CURRENT STUDIES IN BIOLOGY I



Editor

Şifa TÜRKOĞLU

LIVRE DE LYON

2026

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Cover Design • Motion Graphics
Book Layout • Motion Graphics
First Published • July 2026, Lyon

e-ISBN: 979-10-7023-078-7
DOI: 10.5281/zenodo.21453262

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Publisher • Livre de Lyon
Address • 37 rue marietton, 69009, Lyon France
website • http://www.livredelyon.com
e-mail • livredelyon@gmail.com



PREFACE

Biology is a multifaceted scientific discipline that seeks to understand life through the relationships among its different levels of organization. Molecular events are reflected in the functions of cells and tissues, while the responses of organisms shape ecological interactions, environmental health, and biotechnological applications. *Current Studies in Biology I* consists of six chapters that bring together these different levels of biological organization, offering interdisciplinary content that extends from classical and modern analytical methods to chromosomal stability, from secondary metabolites to pesticide toxicology, and from climate change to the functional potential of insect proteins.

The first chapter addresses the classical and modern approaches used in embryological and histological examinations. Traditional methods such as microscopy, histochemistry, and immunohistochemistry are evaluated together with spatial omics, organoid models, and artificial intelligence–assisted analyses, thereby establishing a methodological foundation for integrating morphological structures with molecular information.

The second chapter focuses on the evaluation of chromosomal stability in transgenic plants using classical and molecular cytogenetic methods. The structural and hereditary consequences of genetic transformation are examined through methods such as mitotic index, chromosomal aberrations, the micronucleus test, fluorescence in situ hybridization, and flow cytometry.

The third chapter examines secondary metabolites, and terpenes in particular, in terms of their biological functions, ecological roles, potential benefits, and toxic effects. It emphasizes that a biological effect depends not only on chemical structure but also on dose, duration of exposure, and the characteristics of the target organism.

The fourth chapter evaluates neonicotinoid pesticides with respect to their agricultural uses, mechanisms of action, environmental persistence, and potential risks to human health. The chapter highlights the delicate balance between the production requirements of modern agriculture and concerns regarding environmental and public health.

The fifth chapter examines the effects of climate change on insect–plant interactions at the molecular, physiological, and ecological levels. It

discusses how rising temperatures, atmospheric carbon dioxide, thermal plasticity, and multitrophic interactions may alter ecosystem functioning.

The sixth and final chapter examines the nutritional, functional, and bioactive properties of insect proteins. In addition to protein quality and technofunctional properties, the antioxidant, antimicrobial, and antihypertensive potentials of insect-derived bioactive peptides are evaluated.

As can be seen, although the chapters may appear to address independent topics, they complement one another along a shared biological narrative that extends from insect to plant, from natural compounds to synthetic chemicals, and from genome integrity to developmental analysis. This coherence transforms the book from a mere compilation into a unified work that reflects the multidimensional nature of contemporary biological research.

I would like to express my sincere gratitude to the publishing house that made the publication of this book possible, and to the valued staff of the editorial, technical, design, production, and administrative units who contributed their efforts at every stage of the publication process. I am also grateful to all the chapter authors who contributed by sharing their knowledge and experience; their meticulous work has made an important contribution to the scope, coherence, and academic value of the book.

I hope that *Current Studies in Biology I* will contribute to new scientific questions, to interdisciplinary research, and to the responsible use of biological knowledge, and that it will prove useful to researchers, graduate students, and all readers who wish to follow current developments in biology from an interdisciplinary perspective.

Editor
Prof. Dr. Şifa TÜRKOĞLU

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CHAPTER I

CLASSICAL AND MODERN APPROACHES IN EMBRYOLOGICAL AND HISTOLOGICAL ANALYSIS: SPATIAL OMICS AND ARTIFICIAL INTELLIGENCE-BASED METHODS

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1. INTRODUCTION

Embryological and histological analyses are complementary disciplines that aim to elucidate the developmental processes of living organisms at both morphological and molecular levels. While classical microscopic examination techniques reveal the structural characteristics of tissue architecture and cellular organization, molecular biology and genetics-based approaches illuminate the functional, biochemical, and regulatory mechanisms underlying these structures (Ross & Pawlina, 2019; Bancroft & Layton, 2019; Alberts et al., 2022). The concurrent utilization of these two approaches enables the evaluation of developmental processes through a multi-layered and holistic perspective.

In recent years, advances in high-resolution molecular techniques, particularly spatial transcriptomics, have enabled the analysis of gene expression while preserving its spatial distribution within tissues. These methods have driven a significant transformation in developmental biology

by revealing not only the molecular profiles of cells but also their precise localization within the tissue context (Ståhl et al., 2016; Svensson et al., 2018; Asp et al., 2020). Consequently, the three-dimensional organization and temporal dynamics of embryonic tissues can now be examined in unprecedented detail.

Although classical histological methods provide high accuracy in assessing tissue integrity and cellular organization, they remain limited in analyzing dynamic processes at the molecular level. To overcome these limitations, spatial omics technologies offer substantial advantages; specifically, spatial transcriptomic approaches enable the simultaneous investigation of gene expression profiles alongside the positional information of cells within the tissue (Chen et al., 2023). Furthermore, the employment of deep learning-based algorithms in the analysis of large-scale imaging and omics datasets facilitates cell-type classification, the definition of tissue regions, and the identification of potential biomarkers (Litjens et al., 2017; Zhang et al.,2024). This integrative approach contributes to the high-precision mapping of temporal and spatial dynamics in embryonic development, providing a deeper molecular and structural perspective compared to classical histology.

Another prominent approach in developmental biology is stem cell-based model systems. Organoids and synthetic embryo models, as three-dimensional structures capable of mimicking *in vivo* developmental processes, allow for the investigation of cellular differentiation, tissue organization, and organogenesis (Terhune et al., 2022; Rosner et al., 2025). Within these systems, immunohistochemistry (IHC) and immunofluorescence (IF) techniques play critical roles in identifying cell types and demonstrating protein localization (Ramos-Vara, 2005; Wen & Tang, 2022). Additionally, *in situ* hybridization (ISH) and fluorescence *in situ* hybridization (FISH) methods support histological data at the molecular level by revealing the cellular localization of gene expression (Rehman et al., 2024).

The combined use of these techniques allows for the detailed mapping of the molecular characteristics and organization of cell types within tissues. In particular, the combination of single-cell analysis and spatial transcriptomic approaches contributes to a more nuanced understanding of developmental processes by revealing the heterogeneous structure of embryonic tissues (Chao et al., 2023; Kiessling & Kuppe, 2024; Liu et al., 2024). Furthermore, advanced imaging methods such as light-sheet

microscopy and tissue clearing techniques enable the three-dimensional examination of large-volume biological samples (Delage et al., 2023).

Digital pathology and AI-supported analysis systems are opening new horizons in the holistic evaluation of histological and molecular data. Deep learning-based algorithms, in particular, provide high accuracy and reproducibility in the automated segmentation of histological sections, cell-type classification, and quantitative morphometric analysis. These approaches go beyond classical microscopic evaluation, allowing for the standardized and objective analysis of large-scale datasets (Ballard et al., 2024; Zahedi et al., 2024). Moreover, through contemporary AI approaches based on multimodal data integration, spatial omics data and histological images can be analyzed simultaneously; thus, the relationships between cellular organization, gene expression patterns, and tissue architecture can be elucidated with higher resolution and comprehensiveness (Liu et al., 2026). These approaches hold significant potential for both basic research and translational medicine applications.

This integrated methodological approach enables a detailed temporal and spatial characterization of embryo development at the cellular and molecular levels. It also provides vital contributions to the modeling of early developmental processes, the validation of organoid systems, and a better understanding of the mechanisms underlying human development (Asp et al., 2020; Terhune et al., 2022).

This review aims to comprehensively examine the methodological developments ranging from classical histological techniques to spatial omics technologies and AI-based digital analysis approaches used in embryonic tissue analysis. In this context, the advantages, limitations, and application areas of various techniques are discussed comparatively, evaluating their use-particularly in organoid and synthetic embryo models-to reveal their potential in developmental biology and translational research.

