7 phages, it produces OMVs of different sizes and structural properties. This may help the bacteria evade phage infection (Manning and Kuehn, 2011; Mandal et al., 2021). OMVs released by *E. coli, V. cholerae*, and *Pseudomonas putida* have been found to act as traps in the presence of toxic or antimicrobial compounds such as toluene, polymyxin B, and colistin

(Kobayashi et al., 2000; Manning and Kuehn, 2011; Giacomucci et al., 2022). It has been suggested that OMVs may contribute to the detoxification of environmental toxins or heavy metals through the enzymatic components they contain. Moreover, they carry stress-response proteins which support the remodeling of the cell membrane (Shao et al., 2014; Muñoz-Echeverri et al., 2024). Thus, it can be said that the OMVs can be considered as an extension of bacterial adaptation mechanisms. They also contribute to the physiological stability of the organism under both acute and chronic stress conditions.

Interbacterial Communication

Bacteria can also establish a complex communication network via the OMVs. These vesicles can carry signal mediators, quorum sensing molecules, metabolites, or small RNAs, which regulates specific gene expressions in bacteria (Schooling and Beveridge, 2006; Koeppen et al., 2016; Toyofuku et al., 2019). The role of OMVs in quorum sensing has been gaining increasing significance. It is known that vesicles released by *P. aeruginosa* can carry the PQS signaling molecule. This helps the bacterium regulate its interactions both with other *P. aeruginosa* cells and with other species. When transmitted to other bacterial cells, PQS facilitates group behaviour (Bassler and Losick, 2006). Similarly, it has been shown that the C16-HSL signal, used in intercellular communication by *Paracoccus denitrificans*, is secreted from cells in OMVs. Moreover, findings showing that OMVs fuse with different bacteria have led to the suggestion that these vesicles can recognize specific cell types (Toyofuku et al., 2017). In conclusion, it can be said that OMVs are dynamic structures that regulate the flow of information between bacteria. These structures are molecular messenger systems that facilitates both intra- and interspecies interactions among microorganisms. These properties help the bacteria to organize,

coordinate, and form various relationships within microbial ecosystems (Jan, 2017).

Bacterial Growth

It is known that OMVs also play roles in bacterial growth and metabolic processes. These vesicles are important for bacteria to adapt to different environments, and access essential nutrients, and regulate metabolisms. For example, under iron-limitations *Bordetella pertussis* can acquire iron from the environment through OMVs. Studies have shown that OMVs obtained from an iron-rich culture can enable bacteria, grown in iron-deficient media, to transfer iron into their cells via these OMVs (Gasperini et al., 2017). Moreover, nutrient deficiencies such as iron restriction, cysteine limitation, sulphur scarcity, and magnesium depletion can trigger general stress response pathways (Mozaheb et al., 2020). Under those environmental stress conditions bacteria may increase OMV production. For instance, *Mycobacterium tuberculosis* produces siderophores to cope with iron deficiency. The bacterium releases these siderophores into its environment encapsulated within the OMVs (Prados-Rosales et al., 2014). OMVs of *Porphyromonas gingivalis* contain the HmuY protein, which exhibits binding activity. This protein helps the bacterium persist in iron-limited environments, such as biofilms, by enhancing its iron uptake (Olczak et al., 2010). In addition, to break down complex environmental components into smaller and more accessible form, bacteria secrete hydrolytic enzymes via OMVs. This helps bacteria to degrade complex proteins, polysaccharides, or lipids present in their surroundings (Kulp and Kuehn, 2010). This process is particularly important for maintaining nutrient cycling within multicellular structures in biofilms.

Antibiotic Resistance and Gene Transfer

OMVs are an important component of the defence mechanisms that bacteria develop against antibiotic stress. These structures generally mediate antibiotic resistance through four distinct mechanisms (Liu et al., 2022). They can increase drug efflux by exporting antibiotics out of the cell. In this way, they prevent the accumulation of antibiotics inside the bacterial cell and may support the survival of the bacteria in antibiotic-containing environments. For example, in *Acinetobacter baumannii*, antibiotic stress activates efflux pumps, leading to the encapsulation of large amounts of intracellular components and antibiotics into OMVs (Huang et al., 2020).

OMVs can act as traps by binding or sequestering antibiotic compounds. Because the bacterial membranes are the main components of these vesicles, OMVs mimics their parental bacterial cells and can readily bind and capture peptide antibiotics or toxins (Zhao et al., 2025).

OMVs can also cause transient resistance in susceptible strains by carrying antibiotic-degrading enzymes which can degrade antibiotics extracellularly. It was shown that OMVs of *Moraxella catarrhalis* carrying β-lactamase were able to inactivate amoxicillin. This activity of the bacterium supports the survival of *Streptococcus pneumoniae* and *Haemophilus influenzae* (Schaar et al., 2011). Similarly, vesicles carrying β-lactamase produced by members of *Bacteroides* help to protect certain commensals or enteric pathogens from cefotaxime (Stentz et al., 2015). This also highlights the ecological role of OMVs among microorganisms.

Finally, OMVs can carry resistance genes and by spreading these genes they provide drug resistance to other cells. Gene transfer in bacteria occurs through three mechanisms: conjugation, transduction, and transformation. However, after the discovery in the

1980s that OMVs contain RNA and DNA fragments, it was understood that these structures could also play a role in gene transfer. It has been shown that *A. baumannii* transfers carbapenem resistance genes to susceptible strains via OMVs (Rumbo et al., 2011). Moreover, OMVs have been observed to mediate gene transfer in several studies, including the dissemination of the β-lactamase gene in *Acinetobacter* (Chatterjee et al., 2017) and Enterobacteriaceae (Bielaszewska et al., 2020), as well as the spread of carbapenemase genes in *K. pneumoniae* (Chen et al., 2023). Building on these observations, Soler and Forterre (2020) introduced "vesiduction" as a fourth route of intercellular DNA exchange. Currently this term is used as the fourth category under gene transfer mechanisms (Wachino, 2025).

Pathogenesis

OMVs play key roles in the interactions between pathogenic bacteria and host organism. These vesicles contribute to multiple stages of bacterial pathogenesis by carrying various virulence factors, manipulating the immune system, and facilitating tissue invasion. OMVs are often carry molecules such as proteases, toxins, LPS, phospholipases, adhesins, and DNA/RNA. This enables bacteria to deliver effector molecules to host cells without direct contact (Ellis and Kuehn, 2010; Jan, 2017). After binding to the host cell membrane, these vesicles can be internalized via endocytosis, macropinocytosis, or membrane fusion, subsequently releasing their virulence factors into the cytoplasm (Bomberger et al., 2009). For example, OMVs from uropathogenic *E. coli* carry the toxin CNF1 can causes neutrophil disfunction and weakens the immune system (Davis et al., 2006). OMVs secreted by *Helicobacter pylori* can transport toxins such as VacA (vacuolating cytotoxin A), triggering vacuole formation and apoptosis in gastric epithelial cells (Ismail et al., 2003). OMVs may also target uninfected cells, contributing to the spread of infection. The virulence factors they carry can initiate

pathological processes in new cells. This is an important factor in chronic infections or in diseases associated with systemic inflammation. In a study of Bomberger et al., (2009), it was shown that OMVs from *P. aeruginosa* reached alveolar epithelial cells in the absence of live bacteria and induced cellular damage via various virulence factors (alkaline phosphatase, β-lactamase, phospholipase C). These findings clearly demonstrate that OMVs, through their molecular cargo, can induce inflammation, cell death, and tissue damage in distant target cells. Therefore, OMVs are considered important targets for understanding infectious diseases and for developing new therapeutic strategies.

Immunomodulation

OMVs are notable immunomodulatory structures with the capacity to both activate and suppress the host immune system. In addition to LPS, the main component of OMVs, molecules such as lipoproteins, peptidoglycan, and bacteria-derived DNA/RNA can activate the innate immune system through Toll-like receptors (TLR2, TLR4, TLR9) and Nod-like receptors, thereby triggering proinflammatory responses (Mancini et al., 2020). However, this interaction is not limited to a proinflammatory response; OMVs from many commensal or probiotic bacteria can also exhibit immunosuppressive or immune-balancing (homeostatic) effects. For example, *Bacteroides fragilis* is recognized as a symbiotic bacterium that mediates immune modulation in favour of the host under pathological conditions such as inflammatory bowel disease; this effect is largely mediated by a molecule called capsular polysaccharide A (PSA). PSA is carried within OMVs to the mesenteric lymph nodes, where it is recognized by dendritic cells through TLR2, subsequently increasing the IL-10 production of regulatory T cells. This suppresses inflammation and provides protective effects in colitis-like diseases (Ochoa-Repáraz et al., 2010; Shen et al., 2012). Notably, OMVs lacking PSA do not exert

this regulatory effect, clearly demonstrating that the transport of PSA within OMVs is critical for immune regulation. At the same time, OMVs from some pathogenic or opportunistic pathogenic bacteria may also provide immunomodulatory effects for the host. Vesicles of *Salmonella enterica* serovar *Typhimurium* can act as strong inducers of proinflammatory cytokine release and immune cell activation. These vesicles activate macrophages and dendritic cells resulting increased production of tumour necrosis factor-alpha (TNF-α) and interleukin-12 (IL-12) (Alaniz et al., 2007). These complex immune modulation properties have made OMVs potential therapeutic tools capable of controlled interactions with the immune system. Thus, because their effects on the immune system are complex but can be targeted, bacterial vesicles are attractive candidates for applications such as vaccine design, therapeutic delivery, and immune modulation.

OMV-Based Biotechnological Applications

OMV-Based Vaccine Technologies

Vaccines elicit a specific immune response against pathogens, resulting in the reduction of the incidence and prevalence of infections (Siegrist and Lambert, 2016). The development of OMVs as a vaccine platform has advanced, particularly against meningococcal disease caused by *N. meningitidis* serogroup B (MenB). OMV vaccines developed against MenB have successfully controlled MenB-related meningitis outbreaks in Cuba, New Zealand and Norway. The first licensed OMV-based vaccine, VA-MENGOC-BC, was developed in Cuba in 1989 to control a MenB epidemic. This vaccine is produced by combining OMVs obtained from a local epidemic strain with the serogroup C polysaccharide component. It demonstrated ~83% protective efficacy in children aged 3 months to 6 years (Sierra-González, 2019). Between 1988 and 1992, the MenBVac vaccine produced in Norway used OMVs

specific to the PorA subtype to control long-term MenB outbreaks. Although MenBVac provided strain-specific protection, it provided limited cross-protection against non-homologous strains (Holst et al., 2013). MeNZB was a tailor-made vaccine, specifically designed to induce immunity against the epidemic MenB strain (B:4:P1.7-2,4) circulating in New Zealand. This vaccine was derived from the NZ98/254 strain, which was selected from among the strains representing the epidemic. It provided strain-specific protection but limited cross-protection against other MenB strains (Oster et al., 2005). A significant milestone in the field of OMV-based vaccines was the development and licensing of 4CMenB (Bexsero) in 2013. This vaccine was produced by combining three recombinant protein antigens NHBA, NadA, and fHbp with OMVs obtained from the NZ98/254 strain to achieve broader strain coverage (Serruto et al., 2012). The additional antigens with the OMVs in the vaccine have enhanced its suitability for worldwide. This vaccine evokes a more controlled immune response and provides broader protection against diverse MenB strains compared to vaccines containing only OMVs (O'Ryan et al., 2014). In the United Kingdom, the inclusion of the 4CMenB vaccine in the infant immunization programme resulted in a 50% reduction in laboratory-confirmed MenB cases in the target age group (Parikh et al., 2016).

Currently, OMV vaccines developed against different bacteria are under investigation. AltSonflex1-2-3 is a four-component vaccine based on Genetically Modified Meningococcal Antigen (GMMA) developed against shigellosis. This platform is based on the use of bacterial OMVs as antigen carriers. The vaccine aims to provide protection against *Shigella sonnei* and *S. flexneri* serotypes 1b, 2a, and 3a. Preclinical studies have shown that altSonflex1-2-3 is highly immunogenic in mice and rabbits, producing bactericidal antibodies against heterologous *Shigella* strains (Rossi et al., 2023). Following a Phase 1 trial in adult

participants in Europe, the vaccine progressed to a Phase 2 trial (Launay et al., 2017; Obiero et al., 2017; Launay et al., 2019). OMV vaccines have been developed against *B. pertussis*. A study in mice showed that both subcutaneous and intranasal OMV-based pertussis vaccines provided broad immunity, including Th1/Th17 responses and numerous antibodies (Raeven et al., 2020; Yılmaz Çolak and Tefon Öztürk, 2023). Moreover, Kanojia et al., (2018) developed nasal OMV vaccines that can be transported without cold chain, making them suitable for global use. There are studies using modified OMVs against viruses. Grandi et al., (2023) developed a an OMV-based vaccine containing receptor-binding motifs of the SARS-CoV-2 spike protein. Experiments in mice have demonstrated that the vaccine elicits an effective immune response and is effective against various SARS-CoV-2 variants. In addition to their application against SARS-CoV-2, OMV-based vaccine platforms have also been used against other viral pathogens, such as the Zika virus. Martins et al., (2018) developed an OMV-based vaccine by fusing OMVs from *N. meningitidis* with the Zika virus propagated in C6/36 cells. Researchers have shown that this vaccine activates both TH1 and TH2 cellular immune responses in mice, resulting in antibody levels of up to 1:160. This is a significant increase compared to those in unvaccinated mice.

OMV-based vaccines offer several significant advantages over traditional conjugate, protein subunit, and inactivated vaccines. Pathogen-associated molecular patterns (PAMPs), located on OMVs can activate various pattern recognition receptors (PRRs) in host cells resulting in the activation of multiple immunogenic pathways. It is known that OMVs a can also activate various TLRs on the host cell membrane. These characteristics enable OMV vaccines to stimulate both cellular and humoral immunity (Bottero et al., 2016; Zurita et al., 2019). Because of their smaller size, they can easily enter the lymphatic system and be captured and presented by

antigen-presenting cells (APCs) (Bachmann and Jennings, 2010). Also, because they cannot replicate, they are a safe alternative for individuals with compromised immune systems (Kashyap et al., 2022).

There are some limitations that make it difficult to use OMVs in vaccine technologies. To fulfil demand, routinely used vaccines must have high production capacity. However, yields from OMV production are generally low, and obtaining sufficient quantities can be challenging. Therefore, cost-effective new protocols need to be developed to increase OMV yield (Collins, 2011). Another disadvantage of using OMVs as vaccines is inconsistent protein expression on the cell surface, which can lead to variation between batches. Moreover, the presence of LPS on the surface of OMVs may induce toxic shock syndrome and contribute to endotoxic effects in vaccinated individuals. Using methods such as detergents during OMV isolation can reduce the amount of LPS present in OMVs and prevent vaccine side effects (Lieberman, 2022). Methods such as genetic modification can be used to increase the yield of OMVs and reduce the amount of endotoxin induced by LPS (Balhuizen et al., 2021). In conclusion, the use of OMVs in vaccine technology shows promise.

Use of OMV as Adjuvants

An adjuvant is a substance that, when combined with a vaccine, can stimulate the immune system and promote a stronger, more persistent, and effective immune response. Aluminium salts, oil-based emulsions, and liposomes are some example types of adjuvants. However, aluminium salts are the most preferred among them (McKee and Marrack, 2017). OMVs function as potent immune stimulatory platforms due to the presence of TLRs and PAMPs. These characteristics of OMVs enable APCs activation, enhance antigen capture, strengthen the display of co-stimulatory

molecules, and support T-cell stimulation (Kashyap et al., 2022). Another feature of OMVs is their ability to transport antigens from the injection site to nearby lymphoid tissues via dendritic cells, thereby enhancing adaptive immune responses (Prior et al., 2021). OMVs are internalized by epithelial cells via "raft"-dependent uptake (O'Donoghue et al., 2017). They are then processed in lysosomes and mediate antigen presentation. Therefore, using OMVs as adjuvants is particularly promising in environments where traditional adjuvants often prove insufficient, such as mucosal tissues. The relevant mechanisms include the lipopolysaccharide fraction, which stimulates B lymphocytes through the TLR4 receptor. In some cases, it directs antibody formation through shared signaling pathways with the B-cell receptor (Minguet et al., 2008).

Currently, numerous studies focus on the use of OMVs as adjuvants. Harrell et al., (2021) developed an oral vaccine that uses OMVs isolated from *Burkholderia pseudomallei* as an adjuvant against salmonellosis. Researchers demonstrated that the vaccine using OMVs as an adjuvant provides greater protection in mice than the heat-killed (inactivated) *S. typhimurium* vaccine. This vaccine also induces CD4+ T cells and B cells. Sardiñas et al., (2006) demonstrated that *Neisseria lactamica*-derived adjuvants can function as effective intranasal adjuvants. Co-administration with the model antigen (hepatitis B surface antigen, HBsAg) resulted in a significant increase in IgG and IgA antibody responses against HBsAg. Song et al., (2020) demonstrated that OMVs obtained from *Helicobacter pylori* strain 7.13 induced persistent anti-*H. pylori* immunity for 12 weeks and significantly increased both systemic and gastric mucosal immunity and humoral immunity in vaccinated mice. Furthermore, OMVs effectively supported the Th1 immune response, but the response shifted towards Th2 and Th17 immunity compared to that generated by the CT adjuvant. Additionally, OMVs are promising candidates with self-adjuvant properties. Li et al.,

(2021) developed a vaccine (rOMV-PH) against *P. aeruginosa* by combining the PcrV-HitA T (PH) fusion antigen with OMVs possessing self-adjuvant properties, which were isolated from *Yersinia pseudotuberculosis* (Yptb strain). *In vivo* experiments have demonstrated that the rOMV-PH vaccine provides 73% protection against the cytotoxic PA103 strain and complete protection against the non-cytotoxic PAO1 strain. In contrast, the use of PH or OMV alone did not protect against these strains.

By modifying these vesicles, their adjuvant properties can be enhanced. Combining OMVs with stimulator of interferon genes (STING) agonists is an emerging strategy for enhancing immune responses in infection and cancer vaccines. This co-administration triggers the production of type I interferons (Shen et al., 2025). Advances in genetic engineering and synthetic biology permit the co-display of multiple antigens or epitopes on a single OMV. This facilitates the development of vaccine platforms with broad coverage against multiple pathogens (Piliou et al., 2023). Moreover, researchers have demonstrated that the localization of antigens directly influences the immune response. Antigens located on the surface of OMVs elicit a much stronger immune response than those located within the OMV lumen (Salverda et al., 2016; Necchi et al., 2021). These techniques have been shown to be applicable to both protein and polysaccharide antigens. These developments demonstrate that the adjuvant properties of OMVs can be optimized through design and engineering. Therefore, developing OMVs is important for designing safer and more effective adjuvants in the future.

OMV-Based Drug Delivery Systems

OMVS can also be used as drug delivery systems. These vesicles can encapsulate both hydrophilic and hydrophobic molecules due to their natural nano vesicular structures. Their unique

structure provides a platform particularly suited to the delivery of small-molecule drugs, peptides, nucleic acids, and other nanoparticles (Amalia and Tsai, 2023; Kan et al., 2024; Wu et al., 2025). By using genetic engineering methods, OMVs can target specific cells and tissues. Recent studies have also demonstrated that OMVs can be used effectively to deliver chemotherapeutic agents, antibiotics, and immunomodulatory molecules (Gujrati et al., 2014; Kuerban et al., 2020; Collins and Brown, 2021). OMVs can be loaded with drugs either *in vivo* or *in vitro*. *In vivo* loading involves incorporating drugs into OMVs during their synthesis by bacteria. Antibiotics such as azithromycin, ampicillin, amikacin, ceftriaxone, ciprofloxacin, norfloxacin, and levofloxacin are added to the medium during bacterial growth to produce OMVs containing antibiotics (Xue et al., 2022). Allan and Beveridge (2003) demonstrated that the *P. aeruginosa* PAO1 strain, when treated with gentamicin, secretes MVs containing peptidoglycan hydrolase and gentamicin. They also indicated that these vesicles have bactericidal properties. Editing the parent bacterium, such as by plasmid introduction, allows researchers to reshape OMV content (Xue et al., 2022). *In vitro* loading involves introducing drugs into OMVs after their isolation (Gujrati et al., 2014). Molecules can be delivered to isolated OMVs using electroporation, incubation, chemical modification, or membrane fusion (Xue et al., 2022).

The transport of drugs using OMVs can increase the efficacy of target drugs and prevent systemic spread. Huang et al. (2020) showed that orally administering antibiotic-loaded OMVs to mice retained the antibiotics in the gut for 36 hours thus preventing their spread throughout the body. They also reported that OMVs containing antibiotics exhibited excellent biocompatibility in safety tests. Gong et al., (2025) demonstrated that, orally administered OMVs loaded with enzyme can be cleared from the digestive fluid and enter the bloodstream by catalyzing various detoxification

reactions. They indicated that enzyme-loaded OMVs could be used in the treatment of metabolic diseases. Consequently, the ability of OMVs to encapsulate a variety of molecules makes them suitable as drug delivery systems in the treatment of different diseases. This highlights their potential for a wide range of biomedical applications.

OMV-Based Cancer Treatment Strategies

OMVs also have the potential to be used in cancer treatment. Because they are natural products of bacteria, they are potentially less toxic and more biocompatible than nanoparticles (Li et al., 2025). OMVs are promising for cancer treatment because of their two most important abilities: they serve as direct drug carriers and as immunotherapeutic agents. There are studies showing that OMVs can transform the immunosuppressive tumour microenvironment, trigger the maturation of dendritic cells, resulting in enhanced antigen presentation and increased cytotoxic activity of T cells (Won et al., 2023; Zhuang et al., 2023; Lin et al., 2025). OMV-based approaches have produced promising results in aggressive tumour models: such as triple-negative breast cancer, melanoma, and lung cancer (Kuerban et al., 2020; Peng et al., 2020; Liu et al., 2023). In preclinical models, the combined administration of chemotherapy, photodynamic therapy, and immunotherapy significantly accelerated tumour regression when delivered using OMV-based systems (Kuerban et al., 2020; Li et al., 2023). Furthermore, oncolytic viruses coated with OMVs both enhance viral replication and stimulate systemic anti-tumour immunity (Ban et al., 2023).

OMVs can be designed to contain surface proteins and ligands that recognise specific receptors on cancer cell surfaces. Gujrati et al., (2014) developed OMVs that specifically bind to cancer cells and are less endotoxic to human cells, with the aim of killing cancer cells. The researchers used an *E. coli* strain with the *msbB* mutation, which reduces the number of fatty acids in LPS,

enabling binding to the human epidermal growth factor receptor 2 (HER2). HER2 is highly expressed in breast and stomach cancer cells. The researchers fused an anti-HER2 affibody binding protein, which has high affinity for HER2, to ClyA, thereby transporting the protein to the OMV surface. They then added siRNA targeting kinesin spindle protein (KSP, EG5), which is essential for cancer cell division, into the OMVs, resulting in the death of cancer cells.

OMVs can also be used for the imaging cancer cells. Loading OMVs with nanoparticles or fluorescent dyes enables clearer visualisation of cancer cells in positron emission tomography (PET) scans or fluorescence microscopy, facilitating early diagnosis and monitoring of tumours. Gujrati et al., (2019) engineered a bacterial strain expressing a tyrosinase transgene to produce melanin-containing OMVs, which generated strong signals suitable for optoacoustic imaging of cancer cells. Additionally, the melanin encapsulated within OMVs enabled efficient absorption of laser energy generating substantial heat and mediating pronounced photothermal effects in both *in vitro* and in vivo settings.

In conclusion, the application of OMVs in cancer therapy holds promise, particularly for enabling tumour visualization and promoting tumour regression. However, certain obstacles remain before clinical translation can be achieved. These include production scaling, heterogeneity, immune tolerance, long-term biosafety, and efficacy (Li et al., 2025). Therefore, further research is required before OMVs can be used to treat cancer.

Conclusion

Currently OMVs are important subjects in microbiology, because of their natural biological roles and their biotechnological potential. These vesicles are involved in fundamental processes such as the bacterial stress response, growth, interbacterial communication, antibiotic resistance, gene transfer, pathogenesis,

and immunomodulation. These processes which they are taking place, demonstrate their critical importance for microbial life. They are also biotechnologically important structures because of their diverse application areas. OMV-based vaccine technologies have achieved clinical success due to their ability to generate a strong immune response. OMVs also offer significant advantages over traditional systems because of their natural adjuvant properties. Their biodegradable structures and ability to cross cell membranes make them a promising tool for drug delivery, particularly in targeted treatment strategies. Furthermore, OMVs play a key role in developing innovative cancer treatments due to their capacity to carry tumour antigens and modulate the immune response. Although challenges such as content heterogeneity, the potential for endotoxicity from LPS, and the need to standardize production remain, recent advances in genetic engineering and synthetic biology suggest that these limitations can be overcome. Therefore, OMVs could play an important role in fundamental microbiological research as well as in clinical and industrial applications.

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METAGENOMICS APPROACHES IN DETERMINING MICROBIAL BIODIVERSITY OF ACTIVATED SLUDGE IN WASTEWATER TREATMENT

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Introduction

Water is the most resource need for all living things to sustain their vital activities. Although the Earth is covered with water, only almost 3% of the Earth's surface water is usable. There isn't enough water to meet our basic demands and daily consumption, and access to clean water is gradually decreasing over time (Sathya et al., 2023). Anthropogenic influences such as increasing global warming, rapid population growth, and industrial development significantly contribute to the depletion of water resources. In particular, wastewater poses a more serious environmental problem by

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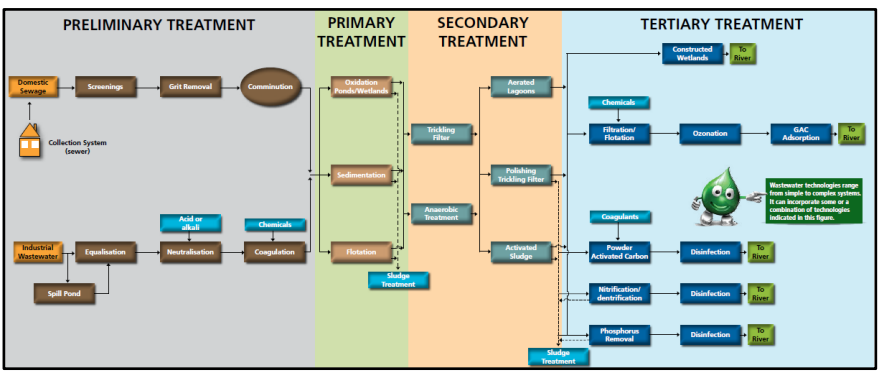
contaminating groundwater and freshwater resources (Iqbal et al., 2024).

Wastewater contains organic and inorganic pollutants, pharmaceutical waste, heavy metals, dyes, and various chemicals. Discharging this wastewater without treatment into receiving environment such as lakes, rivers, and streams has negative impacts on the environment and humans. In addition, since these wastewaters are rich in nutrients such as nitrogen and phosphorus, they cause eutrophication when discharged into receiving environments. Therefore, wastewater must be treated before being discharged into receiving environment (Obaideen et al., 2022; Mishra & Sundaram, 2024; Shah, Walia & Kazemian, 2024).

Wastewater Treatment

Wastewater treatment processes are advanced systems used to remove solids, organic, and inorganic contaminants from wastewater. Wastewater treatment consists of primary, secondary and tertiary stages (Figure 1) (Kato & Kansha, 2024).

Figure 1. *Wastewater treatment process stages*



Islam, 2025: 30

Primary Treatment

Primary treatment is the first stage of wastewater treatment. Primary treatment involves the removal of suspended solids from the wastewater by gravity. Solid substances are settled by adding chemicals such as coagulants and flocculants. In the sedimentation tank, heavy solids settle to the bottom while slight solids rise to the surface of the tank. While the solids that settle to the bottom are kept in the tank, the remaining wastewater is sent to secondary treatment (Solcova, Dlaskova & Kastanek, 2024).

Secondary Treatment

Secondary treatment is applied to remove inorganic and organic pollutants in wastewater. In this treatment process, organic substances are removed biologically from wastewater using microorganisms. Because microorganisms remove dissolved and organic matter, they reduce the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) in the wastewater. Furthermore, microorganisms decompose organic matter, converting it into byproducts such as water, carbon dioxide, ammonia gas, and nitrate. It is widely applied in secondary treatment in activated sludge process (Islam, 2025).

Tertiary Treatment

Tertiary treatment is applied to reuse wastewater that cannot be treated in primary and secondary treatment processes and to meet discharge limits. Tertiary treatment utilizes adsorption, membrane filtration, UV and advanced oxidation processes (Kato & Kansha, 2024).

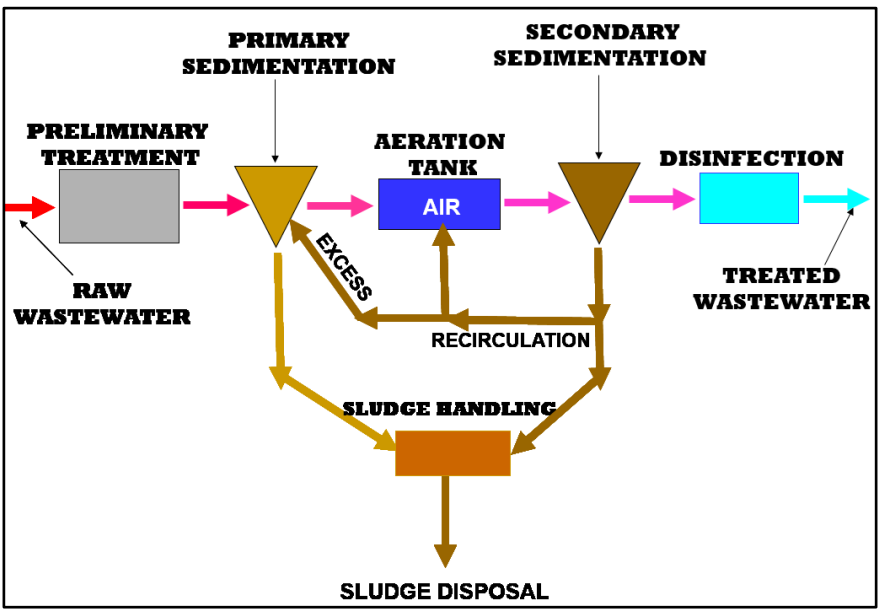
Activated Sludge Process

The activated sludge process is used treatment method for the biological nutrient removal of nutrient from wastewater (Figure 2)

(Awasthi et al., 2023). This treatment process is the process of removing organic matter through biological oxidation by aerobic microorganisms in the aeration tank (Eq. 1). By introducing air into the system through bottom diffusers or mechanical aerators located in the aeration tank, both thorough mixing is achieved and dissolved oxygen levels are maintained (Tsalas et al., 2024).

$$\text{Organic matters} + O_2 + N + P + \text{ microorganisms} \rightarrow \text{new cells} + CO_2 + H_2O + \text{inert matters} \quad (1)$$

Figure 2. *Activated sludge process*



Tsalas et al., 2024

Biological Nitrogen Removal Processes

Activated sludge is rich in biodiversity, including bacteria, actinomycetes, fungi, and protozoa. Bacteria with the largest microbial populations play an active role in the removal of pollutants from wastewater. The complex structure of the microbial community and the interactions among microbial groups had a major

function in nitrogen removal from wastewater. The fundamental processes in wastewater treatment are nitrification and denitrification (Meng, Huan & Ge, 2024).

Nitrification

Nitrification is the oxidation of ammonia in wastewater, first to nitrite and then to nitrate. Nitrification occurs in two stages. In the first stage, ammonium-oxidizing bacteria (AOB) convert ammonia to nitrite (Eq. 2). In the second stage, nitrite-oxidizing bacteria (NOB) convert ammonia to nitrate (Eq. 3). *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira* are bacteria that comprise AOB. *Nitrobacter, Nitrospina, Nitrococcus, and Nitrospira* are bacteria that comprise NOB (Orman, 2019; James & Vijayanandan, 2023).

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^- \quad (2)$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (3)$$

Denitrification

Denitrification is the reduction of nitrate to nitrogen gas by anaerobic heterotrophic bacteria (Eq. 4). They use the nitrate or nitrite produced during the nitrification process as electron acceptors (Dai et al., 2022; Song et al., 2023). Pre-denitrification, post-denitrification, and simultaneous nitrification-denitrification processes are the processes used in biological nitrogen removal.

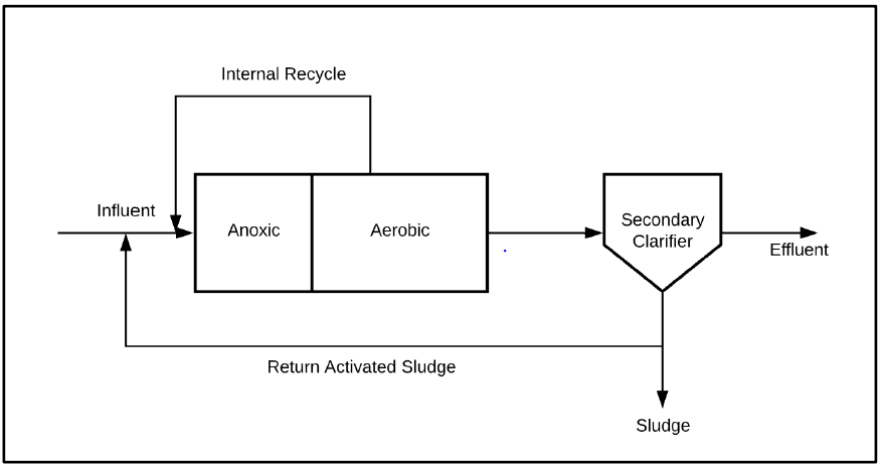
$$NO_3^- + 5CH_3OH \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \quad (4)$$

Pre-denitrification process

Pre-denitrification process includes anoxic and aerobic tanks. It involves feeding activated sludge back to the anoxic tank via internal return line (Figure 3). Nitrate formed as a result of nitrification in the aerobic tank is returned to the anoxic tank via a recycle line. Anaerobic bacteria use nitrate as an electron acceptor to

perform the denitrification process (Lim et al., 2012; Qian et al., 2023).

Figure 3. *Flow diagram of the pre-denitrification process*

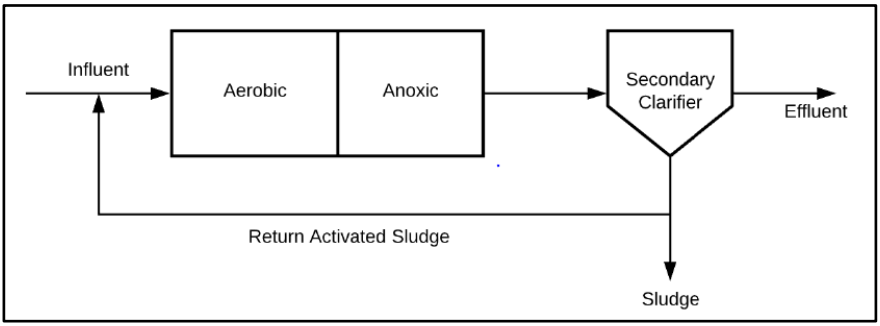


Orman, 2019: 120

Post-denitrification process

The post-denitrification process follows the anoxic tank and the aerobic tank. In this process, nitrification occurs in the aerobic tank (Figure 4). Additionally, some of the carbonaceous material in the influent is removed in this tank. When the carbonaceous organic substances in the wastewater coming to the anoxic tank after the aerobic tank are insufficient, carbon-based substances must be added to the anoxic tank from outside (Yin & Guo, 2022).

Figure 4. *Flow diagram of the post-denitrification process*



Orman, 2019

Simultaneous Nitrification-Denitrification (SND) process

The simultaneous nitrification-denitrification (SND) process combines the two-stage nitrogen removal process into a single stage. In the SND process, nitrate produced in the aerobic zone occurs denitrification in the anoxic zone. In this process, there is no need to add carbon substances to complete denitrification, which makes it cost advantageous (Bhattacharya & Mazumder, 2023; James & Vijayanandan, 2023).

Biological Phosphorus Removal Process

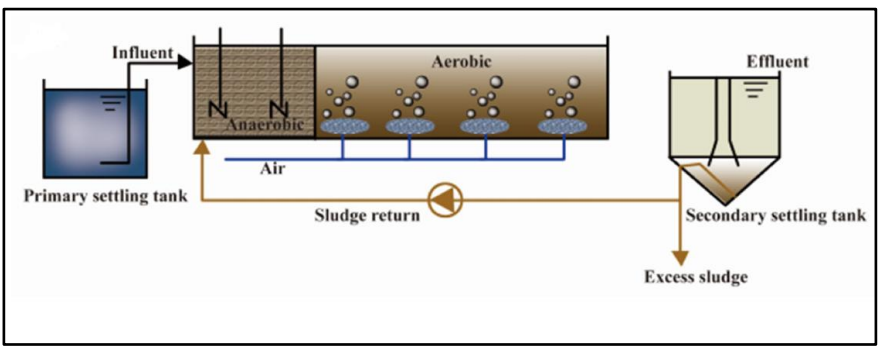
Phosphorus is an essential nutrient for all living things. After phosphorus is used, it is discharged into wastewater. When phosphorus-containing wastewater is discharged untreated into receiving environments, it causes eutrophication, which is a serious environmental problem. Therefore, this wastewater must be treated before being discharged into receiving environments. These wastewaters are treated using the biological phosphorus treatment method. PAOs play a key role in the biological phosphorus treatment method because PAOs gather phosphorus and store it as polyphosphates. Thus, PAOs remove phosphorus from wastewater. PAOs, such as *Gemmatimonas, Halomonas,* and *Accumulibacter*, are

microorganisms that play an important role in phosphorus removal (Zahed et al., 2022; Zheng et al., 2023). The A/O process is the most commonly used biological phosphorus removal process.

Anaerobic/Oxic Process

Anaerobic/Oxic (A/O) process is applied to the removal of carbonaceous matter and phosphorus from wastewater in sequential anaerobic/ oxic tanks (Figure 5). Carbonaceous matter removal is achieved by introducing oxygen into the aerobic tank. It feeds activated sludge from the final sedimentation tank to the anaerobic tank via the sludge return line. The phosphorus contained in the activated sludge is released into the anaerobic tank. This allows the phosphorus delivered to the anaerobic tank to be hold by microorganisms in the aerobic tank, enabling phosphorus removal from the wastewater (Chen et al., 2022).