2. EMBRYO HISTOLOGICAL PREPARATION TECHNIQUES

The histological analysis of embryonic tissues is a fundamental approach that enables the morphological evaluation of developmental processes. In this context, the accurate demonstration of cellular organization, tissue architecture, and stages of developmental differentiation is directly dependent on the success of the specimen preparation phases. While

histological methods aim to examine tissues by preserving their structural integrity as close to their natural state as possible, molecular techniques provide complementary information to these findings (Bancroft & Layton, 2019; Ross & Pawlina, 2019).

Spatial omics approaches developed in recent years allow for the determination of the spatial distribution of gene expression directly on histological sections, thereby enabling the simultaneous evaluation of morphological and molecular data (Ståhl et al., 2016). In addition, histochemical methods facilitate the selective visualization of tissue components, supporting the detailed examination of the chemical and structural properties of embryonic tissues.

The reliability of the obtained data depends on the preservation of tissue integrity throughout all steps of the specimen preparation process. Fixation, the first and most critical stage of this process, stabilizes the structural and biochemical properties of tissues, directly influencing the accuracy of subsequent staining and analysis phases.

2.1. Fixation

Fixation is one of the most critical stages of histological examination, ensuring the preservation of the morphological and biochemical integrity of tissues. During this process, cellular structures are immobilized through the chemical stabilization of proteins, enzymatic activity is halted, and autolysis and microbial degradation are prevented. The efficacy of fixatives varies depending on parameters such as pH, temperature, duration of fixation, and tissue thickness. Suboptimal fixation conditions can lead to artifacts—such as protein denaturation, cell shrinkage, or inadequate penetration—which adversely affect histological evaluation (Bancroft & Layton, 2019).

2.1.1. Fixation for Light Microscopy

The fixatives used in light microscopy directly influence the success of histochemical and immunohistochemical stainings, in addition to preserving tissue morphology. Therefore, the chosen fixative must be compatible with the intended analytical method. Formaldehyde-based solutions are the most commonly used fixatives in routine histological examinations.

10% Neutral Buffered Formalin (NBF): With an approximate formaldehyde content of 4%, it ensures the preservation of tissue architecture by cross-linking proteins. Due to its versatile application, it

is the fundamental fixative of routine histology. However, as it may cause the masking of certain protein epitopes, the application of antigen retrieval methods may be necessary for immunohistochemical analyses (Bancroft & Layton, 2019).

4% Paraformaldehyde (PFA): Particularly preferred for immunohistochemistry and immunofluorescence studies, PFA can provide more controlled results in preserving antigenic structures compared to standard formalin (Bancroft & Layton, 2019).

Bouin's Fixative: Composed of a mixture of picric acid, formaldehyde, and acetic acid, it allows for the distinct preservation of nuclear and cytoplasmic details, especially in delicate specimens such as embryonic tissues. However, appropriate washing procedures are required before staining to remove the yellow discoloration caused by picric acid (Carson & Hladik, 2009).

Zamboni's Fixative (Paraformaldehyde–Picric Acid mixture): Preferred in immunohistochemical applications due to its ability to preserve both morphological integrity and antigenicity (Stefanini et al., 1967).

Alcohol-based Fixatives (Ethanol, Methanol): These ensure rapid fixation by precipitating proteins. However, as they can cause tissue shrinkage and morphological deformations, they are generally preferred for enzyme histochemistry and nucleic acid analyses (Fischer et al., 2008; Bancroft & Layton, 2019).

2.1.2. Fixation for Electron Microscopy

In electron microscopic analyses, preserving the cellular ultrastructure at high resolution without compromising its metabolic and physiological characteristics is of paramount importance. Consequently, potent cross-linking aldehyde-based fixatives are employed during the preparation of tissue and cellular specimens. These fixatives stabilize proteins and other macromolecules, ensuring the preservation of cellular organelles, membrane structures, and tissue architecture at the ultrastructural level, thereby facilitating detailed morphological examination.

Glutaraldehyde: A monomeric aldehyde fixative widely utilized in electron microscopy preparation. When applied at a concentration of 2.5%, it ensures the detailed preservation of cellular organelles and membrane structures through protein cross-linking. Although glutaraldehyde effectively stabilizes the cytoskeleton and organelles, its potent cross-linking properties

may hinder the detection of certain antigens. Therefore, it may be used in conjunction with specialized protocols for immunohistochemical analyses (Leica Biosystems, 2021).

Osmium Tetroxide (OsO_4): Typically applied as a post-fixative following primary aldehyde fixation. Osmium tetroxide increases the stabilization of membrane structures by reacting specifically with lipid components and enhances contrast by providing high electron density to the membranes. This property enables the detailed investigation of lipid-rich ultrastructures, such as mitochondria, the endoplasmic reticulum, and the plasma membrane (Hayat, 2000). Post-fixation with OsO_4 following glutaraldehyde is considered a classical protocol for maintaining membrane integrity and ensuring clear visualization of the cellular ultrastructure (Thiéry, Bernier, & Bergeron, 1995; University of Gothenburg, n.d.).

Karnovsky's Fixative: A combination of paraformaldehyde and glutaraldehyde, this is widely used as a balanced fixative that provides both morphological and ultrastructural preservation (Karnovsky, 1965).

2.2. Tissue Processing

Following fixation, tissue processing and embedding procedures are performed to render the tissues suitable for sectioning. This process comprises dehydration, clearing, and embedding stages.

Dehydration is conducted after fixation to remove water from the tissue. This procedure is carried out using a series of ascending alcohol concentrations to ensure that the tissue can subsequently be infiltrated by the embedding medium. Typically, this series starts from low concentrations and increases to 100% ethanol, allowing for the gradual removal of water; thus, the morphological integrity of the tissue is preserved, making it suitable for embedding and sectioning (Hewitson & Darby, 2010; Junqueira & Carneiro, 2018).

During the clearing stage, xylene or similar organic solvents are used to facilitate the transition between the alcohol and the embedding medium (Hewitson & Darby, 2010).

In the embedding stage, tissues are placed into an appropriate medium to form blocks. Paraffin embedding is the most widely used method for light microscopic analysis, offering advantages for long-term storage and serial sectioning (Hewitson & Darby, 2010; Junqueira & Carneiro, 2018). Resin embedding, on the other hand, is specifically preferred for electron microscopy and allows for the acquisition of ultra-thin sections; this method

is essential for ultrastructural analyses requiring high resolution (Thermo Fisher Scientific, 2021).

2.3. Sectioning and Specimen Preparation

In histological analysis, the sectioning process is performed using different techniques depending on the embedding material and the objective of the analysis.

The microtome allows for sections typically 3-10 µm in thickness to be obtained from paraffin blocks and is the most commonly used device in routine histological examinations. These sections are suitable for histochemical staining and immunohistochemical analyses. Thinner sections can be obtained using ultramicrotomes (for electron microscopy), while thicker sections can be produced using specialized microtome types such as the cryostat (Bancroft & Layton, 2019; Ross & Pawlina, 2019).

The ultramicrotome enables the ultrastructural examinations required for electron microscopy by taking sections 50–100 nm thick from resin-embedded tissues. These sections are generally collected and examined on grids (metallic support structures) (Bozzola & Russell, 1999; Barresi & Gilbert, 2023).

The vibratome is primarily used to obtain thick sections and allows for the preservation of cellular integrity in live or minimally processed tissues. This method provides advantages for physiological studies and certain imaging techniques (Ross & Pawlina, 2019).

The cryostat produces sections 5–20 µm thick from flash-frozen tissues and is particularly preferred for immunohistochemical analysis, enzyme histochemistry, and rapid diagnostic applications. Although the tissue structure is largely preserved, morphological resolution may be limited compared to paraffin sections (Hira et al., 2019).

Sections are typically mounted onto glass slides, dried, and subsequently subjected to histochemical staining, immunohistochemistry, or immunofluorescence procedures. In the final stage, they are covered with a coverslip using an appropriate mounting medium and made ready for microscopic examination.

3. HISTOLOGICAL STAINING and IMMUNOHISTOCHEMISTRY METHODS

In histological analyses, evaluating tissue architecture and cellular organization alone is insufficient; it is also of paramount importance to elucidate cellular functions and molecular components. Histochemical

and immunohistochemical techniques employed for this purpose allow morphological data to be complemented at the biochemical and molecular levels (Bancroft & Layton, 2019). While classical staining methods reveal the general tissue structure, advanced techniques provide information directed toward determining the localization and functional status of specific molecules (Ross & Pawlina, 2019).