Figure 5. *Flow diagram of the A/O process*



Chen et al., 2022

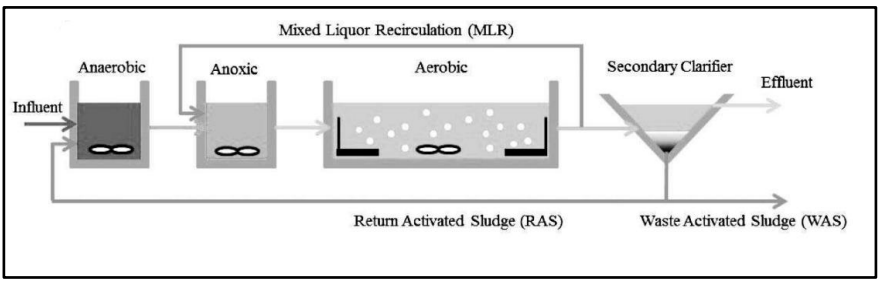
Biological Nutrient Removal Process

Biological nutrient removal process is combined of nitrogen and phosphorus. These processes utilize sequential systems involving anaerobic/anoxic/oxic (A^2/O) tanks to achieve nutrient removal. The A^2/O process is widely used for biological nutrient removal.

Anaerobic/Anoxic/Oxic Process

Anaerobic/anoxic/oxic (A^2/O) process is a modification of the A/O process, with the anoxic tank located between the anaerobic and oxic tanks (Figure 6). Nitrate is needed for denitrification to occur in the anoxic tank. The nitrate formed as a result of nitrification in the oxic tank is given to the anoxic tank through the internal recycle line, thus performing the denitrification process. In the anaerobic tank, PAOs hold the organic substrate and release phosphorus into the wastewater. In the aerobic tank, the organic substrate taken by PAOs is converted into energy, and the bacteria receive the phosphorus necessary to meet their nutritional needs. In this way, phosphorus is removed from wastewater (Sheik et al., 2023).

Figure 6. *Flow diagram of the A^2/O process*



Xu, Bernards & Hu, 2014: 8

Metagenomics Approaches in Wastewater Treatment

Metagenomic approaches have recently provided a broader understanding of microbial communities. Metagenomics is defined as the culture-independent genomic analysis of microbial communities based on DNA isolated from environmental samples (Nowrotek et al., 2019).

Metagenomics provides extensive information about the microbial community in mixed culture wastewater treatment plants (Albertsen et al., 2013). The activated sludge process utilizes the

activities of various microbial communities to remove organic matter and nutrients (Fan et al., 2020). Because the performance of this process is directly related to the structure and functionality of the microorganisms that perform the treatment process, elucidating the biological properties of the sludge, as well as its technological properties, is crucial for developing sustainable processes (Ju et al., 2014; Dottorini et al., 2023). In this context, investigating the microbial biodiversity in the activated sludge process helps elucidate its biological mechanisms (Xie et al., 2021).

Metagenomic studies have been conducted to determine the biodiversity of microorganisms effective in biological nutrient removal in the literature. Speth et al. (2016) investigated metagenomic studies on the partial nitritation-anammox (PNA) process. In this study, they obtained the metagenome composition of 23 denitrifying microorganisms and classified these genomes in terms of functional genes. Zhao et al. (2019) conducted metagenomic studies to determine the substrate competition mechanism between *Ca. Jettenia* and *Ca. Brocadia* in a stationary anaerobic chamber reactor. Their study found that *Ca. Brocadia* had a higher gene count for nitrogen metabolism and chemotaxis than *Ca. Jettenia*. These findings suggest that *Ca. Brocadia* increased ammonia nitrogen removal due to its effect on substrate competition. Kolakovic et al. (2021) conducted a study on *Accumulibacter* biodiversity by adjusting operating parameters. This study examined the effect of the dominance of different clades on phosphorus removal. The results of this study indicated that determining *Accumulibacter* biodiversity plays a role in affecting phosphorus removal performance in the reactor.

As a result, metagenomics plays a fundamental role in the study of microorganisms in uncultured wastewater and their metabolic pathways during the treatment process. It also provides a unique tool for elucidating the responses of microbial communities

in activated sludge to changing environmental conditions. Thanks to this method, it is possible to elucidate the dynamics of microbial biodiversity, better understand and optimize treatment processes, and design more efficient and controlled bioreactors.

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EXPLORING BIOPLASTICS FROM PSYCHROPHILIC MICROALGAE: UNLOCKING COLD-ADAPTED BIOMASS FOR INDUSTRIAL APPLICATIONS THROUGH ADVANCED FERMENTATION TECHNOLOGIES

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Introduction

The environmental problems caused by the widespread use of petroleum-derived plastics are becoming increasingly troubling. Unfortunately, the production and use of these plastics contribute to the global problem of plastic pollution, the accumulation of microplastics in our ecosystems, and the rise of greenhouse gas emissions, all of which pose serious threats to environmental, human health, and economic sustainability (Sharma et al., 2023; Ziani et al., 2023). Therefore, stakeholders, particularly scientists, as well as national and international non-governmental organizations,

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associations, foundations, schools, and industrial actors, are undertaking activities to develop sustainable, renewable, environmentally friendly alternatives that are compatible with circular economic principles (Arora et al., 2023).

Recent developments have focused not only on finding alternative raw material sources to petroleum-based plastics, but also on increasing biodegradability, reducing the carbon footprint and increasing the mechanical durability of the materials to be used and in this regard, bioplastics have become attractive alternatives in terms of both environmental and industrial performance (Adetunji & Erasmus, 2024).

Microalgae are increasingly gaining prominence as a raw material source in biofuel and bioplastic studies due to their high growth rates, direct integration potential through CO_2 capture systems, and their lack of lignin, a structurally complex polymer, to be used in biomass processes, it must first be separated, degraded, and transformed into its basic components. This is a very difficult process, requiring high energy and chemical requirements. The fact that microalgae do not contain this difficult-to-transform polymer gives them the advantage of being more adaptable to fermentation and hydrolysis steps compared to other biomass sources in the ecosystem. This advantage, in particular, leads to the reduction of high-cost processes to more economically viable costs and the increase in material efficiency (Ashour et al., 2024).

Recent scientific studies have shown that microalgae can be used as a "third-generation biomass" source in bioplastic production. With their carbohydrates, lipids, and various polymeric precursors, microalgae are not only used as raw materials in the production of sustainable, environmentally friendly bioplastics, but also find wide applications in a wide variety of sectors such as energy,

pharmaceuticals, and biochemistry (Sarwer et al., 2022; Ashour et al., 2024; Saratale et al., 2024).

Currently, the majority of microalgae used as raw materials in bioplastic production are species cultivated in temperate or tropical climates. However, scientific studies have shown that these temperate/tropical species do not perform sufficiently in terms of biomass production and metabolic efficiency in cool climates or regions with significant temperature fluctuations (García et al., 2018; Montuori et al., 2023; Novoveská et al., 2023). This limitation has led to the search for new raw materials for bioplastic production.

Alpine ecosystems (high altitude, temperature 0-10 °C, sudden day/night temperature changes), glacial lakes (temperature 0-4 °C, limited light, nitrogen and phosphorus are very limited) and polar regions (temperature °below 0 °C, high salinity change, high sub-ice pressure, intense ice movements and strong winds) have unique advantages such as showing high metabolic activity even at low temperatures, being able to continue their vital activities even with low energy input and accumulating fatty acids, polysaccharides, EPS biotechnologically valuable metabolites under stress conditions (Suzuki et al., 2019; Lauritano et al., 2020; Chia et al., 2025). Therefore, these microalgae have unique advantages such as showing high metabolic activity even at low temperatures, being able to continue their vital activities even with low energy input and being able to accumulate fatty acids, polysaccharides, EPS biotechnologically valuable metabolites under stress conditions (Suzuki et al., 2019; Lauritano et al., 2020; Chia et al., 2025). The use of bioplastics in production is considered a promising approach in terms of both diversifying new generation raw material sources and supporting environmentally friendly, low-carbon footprint production processes (Li & Yao, 2024).

Ecological and Physiological Properties of Microalgae

Microalgae are chlorophyll-containing organisms that live in fresh and salt waters such as streams, lakes, and seas, or on moist substrates (Wang et al., 2024). Unlike terrestrial plants, they do not exhibit organ differentiation such as roots, stems, or leaves. They perform all their metabolic and photosynthetic activities at the cellular level. Their high photosynthetic efficiency, ability to adapt to a wide variety of environments, and ability to reproduce quickly and easily lead to their use and research in many biotechnological applications, including biofuels, bioremediation, valuable metabolite production, bioplastics, and biofilm raw materials (Wang et al., 2024).

Cold-adaptive microalgae, also known as psychrophilic or psychotropic, on Earth's surface is particularly evident in extremely cold habitats. In these environments, daily temperatures range from −2 to +4 °C, and salinity is high or variable. Light levels are low or fluctuating (Lund-Hansen et al., 2024). From a physiological perspective, psychrophilic Microalgae possess several prominent adaptation mechanisms. These include the ability to maintain membrane fluidity at low temperatures, adaptation osmolytes, antifreeze proteins, and stress regulator genes (Chia et al., 2025, Zakaria et al., 2025).

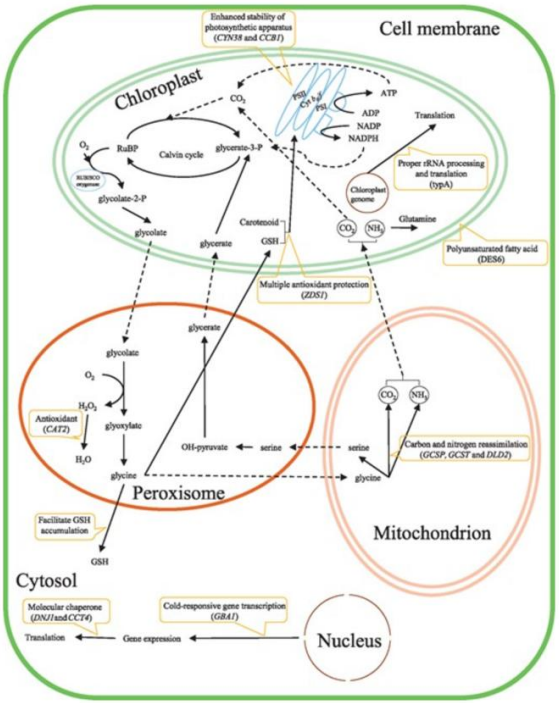
Psychrophile Microalgae: Diversity and Adaptations

Psychrophilic microalgae can live in the coldest habitats on earth, such as glaciers, glacial lakes, under-ice habitats, alpine lakes and the poles. Despite quite harsh environmental conditions such as strong winds, dryness, drought and high ultraviolet radiation, a rich diversity of microalgae is seen in these regions (Smith et al., 1994; Teoh et al., 2004). Optimum growth temperatures are generally between 0-15 °C; some species can remain metabolically active even at temperatures close to freezing point. They form microalgal

biofilms in the channels formed by sea glaciers in the Arctic and Antarctic regions. These organisms can maintain their viability by adapting to harsh conditions such as salinity fluctuations, light limitations, freezing and thawing, thanks to the biofilms they form (Montuori et al., 2023; Chia et al., 2025). Microalgae living at low temperatures have developed various structural and biochemical adaptations in order to adapt to these extremely cold habitats. While their enzyme systems show cold-active properties; They have low activation energy, high substrate binding affinity, and flexible active sites that facilitate binding, which help maintain their catalytic efficiency at low temperatures (Liu et al., 2023; Kuddus et al., 2024). They can also survive even in harsh conditions by producing specialized compounds such as ultraviolet radiation screening compounds, antifreeze proteins, antioxidants, and polyunsaturated fatty acids (Montuori et al., 2023; Chia et al., 2025).

Psychrophile at the membrane level microalgae rearrange their lipid classes to maintain their fluidity, accumulate polyunsaturated fatty acids in their structures, and synthesize carotenoids and antifreeze proteins to protect their membrane structure (Hernando et al., 2021; Chia et al., 2025). Transcriptomic studies have shown that psychrophile microalgae have been shown to selectively regulate the pentose phosphate pathway, carbon metabolism, and stress response genes during sudden temperature drops, reduce oxidative stress using their existing antioxidant defense systems, and create alternative enzyme variants (Peng et al., 2021). Unfortunately, despite all this, energy efficiency under low-temperature conditions remains a significant challenge. Psychrophilic is observed that microalgae species grow very slowly compared to temperate microalgae in terms of energy efficiency, but they can synthesize extracellular polysaccharides and lipids, which make them very valuable from an industrial perspective, thanks to the cold stress they are exposed to (Gao et al., 2023).

Figure 1. *Summary of adaptive mechanisms in Antarctic psychrophilic algae. (This scheme shows the principal cell components and pathways associated with cold adaptation. The solid arrows indicate the reactions; the dashed arrows represent the transport; and rounded orange rectangles denote the putative adaptive strategies).*



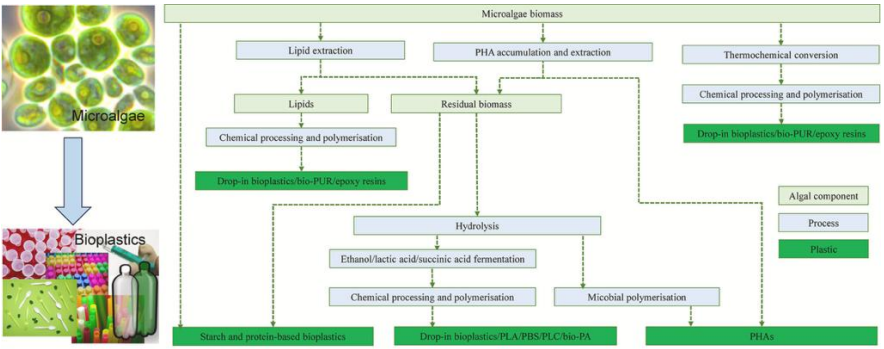
(Zhang et al., 2019).

Bioplastic Production Pathways from Microalgal Biomass

Microalgal biomass is a versatile resource that can be used in bioplastic production due to its diverse components, including proteins, lipids, carbohydrates, cell wall components, and extracellular polysaccharides (Figure 2). In recent studies, direct conversion, also known as blending or formulation, and biocatalysis /fermentation-assisted conversion methods are two prominent

strategies that aim to optimize the production of useful polymeric building blocks from raw biomass (Arora et al., 2023).

Figure 2. *Bioplastic production from microalgae*



(Chaudry et al., 2025)

Direct Conversion and Blending Methods

In the direct conversion method, which is the first of the two widely used methods in bioplastic production, microalgae biomass is incorporated into the structure with minimal processing or by mixing directly into the bioplastic matrix or biopolymers. This method, called blending, aims to increase raw material efficiency by integrating biopolymer recipes into microalgae biology. The blending method provides the advantages of a simpler production line and lower processing costs compared to other biocatalysis processes (Arora et al., 2023). Some studies have reported the use of microalgae without blending. They propose using biomass with minimal processing or by incorporating it directly into the bioplastic matrix. This strategy stands out because it supports the idea of zero waste and reduces extraction losses (Sudhakar et al., 2024).

Fermentation / Biocatalytic Conversion Methods

Biocatalytic conversion methods, a more sophisticated and efficiency-focused method, first break down microalgae into

monomers and then convert the separated monomers into bioplastic precursors using enzymatic systems or microorganisms. This pathway is most commonly associated with PHA/PHB production. Here, the sugars, carbohydrates, or organic compounds from the isolated microalgae are fed to PHA-producing bacteria. Which are then targeted to produce lactic acid for PLA synthesis from microalgae- based substrates (Sharma et al., 2024).

In recent years, methods that extract sugar monomers from microalgae biomass via enzymatic hydrolysis and convert them directly into polymer synthesis have attracted attention. Factors such as temperature optimization, enzyme selection, and substrate inhibition play critical roles in these methods (Adetunji & Erasmus, 2024).

Metabolic manipulation approaches and genetic engineering applications that enable direct PHA production from microalgae by enhancing polymer-producing genes within microalgae are also prominent methods (Arora et al., 2023).

Combined and Integrated Approaches

Recent studies also include hybrid strategies combining direct conversion/blending and fermentation/biocatalytic conversion methods. For example, in a biofuel production system, lipid extraction from microalgae can first be performed, and then the proteins and carbohydrates from the remaining biomass can be used to feed bioplastic production lines. This increases both resource utilization efficiency and product diversity (Iqbal et al., 2025). Another combined approach involves the extraction of microalgae grown in wastewater. The use of bioplastics as an input in bioplastic production provides an example of a dual-benefit integrated biorefinery concept by providing water treatment while providing waste resource assessment (Sharma et al., 2024).

Advanced Fermentation Technologies for Cold-Compatible Biomass

Cold-adapted microalgal biomass offers unique opportunities for use in bioprocess lines thanks to its high catalytic efficiency at low temperatures, energy-efficient process conditions, cold-active enzymes, and low thermal stress. Cold-active enzymes, with their increased structural flexibility and low activation energies, exhibit high specific activity at temperatures between 0 and 20 °C, thus minimizing the carbon footprint by reducing the required heat requirements. This makes the fermentation steps of hydrolysis → saccharification → fermentative conversion in low-temperature regimes attractive from both technical and environmental perspectives (Liu et al., 2023).

Bioreagent and process designs in cold-adapted biomass, the increase in ambient viscosity and the decrease in the diffusion coefficient at low temperatures limit mass transfer, particularly O_2 transfer. Therefore, pneumatic/air-lift designs and low-shear mixing strategies are gaining prominence. Recent studies highlight pneumatic/air -lift geometries due to their advantages in balancing low energy and high mass transfer and in industrial production. Computational fluid dynamics (CFD), another engineering field used in bioreactors for cold-adapted biomass, is recommended as a tool for optimizing the mixing-gas transfer-cooling triangle, accelerating design decisions. Single-use bioreactors are becoming increasingly attractive due to their advantages such as reducing the risk of contamination that may occur in low-temperature, long-run processes and enabling flexible production (Palladino et al., 2024).

Microalgal carbohydrate hydrolysates into bioesters such as PHA/PHB Fermentative transformation is possible even at low temperatures. However, low-temperature conditions can slow down polymer aggregation kinetics and growth rate, resulting in pulse

feeding strategies such as feeding, fed-batch, psychrotolerant. This can make calcium selection and nitrogen/phosphorus limitation critical. Indeed, studies have shown that low temperatures sensitize the structure of PHA accumulation and affect the performance of the feeding regime. Conversely, the availability of microalgal biomass as a cheap and abundant feed for bacterial PHA production in cold-chain biorefinery lines provides a cost-cutting leverage (Trego et al., 2024).

From the perspective of low-temperature process intensification and integration, increasing sugar yield through pretreatment with cryo-adaptive hydrolysis, followed by moderate-temperature fermentation, and, where possible, using hybrid open cultivation/closed fermentation methods are prominent. Integrating waste - to -PHA and microalgal fermentation, which offers a waste - to - product approach, is recommended to reduce the total energy input of the system and increase carbon efficiency (Kusuma et al., 2024). The strategy combining cold-activated biocatalysis, low-energy bioreactor designs and suitable feeding configurations has shown increased scalability in the fermentative utilization of cold-adapted biomasses (Imran et al., 2025).

Advanced Fermentation Technologies for Cold-Adapted Biomass

The use of cold-adapted biomass in biotechnological applications, unlike traditional fermentation technologies, presents several limitations due to challenges such as slow substrate turnover, low metabolic activity, mass transfer efficiency, and reduced diffusion rates. The development of new approaches and strategies to overcome these challenges is crucial.

Fermentation technologies developed for cold-adapted biomasses. The most widely used of these: (Joshi & Satyanarayana, 2013; Liang et al., 2023; Liu et al., 2023).

- Cold active enzymes and microorganisms
- Optimized fermentation processes at low temperature
- Reactor and engineering optimization
- Integration and process intensification methods.

Each of these methods has its advantages and limitations. Therefore, the fermentation technology that provides the best results for cold-adapted biomass is the method that combines the following steps in a balanced manner: cold-active enzyme, suitable microorganism, reactor optimization, and process integration.

Industrial Applications and Opportunities

Cold-adaptive microalgae with many advantages in bioplastic production They are candidates for not only laboratory-scale but also large-scale, industrial-scale applications. Some of these advantages are as follows:

- **Low energy requirements and low environmental impact**

One of the biggest drawbacks limiting the use of industrial-scale bioprocesses is their high cost. Heating and cooling to high temperatures, in particular, is a costly process step in the industry. However, the ability of cold-adaptive microalgae to maintain normal metabolic activity at low temperatures is a significant advantage in reducing energy consumption costs and carbon footprints in industrial-scale bioplastic production processes (Liu et al., 2023). Furthermore, selecting locations for biotechnological facilities established for bioplastic production in cold regions can also provide advantages in meeting cooling needs. Low ambient temperatures minimize the environmental cooling load, offering advantages in terms of production costs, especially in extremely cold habitats such as the Alps, Arctic, and Antarctic regions (Chia et al., 2025).

- **Providing holistic added value with hybrid or modular approaches**

From microalgae bioplastic production need not be limited to a single product. Various industrial-scale scenarios can be implemented, first by extracting lipids, then by utilizing the remaining biomass for protein and polysaccharides. This multi-product biorefinery approach increases production efficiency while also ensuring economic sustainability. Furthermore, with advanced bioreactor designs, bioplastic precursors can be obtained from industrial waste and polluted water in microalgae development (Sharma et al., 2024; Iqbal et al., 2025).

- **Suitable application sectors and market potentials**

Cold-adaptive microalgae-based bioplastics as packaging materials, particularly for products requiring a cold chain such as frozen foods and biomedical refrigerated transportation, biological sensor-based applications, and medical supplies requiring low-temperature storage, offers significant advantages. Furthermore, from a market perspective, cold-adaptive microalgae-based bioplastics have a promising future if supported by various biological and economic trends. This is why large communities like the European Union have recently adopted the "Plastics" Convention. They encourage the production of bioplastics by developing various strategies such as “Economy” (2023) and developing new incentive and investment mechanisms in this field (Ghasemlou et al., 2024).

Challenges and Future Perspectives

The primary bottleneck for cold-adaptive microalgae is the limited productivity and production rate at low temperatures, due to factors such as lack of light, photoprotective mechanisms consuming available energy, and fluctuating salinity. While the low optimum

growth temperature is an advantage for microalgae isolated from the Arctic, the lower specific growth rates compared to temperate species pose a challenge that limits volumetric productivity (Chia et al., 2025).

The decrease in viscosity and diffusion rate with decreasing temperature lowers fermentation parameters, leading to the recommendation of methods such as low-shear mixing, air-lift / pneumatic configurations, and optimized gas distribution. However, the use of cold-active enzymes, such as hydrolases, is an important feature of the process, as it reduces the need for heating by moderating pretreatment steps and thus lowering energy requirements and carbon footprint (Liu et al., 2023).

Cold-adaptive microalgae, when we look at the sustainability and market share of bioplastic production, we immediately see a rising trend. The EU's roadmaps and packaging regulations, in particular, regarding plastic use and production, are seen as encouraging bioplastic production and shaping the global bioplastics market.

Conclusion

Cold-adaptive microalgae as a raw material source in bioplastic production presents a unique intersection not only with industrial significance but also with ecological and sustainable environmental requirements. Unlike the petrochemicals commonly used in plastic production, cold-adaptive microalgae masses stand out as a promising alternative due to their renewability, low energy requirements, and environmental friendliness. Thanks to their cold-active enzymes, these microalgae possess physiological properties such as metabolic plasticity and membrane flexibility, allowing them to survive in extreme cold conditions and produce valuable industrially important metabolites. This offers significant advantages in terms of their usability in bioplastic production.

Despite the advantages of using cold-adaptive microalgae in bioplastic production, there are also significant challenges that must be overcome, limiting their industrial-scale production. These include low growth rates, engineering constraints related to oxygen transfer and mass diffusion, and limited volumetric efficiency. To overcome these challenges, new fermentation technologies have been introduced, including computational fluid dynamics (CDF)-based designs, air-lift reactors, hybrid systems using cold-activated biocatalysts, and combinations of these in specific proportions. Furthermore, efforts are being made to increase system efficiency through adaptive laboratory, synthetic biology applications, and process intensification approaches.

From a sustainability and market perspective, bioplastics derived from cold-adaptive microalgae offer new opportunities for use as packaging materials for products requiring a cold chain and cold storage, particularly in global sustainability plans such as the EU Plastics Strategy for a Circular Economy. Both the development of algal biology and studies on bioprocess optimization and metabolic engineering are critical to unlocking this potential.

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APPLICATIONS OF BACTERIALLY SYNTHESIZED NANOPARTICLES IN ELECTRONICS AND OTHER FIELDS

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Introduction

The design and application of materials in the domains of biology, chemistry, physics, and engineering have been completely transformed by nanotechnology. The field has made steady development over the past 20 years towards bio-inspired and sustainable synthesis pathways, which are collectively referred to as "green nanotechnology." These eco-friendly methods steer clear of the hazardous chemicals and large energy inputs that are typical of conventional chemical synthesis by emphasizing the use of biological organisms, extracts, and biomolecules as reducing and stabilizing agents.

Bacterially mediated nanoparticle synthesis has become one of the most promising green approaches for creating nanoscale materials with regulated stability, functionality, and shape. Under

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ideal conditions, bacteria can use enzymatic mechanisms to convert metal and metalloid ions into nanostructures, making them operate as natural nanofactories. They are extremely effective and ecologically friendly synthesis platforms because of their capacity to control redox processes, release reductase enzymes, and produce biocompatible capping compounds.

The evolution of this research area has been strongly interdisciplinary. Initial studies in the 2000s showed that metal ions such as Ag^{+} and Au^{3+} are microbially reduced by Pseudomonas and Bacillus species. In recent years, this concept has expanded to include complex chalcogenides and carbon-based hybrid materials, enabling applications that bridge biotechnology and materials science. Between 2017 and 2025, Çakıcı Can and his associates carried out a number of investigations that methodically improved bacterial synthesis techniques for Se, Ag, Zn, ZnSe, CuSe, and GO-based hybrid nanoparticles, demonstrating both biological comprehension and functional performance.

Because of their consistent enzymatic activity and metabolic restrictions, bacterial systems offer greater control over nucleation kinetics and phase development than plant or fungal-mediated synthesis. In the unique biochemical conditions that each bacterial strain provides, the form and crystallinity of nanoparticles are influenced by pH, redox potential, and nutritional balance. This precise control is crucial for applications in biomedicine, catalysis, and environmentally friendly electronics, which are increasingly focused on materials with lower toxicity and higher efficiency.

Global environmental goals, especially the decrease of hazardous waste and carbon footprint in the creation of nanomaterials, are also in line with the move towards biogenic synthesis. The microbial technique complies with the core tenets of Green Chemistry by using renewable growth media, operating at

room temperature, and producing few byproducts. For the next generation of functional materials produced from biological sources, the combination of biological reduction systems and nanomaterial design is not only an alternative but a revolutionary step.

Microbial Synthesis of Nanoparticles

The internal metabolic mechanisms of bacteria can reduce metal and chalcogen ions to their elemental or compound nanoparticle forms. Enzymes including nitrate reductase, NADH-dependent reductase, and hydrogenase catalyze these reduction processes by assisting in the transfer of electrons from metabolic cofactors (NADH/NADPH) to ionic precursors (e.g., Ag^{+}, SeO_3^{2-}, Cu^{2+}, Zn^{2+}). There are two main ways that the nanoparticles can form:

-Intracellular synthesis, where ions are transported into the cell-specific space and reduced within controlled cellular compartments.

- Extracellular synthesis, in which secreted enzymes reduce ions outside the cell, leading to nanoparticles that are easier to harvest and purify.

Proteins, polysaccharides, and peptides are typical biomolecules that function as capping and stabilizing agents during these processes by preventing aggregation and offering helpful surface groups. The dispersion, chemical reactivity, and biocompatibility of nanoparticles are all improved by these biomolecular layers.

Electron microscopy and spectroscopic studies have demonstrated that the synthesis method (intracellular vs. extracellular) has a substantial impact on the form and size distribution of nanoparticles. *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Lactobacillus* sp. are some of the most effective

bacterial producers, producing nanoparticles with different crystalline phases that are between 20 and 60 nm in size. The resulting materials are remarkably stable and pure when compared to materials produced by purely chemical reductions.

Characterization and Biological Perspectives

The physicochemical characterization of bacterially synthesized nanoparticles has revealed a strong and fundamentally related to between **biological synthesis conditions**—including enzymatic activity, precursor concentration, and pH—and the resulting **structural, morphological, and electronic properties** of the produced materials. All of these results show that the microbiological microenvironment is important for determining the crystallinity, defect structure, electronic, and optical behaviour of nanoparticles in addition to their creation

The crystalline nature and phase identity of biogenic nanoparticles, including copper selenide (CuSe), zinc selenide (ZnSe), silver (Ag), and selenium (Se), are confirmed by X-ray diffraction (XRD) research. The hexagonal phase's diffraction peaks are primarily seen in the Se nanoparticles, suggesting somewhat ordered crystallisation aided by enzymatic reduction.

The hexagonal phase's diffraction peaks are primarily seen in the Se nanoparticles, suggesting somewhat ordered crystallisation aided by enzymatic reduction. Ag nanoparticles exhibit sharp reflections at $2\theta = 38°$, 44°, 64°, and 77°, which correlate to the (111), (200), (220), and (311) planes of the face-centered cubic (FCC) structure (JCPDS 04-0783). Preferred orientation in this direction is suggested by the high intensity of the (111) plane, which is characteristic of physiologically reduced metallic silver. The hexagonal close-packed (HCP) phase reflections in the Zn nanoparticles verify that Zn is in a metallic state with well-developed lattice periodicity (JCPDS 04-0831).

While CuSe exhibits distinct reflections at 30.4°, 32.8°, and 47.7°, indicating an orthorhombic lattice, ZnSe's XRD patterns, which correspond to the (111), (220), and (311) planes of the zinc blende cubic structure, show sharp peaks at 27.3°, 45.1°, and 53.5° in the case of binary chalcogenides (JCPDS 06-0429). The crystallite size is estimated using the Debye-Scherrer equation to be between 18 and 40 nm, which is consistent with nanoscale growth in the presence of bacterial reduction. These results suggest that bacterial enzymatic pathways promote uniform growth and progressive nucleation, resulting in narrower crystallite borders, in contrast to conventional chemical techniques.

These structural findings are further corroborated by scanning electron microscopy (SEM). As a result of uniform nucleation in the enzyme environment, the biogenic Ag and Se nanoparticles have spherical and quasi-spherical morphologies with narrow size distributions (usually 20–40 nm). During the reduction of Zn^{2+} and SeO_3^{2-} ions, ZnSe nanoparticles exhibit polyhedral and faceted grains, indicating anisotropic development affected by biomolecular interactions. The thin, sheet-like structures or nanosheets seen in CuSe samples point to a two-dimensional growth propensity brought on by the bacterial proteins' selective adsorption on particular crystallographic planes. Such biomolecular capping improves surface stabilisation and prevents unchecked aggregation.

The chemical composition of the bacterially produced nanoparticles is confirmed by energy-dispersive X-ray (EDX) spectroscopy, which displays strong peaks for Ag, Se, Zn, Cu, and O along with trace signals of carbon and nitrogen coming from the bacterial matrix. The identification of organic components confirms the existence of biomolecular capping layers, which are mostly made up of amino, carboxyl, and thiol groups. These layers improve the stability of nanoparticles and make it easier for them to be subsequently integrated into hybrid materials like GO composites.

Raman spectroscopy reveals more information on the lattice disorder and local bonding environment. Se–Se stretching modes of trigonal Se chains are represented by distinctive vibrational bands seen for elemental Se nanoparticles in the vicinity of 235–255 cm^{-1}. While CuSe displays separate bands between 190 and 210 cm^{-1}, which are attributed to Cu–Se bond vibrations in the orthorhombic phase, ZnSe nanoparticles exhibit longitudinal optical (LO) phonon modes about 250–260 cm^{-1}, which is consistent with high crystallinity. The production of stoichiometrically well-defined chalcogenides during microbial reduction is confirmed by these vibrational modes.

The D (1345 cm^{-1}) and G (1595 cm^{-1}) bands, which stand for disordered and graphitic sp^2 carbon, respectively, dominate the Raman spectra of GO-based composites. A shift in the degree of graphitic ordering and the formation of novel electronic states at the GO–nanoparticle interface is shown by the difference in the I_D/I_G ratio before and after nanoparticle integration. For GO:Ag composites, a decreased I_D/I_G ratio reflects partial restoration of graphitic domains due to charge transfer from Ag nanoparticles. Conversely, GO:Se and GO:Cu exhibit higher D-band intensities, indicating more robust structural disorder brought on by the interfacial bonds between Se and Cu. Together, these spectral characteristics show that nanoparticles may successfully anchor onto the GO surface, improving interface conductivity and electron mobility—two critical characteristics for sensor and diode applications.

The optical transitions and energy bandgaps of the bacterially produced nanoparticles are revealed by UV-visible spectroscopy (UV-Vis). The Ag nanoparticles' significant surface plasmon resonance (SPR) band, which is centred at 430–440 nm, confirms the existence of metallic nanoparticles and their collective electron oscillations under visible light. Se nanoparticles' broad absorbance between 500 and 650 nm is a sign of their quantum-size effects and

semiconducting nature. While Zn and ZnSe show clear absorption edges around 370 nm and 460 nm, respectively, corresponding to optical bandgap values of 3.2 eV and 2.7 eV, CuSe has a red-shifted edge near 580 nm, implying a narrower bandgap around 2.1 eV.

Crucially, the biological synthesis circumstances, specifically the enzyme concentration and cell wall chemistry, which regulate nanoparticle size and defect density, have an impact on these optical behaviours. Light absorption, charge separation, and electron transfer efficiency are crucial characteristics in optoelectronic and sensing applications, and the variable bandgap energies obtained through bacterial synthesis allow for precise design. When coupled with GO or other carbon frameworks, these nanoparticles exhibit broadened absorption spectra, demonstrating synergistic **plasmonic–semiconducting coupling** effects that further enhance photoresponse. These structural and optical investigations taken together demonstrate that bacterial synthesis offers a mechanism for creating high-quality nanomaterials that is reproducible, physiologically controlled, and functionally flexible. Incorporating these materials into sustainable nanoelectronic and bio-interactive devices is made possible by the biomolecular stabilisation that is accomplished during synthesis. This stabilisation guarantees long-term dispersion stability, environmental compatibility, and the preservation of electronic and optical properties.

Table 1 lists the device-level performance, key structural characteristics, and biosynthetic origin of a number of bacterially synthesised nanomaterials developed between 2019 and 2025.The materials include elemental and hybrid systems such as Ag, Se, Zn, ZnSe, CuSe, MgSe, $AgInSe_2$, and composites based on graphene oxide.

Tablo 1. Bacterially Synthesised Nanomaterials: Structural Properties, and Device-Level Functionality (2019–2025).

Material	Source	Structural Features	Functional Behavior	Φ_B / Eg / Sens.	Application	Ref.
Ag, Se, and Zn NPs	*Bacillus sp.* and *Pseudomonas aeruginosa* enzymatic reduction via nitrate reductase and NADH-dependent pathways	Mixed FCC (Ag), hexagonal (Se), and HCP (Zn) phases; 20–50 nm	Antimicrobial, antibiofilm, antiurease; plasmonic and photoconductive	Eg(Se) ≈ 2.0–2.2 eV	Biomedical coatings; antibacterial, photoconductive layers	Gurkok et al. (2025)
ZnSe and CuSe NPs	Bacterial co-reduction and selenization of Zn^{2+}/SeO_3^{2-} and Cu^{2+} ions	Zinc blende (ZnSe), orthorhombic (CuSe); 2.1–2.7 eV Eg	Photoactive, p-type; strong H_2S response	ΦB ≈ 0.75 eV / Sens. ≈ 35–40 % @ 100 ppm H_2S	UV/gas sensors; thermoelectric, optoelectronic	Çakıcı et al. (2019); Gurkok et al. (2025)
MgSe NPs	Bacterial reduction of Mg^{2+} and SeO_3^{2-} (aerobic)	Polycrystalline; uniform nanograins	Stable I–V; photoresponse under illumination	ΦB ≈ 0.68 eV	Optoelectronic diodes; heterojunctions	Çakıcı et al. (2023); Çakıcı & Özdal, (2023)
$AgInSe_2$ NPs	Multimetallic reduction of Ag^+, In^{3+}, SeO_3^{2-}	Chalcopyrite-type; spherical	Negative capacitance; dielectric enhancement	—	High-dielectric Schottky diodes	Çakıcı et al. (2023)
GO:Ag	GO matrix with Ag^0 NPs (bacterial route)	rGO sheets with Ag clusters	Enhanced rectification; improved C–V; NO_2 sensing	ΦB ≈ 0.72 eV / Resp. ≈ 50 % @ 5 ppm NO_2	Multifunctional diode + gas sensor	Çakıcı Can et al. (2025)
GO:Se	GO decorated with Se clusters	High ID/IG; hydrophilic surface	VOC-sensitive; resistance modulation	Resp. ≈ 40 % @ 50 ppm EtOH	Chemical gas sensors; flexible devices	Çakıcı, (2019); Çakıcı et al. (2019)
GO:Cu	GO matrix reduced with Cu^{2+} ions	Smooth thin film; Cu–C interface	High conductivity; redox-active	—	Electrochemical sensors; energy devices	Çakıcı (2020)

Each of these systems exhibits distinct optoelectronic, electrical, and crystalline morphologies.

The findings presented here demonstrate the close connection between the characteristics of the resultant nanostructure

and the bacterial reduction pathways. The sensitivity (Sens.), optical bandgap (Eg), and barrier height (Φ_B) measurements show how biologically mediated synthesis allows for adjustable semiconducting and sensing capabilities. From optoelectronic, dielectric, and electrochemical device applications to biological coatings and antimicrobial surfaces, these nanomaterials have multifunctional promise.

Discussion and Future Perspectives

The systematic development of bacterially synthesized nanomaterials between 2019 and 2025 has clearly demonstrated that biological systems can act as efficient, controllable, and sustainable nanofactories. According to the findings reported in Table 1, the enzymatic pathways engaged in metal or chalcogen ion reduction and the biological makeup of the microorganisms directly influence the physicochemical characteristics of these nanostructures.In particular, the formation of unique crystalline phases, including the hexagonal selenium, face-centered cubic silver, and hexagonal close-packed zinc structures, each with well-defined size distributions and stability profiles, has been made possible by the selective reduction mediated by *Bacillus sp.* and *Pseudomonas aeruginosa.*

The first evidence that bacteria may reduce mixed metal and chalcogen precursors to generate binary and ternary compound nanoparticles (ZnSe, CuSe, MgSe, and $AgInSe_2$) is a significant contribution of the current research series. These findings provide compelling evidence that microbial enzymatic systems possess sufficient reducing and coordinating capacity to drive not only elemental metal reduction but also **complex compound formation under mild biological conditions**. The produced compound nanoparticles were effectively converted into superior **thin films, which were then included into Schottky diodes and**

heterojunctions, two components of electronic circuits. According to structural and morphological investigations, these biogenic thin films had outstanding adherence to semiconductor substrates, low defect density, and amazing surface uniformity—qualities that were on par with or excellent compared to those attained by traditional physical deposition methods. Thus, complex nanostructures with high structure–property correlations have been successfully fabricated by combining biogenic reduction methods with multimetallic precursors.