3.1. Hematoxylin and Eosin (H&E)

Hematoxylin and eosin (H&E) staining is the most widely utilized fundamental staining method in histological examinations. In this technique, hematoxylin acts as a basic dye that binds to nucleic acids, staining cell nuclei in blue-purple tones; conversely, eosin, as an acidic dye, binds to cytoplasmic proteins, staining the cell cytoplasm and extracellular matrix in shades of pink **(Figure 1).** Due to this differential staining property, H&E is recognized as the standard method for assessing tissue architecture, cellular organization, cell density, and pathological changes (Bancroft & Layton, 2019; Ross & Pawlina, 2019). H&E staining offers significant advantages, particularly in monitoring cell differentiation and tissue stratification in embryonic tissues. When integrated with modern molecular techniques, it enables the spatial investigation of the gene expression profiles and functional states of tissue specimens (Ståhl et al., 2016).

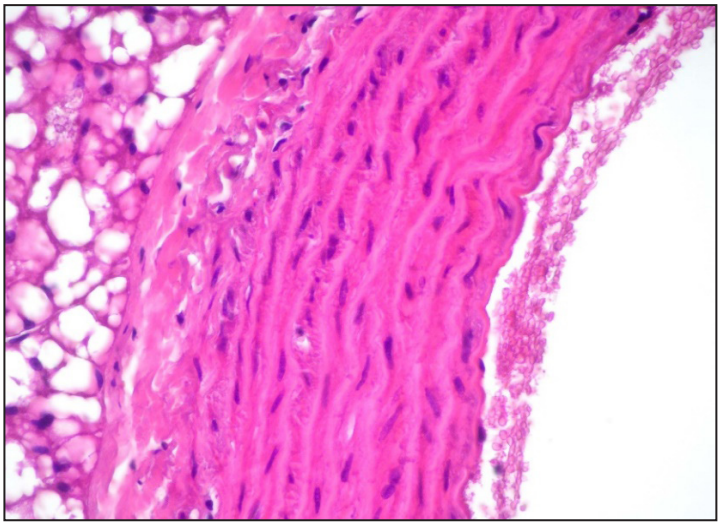

Figure 1: Histological appearance of elastic connective tissue and elastic artery obtained via hematoxylin-eosin staining. (Başımoğlu Koca, 2012)

3.2. Special Histochemical Stains

Beyond the general morphological information provided by H&E staining, special histochemical stains are employed for the selective demonstration of specific cellular components or chemical structures. These stains play a crucial role in identifying cellular differentiation and tissue-specific components that emerge during embryonic development (Kiernan, 2015; Bancroft & Layton, 2019).

Examples of these stains are provided below:

Toluidine Blue is a basic dye exhibiting metachromatic properties; it is specifically used to demonstrate mast cell granules and acidic mucopolysaccharides. Due to its metachromasy, certain structures can be distinguished by staining in different color tones.

Alcian Blue specifically stains acidic mucopolysaccharides and glycosaminoglycans, enabling the examination of mucus content and connective tissue components.

Periodic Acid-Schiff (PAS) staining is an essential histochemical method used to identify carbohydrate structures. This technique reveals the distribution of intracellular and extracellular carbohydrates by staining glycogen, neutral mucopolysaccharides, and basement membrane components in pink-purple tones.

Mallory's Trichrome stain facilitates the differentiation of tissue components by staining collagen fibers, muscle tissue, and cytoplasmic proteins in distinct colors; it is particularly used for evaluating connective tissue organization.

Silver staining (Silver impregnation) is utilized to visualize fine structures such as reticular fibers, neurons, certain microorganisms, and nuclear details. These techniques are based on the principle of precipitating silver ions onto specific structures within tissue specimens and subsequently reducing them to metallic silver. The delicate network of reticular fibers, neuronal processes, and occasionally nuclear details can be visualized with high contrast using silver staining. Silver staining methods remain a vital histological tool, especially for the morphological evaluation of connective tissue and nervous system structures (Bancroft & Layton, 2019; Ross & Pawlina, 2019**) (Figure 2).**

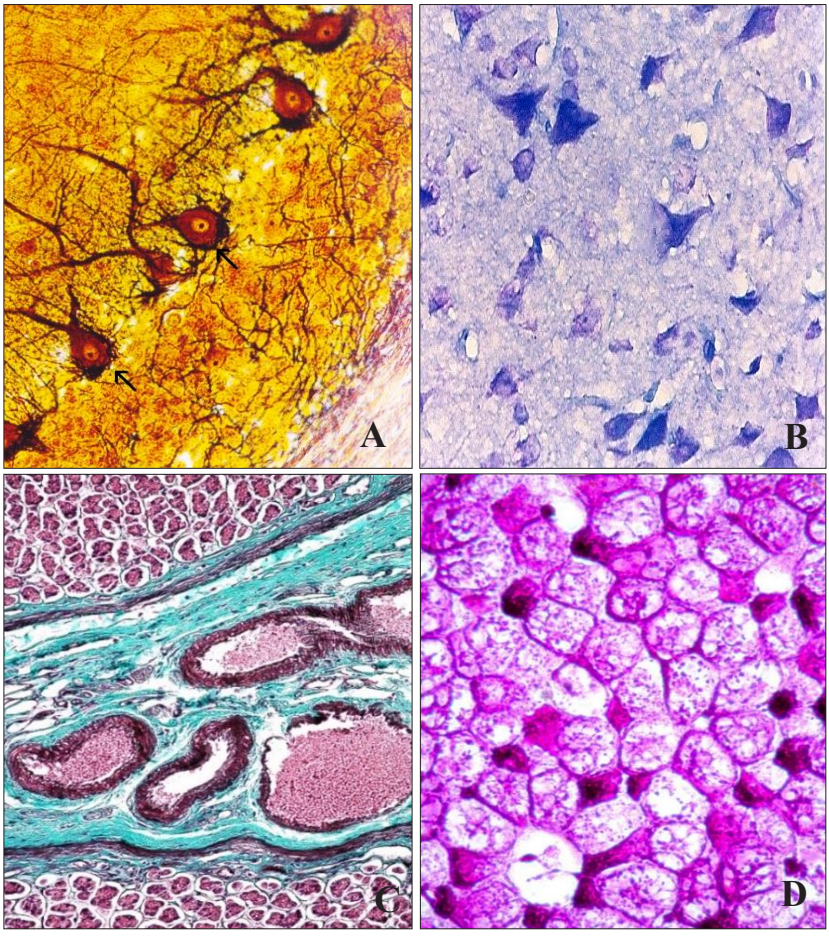

Figure 2: Sections obtained with various histochemical stains; Purkinje cells – cerebellum (A: Silver staining), pyramidal cells – cerebrum (B: Toluidine blue), loose connective tissue – gastric submucosa (C: Gomori's trichrome), glycogen – liver hepatocytes (PAS). (Başımoğlu Koca, 2012)

3.3. Immunohistochemistry (IHC) and Immunofluorescence (IF)

Immunohistochemistry (IHC) is a staining method based on antigen–antibody interactions that demonstrates the distribution and localization of specific proteins within tissue sections. In this technique, primary antibodies specific to the target protein are utilized, followed by secondary antibodies conjugated with enzymes that bind to these primary antibodies. A chromogenic reaction product is then generated, rendering the protein of interest visible under the microscope. The most frequently used enzyme

systems include peroxidase and alkaline phosphatase (Ramos-Vara, 2005; Bancroft & Layton, 2019) (**Figure 3**).

Immunohistochemistry (IHC) is widely employed in numerous fields, such as cellular differentiation, tissue-specific protein expression, tumor classification, and biomarker identification. Furthermore, it is a critical tool for the validation of cell types and the execution of functional analyses in organoid and synthetic embryo models. When used in conjunction with spatial transcriptomics or *in situ* hybridization techniques—which reveal the spatial distribution of gene expression—this method enables the holistic evaluation of morphological and molecular data (Ståhl et al., 2016; Marx, 2021; Levy-Jurgenson et al., 2020).

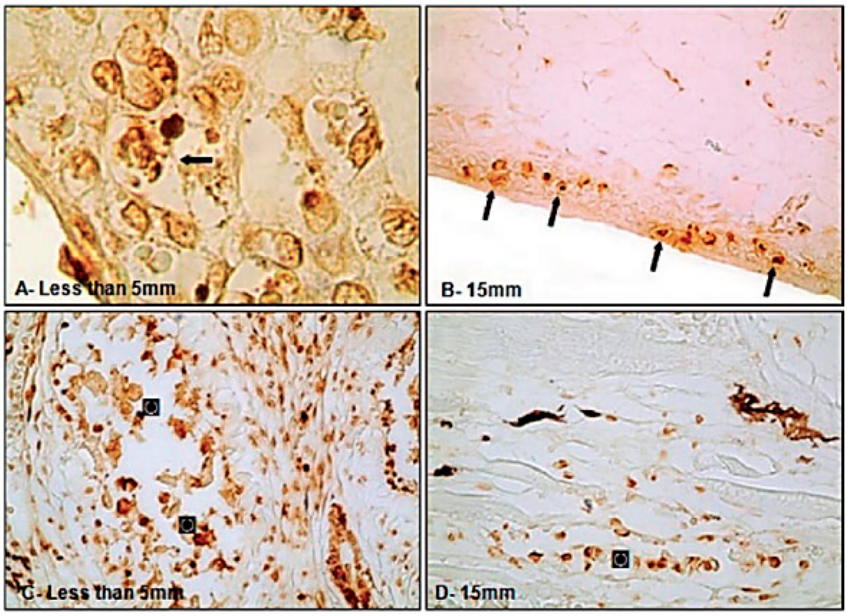

Figure 3: Demonstration of apoptosis at the tissue level via immunohistochemical (IHC) analysis. Distribution of TUNEL-positive apoptotic cells across different tissue types during the metamorphosis process in *Pelophylax ridibundus* tadpoles. (Başımoğlu Koca & Koca, 2013)

Immunofluorescence (IF) methods enable the detection of target proteins through antibodies labeled with fluorescent dyes. In this approach, antibodies may be directly fluorescently labeled (direct IF) or detected indirectly via fluorescently labeled secondary antibodies (indirect IF). The resulting signals are visualized using fluorescence or confocal microscopy.

The IF method offers high sensitivity and specificity, particularly for multi-protein analysis, intracellular localization studies, and the investigation of protein-protein interactions (Odell & Cook, 2013). Furthermore, its capacity for three-dimensional imaging and co-localization analyses has rendered this method indispensable in developmental and cell biology research. When integrated with approaches such as multiplexed antibody-based imaging, this technique facilitates the simultaneous detection and analysis of multiple target proteins (Hickey et al., 2022).

3.4. Limitations of Classical Staining Methods and Integration with Modern Approaches

Every stage of the histological specimen preparation process has a direct impact on the accuracy and reliability of the data obtained. Technical errors that may occur, particularly during the fixation and embedding phases, can lead to the formation of artifacts, adversely affecting both morphological evaluations and molecular analyses. Today, histological methods are no longer limited to morphological analysis; they enable multi-dimensional data generation by being integrated with spatial omics technologies and digital imaging approaches. In this context, recognizing the limitations of classical histological staining methods and implementing them in integration with modern analytical methods stands out as a fundamental requirement for obtaining reliable and comprehensive data in embryonic tissue studies.

While classical histological and histochemical staining methods provide significant information regarding tissue architecture and cellular organization, they are generally limited to two-dimensional evaluation and may not fully reflect the molecular heterogeneity within the tissue. Additionally, the simultaneous high-resolution analysis of molecular parameters, such as protein and gene expression, remains restricted with these methods (Kiernan, 2015).

Spatial omics approaches and digital imaging technologies developed in recent years allow these limitations to be overcome. Spatial transcriptomics and spatial proteomics methods enable the integration of morphological and molecular data by revealing the spatial distribution of gene and protein expression directly on histological sections (Ståhl et al., 2016; Svensson et al., 2018; Guo et al., 2026). These methods uncover the localization of different cell types, developmental process-specific molecular patterns, and microenvironmental interactions in embryonic tissues at high resolution.

Moreover, Artificial Intelligence (AI)-supported image analysis systems increase both the speed and accuracy of analytical processes by

automating tasks such as cell classification, morphometric measurements, and three-dimensional reconstruction in histological sections. AI-based approaches—particularly in the integration of spatial omics data with histological images—allow for a clearer elucidation of the relationships between molecular profiles and morphological structures (Chen et al., 2025; Li et al., 2026).

Histological staining and immunohistochemical methods are fundamental tools that enable the joint evaluation of morphological and molecular data in embryological research. While classical staining techniques reveal tissue organization, advanced methods such as IHC and IF contribute to the understanding of cellular functions and molecular mechanisms. The integration of these techniques with spatial omics technologies and AI-supported analysis allows for the multi-dimensional and high-resolution examination of embryonic development, offering new research perspectives in the field of developmental biology (Marx, 2021; Desta & Birhanu, 2025).

4. TISSUE ANALYSIS AT THE MOLECULAR LEVEL: MODERN APPROACHES

In understanding embryonic development, morphological analyses alone are insufficient; the dynamic processes occurring at the cellular and molecular levels must also be elucidated. Consequently, molecular analysis techniques developed in recent years complement histological methods by enabling the high-resolution examination of gene and protein expression within tissues. Immunohistochemistry, *in situ* hybridization, and spatial omics technologies allow for a holistic evaluation of both the structural and functional organization of embryonic tissues.

4.1. Immunohistochemistry and In Situ Hybridization

Immunohistochemistry (IHC) and in situ hybridization (ISH) techniques are fundamental molecular methods aimed at determining the localization of proteins and nucleic acids within tissues, respectively, with high specificity. These techniques play a critical role in identifying cell types, characterizing the tissue microenvironment, and analyzing gene–protein interactions during embryonic developmental processes (Oliver et al., 2023; Ball et al., 2023; Rehman et al., 2024).

While the IHC method reveals the distribution of specific proteins in tissue through antigen–antibody interactions, ISH techniques determine the cellular localization of gene expression by using probes complementary to DNA or RNA sequences. Specifically, methods such as fluorescence *in situ*

hybridization (FISH) and RNA-FISH ensure the detection of target genes with high sensitivity.

The combination of these techniques with fluorescent labeling methods and confocal microscopy enables the three-dimensional imaging of intracellular organelles and protein localizations. Thus, cell differentiation, morphological changes, and tissue organization processes in embryonic tissues can be analyzed at high resolution (Elagöz et al., 2022; André et al., 2024). Notably, the concurrent use of FISH and immunofluorescence techniques allows for the simultaneous analysis of gene and protein expression (**Figure 4**).

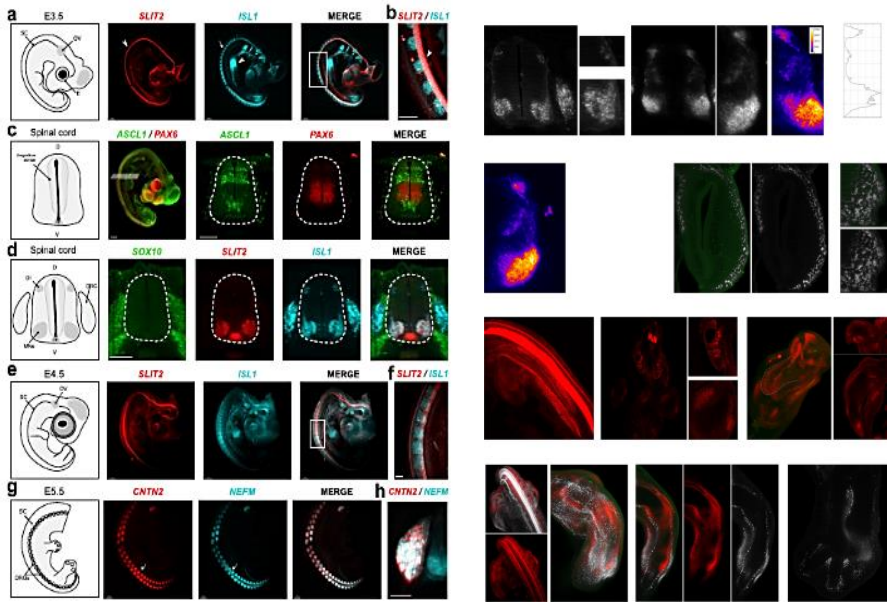

Figure 4: Spatial expression patterns of different genes during embryonic development. Gene localization via RNA-FISH (red) and protein expression via immunofluorescence staining (green) were visualized in three dimensions using confocal microscopy. Nuclei were counterstained blue with DAPI. The tissue distribution of various genes (e.g., SLIT2, ISL1, SOX10) across different developmental stages (E3.5–E5.5) is demonstrated.
(André et al., 2024)

RNA-based hybridization methods and spatial transcriptomic approaches reveal not only the presence of gene expression but also its spatial distribution within the tissue. This allows the time- and space-related dynamics of genetic programs during embryonic development

to be examined in detail at both the cellular and tissue levels (Desta & Birhanu, 2025).