The zinc blende and orthorhombic phases of ZnSe and CuSe provided tailored semiconducting behavior, while MgSe and $AgInSe_2$ compounds demonstrated stable rectifying behavior and enhanced dielectric performance. Such outcomes confirm that biological synthesis offers not only an eco-friendly alternative to chemical or physical methods but also precise control over crystallinity and interface quality, which are crucial for device integration.

Another significant trend observed across these studies is the transition from elemental nanoparticles to **hybrid graphene oxide–based systems (GO:Ag, GO:Se, and GO:Cu)**. These hybrids combine the biocompatibility of bacterial synthesis with the high electrical conductivity and large surface area of graphene derivatives. The resulting materials display multifunctionality: GO:Ag enhances rectification and charge transport in Schottky junctions, GO:Se exhibits pronounced VOC sensitivity, and GO:Cu provides a stable electrochemical platform for energy and sensing devices. This progression toward hybrid biogenic nanomaterials reflects a broader movement in nanoscience—from simple biological synthesis to **biologically engineered functional interfaces**, bridging biology and electronics.

From a materials perspective, bacterial synthesis has proved capable of tuning **barrier height (Φ_B)** and **optical bandgap (E_g)** values within ranges comparable to those achieved by high-temperature physical techniques. The observed barrier heights between 0.68 and 0.75 eV and bandgap energies from 2.0 to 2.7 eV indicate that bacterial routes can deliver semiconducting quality sufficient for electronic and optoelectronic applications. Moreover, the consistent enhancement in current–voltage (I–V) and capacitance–voltage (C–V) characteristics observed in bacterially derived diodes underlines the effectiveness of the biological approach in minimizing interfacial defects and promoting uniform metal–semiconductor contact formation.

In practical terms, these findings establish a strong foundation for the utilization of biogenic nanomaterials in **biosensors, photodetectors, energy harvesters, and low-cost electronic components**. The antibacterial and antibiofilm properties of silver- and selenium-based nanoparticles further expand their usability to biomedical and environmental technologies. The ability to achieve such multifunctionality under mild conditions—without toxic reagents or high energy consumption—makes bacterial synthesis a pivotal step toward the realization of circular, low-carbon nanomanufacturing.

Looking forward, the next decade of research is expected to move beyond proof-of-concept demonstrations toward **scalable, genetically optimized microbial production systems**. Advances in synthetic biology and metabolic pathway engineering will allow selective tuning of nanoparticle composition, shape, and size through the manipulation of specific enzymatic expressions. Furthermore, coupling bacterial synthesis with microfluidic and real-time monitoring platforms could enable continuous, reproducible nanoparticle production for industrial use.

In conclusion, the collective results between 2019 and 2025 highlight an important scientific milestone: for the first time, binary and ternary compound nanoparticles were biologically synthesized and transformed into functional thin films exhibiting excellent structural quality and electronic performance. This achievement positions bacterial synthesis not only as a sustainable alternative but also as a technologically viable route for producing next-generation green nanoelectronic materials and biosensing interfaces, where biological precision and material functionality coexist in harmony.

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BIOFILM-FORMING BIOFERTILIZERS AS A PROMISING ALTERNATIVE TO CHEMICAL FERTILIZERS

SEDAT ÇAM[1]

Introduction

Soil infertility is one of the most notable constraints in enhancing agricultural productivity (Jote, 2023). Natural soils are poor and inefficient in nutrients (Jote, 2023) since their organic matter concentration is between 0.8 and 2.0% (Davey & O'Toole, 2000). Limitation in soil organic matter adversely affects soil microbes that drive nutrient cycling and decrease the availability of critical nutrients to plants (Oldfield et al., 2018). Nutritionally poor soils require the utilization of chemical fertilizers to increase plant growth and crop productivity. Therefore, today's agriculture still mostly depends on the overuse of agricultural chemicals (Hossain et al., 2022). In addition, to meet the escalating demands for crop production due to the rapidly increasing world population, the use of such chemicals will be more important than ever before, but they have hazardous impacts on soil properties and health, the utility of

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groundwater, and human health (Hossain et al., 2022). Thus, there is a need for an ecologically safe application to replace the use of agricultural chemicals.

Plants are colonized by a wide range of organisms such as protozoa, fungi, bacteria, and algae but 95% of microorganisms are bacteria (Timmusk et al., 2017). Bacteria residing inside or on plant surfaces can exert beneficial impacts on plant growth and development. Plants are involved in very complex interactions with such plant-associated microbial communities, grouped as biofertilizers, that are capable of supporting the growth and nutrition of host plants by providing necessary macro- and/or micro-nutrients to plants through direct and/or indirect mechanisms (Timmusk et al., 2017; Çam, 2024a; Çam et al., 2024). PGP bacteria can do so through various ways, alone or in combination, such as nitrogen fixation, nutrient solubilization, the production of siderophore and hydrolytic enzymes, exopolysaccharide (EPS) synthesis, and biofilm formation (Basu et al., 2021; Santoyo et al., 2021; Çam et al., 2022, 2023). Their use reduces reliance on the chemicals. In a recent study, application of PGP bacteria reduced the excessive utilization of nitrogen fertilizers by ~30% (Geries & Elsadany, 2021). Therefore, the integration of plant and beneficial microbe interactions appears to be a promising solution to enhance crop production in an eco-friendly manner (Timmusk et al., 2017).

Agricultural soils represent a heterogeneous environment in which various environmental factors, including nutrient limitation, play a crucial role in microbial growth and survival (Davey & O'Toole, 2000). The use of agricultural chemicals also has negative impacts on the diversity of soil microbial communities that fuel nutrient cycling in the soils (Hossain et al., 2022). PGP microorganisms must obtain enough nutrients from their surroundings to proliferate in soils, compete with other communities, and colonize on plant surfaces (Santoyo et al., 2021). Therefore, soil

organisms need to constantly combat nutrient deficiencies (Davey & O'Toole, 2000) and other adverse environmental parameters (Çam, 2024b). To contend with such conditions, soil microorganisms prefer to establish a biofilm lifestyle rather than their free-living forms (Çam, 2021). Biofilm formation is very common among soil microbial communities (Wu et al., 2019) because biofilm mode of growth extends the survival and existence of soil microbes in the rhizosphere (Çam, 2023), provides rapid microbial responses and adaptation to fluctuating external stress factors, inhibits pathogen attacks, and contributes to nutrient cycling (Singh et al., 2019). Such benefits offer ecological advantages to biofilm-forming organisms, thus biofilm formation has been recognized as an effective strategy for the survival of microorganisms in nature (Çam & Brinkmeyer, 2020a).

Biofilms produced by agriculturally important microbes are very important for crop production and improvement and have gained more attention in agriculture recently (Velmourougane et al., 2017). The positive impacts of biofilm formation on the alleviation of abiotic stress factors in plants have been well-documented in literature (Morcillo & Manzanera, 2021; Çam, 2024b). Plant-associated biofilms substantially increase plant tolerance to salinity (Çam, 2022a), drought (Çam, 2023), and heavy metal stresses (Çam, 2022b). Biofilm formation also enhances the biocontrol activity of PGP microorganisms against phytopathogen-induced diseases (Çam, 2022c). Biofilm-forming microbial-based fertilizers are also becoming popular among farmers in recent years to favor crop yield (Rathnathilaka et al., 2023) but their beneficial effects on plant nutrition status have not been well-clarified. Therefore, in this chapter, the advantageous roles of biofilm formation on both biofilm-forming PGP microorganisms and their contribution to soil fertility and plant nutrition are discussed.

Degradation of global agricultural lands

Climate is among the most important global concerns in altering or reshaping environments, so changes in the climate over the last century have dramatically increased desertification and nutrient-limited soils around the globe (Arora, 2019). In addition to climate change, human activities, including the use of chemical fertilizers, also deteriorate the fertility of arable lands (Gupta, 2019). Land degradation was a major problem in the past century and will still be a global issue in the 21st century (Utuk & Daniel, 2015). According to Arora (2019), a quarter of world's land area has been degraded, which continues at a very high rate day by day, influencing the lives of billions of people globally, and eventually leading to a major global threat. It was estimated that about 37 million km^2 (>25%) of the world's land area is affected by land degradation, decreasing biological productivity because of physical and chemical changes in soils (Webb et al., 2017).

The occurrence of infertile lands as a consequence of land degradation also results in significant economic losses and social problems (Arora, 2019). Extreme conditions create barren lands by causing nutrient immobilization and salinization in soil (Arora, 2019), therefore posing serious risks to food security worldwide (Utuk & Daniel, 2015). If climate changes continue at the current rate, there will be substantial decreases in the yields of crops such as wheat, maize, and rice in the near future (Arora, 2019). Moreover, it has been proposed that world population will reach about 10 billion by 2050, so current crop production will not meet the growing needs of the populations (Arora, 2019; Hossain et al., 2022). A study conducted in 2017 estimated that agricultural production needs to considerably increase by approximately 2.4×10^9 tonnes per year to meet the growing human population by 2025 (Timmusk et al., 2017). In many countries, even though considerable attention was taken for the production of adequate food, the growth rate of crop production

is still much less than that of the human population (Utuk & Daniel, 2015). It appears that the loss of soil fertility and rising demands for food production necessitate the utilization of more agricultural lands, thus plant cultivation might expand to nutrient-deficient barren soils to ensure global food security (Çam, 2024a).

Disadvantages of chemical fertilizers

Soil deterioration is one of the most important problems associated with chemical-intensive farming practices because agrochemicals increase the acidity of soils, which limits phosphate uptake and accumulates toxic ion concentrations in soils, eventually interfering with plant development and crop growth (Bishnoi, 2018; Jote, 2023). A recent study states that the extensive use of chemical fertilizers deteriorates biological and physico-chemical structure of arable soils, decreasing global productivity in agriculture in the last decades (Basu et al., 2021). Application of nitrogen in large amounts may destroy nutrient balance over time, damaging topsoil, and reducing crop yield (Jote, 2023). The utilization of agricultural chemicals resulted in the loss of, on average, up to 2% organic matter of top 30 cm of soil, washing away 24 billion tonnes of fertile soil every year, and posing a serious threat to humanity (Bishnoi, 2018). Synthetic fertilizers also reduce soil fertility and productivity by destroying plant-associated beneficial soil organisms, including bacteria (Bishnoi, 2018).

Nitrogenous fertilizers cause detrimental effects on water quality, ecosystem, and biodiversity (Yang & Fang, 2015). The nitrogen in nitrogenous fertilizers breaks down into toxic nitrates that reach the water supplies and consequently cause water pollution by enhancing their levels in the groundwater (Bishnoi, 2018). Evidence in a recent study show that nitrate levels have increased in agricultural areas compared to Federal drinking-water standards (Dubrovsky et al., 2010). As in the case of many developed

countries, the main source of water pollution in China, the world's biggest consumer of artificial agrochemicals, was attributed to agricultural runoff because of extreme use of chemical fertilizers to favor plant productivity (Yang & Fang, 2015). Nitrates and phosphorus in the fertilizers can also flow into lakes and oceans, subsequently leading to the death of aquatic organisms (Bishnoi, 2018).

Artificial chemicals are persistently present in the natural environment, degrading the utilization of groundwater; therefore, humans, subjected to agrochemical-contaminated water for a long period, have various health hazards such as cancer, hormone disruption, reproductive abnormalities (Hossain et al., 2022), and other health problems (Jote, 2023). Nitrates in the drinking water can be accumulated in salivary glands of humans; as a result, the mouth reduced nitrate into nitrite in an anaerobic environment that causes several disorders in human body (Jote, 2023). Soil and water are of utmost importance for all the forms of life on our planet, thus significant measures have to be taken to solve the problems associated with environmental and economic concerns raised with the interminable use of synthetic agricultural chemicals (Bishnoi, 2018).

The roles of biofertilizers on plant growth

Beneficial microbial communities in the agricultural soils are naturally interacting with their host plants, improving plant growth and development without the side effects on the environment, humans, and animals (Santoyo et al., 2021). For a long time, biofertilizer treatments have been applied to agriculture under *in vivo* and *in vitro* conditions as an effective alternative to the adverse impacts of synthetic chemical fertilizers to improve plant growth and yield in an environmentally friendly manner (Basu et al., 2021; Çam et al., 2024). Beneficial soil organisms can contribute to soil fertility

by making nutritionally important nutrients available for plants through biological processes, creating a suitable rhizosphere environment for not only plant growth but also their survival (Vejan et al., 2016). For example, nitrogen is the most important element for plant growth and development (Aasfar et al., 2021) but the most-limiting macronutrient in agricultural soils (Vejan et al., 2016). However, PGP organisms such as *Azotobacter*, *Rhizobium*, and *Azospirillum* can fix atmospheric nitrogen into organic forms which can be acquired by plants (Aasfar et al., 2021). Following nitrogen, phosphorus (P) is the most essential nutrient for plant growth and metabolism (Rawat et al., 2021). P levels are abundant in soils in organic/inorganic forms, but it is unavailable for plants because of its complexation with metals in the soils (Rawat et al., 2021). However, some PGP rhizobacteria, called phosphate-solubilizing bacteria, can increase soil P levels by solubilizing insoluble phosphates into available forms for plant uptake (Rawat et al., 2021).

Biofertilizers are considered an important aspect of organic farming and a main player for global agricultural production, therefore some PGP species such as *Bacillus*, *Azotobacter, Pseudomonas,* and *Azospirillum* are commercialized (Vejan et al., 2016). Commercial application of microbial inoculants was initiated many years ago and has increased for the last decades due to their positive effects on soil biology and fertility. The market data in 2017 indicated that nitrogen-fixing and phosphate-solubilizing biofertilizers dominate the global markets with a share of ~ 80% and 14%, respectively (Basu et al., 2021). However, global biofertilizer markets still represent only a small fraction (~5%) of the total artificial chemical fertilizers (Timmusk et al., 2017). This was attributed to the inconsistency of inoculated PGP microorganisms under natural conditions (Vejan et al., 2016). They suggested that a successful use of PGP organisms depends on their survivability in soils, compatibility with the crops, interactions with indigenous soil

communities and environmental parameters. Climate change and ever-increasing world population necessitate enhanced crop production, thus commercial use of microbial-based products needs to be intensified for eco-friendly sustainable agricultural production (Timmusk et al., 2017).

The impact of environmental factors on biofertilizers

Application of effective biofertilizers selected under greenhouse conditions has some drawbacks in terms of their survival, colonization, and efficacy in the field. Inoculation of microbial species to plants may work well under laboratory conditions but they often fail to exert their expected potential on plant growth under complex field conditions (Basu et al., 2021). Environmental parameters influence the ability of microbial survival and colonization under natural conditions, thus PGP microorganisms may not consistently perform their potential in the field (Çam, 2024b). Plants affect the existence and persistence of microbial communities nearby the plant roots via root exudation (Timmusk et al., 2017). Plant root exudates are very rich in nutrients such as amino acids, sugars, fatty acids, organic acids, nucleotides, phenolic compounds, and vitamins (Santoyo et al., 2021), and their chemical composition varies depending on the health, fitness and genotype of plants (Timmusk et al., 2017). Such nutrients exuded by the plant roots allow the soil microbial communities to successfully colonize the host plants by acting as chemoattractants (Santoyo et al., 2021). Root exudates create a highly favorable rhizosphere environment, which requires high competition for the secreted nutrients between the inhabiting microorganisms (Santoyo et al., 2021). Potential PGP strains need to replace the native inefficient microbial communities in the rhizosphere and should not exhibit antagonistic activity against other beneficial microbiota (Basu et al., 2021). Therefore, a rational approach needs to be developed for the successful delivery of promising PGP organisms to the field (Timmusk et al., 2017).

In addition to root exudates, a great variety of environmental factors play important roles in the proliferation of plant-associated microbes in the vicinity of plants under the highly fluctuating environmental conditions such as salinity, drought, metal ion toxicity, and nutrient deprivation (Çam, 2024b). Such conditions may adversely influence the ability of PGP organisms to survive and colonize in the stressful field environment, thus hampering plant growth and production (Santoyo et al., 2021). Survival and colonization of PGP microorganisms around or inside the host plants are the first step to exert their beneficial effects on plant growth (Çam, 2022c; Çam, 2024b). Efficiency of bioinoculants can be reduced by their low survival and colonizing capacity in the rhizosphere (Santoyo et al., 2021). Microbial survival and colonization on the plants under complex field environments seem to be vital for successful plant-microbe interactions (Çam, 2022c). Further, environmental parameters such as salt affect plant growth-promoting activities (Çam & Küçük, 2020). An ideal PGP organism should be effective in plant growth promotion, be compatible with other soil communities, be safe for the environment, be highly competent, and be tolerant to environmental factors (Vejan et al., 2016). Thus, such factors should be taken into account during the selection of potential PGP inoculants for better performance under field conditions.

The importance of biofilm formation for microorganisms

Microorganisms produce biofilms by aggregating themselves in a self-produced extracellular matrix attached to living or non-living surfaces, especially under unfavorable conditions (Çam, 2021). Microbial cells have a tendency to live as groups in biofilms in nature (Çam, 2023) because biofilms offer a reproductive and fitness advantage to biofilm organisms (Velmourougane et al., 2017). The gene expression levels of biofilm-producing cells can remarkably change during transition from free-living to sessile

lifestyle, even under different environmental conditions and at the different steps of biofilm development (Çam & Brinkmeyer, 2020b). Such alterations in gene expressions make phenotypically and metabolically distinct cells in biofilms (Velmourougane et al., 2017). The changes in phenotypic and metabolic activities during biofilm formation may increase microbial survival in stressful environments (Çam, 2023). Biofilm mode of life protects microbial cells from a great variety of factors such as nutrient deprivation, pH changes, osmotic stress, disinfectants, antibiotics, oxygen radicals, ultraviolet radiation, desiccation, and predations (Çam & Bicek, 2023; Çam & Badıllı, 2024). Protection against the environmental parameters makes biofilm-forming cells less vulnerable compared to their planktonic homologues, therefore microbial communities switch from free-living forms to biofilm formation to sustain their survival and persistence under stringent natural conditions (Çam, 2022b).

The advantageous roles of biofilms lie in the composition and functions of the self-produced biofilm matrix. The extracellular polymeric substance in the matrix is composed of mainly exopolysaccharides, nucleic acids, proteins and lipids (Flemming & Wingender, 2010). Such components provide several advantages to biofilm-forming cells, such as 1) increased colonization by facilitating the adhesin and long-term attachment of microbial cells to biotic/abiotic surfaces; 2) aggregation of microbial cells in close proximity, so allowing bridging between biofilm organisms for communications; 3) increasing cohesion of biofilms, therefore mediating the mechanical stability and determining biofilm architecture; 4) enhancing the retention of water by maintaining a highly hydrated micro-environment around the cells; 5) acting as a protective barrier, thus conferring resistance to various antimicrobial compounds; 6) allowing the exchange of genetic information between biofilm-forming organisms; 7) providing a sink for excess energy; and 8) functioning as an electron donor or acceptor,

permitting redox activities in the matrix (Flemming & Wingender, 2010). Apart from such functions, the biofilm matrix also accumulates the organic and inorganic nutrients from the environment, enabling the digestion of macromolecules through enzymatic activities, and providing a source of energy and nutrient for the biofilm-forming organisms (Flemming & Wingender, 2010; Çam, 2024b). For those reasons, microorganisms tend to form biofilms in nature for better adaptation and survival under unfavorable conditions.

Microorganisms can form single- or mixed-species biofilms under natural conditions (Velmourougane et al., 2017). Recently, scientists have focused on multispecies biofilms because they offer more advantages over single-species biofilms or free-living counterparts, such as maintaining ecological balance in soils, enhanced resistance to antimicrobial compounds, increased protection against desiccation and predators, facilitating horizontal gene transfers, and forming a higher amount of biomass because of synergism between biofilm organisms (Velmourougane et al., 2017). Mixed-species biofilms produce new types of exopolysaccharides with different compositions in the biofilm matrix in comparison with single-species ones, illustrating the interactive and synergistic interactions between the partners in the biofilms; such interactions have great effects on the physiology and metabolisms of biofilm cells (Velmourougane et al., 2017). In multispecies biofilms, the metabolites produced by one kind of species in the biofilm communities are used by the other species, thus enhancing their resistance to adverse conditions (Velmourougane et al., 2017).

In the past, when applying PGP microorganisms to plants, their biofilm-forming potential had been ignored but recent studies have also focused on the biofilm-producing capacity of PGP inoculants (Morcillo & Manzanera, 2021). However, it is not clear whether the biofilm-forming PGP bacteria, selected in the laboratory

based on their growth promotion, will be able to reside and form biofilms in natural soils. It was proposed that more than 99% of bacterial cells live in the biofilm matrix in natural environments (Wu et al., 2019) but this ratio is very high compared to the results of the *in vitro* biofilm formation by some rhizobacteria under laboratory conditions in recent studies (Çam et al., 2023; Çam & Bicek, 2023). The *in vitro* conditions when growing biofilms are generally rich in nutrition, therefore laboratory results may not reflect the nutritionally poor field conditions. As we know, biofilm formation is triggered under stressful conditions (Çam & Badıllı, 2024). Those studies also found that not all bacterial strains tend to make biofilms, and their biofilm-producing degree considerably varies based on the genetic and environmental factors. A great number of factors affect biofilm formation such as nutrients, temperature, pH, oxygen, osmolarity, biocides, antimicrobials, chemicals, metals, enzymes, plant volatiles, mechanical and host-derived signals, quorum-sensing, and other genetic factors (Velmourougane et al., 2017). When considering all the factors above, during the application of biofilm-formulated bioinoculants to the field, biofilm formation-affecting factors are of utmost importance to largely mimic the field conditions.

Biofilmed biofertilizers on soil fertility and plant nutrition

In addition to the advantageous roles of biofilm formation on the survival and colonization of PGP bacteria, it also contributes to crop growth and productivity by augmenting the nutrient availability in soils. Plant-microbe interactions are essential for plant nutrition since they drive biogeochemical cycles that facilitate nutrient balance in soils (Velmourougane et al., 2017). It was suggested that root-attached microbial biofilms play a significant role in improving soil fertility (Gupta et al., 2017). Application of biofilm-based biofertilizers decreased the recommended amounts of chemical fertilizers by ~50% (Seneviratne et al., 2009) and even restored the

agricultural soils deteriorated by the conventional practices (Seneviratne et al., 2011). Soil biofilms sustain highly active microorganisms that rapidly respond to nutrient changes compared to their planktonic counterparts, which contributes to enhanced organic turnover rate in soil (Wu et al., 2019). That study indicated that biofilm formation shows considerable impacts on the soil microbial and biogeochemical processes. Similarly, the application of biofilm fertilizers enhanced soil quality and microbial communities as well as grain yields in rice cultivation over chemical fertilizer treatment (Rathnathilaka et al., 2023). Another study with cyanobacterial biofilmed biofertilizers demonstrated an increase in soil nutrient contents and plant nutrition (Swarnalakshmi et al., 2013). Interestingly, the application of bradyrhizobial-fungal biofilmed inoculants with nitrogenase activity improved nitrogen-fixing symbiosis in soybean and contributed to soil N fertility in comparison with conventional inoculants (Jayasinghearachchi & Seneviratne, 2004). Further, *Trichoderma-Azotobacter* biofilm inoculation increased the availability of soil nutrients by up to 40%, most likely through the nutrient solubilization and immobilization into available forms (Velmourougane et al., 2019). Likewise, biofilm formation by co-inoculation of a *Bradyrhizobium japonicum* strain and *Penicillium* species enhanced soil fertility by increasing N and P mineralization and nitrogenase activity in soils (Seneviratne & Jayasinghearachchi, 2005).

Several studies have shown that plants increase their nutrient uptake with the help of biofilm-forming organisms. For instance, biofilm-producing PGP bacteria enhanced the uptake of plant nutrients (N, K, and P) in sunflower plants under salt stress (Yasmeen et al., 2020). Similarly, consortium treatment of EPS-producing rhizobacteria and salicylic acid augmented the levels of Ca, Na, Mg, and K in wheat plants under rainfed conditions (Khan & Bano, 2019). In a most recent study, inoculation of biofilm- and

EPS-producing bacterial strains promoted wheat growth by boosting the concentrations of plant micro- and macro-elements (Fe, Mn, Cu, Zn, Mg, K, and Ca) in a nutrient-limited soil (Çam, 2024a). Similar results were also obtained by the combined inoculations of different plant-associated microbes through biofilm formation (Singh et al., 2019). They observed that dual inoculation of *Trichoderma harzianum* and *Brevibacterium halotolerans* showed the highest uptake of nutrients (N, K, and P) in *Mentha arvensis* under the field conditions. Further, cyanobacteria-based biofilm inoculants increased the nutrient availability and plant growth (Prasanna et al., 2015). Likewise, cyanobacterial biofilms enriched rice crops in Cu, Mn, Zn, and Fe under field conditions (Adak et al., 2016). Such studies clearly indicate that biofilm formation capability of PGP microorganisms favors crop growth and productivity by enhancing the acquisition of plant nutrients.

How do microbial biofilms contribute to plant nutrition?

The underlying mechanism of how biofilm formation increases the nutritional status of plants most probably lies under the presence of exopolysaccharides in the biofilm matrix. EPS is an indispensable part of the matrix since EPS-defective mutant organisms cannot produce well-structured mature biofilms (Flemming & Wingender, 2010). EPS improves soil physical structure by increasing soil aggregation and the retention capacity of nutrients and water around the roots (Velmourougane et al., 2017). It is well-established that bacterial EPS stimulates soil aggregate formation in the rhizosphere (Çam, 2022a). EPS production allows cell-cell aggregation that facilitates bacterial adhesion to plant roots, which in turn is involved in the formation of soil aggregates in the vicinity of root surfaces (Morcillo & Manzanera, 2021). Inoculation of EPS-producing bacterial strain to sunflower plants significantly enhanced root-adhering soil by up to 100% and soil macropore volume as well as N uptake (Alami et al., 2000). Enhancement in

soil aggregation and porous soil structure allows for greater water retention around plant roots, therefore supporting water and nutrient uptake by the roots (Morcillo & Manzanera, 2021). Application of EPS-based bioformulation considerably improved plant growth attributes under stressful and non-stressful conditions, suggesting that such formulation increases root-adhering soil, soil texture and porosity, plant nutrient uptake, and consequently plant growth (Tewari & Arora, 2014).

In a recent study, more biofilm-producing bacterial strains inoculated with macroalgae extract were found to be significantly more effective in barley growth parameters under organic matter-limited field conditions compared to other treatments, including chemical fertilizer (Çam et al., 2024). In that study, it was suggested that the inoculating bacteria most likely converted the algal extract into forms which can be readily utilized as a nutrient source by both biofilm-producing strains and barley seedlings in the barren soil. Bacterial species serve as decomposer and recycler in agricultural soils, thus contributing to processes of nutrient cycling and energy flow (Kang et al., 2021) and driving the biogeochemical cycling that protects the biosphere (Davey & O'Toole, 2000). Microorganisms secrete several enzymes into their environments that catalyze various biochemical reactions such as breakdown of complex organic residues into simple forms, mineralization of nutrients, and transformation of soil organic compounds, thereby increasing the availability of absorbable molecules to plants (Adetunji et al., 2017). Such processes necessitate the combined efforts of microbes with different metabolic activities, so the biofilm organisms can perform those complex reactions in the biofilm matrix (Davey & O'Toole, 2000), which enhances soil productivity and plant nutrition. Further, the anionic nature of EPS layer captures the nutrients from the surroundings (Morcillo & Manzanera, 2021). Biofilm matrix allows the accumulation and digestion of exogenous macro-molecules for

nutrient acquisition (Flemming & Wingender, 2010) as well as the exchange of nutrients and metabolites in close proximity (Prasanna et al., 2014). In the study conducted by Çam et al. (2024), it was proposed that nutrients most likely obtained as a result of the degradation of algal biomass by the cycling bacteria were accumulated and digested in the biofilm matrix, which then promoted barley growth in the unproductive soil. As stated by Singh et al. (2019), biofilmed microorganisms have a good performance in nutrient cycling. It appears that biofilm formation in soils contributes to soil fertility by participating in the process of nutrient cycling.

Rhizosphere represents a stressful environment for plant growth-promoting organisms, which has an adverse impact on the existence of their planktonic forms, therefore free-living microbes must deal with environmental stresses and increase their survival in the rhizosphere in order to show their beneficial effects on plant growth (Çam, 2024a). In other words, the limitations in the colonization and survival of PGP microbes reduce their expected PGP performance, depending on their competence in stressful rhizospheric soils (Swarnalakshmi et al., 2013). According to Santoyo et al. (2021), PGP organisms must first attach to plant roots to more easily perform their beneficial roles in plants; their biofilm formation ability enhances the growth-promoting activities. For example, biofilm formation increases plant nutrient uptake and N_2-fixing ability of PGP bacterial species as well as other growth-promoting attributes (Seneviratne et al., 2010). Likewise, in a recent investigation, the enhancement in plant nutrient uptake was attributed to increased microbial survival and colonization with the help of biofilm formation on plant roots (Singh et al., 2019). A similar conclusion was also made in the results of inoculation of high-biofilm and EPS-producing rhizobacterial strains in wheat nutrition under nutrient-limited soil (Çam, 2024a). This is because biofilm production provides an appropriate environment for the

growth and survival of agriculturally important microbes in the rhizosphere soils (Swarnalakshmi et al., 2013).

Synergism among biofilm communities may also contribute to plant nutrition by increasing their PGP activities. Evaluation of biochemical attributes of *Trichoderma*-based biofilms with rhizobacteria demonstrated that biofilm formation exhibited higher plant growth-promoting values over individual/dual cultures, enhancing seed germination and biochemical traits by mediating signaling molecules (Triveni et al., 2013). They revealed that synergistic interactions between biofilm cells may make PGP inoculants a superior application. Further, in a study, biofilmed preparation with *Anabaena-Pseudomonas* species increased N and P uptake by plants, showing the relationship between N fixation and higher phosphate uptake by the roots, which was attributed to the synergism among biofilm-producing microorganisms (Swarnalakshmi et al., 2013). Likewise, a higher amount of biofilm production by co-inoculation of *Bacillus* and *Azotobacter* species was more effective in enhancing nutrient uptake in wheat plants compared to less biofilm-forming treatments and the control group under nutrient stress (Çam, 2024a), showing the importance of biofilm formation and synergism between biofilm-forming organisms. Application of a consortium of plant-associated microorganisms enhanced the uptake of plant nutrients in the field, indicating a synergistic interaction between the microorganisms as a result of biofilm formation (Singh et al., 2019). The growth promotion performance of bacterial strains may be achieved by more than one growth-promoting activity that may not be found in a single species, thus biofilm formation by more than one organism may offer a definite advantage (Triveni et al., 2013). According to Velmourougane et al. (2017), there is a growing interest in studying mixed-species biofilms due to their advantages over single-species biofilms, including synergism between biofilm-forming partners.

Compatibility among biofilm organisms enhances signaling between the cells, which contributes to synergistic effects (Prasanna et al., 2014). They observed that efficacy of mixed species-biofilmed inoculants considerably differs in terms of soil nutrient availability, microbial activity, and plant growth parameters. It may be concluded that co-inoculated microbes can increase soil nutrient levels toward the plants by combining their benefits through synergistic actions (Emmanuel & Babalola, 2020), most probably, in compatible multispecies biofilms.

Conclusions

Increasing global land degradation and human population growth require the use of more fertile lands to ensure the rising needs for food security. The utilization of chemical fertilizers can meet the growing food demands but poses significant challenges to soil and human health. The application of biofertilizers has been raised as an effective safe alternative to the utilization of agrochemicals to improve crop growth and productivity in an eco-friendly manner. Various environmental factors can adversely affect the existence and persistence of planktonic microbial inoculants under natural conditions, especially in rhizosphere soils, therefore PGP organisms may not exert their growth-promoting potential on crop quality and quantity. Biofilm production provides several advantages to biofilm-forming organisms, thus PGP microbes may prefer biofilm mode of growth over planktonic lifestyle in nature. In addition to the existence of PGP microorganisms, biofilm formation by beneficial microbial communities directly or indirectly contributes to plant growth and development by enriching soil nutrient contents and increasing the uptake of essential nutrients. Biofilm-forming PGP microbes can do so through the formation of soil aggregates around the roots, facilitating the nutrient cycling process in soils, increasing their survival and colonization in the rhizosphere, and establishing synergistic interactions in the biofilm matrix. Biofilm formation,

particularly by a group of compatible PGP organisms, appears to be superior to chemical fertilizers for improving soil fertility and plant nutrition, even in nutrient-deficient soils.

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BIOMATERIALS WITH ANTIMICROBIAL PROPERTIES AND THEIR AREAS OF APPLICATION

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Introduction

Antimicrobial resistance, the development of resistance by microorganisms to antimicrobial drugs, especially antibiotics, has become one of the greatest global health threats to modern medicine. Millions of people worldwide die from it each year, making infections increasingly difficult to treat (Prestinaci et al., 2015). In the 20th century, antibiotics made a big difference in the number of people who died from infections. However, microorganisms can quickly adapt, and the wrong and too much use of antimicrobial drugs has led to the rise of resistant pathogens. Today, antimicrobial resistance is called a silent pandemic that threatens healthcare systems in both developed and developing countries. The rapid

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spread of resistant microorganisms is caused by things like poor hygiene, not enough infection control, the use of antimicrobials in livestock, and global trade and travel (Ferri et al., 2017). The challenges in developing new antimicrobial drugs have necessitated a multidisciplinary and international approach, including global screening and monitoring, controlled drug use, sustainable food and livestock policies, and effective infection control measures (Bertagnolio et al., 2024). Antimicrobial biomaterials have become more important in recent years, especially in the healthcare field, because infections are becoming more common and people are becoming resistant to traditional antibiotics. Biomaterial-associated infections make hospital stay longer, raise the cost of treatment, and lower the chances of success (Nowotnick et al., 2024). Conventional antibiotics cannot completely prevent microorganisms from adhering to or forming biofilms on biomaterials used in wound care, implants, and prosthetics. Chronic infections highlight the importance of prioritizing biomaterials in treatment plans, especially for those caused by antibiotic-resistant fungi and bacteria. Recent advancements in contemporary biomaterials include metal ions, antimicrobial peptides, and nanostructured surfaces. When bacteria encounter these features, they are killed (Barman et al., 2024; Kasapgil et al., 2024). Biomaterials, especially those that are protein-based or carbon-based, work through key mechanisms and are effective against biofilms, helping to prevent the development of resistance by reducing antibiotic use (Lewandowski et al., 2025). Multifunctional antimicrobial coatings and slow-release systems prevent microbial adhesion and provide a safe, compatible environment for surrounding tissues (Kasapgil et al., 2024). Antimicrobial biomaterials have innovative and effective applications in wound healing, prevention of bone infections, dentistry, and surgery (Wang et al., 2025). Recent research has shown that antimicrobial biomaterials are very important for

stopping biofilm formation, lowering the risk of infection, and providing a next-generation defense against pathogens that are resistant to traditional drugs. Because of this, antimicrobial biomaterials are necessary for healthcare technologies to last in both hospitals and homes (Su et al., 2025). Biomaterials are any natural or man-made substance that interacts with biological systems and is usually used to replace, repair, or support damaged organs or tissues. These materials need to have certain important properties, like biocompatibility to avoid bad immune responses, biodegradability to safely break down after they have done their job, and bioactivity to get the tissue to respond in the way you want it to (Eldeeb et al., 2022). Biomaterials include a wide range of materials designed to closely mimic the structure and function of native tissues, with properties such as mechanical strength, chemical stability, and suitable surface characteristics that influence cell behavior and biological integration (Agrawal et al., 2023). The growing field of biomaterials is making great strides in tissue engineering and regenerative medicine by using smart or stimuli-responsive materials that can interact with their surroundings and respond to changes in pH, temperature, or biochemical signals (Eldeeb et al., 2022).

Antimicrobial Mechanisms

Antimicrobial biomaterials use a number of different ways to stop or kill microorganisms. This makes them especially important for keeping medical devices and implants from getting infections. Recent scientific reviews have put together a list of the most important antimicrobial mechanisms and their effects (Kadirvelu et al., 2024). One of the main ways that antimicrobials work is by damaging the cell wall or membrane of the microbes. Certain biomaterials interact with the lipid bilayer of bacteria, enhancing permeability, inducing leakage of cellular contents, and ultimately resulting in cell death. Graphene-based materials, for instance, have

sharp edges and strong interactions that can break through bacterial membranes and damage their integrity (Mohammed et al., 2020). Similarly, silver nanoparticles bind to bacterial membranes, destabilizing them, increasing permeability, and causing structural damage (Roy et al., 2022).

Antimicrobial agents disrupt metabolic pathways necessary for bacterial survival by interfering with essential bacterial proteins and enzymes. Nanoparticles can get inside bacteria and bind to enzymes or proteins that contain sulfur and are involved in DNA replication. This stops them from working normally. These biomaterials stop bacteria from growing and multiplying by stopping enzyme activity (Roy et al., 2022). Some antimicrobial biomaterials can get into microbial cytoplasm and interact with nucleic acids. This can cause degradation or problems with replication and transcription. Graphene materials have been demonstrated to bind to bacterial DNA/RNA, obstructing accurate replication and inducing dysfunction. This condition causes protein synthesis to stop and reproductive ability to be lost (Mohammed et al., 2020). Reactive oxygen species, like hydroxyl radicals, superoxide anions, and hydrogen peroxide, are a keyway that antimicrobials work. These reactive oxygen species (ROS) cause oxidative stress, which damages lipids, proteins, and DNA, killing bacteria. Metal-based nanoparticles, like silver and zinc oxide, greatly boost antimicrobial activity by making reactive oxygen species (ROS) when they touch bacteria (Roy et al., 2022). The physical and chemical surface properties of biomaterials are crucial for antimicrobial activity. Surface roughness affects bacterial adhesion; certain rough or nanostructured surfaces mechanically disrupt bacteria or inhibit biofilm formation (Kasapgil et al., 2024). Since many bacterial membranes are negatively charged, surface charge is important, and positively charged biomaterials can attract and disrupt microbial cells (Sam et al., 2023). Additionally, hydrophilic or hydrophobic

surface structures influence bacterial colonization patterns, with some hydrophilic coatings reducing bacterial adhesion (Wang et al., 2025).