In parallel, single-cell RNA sequencing (scRNA-seq) technologies uncover the heterogeneity among cell populations. The fundamental differences between single-cell RNA sequencing and bulk RNA sequencing (bulk RNA-seq) approaches are illustrated in **Figure 5**.

The utilization of these techniques alongside spatial transcriptomic methods allows for the evaluation of cellular gene expression profiles in conjunction with their positions within the tissue, thereby enabling the analysis of cellular heterogeneity within a spatial context (Peng et al., 2020).

The combined application of these approaches ensures the elucidation of not only cellular structures but also the molecular mechanisms of gene–protein interaction networks and differentiation processes in embryonic tissues. In particular, three-dimensional images obtained via confocal microscopy provide significant contributions to the detailed analysis of cellular positional relationships and tissue organization (André et al., 2024).

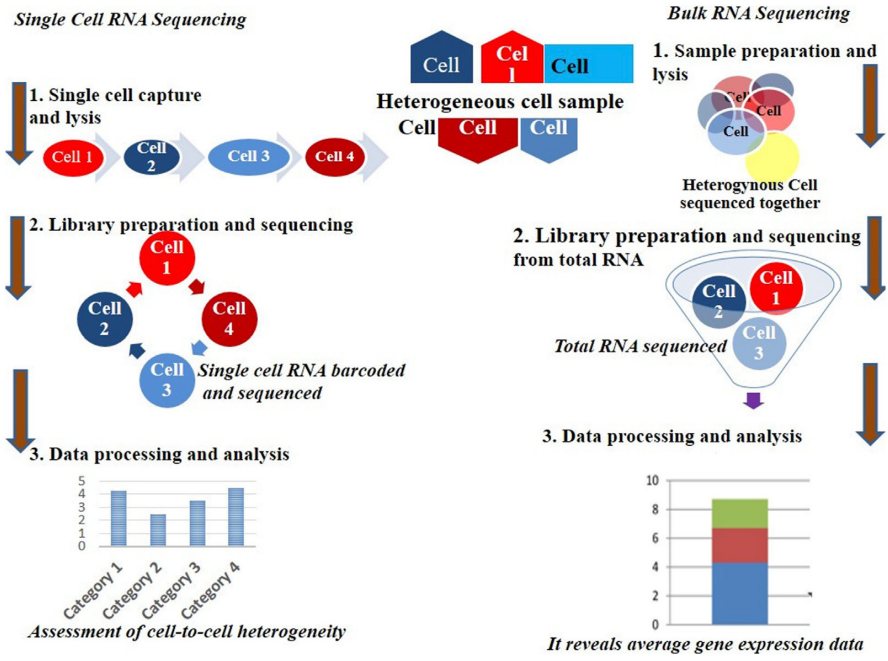

Figure 5: Comparison of single-cell RNA sequencing (scRNA-seq) and bulk RNA sequencing (bulk RNA-seq) approaches. While single-cell RNA sequencing allows for the analysis of individual cells separately to determine cellular heterogeneity, the bulk RNA sequencing method reflects the average gene expression profile of cell populations. (Desta & Birhanu, 2025)

4.2. Spatial Transcriptomics and Proteomics with Artificial Intelligence Analysis

The effective implementation of spatial omics approaches relies on the analysis of high-resolution data obtained through advanced imaging techniques. In this context, the integration of sophisticated imaging and omics technologies enables the multi-dimensional investigation of the molecular and structural organization of embryonic tissues. While fluorescent antibodies and confocal microscopy allow for high-resolution imaging of cellular substructures, three-dimensional light-based microscopy techniques facilitate a more comprehensive evaluation of tissue organization. Advanced imaging methods, such as Light-Sheet Fluorescence Microscopy (LSFM), combined with tissue clearing techniques, enable rapid and long-term 3D imaging of large tissue specimens. These approaches significantly contribute to revealing the spatial relationships between cell types and microenvironments, particularly in embryonic and human tissue samples (Liu et al., 2023; Delage et al., 2023).

Spatial omics technologies integrate molecular data with positional information within the tissue, uncovering not only the presence of cellular heterogeneity but also its spatial organization within the tissue architecture. While traditional omics approaches provide the average molecular profiles of cell populations, spatial omics methods allow for the evaluation of gene and protein expression on a per-cell basis and within a tissue context. Consequently, the positions of different cell types in embryonic tissues, intercellular interactions, and gene expression patterns specific to developmental processes can be analyzed with high spatial resolution and accuracy (Alexandrov et al., 2023; Chen et al., 2023; Liu et al., 2024; Guo et al., 2026).

Spatial transcriptomics approaches enable the mapping of gene expression alongside positional information on tissue sections, allowing for the identification of cellular niches and developmental microenvironments. These techniques stand out as a vital tool for understanding the spatial dynamics of gene regulatory networks involved in cell fate determination during embryonic development. Spatial proteomics, on the other hand, performs molecular mapping at the protein level, enabling the examination of cellular functional states, signal transduction mechanisms, and intercellular communication networks within a spatial context. These protein-level analyses contribute to the interpretation of the functional outputs of

transcriptional data, facilitating a more holistic evaluation of multi-layered biological processes (Horvath & Coscia, 2025).

The concurrent use of these technologies ensures the integration of gene and protein expression, enabling the creation of a multi-layered map of embryonic development at both molecular and cellular levels. Thus, it becomes possible to investigate cellular heterogeneity not only at the molecular level but also in relation to tissue organization and morphological structure. The fundamental principle of spatial omics approaches is based on integrating gene and protein expression data with positional information within the tissue, a process schematically illustrated in **Figure 6**.

The integration of these data with Artificial Intelligence (AI) and deep learning algorithms has established a new paradigm in biomedical analysis. Deep learning-based approaches provide high accuracy and speed in areas such as cell type classification, tissue segmentation, 3D and 4D reconstruction, multi-omics data integration, and biomarker discovery (Zahedi et al., 2024; Li, Y. et al., 2025; Li, Z. et al., 2026).

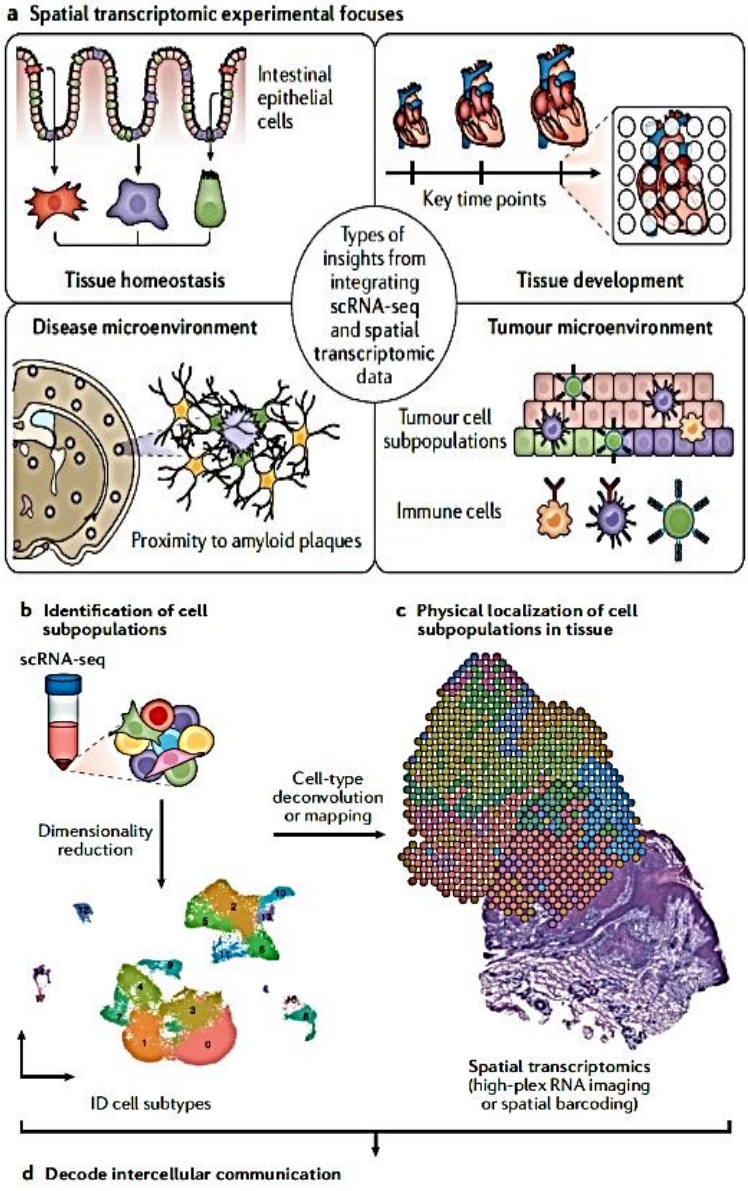

Figure 6: Schematic representation of spatial omics workflows (spatial transcriptomics and spatial proteomics) and multi-layered data integration (Longo et al., 2021, *Nature Reviews Genetics*).

In conclusion, the combination of spatial omics technologies with AI-based analysis enables high-resolution mapping of embryonic developmental processes at both molecular and morphological levels. This

synergy contributes to a deeper understanding of biological mechanisms and the more effective development of disease models. These holistic approaches hold significant potential for future precision medicine applications and developmental biology research (Marx, 2021; Desta & Birhanu, 2025).

4.2.1. High-Resolution Evaluation of Spatial and Molecular Data

The high-resolution evaluation of spatial and molecular data enables a deeper understanding of embryonic developmental processes. Spatial transcriptomics and spatial proteomics approaches allow for the detailed investigation of cell type localization, developmental-specific molecular patterns, and microenvironmental interactions by mapping gene and protein expression within a spatial context (Ståhl et al., 2016; Xu et al., 2024; Zhang et al., 2024).

Recent reports provide comprehensive evaluations of the platforms and analytical tools used in spatial omics data generation. For instance, Guo et al. (2026) summarized the methodological advantages of platforms such as Slide-seqV2, Stereo-seq, and CosMx SMI for spatial transcriptomics, demonstrating their utility in embryonic tissue analysis. Such methodological reviews guide researchers by clarifying the strengths and limitations of these technologies. Furthermore, AI-supported bioinformatic tools facilitate the processing of high-dimensional data, enabling biological interpretation while maintaining molecular resolution. Consequently, the relationship between the molecular profiles of cells and morphological structures during embryonic development can be elucidated more clearly (Desta & Birhanu, 2025; Chen et al., 2025).

4.2.2. Cell Type Classification and Tissue Segmentation

Artificial intelligence (AI) and machine learning algorithms play a critical role in the joint analysis of spatial omics data and histological images. These algorithms provide high accuracy and reproducibility in complex tasks such as cell type classification and tissue segmentation. Deep learning-based methods enable the differentiation of various cell types and the identification of tissue regions by integrating histological images with molecular data (Litjens et al., 2017; He et al., 2016).

Convolutional Neural Networks (CNN), graph neural networks, and other next-generation model architectures ensure the effective analysis of

spatial omics and imaging data across large datasets. These classification and segmentation capabilities help clearly elucidate **cell lineages**, differentiation patterns, and microenvironmental interactions during embryonic developmental processes. Furthermore, AI models shorten analysis times and increase the reliability of results by enabling the automation of tissue segmentation workflows (Kim et al., 2024; Gao et al., 2025; Coleman, Schroeder, & Li, 2024).

4.2.3. 3D/4D Reconstruction and Multi-omics Data Analysis

3D and 4D reconstruction allow for the detailed examination of the spatial and temporal dynamics of embryonic development. Light-based 3D microscopy and tissue clearing approaches ensure the high-resolution imaging of large tissue blocks (Liu et al., 2023; Delage et al., 2023) (**Figure 7**). Tissue clearing is a process where lipids and other light-scattering molecules are removed to render the blocks optically transparent. This makes it possible to obtain high-resolution images deep within large tissue specimens (Ertürk, 2024; Susaki et al., 2014).

AI-based analytical methods facilitate the automated processing of these complex datasets and the generation of 3D/4D reconstructions. Consequently, the temporal patterns of developmental events such as cell movements, tissue remodeling, and organogenesis can be revealed in detail (Pandey & Rathore, 2025). Multi-omics integration, on the other hand, ensures the resolution of biological processes at molecular and cellular levels by combining genomic, transcriptomic, and proteomic data layers (Al Marouf, Rokne & Alhajj, 2025).

While the integration of spatial omics and AI provides significant advancements, several limitations persist. High costs, the computational power required for big data analysis, a lack of data standardization, and limited data in human embryological models constitute the primary challenges (Mulholland & Leedham, 2024; Wen & Tang, 2022). The merging and interpretation of multimodal spatial omics data remain issues that need to be addressed.

In the future, the advancement of AI-based analyses and the standardization of multimodal data integration tools will enable a more detailed, dynamic, and reliable investigation of embryonic developmental processes. User-friendly analytical platforms, open data-sharing networks,

and extensive non-human model systems will strengthen the translational applications of these technologies.