Antimicrobial Biomaterials

Biopolymers functionalized with natural biomaterials (chitosan, cellulose, alginate, gelatin, collagen) and plant extracts or essential oils enable versatile and innovative applications using the latest approaches in the biomedical, food, and environmental sectors (Manivannan et al., 2024). Chitosan, due to its high biocompatibility, antimicrobial and biodegradable properties, is used in tissue engineering, wound-healing hydrogels and drug delivery systems. Recently, 3D celled scaffolds and controllable drug delivery systems have been developed by combining biopolymers such as chitosan, alginate, gelatin and cellulose (Zöller et al., 2025). Cellulose of bacterial or plant origin is used in innovative medical applications (e.g., cardiac, cartilage, and wound scaffolds); its biomedical properties are enhanced with materials such as its derivatives, carboxymethylcellulose and cellulose acetate (Brovold et al., 2018). Alginate, derived from seaweed, is commonly used in cell capsules, wound dressings, and tissue engineering scaffolds. It is notable for its high water-holding capacity, gel-forming properties, and cell-friendly structure (Manivannan et al., 2024). Biomaterials made from proteins, especially collagen, are very important for technologies that help repair soft tissue, bones, and skin because they look like the natural cell matrix. Gelatin is used to make drug delivery systems, hydrogels, and biodegradable films. It is also possible to make hybrid scaffolds that are made of chitosan and collagen (Amal & Rajasree, 2025). Plant extracts and essential oils (e.g., ginger, thymol, thyme, tea tree, and sage extracts) are incorporated into biopolymer matrices (e.g., cellulose, chitosan, and gelatin) to confer antimicrobial, antioxidant, and functional properties. These extracts are widely used in biopolymer-based

edible films and coatings, delivering effective results in food safety and microbial protection of medical products. Essential oils enable controlled release and long-term activity in biopolymers, either directly or via nanoencapsulation. Components such as phenolics, terpenes, and aldehydes contribute to the chemical stability and antimicrobial activity of biofilms (Özbek et al., 2025; Tomić et al., 2023). Natural biopolymers provide bioactive surfaces for developing biomimetic and immunomodulatory tissue scaffolds and optimize biological processes such as cellular proliferation and adhesion. Smart materials, in situ engineering, and hybrid natural-synthetic systems are significantly improving the clinical efficacy of biomaterials such as collagen, chitosan, alginate, and cellulose. Nanotechnological or chemical alterations are still being used to fix problems with naturally generated biomaterials, such as differences in quality from batch to batch, mechanical weakness, and functional instability (Brovold et al., 2018; Fan et al., 2022).

Synthetic biomaterials, particularly polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactone (PCL), are biodegradable polymers widely used in biomedical applications such as tissue engineering, implants, drug delivery systems, and surgery. Additionally, polymers modified with antimicrobial agents are rapidly being developed to reduce the risk of infection and increase biocompatibility and functionality (Prestinaci et al., 2015). Polylactic Acid (PLA) is produced from renewable plant sources such as maize starch. It is both biodegradable and biocompatible. PLA has a strong yet somewhat brittle structure, with a tensile strength of 50–70 MPa and an elastic modulus of 3.5–4 GPa. PLA is widely used in 3D tissue scaffolds, dissolvable surgical sutures, and implants. Its thermal stability depends on molecular weight; longer-chain PLA is stronger and performs better. Polyglycolic Acid (PGA) is a strong gas barrier that is durable and degrades rapidly. Its main uses are temporary implants and surgical suture materials. PGA is

extremely tough and long-lasting due to its highly crystalline structure, but it is also very brittle. When blended with PLA and PCL, its mechanical properties improve, costs are reduced, and degradation becomes easier to control. Polycaprolactone (PCL) is a semi-crystalline, biodegradable polyester with a low melting point (around 60°C) and high flexibility. It degrades slowly and is commonly used in drug delivery systems for sustained release, soft tissue scaffolds, and orthopaedical implants. When combined with other polymers, their mechanical and biological properties are enhanced. These polymers are often customized, and their functionality improved by blending with other materials and through modifications such as melt blending, nanoparticle addition, and cross-linking. (Prestinaci et al., 2015; Grande-Tovar et al., 2023). Synthetic biopolymers are functionalized with antimicrobial agents (e.g., silver, zinc oxide nanoparticles, or essential oils) to produce biocompatible medical products with a low risk of infection (Grande-Tovar et al., 2023). PLA/PCL biopolymers modified with zinc oxide nanoparticles and tea tree essential oil are used in wound dressings, suture materials, tissue blocks, and implants due to their mechanical strength and antibacterial activity. This combination provides effective protection against common pathogens such as Staphylococcus aureus and Escherichia coli. These modifications are achieved by adding antimicrobial additives to the polymer matrix, either physically or chemically, to prevent microorganism colonization and minimize the risk of inflammation (Grande-Tovar et al., 2023).

Nanostructured biomaterials are transforming medicine and biomedicine due to their large surface area and multifunctional capabilities. Nanoparticles composed of metals and metal oxides, such as silver nanoparticles, copper, zinc oxide, and titanium dioxide; carbon-based nanomaterials, including carbon nanotubes and graphene oxide; and nanocarrier systems loaded with

antimicrobial peptides are increasingly used in areas such as infection control, tissue engineering, and targeted drug delivery (Li et al., 2022). Silver nanoparticles (AgNPs) are used in wound dressings, implant coatings, and surgical equipment due to their potent antimicrobial properties. They disrupt bacterial cell walls and generate reactive oxygen species. They offer promising solutions against bacterial resistance in clinical settings (Yin et al., 2020). Copper, zinc oxide (ZnO), and titanium dioxide (TiO_2) not only reduce the harmful effects of fossil fuels but also offer antibacterial, antiviral, and UV-protective properties, leading to their use in medical coatings and biosensors. ZnO and TiO_2 nanoparticles are also effective in managing oxidative stress (Habib et al., 2023). Because they are electrically conductive, strong, and biocompatible, carbon nanotubes (CNTs) are the best choice for tissue engineering scaffolds and portable biosensors. They are also utilized in nano scavenger systems to deliver drugs and genes. Graphene oxide has a huge surface area and a surface that can be made functional, which makes it useful for biosensors, targeted drug delivery, and tissue engineering. It has antibacterial properties because it breaks down bacterial membranes (Shin et al., 2016). Antimicrobial peptide-loaded nanocarrier systems enable specific targeting of introduced peptides to pathogenic cells, increasing efficacy and minimizing side effects. Peptide-loaded nano formulations are being developed as promising therapeutics, particularly to address antibacterial resistance (de Oliveira et al., 2024). Nanocarrier technology provides controlled, target-specific, and biodegradable carriers, enabling therapeutic dose optimization and high local tissue concentrations. Nanostructured biomaterials offer significant advances in preventing hospital-acquired infections, accelerating wound healing, and reducing toxicity and side effects (Fadaka et al., 2021). Implants coated with silver and metal oxide nanoparticles extend implant life and reduce inflammation due to their

antibacterial properties. Carbon nanotubes and graphene-based nanomaterials enable highly functional systems in tissue engineering and biosensors (Haugen et al., 2022).

Hybrid and composite biomaterials combine the advantageous characteristics of different material categories to provide enhanced mechanical strength, biocompatibility, and functionality in biomedical applications. Orthopaedical implants and bone healing employ polymer matrices reinforced with metal particles or nanoparticles. For example, poly(ε-caprolactone) (PCL)-based composites are reinforced with inorganic fillers such as bioglass and demineralized bone matrix (DBM) to enhance both mechanical support and tissue regeneration (Copur et al., 2025). Ceramic powders (hydroxyapatite, calcium sulfate, etc.) are added to the biopolymer matrix to enhance mechanical properties and biocompatibility. These combinations are frequently used, particularly in bone and dental tissue engineering. Ceramic–biopolymer scaffolds produced using 3D printing technologies provide an ideal environment for both stability and cellular activity in the biological environment (Gharibshahian et al., 2023). Hydrogels are preferred for wound dressings, tissue engineering, and controlled drug release because of their high-water retention capacity and tissue-like mechanical properties. Polymeric hydrogels, used alone or in hybrid structures with additives such as ceramics and nanoparticles, have been employed to enhance mechanical strength and biological functions. The use of natural polysaccharides such as hyaluronan imparts cell-enhancing properties to hydrogels, enabling success in tissue regeneration applications (Cui et al., 2022). Hybrid composites accelerate bone repair by providing the necessary mechanical strength and bioactivity. Biomaterial design employs cross-linking, in situ polymerization, and nano reinforcements. Combinations of natural and synthetic polymers

with various ceramics are important for personalized implant and tissue engineering solutions (Tian et al., 2025).

Conclusion

In the future, smart and innovative antimicrobial biomaterial designs will enable the development of new products that contribute to public health through more effective management of infection risks and innovative solution models. Antimicrobial biomaterials are becoming increasingly important in environments and industrial applications where infection risk is high. Various classes, such as metal and metal oxide nanoparticles, carbon-based nanostructures, and antimicrobial peptide-loaded systems, offer critical advantages, including preventing infection, inhibiting biofilm formation, and accelerating tissue healing. With a wide range of applications in medicine and healthcare, from implant coatings and wound dressings to surgical materials and catheters, these biomaterials are effectively used to improve treatment success and reduce hospital-acquired infections. Similarly, the use of antimicrobial biomaterials in food packaging and water treatment systems plays a crucial role in extending shelf life and ensuring microbiological safety.

Thus, antimicrobial biomaterials are advanced solutions interfacing with a safety-biocompatibility-efficiency chain, used in clinical as well as industrial settings. Due to developments in materials science and nanotechnology, antimicrobial biomaterial designs are increasingly becoming sophisticated as well as functional. This progress is expected to benefit human health and environmental conditions over the coming years.

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THE UTILIZATION OF GENETICALLY MODIFIED WHEAT IN AGRICULTURE

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Introduction

Wheat is a crucial staple food worldwide, providing a significant portion of individuals' daily calories and protein requirements. The production of wheat is under threat due to the rapid increase in population, changes in eating habits, climate change, soil degradation, and depletion of water resources (Menz et al., 2020; Bapela et al., 2022). Using modern molecular strategies like genetic modification and genome editing in wheat plants is required due to this situation. Bread wheat (*Triticum aestivum* L.) possesses a genome that is roughly 17 gigabases in size, with repetitive sequences and multiple genes accounting for the majority of it. Classical breeding methods are slow to progress and targeted

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genetic modifications are technically challenging due to this structure. Agronomically critical traits such as drought tolerance, disease resistance, and grain quality are controlled by multiple genes. Furthermore, the interactions between genotype and environment (G×E) are strongly influenced. To achieve lasting improvements in these traits, it is necessary to regulate gene expression or make simultaneous multiple genetic changes (Menz et al., 2020; Smedley et al., 2021).

Genome editing techniques, specifically CRISPR/Cas-based approaches, enable wheat to undergo multiple and targeted genetic modifications. These methods can be utilized to deactivate genes (knockout), edit promoter regions, or make single base changes. Obtaining plants without carrying stable foreign DNA in certain applications is possible, which has significant advantages for both regulatory agencies and agricultural practices (Smedley et al., 2021; Laforest & Nadakuduti, 2022). Despite the significant potential of genetic modification and genome editing methods for wheat, their commercialization has not kept up with other staple crops such as maize, soy, and rice. The primary underlying factors for this are the decreased productivity of elite wheat varieties, the polyploid genome structure, which necessitates simultaneous editing of homologous genes, stringent regulatory policies, and public concerns. Genome editing applications in wheat are now possible due to the recent development of gene transfer systems, morphogenic gene-mediated regeneration methods, and DNA-free ribonucleoprotein (RNP) based editing. The development of the HB4 drought-resistant wheat variety has sparked a rise in scientific and political interest in this method. Certain researches have highlighted the obstacles to field application and market acceptance of the product (Menz et al., 2020; Laforest & Nadakuduti, 2022; Rafiei et al., 2024).

The availability of molecularly engineered wheat to farmers and markets is contingent on three key factors. The factors that are

important are technical feasibility, reliability of the feature, and environmental effect performance. Thus, the successful commercialization of cereals can be achieved (Laforest & Nadakuduti, 2022; Rafiei et al., 2024; Zhou et al., 2023). This situation is a result of significant innovations, particularly in wheat breeding. Single base changes can be made without the need for homology-directed repair using new approaches like improvements in guide RNA design algorithms, base and primer editors, transient RNP transfer and the use of high-fidelity Cas variants. Targeted and multiple gene editing, previously unthinkable in hexaploid wheat, may now be achieved in a research setting, as demonstrated by these novel approaches (Smedley et al., 2021; Laforest & Nadakuduti, 2022; Zhou et al., 2023). Although some countries exclude non-DNA genetic editing from GMO legislation, a majority of countries consider genome editing to be within the scope of GMOs. This situation has a direct impact on investment incentives, research strategies, and commercialization processes. This leads to either facilitation or hindrance in the equitable and widespread distribution of genetically modified wheat (Menz et al., 2020; Kanchiswamy et al., 2020; Rafiei et al., 2024).

To sum up, this chapter covers the potential of genetically modified wheat in agricultural production and the techniques used to achieve it. Achieving greater wheat tolerance to abiotic and biotic stresses, yield, quality, and general agricultural performance are the main goals of new molecular strategies. It is crucial to develop sustainable and efficient breeding strategies for this purpose. Also discussed is the field application of wheat through genetic modification and genome editing methods, along with the barriers to market acceptance and potential opportunities. Their future contributions are presented.

Methods of Obtaining GMO Wheat

Agrobacterium-Mediated Method

The method is known as a classical one for integrating T-DNA vectors into immature embryos or callus tissue with the help of *Agrobacterium tumefaciens*, the expression of a transgenic gene, or CRISPR/Cas constructs. This method typically causes a lesser amount of tissue damage, allows for single-copy transfer, and has high permanence (stability). Protocols that utilize morphogenic regulators such as BBM/WUS have resulted in a reduction in genotype dependency and facilitated the application of CRISPR-based vectors in diverse wheat varieties (Johnson et al., 2023).

Particle Bombardment (Biolistics) and RNP Applications

The biolistic technique involves transferring DNA, RNA, or protein complexes directly into plant tissues by means of gold or silver particles. Obtaining DNA-free engineered plants has become a significant strategy in recent years due to the biolistic transfer of RNP complexes. The risk of permanent transgenic DNA integration can be eliminated by using this method specifically for Agrobacterium-resistant genotypes (Poddar et al., 2023).

Protoplast Transfer and ITER-like Validation Platforms

Plant cells can take up gene editing components directly through protoplasts, making them a valuable tool. The rapid testing and validation of genetic modifications is possible through this system. The effectiveness of different guide RNA (gRNA) sequences may be rapidly elucidated through protoplast transfer, especially in gene editing technologies such as CRISPR/Cas9. The time it takes to obtain results in this method is significantly shorter than in traditional transgenic approaches and field trials (Gaillochet et al., 2022).

ITER and similar validation platforms make it possible to compare experiments performed at the protoplast level in a systematic manner. Using these platforms, the optimal gRNA design may be determined by measuring the performance of base and forward regulator configurations. Developing strategies can achieve high efficiency and effectiveness not only in laboratory conditions but also in the field environment (Ni et al., 2023).

The efficiency of gene editing can be increased and plant regeneration processes can be directly impacted by protoplast-based optimization studies. The success rate in field trials is increased by experimentally determined effective gRNA and regulator combinations that increase plant regrowth and development capacity. Therefore, protoplast transfer and ITER-like platforms are considered indispensable tools in both basic research and applied plant biotechnology studies (Gaillochet et al., 2022; Ni et al., 2023).

gRNA Transfer via Viral Carriers

The transfer of single guide RNA (sgRNA) to the plant is the basis of this method in the gene editing process. The Cas9 protein is already present in wheat lines that have been genetically engineered with it. Direct editing on plant tissue can be performed by eliminating DNA transport or stable transformation steps in this way. Such a strategy offers the advantage of being both faster and less dependent on regeneration compared to protocols requiring classical Agrobacterium-based transformation or tissue culture.

The study of vectors that are based on Barley Stripe Mosaic Virus (BSMV) is an important example of this system. Wang et al., (2022), somatic and heritable gene editing was successfully achieved in transgenic wheat lines expressing Cas9 protein through the use of BSMV vector to deliver sgRNA into wheat tissues. The virus in this system transports the sgRNA to the target cells while spreading systemically in the plant, and then interacts with the Cas9 protein to

generate double-strand breaks in the target gene regions. The researchers have demonstrated that this method may simultaneously edit not only single genes but also multiple target genes. Species with a polyploid structure, such as wheat, are particularly benefited by this multiple targeting feature. Because, multiple homologous copies of the same gene can be edited simultaneously. Furthermore, the absence of tissue culture or regeneration steps in this approach offers a significant advantage in terms of both time and resources (Wang et al., 2022).

Ribonucleoprotein (RNP) Strategies

Ribonucleoprotein (RNP)-based gene editing involves the use of pre-synthesized Cas protein to deliver gRNA directly into plant cells. The editing process is done without the incorporation of foreign DNA into the plant's genome due to the absence of a DNA plasmid or genetic carrier system in this approach. This is a great advantage for both biosafety and legal regulations. Because the resulting plants are generally classified as non-transgenic. As stated by Poddar et al., (2023), the RNP method is prepared by purifying cutting proteins such as Cas9 or Cas12a and complexing these proteins with target-specific guide RNAs in vitro. Physical methods like electroporation, microinjection, or biolistics are used to transfer this complex to target cell types like protoplasts, embryo cells, or callus tissues. In consequence, the Cas protein and gRNA aim for the targeted DNA region in the cell and generate double-strand breaks (DSBs). Permanent mutations occur in the target gene region when the plant's natural DNA repair mechanisms (especially non-homologous end joining, NHEJ) repair these breaks. The Cas protein and gRNA are only present temporarily in the cell, which is one of the most significant advantages of this method. The RNP complex's rapid disintegration reduces the risk of off-target mutations and provides an advantage in regulatory processes due to the absence of any transgenic DNA residue. Furthermore, Poddar et al., (2023)

emphasize that the RNP method demonstrates high editing efficiency in cereals such as wheat, rice and maize and generally offers a faster, safer and environmentally friendly alternative compared to classical Agrobacterium or viral carrier systems. The gene editing process is significantly accelerated, particularly when it is used with systems that don't require regeneration and tissue culture (Poddar et al., 2023).

Morphogenic Gene-Mediated Regeneration Protocols

Morphogenic gene-mediated regeneration protocols are not a separate gene editing method, but rather an auxiliary strategy that enhances the efficiency of existing Agrobacterium-mediated gene transfer systems. In this application, somatic embryogenesis is induced in plant tissues by transiently expressing certain transcription factors, particularly BABY BOOM (BBM) and WUSCHEL2 (WUS2). As a result, it significantly enhances the success rate of genetic transformation by enhancing regeneration capacity (Johnson et al., 2023). This system is incorporated into the traditional Agrobacterium-mediated transformation process.

The tissue culture stage of species such as wheat is genotype dependent and many lines are resistant to transformation. However, transient activation of the BBM and WUS2 genes provides a genotype-independent embryogenesis response, effectively eliminating this problem. The reprogramming of cells is facilitated by these transcription factors, which enable differentiated tissues to form embryo-like structures. CRISPR/Cas9 or similar editing components carried by Agrobacterium can be more efficiently accepted by target tissues.

Johnson et al. (2023) created the QuickWheat protocol as a prime example. This protocol involves using Agrobacterium-mediated helper vectors to transiently express the BBM and WUS2 genes. Plant tissues are able to form somatic embryos with greater

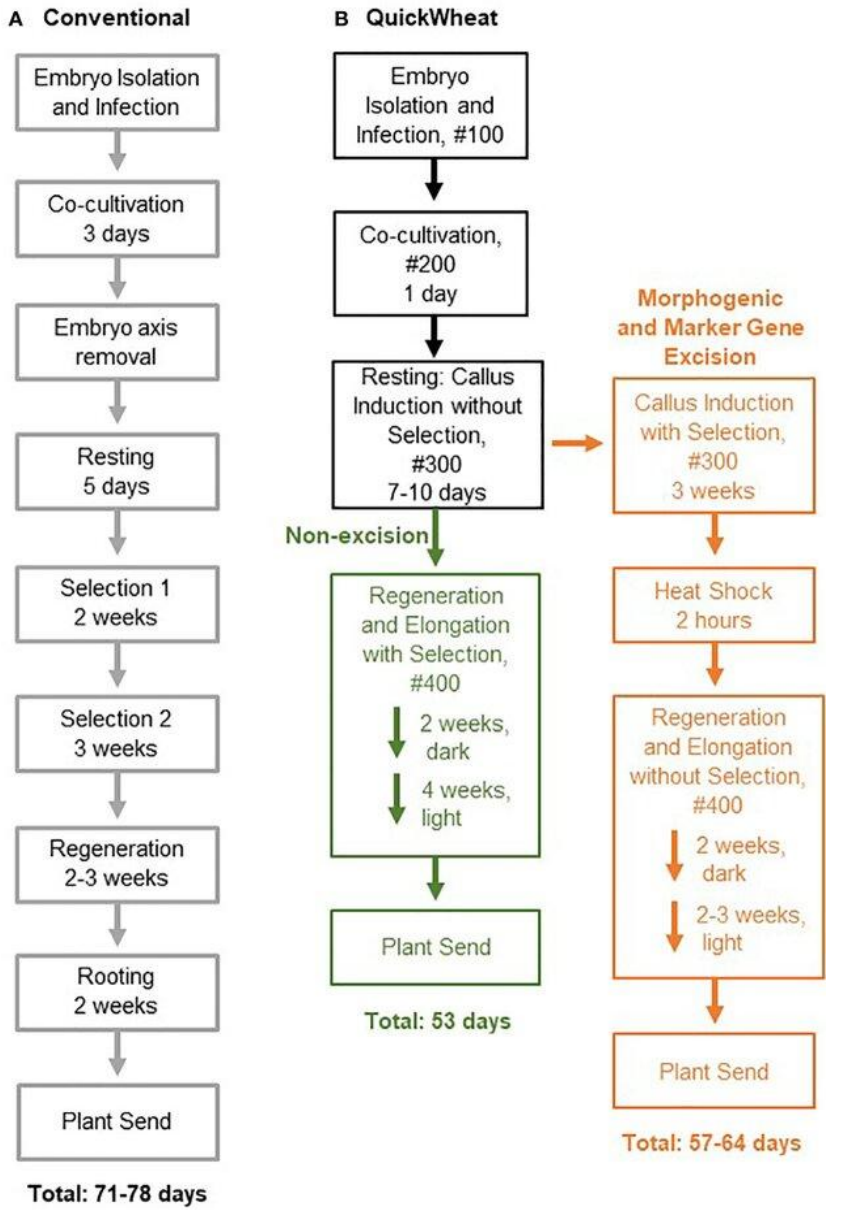
speed and the regeneration time after transformation is significantly reduced. The QuickWheat protocol not only increased yields but also made the transformation process genotype-independent, allowing high success rates to be achieved even in wheat lines that were previously difficult to transform. Additionally, morphogenic gene-mediated regeneration protocols are able to expedite the production of transgenic lines and reduce losses during regeneration steps when used in tandem with CRISPR/Cas-based gene editing applications (Johnson et al., 2023).

CRISPR-Based Systems

CRISPR systems are being used in genome editing studies in wheat. Recent research suggests that CRISPR-Cas systems are not restricted to gene targeting; with their different variants, they can be used for a wide range of applications, including single base changes and multiple allelic edits (Smedley et al., 2021; Gaillochet et al., 2022).

Targeted nucleotide changes are introduced directly into the genome by base editors who fuse a deaminase with a Cas-nickase enzyme. By editing without creating double-strand breaks, this method reduces the risk of major rearrangements caused by DNA repair and allows simultaneous changes to alleles. The optimization process of Cas12a-derived base editors using high-throughput validation systems such as ITER has resulted in permanent editing success rates of 40-55% in gene regulatory regions, and they have demonstrated high efficiency in producing functional single base changes (Ni et al., 2023). Prime editing (pe) can be done via custom-designed pegRNA using a combination of Cas9-nickase enzyme and reverse transcriptase. Despite the low efficiency of the previous forward regulator generation in plants, upgraded versions like ePPEplus have achieved high editing rates at multiple targets in hexaploid wheat. The forward editing method is particularly useful

Figure 1. Flowchart of Conventional to QuickWheat conversion procedures (Johnson et al., 2023)



for multiple traits stacking because it allows for simultaneous small insertions, deletions, or single-nucleotide changes on multiple alleles without the need for HDR (homology-based repair). PegRNA design, reverse transcriptase variants, and delivery strategies play a crucial role in these methods. Optimizing transfer of RNP or plasmids in protoplast or immature embryo systems may increase editing and regeneration success (Poddar et al., 2023).

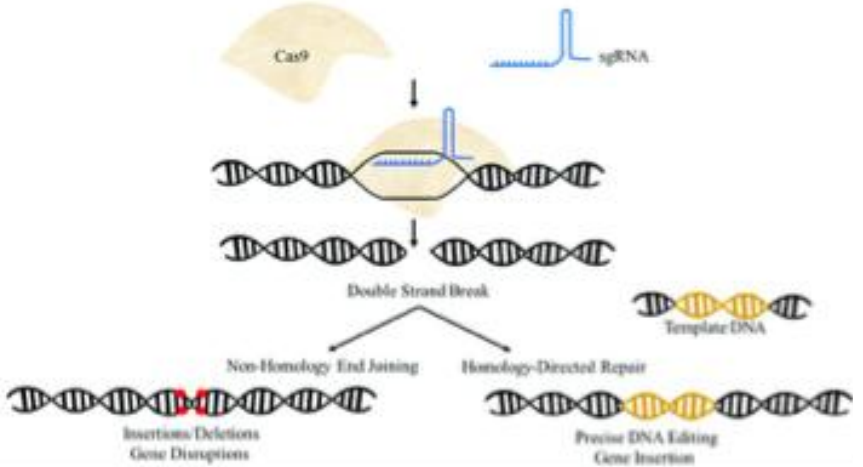
The editor construct utilized and the properties of the target gene sequence, chromatin accessibility, and homogeneous arrangements are all important factors in the success of CRISPR systems. Cas12a-derived systems are amenable to multiplex editing due to their inherent gRNA processing capacity, which allows simultaneous editing of homogeneous targets with multiple gRNA sequences. High-fidelity Cas9 variants (like HiFi and eSpCas9) and transient gene expression strategies are the preferred methods for reducing off-target effects. RNP-based transient gene expression has regulatory advantages that prevent permanent transgenic DNA transfer. Validations are carried out using ITER-like systems or protoplast experiments, and the most appropriate gRNA/pegRNA combinations are identified and refined for field usage. The use of base and forward modifiers, simultaneous editing of multiple targets, and high efficiency in polyploid genotypes are all possible through this process (Gaillochet et al., 2022; Ni et al., 2023).

Multiplexing and Off-Target Control

Multiple gRNA designs are necessary to regulate homologous genes in hexaploid wheat simultaneously. This objective is achieved using methods such as tRNA-processing arrays, Csy4, or ribozyme-based assemblies. Cas12a-derived systems are a suitable choice for multiple targeting because of their guide RNA processing capabilities. Special scoring systems that are

compatible with polyploid plants are being developed to reduce the risk of mistargeting by taking into account chromatin accessibility.

Figure 2. Diagram of the CRISPR/Cas9 mechanism (Ahmad et al., 2021)



Regeneration and Agronomic Trials

The successful application of gene editing requires high regeneration rate and environmental interaction (GxE) tests. Morphogenic gene-mediated regeneration protocols make it easier to regenerate plants from cells in vitro and boost productivity. High efficiency may be achieved through these approaches, particularly in Agrobacterium-mediated or gene editing-based delivery systems (Rafiei et al., 2024). Moreover, agronomic experiments conducted in several locations ensure that phenotypes obtained in the laboratory are preserved under field conditions. Thus, morphogenic gene-mediated regeneration protocols enhance the stability and application success of genetic modifications, not only in laboratory settings but also in field conditions. This holistic approach is crucial in guaranteeing the practical effectiveness and sustainability of gene editing strategies (Rafiei et al., 2024).

Current research on the development of genetically modified wheat varieties

The production of wheat (*Triticum aestivum*) has been seriously threatened by environmental pressures such as climate change, drought, and disease factors in recent years. In this context, modern genome editing technologies, especially CRISPR/Cas9-based approaches, are used extensively to increase both durability and efficiency. The CRISPR/Cas9 system allows for mutation or direct gene editing by creating precise cuts in target gene regions. This method offers high accuracy and efficiency, especially when simultaneous editing of three subgenomes (A, B and D) is required in wheat with hexaploid structure (Zhou et al., 2023).

Enhancing Resistance to Water Stress

Wang et al., (2023) edited the TaATX4 gene with the CRISPR/Cas9 system to improve water stress tolerance. This gene encodes a regulatory protein involved in abiotic stress responses. Following regulation of the target gene via guide RNAs, embryos were regenerated from the protoplast and immature embryo systems. It was determined that the edited wheat lines provided higher survival rate and yield under drought conditions compared to the control groups. In addition, it has been emphasized that the integration of foreign DNA is prevented by the transient expression of CRISPR/Cas9, which provides a regulatory advantage (Wang et al., 2023).

Improving Resistance to Diseases

Disease resistance is a critical element in wheat production. Liu et al., (2024) developed resistance to common diseases such as powdery mildew and yellow rust by inactivating the TaMPK1 gene with CRISPR/Cas9. In study, multiple gene targeting was performed using protoplast and immature embryo systems, and the resulting

lines were phenotypically tested. It has been observed that disease symptoms decrease in regulated plants after pathogen infection. It has been stated that editing specificity is increased by optimizing the Cas9 variants used to reduce mistarget effects (Liu et al., 2024).

Increasing Productivity

According to Poddar et al., (2023), CRISPR/Cas9 can also be employed to control genes that increase yield. By regulating genes that affect the growth and development processes of the plant, higher yielding wheat lines have been obtained. Multiple targeting was achieved by done multiple guide RNAs, regeneration and regulating efficiency were optimized with different Cas9 variants. Thus, both phenotypic improvement and simultaneous regulating in subgenomes were provided. This approach is critical in terms of multi-property acquisition in hexaploid wheat (Poddar et al., 2023).

Simultaneous Regulating of Multiple Properties

According to the comprehensive review by Zhou et al., (2023), CRISPR/Cas9 technology is an effective tool to introduce multiple traits simultaneously. In studies have developed wheat lines that have enhanced disease resistance along with drought tolerance. At this process involved precise modifications using both single-base editing (base regulators) and forward-editing strategies (primary regulators). Findings show that these methods work with high accuracy and low false target effect, providing high efficiency despite the polyploid structure (Zhou et al., 2023). In general, current literature reveals that CRISPR/Cas9 and its derivatives (base regulators, first regulators) are effective in increasing resistance to both abiotic and biotic stresses in wheat, increasing yield and regulating multiple traits simultaneously. In particular, transient expression systems, DNA-free ribonucleoprotein applications and morphogenic regulator-assisted regeneration methods enable the to

obtain of regulated plants in a safe and high-yield manner. (Poddar et al., 2023; Wang et al., 2023; Liu et al., 2024; Zhou et al., 2023).

Agricultural Applications of Genetically Modified (GMO) Wheat

Agricultural applications of GMO wheat focus particularly on disease resistance, drought tolerance and yield increase. Genome editing technologies, particularly CRISPR/Cas9-based systems, allow for rapid and efficient in targeted changes to the genetic structure of wheat (Waites et al., 2025). A study in 2025 used CRISPR-based editing methods to increase disease resistance; these methods are aimed to reduce the impact of pathogens through resistance (R) genes and NLR receptors (Waites et al., 2025). HB4 wheat, developed for drought resistance, is one of the genetically modified first drought-tolerant varieties. HB4 wheat, registered in Argentina in 2020, was approved in the US in 2025 and has been deemed fit for human consumption by nine different countries. However, it has not yet been commercialized in Australia (Carey-Fung & Johnson, 2025).

Agricultural Use Areas

Genetically modified wheat varieties provide many advantages when integrated with traditional agricultural practices. Increasing disease resistance makes it possible to minimize environmental impacts by reducing the use of chemical medicine (Waites et al., 2025). Drought-tolerant varieties contribute to production sustainability in regions where water resources are limited (Carey-Fung & Johnson, 2025). However, there are certain challenges at the field applications of GMO wheat. The complexity of wheat tissue culture and genetic transformation processes limits the widespread use of these methods (Waites et al., 2025). Legal regulations regarding genetic engineering and genome editing

applications are diverse in each country, and this has a direct impact on commercialization processes (Carey-Fung & Johnson, 2025).

Future-Oriented Perspectives

It has been found by research that the use of genetically modified wheat varieties will increase in the upcoming years (Waites et al., 2025). Advances in genome editing technologies and a deeper understanding of the wheat genome will enable the development of varieties with higher yield potential and greater resistance to environmental stress conditions in the future (Carey-Fung & Johnson, 2025). However, for the process to proceed sustainably, manufacturers' adaptation to new technologies, harmonization of legal and ethical regulations, and increasing consumer acceptance stand out as critical factors.

Conclusions

Literature indicates that genetically modified wheat offers significant opportunities in agriculture. Current research suggests that CRISPR/Cas9 and its derivatives, base and first regulators, can be successfully used to enhance stress tolerance, increase yield, and regulate multiple traits in hexaploid wheat. Morphogenic regulator-assisted regeneration, DNA-free ribonucleoprotein applications, multiple guide RNA designs, and thanks to high-fidelity Cas variants make it possible to safely transfer laboratory findings to agricultural applications. In the future, with advances in genome editing technologies and a detailed understanding of the wheat genome, it is envisaged that varieties with greater yield, resistance to environmental stresses and disease resistance will be developed. These advances, when supported by technical feasibility, regulatory compliance and consumer acceptance, will be one of the basic components of sustainable agriculture strategies.

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SOME BIOMONITOR PLANTS IN DIFFERENT ECOLOGICAL AREAS

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Introduction

Due to reasons such as the ever-increasing population and industrialization, the earth and atmosphere are becoming polluted, and the increasing amount of heavy metals continues to negatively affect life.

Heavy metals (HvM) can accumulate in various organs of organisms and have toxic effects. Humankind is the primary culprit in this situation, which seriously threatens the ecological balance. Humankind not only endangers its own lives but also negatively impacts all organisms. Despite these negative consequences, some plants accumulat and tolerate toxic effects.

In recent years, metal-sensitive plants have been frequently used as biomonitors and bioindicators. While biomonitor show that

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provide quantitative environmental information, bioindicator show that provide environs cantitie (Markert 1993).

HvM released into the environs sources are pasıple causes of environmental problem due to their toxic properties (Goyer, 1991).

The accumulation of heavy metals that are harmful in soil, water, air, and plants because of increased industrialization and antroponic activities. These heavy metals, which are toxic substances, have a toxic effect on living things (Türküm, 1998).

HvM are defined density greater than 5 g/cm3 in terms of their physical properties. This group, which includes elements such as iron (Fe), lead (Pb), cobalt (Co), zinc (Zn), copper (Cu), chromium (Cr), nickel (Ni), and cadmium (Cd), comprises nearly 70 metals. While attempts are made to determine the effects of metals on the ecological system based on their density values, it is not actually possible to reveal the environmental damage they cause. While an element's density indicates its place on the periodic table, its chemical properties are the properties of the group to which it belongs. Therefore, when examining an element's impact on the ecological cycle (Kahvecioğlu et al., 2009).

If we were to classify heavy metals according to their participation in biological processes, we could classify them as necessity and non-necessity HvM. Necessity HvM (Fe, Cu, Zn, Ni, and Se), which participate in biological reactions and must be present in certain amounts in the body, must be consumed regularly through diet, while non-necessity hvm (Hg, Cd, and Pb) shown to cause important problems even at minimum concentrations (Dökmeci and Dökmeci, 2005; Klassen et al., 2009).

HvM are known to leach into water, soil, and air. It is known that the accumulation of metals in living organisms lead to some problems (Karpuzcu, 1999).

It is crucial to reveal how stress from heavy metals affects metabolic processes in living organisms and to determine how plants respond to these polluting heavy metals and how they develop adaptation mechanisms.

In recent years, the use of natural bioindicators to monitor the quality of the atmosphere in urban and rural areas has increased significantly in order to protect against the harmful effects of toxic metals and to minimize their toxic effects (Çavuşoğlu et al., 2009).

Plants growing in soil contaminated with heavy metals experience three different types of reactions:

1. Metal excluders; they keep huge amounts of metals under control in their roots.

2. Metal indicators: Plants that accumulate and levels in tissues reflect the metal concentration in soil.

3. Hyperaccumulators: Plants that store concentrated of metals from the soil in their tissues. These are plants that contain more than 0.1% Ni, Co, Cu, Cr, Pb, or 1% Zn by in their leaves (Baker and Walker, 1990).

Some plants have higher heavy metal contents and requirements than non-accumulator species. These plants include more than 10 ppm Hg, 100 ppm Cd, 1,000 ppm Co, Cr, Cu, and Pb, and 10,000 ppm Ni and Zn.

Today, there are 400 known heavy metal accumulating plants (Reeves and Baker 1999). The most well-known plant is *T. caerulescens* (*Alpine pennycress*). While many plants show toxic symptoms at 100 ppm Zn accumulation, *T. caerulescens* has been reported in the literature to accumulate 26,000 ppm Zn (Lasat, 2000). The tolerance mechanisms of these plants can be summarized as follows:

a) Metal binding to cell walls: retained as Pb-carbonate

b) Decreased transport to cell membranes: Transport is reduced by trapping heavy metals in plant roots and preventing their transport to stems and shoots.

c) Storage in vacuoles: Zn is stored in vacuoles as low-molecular-weight organic compounds such as Zn phytate, malate, and oxalate. Cd binds to thiol groups, and Ni binds to histidine.

d) Chelation: Cadmium binds to thiol groups, Pb binds to glutathione and amino acids, forming phytochelates. They also combine with organic acids such as citrate, malate, and malonate to form phytochelates. Metallothioneins are proteins found in many animals and plants. They form protein compounds by binding with heavy metals (Aksu and Yıldız, 2004).

Elements necessary for plants to perform certain activities are called "plant nutrients" (Yıldız, 2003). Some heavy metals, as Cu, Mo, and Ni, are beneficial and necessity micronutrients for higher plants. Zn+2 and Cu+2 cofactors for the structure of proteins and enzymes, which play important roles in plant growth and development.

Accumulatıon excessively in plant tissues cause various abnormalities in the organism. Some of the abnormalities observed in plants include changes in vital processes (Zengin and Munzuroğlu, 2004).

There are several basic criteria for a species to be used as a biomonitor to determine heavy metal pollution. These include being represented in large numbers in the collection area, having a wide geographic range, being easy to sample, and having no identification problems (Aksoy et al., 1999).

Excessive accumulation of HvM in plants can, in some cases, slow down plant physiological activity, reduce productivity, and

eventually lead to plant death. Factors affecting plant tolerance to heavy metal toxicity include the plant species, the type of element, the duration of plant exposure to the heavy metal, and the structure of the tissue or organ exposed to the heavy metal.