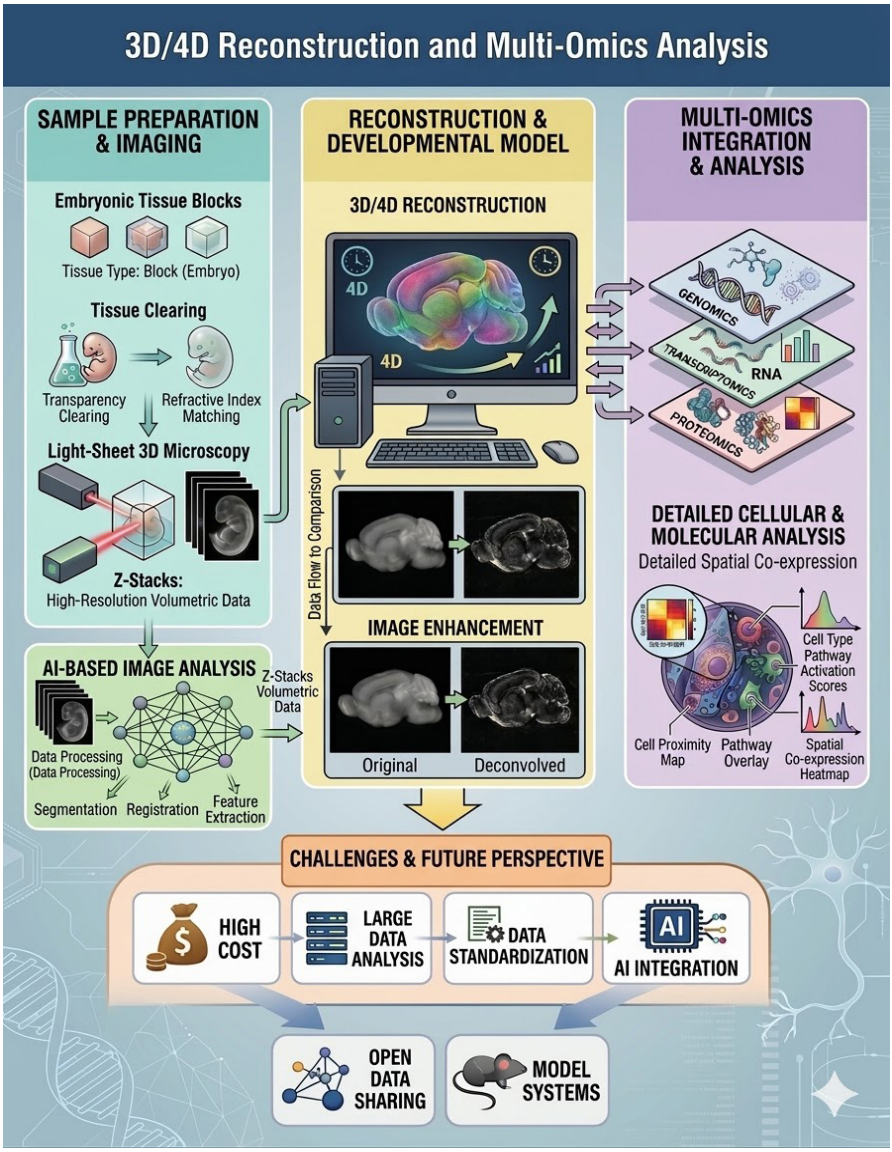

Figure 7: Data workflow in AI-supported 3D/4D reconstruction and multi-omics data analysis: Embryonic tissue blocks are imaged at high resolution using light-based 3D microscopy and tissue clearing; data is processed via AI-based analyses to generate 3D/4D reconstructions; and detailed cellular and molecular analysis is performed by ensuring integration with multi-omics layers (genomics, transcriptomics, proteomics).

5. HISTOLOGICAL EVALUATION IN ORGANOID and SYNTHETIC EMBRYO MODELS

Organoids and synthetic embryo models are systems that allow for the *in vitro* recapitulation of embryonic developmental processes and represent some of the most powerful experimental platforms in modern developmental biology. These stem cell-based models, in particular, enable the investigation of cellular differentiation, tissue organization, and morphogenetic processes under controlled conditions (Shahbazi & Pasque, 2024). These approaches go beyond classical embryological analyses, contributing to the detailed examination of developmental mechanisms at both cellular and molecular levels. Furthermore, organoid and synthetic embryo models offer the opportunity to observe tissue responses under various genetic manipulations and pharmacological treatments, providing an ideal platform for embryogenesis studies and drug testing.

Synthetic embryo models (also known as embryoid systems) offer significant advantages for studying the interactions between embryonic and extraembryonic tissues. These systems facilitate the laboratory modeling of fundamental biological events occurring during early embryonic stages, such as cell migration, axis formation, tissue specification, and organogenesis (Shahbazi & Pasque, 2024). The effects of intercellular communication, signaling pathways, and microenvironmental factors on embryonic development can be analyzed in detail through these models.

Advancements in specimen preparation and sectioning techniques play a vital role in the histological evaluation of organoids. Newly developed sectioning and culture protocols ensure the preservation of the three-dimensional architecture of organoids and allow for high-resolution histological examinations. These systems, integrated with spatial omics and transcriptomic approaches, contribute to the analysis of cellular organization and gene expression patterns in a 3D plane (Ciprietti et al., 2025; Wang et al., 2025a) (**Figure 8**). Consequently, morphological features such as tissue layer morphology, epithelial organization, cell layer arrangement, and tissue polarity can be reliably analyzed.

The integration of histological analyses with spatial omics technologies establishes a new paradigm in the field of developmental biology and modeling for the evaluation of organoid and synthetic embryo models. Spatial transcriptomics and spatial proteomics approaches reveal

the positional distribution of gene and protein expressions within the tissue, enabling high-resolution analysis of cellular heterogeneity and microenvironmental interactions (Ståhl et al., 2016; Wang et al., 2018). This integration is of critical importance for assessing the physiological accuracy of organoids, biomarker discovery, and the characterization of disease models.

Particularly in translational research, organoid models are regarded as essential tools in basic and clinical research for investigating disease-specific phenotypes, evaluating drug responses, and developing personalized treatment strategies. The concurrent use of spatial omics and AI-supported analysis enhances the accuracy of data obtained from these models and ensures the multi-dimensional interpretation of biological processes. Thus, organoid and synthetic embryo systems are assuming an increasingly central role in both fundamental science research and clinical applications (Noorbakhsh et al., 2025; Coleman, Schroeder, & Li, 2024).

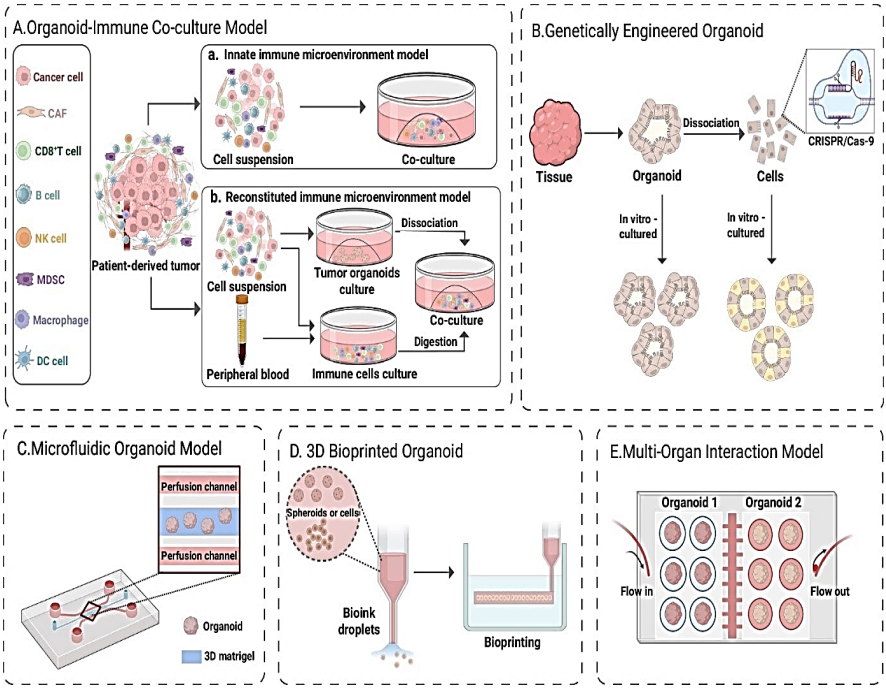

Figure 8: Schematic representation of different organoid models: (A) organoid–immune cell co-culture model, (B) genetically edited organoids, (C) microfluidic organoid system, (D) 3D bioprinting organoid model, and (E) multi-organ interaction model (Wang et al., 2025a-b).

6. CLINICAL and RESARCH APPLICATIONS

The integration of histological analyses with spatial omics technologies has led to significant advancements in both fundamental research and clinical applications. In particular, spatial transcriptomics and spatial proteomics methods enable the high-resolution analysis of tissue organization and the detailed characterization of cell types and microenvironments (Ståhl et al., 2016; Svensson et al., 2018). These technologies transcend classical histological approaches by making it possible to interpret molecular data within a spatial context.

Artificial intelligence and deep learning algorithms are playing an increasingly central role in the analysis of histological and omics data. These algorithms can perform complex tasks such as cell type classification, tissue segmentation, image analysis, and 3D/4D reconstruction with high accuracy (Litjens et al., 2017; He et al., 2016). Especially in the analysis of large datasets, they reduce human error and enhance the reproducibility of results.

In recent years, organoid and AI-based approaches have gained prominence in clinical biomarker discovery and personalized medicine applications (Marei et al., 2025; Morizane & Lamers, 2025). Organoid models offer powerful platforms for modeling disease-specific phenotypes and assessing treatment responses (Marei et al., 2025). Furthermore, utilizing these models alongside spatial omics and AI facilitates the identification of both diagnostic and prognostic biomarkers, contributing to the individualization of therapeutic strategies (Morizane & Lamers, 2025).

In parallel, the use of these technologies in the field of prenatal diagnosis is steadily increasing. Examining embryonic tissues at the molecular level allows for the early detection of developmental anomalies and supports clinical decision-making processes. Additionally, quality control processes in organoid models are becoming more reliable through the combined use of histological and molecular analyses.