HvM contain toxic substances, and high concentrations, particularly copper (Cu), zinc (Zn), nickel (Ni), and lead (Pb), leave sludge on the soil surface (Schmidt 1997). Because of their high toxicity, they pose a danger to living organisms when they enter the food chain. Heavy metals can enter the food chain from air, water, and agro-ecological systems, causing toxic effects on consumers (Chen et al., 2001).

Cement factories, glass factories, and waste and sludge incineration facilities also release heavy metals into the environment. HvM released into the air reach the terrestrial environment and from there, through plants and the food chain, reach animals and humans. They also reach humans and animals through inhalation, either as aerosols from the air or as dust (Kahvecioğlu et al., 2001). These metals, with densities greater than 5 g/cm3, contain more than 60 elements, including Pb, Cd, Cr, Fe, Co, Cu, Ni, Hg, and Zn. In nature, these elements are generally found as stable compounds in the form of carbonates, silicates, and sulfur, or bound within silicates (Kahvecioğlu et al., 2007). For this reason, it has been reported that knowing the rate of HvM, the dosage and type of toxicity, and the toxicity process are important for plant growth and development (Okcu et al., 2009).

As a result of the population growth that has occurred in Turkey over the last 40-50 years, uncontrolled and unplanned urbanization and industrialization have triggered each other. This has caused serious environmental problems in urban areas (Yılmaz et al., 2006).

Table 1. Toxic HvM Sources Mixed into the Ecosystem

Particles and Fumes in the Biosphere	• Vehicles (Cd, Pb, Mo) • Fossil Fuels (As, Cd, Cr, Cu, Mn, Ni, V, U, Pb, Sr, Zn, Ti) • Cities and Factories (Cd, Cu, Pb, Sn, Hg, V • Vehicles (Cd, Pb, Mo) • Fossil Fuels (As, Cd, Cr, Cu, Mn, Ni, V, U, Pb, Sr, Zn, Ti) • Cities and Factories (Cd, Cu, Pb, Sn, Hg, V
Industry	• Plastics (Co, Cr, Cd, Hg) • Textiles (Zn, Al, Ti, Sn) • Wood Processing (Cu, Cr, As) • Refinery (Pb, Ni, Cr) • Household Appliance Production (Cu, Ni, Cd, Zn, Sb)
Metal and Mining Industry	• Iron and Steel Industry (Zn, Cu, Ni, Cr, Cd) • Metal Processing (Zn, Cu, Ni, Cr, Cd, Hg, Pb, As) • Metal Smelting (As, Cd, Hg, Pb, Sb, Se)
Agriculture	• Irrigation (Cd, Pb, Zn) • Chemical and Animal Fertilizers (As, Cd, Cu, Mn, Zn, U, V) • Lime (As, Pb) • Metal Corrosion (Fe, Pb, Zn)
Waste	• Sewage (Cd, Cr, Cu, Hg, Mn, Mo, Ni, V, Pb, Zn) • Digging and Drilling (As, Cd, Fe, Pb) • Ashes (Cu, Pb)

(Markert 1993)

Buszewski et al., (2000) used popular bioindicators to determine heavy metal pollution in the city of Torun, Poland. Environmental monitoring of the identified metals was conducted using biota and soil samples. They applied conventional extraction and microwave mineralization techniques for the isolation of analytes. They used Atomic Absorption Spectroscopy (AAS)

equipped with a Graphite Furnace (GF) for the determination of heavy metals and macroelements. In this study, they observed that heavy metals and macroelements accumulated in the organs and roots of selected plants. They determined that the metal concentrations in the measured soils did not exceed the limit values set by Polish standards. They concluded that the metal concentrations in the examined *Pinus silvestris* L. (pine needles), *Festuca protensis* L. (grass), and *Pleurozium schreberi* (moss) samples were lower than the permitted limits. Only Pb exceeded this level. They reported that the highest concentration was found in plant samples collected in the spring.

Güleryüz et al. (2002) the effects of Etibank Wolfram mining waste on plants in Bursa's Uludağ National Park. They used soil and plant parts from four different plant species in their study. They examined the samples for the metals Ca, K, Mg, Fe, Zn, and Cu. The study concluded that wolfram mining altered the soil composition in this area. In plants, they determined that these heavy metals were present in different forms and at high concentrations in different plant parts. This study demonstrated that plant element content is species-specific.

Kochian et al. (2002) investigated the mechanisms and strategies that plants use to tolerate metal toxicity in soil. A metal-accumulating plant species, the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (Shepherd's spear), was investigated. The focus was on identifying genes that confer Al resistance to heavy metals to help develop plants more suitable for growth in acidic soils. Although there are different strategies, the most common Al resistance mechanism for Al is the removal of Al from the root apex.

Brej et al. (2006) studied five species representing regional hvm accumulators in Lower Silesia, Poland. The Sudeten flora consists of several plants known to be heavy metal accumulators.

Others inc *caerulescens, Arabidopsis halleri, Armeria maritima ssp. halleri s.l.,* and possibly the endemic fern Asplenium onopteris var. silesiaca. No Lower Silesian Sudeten plant has developed the typical characteristics of hyperaccumulators known from other parts of Europe. This may be due to the influence of time-dependent natural factors, the plant variety of the metal-contaminated land, and its ecological inaccessibility. Heavy metal concentrations in plants observed over time are due to soil element diversity and mechanisms developed by plants that allow element accumulation. It is recommended to prevent the degradation of the study areas.

They noted that a second mechanism, involving the lateral deoxidation of Al accumulated by organic acids (citrate and oxalate), resulted in high Al accumulation in the leaves of hydrangea and buckthorn plants.

Çavuşoğlu et al. (2009) investigated the effects of vehicle-induced lead pollution on the leaf anatomy of pine *(Pinus nigra*) and cedar (Cedrus libani) trees along the 10-km road between the city center of Isparta and Süleyman Demirel University. They found the lowest lead pollution in samples taken from the Süleyman Demirel University campus entrance, while the highest lead pollution was found in samples collected from the city center. They concluded that the lead pollution rate, which was lower at the university located on the outskirts of the city, was higher in the city center due to vehicle density.

Hussain and Khan (2010) conducted a study on *Eclipta alba* L., a medicinally important plant, collected from three different regions in Peshawar, Pakistan. Heavy metals such as Cr, Fe, Zn, Mn, Ni, Pb, Cu, and Cd were measured in the plant collected from its natural habitat and in soil where it was grown using atomic absorption spectroscopy. All heavy metals examined, except Cd, were found in all plant materials at concentrations consistent with

the soil concentration. Fe, Mn, and Zn were observed in high amounts, while the others were observed in low amounts. This study demonstrated that *Eclipta alba* L. can be used as a bioindicator. However, they recommended that materials intended for medicinal use be harvested from cleaner areas, free from heavy metal pollution.

Yener and Yarcı (2010) collected *Alcea pallida* plant samples from five different regions in three districts of Istanbul during the summer of 2005. The biomonitoring properties of *Alcea pallida* were examined for hvm Pb, Cd, Cu, and Zn in four organs: flowers, leaves, stems, and roots. Hvm content in *Alcea pallida* organs was measured using the wet digestion method and atomic absorption spectrophotometer. The highest average Pb levels were observed in the leaves, while the lowest were observed in the stems, Cd in the roots and flowers, Cu in the leaves and stems, and Zn in the leaves and flowers. The study concluded that *Alcea pallida* is a reliable biomonitor for Cd, Cu, and Zn, especially in root zone.

Demirayak et al. (2011) conducted a study on some native and exotic trees and shrub species found in and around Samsun. They investigated the accumulation levels of certain metals (Pb, Cd, Zn, and Cu) in the samples across different seasons. They measured metal concentrations in the leaves, needles, and branches of the plants. They attempted to determine the biomonitoring properties of plants in an area polluted by heavy traffic and fossil fuels. Based on the data obtained in the study, they determined that Magnolia grandiflora, *Ligustrum vulgare*, and *Phoenix dactylifera* could be used as biomonitors.

Duru et al. (2011), they investigated the extent of heavy metal pollution caused by vehicles in the leaves of *Verbascum sinuatum* L. (Scrophulariaceae) (mullet) collected from the Black Sea Coastal Highway, which runs between the provinces of Samsun, Ordu, Giresun, Trabzon, and Rize, and the district of Hopa. They collected

samples from 23 designated stations along the road. They determined the amounts of heavy metals in the leaf samples collected from each station using a Perkin Elmer Optical Emission Spectrometer (ICP-OES). HvM concentrations in the leaf samples of *Verbascum sinuatum* L. were determined as follows: Lead (Pb) > Zinc (Zn) > Chromium (Cr) > Nickel (Ni) > Copper (Cu) > Cadmium (Cd). The results of this study showed that heavy metal accumulation in leaves increases with traffic density and that *Verbascum sinuatum* L. can be used as a biomonitor to detect this accumulation.

Delavar et al. (2011) measured the concentrations of Al, As, Pb, Cd, and similar metals originating from pollution in the Iranian city of Arak, where industrial facilities are concentrated, using atomic absorption spectrometry in five selected medicinal plants. They found that all plants in this region were contaminated with lead, cadmium, arsenic, and aluminum, but these levels were within legal limits. Due to the difference between the contaminated and washed samples, it was recommended that the medicinal plants be washed before use.

Colpaert et al. (2011) reported that HvM pollution is a trigger for aboveground and belowground communities and is the cause of evolutionary adaptation in living organisms. This review provides an overview of the effects of toxic concentrations of metals on ectomycorrhizal populations and communities. The selection and adaptations exhibited by ectomycorrhizal species colonizing host plants in polluted environments are examined. In particular, the metal exclusion technique of Suillus species is carefully observed. It was observed that it reduces the transfer of large amounts of metals to the plant without inhibiting nutrient transfer to the host plants. They stated that the evolutionary adaptation in Suillus species contributes to the survival of host trees in metal-contaminated soils and could be used in phytostabilization strategies for heavy metal-contaminated soils.

Rahimi et al. (2012) examined medicinal, aromatic, and spice plants growing in different regions of Austria for their Fe, Cu, Cd, Mn, Pb, As, and Zn concentrations. Medicinal plants are used in the production of life-saving medicines for all people. Heavy metal pollution rates have increased significantly due to mining, smelting, and the tanning industry. As a naturally occurring toxic element in environment.

Phytoremediation is an environmentally friendly and inexpensive technique that cleans heavy metal-contaminated environments by using metal-accumulating plants. The study indicated that plants such as mint, lavender, cannabis, and garden sorrel can accumulate high-ammonia heavy metals and participate in phytoremediation

Pei et al. (2015) investigated the Pb and Cd amount in soil and water of 13 plantation tree species in the coastal afforestation stand of the Nansha district of Guangzhou, South China. They found that Pb content accumulated more in branches than in leaves. They examined mechanisms of HvM accumulation in trees but no found significant differences between different tree taxa. They noted that the variation in the detected HvM amounts was related to the individual of different species and plant organisms. The study demonstrated that trees would be useful for remediating areas containing Pb and Cd in urbanized areas.

Özyürek and Leblebici (2016) identified three stations in each of the different regions of the Nevşehir region (Avanos, Kavak, Sulusaray, and Nar) where contamination might occur. They used samples *of Lycopersicon esculentum Mill. (tomato), Capsicum annuum L. (pepper), Allium cepa L. (onion), and Phaseolus vulgaris L. (bean),* along with soil and irrigation water collected during the fall of 2012-2013, as materials. The amounts of Nickel (Ni), Copper (Cu), and Lead (Pb) in the samples were determined using an ICP

OES device. They determined that accumulation in the plants occurred in the following order: root > stem > leaf > fruit. Their study concluded that the amount of HvM in vegetables and soil in which they were grown was within the required limit values. They stated that necessary precautions should be taken to prevent these heavy metal levels from reaching higher levels. Vegetables should be grown away from roadsides with heavy traffic, industrial facilities, and city centers where pollution may be higher. They also stated that caution should be exercised in watering vegetables and using pesticides.

The aim of the study conducted by Ozturk et al. (2017) was research the Pb, Cd, Cu, and Zn pollution levels in Istanbul using *Celtis australis*, a member of the Ulmaceae family, which has a wide geographical distribution in Southern Europe and North Africa. Nettle leaves, branches, and bark to take from 40 different locations in Istanbul, particularly in summer months of July and August, were also used to determine the levels of heavy metal pollution in soil samples where the nettle grows, and to investigate whether this plant species could serve as a monitoring plant. The amounts of heavy metals in the samples were measured using atomic absorption spectroscopy. HvM accumulation is directly proportional to traffic density and proximity to the roadside. It was determined that *Celtis australis* could be used as a biomonitor for Pb, Zn, Cu, and cadmıum. They also observed the usability of nettle shells in long-term heavy metal absorption measurement.

The toxicity levels of heavy metals for plants and soil, according to international standards, are shown below.

Element Toxicity Values mg/kg

Pb 30-300 mg/kg

Cu 20-100 mg/kg

Fe 50-500 mg/kg

Mn 300-350 mg/kg

Ni 25-40 mg/kg

Zn 80-200 mg/kg

Toxicity amounts of heavy metals for soil according to international standards

Toxicity Values mg/kg

Pb 50-100 mg/kg

Cu 100 mg/kg

Fe 10-200 mg/kg

Mn 30-300 mg/kg

Ni 30-75 mg/kg

Zn 150-300 mg/kg

Heavy metals are involved in biochemical processes in plants. Factors that determine the amount of HvM in plants include plant species, the type of soil in which it grows, climatic conditions, rainfall, and environmental factors.

It has been observed that various factors influence HvM collected in plants. These factors include the plant's genetic characteristics, proximity to pollution sources, soil characteristics, wind, and precipitation. Proximity to emission sources has been determined to be a significant factor in the contamination of soil and plant samples with HvM.

By utilizing these properties of plants, it is possible to clean heavy metal-contaminated soil and water using phytoremediation. Furthermore, this method is both easier and more economical than many other methods.

Plants tolerant to heavy metals are crucial in solving this problem. Numerous studies have shown that many plants can tolerate these toxic elements by absorbing them.

Conclusion

Studies on environmental pollution and plant interactions, as well as the biomonitoring properties of plants, have become increasingly important in recent years to ensure that the rapidly destructive ecosystem and the irreversibly polluted Earth remain in a more livable state for future generations.

HvM have positive and negative effects on human life. While metal Fe, Zn, and Cu are absolutely essential for life, metals Cd, Pb, and Hg are toxic very small doses. These toxic metals enter living organisms through various means and negatively affect their metabolism. Soil, air, water, industrial waste, exhaust fumes, factory emissions, and mines are all factors contributing to the increase in heavy metal levels on Earth. Toxic elements that enter our bodies through the food chain also cause organ damage.

Today, rapid population growth and heavy traffic are among the primary causes of environmental pollution, especially in large cities. Environmental pollution has become one of the world's most important problems, urgently awaiting a solution. In today's world, where cancer and a wide variety of fatal diseases are rapidly increasing, environmental pollution caused by heavy metals is a problem that urgently needs to be solved. Humanity must stop this pollution and ensure access to clean air, water, and food. Otherwise, pollution will pose a major threat not only to humanity but to all biological life. Purifying polluted environments from these toxic elements is the most important issue awaiting solution in recent years.

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FROM ENVIRONMENTAL POLLUTANTS TO MICROBIAL CRISIS: THE MULTILAYERED EFFECTS OF ENVIRONMENTAL TOXICITY

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Introduction

Today, as a result of industrialization, rapid population growth, and changes in consumption habits, there has been a significant increase in the quantity and diversity of environmental waste. This inevitably leads to pollution in the ecosystem and toxic effects on organisms. Environmental wastes, such as organic pollutants, pharmaceutical waste, heavy metals, and microplastics, which can persist in the environment for long periods and have a high bioaccumulation potential, constitute the primary risk group. The effects of these wastes increase the risk not only at the individual organism level but also at all levels, including physiological, morphological, and genetic levels (Riyazuddin et al., 2021). Therefore, addressing toxic wastes at the ecosystem level and

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assessing their impacts from a multidisciplinary perspective is crucial.

Environmental Waste Classification

To understand the toxicological profiles and exposure pathways of environmental wastes, waste classification can be made based on various criteria, such as the physical and chemical properties of the waste, its degree of hazard, or its source. For example, based on physical and chemical properties: Wastes such as plastic and electronic waste can be classified as solid, wastes such as sewage and industrial wastewater as liquid, and wastes such as volatile organics and exhaust as gaseous. Based on the degree of hazard, wastes such as inert materials and organic kitchen waste are classified as non-hazardous, while pollutants such as radioactive waste, pesticides, and heavy metals are classified as hazardous. Based on their source, they are generally classified as domestic, industrial, and agricultural (Li et al., 2024).

Sources of Environmental Wastes

The main sources of environmental waste are primarily domestic, industrial, and agricultural activities. Domestic waste releases large amounts of pollutants such as plastic, microplastics, and chemical waste into the environment. Domestic waste primarily includes textiles, food waste, plastic packaging, detergents, surfactants, phthalates, bisphenols, and cosmetics. Waste from agricultural activities generally consists of various pesticides such as organophosphates and carbamates, chlorinated compounds, fertilizers, agricultural film, and microplastics. Depending on the type of industrial activity, industrial wastes, electronic wastes, chemical and textile wastes, mining and construction wastes, and petrochemical wastes are released into the ecosystem as environmental waste. The main waste types resulting from these activities include: synthetic solvents, dyes, organic solvents, circuit

boards containing Pb, Hg, Cd, brominated flame retardants, batteries, cell phones, computers, and fluorescent lamps; acidic mine drainage, metal-laden muds, asbestos, and dust particles, polycyclic aromatic hydrocarbons, VOC emissions; infected medical wastes, chemical solutions, hormone-like substances, and pharmaceutical residues such as painkillers, antibiotics, and cytotoxic drugs (Kato & Kansha, 2024).

Paths of Exposure to Environmental Wastes

Exposure of organisms to waste materials occurs through air, soil, water, the food chain, and direct contact.

Humans have been reported to ingest environmental wastes through the respiratory and gastrointestinal systems (Ruggles & Benakis, 2024). For example, it has been reported that human microplastic exposure can occur through inhalation (air, dust particles), ingestion (food, drinking water), and dermal contact (especially through cosmetics or personal care products), depending on the dose, particle size, shape, and surface chemical coverage (Nawab et al., 2024).

Direct contact with contaminated soil and irrigation water in plants has been observed to result in the uptake of toxic substances such as pesticides, heavy metals, or microplastics through the root system and their translocation to leaf and stem tissues. This leads to the activation of biochemical stress markers and impairments such as decreased photosynthetic efficiency (Li et al., 2024).

For aquatic organisms such as the duckweed *Lemna minor* and *Daphnia magna*, and the zebrafish Danio rerio, exposure to waste substances occurs both through direct uptake of dissolved contaminants in water and through particulate transporters. In fish, pollutants can be absorbed through the skin and gills, as well as by ingestion (Lanzarin et al., 2023). Exposure to glyphosate and a metal

mixture in dissolved and particle-bound forms in water has been reported to cause behavioral changes and disruption of central nervous system developmental genes in *Danio rerio* (Babich et al., 2024). In *Lemna minor*, nanoplastics were detected in contact with suspended particles in water and taken up from the water surface or root zone; this exposure was reported to be associated with physiological changes such as growth inhibition and decreased chlorophyll on the leaf surface (Iannelli et al., 2022; Souza et al., 2021).

Toxicity and Types

Toxicity is a fundamental concept that can be defined as the totality of the adverse effects of any chemical, biological, or physical agent on an organism. Essentially, the principle of "dose determines the poison," introduced by Paracelsus and considered a founding principle of science, has formed the basis of toxicological assessment. This phenomenon, central to the science of toxicology, is of critical importance for both human health and the sustainability of ecosystems. According to this principle, any substance can be beneficial or harmless at low doses, but can exhibit toxic effects at high doses. For example, even substances essential for life, such as vitamins, water, and even oxygen, can exhibit toxicity at high concentrations.

The emergence of toxic effects in living systems can vary depending on the interaction of complex factors such as the chemical structure of the substance, the dose and duration of exposure, routes of exposure such as inhalation, oral, and dermal, and the organism's sensitivity (Casarett, 2008). Therefore, toxicity is considered a multidimensional phenomenon that can manifest itself at different levels and organ systems. In this context, toxicity constitutes an indispensable parameter in the safety assessment of environmental pollutants.

Because the concept of toxicity is multidimensional, it requires scientific classification based on the duration, route of exposure, and mechanism of action on the organism. This classification determines the fundamental parameters used in toxicological risk assessment and details the effects at the temporal, anatomical, and biological levels. The types of toxicity and their associated characteristics are briefly presented below under subheadings.

Acute Toxicity

• Occurs after short-term (single or 24-hour) high-dose exposure.

• Symptoms are usually observed within minutes to days.

Subacute and Subchronic Toxicity

• Subacute toxicity: Based on repeated exposure over 28 days.

• Subchronic toxicity: Develops after repeated exposure over 1–3 months.

• These types of toxicity studies are standard methods for safety testing.

Chronic Toxicity

• Develops after low-dose but prolonged exposure (months to years).

• Chronic effects are generally associated with long-term consequences such as organ damage or cancer.

Local and Systemic Toxicity

• Local toxicity: The substance causes direct damage to the exposed area (e.g., skin burns from caustic acids).

• Systemic toxicity: The substance enters the circulation and damages various organs (e.g., neurotoxic effects of lead (Pb) in the central nervous system).

Target Organ Toxicity

Some substances target specific organs. For example:

Hepatotoxicity: Liver damage (e.g., alcohol, aflatoxin).

Nephrotoxicity: Kidney damage (e.g., cadmium, some antibiotics).

Neurotoxicity: Nervous system damage (e.g., mercury, organophosphates).

Cardiotoxicity: Heart tissue damage (e.g., anthracycline drugs).

Genetic and Developmental Toxicity

• Mutagenic toxicity: The substance causes permanent changes in DNA (e.g., benzene, radiation).

• Carcinogenic toxicity: The substance causes cancer (e.g., arsenic, asbestos).

•Teratogenic toxicity: The substance causes abnormalities in embryonic/fetal development as a result of exposure during pregnancy (e.g., thalidomide, alcohol).

Toxic wastes exert their effects through oxidative stress and DNA damage, disruption of the endocrine system, and bioaccumulation and biomagnification. Many toxic pollutants cause oxidative stress in cells by increasing the production of reactive oxygen species (ROS). Organisms defend themselves against waste products by activating enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). However, under high toxicity conditions, these mechanisms become insufficient

(Riyazuddin et al., 2021). This can lead to lipid peroxidation, protein denaturation, DNA damage, and cell death (Zhang et al., 2016). Plastic additives such as bisphenol A, phthalates, and some heavy metals disrupt the endocrine system by mimicking or blocking hormonal receptors. Toxic substances accumulate in organisms' tissues (bioaccumulation) and their concentrations increase along the food chain (biomagnification). This poses a higher risk to higher-level organisms. Bioaccumulation is particularly evident in lipophilic and persistent substances such as PFAS, PCBs and heavy metals, and accumulation is observed in the gills, kidneys, muscle tissue, fat tissue, liver and bone tissue (Ali & Khan, 2019).

Toxic Effects of Domestic Waste

Household waste, detergent residues, plastic products, and their derivatives generate Endocrine Disrupting Chemicals (EDCs) and nanoparticle-based toxicity. Detergent residues can cause potential toxicity in the kidneys and liver, while plastic additives such as bisphenols and phthalates can disrupt hormonal balance (Nawab et al., 2024).

Detergents and surfactants have been observed to disrupt membrane integrity, increase lipid peroxidation, and affect respiratory and feeding behaviors, even at low concentrations (mg/µg/liter) (Sobrino-Figueroa, 2018). Studies have shown that ingestion of chemicals leached from detergents through food and drinking water can cause skin and respiratory sensitization, gastrointestinal irritation, and potential endocrine-disrupting effects in humans (Brunning et al., 2025).

The impacts caused by microplastics (<5 mm) and nanoplastics (<100 nm) in household plastic waste are drawing global attention to environmental toxicity. These particles lead to multidimensional effects on both terrestrial and aquatic organisms, from the molecular level to the population level. In freshwater

ecosystems, plastic particles such as polyethylene and polystyrene cause serious cellular damage by increasing the activity of oxidative stress biomarkers (Ding et al., 2020). In aquatic macrophytes, they can cause growth retardation and a decrease in photosynthetic pigment concentrations through fiber accumulation in root and leaf tissues (Mateos-Cárdenas et al., 2021). Microplastics have been observed to increase combined toxicity because they can carry other toxic chemicals (Wootton et al., 2021). Microplastic ingestion leads to disruptions in energy metabolism and disruptions in intestinal flora in fish (Wootton et al., 2021). When invertebrates living in terrestrial soils are exposed to soil containing microplastics, their mobility may be reduced, particularly with small particle sizes and long-term exposure, and physiological effects such as delayed reproductive cycles and growth retardation may be observed (Huo et al., 2022). Similarly, nanoplastics can increase the production of reactive oxygen species at the cellular level, leading to DNA damage, apoptosis, and developmental disorders (Souza et al., 2021). In humans, evidence that nanoplastics can cross the brain-placental barrier suggests the potential for neurotoxicity and immune response disorders (Vogel et al., 2024).

Toxic Effects of Agricultural Wastes

Agricultural wastes pose a serious threat to ecosystems due to the globally used pesticides such as organophosphates, carbamates, pyrethroids, and neonicotinoids, as well as excessive fertilizer loads.

Recent studies have shown that pesticide exposure in aquatic ecosystems can cause altered gene expression, reproductive disorders, and behavioral changes in water fleas (Aksakal et al., 2025; Li, 2025). In fish, pesticides can cause abnormalities in gonadal development, leading to population declines (Zhou et al., 2025a). Exposure to pesticides in *Danio rerio* larvae has been shown

to cause significant changes in antioxidant enzyme activities and increased lipid peroxidation (Jiang et al., 2022). Pesticide exposure in zebrafish embryos resulted in pericardial edema, spinal deformities, and embryonic developmental delays (Meng et al., 2021). It is known that increases in transaminase enzyme activities such as ALT and AST can be observed in fish liver tissues following pesticide exposure (Bojarski & Witeska, 2020).

Furthermore, insecticide exposure has been shown to cause midgut damage in bees and significantly affect survival (Aljedani, 2021; Benito-Murcia et al., 2024). Histopathological damage, such as intestinal epithelial degeneration, has been observed in earthworms (*Eisenia fetida*) (Gunstone et al., 2021). Chronic industrial and agricultural chemical surface water pollution has caused DNA breaks and erythrocytic anomalies in wild populations of the marsh frog (*Pelophylax ridibundus*), threatening genetic integrity (Mitkovska et al., 2024). It has been reported that long-term exposure to glyphosate in humans may be associated with endocrine disorders, DNA damage, and cancer risks (Zhou et al., 2025b).

Toxic Effects of Industrial and Healthcare Wastes

Industrial wastes include persistent and biologically active environmental pollutants such as heavy metals, organic solvents, and dyes. These toxic compounds pose a chronic toxicity risk to humans through direct contact and inhalation, or through the water and food chain. Industrial metal wastes have been shown to induce oxidative stress in aquatic model organisms such as Daphnia. Sublethal concentrations of ionic metals such as Cu and Zn lead to increased production of ROS, elevated levels of Malondialdehyde (MDA), and imbalances in antioxidant enzyme activities (SOD, CAT, GST, GPx) (Paylar et al., 2024; Mona et al., 2023). Heavy metals such as Pb, Cd, and Hg affect plant root and shoot development, inhibit seed germination and leaf growth, reduce photosynthetic capacity, and

suppress chlorophyll synthesis, causing changes such as chlorosis and necrosis (Kaur et al., 2021). On the other hand, heavy metals can accumulate in the kidneys, liver, and nervous system, while some dyes have genotoxic and carcinogenic potential (Kato & Kansha, 2024).

Heavy metals and dyes cause significant biochemical disruptions in organisms. Increases in transaminase (ALT/AST) activities and changes in the expression of detoxification enzymes (CYP450) and metallothionein can be observed in fish liver tissue (Mona et al., 2023). Studies with synthetic dyes have revealed teratogenic effects in zebrafish embryos, along with signs of genotoxicity such as DNA breaks and micronucleus formation. Furthermore, omics analyses have reported changes in the expression of genes regulating the stress response, DNA repair, and apoptotic pathways in *D. magna* due to metal exposure (Porkodi et al., 2024; Paylar et al., 2024).

The phenotypic consequences of industrial wastes have been demonstrated through growth and developmental disorders and histopathological findings. For example, exposure to organic dyes and solvents in zebrafish embryos has been associated with decreased growth rates, pericardial edema, and spinal deformities (Porkodi et al., 2024). Histopathological damage such as hepatic steatosis, necrosis, renal tubular degeneration, and gill lamella hyperplasia are frequently observed in target tissues such as the liver, kidney, and gill (Mona et al., 2023). These damages limit the organism's growth capacity by disrupting gas exchange and general homeostasis, and the multigenerational effects of chronic low-dose exposures can lead to reduced productivity at the population level (Paylar et al., 2024).

Hospital wastewater contains a variety of pollutants, such as organic pharmaceutical compounds, antibiotics, and disinfectants.

These compounds alter the oxidative stress responses of aquatic organisms. Studies on *D. magna* have shown that exposure to wastewater causes changes in antioxidant enzymes (such as CAT, GPx, and GST) and increases in MDA levels, a marker of lipid peroxidation. At the same time, elevations in cholinesterase activity and inflammatory marker levels indicated neurological and metabolic disorders (Afsa et al., 2022). Under chronic exposure conditions, reproductive failures, growth retardation, and significant changes in genotoxicity markers can occur in *Daphnia similis* (Tominaga et al., 2022). Morphological anomalies (edema, spinal curvatures) and histopathological changes (cell necrosis, apoptosis in liver and kidney tissues) can also occur in fish during embryonic development; these findings point to the synergistic effect of metal and organic pollutants combined with pharmaceuticals (Ishaq et al., 2023).

Mining wastes (acidic drainage, mine tailings) contribute a significant toxic burden to both aquatic and terrestrial ecosystems through heavy metals such as arsenic, mercury, lead, and cadmium. In fish species such as *Salmo trutta*, metal accumulation in tissues such as the gills and liver, increased lipid peroxidation, and oxidized protein carbonyl contents have been detected, and histopathological analyses have reported changes such as necrosis, vasodilation, and infiltration (Kurhaluka et al., 2025). High percentages of nuclear anomalies were detected in fish chronically exposed to heavy metals from mining, indicating significant genotoxic damage due to environmental wastes (Córdoba-Tovar et al., 2023).

General Consequences of Environmental Waste Toxicity and Ecological Threats

Toxicity caused by environmental wastes presents a multifaceted hazard that directly threatens global ecosystem health

by causing permanent damage to humans, animals, plants, and microorganisms.

Exposure to environmental wastes has critical consequences for human health. Sudden, high-dose exposure to wastes—as in industrial accidents or pesticide inhalation—results in acute poisoning. In such cases, severe skin and eye irritation, respiratory failure, neurological collapse, and multiple organ failure are common (Hernández et al., 2021). These acute consequences develop rapidly and require immediate medical intervention. In contrast, long-term, low-dose exposures produce chronic effects. Chronic exposure to heavy metals and pesticides, along with endocrine-disrupting chemicals, can lead to liver and kidney failure, immune suppression, and neurodegenerative disorders (Cao et al., 2023; Ramírez Ortega et al., 2021). Metals such as lead, mercury, and cadmium have direct neurotoxic effects, while pesticides and plastic additives affect the endocrine system by mimicking hormone receptors and increase the risk of reproductive health problems such as infertility, miscarriage, and polycystic ovary syndrome (Amir et al., 2021; Kawa et al., 2021). Epidemiological studies have revealed that chronic exposure to environmental waste causes epigenetic changes and long-term health problems. Benzene, arsenic, asbestos, dioxins, and polyaromatic hydrocarbons can lead to cancer through processes such as alterations in DNA methylation, oxidative DNA damage, and suppression of repair mechanisms (Van Horne et al., 2023). Furthermore, long-term exposure to PFAS and microplastics can result in metabolic syndrome, thyroid dysfunction, and immunodeficiency (Cserbik et al., 2023; Li et al., 2024). These findings suggest that exposure to environmental wastes can have both acute and chronic low-severity systemic consequences. Therefore, both controlling exposure sources and developing early toxicity biomarkers should be among the priority targets of environmental health policies (Fuller et al., 2022).

Animals are exposed to both the direct and indirect effects of environmental waste substances that disrupt population dynamics. Heavy metals and pesticides can cause gill and liver tissue damage, reproductive disorders, and population declines in fish (Dane & Şişman, 2017). From a toxicological perspective, the toxic effects of environmental wastes on organisms manifest first as organ dysfunction, then as systemic effects that compromise fitness, growth, reproductive potential, and survival. For example, oil spills can cause impairments in locomotion, stress responses, adrenal gland function, immune system function, and blood cell and blood cell functions in organisms such as fish, birds, and turtles; at the highest doses, they can trigger a series of reactions leading to multiple organ failure (Troisi et al., 2016; Takeshita et al., 2021).

The movement of environmental wastes, including pesticides, plastic waste, and persistent organic pollutants, up the food chain through bioaccumulation and biomagnification processes in ecosystems, causing serious toxic effects, particularly in organisms at higher trophic levels, is a significant problem. This accumulation results in neurotoxicity, immune system suppression, hormonal imbalance, and dramatic decreases in reproductive success (Saikumar et al., 2024).

Plants are considered the primary entry point for toxicity in ecosystems because they are the first living groups exposed to toxic wastes in soil and water. Heavy metals accumulated in soil significantly negatively impact plant nutrient uptake, photosynthetic capacity, and root development (Sperdouli, 2022). They disrupt ion balance in root cells, leading to the accumulation of ROS, which can lead to lipid peroxidation in the cell membrane through oxidative stress and ultimately DNA damage (Jawad et al., 2020). This leads to agricultural yield losses, the transfer of toxic elements into the food chain, and indirectly to problems for animal and human health (Gupta et al., 2021). Similarly, excessive pesticide use causes

increased antioxidant enzyme activity, decreased chlorophyll content, and mitochondrial dysfunction during the detoxification process in plants (Sule et al., 2022). Studies show that microplastics also pose a significant environmental threat to plants. Microplastics can cause mechanical stress by physically damaging the root cell wall and secondary toxicity through chemical additives on their surfaces (Qiu et al., 2021). Growth and development can be limited by changes such as reduced water and mineral uptake in the root system, chloroplast deformation, and photosynthetic insufficiency (Azeem et al., 2021).

Persistent Threats to Microbial Ecotoxicology and Environmental Cycles

Because microorganisms play a critical role in ecosystem functioning by forming the foundation of biological cycles, environmental wastes directly affect the diversity and functions of these organisms, threatening ecosystem services. Microorganisms are highly sensitive to environmental changes, and toxic wastes such as heavy metals, pesticides, drug residues, and microplastics can radically alter microbial community composition. This is critical for the collapse of ecosystem functions.

Toxic substances can cause mutations in DNA, leading to a decrease in genetic diversity in the long term. Furthermore, an increase in horizontal gene transfer (plasmid, transposon, integron) can lead to the spread of antibiotic resistance genes throughout the environment (Lin et al., 2024).

Heavy metal pollution reduces the diversity of beneficial bacteria and fungi in the soil, leading to disruptions in biological nitrogen fixation, organic matter decomposition, and nutrient cycling (Naz et al., 2022). Metals such as Cd, Pb, and Zn bind to the active sites of metalloenzymes in microbial enzyme systems, inhibiting catalytic activity. This results in decreased dehydrogenase,

phosphatase, and urease activities in the soil and a decrease in soil respiration rates (Samuel et al., 2012). On the other hand, under high metal concentrations, some symbiotic bacteria (e.g., *Rhizobium*, *Azotobacter*) and mycorrhizal fungi exhibit sensitivity, while some metal-tolerant strains (e.g., *Pseudomonas*, *Bacillus*) gain a selective advantage, resulting in changes in soil microbial community structure (Li et al., 2017; Riyazuddin et al., 2021). Disruption of microbial diversity directly results in the weakening of ecosystem functions and disruption of vital biogeochemical cycles. Heavy metals suppress nitrifying *Nitrosomonas* and *Nitrobacter* species, leading to a decrease in nitrate production, a slowdown in soil nitrogen mineralization, and a decrease in agricultural yields. This disrupts the nitrogen cycle, which is fundamental to agricultural yield and food security. In the long term, disruption of the nitrogen cycle poses a serious threat to global food security.

Pesticides can have direct toxic effects on microbial communities. Frequently used organophosphate and carbamate pesticides can suppress microbial carbon and nitrogen cycling enzymes and increase microbial mortality by increasing membrane permeability and lipid oxidation in the cell membrane (Bansal, 2015). Due to these effects, the metabolic activity of soil microbiota and soil fertility are reduced, indirectly affecting plant growth and ecosystem resilience (Ni et al., 2025). When pesticides and organic solvents suppress the activity of lignocellulose-degrading fungi and bacteria (*Trichoderma*, *Actinobacteria*), organic matter degradation is slowed, thereby disrupting the carbon cycle. In the long term, increased greenhouse gas emissions may occur because soil carbon sequestration capacity decreases. Similarly, a decrease in phosphate-solubilizing bacteria leads to phosphorus scarcity for plant development, while the loss of sulfate-reducing bacteria (*Desulfovibrio*) disrupts the sulfur cycle and energy flow in anaerobic ecosystems (Raklami et al., 2021).

Research indicates that microplastics cause significant changes in microbial ecology. Microplastics can disrupt intercellular signaling and gene transfer by altering biofilm formation on microbial cell surfaces (Miao et al., 2021), and additive chemicals and adsorbed heavy metals found on the surface of microplastics can lead to oxidative stress and DNA damage (Qin et al., 2023). These changes not only compromise the survival of microorganisms but also plant nutrition, soil health, and overall ecosystem functions, ultimately leading to indirect impacts on plant, animal, and human health.

The current toxicity of environmental wastes on microbial systems provides insight into future environmental and public health risks:

• With a decrease in microbial diversity, ecosystems lose their natural purification capacity (self-purification), and the oil, pesticide, or metal load in polluted waters becomes unable to spontaneously degrade, meaning the pollution becomes persistent.

• Drug residues and microplastics accelerate the transmission of resistance genes. In the future, when these genes are transferred to pathogenic bacteria such as *Clostridium*, *Klebsiella*, and *Candida*, losses in agricultural production and untreatable infections in humans and animals may occur (Shankar & Balasubramanium, 2014).

• Disruption of microbial cycles creates a feedback loop that accelerates global warming by increasing greenhouse gas (CO_2, CH_4, N_2O) emissions.

This section demonstrates with scientific data that toxicity caused by environmental waste is not a singular problem but rather a multifaceted, bioaccumulative, global threat that fundamentally disrupts ecosystem functions. Toxicological effects cover a wide spectrum, from acute toxicity to significant health problems such as

neurotoxicity, carcinogenic risks, and endocrine disorders, as well as ecosystem collapse scenarios such as disruption of biological cycles and the antibiotic resistance crisis. Oxidative stress, genotoxicity, and histopathological damage caused by heavy metals, pesticides, and organic solvents threaten population sustainability in all living groups. Furthermore, the ability of wastes such as micro- and nanoplastics to cross biological membranes and accelerate horizontal gene transfer carries the potential to transform the environment into a "resistance gene bank" that is difficult to reverse. In this context, toxicity management requires not only cleaning up pollution but also preserving the natural purification capacity of ecosystems (self-purification).

To reduce risks and ensure long-term environmental sustainability, radical and holistic approaches to toxicological risk management should be adopted:

• Zero discharge can be targeted for industrial and hospital wastewater; advanced treatment technologies—such as membrane bioreactors and advanced oxidation processes—can be mandated to neutralize pharmaceutical residues and resistance genes.

• The use of toxic chemicals in agriculture and industry should be replaced with less harmful alternatives; integrated pest management and biological control methods can be promoted.

• Bioremediation and phytoremediation methods can be activated to protect soil microbiota and restore the functionality of the nitrogen and carbon cycles.

• Contaminants such as microplastics and nanoplastics can contribute to environmental They can be classified as hazardous waste in legislation, and their use and disposal can be updated accordingly.

• Occupational health and safety protocols can be implemented for workers in the agricultural and mining sectors to prevent exposure to toxic substances, and the use of protective equipment can be made mandatory.

• Public awareness can be increased and toxicological risk education can be disseminated to change consumer behavior (such as limiting the use of chemical-containing detergents and reducing plastic consumption).

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TRENDS IN FRUIT WINES

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Today, due to the wide range of aromas and flavors that fruits possess, wine production from fruits other than grapes is carried out in academic and industrial fields. One of the appealing aspects of fruit wines is the diversity of their compounds. Due to their rich content of health-promoting compounds, fruit wines are gaining recognition as functional foods. Another factor encouraging the production of fruit wines is their ability to extend the shelf life of products and increase their nutritional value. The production process for fruit wines is similar to that of grape wines, but various adjustments are made depending on the content of the fruit used. Therefore, these adjustments are made according to the condition of the fruit used before the fermentation process begins. Fruit wines are composed of volatile (alcohols, esters, fatty acids) and non-volatile (organic acids, amino acids, sugars, phenolic compounds) components from a chemical perspective. The type of fruit used and the fermentation process play an effective role in the ratio and variety of these compounds. A recent trend in fruit wines was identified through bibliometric analysis. The bibliometric analysis's findings indicate that fruit wines have been the subject of a significant

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increase in research, particularly since the 2000s. The most productive countries are China, the United States, Spain, and Italy, while Turkey has been among the countries increasing their publication productivity in recent years. In conclusion, depending on the type of fruit used, fruit wines are likely to find a wide place for themselves in academic and industrial fields in the future due to their broad aroma profiles, functional component contents, and positive effects on health.

Fruit Wines

Fruit wines are alcoholic beverages produced using fruits other than grapes, with their own unique flavors and aromas. In addition to their unique flavors, they attract consumers' interest due to their rich nutritional content. For this reason, the variety of wines produced in this field is increasing. However, fruits have a wide range of aromas and flavors due to their variety and different nutritional compounds (Tan et al., 2024; Velic et al., 2018; Zeng et al., 2025). Unlike grape wines, fruit wines are named after the fruit used to make them(Tan et al., 2024). Due to the prominence of the functional food properties of the fruits used in fruit wines, fruit juice production facilities have found their place in the industrial sector. Indeed, this situation also contributes to minimizing losses in these facilities (Velic et al., 2018). It is particularly important that products that ripen quickly are included in the production process as soon as possible. If these foods are not included in the processing process quickly, they may spoil. This can result in significant product loss. The use of fruit wines not only prevents fruit loss but also preserves the nutritional value of the food. In addition, the nutritional value of fruit wines is increased through microbial activity (Jagtap & Bapat, 2015). The benefits of fruit wines (or fermentation) for producers (Jagtap & Bapat, 2015; Swain et al., 2014; Yang et al., 2021);

- Enabling food to be stored for long periods by utilizing the preservative properties of fermentation,
- The fermentation process results in the production of numerous aromatic compounds due to the activity of microorganisms, enabling fruit wines to acquire different flavors and tastes. In addition, it causes changes and increases in nutritional values,
- Reducing post-harvest product loss and creating an alternative source of profit,
- One of the reasons for producing fruit wines is to create a new market environment in terms of economics, health, and taste by using fruits that have very low economic value and do not appeal to a wide range of sectors. For example, Chinese chestnuts are a fruit with many health benefits (such as nourishing the body's internal organs and its positive effect against hypertension). Currently, the processing of chestnuts relies on heating methods, which negatively affect their nutritional value. Therefore, the aim is to improve and preserve their nutritional value by incorporating them into the fermentation process (Li et al., 2021).
- The fermentation process encompasses a complex process in which biotransformation occurs, substrates are converted into different forms, and consequently, the nutritional value of foods increases. At the same time, it acquires probiotic properties due to the microorganisms it contains (Marco et al., 2017).

Fruits Used in Fruit Wine Production

Fruit wine is made from a wide variety of fruits today. The most commonly used fruits are apples, pears, apricots, kiwis, pineapples, plum, blueberries, bananas, orange, mazhanje, masau,

and strawberries (Saranraj et al., 2017; Tan et al., 2024; Tarko & Duda, 2024; Zeng et al., 2025). Although the basic production process for fruit wines is similar, they are prepared for fermentation with some basic adjustments depending on their sugar content, acidity, and water content. In addition, enzyme additions may be necessary to improve the quality of wines. This is because enzymes derived from fruits or microorganisms may lose their effectiveness in the fermentation environment. Therefore, enzymes such as pectinase, B-glucosidase, or protease must be added to the fermentation environment. Indeed, the addition of these enzymes improves the quality of the wine, helps it become clearer, and contributes to its aromatic richness (Yang et al., 2021). Fruit wine production process are similar to those in grape wine. These stages include, in order, the preparation of the fruit, the preparation of the must, the fermentation process, and the aging process (Tan et al., 2024).

Preparation of Fruits

One of the most important elements of this process is the quality of the selected fruit. Indeed, the first parameter that contributes to the aroma and flavor of fruit wines is the selected fruit(Velic et al., 2018). There are some differences depending on the fruit or fruit-based product used. The first step in preparing the fruit is harvesting it from the field. The process begins with the harvest of fruit that has reached the appropriate ripeness. After this stage, the fruit must be cleaned and washed. This is because various contaminants may be present in products harvested from the field. Another important point is the removal of products that are not suitable for wine production. After sorting, the skins of some fruits must be peeled. Therefore, the skins must be peeled, or if there are seeds, the seeds must be removed. After cleaning, sorting, and crushing, the fruits are pressed to extract their juice. Thus, the preliminary processes before preparing the must are completed(Tan

et al., 2024). Sometimes, this sorting process is not necessary, and the must is prepared by washing and extracting the juice from the fruit. For example, the skins and seeds of black grapes are not removed and remain in the must throughout the fermentation process. This allows the pigments and other components to pass into the fermentation environment (Saranraj et al., 2017).

Preparation of the Must

It is preferable that the fruits used in fruit wines contain an appropriate amount of sugar and have low acidity levels. However, if these criteria are not met, the necessary adjustments are made (Velic et al., 2018). During the preparation of the must, the pH, sugar, and water levels must be adjusted to suitable values for fermentation. This is because some fruits may have low water and sugar content. Therefore, the sugar and water content must be adjusted to suitable levels. In addition, the pH value must also be adjusted, as the pH value of fruits varies (Tan et al., 2024; Velic et al., 2018). Depending on the acidity level of the fruit used for fruit wine, various substances are added to adjust the pH. This is because some fruits have a very low pH value, which can cause the wine to have a sour taste. To prevent this situation, calcium carbonate is added to adjust the pH value(Tan et al., 2024). However, harvesting fruits with excessive pigment content before they are fully ripe helps to reduce pigment levels. Indeed, excessive pigment content can affect the color of the wine and cause residues to form during bottling. This can negatively affect consumer preferences (Velic et al., 2018). Finally, the pasteurization stage comes next. Before yeast inoculation, the must must be pasteurized to prevent the growth of unwanted microorganisms (Tan et al., 2024).

Fermentation

The fermentation process is initiated by using appropriate yeasts. The most commonly used yeast type is *S. cerevisiae*. Today,

non-*Saccharomyces* species are also widely used. Different yeasts are also used to achieve different aromas and flavors (González et al., 2018; Larroque et al., 2021; Li et al., 2021). Indeed, in addition to the unique aromas and flavors of the fruits themselves, microorganisms make an important contribution to the aroma and flavor of fruit wines (Tan et al., 2024). Microorganisms and fruits alone are not sufficient to enhance the aroma and quality of wine. Fermentation conditions must also be appropriate. Therefore, temperature, pH, and sugar parameters must also be appropriate. For example, the fact that alcoholic fermentation generally occurs at low temperatures helps to produce ester compounds (Saranraj et al., 2017).

Aging

This process sometimes continues with a few steps of aging and transfer to another tank. This increases the clarity of the wine and allows small particles to settle. Filters are used to remove unwanted residues. In addition, pasteurization is applied to prevent spoilage (Saranraj et al., 2017). After the fermentation process is complete, the aging process begins, as it does with grape wines. During this process, changes occur in the acidity, aroma, and flavor of the wine (Tan et al., 2024).

Chemical Composition of Fruit Wines

The chemical components of fruit wines are basically divided into two groups: volatile organic and non-volatile compounds. Non-volatile compounds include sugars, organic acids, amino acids, and secondary metabolites. Volatile compounds are mainly formed from compounds converted by microorganisms during the fermentation process. The most common of these are alcohols and esters. These compounds affect the aroma and flavor of wines. The chemical composition of wine can vary depending on the type of fruit and the fermentation process. This is an important factor in the use of fruit

wines in the wine industry (Feng et al., 2015; Ivanova et al., 2012; Tarko & Duda, 2024). Fruit wines contain a wide variety of acid compounds in different forms. These acids have a significant effect on the taste and aroma of wines. Commonly found acid compounds include malic acid, citric acid, tartaric acid, and lactic acid (Zeng et al., 2025).

Volatile Organic Compounds

The compounds predominantly found in this group are alcohols, esters, and fatty acids. As with grape wines, the quantity and variety of compounds contained in fruit wines are analyzed in order to evaluate their aroma and taste. This allows for a more accurate assessment of the taste and flavor of the wine produced, as each compound has its own unique taste and aroma (González et al., 2018; Sumby et al., 2010; Tarko & Duda, 2024). Depending on the amount of these compounds in fruit wines, consumers can perceive different aromas such as fruity, earthy, or floral. Indeed, the amounts of volatile organic compounds (VOCs) can vary in different fruit wines (Liu et al., 2023; Tarko & Duda, 2024). The number of volatile organic compounds produced by fruit wine varies greatly. Indeed, analysis of mango has revealed the presence of numerous ester compounds (40) and various alcohols (15). This demonstrates the significant contribution that alcoholic fermentation makes to fruit wines (Pino & Queris, 2011). Similarly, it was determined that the main component of wines made from different fruits (raspberry, strawberry, and mulberry) is alcohol. It was stated that the dominance of alcohols among aromatic compounds was remarkably high. The alcohol found in the highest concentration is 3-Methyl-1-butanol. When considering the numerically occurring compounds, it has been determined that the number of alcoholic compounds (raspberry= 15, strawberry= 13, and mulberry= 13) is greater than that of ester compounds (raspberry= 9, strawberry= 9, and mulberry= 12). In addition, the percentage of alcohols, which

constitute the majority of the aromatic compounds produced, exceeded 90% (Feng et al., 2015). This shows that fruit wines have different compositions and aromas. It allows for greater variety in fruit wines and the creation of new fruit wines. The number and intensity of aromatic compounds produced will vary depending on the fruit used.

Non-volatile Compounds

One of the components that affect the aroma and taste of fruit wines is the organic acids they contain. The amount of organic acids can vary depending on the climate and temperature in which the fruit is grown. In addition to affecting the taste and aroma of wine, organic acids also have a significant effect on aging and the stability of wine (Samarasekara et al., 2018). Organic acids are the main source for determining the acidity level of wines. Malic acid and tartaric acid constitute the most dominant group among organic acids (Cioch-Skoneczny et al., 2021). Malic acid, it is one of the most common organic acids found in fruit wines. Generally, it has a heavy and harsh taste, so it is converted to lactic acid during malolactic fermentation. This process is carried out to obtain a milder taste (Zeng et al., 2025). High levels of malic acid are not desirable. This is because it provides a source of certain compounds needed by microorganisms that cause wine to spoil and lose its flavor (Cioch-Skoneczny et al., 2021).

Nutritional Value and Health Benefits

Many fruits found in the mountains naturally contain phenolic compounds. These compounds have antioxidant effects (Molole et al., 2022). Among the plant-derived components with antioxidant activity are polyphenols, carotenoids, and vitamins (Xu et al., 2017). Antioxidants found in natural foods are preferred to minimize the effects of free radicals. These food-based, non-synthetic antioxidants help minimize the damage that free radicals

can cause. For this reason, various fruits used in wine production are preferred to achieve this goal (Feng et al., 2015; Lobo et al., 2010; Molole et al., 2022). Various experimental studies have shown that when consumed in moderation, it has a positive effect on human health (such as cardiovascular diseases and neurological disorders). Polyphenols, found particularly in wine and classified as flavonoids and non-flavonoids, have numerous effects, such as reducing LDL oxidation and atherosclerosis. For example, polyphenols such as resveratrol have effects on inhibiting LDL oxidation. It also plays a role in regulating lipid metabolism. One of wine's effects on lipid metabolism is that it increases HDL levels, while causing LDL levels to decrease. Moreover, among the positive effects of wine on human health, it can regulate immune responses and help reduce problems such as cardiovascular diseases, antiplatelet activities, endothelial function, atherosclerosis, hypertension, cancer, and type 2 diabetes (Guilford & Pezzuto, 2011).

Trends in Fruit Wines

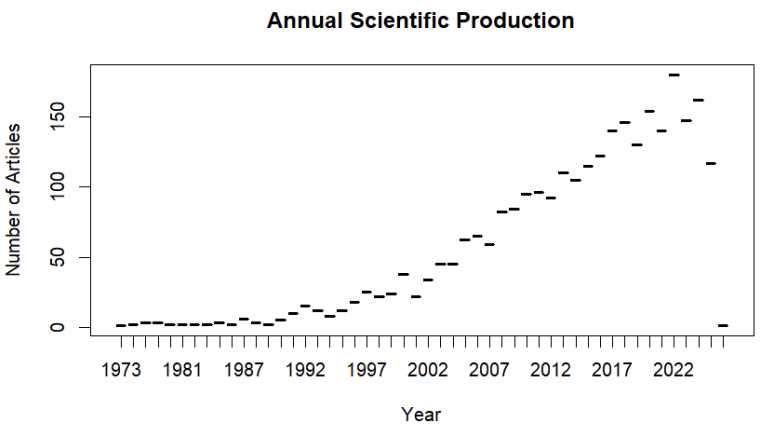
Web of Science was used to identify trends in fruit wines. Articles were analyzed for trends based solely on WoS. WoS has been chosen because it is one of the most important and largest databases in the scientific world. Indeed, it contains many studies and journals in the academic field. For this reason, WoS is an important resource for researchers. As with any database, in order to search the Wos database, it is necessary to select keywords related to the relevant topic. In this context, a search was conducted using the keywords "*TS=((fruit wine OR wine OR fruit) AND (apple wine OR Malus domestica wine OR cherry wine OR sour cherry wine OR Prunus cerasus wine OR blueberry wine OR Vaccinium wine OR pomegranate wine OR Punica granatum wine OR peach wine OR Prunus persica wine OR pineapple wine OR Ananas comosus wine OR strawberry wine OR "Fragaria wine" OR mango wine OR Mangifera indica wine OR pear wine OR Pyrus wine OR blackberry*

wine OR raspberry wine OR Rubus wine)) " related to fruit wines. This search produced 3326 results. These results include many types of documents such as articles, books, and reports. After this selection, only articles were considered (N=2886). Finally, English is selected as the language (N=2772). After this stage, the data was downloaded in "Plain Text File" (*.txt*) format. The data was analyzed in R.

Scientific Production

When considering the distribution of research conducted on fruit wines (Table 1), an upward trend can be observed over the years. In particular, a rapid increase can be observed after the 1994. In contrast, a more stable trend continued in the period before the 2000s. The highest number of publications was reached in 2022 (f=180, 6.49%). However, the upward trend on an annual basis continues. Considering the percentage distribution by years, the distribution was below 1% in the years prior to 1999 (Highest percentage value is 0.90%). As of 2015, the distribution of percentage values has changed between 4.15% and 6.49%.

Figure 1. Annual scientific production by year



Country Production

The distribution of the ten countries with the highest productivity levels is shown in Table 1, which was prepared to determine the productivity values of countries by year. To reveal the cooperation between countries individually and with other countries, the number of publications SCP (Single Country Publications) and MCP (Multiple Country Publications) has been arranged comparatively (Table 1). As shown in Table 1, the top 10 most productive countries are listed (China, USA, Spain, Italy, India, Brazil, France, Turkey, Germany, and Korea). In terms of publication productivity, China (f=455), USA (f=338), Spain (f=253), and Italy (f=213) are among the dominant countries. In percentage terms, the productivity of these countries is 16.86%, 12.53%, 9.38%, and 7.89%, respectively. In other words, it comprises 46.63% of all publications made by all countries. However, the least productive countries among the top ten are Turkey (f=82, 3.04%), Germany (f= 76, 2.82%), and Korea (f=76, 2.82%). In the comparison of countries' productivity in terms of SCP, China (f=382), the USA (f=264), and Spain (f=210) are seen to be in the top rankings. However, when considering the MCP Ratio, the highest values are 0.22 (USA), 0.21 (France), and 0.17 (Spain), respectively. In other words, it shows that they rank first among the countries that cooperate most with other countries. The least cooperative countries are India (0.0612), Turkey (0.0610), and Korea (0.0526).

Table 1 Country production

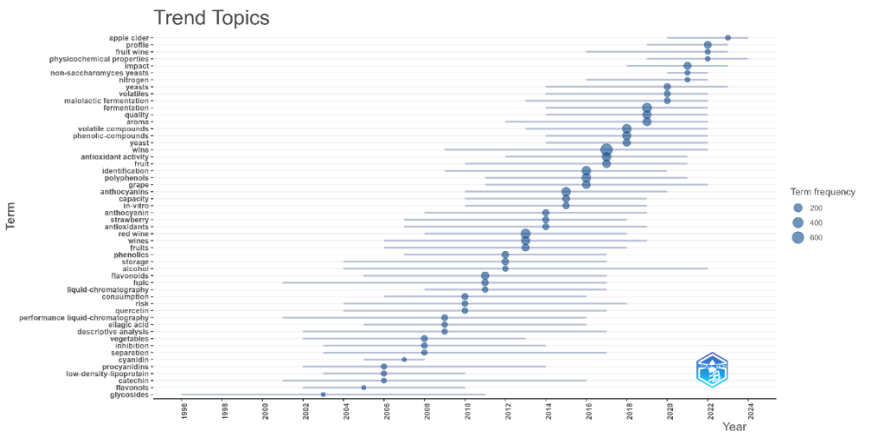
Country	Articles	%	SCP	MCP	MCP Ratio
1 CHINA	455	16.86	382	73	0.1604
2 USA	338	12.53	264	74	**0.2189**
3 SPAIN	253	9.38	210	43	**0.1700**
4 ITALY	213	7.89	177	36	0.1690
5 INDIA	98	3.63	92	6	0.0612
6 BRAZIL	92	3.41	79	13	0.1413
7 FRANCE	90	3.34	71	19	**0.2111**
8 TURKEY	82	3.04	77	5	0.0610
9 GERMANY	76	2.82	64	12	0.1579
10 KOREA	76	2.82	72	4	0.0526

The top ten journals in which researchers publish the most articles on fruit wine are as follows: Journal of Agricultural and Food Chemistry, Food Chemistry, lWT-Food Science and Technology, Foods, Food Research International, Journal of the Science of Food and Agriculture, Molecules, Journal of Food science, American Journal of Enology and Viticulture, and European Food Research and Technology (Table 2). Among the top ten journals, the most preferred journal by researchers is Journal of Agricultural and Food Chemistry (f=178), and the least preferred are American Journal of Enology and Viticulture (f=39) and European Food Research and Technology (f=39).

Table 2. Journals with the most publications

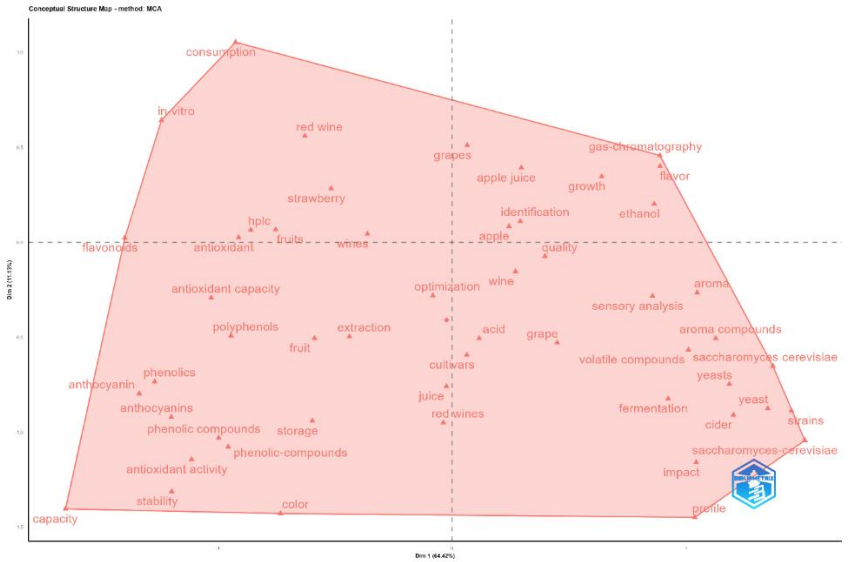
Sources	Articles
1 JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY	178
2 FOOD CHEMISTRY	176
3 LWT-FOOD SCIENCE AND TECHNOLOGY	90
4 FOODS	70
5 FOOD RESEARCH INTERNATIONAL	56
6 JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE	51
7 MOLECULES	51
8 JOURNAL OF FOOD SCIENCE	50
9 AMERICAN JOURNAL OF ENOLOGY AND VITICULTURE	39
10 EUROPEAN FOOD RESEARCH AND TECHNOLOGY	39

Figure 2. Trends topics by year



From 1996 to 2011, the concept that was primarily focused on was glycosides. However, it can be said that the frequency of the word was low. Research between 2010 and 2006 appears to have focused on *low-density lipoprotein, catechin, flavonols, cyanidin*, and *procyanidin*. The concept of *catechin* has been among the concepts that have been given priority from 2000 to 2016. Similarly, the concept of *procyanidin* has also been widely preferred by researchers during these years. In contrast, *cyanidin* has been used within a narrow time span (2005-2008). The most frequently used words in research between 2002 and 2018 were: *phenolics, storage, alcohol, flavonoids, HPLC, liquid chromatography, consumption, risk, liquid chromatography, quercetin, performance liquid chromatography, ellagic acid, descriptive analysis, vegetables, inhibitor*, and *separation*. However, it is noticed that the concepts of *flavonoids, performance liquid chromatography,* and *quercetin* are among the concepts with the highest weight in the specified time period. As 2020 approaches, concepts that are gaining more focus; *phenolic-compounds, yeast, wine, antioxidant activity, fruit, identification, polyphenols, anthocyanin, antioxidants*, and *capacity*. In recent years, it can be said that concepts such as *aroma, quality, profile, non-saccharomyces yeasts, volatile compounds, nitrogen*, and *malolactic fermentation* have begun to feature prominently in publications. In other words, it shows that researchers have focused on studies aimed at improving aroma and quality in recent years.

Figure 3. Conceptual structure map



Multiple Correspondence Analysis (MCA) was used to reveal the patterns of co-occurrence of concepts found in the literature. The most significant contribution of the analysis is to identify clusters of the most frequently used keywords or concepts in studies, taking into account the research in which they are used. This enables a more accurate understanding of the relationships between the topics studied. As shown in the Figure 3, concepts can be grouped under five headings according to their areas of convergence; Phenolic & Antioxidant area (bottom left), Consumption & biomedical (top), Aroma & sensory quality (top right), Fermentation & biotechnology (bottom right), and Center & intersection area. Central concepts (wine, grapes, apple, quality): The core topics of the field are connected to all other clusters Because the focus of the research is fruit wines. Chemical/antioxidant studies and Fermentation/aroma studies, which explain a large majority of the variance (64.42%), are seen to

be related to each other. The second dimension (11.51%) is *consumption/health effects* and *technological quality/stability*.

Conclusion

Fruit wines are produced using fruits other than grapes and are made by including them in the fermentation process in the same way as grapes. Today, they attract the interest of industrial, academic, and amateur producers. In other words, there has been a trend towards sources other than grapes in recent years. One of the most important advantages of the fruits used in fruit wines is that they offer different flavor and aroma profiles. However, depending on the choice of different fruits, fruit wines are also considered functional foods because they contain phenolic compounds, vitamins, organic acids, and antioxidants. Therefore, by including different fruits in the fermentation process, they go beyond classic wines. Due to their functional food properties, they can meet consumers' demands for both taste and health benefits.

The production process of fruit wines is similar to that of grape wines, but the water, sugar, and acid levels vary depending on the content and structure of the fruit used. This necessitates certain adjustments before the fermentation process begins. This is because some fruits do not have sufficient water and sugar content like grapes. In particular, factors such as enzyme additions, pH adjustments, and appropriate yeast selection must be considered to enhance aroma and product quality. This is because these factors directly contribute to product quality, similar to fruit variety. Another factor that affects product quality, especially during the fermentation process, is temperature. This is because fermentation carried out at low temperatures leads to an increase in the formation of ester compounds. One of the factors that determine wine quality and aroma is the aging period and process. This is because the aging period can play a decisive role in shaping the clarity, acid balance,

and aromatic structure of wines. Therefore, methods and technologies aimed at improving the quality of fruit wines will show an increasing trend in the coming years. In industrial and academic fields, the use of new products with functional food value, along with aroma and taste, in wine production will become more widespread.

Fermented beverages produced from fruit wines (especially alcoholic beverages) not only satisfy the community's taste preferences but also elevate fruit wines to the category of functional foods. Specifically, chemicals like polyphenols, flavonoids, and resveratrol have the ability to neutralize free radicals, which may help avoid cardiovascular illnesses, boost the immune system, and lessen neurological issues. However, consumption must be balanced in order for them to effectively demonstrate or reveal the specified health benefits.

Studies on fruit wines have accelerated, especially during the 2000s, while examining scientific production and research trends. China, the US, Spain, and Italy are the top four countries in this sector, according to Web of Science data, while Turkey has recently made more scholarly contributions. The research's main subjects include enhancing scent and quality, preserving phenolic compounds, using various yeast strains, antioxidant activity, and consumer preferences. According to these results, fruit wines will likely be the focus of future studies from a scientific and production technology standpoint. According to future projections, assessing microbial diversity—especially using biotechnological methods—will greatly enhance wine quality. Furthermore, fruit wines are essential for lowering post-harvest losses and turning fruits into products with added value in the modern food business, where sustainability is becoming more and more significant. They are unique in this regard as a product category that offers both financial and ecological advantages.

In conclusion, fruit wines have become a rapidly growing field today due to their diverse aromatic and flavor profiles, production techniques, rich nutritional components, numerous positive effects on human health, economic contributions, and increasing interest in academic research. In future research, fruit wines will not remain merely an alternative beverage to grape wines. They are also likely to gain further importance as an alcoholic beverage product of strategic importance from both a scientific and industrial perspective. In addition to further developing their aroma and flavor, it will also enable research efforts aimed at preserving the components that give them their functional food properties and revealing their health effects through clinical studies.

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ENZYME-BASED CANCER THERAPIES: MOLECULAR APPROACHES AND CLINICAL APPLICATIONS

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Introduction

Significant progress has been made in cancer treatment thanks to studies conducted from the past to the present. Treatment strategies have advanced toward targeted and personalized methods that offer alternatives to traditional approaches. Among these modalities, enzyme-based therapies stand out as a highly innovative strategy that targets the metabolism of cancer cells while causing minimal damage to healthy cells and fewer side effects compared to conventional treatments.

Although many enzymes are being studied for enzyme-based cancer therapies, the number of enzymes used in clinical treatment

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remans limited. L-asparaginase, which is used clinically to treat acute lymphoblastic leukemia, is the best known of these enzymes.

L-asparaginase (EC 3.5.1.1.1) targets tumor cells by hydrolyzing L-asparagine, which is required for protein synthesis in leukemic cells (Díaz-Barriga et al., 2021; Tsegaye et al., 2024). The arginase enzyme may suppress growth in arginine-dependent tumors by targeting arginine consumption (Feng et al., 2025). The use of glutaminase enzyme also offers promising results, especially in aggressive tumors and hematological malignancies (Gross et al., 2014).

Catalase is an essential enzyme that protects cells from oxidative stress by breaking down hydrogen peroxide into water and oxygen and has shown potential for cancer therapy (Bauer and Graves, 2016). The horseradish peroxidase (HRP) enzyme can be used in enzyme-prodrug systems to convert prodrugs into toxic agents, enabling targeted low-toxicity therapy (Greco et al., 2000). Laccases, a copper-containing oxidase, shows anticancer effects such as inducing apoptosis and DNA damage in various cancer cells due to its capacity to oxidize phenolic compounds (Elsaba et al., 2023).

Enzyme-based therapies are becoming increasingly important in cancer treatment. Enzymes are used alone or in combination with traditional methods. However, clinical use remains limited for many enzymes. The short half-life of enzymes in clinical use and the fact that some require high doses to be effective increase the risk of toxicity and limit their use. With ongoing efforts to overcome these and other limitations, enzymes will become safer, more effective, and clinically applicable biotherapeutics in cancer treatment. In this chapter, the molecular mechanisms, clinical applications, and challenges of enzymes with therapeutic activity in cancer treatment are discussed.

L- Asparaginase

L-asparaginase (EC 3.5.1.1.1) is an important therapeutic enzyme used in the treatment of specific hematological malignancies, namely acute lymphoblastic leukemia (ALL) and lymphomas. ALL is a hematological cancer characterized by the uncontrolled proliferation of immature lymphocytes. L-asparaginase is one of the most widely used agents in the treatment of ALL and is approved by the FDA for this indication (Díaz-Barriga et al., 2021; Tsegaye et al., 2024). First discovered in 1922 as an enzyme with anticancer properties (El-fakharany et al., 2020). This enzyme catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia, eliminating the source of asparagine critical for protein synthesis in leukemic cells (Díaz-Barriga et al., 2021). Since lymphoblasts do not express asparagine synthetase, these cells must take up extracellular asparagine to survive. L-asparaginase therefore reduces the availability of L-asparagine and inhibits the proliferation of cancer cells (Tsegaye et al., 2024). Several studies have highlighted the enzyme's potential not only in pediatric oncology but also in the treatment of other malignancies, including some solid tumors where asparagine dependence plays a role (Brumano et al., 2019).

Isolated from guinea pig serum in the 1950s, L-asparaginase has been a key component of modern ALL therapy since the 1970s, with forms later purified from *Escherichia coli* and *Erwinia chrysanthemi* (Pokrovsky et al., 2016). L-asparaginases from *E. coli* and *E. chrysanthemi* show 77% structural similarity in terms of amino acid sequences and have similar pharmacological properties (Alexandrova et al., 2022). L-asparaginases from these two sources also possess L-glutaminase activity. Although this provides an additional antitumor effect, it can also be associated with serious toxicities. Therefore, the clinical use of L-asparaginase is often

limited by side effects such as hypersensitivity reactions, hepatotoxicity and development of resistance (Tsegaye et al., 2024).

Despite its short half-life, L-asparaginase is associated with complications such as hypersensitivity, antigenicity and undesirable L-glutaminase-dependent neurotoxicity, necessitating the development of more suitable alternatives (Tsegaye et al., 2024). Current studies are focused on developing modified L-asparaginase variants to reduce these adverse effects while maintaining therapeutic efficacy, and to obtain them from different sources (Andrade et al., 2014). In particular, these variants with reduced glutaminase activity may improve treatment safety by targeting the elimination of side effects associated with existing therapies (Nguyen et al., 2018). Genetically engineered enzyme variants have been shown to improve the therapeutic profile by limiting side effects while maintaining potent antitumor efficacy (Nguyen et al., 2018).

Natural enzymes from *E. coli* and *E. chrysanthemi* and their PEG-conjugated preparations are the form most frequently and safely used in clinical practice (Alexandrova et al., 2022). The primary treatment for most ALL patients is PEG-conjugated *E. coli* L-asparaginase. Unfortunately, many patients develop hypersensitivity reactions to these preparations. These reactions are associated with immune responses against the enzyme or the formation of neutralizing antibodies (silent inactivation) (Modi and Gervais, 2022). Glutaminase activity is also known to play a role in toxic effects such as hyperglycemia and pancreatitis (Radadiya et al., 2020). Although the enzymes in clinical use are similar, native L-asparaginase from *E. chrysanthemi* is immunologically distinct from PEG-conjugated *E. coli* L-asparaginase. Therefore, in patients allergic to PEG-conjugated *E. coli* L- asparaginase, natural *E. chrysanthemi* L- asparaginase or its recombinant form, available from July 2021, was preferred as an alternative (Tsegaye et al.,

2024). Indeed, some findings suggest that glutaminase activity may not be essential for therapeutic efficacy (Chan et al., 2014).

L-asparaginase remains a key agent in the treatment of various hematologic malignancies, especially ALL, due to its unique mechanism of action against L-asparagine-dependent cancer cells. Despite limiting factors such as immunogenicity, short half-life, development of resistance and metabolic toxicities due to glutaminase activity, ongoing research aims to overcome these limitations to improve clinical efficacy and provide safer treatment options. These efforts are likely to reshape the future role of L-asparaginase and similar enzyme-based agents in cancer treatment, clearly demonstrating the ongoing need for innovation and individualized treatment in oncological therapeutics.

Arginase

Arginine is an amino acid that many tumor cells require for growth and proliferation (Al-Koussa et al., 2020). Arginine plays a critical role in the proliferation of tumor cells and their escape from the immune system. Some cancer cells are unable to biosynthesize arginine due to their low expression of arginosuccinate synthetase (ASS1), making them dependent on external sources for arginine (Delage et al., 2010). This metabolic vulnerability constitutes a target for arginine deprivation therapies. Certain types of cancers have been shown to rely on the source of arginine, and it has been suggested that arginine reduction may be a potential treatment for these types of cancer, called arginine auxotrophic cancers (Feng et al., 2025).

The enzyme arginase is an enzyme containing manganese (Mn^{2+}). Hydrolyzes L-arginine to L-ornithine and urea (Caldwell et al., 2015). Arginase is a ubiquitous enzyme found in bacteria, yeasts, plants, invertebrates and vertebrates. (Dzik, 2014). In mammals, the enzyme arginase exists in two isozyme forms, arginase 1 (ARG1)

and arginase 2 (ARG2). Although both are functionally similar, their coding genes, tissue distribution, subcellular localization and molecular regulation are different (Niu et al., 2022).

Recently, basic and clinical studies have shown that arginase is also highly expressed in various cancers such as breast, gastric, colorectal and liver cancers. Arginases are recognized as a promising target in cancer therapy by targeting arginine metabolism (Niu et al., 2022). Studies showing potential anti-tumor activity of arginase 1 in advanced hepatocellular carcinoma patients are available in the literature. (Yau et al., 2013). Studies have shown that arginase inhibitors inhibit the growth of lung carcinoma. High levels of arginase I have been found in tumor samples from patients with non-small cell carcinoma. (Rodriguez et al., 2004). One of the important applications of arginase is in solid tumors such as tumors of myeloid origin and melanoma. Studies have shown that after injection of an arginase inhibitor or genetic disruption of the arginase enzyme leads to a reduction in tumor growth, thus the arginase enzyme is pro-tumorigenic (Rodriguez et al., 2004). A study shows that arginase inhibition with agents such as CB-1158 blunts myeloid cell-mediated immune escape and reduces tumor growth by reducing immune suppression (Steggerda et al., 2017). This study also suggests that arginase blockade with CB-1158 may be an effective treatment in many types of cancer and that combining it with chemotherapy or other immunotherapies may provide significant clinical responses (Steggerda et al., 2017).

However, there are some challenges that arginase-based therapeutic approaches face in clinical practice. The most important limitations include the short half-life of the enzyme in mammals and the risk of systemic toxicity as high doses must be administered daily to be effective (Barzkar et al., 2025). To overcome these problems, systems have been developed in which the enzyme is bound to polyethylene glycol (PEG), resulting in low antigenicity, low

toxicity and extended circulating half-life (Yau et al., 2013). In Phase I studies, PEGylated arginase (Peg-rhArg1) induced dose-dependent arginine depletion in patients with advanced hepatocellular carcinoma, showing promising antitumor activity (Yau et al., 2013).

In conclusion, therapeutic strategies targeting arginine metabolism are emerging as an important treatment approach, especially in arginine auxotrophic tumors. Arginine-metabolizing enzymes, such as arginase, have attracted attention for their direct suppression of tumor cell growth as well as their potential to reshape the immune response. However, challenges such as short half-life, systemic toxicity and immune modulation are important factors limiting the clinical use of these agents. Bioengineering approaches such as PEGylation show promise in reducing these limitations and increasing therapeutic efficacy. Future comprehensive clinical trials will further clarify the feasibility and clinical utility of arginase-based therapies alone or in combination with immunotherapies and chemotherapy.

Gulutaminase

Glutaminase (GLS) is an enzyme that catalyzes the conversion of glutamine to glutamate, which is critical for cellular energy and biosynthesis. This reaction, together with the production of glutamate, which plays a key role in many cellular processes, is important for cell proliferation and cell survival (Anthony et al., 2024). Glutamine metabolism is one of the metabolic pathways that can be successfully targeted in cancer therapy (Halama Suhre., 2022). Glutaminase exists in two different isoforms, GLS1 (kidney-type glutaminase) and GLS2 (liver-type glutaminase), depending on the site of secretion. Cancer cells, especially rapidly dividing types, become dependent on glutamine to meet their increased energy and biosynthetic needs. Compared to GLS2, GLS1 plays an important role in tumorigenesis with its overexpression in aggressive cancer

types to meet the high glutamine demand (Yu et al., 2021). Re-expression of GLS2 silenced by promoter methylation in liver cancer, colorectal cancer and glioblastoma has been shown to have tumor suppressor activities (Altman et al., 2016).

Allosteric inhibitors of GLS have shown promise in preclinical cancer models and CB-839, a highly potent compound in this class, is a strong candidate to move into clinical trials (Gross et al., 2014). A preclinical compound, bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide (BPTES), is an inhibitor of GLS (Gross et al., 2014). CB-839 has shown antiproliferative activity against triplenegative breast cancer in preclinical studies (Gross et al., 2014). Studies have shown that the use of glutaminase inhibitors such as BPTES sensitizes cancer cells resistant to paclitaxel and cisplatin to chemotherapy (Masamha and LaFontaine, 2018).

In conclusion, glutaminase is a clinically targetable enzyme that plays a central role in cancer metabolism. The discovery of glutaminase as a therapeutic target reflects a significant advance in the understanding of cancer metabolism, and ongoing research is likely to expand the potential applications of glutaminase inhibitors in various cancer types. This holistic view of targeting glutaminase is essential in formulating new strategies to effectively combat cancer.

Catalase

Catalase (EC 1.11.1.1.6) is an essential antioxidant enzyme that protects cells from oxidative damage by catalyzing the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen (O_2). Catalase is a common antioxidant enzyme found in all aerobic organisms (Sharma and Ahmad, 2014). Although cancer cells are adapted to high levels of reactive oxygen species (ROS), excessive accumulation of ROS can trigger apoptotic signals in these cells. Therefore, the relationship between catalase and ROS is particularly

important in the context of cancer therapy (Glorieux and Calderon, 2017).

The role of catalase in cancer is dual: While protecting cancer cells from oxidative stress by preventing apoptosis, especially in the early stages of tumorigenesis (Bauer and Graves, 2016), high antioxidant defense mechanisms may also prevent carcinogenesis (Pongsavee, 2022). This paradoxical situation causes catalase modulation to present both challenges and opportunities in therapeutic strategies. For transformed cells to escape intercellular signaling that would normally trigger cell death, i.e. tumor progression, requires the expression of membrane-bound catalase (Bauer and Graves, 2016). This protective mechanism may hinder the efficacy of therapies aimed at inducing oxidative stress in tumor cells. Modulation of catalase levels therefore presents both challenges and opportunities in therapeutic strategies aimed at reducing tumor growth and improving patients' survival outcomes. Studies have shown that high antioxidant defenses, including the enzyme catalase, can prevent carcinogenesis (Pongsavee, 2022). A study has shown that exogenous catalase administration reduces the aggressiveness of tumor cells in lung adenocarcinomas (de Oliveira et al., 2016). A different study showed that intravenous administration of catalase significantly inhibited the development of metastatic tumors (Hyoudou et al., 2006). *In vivo* studies have revealed that catalase activity may be an effective strategy for the prevention of lung metastasis (Nishikawa et al., 2002). It has also been reported that catalase may support the efficacy of chemotherapeutics and contribute to the reduction of potential side effects (Glorieux and Calderon, 2017).

The tumor microenvironment plays a critical role in tumor progression and increases resistance to conventional therapies. Hypoxia is common in solid tumors. When tumor cells do not receive enough oxygen, they adapt to the hypoxic state by

establishing genetic differences before going to cell death. In cancer therapy, catalase aims to block the tumor cell-promoting effects of oxidative stress, particularly by reducing the accumulation of high levels of H_2O_2 in the tumor microenvironment (Wan et al., 2022). A possible candidate for O_2 production and attenuation of hypoxia in the tumor microenvironment is the utilization of the H_2O_2 molecules produced. Catalase enzyme helps attenuate hypoxia by increasing O_2 production in the tumor microenvironment (Wan et al., 2022).

Studies have shown that catalase is characterized by high catalytic specificity and activity; however, poor stability and limited cellular transit limit the therapeutic potential of this enzyme (Najafi et al., 2022). Research is focusing on nanocarrier technologies and liposome-loaded forms of catalase to improve and maintain the biological activity of catalase and ensure its intracellular targetability (Shi et al., 2020).

Clinical applications of catalase are still at an early stage. Catalase and similar antioxidant enzymes may offer new perspectives in cancer treatment, especially in combination with conventional therapies. In the future, more preclinical and clinical studies are needed to make catalase-based therapeutic approaches more specific, safe and effective.

Horseradish Peroxidase

Horseradish peroxidase (HRP, EC 1.11.1.7) is a heme-containing enzyme isolated from the roots of *Armoracia rusticana* and used in immunological applications for many years (Veitch, 2004). Enzyme-drug strategies for targeted cancer therapy have historically been of interest in the context of targeted therapies and there are many examples of prodrugs used in this context (Wardman, 2002). HRP-based enzyme-prodrug systems offer a targeted approach to cancer therapy. The enzyme-prodrug system is based on a tumor-specific targeted enzyme converting a systemically

administered inactive prodrug into an active one (Napier et al., 2000). HRP is advantageous in this strategy because it can rapidly oxidize small molecule substrates into toxic products. The HRP enzyme can also be specifically targeted to tumor tissue and then a systemically administered prodrug is converted by the enzyme into active chemotherapeutic agents. For example, prodrugs such as indole-3-acetic acid (IAA) can be converted into cytotoxic radical species via oxidative decarboxylation in the presence of HRP and H_2O_2 (Greco et al., 2000). IAA produces free radicals when oxidized by HRP, which can damage cell membranes, DNA and other cellular components, leading to apoptosis. A study shows that the delivery of HRP to human tumors followed by IAA treatment may provide a novel cancer enzyme prodrug therapy approach with the potential to target hypoxic cells (Greco et al., 2000).

HRP can be functionalized on nanoparticles targeting cancer cells. For example, in one study, HRP conjugated to gold nanoparticles (HRP-AuNCs) was shown to cause cancer cell death by activating the prodrug IAA. In the study, the HRP/IAA system showed high efficacy in killing cells in both 2D and 3D breast cancer models and significantly reduced tumor cell viability. With nanoformulation, it can overcome the limitations of conventional therapies by improving enzyme stability and delivery to tumor cells (Vivo-Llorca et al., 2022).

In summary, the application of HRP in cancer therapy exploits its enzymatic oxidation ability to locally activate prodrugs to cancer cell toxic agents and shows significant potential as a targeted and low-toxicity therapeutic strategy in both nanoparticle-based drug delivery and gene therapy contexts. HRP's substrate diversity, oxidizing capacity and ability to interact with the tumor microsystem have placed it in an important position among therapeutic enzymes.

Laccase

Laccases (EC 1.10.3.2) are copper-containing oxidase enzymes of wide interest in biotechnology and medicine for their capacity to oxidize phenolic, aromatic and various organic compounds (Dana et al., 2017).

Their ability to oxidize various phenolic and non-phenolic compounds using molecular oxygen offers a unique mechanism that can be used in therapeutic strategies against cancer. Recent studies have shown promising results with laccase's ability to selectively kill cancer cells, induce apoptosis and cause DNA damage in various cancer cell lines while sparing normal cells (Kale Bakir et al., 2025).

Studies with laccase from different sources (e.g. *Trametes versicolor*, *Cerrena unicolor*) have shown significant cytotoxic and antiproliferative effects in thyroid, endometrial, ovarian, colon and breast cancer cell lines (Sondej et al., 2025; Kale Bakir et al., 2025; Pigon-Zajac et al., 2025). In some studies, laccase has also been reported to exert a stronger inhibitory effect on cancer cells compared to some standard chemotherapy agents, without causing serious toxicity in healthy cells (Elsaba et al., 2023).

Although the current findings are promising, the vast majority of studies on the anticancer potential of laccase remain at the *in vitro* cell culture level or at the preclinical stage with a limited number of animal models (Sondej et al., 2025). Molecular pathways, potential side effects and clinical efficacy in humans have not yet been fully elucidated. Therefore, increasing enzyme stability, optimizing tumor targeting strategies and improving pharmacokinetic properties are required to enhance the therapeutic use of laccase. Current research suggests that formulation approaches, such as transporting laccase in nanoparticles, immobilizing it or reconstituting it by genetic engineering methods,

may accelerate its transition to clinical use in the future (Chauhan et al., 2019).

Conclusion

Enzyme-based cancer therapies have become an important part of targeted and personalized medicine. Besides classical enzymes such as L-asparaginase, new candidates such as arginase, glutaminase inhibitors and antioxidant enzymes show promise in targeting metabolic vulnerabilities of cancer cells. Bioengineering approaches, such as nanocarrier systems and PEGylation, extend the half-life of these enzymes, increasing their efficacy and reducing side effects.

However, challenges such as immunogenicity, toxicity and resistance development remain. Future studies should focus on the development of more specific enzyme variants and combinations of enzymes with chemotherapy and immunotherapy. In this way, enzyme-based therapies may find a wider application in cancer treatment and become a safer and more effective option for patients.

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NOVEL APPROACHES IN EPIGENETIC MECHANISMS AND BIOLOGICAL RESEARCH

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Introduction

Epigenetic mechanisms play as key regulators of gene expression, enabling heritable alterations in gene activity without changes to the DNA sequence itself. These changes are often influenced by environmental conditions, lifestyle factors, aging, and pathological states. In recent decades, epigenetics has emerged as a rapidly advancing field, with growing insights derived from human and plant population research, as well as in vitro and in vivo experimental models, enhancing our comprehension of their biological significance (Clark & Rager, 2020). Insights into these processes have facilitated the development of innovative strategies

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in medicine, biotechnology, and scientific research (Al Aboud et al., 2018; Ostaiza-Cardenas et al., 2025).

Epigenetic regulation is a key factor in these adaptations, contributing to both heritable and non-heritable modifications that may persist across generations (Tresas et al., 2025). Although plants and animals (humans) have long diverged evolutionarily, many epigenetic components remain conserved; that is, they utilize similar molecular pathways. At the same time, biological differences such as life cycle, germline segregation, and reproductive modes alter the implementation and effectiveness of these mechanisms.

Epigenetics regulates chromatin state dynamics through principal mechanisms (Dai et al., 2024). These principal epigenetic mechanisms involved include DNA methylation (CpG), histone modifications (H3K4me3, H3K27me3, etc.), the activity of ubiquitin ligase complexes, chromatin remodeling, and the function of regulatory non-coding RNAs (siRNA, miRNA) (Clark & Rager, 2020; Tresas et al., 2025). Thanks to these similarities, plants are used as model organisms in epigenetic research (e.g., *Arabidopsis thaliana*). Plant studies contribute to the understanding of epigenetic mechanisms in humans, such as stress response, aging, gene silencing, and epigenetic memory (D'Urso & Brickner, 2017).

Epigenetic regulation mechanisms and functions

Epigenetic changes are proposed to serve as a crucial role between environmental exposures-chemicals, infections, aging, and genetic susceptibility in disease pathogenesis. In autoimmune conditions, such modifications can play a direct role in disease progression by regulating immune system activity (Bagni et al., 2025). The regulation of gene expression plays a central role to the survival and reproduction of all living organisms, as it adaptation to environmental stresses (Altayli, 2020).

DNA methylation involves the addition of a methyl group (CH_3) to cytosine bases in DNA (usually in CpG islands), leading to the silencing or regulation of gene expression. DNA methylation typically represses gene expression when occurring in promoter regions; global hypomethylation is lead to genomic instability. Important in development, X-chromosome inactivation, imprinting, and tumor suppression (Łach et al., 2024). One of the most studied epigenetic regulation mechanisms in plants is 5-methylcytosine (5mC) methylation; however, in recent years, N^6 adenine DNA methylation (6mA, or N6-methyl deoxyadenine) has also begun to attract attention (Liang et al., 2018; Liang et al., 2020; Wu, 2020). 6mA is formed by the attachment of a methyl group at the N^6 position of adenine. This modification has long been well known, particularly in prokaryotes; now, there are a growing number of studies on its presence, abundance, regulation, and functional effects in eukaryotes including plants (Zhou et al., 2018; Wu, 2020). Plants alter 6mA levels under stress; these changes can have important effects such as stress tolerance, gene expression, and developmental phenotypes. As gene regions 6mA is found in TEs and promoters in plants. However, the precise role of 6mA in stress perception, signal transduction, and post-stress adaptation (especially stress memory and transgenerational effects) has not yet been fully determined. 6mA is present in human DNA at very low levels (~0.0001–0.00001%) it is more prevalent in mitochondrial DNA and some heterochromatic regions. However, its function is still largely under investigation. In humans, it plays roles such as gene regulation, the expression of cancer-related genes, and embryonic development. Some studies have also suggested that 6mA may play a role in neurological processes in human brain tissue. However, the precise physiological and pathological roles of 6mA in humans remain controversial (Xie et al., 2023).

Histone modification refers to the post-translational modifications of particular amino acids, which subsequently affect the structure of histone proteins. Modifications affect how tightly DNA is wound. These changes can loosen or tighten chromatin structure, thus affecting transcription (Bure et al., 2022). Histone modifications, changes include such as acetylation, methylation, phosphorylation, ubiquitination and, less frequently, ribosylation, sumoylation, and citrullination on amino acids such as lysine and arginine in histone proteins. These modifications serve as signals to recruit or repel transcription machinery. Roles varies depending on the specific modification. Acetylation typically activates transcription while methylation can either activate or repress, depending on the specific amino acid residue and extent of methylation. Phosphorylation plays role in chromosome condensation during cell division, DNA damage repair, and transcriptional regulation. In addition, ubiquitination involves DNA damage response. Monoubiquitylation of H2A is associated with gene silencing, while H2B ubiquitination correlates with transcription activation (Sherif et al., 2025). Ubiquitination, entail chemical alterations to histone proteins that influence chromatin structure and gene accessibility (Acharjee et al., 2023). On the other hand, ADP-ribosylation is a reversible post-translational modification that is involved in many cellular processes (including cell signaling, DNA repair, gene regulation and apoptosis). Sumoylation induces alterations in the protein's surface properties or conformational structure, thereby modulating its interactions with other macromolecules (Fuhrmann & Thompson, 2016). Moreover, citrullination, which is also known as deimination, is one type of irreversible histone modification that leads to a reduction in hydrogen-bonding and a looser chromatin structure.

Chromatin remodeling, dynamic modification of chromatin architecture through repositioning, ejection, or restructuring of

nucleosomes (Murawska & Brehm, 2011). This mechanism either activate or repress gene expression by altering DNA accessibility to transcription factors and other regulatory proteins (Sherif et al., 2025). Nucleosome repositioning is a crucial aspect of the epigenetic regulation of gene expression. Chromatin remodelers alter nucleosome spacing by sliding nucleosome core particles (NCPs) along the DNA or by removing or replacing histones within the NCP and also regulate various cellular processes, including transcription and DNA repair, by modulating the accessibility of genomic DNA (Morgan et al., 2021).

RNA modifications, including naturally occurring chemical alterations such as 5-methylcytidine (m5C) and pseudouridine (Ψ) (Cohn, 1960; Delaunay et al., 2024). Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into proteins, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Non-coding RNAs (ncRNAs) play crucial roles in the regulation of gene expression, including chromatin remodeling, RNA splicing, protein synthesis, cell differentiation, and development, functioning as either activators or repressors through diverse molecular mechanisms. One of the ncRNAs is miRNAs predominantly suppress gene expression by inhibiting translation or promoting mRNA degradation, whereas lncRNAs often modulate transcription by interacting with chromatin-modifying complexes. In addition to the well-characterized transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), the ncRNA repertoire also includes small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), Piwi-interacting RNAs (piRNAs), and circular RNAs (circRNAs), each contributing uniquely to the complexity of post-transcriptional and epigenetic regulation (Sherif et al., 2025). These mechanisms, along with their associated enzymes, transmit genetic information independently of the DNA

nucleotide sequence and are crucial for regulating development and maintaining physiological homeostasis (Dai et al., 2024).

Epitranscriptomic modifications are chemical modifications of mRNA, tRNA, and other RNAs e.g., N6-methyladenosine (m6A) are an increasingly studied area and have RNA-specific regulatory functions (Ruden, 2025). The effects of m6A and other RNA modifications on gene control, how they change in diseases, and their potential therapeutic targets are a growing area of interest. Epitranscriptomic modifications, especially m6A play a pivotal role in the regulation of gene expression by modulating RNA metabolism, including its stability, splicing, and translational efficiency but also in processes such as cell fate, stress response, and tumor development (Liu et al., 2025; Smith et al., 2025). Selecting or editing plants based on their 6mA profiles could help produce more resistant varieties. Furthermore, 6mA, as an epigenetic marker, could serve as a molecular indicator of stress adaptation in agriculture. Targeting 6mA with gene editing systems such as CRISPR/dCas9 may also be possible in the future. In addition, RNA-directed therapies such as RNAi (siRNA, shRNA), antisense oligonucleotides (ASO), and locked nucleic acids (LNA) are used to modulate gene expression at the epigenetic level (Amirmahani et al., 2025a, b).

Epigenetic inheritance during cell division/replication is great importance about how DNA methylation and histone marks are transferred, maintained, and degraded on the cell's new DNA strands/chromatin structures during cell division (Almouzni & Cedar, 2016).

Recent awareness and modern epigenetic technologies in humans and plants

Major roles of epigenetic mechanisms include cell differentiation and development, X-chromosome inactivation,

genomic imprinting, response to environmental factors (diet, stress, toxins), cancer and disease tolerance mechanisms. In plants, epigenetic changes (especially methylation) can be transmitted across generations; germline differentiation is late; epigenetic resetting is more limited (Quadrana & Colot, 2016). With the understanding of epigenetic mechanisms, new technologies and therapeutic strategies have emerged that target these mechanisms (Dai et al., 2024). In addition, various current approaches and techniques have been used to elucidate epigenetic mechanisms in humans and plants in recent years (Li, 2021). The field of epigenetics is now gaining a strong foothold not only as a "gene expression regulator" but also as a treatment target, diagnostic criterion for agricultural productivity, enriched nutrient sources, and biomarker source (Zhang et al., 2022; Muroddinova, 2025; Song & Li, 2025; Xue et al., 2025).

Priming mechanisms "Epigenetic Preparedness"

A recent study has examine the ways cells "prime" genes before signals trigger gene expression arrive. These priming mechanisms are respectively, Priming; the activating state of specific gene promoters or regulatory regions to respond more quickly to future signals, Reining; maintaining a high activation barrier; that is, preventing premature or excessive activation of genes, Transcriptional memory; genes that have been activated in the past respond more quickly or strongly when stimulated again, Transcriptional tolerance; the "habitual" or tolerance-induced decrease in gene activation after a strong or persistent stimulus; for example, to prevent excessive reactions in inflammatory responses. Topics such as the persistence of these preparatory states between cell divisions, dynamic changes in regulatory barriers, and gene selectivity are still among the areas of intensive study (Xiong & Zhu, 2025).

Histone variants, chromatin dynamics, and structural organization

New data continues to emerge regarding the genome regions where histone variants (e.g., H3.3 vs. H3.1) are used, how they are stored, and their effects on chromatin organization. It has been reported that the boundaries of histone variants H3.1/H3.3 are associated with early replication sites, and chaperone proteins play a critical role in this distribution (Smith et al., 2025). Chromatin topology, or the three-dimensional chromatin structure, is considered an important modulator for the stability of epigenetic organization and its response to change. For example, during the relocalization of histone-modifying enzymes (e.g., sirtuins) during stressful situations such as DNA damage, the folding/integrity of chromatin can help maintain epigenetic marks.

DNA methylation & active turnover cycles

DNA methylation has been shown to be more than just a "fixed repressive mark," but rather to create spatiotemporal patterns through the active cycling of methylation-demethylation activities. For example, some models propose that long-range chromatin interactions, due to genome heterogeneity, create locally synchronized "turnover domains". DNA methylation changes are used as powerful biomarkers for aging, disease development (especially cancer and neurodegenerative diseases), and biological age measurements (epigenetic clocks) (Olmeda et al., 2025).

Therapeutic approaches for epigenetic profiling

Following the understanding of epigenetic mechanisms, new techniques and therapeutic strategies targeting or exploiting them are rapidly advancing (Sherif et al., 2025).

Single-cell epigenomics research epigenetic changes at individual cell level important in cancer, stem cells (Berkel & Cacan,

2019; Casado-Pelaez et al., 2022). To date, the majority of studies involving single-cell omics and multi-omics techniques have focused on proof-of-concept investigations, with reports detailing their application across different areas of cancer research only recently starting to appear (Berkel & Cacan, 2019). Single-cell mono-omic technologies for investigating cancer epigenomics represent an approach that characterize complex biological systems by looking individual cells. Single-cell methodologies are crucial for accurately investigating the intrinsic complexity of tumors and for dissecting cellular heterogeneity across multiple dimensions. Since the introduction of single-cell RNA sequencing (scRNA-seq), the transcriptome has emerged as the most extensively studied molecular layer within single-cell research frameworks (Casado-Pelaez et al., 2022).

Multi-omics approaches consist of a range of disciplines, including genomics, epigenomics, transcriptomics, proteomics, glycoproteomics, glycomics, metabolomics, and lipidomics, among others, offering a comprehensive analysis of the associated biomolecular profiles (Gutierrez-Reyes et al., 2024). Omics represent an interdisciplinary research domain that aim to comprehensively understand biological systems by examining their components and interactions as integrated wholes, rather than in isolation. This field prioritizes the application of high-throughput technologies to generate large-scale molecular data, which are subsequently integrated and analyzed using computational tools to advance the understanding of complex biological processes. The integration of omics technologies into natural product research has significantly advanced the field by facilitating high-throughput screening, expediting the discovery of novel bioactive compounds, and elucidating complex biological interactions within producing organisms (Sahana et al., 2025). A holistic approach to cancer research fosters transformative progress by providing a

comprehensive understanding of the disease. A popular application of next-generation sequencing (NGS) is epigenomic profiling, which provides a mechanistic context for genome regulation in cancer (Sherif et al., 2025).

Spatial epigenomics involves the investigation of the spatial organization of epigenetic modifications within the genome and their impact on gene expression and cellular function, playing a critical role in cancer biology by elucidating how epigenetic alterations contribute to tumor progression and metastasis (Liu et al., 2024; Olmeda et al., 2025).

Long-read sequencing technologies provide novel opportunities for investigating complex epigenetic patterns and detecting structural genomic variations with greater accuracy and resolution.

Nanopore sequencing represents a robust approach for the direct detection of DNA modifications, bypassing the conventional requirement for bisulfite conversion in methylation analysis, and enabling the simultaneous capture of both genetic and epigenetic data to advance our understanding of gene regulatory processes (Lucas & Novoa, 2023). Recent advancements in nanopore technologies for sequencing individual long DNA and RNA molecules have significantly enhanced accuracy, read length, and throughput. These improvements have necessitated the development of sophisticated experimental protocols and bioinformatics tools to fully leverage nanopore long-read data for comprehensive analyses of genomes, transcriptomes, epigenomes, and epitranscriptomes. Nanopore sequencing is increasingly utilized in applications such as genome assembly, full-length transcript identification, base modification detection, as well as specialized fields including rapid clinical diagnostics and infectious disease outbreak monitoring (Wang et al., 2021).

CRISPR-based epigenome editing and screening technologies have been adapted for precise epigenome editing, manipulation, and high-throughput screening of epigenetic regulators, allowing researchers to modify specific epigenetic markers at targeted genomic loci (Sherif et al., 2025). CRISPR dCas9 systems partially inactive Cas9 variants ("dead Cas9") allow for the modification of epigenetic marks at specific gene loci by adding effector domains such as methyltransferase (DNMT), demethylase (TET), histone acetyltransferase/deacetylase, and histone methyltransferase/demethylase without binding to DNA (Cai et al., 2023; Smith et al., 2025). Furthermore, different Cas protein derivatives, such as dCas12 and dCas13, are now being used for RNA-directed modifications or targeting different targets (Huang et al., 2025). These approaches can be reversible, meaning they do not alter the DNA sequence, making potential side effects more manageable (Huang et al., 2025). TALE and zinc finger protein (ZFP)-based systems are also being used in a similar manner (Lavoro et al., 2025).

The growing complexity of epigenomic data has driven the development of sophisticated computational approaches. This rapidly evolving area is crucial for interpreting vast amounts of complex data generated by modern epigenomic studies. These methodologies are essential for extracting meaningful insights from large-scale multidimensional epigenomic datasets in cancer research. Machine learning and artificial intelligence have increasingly been employed in the analysis of epigenetic data, with deep learning models such as convolutional neural networks (CNNs) and recurrent neural networks (RNNs) demonstrating efficacy in predicting epigenetic states and their regulatory effects on gene expression in cancer, using DNA sequence information (Zhang & Wang, 2024).

Epigenetic biomarkers

Epigenetic biomarkers are used for early diagnosis of cancers, neurodegenerative disorders, and metabolic diseases (Martínez-Iglesias et al., 2021). Epigenetic biomarkers (cf (methylated) DNA, histones, and miRNAs) analyzed from multiple biospecimens (i.e. liquid biopsy, fresh tissue, and FFPE tissue) may allow simultaneous conduction of diagnosis and targeted therapy, therefore, contributing to theragnosis and precision medicine (Skinner, 2024).

Pharmacological interventions; epigenetic drugs (epi-drugs) and combination strategies

Epigenetic-drugs target epigenetic enzymes, e.g., DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors are currently the most widely used classes (El Bahhaj, 2014; Miranda Furtado et al., 2019). Additionally, new small molecules targeting histone methyltransferases or demethylases, such as EZH2 inhibitors, are being developed (Eich, et al., 2020; Cantone et al., 2022). Agents that target more specific enzymes, such as histone methyltransferase/demethylase inhibitors (e.g., EZH2 inhibitors) and PRMT inhibitors, are used both to increase sensitivity to existing treatments and to overcome resistance mechanisms. They exhibit effects in cancer treatment, particularly in reactivating suppressed tumor suppressor genes and altering the tumor microenvironment (Prabhakaran et al., 2024).

Nanotechnology–based drug delivery systems

Nanoparticles can be used for targeted drug delivery to the site of disease, increasing the absorption of poorly soluble drugs, targeting drugs to a specific site, and increasing drug bioavailability. There are studies on nanoparticles, liposomes, and other delivery

systems for the specific distribution of epigenetic modulatory drugs to target tissues or cell types (Li et al., 2024; Islam et al., 2025).

Timing and cell-specific control

Optogenetic tools offer the opportunity to control the timing of epigenetic regulation through light-controlled domains (Day, 2014; Polesskaya, et al., 2018). There is work to develop regulators that use cell-type-specific promoters or target tissue-specific delivery systems (Lee et al., 2025).

Diagnostic tools and age measurement (epigenetic clocks)

Epigenetic clock models (DNA methylation, CpG sites, etc.) are used to estimate differences between biological and chronological age and to assess disease risk (Smith et al., 2025). In recent years, various fields have emerged with the increasing number of individual-specific epigenetic studies. Biological age, which reflects a particular organism's state, functional capabilities, social well-being, and risk of premature death from various causes, often does not coincide with chronological age. Therefore, there is a growing number of studies on the development of specific DNA methylation patterns, called "epigenetic clocks" (ECs), to determine the biological age and rate of aging of a specific individual (Kiselev et al., 2025; Smith et al., 2025).

Combination therapies and personalized epigenetic therapy

Combination therapies using epigenetic drugs in conjunction with immunotherapy, chemotherapy, etc.; in some models, epigenetic modulators also directly modulate the immune response (Smith et al., 2025). Clinical and preclinical studies on the combination of epigenetic drugs with conventional chemotherapy, immunotherapy, etc. are increasing. Creating a patient-specific epigenetic profile and tailoring treatment accordingly (precision medicine approach) is of particular importance. The combination of

epigenetics and chemotherapy aims to enhance DNA damage, cell death, and synergy; the combination of epigenetics and immunotherapy aims to enhance immune response and overcome resistance; and the combination of epigenetics and targeted therapy aims to reverse resistance (Majchrzak-Celińska et al., 2021; Navakauskienė, 2023).

Lifestyle, environment, plant epigenetics and drug development (plant biotechnology – human applications)

Nutrition, exercise, stress management, and environmental factors influence epigenetic marks; however, the implications of these effects for long-term health outcomes are increasingly being studied (Jose Ostaiza-Cardenas et al., 2025). Furthermore, psychological interventions such as mindfulness and meditation can influence biological age and health outcomes by altering the epigenetic patterns of genes linked to stress and inflammation (Jose Ostaiza-Cardenas et al., 2025). Plant research has substantially advanced the field of epigenetics through key discoveries such as paramutation, parental imprinting, nucleolar dominance, and the RNA-directed DNA methylation (RdDM) pathway (Kumar & Rani, 2023; Wu et al., 2025). Plants, as sessile organisms, are continuously exposed to environmental stresses, including abiotic (e.g., drought, salinity, extreme temperatures, heavy metals) and biotic (e.g., pathogens, herbivores) stressors. To survive and adapt, plants have developed complex regulatory networks, and epigenetic mechanisms play a crucial role in this process (Miryeganeh, 2021; Leisner et al., 2023; Du et al., 2024). Epigenetic regulation plays various roles in plants. Some of these are stress response (drought, salinity, and temperature), regulation of flowering time, seed development, transposon silencing, defense against pathogens and adaptation to environment (transgenerational memory) (Abdulraheem et al., 2024; Essemine et al., 2024). In recent years, various current approaches that modulate and deepen plant gene

expression and stress tolerance mechanisms have become increasingly important (Mishra et al., 2021).

Nutraceutical and pharmaceutical products are being developed by increasing the production of natural compounds with epigenetic effects (e.g., resveratrol, apigenin, luteolin) in plants (Zhang et al., 2018). Epigenetically modified plants can be designed as personalized nutrition and epigenetic therapy support products. Possibility of transgenerational effects, stress-induced epigenetic changes in plants can be transmitted to subsequent generations (transgenerational epigenetic inheritance). This mechanism is still limited and controversial in humans, but it is thought that some environmental effects can be epigenetically transmitted to children (Hossain et al., 2025). These model systems in plants can be used to understand the mechanisms of transgenerational effects in humans (Miryeganeh, 2021).

Some plant components and phytochemicals (e.g. flavonoids, polyphenols, isothiocyanates) taken from through diet effect human epigenetics (Mansoor et al., 2025). Sulforaphane, sourced from broccoli and cabbage, has been found to inhibit DNMT and HDACs and can activate tumor suppressor genes (Kaufman-Szymczyk et al., 2015; Hyun, 2020). Genistein, sourced from soybeans, has been found to alter the expression of cancer-related genes by affecting DNA methylation patterns (Sharifi-Rad et al., 2021; Naponelli, et al., 2025). Epigallocatechin gallate (EGCG), sourced from green tea, has been reported to inhibit DNMT and HDAC activity and activate cell cycle regulatory genes (Singh et al., 2011; Dabre et al., 2025). It has been determined that curcumin, which is sourced from turmeric, plays a regulatory role on histone modifications and miRNA profile (Boyanapalli et al., 2015; Ahamad et al., 2017; Bakrim et al., 2025). These compounds do not originate directly from plant epigenetics, but from the plants' own metabolic products. However, these components may have pharmacoepigenetic effects on human

epigenetics. Epigenetically enhanced plants (with more antioxidants, vitamins, and lower allergen content) may positively impact human epigenomic responses. Moreover anthocyanin-enriched tomatoes → regulatory effect on aging-related epigenetic pathways.

Epigenetic changes in plants are indirectly related to the soil microbiome, plant-microorganism interactions, and ultimately the human gut microbiota. The microbial composition of agricultural products may influence epigenetic regulation in the human gut (Rubas et al., 2025).

Plant epigenetics and its effect on humans can be summarized as follows: Phytochemicals → epigenetic regulators DNMT, HDAC, etc. may affect enzyme activity. Plant RdDM, miRNA, and lncRNA studies also help understand common epigenetic pathways, and epigenetically modified foods may contribute to epigenetic regulation through nutrition (Zhang et al., 2018).

Mechanistic similarities and differences in plant and human epigenetics

DNA methylation motifs in plants are CG, CHG, and CHH (non-CpG methylation is common in plants). In humans and animals, CpG contexts generally predominate; non-CpG methylation is seen in a limited number of cell types or contexts (e.g., root/neuronal cells). Both have DNA methyltransferase (DNMT)-like enzymes in common, and methylation is used for gene repression, transposon control, and developmental regulation (Fresnedo-Ramírez, et al., 2023).

Genomic imprinting in plants is the expression of dominant maternal or paternal alleles in the endosperm. Plants have imprinting and related methylation pathways. However, mammals (and especially humans) have a very widespread imprinting system, important for development, growth, and metabolic balance. DNA

methylation, histone modifications, and ncRNAs play a role in imprinting in both, but the evolutionary differences in the imprinting gene list and control patterns are significant (Köhler et al., 2012).

DNA methylation-directed pathways are more limited, lack RdDM-like pathways or operate in different ways in humans while RdDM is a powerful pathway unique to plants (Fresnedo-Ramírez et al., 2023).

Epigenetic inheritance / transgenerational epigenetics mechanisms play critical roles in processes such as development, tissue differentiation, cell type-specific expression, environmental influences, diseases (e.g., cancer), and aging in humans. But in plants, epigenetic regulation is crucial in areas such as developmental processes, adaptation to environmental stresses, immunity (pathogen interactions), phenotypic plasticity, and transposable element control (Williams & Gehring, 2020; Fresnedo-Ramírez et al., 2023).

From an application and human health perspective, plant epigenetics studies inform and contribute to human epigenetics research. Model systems, plants (especially *Arabidopsis thaliana*) facilitate the dissection of genetic and epigenetic mechanisms. Because humans have complex systems and ethical constraints, learning from plants provides fundamental principles. Epigenetic medicine/nutrition strategies, Natural compounds (plant compounds) that drive methylation/histone pattern changes in plants are being investigated; this provides insights into epigenetic therapy or nutrition-based interventions using similar compounds in humans.

Technology and methodology sharing, as methods such as DNA methylation mapping, histone modification analyses, and chromatin restructuring techniques are developed in plants, similar techniques are also being applied to the human genome. This can provide benefits in terms of precision, output quality, etc.

Conclusions

Comparative genomic and epigenomic studies are important in large-scale comparisons of plant and animal epigenomes (especially the location, frequency, and function of epigenetic markers) and need further development. Parallel studies of epigenetic responses to factors such as environmental stress, nutrition, and toxin exposure in both groups are needed (e.g., DNA methylation or ncRNA changes in plant and human samples under similar stress conditions). Potential use of plant compounds in epigenetic therapies; further investigation of the effects of dietary epigenetic interventions on human health is needed. Stress-induced epigenetic changes in plants can be passed on to subsequent generations (transgenerational epigenetic inheritance). This mechanism in humans is still more limited and controversial, but it is thought that some environmental influences may be epigenetically transmitted to children. therefore These model systems in plants can be used to understand the mechanisms of transgenerational effects in humans.

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CELLULAR ROLES AND MEDICAL APPLICATIONS OF PROTEASES

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Introduction

Enzymes are biological catalysts, usually protein-based, that increase the speed of chemical reactions. They significantly accelerate reaction rates by providing an alternative reaction pathway with lower activation energy. Enzymes bind to their substrates and catalyze the reactions. The substrate binds to the active site of the enzyme and is converted into products at the end of the reaction. Then, enzymes remain unchanged and return to their original state, ready to bind to new substrates to catalyze subsequent reactions. Enzymes are vital for all stages of biochemical reactions in living cells. They have the ability to catalyze reactions both intracellular and extracellular.

In the enzyme market, enzymes are generally classified as industrial enzymes and specialty enzymes based on their application areas, production/usage volumes, and economic values. Industrial enzymes include hydrolytic enzymes such as proteases, lipases,

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cellulases, and amylases, which are primarily used in detergents, textiles, food & beverage, etc (Sharma et al., 2019; Hemsinli and Gurkok, 2024; Fatima et al., 2025). Specialty enzymes, on the other hand, are a class of enzymes produced for low-volume but high-value applications rather than large-scale, bulk industrial use. Prominent examples of specialty enzymes include asparaginase, hyaluronidase, glucose oxidase, peroxidase, urease, alkaline phosphatase, dehydrogenases, DNA polymerase, protease, reverse transcriptase, restriction endonucleases, ligases, RNase, Taq polymerase, transaminases, and nitrilases, which are used in the fields of medicine, diagnostics, treatment, research, and biotechnology (Jamal et al., 2025). According to "Grand View Research" 2024 reports, industrial enzymes accounted for approximately 57% of the total enzyme market, while specialty enzymes accounted for the remaining 43%. Industrial enzymes hold a larger share of the market and are in high demand across various application areas. However, the market for specialty enzymes is also continuing to grow rapidly with greater momentum.

Proteases

Globally, proteases are the most sought-after enzymes in the enzyme market (Kumar et al., 2014). Among industrial enzymes, proteases stand out with a 25-30% share. They have taken the top spot, surpassing carbohydrase enzymes such as amylase and cellulase, which break down carbohydrates, and lipase enzymes, which are responsible for breaking down fats and oils (Kumar et al., 2014). According to Mordor Intelligence data, the global protease market is projected to be worth $2.03 billion in 2025 and reach $4.15 billion by 2030, with a compound annual growth rate of 10.23% (https://www.mordorintelligence.com/industry-reports/proteases-market).

Proteases, also known as peptidases or proteinases, are a subclass of enzymes that catalyze the hydrolysis of protein molecules. They cleave the peptide bonds connecting two amino acids and break down long protein chains into smaller polypeptides or amino acids (Dhillon et al., 2017).

The functions of proteolytic enzymes within the cell are not limited to being simple destructive enzymes necessary for protein catabolism and amino acid production; they also play critical roles in regulating processes such as protein quality control, signal transduction, the cell cycle, inflammation, hormone secretion, blood clotting, the transport of secretory proteins and peptides across membranes, autophagy, apoptosis, and immunity (López-Otín et al., 2008; McShane and Selbach, 2022; Zhang et al., 2023).

Classification of Proteases

Proteases can be classified based on several criteria, including their source, specific site of action on the substrate, the nature of the catalytic amino acid residue, and the optimal pH range for activity (Rawlings, at al., 2020).

Proteases based on the biological sources: Proteases are found in all life forms, including animals, plants, fungi, bacteria, and archaea, and even in viruses (Morazzani et al., 2019; Gagaoua et al., 2021; Yu and Feng, 2025; Moussi et al., 2025; Saxena et al., 2025). Based on the source of the origin, proteases are divided mainly into three gropus including microbial proteases produced by bacteria and fungi (Song et al., 2023), plant proteases and animal proteases (Heu et al., 1995).

Papain, bromelain, ficin, and keratinases are among the best-known plant-based proteases (Venetikidou et al., 2025). They can be used in beer production, meat tenderization, milk coagulation, cancer treatment, digestion, and viral disorders (Gonzalez-Rabade et

al., 2011). The production of protease enzymes from plants has disadvantages such as the need for large cultivation areas, sensitivity to climate and seasonal changes, and long production times.

Among animal proteases, trypsin, chymotrypsin, pepsin, and renin are the most widely known (Patil et al., 2022). Trypsin is found in the small intestine, while chymotrypsin is found in the pancreas, and pepsin is produced in an inactive precursor form in the stomach.

Microbial proteases are the most researched and industrially valuable enzymes due to their ability to function under harsh conditions, their short production time, low cost and space requirements, high yield, and ease of acquisition (Sharma et al., 2019). As proteases are an essential enzyme class for cellular metabolism, they are synthesized by almost all microorganisms. However, certain species possess higher potential for commercial production and usage due to their high yield and suitability for industrial conditions. Among these microorganisms, bacterial species belonging to the genus *Bacillus* (Zeng et al., 2025) and fungal species of the genus *Aspergillus* (Ajayi & Lateef, 2025) are the most common protease producers.

Proteases based on their site of action: Proteases are divided into two groups based on the site where the enzyme acts on the substrate: exopeptidases and endopeptidases (Mahler and Cordes, 1966).

Exopeptidases hydrolyze one or more amino acids from the free amino group or free carboxyl group of a peptide (Sawant and Nagendran, 2014). Exopeptidases are divided into three types: aminopeptidases, carboxypeptidases, and omega (Ω) peptidases. Aminopeptidases, which are quite common in nature, are one of the main subclasses of exopeptidases. They catalyze the cleavage of amino acids by acting on peptide bonds at the amino terminus of proteins or peptides. Most remove one amino acid at a time, while a

small percentage of aminopeptidases remove two or three amino acid residues at a time (Bradshaw, 2013). Dipeptidyl peptidases cleave a dipeptide from the free amino end of the peptide chain, while tripeptidyl peptidases cleave a tripeptide from the free amino end of the peptide chain. Carboxypeptidases act on the carboxyl terminus of the amino acid chain, releasing an amino acid or a dipeptide. Omega peptidases, which are less common than others, are a class of enzymes that hydrolytically remove specific modified amino acids and amino acid derivatives from the N-terminus of polypeptides. Unlike classical aminopeptidases, these enzymes target modified residues such as pyroglutamate (pGlu) (Sánchez et al., 2025), N-acetyl amino acids, or N-formyl (Suda et al., 1980) amino acids, rather than free N-terminal amino acids. Omega peptidases, such as pyroglutamyl peptidase I/II, acylaminoacyl-peptidase, and N-formylmethionyl-peptidase, play critical roles in biological processes.

Endopeptidases target specific internal amino acid sequences of the substrate and catalyze the hydrolysis of internal peptide bonds within a polypeptide chain, resulting in the formation of smaller peptide fragments (Sawant and Nagendran, 2014). Trypsin, pepsin, and subtilisin are the examples of endopeptidases.

Proteases based on the nature of the catalytic amino acid residue: Proteases based on the nature of the catalytic amino acid residue are divided into four groups: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases.

Serine proteases such as trypsin, chymotrypsin, subtilisin constitute one-third of known proteolytic enzymes. Their name comes from the nucleophilic serine (Ser) residue located in the enzyme's active site (Mahajan and Badgujar, 2010). Aspartic proteases such as pepsin, renin contain aspartic acid in their active sites. Cysteine proteases such as papain, cathepsins also known as

thiol proteases are named after the cysteine found in their active site. (Nadeem et al., 2013). Metalloproteases such as thermolysin, collagenase are protease enzymes that use metal ions as cofactors. The activity of metalloproteases depends on divalent metal ions, typically zinc, for activity in the medium (Rao et al., 1998). Cobalt and calcium are also metal ions that can be present in proteases.

Proteases based on the optimal pH range for activity: Based on the pH range in which they are active, they are further divided into three groups: acidic proteases, neutral proteases, and alkaline proteases (Singh et al., 2001). Acid proteases (e.g., pepsin) show maximum activity under acidic conditions (pH 2–5) (Singh et al., 2016); neutral proteases (e.g., thermolysin) at neutral pH (pH 6–8) (Rao et al., 1998) and alkaline proteases (e.g., subtilisin) in alkaline environments (pH 8–12) (Hemsinli and Gurkok, 2024).

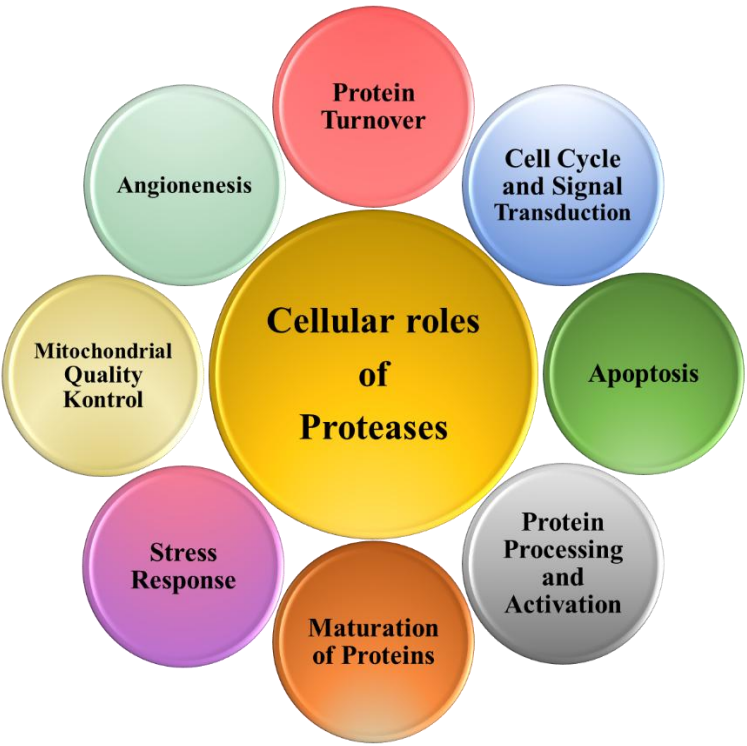
Cellular Roles of Proteases

Proteases are essential biomolecules for life. It has been determined that more than 2% of the human genome encodes proteases, indicating that proteases play a wide variety of structural and physiological roles in the body (Shankar et al., 2021). The functions of proteases within cells are not limited to their role in protein digestion by hydrolyzing peptide bonds. Due to their structural and functional diversity, they perform fundamental physiological processes within the cell, such as protein turnover, regulation of the cell cycle and signal transduction, apoptosis (programmed cell death), processing and activation of proteins, maturation of extracellular and membrane-associated proteins, defense and stress response, quality control within the mitochondria, and angiogenesis as summarized in Figure 1.

1. Protein turnover: Intracellular protein degradation is an active and highly regulated process. Lysosomal, proteasomal, and mitochondrial degradation systems have been found to play a role in

numerous biological functions (McShane and Selbach, 2022). Cells continuously breaks down proteins that have lost their function or are misfolded. This degradation is carried out by lysosomal proteases (cathepsins) or the proteasome complex (especially the 26S proteasome). Through this process, the cell eliminates defective/harmful proteins and recovers amino acids and uses them for new protein synthesis. The ubiquitin–proteasome system tags proteins for degradation with ubiquitin and directs them to their target (Goll et al., 2008). The ubiquitin-proteasome system (UPS) is the mechanism responsible for the degradation of intracellular proteins. In this system, a small protein called ubiquitin is covalently attached to the lysine residues of the target protein through the sequential action of ATP-consuming enzymes (E1, E2, and E3). The E3 ligase typically recognizes a specific amino acid sequence (degron) in the protein. Ubiquitin chains attached to the target protein, particularly K48-linked polyubiquitin chains, are recognized by the proteasome and lead to its degradation. The 26S proteasome, which carries out this degradation, is the primary protein-degrading complex in eukaryotic cells (Bard et al., 2018; McShane and Selbach, 2022).

Figure 1. The roles of proteolytic enzymes in key cellular processes



2. Regulation of the cell cycle and signal transduction: Proteases play roles in protein activation or inactivation. The degradation of specific proteins in the cell cycle allows the cell to transition from one phase to another (Reed, 2005). Ubiquitin-mediated proteolysis is a key mechanism in cell cycle control, removing negative regulators and unnecessary or harmful proteins. Ubiquitin-conjugating enzymes and ligases tag proteins with multi-ubiquitin chains for degradation by the proteasome. Most regulated proteolysis is carried out by two ligase families. Anaphase-Promoting Complex/Cyclosome (**APC/C)** is active during mitosis and G1 to eliminate proteins that impede mitosis or are harmful in G1. Skp1–Cullin–F-box protein complex (**SCF) ligases** target

phosphorylated proteins at phosphodegrons and link protein degradation to cell cycle signaling pathways (Reed, 2005).

3. Programmed Cell Death (Apoptosis): The most important regulators of apoptosis are the caspase (cysteine-aspartic protease) family. These enzymes exist in an inactive pro-caspase form and are activated upon receiving the proper signal. Caspases are activated through proteolytic cleavage in response to apoptotic signals. Firstly, initiator caspases (i.e., caspase-8 and caspase-9) are activated by death receptors or mitochondrial pathways, subsequently activating executioner caspases (i.e., caspase-3, -6, and -7), which cleave structural and regulatory proteins, leading to the characteristic morphological and biochemical features of apoptosis. In this way, proteases ensure the controlled dismantling of cellular components during programmed cell death.

4. Protein processing and activation: Several proteins including proteolytic enzymes are synthesized as zymogens, or inactive precursors, to allow for the spatial and temporal control of proteolytic activity and to stop undesired protein destruction (Khan, and James, 1998). These forms are inactive and need to be cleaved by proteases to become active enzymes or proteins such as hormones. For example, trypsin is activated by the autoproteolysis of pancreatic trypsinogen (Freiburghaus et al., 2021). Similarly, pepsin and chymotrypsin, which are secreted in zymogen forms, are also activated by the action of proteases (Smeekens, 1993). Insulin hormone is produced as proinsulin and become functional after proteolytic cleavage.

5. Maturation of extracellular and membrane-associated proteins: Proteins (e.g., enzymes, hormones, antibodies) that will be secreted in the cell or located on the membrane are mostly produced in precursor form and must be processed by proteases in order to function properly. Newly synthesized secretory proteins carry a short

sequence called a signal peptide as soon as they leave the ribosome. This signal peptide is cleaved and removed by signal peptidases. As a result, the protein enters the endoplasmic reticulum lumen and progress toward its mature state. In the Golgi apparatus, certain proteases cleave the terminal regions of proteins, making them suitable for glycosylation and shaping them according to their destination (lysosome, plasma membrane or extracellular environment) (Blobel, 2000; Brown et al., 2000; van der Goot and Gruenberg, 2006).

6. Stress response: There are various environmental stress factors, and different tissue and cell types are affected by these stresses to varying degrees and respond differently to stress factors. Cells constantly exposed to internal and external stress factors that threaten cellular integrity activate stress sensing and signaling pathways. This response generally occurs in the form of repairing damage or breaking down macromolecules that have been irreversibly damaged. When repair mechanisms fail, programmed cell death processes such as apoptosis are triggered to protect the organism. Proteolysis, particularly via the ubiquitin-proteasome system (UPS), plays a crucial role in these stress responses by eliminating oxidized, misfolded, or otherwise damaged proteins and regulating important transcription factors such as p53, Nrf2, and HIF1. The UPS coordinates cellular adaptation, DNA damage responses, and the initiation of apoptosis when necessary through the selective ubiquitination and degradation of regulatory proteins, thereby maintaining cellular homeostasis under stress conditions (Flick and Kaiser, 2012). In addition, lysosomal proteases, particularly cathepsin D, go into the cytosol and function as early apoptotic mediators when exposed to oxidative stress. By encouraging the clearance of irreversible damaged components and inducing programmed cell death when healing is no longer feasible,

their activation supports the cellular defense and stress response (Flick and Kaiser, 2012).

7. Mitochondrial Quality Control Proteases: Mitochondrial proteases are important in maintaining protein quality and preserving mitochondrial function by degradation of misfolded or damaged proteins. Mitochondrial proteases are classified into three main types: ATP-dependent proteases (iAAA, mAAA, LonP, ClpP)**,** ATP-independent proteases (ATP23, HtrA2/OMI), and oligopeptidases (PITRM1, MEP17) and they break down misfolded or oxidatively damaged proteins, ensuring the continuation of mitochondrial function (Zhang et al., 2023). Targeting mitochondrial quality control proteases has become a promising approach in cancer treatment and the creation of new anticancer medications since dysregulation of these proteases aids in the growth of cancer (Zhang et al., 2023).

8. Angiogenesis: During the angiogenesis (new blood vessel formation) process, proteases play a critical role in events such as the remodeling of the extracellular matrix, cell migration, and the release of growth factors. Three main groups of proteases are involved in this process: matrix metalloproteinases (MMPs), cysteine proteases (especially cathepsins), and serine proteases. The activities of these enzymes are regulated by specific inhibitors such as tissue inhibitors of metalloproteinases (TIMPs), cystatins, and serpins. Aminopeptidases have also been found to play a role in angiogenesis. The function of proteases is not limited to the degradation of the basement membrane and ECM, they also activate other proteases and pro-angiogenic molecules, release growth factors stored in the ECM, and in some cases can also produce molecules that inhibit angiogenesis. Among serine proteases, the plasmin/plasminogen activator system plays a major role in angiogenesis (Shankar et al., 2021).

Medical Applications of Proteases

Medical applications of proteases are quite diverse (Shankar et al., 2021). Alkaline proteases derived from *Bacillus subtilis* are used for therapeutic purposes in gauze, nonwoven fabrics, new bandage materials, and ointment formulations. Alkaline proteases administered orally are prescribed for the treatment of digestive disorders such as lysozyme deficiency (Jamal et al., 2025). Proteases derived from *Aspergillus oryzae* are similarly used in digestive system diseases (Rani et al., 2012). The high diversity and selectivity of fungal proteases offer significant advantages in the development of potent medicinal compounds. Furthermore, fibrinolytic alkaline proteases are being evaluated for use in thrombolytic therapies and as potential anticancer drugs due to their ability to break down fibrin (Mukherjee and Rai, 2011). Alkaline proteases, when used in combination with collagenases, are also commonly found in sustained-release drug formulations (Barthomeuf et al., 1992; Alipour et al., 2016).

Proteases are used as therapeutic agents in the treatment of many diseases (Shankar et al., 2021). In cardiovascular disorders, urokinase (u-PA), the first FDA-approved enzyme drug, was used to dissolve blood clots by converting plasminogen to plasmin (Mosnier and Bouma, 2006). Subsequently, t-PA (Alteplase, Activase®) and its variants Reteplase and Tenecteplase were developed due to their fibrin selectivity and long half-life (Semba et al. 2001; Mosnier and Bouma, 2006; Howard et al., 2007). In the treatment of hemophilia B, the recombinant form of clotting factor IX, Benefix® (Wyeth), was approved in 1997 and has been effective in controlling bleeding (Di Cera 2008; Monahan and Paola 2010). In surgical applications, recombinant human thrombin (Recothrom®, Zymogenetics) was approved in 2008 and found to be effective in postoperative bleeding with low immunogenicity (Di Cera, 2007; Bowman et al., 2010). In septic patients, recombinant Activated Protein C (Xigris®, Eli Lilly),

with its anticoagulant and anti-inflammatory properties, was approved in 2001. For the treatment of malabsorption due to pancreatic enzyme deficiency, enzyme replacement therapy based on mixtures of amylase, lipase, and protease has been used, and Zenpep® was the first FDA-approved product (Littlewood et al. 2006). Additionally, Wobe-Mugos®, containing a proteolytic enzyme mixture, received orphan drug status from the FDA in 2000 by breaking down folic acid derivatives in tumor cells and triggering their death. Finally, Xiaflex® (collagenase from *Clostridium histolyticum*) was approved for treatment of Dupuytren's disease, characterized by the shortening and thickening of the ligaments that attach the skin to the palmar fascia, in 2010 and Peyronie's disease, a penile problem caused by scar tissue called plaques that form inside the penis, in 2013 (Shin et al., 2018).

To accelerate healing by removing necrotic tissue during wound healing, debridement process is performed. Currently, enzymes such as collagenase and bromelain are being clinically tested for wound debridement (Klasen, 2000).

Serratiopeptidase is used in various clinical settings due to its anti-inflammatory, analgesic, anti-biofilm, and anti-edemic effects and is even promoted as a supplement for preventing cardiovascular disease (Jadhav et al., 2020).

Human lysosomal proteases are being studied in the preclinical phase for the rejuvenation of fibrous tissues. Penzyme®, a therapeutic formulation possessing a mixture of trypsin and chymotrypsin obtained from the moray eel, is applied in the cure of psoriasis and other dermatological conditions (Shankar et al., 2021).

Exfoliation by using enzymes is a method that mimics the natural desquamation process by targeting skin proteins (keratin, desmosomes, collagen, and elastin). Protease enzymes are promising candidates for this process. Proteases break down these proteins,

removing dead cells and clogged pores and contributing to the renewal of the skin surface. It is important that the protease to be used exhibits catalytic activity at the appropriate pH and temperature in the skin; in addition, proteases that have been previously researched, stable, and reliable are preferred. An alternative approach is to select proteases that are structurally and functionally similar to endogenous enzymes involved in natural desquamation in the skin. Protease-based exfoliation offers an alternative to chemical exfoliation, especially for sensitive skin, and stands out as an environmentally friendly, biologically compatible application (Trevisol et al., 2022).

Conclusion

Proteolytic enzymes are essential for regulating the size, shape, transformation, and structure of key proteins in all living organisms. Although numerous studies and reviews have focused on their industrial applications, the metabolic and cellular functions of proteases remain insufficiently explored.

Proteases can be classified according to their sources as plant, animal, or microbial proteases. Among these, microbial proteases have gained particular attention due to their ease of production, cost-effectiveness, and the absence of ethical concerns associated with plant- and animal-derived enzymes. Therefore, microorganisms have become preferred sources for large-scale protease production with high yield and stability. While the industrial relevance of proteases has been widely reviewed, gaps persist in the literature regarding their diverse biological roles at the cellular level.

In the present book chapter, we aimed to address these gaps by highlighting the cellular and physiological functions of proteases, which extend far beyond their well-known role in protein hydrolysis. We discussed their involvement in protein turnover, cell cycle regulation and signal transduction, apoptosis, protein processing and

activation, maturation of extracellular and membrane-associated proteins, cellular defense and stress responses, and mitochondrial quality control, emphasizing their central importance in maintaining cellular homeostasis.

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EPIGENETIC MODIFICATION AND HEALTH WITH NUTRACEUTICAL COMPOUNDS

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MEDİNE GÜLLÜCE[2]

Introductıon

Both DNA sequence-dependent and -independent mechanisms have been shown to affect the rates of change in organismal phenotype (Belton & Kelsey, 2025). Nevertheless, the two primary factors that determine individual differences, genetic and environmental, can also be impacted by epigenetic changes, which are regulatory changes in gene expression that persist across cell divisions and generations (Webster and Phillips, 2025).

A further stage in the maturation process of forming the genome and transferring it from an individual's genotype to their phenotype is epigenetics, a field of biology that focuses on the interactions between genes and their biological products. Without altering the deoxyribonucleic acid (DNA) sequence, epigenetic

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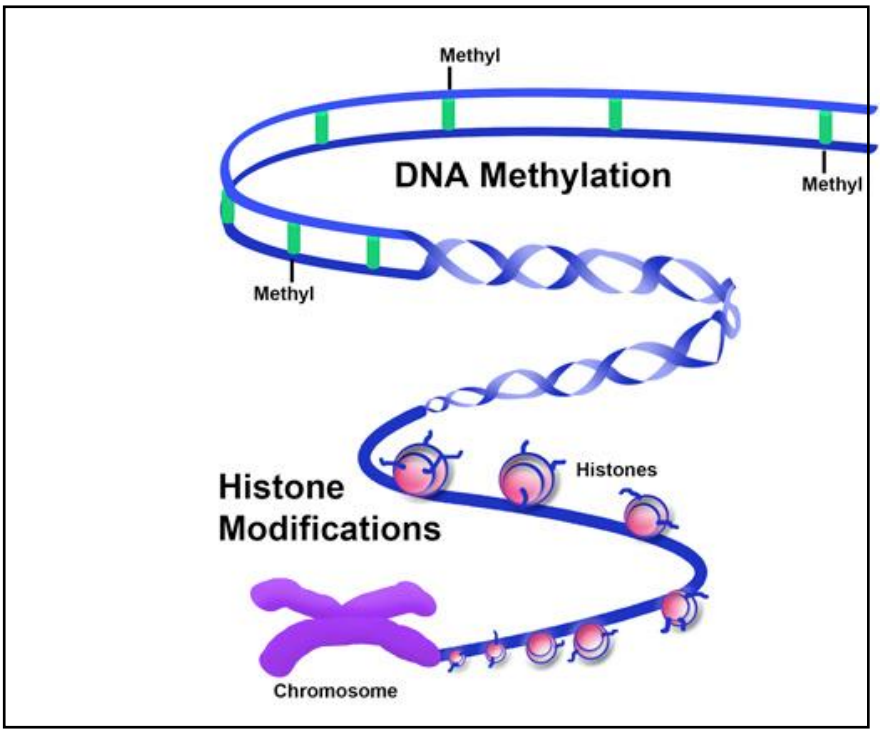
mechanisms cause changes in gene expression by reversibly activating or silencing genes in response to environmental conditions like nutrition availability (Gkiouleka et al. 2025; Gurusamy et al. 2025).

In the early 1940s, Conrad Waddington defined "epigenesis," which means "above genes" in ancient Greek, as "the branch of biology that studies the interactions between genes and their products that create the phenotype." Originally used to describe all molecular mechanisms that control the conversion of a genotype into a certain phenotype, the word was later renamed epigenetics (Dupont et al. 2009; Farsetti et al. 2023). Especially in the past 70 years, one of the most fascinating areas of biological research has been epigenetics. Although the field's pioneers were taken aback when they discovered occurrences that defied the standard rules of Mendelian heredity, there is currently interest in the use of epigenetic regulation through behavioral modifications to treat illness.

Without directly changing the DNA sequence, epigenetic mechanisms are known to control gene expression via altering the chromosomal superstructure into which DNA is packaged and chemically changing the bases of DNA (Al Abud et al. 2018). Histone and DNA alterations are the primary mechanisms underlying epigenetic processes (Figure 1). According to Koç (2015), histone modifications include phosphorylation, ubiquitination, and proline, which can be further broken down into isomerization, SUMOylation, ADP-ribosylation, acetylation, and methylation (Figure 2).

Figure 1. *DNA methylation and histone modifications*

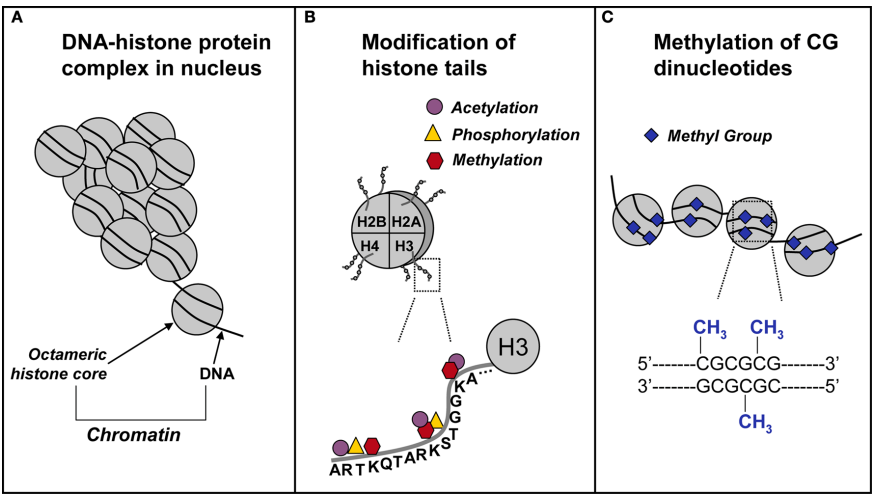


(Walker and Gore, 2011).

It is believed that throughout an organism's existence, epigenetic mechanisms control cellular functions at a high level (Ewen, 2015). Through these processes, cells generate messenger RNA and accurately modify protein levels in response to every environmental stimulation (Raleigh, 2021). Among the most significant of these environmental stimuli are nutrients and bioactive food ingredients, which seem to have the ability to affect epigenetic mechanisms by either directly blocking enzymes that catalyze DNA methylation or histone modifications or by changing the availability of substrates required for these enzymatic reactions. For instance, scientists have studied DNA methylation in great depth because folate has a methyl group and supplies this methyl group for the

creation of AdoMet, the special methyl donor for DNA methylation reactions (Choi & Friso, 2010).

Figure 2. *Schematic representation of epigenetic mechanisms. (A) In the nucleus, DNA coils and condenses around histones. Each octameric histone core contains two copies each of histones H2A, H2B, H3, and H4. The DNA-protein complex is called chromatin. (B) DNA-histone interactions occur at the N-terminal tail of a histone; for example, the H3 N-terminal tail contains several sites for epigenetic marking via acetylation, methylation, and phosphorylation. (C) Methyl groups are transferred to CpG sites in and around gene promoters' rich in cytosine-guanine nucleotides (CpG islands). This process, called DNA methylation, is catalyzed by a class of enzymes known as DNA methyltransferases*



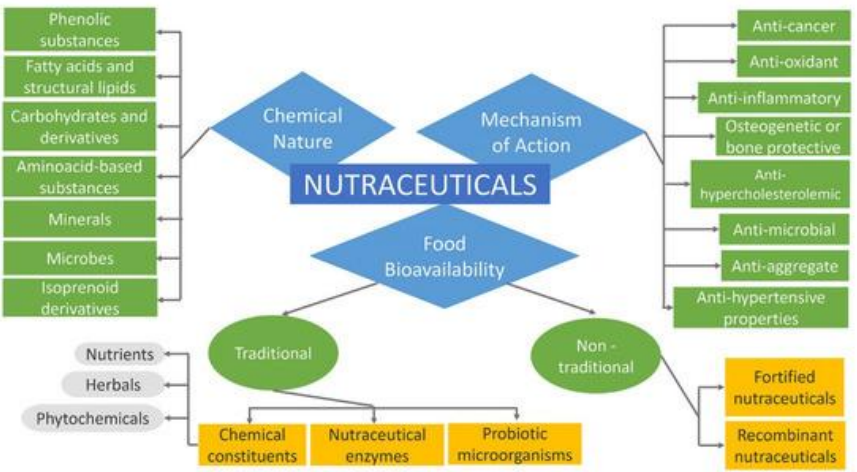
(Penner et al. 2010).

What is nutraceutical?

Nutraceuticals, specifically designed preparations, are formulated to meet specific dietary requirements and/or provide preventive healthcare. The term "nutraceutical," a formulation of nutrients that aid in the prevention and treatment of certain diseases, was coined in 1989 by Dr. Stephen De Felice, combining the words

"nourishment" and "drug." These are foods or parts of foods that are beneficial in providing various health benefits, including the treatment and/or prevention of disease (Puri et al., 2022). However, in the United States, nutraceutical products are also recognized as drugs, food ingredients, and dietary supplements (Nasri et al., 2014). The term, which combines nutritional and pharmaceutical ingredients, is interpreted as "more than food, less than drugs" (Gunarathne et al., 2023). Nutraceuticals are broadly classified into several subcategories based on food bioavailability, mechanism of action of the active ingredient, and chemical nature, as shown in Figure 3 (Hoti et al., 2022).

Figure 3. *Classification of Nutraceuticals*



(Hoti et al., 2022).

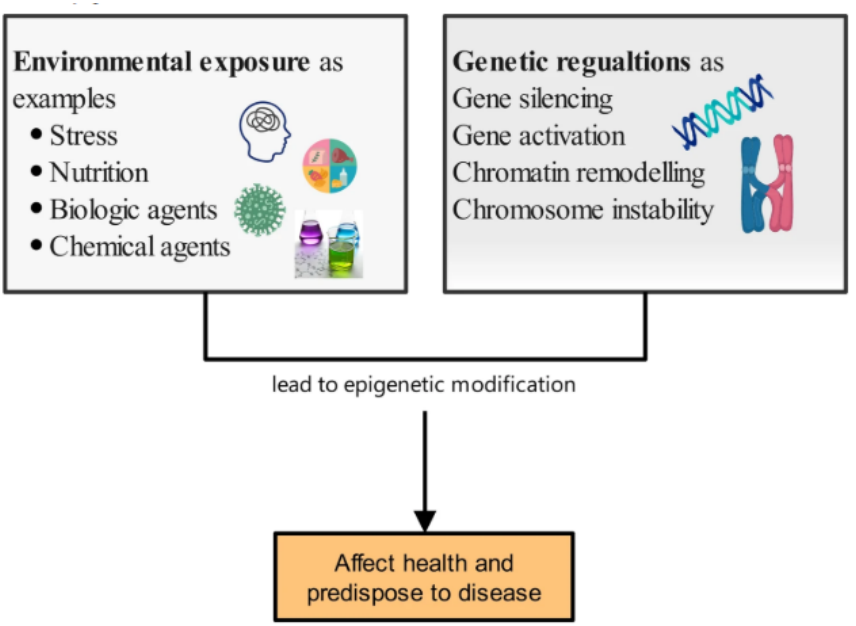
Nutraceuticals have become more popular in the treatment of numerous illnesses due to their possible health advantages. According to Takefuji (2025), the worldwide nutraceutical market is projected to grow from its 2022 valuation of US$540 billion to US$1025 billion by 2030. Furthermore, as interest in alternative medicines employing cutting-edge technologies and scientific

methodologies grows, so does the significance of nutraceuticals in the scientific community. Nutraceuticals have the ability to prevent or lessen the onset of age-related oxidative damage, epigenetic DNA damage, and a number of inflammatory and metabolic illnesses, according to a study by Comhaire and Decleer (2020).

Epigenetic modification with nutraceutical compounds

In the early stages of pregnancy, intrauterine variables and environmental factors—particularly nutrition—have a significant impact on epigenetic modifications. Figure 4 reports that epigenetic alterations can also be influenced by the microenvironment (Kaminsky et al., 2009, El-Sayed et al., 2023). DNA methylation and mental health have been shown to be epigenetically impacted by the amount and quality of nutrition, one of the most significant environmental factors. Additionally, Wang et al. (2018) looked at whether nutrition directly triggers the catalytic activity of specific enzymes that alter epigenetics. In this study, it was demonstrated that adding dihydrocaffeic acid (DHCA) and malvidin-3′-O-glucoside (Mal-gluc) to drinking water decreased the levels of inflammatory cytokines in the blood that cause depression. By decreasing the expression of DNA methyltransferase 1 (DNMT1), it decreased the amount of cytokines in mice, hence alleviating depression (Wang et al., 2018).

Figure 4. *Epigenetic modifications and genetic rearrangements affect health and disease*



(El- Sayed et al., 2023).

Human liver cell line using nutraceuticals Four nutraceuticals were examined in a recent study on the impact of these genes on epigenetic alteration. This work unequivocally showed that combining phytochemicals with established bioactivities with herbal extracts can result in products that have potent epigenetic effects on the transcription of genes that play a crucial role in regulating metabolism. RNA was extracted from the human liver cell line (THLE-2 cells) and unsupplemented control cells after the expression of six genes (ppar-α, glp-1, bdnf, sirt-1, nrf1, and sod-1) was cultivated for 24 hours in the presence of Vitalité, Reviv, Revíve, and Collagène (products of THREE International). RT-PCR was used to assess the transcript levels of these genes in these isolates, and variations in the amounts of mRNA in supplemented cells were

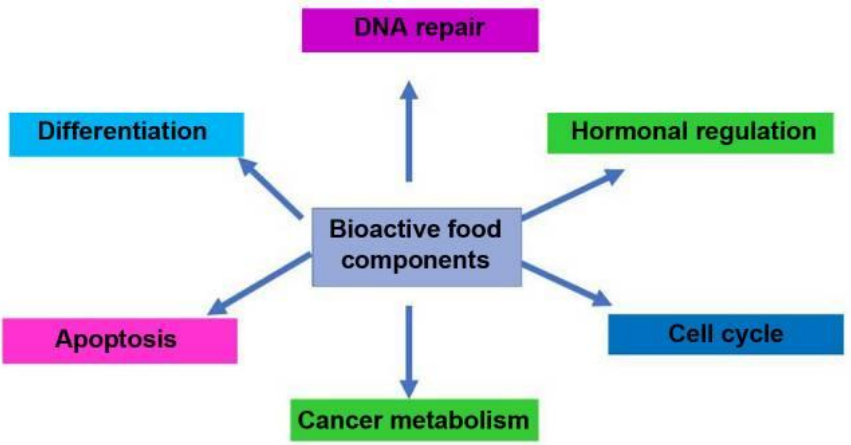
contrasted with reference cells. The transcription of the genes glp-1, bdnf, sirt-1, nrf1, and sod-1 was elevated by Revíve, whereas Éternelin It was discovered that Vitalité raised the transcription of glp-1, bdnf, nrf1, and sod-1 genes, ppar increased the transcription of -α and sod-1 genes, and Collagène boosted the expression of sod-1 and bdnf genes. According to Davis et al. (2025), the study highlighted the potential of nutraceutical preparations as significant options for the prevention and treatment of numerous chronic health issues.

Studies have shown that epigenetic regulation in Alzheimer's disease, specifically the role of dietary factors in regulating epigenetic marks and changes in Alzheimer's disease, even though the potential for developing therapeutic and preventative potential of epigenetics in Alzheimer's disease is limited. In this context, a 2014 study by Davinelli and associates examined the possibly pertinent roles of dietary epigenetic chemicals in Alzheimer's disease. The table below shows the epigenetic processes impacted by dietary components (Davinelli et al., 2014).

Clinical and epidemiological research suggests that nutrition actively contributes to the onset and spread of a number of cancers, including breast, prostate, and colon cancer, and that there is a connection between diet and these tumors (Figure 5). Accordingly, the Mediterranean diet is regarded as a nutritional pool made up of different bioactive substances and nutraceuticals that are present in food and have the potential to directly and indirectly improve health through their own epigenetic processes. The mammalian target of rapamycin (mTOR) pathway and insulin-like growth factor-1 (IGF1) levels, which are known to affect longevity and aging, may be directly modulated by the Mediterranean diet due to its low glycemic index and low animal protein intake. In instance, cutting less on animal protein can dramatically lower serum IGF1 levels and forkhead by downregulating signaling that activates box O3

(FOXO3A) and subsequently the transcription of homeostatic genes that promote lifespan, it can suppress mTOR activity. Many of the chemicals found in the Mediterranean diet have anticancer potential and may be actively involved in cancer prevention, according to Divella et al.'s (2020) study. The majority of research corroborating these conclusions demonstrates that certain dietary substances have epigenetic targets on cancer cells during their genesis, growth, and multiplication. Therefore, incorporating nutraceuticals that can trigger epigenetic regulation is the goal of the "epigenetic" strategy being used today. Diet can be discussed in all its aspects (Divella et al., 2020).

Figure 5. *Diet may influence genetic and epigenetic events associated with several cancer process*



(Divella et al., 2021).

A wide range of diseases can be prevented, suppressed, and treated with the help of the developing discipline of nutritional epigenomics, which modifies different epigenetic variables. Even during cellular differentiation in embryonic and fetal development, nutritional variables can modify our epigenomes throughout our lives. This is known as nutritional epigenomics. It also describes

how bioactive compounds can affect and change transcriptional levels of gene expression. For instance, methyl group donors and cofactors included in food are necessary for DNA methylation. Thus, dietary modifications during a sensitive and crucial time like embryogenesis can change gene expression and methylation processes, which in turn can change a person's metabolism and physiology and possibly set off lifelong pathological processes (Choi et al., 2010; Vahid et al., 2015; Kumari et al., 2022; Villagrán-Andrade et al., 2024).

For the treatment of chronic diseases, the quickly developing science of epigenetics is showing promise as a non-invasive, sustainable, and customized substitute for gene editing. The great majority of diseases are caused by genetic differences that result in aberrant gene expression, differential or abnormal methylation patterns, and epigenetic inflexibility (Sedley et al., 2020).

Rapid technological advancements have improved our understanding of the ongoing biological evolution and biological uniqueness of the human genome, but environmental factors, particularly nutrition, can change epigenetic mechanisms that alter human physiology and biochemistry by altering gene expression (Henikoff & Greally, 2016). To keep up with changing biology, metabolic processes must therefore be acquired and relearned. This is due to the fact that the majority of metabolic pathways, including nutrigenomics research, were first studied without a thorough grasp of how the epigenome functions (Sedley et al., 2020).

Therefore, a crucial field of study that shows the regulatory effects of dietary components on gene expression is the interplay between nutraceuticals and epigenetic mechanisms. The pathophysiology of many chronic diseases and the preservation of cellular homeostasis are significantly influenced by the impact of nutraceuticals on epigenetic processes such DNA methylation,

histone alterations, and microRNA-mediated gene silencing. These results have substantial scientific and clinical implications for the creation of new therapeutic techniques for disease prevention as well as tailored dietary approaches. Thus, nutraceuticals In-depth research on the consequences of epigenetic regulation will serve as the foundation for novel future applications in molecular medicine and nutritional science.

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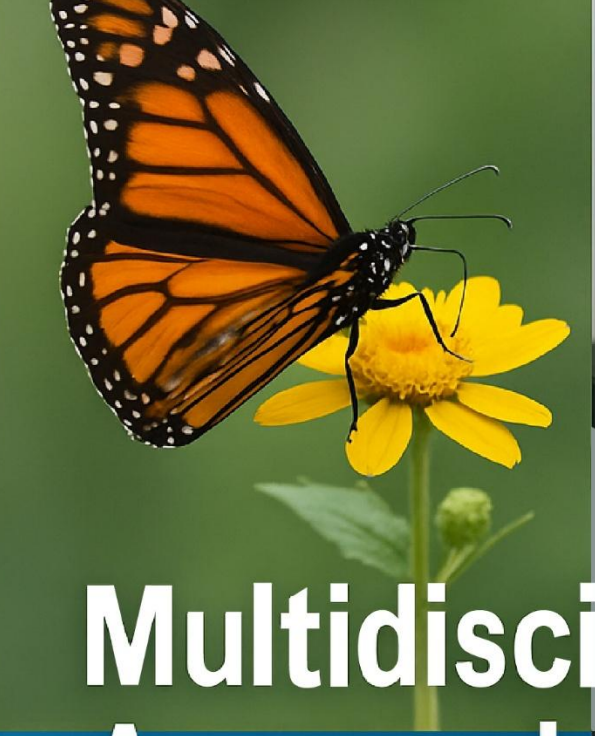

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