7. CONCLUSION and FUTURE PERSPECTIVES

Over the last quarter-century, the integration of classical histological techniques with spatial omics approaches and AI-supported analysis has sparked a fundamental transformation in the fields of embryology and developmental biology. The tissue and structural information obtained through morphological evaluations can now be merged with high-resolution molecular data that reveal gene expression, protein distribution, and cellular

heterogeneity. This integrated approach enables the multi-dimensional and dynamic investigation of embryonic development (Marx, 2021; Liu, 2025).

The integration of AI algorithms into these processes ensures the rapid and reliable analysis of massive datasets. Applications such as cell classification, tissue segmentation, and 3D/4D reconstruction contribute to a more detailed and objective evaluation of developmental processes. These advancements have facilitated significant progress in biomarker discovery, disease stratification, and the development of personalized treatment strategies in both basic research and clinical settings.

Nevertheless, current technologies possess certain limitations. Spatial omics approaches and AI-based analyses remain high-cost, and data processing workflows are complex, requiring specialized expertise. Moreover, a significant portion of existing datasets relies on model organisms or *in vitro* systems. High-resolution and multimodal datasets for human embryonic tissues are still limited, which constrains translational applications.

In the future, with the integration of multi-omics data and more advanced use of AI algorithms, the spatial and temporal dynamics of embryonic developmental processes will be elucidated more comprehensively. Furthermore, the development of ethical standards and the expansion of human model systems will increase the clinical applicability of the data obtained. Consequently, the convergence of histology, molecular biology, and data science will lead the way in developing more holistic, high-resolution, and clinically meaningful approaches in embryology and developmental biology.

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CHAPTER II

EVALUATION OF CHROMOSOMAL STABILITY IN TRANSGENIC PLANTS USING CLASSICAL AND MOLECULAR CYTOGENETIC METHODS

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1. Introduction

Molecular biology and genetic engineering techniques allows to integrate foreign, rearranged, or synthetic genetic material into an organism's genome when it cannot be transferred naturally. The stable integration of this genetic material into the plant genome and its transmission to plant generations form the basis of stable genetic transformation (Hwang et al., 2017). The concept of genetic transformation has its historical roots in studies

showing that hereditary traits can be transferred between bacteria and that DNA is the key molecule responsible for this transfer (Griffith, 1928; Avery et al., 1944). These discoveries helped form the molecular basis of heredity and opened the way for modern genetic engineering studies.

Transgenic plants are produced by introducing foreign or rearranged genetic material into the plant genome using genetic engineering techniques. Their stability is usually considered at different, yet closely connected levels. Chromosomal stability refers to the maintenance of chromosome number, structure, and orderly segregation during cell division, whereas genetic stability covers the persistence of the transgene sequence, copy number, and genomic integration site across generations. Epigenetic stability involves the continuity of DNA methylation and chromatin organization at the transgene locus, while expression stability refers to the stable expression of the introduced gene throughout development and across generations. Distinguishing these concepts is important because the mere presence of a transgene does not, by itself, demonstrate chromosomal or phenotypic stability. Genetically modified organisms have become important tools for addressing a wide range of basic and applied challenges, from clarifying gene function and designing new metabolic pathways to developing varieties with improved nutritional profiles and enhanced resistance to biotic and abiotic stresses (Marenkova et al., 2026). Although the main goal of this process is the introduction of a target gene into the plant genome, unwanted numerical and structural changes may also occur. For example, during *Agrobacterium tumefaciens*-mediated transformation, T-DNA integration can interact with plant DNA repair mechanisms, leading to chromosomal rearrangements, DNA breaks, and genomic instability (Thomson et al., 2024). T-DNA integration often takes place during the repair of double-strand breaks in the genome. The involvement of non-homologous end joining and microhomology-mediated repair can generate deletions of plant DNA at T-DNA borders, short filler sequences, vector-backbone fragments, and complex integration structures in which multiple T-DNA copies are joined in tandem. Therefore, the molecular structure of the integration site should be evaluated not only for the presence of the transgene but also for the integrity of the surrounding chromosomal region. In Agrobacterium-mediated transformation or particle bombardment, the genomic integration

site, copy number, and structure of the transgene can cause unintended genetic, epigenetic, and phenotypic changes in plants. Random insertion of a transgene may disrupt the coding or regulatory region of an endogenous gene. In addition, promoter, terminator, or enhancer sequences within the transgene cassette may increase, reduce, or alter the tissue-specific expression patterns of nearby genes. Direct disruption of gene function or modification of the regulation of the surrounding genomic region by transgene integration is defined as insertional mutagenesis (Filipecki and Malepszy, 2006; Latham et al., 2006). This is considered an important mechanism that may underlie unexpected phenotypes in transgenic plants. In addition, tissue culture, regeneration, selection pressure, and *in vitro* conditions used during transformation can impose stress on plant cells. Such stress may lead to somaclonal variations, including point mutations, ploidy changes, transposon activation, and alterations in DNA methylation. Thus, some unintended effects observed in transgenic plants may result not only from the introduced gene itself but also from genetic and epigenetic changes generated during transformation and regeneration (Filipecki and Malepszy, 2006). Preserving chromosomal structure does not necessarily ensure successful transgene expression. For this reason, transgene expression stability should be evaluated as a complementary but separate aspect of chromosomal and genetic stability. Multicopy integration, insertion into an unfavorable genomic region, or epigenetic regulation may lead to transgene silencing. In such cases, expression of the introduced gene may decrease, disappear completely, or vary across generations. The transgene product may also interact with the plant's own metabolic and regulatory networks. As a result, a single genetic change may produce pleiotropic effects, influencing several traits such as growth, development, yield, stress responses, and metabolite composition (Filipecki and Malepszy, 2006; Ohnishi and Kawashima, 2023).

Chromosomal stability in transgenic plants is evaluated using complementary cytogenetic approaches, including metaphase chromosome analysis, mitotic and meiotic aberration assessment, the micronucleus test, and FISH. The combined use of these methods, allows the detection changes in chromosome number, numerical deviations such as aneuploidy and polyploidy, and obvious structural differences (Wang et al., 2026). In addition, measurements of the mitotic index and the evaluation of chromosomal aberrations observed at anaphase and telophase provide

important indicators of genome instability at the cellular level (Nicuță et al., 2025). The micronucleus test is also used as a sensitive indicator of chromosomal damage because it reveals chromosome or chromatid loss occurring during cell division. Micronuclei form when whole chromosomes or chromosome fragments fail to join the main chromosome group during anaphase and remain enclosed in a separate nuclear structure (Torres-Bugarín et al., 2026). At a more advanced level, molecular cytogenetic methods such as fluorescence in situ hybridization (FISH) allow the direct visualization of introduced DNA sequences on chromosomes in transgenic plants. This approach can identify the chromosome or chromosomal region into which the transgene has integrated, determine whether integration occurred at a single or multiple loci, and show how transgene copies are organized on chromosomes (Schwarzacher, 2009).

The assessment of chromosomal stability in transgenic plants is essential not only to confirm transformation success but also to verify the genetic integrity, inheritance stability, and biosafety potential of the developed lines. Classical karyological analyses and molecular cytogenetic approaches help detect numerical and structural chromosome changes that may occur in transgenic plants, providing a broader understanding of how transformation affects cells and chromosomes. In this review, metaphase chromosome analysis, mitotic index and chromosomal aberration assessment, the micronucleus test, meiotic chromosome analysis, FISH-based approaches, and next-generation molecular cytogenetic applications used in the evaluation of chromosomal stability in transgenic plants are discussed in light of the current literature. The review also emphasizes the importance of interpreting cytogenetic findings together with molecular data on transgene copy number, integration structure, and inheritance.

2. Chromosomal Stability and the Effects of Transformation

In transgenic plants, chromosomal stability can vary depending on the transformation technique used, the mode of transgene integration, the duration of *in vitro* culture, and the plant species. When chromosomal stability is evaluated, numerical and structural changes should be considered separately. Numerical changes include deviations in chromosome number, such as aneuploidy, polyploidy, and chromosome loss, whereas structural changes include deletions, duplications, inversions, translocations, and chromosomal breaks that affect chromosome organization. These

changes may arise directly from transgene integration or from the tissue culture and regeneration processes that accompany transformation. One mechanism influencing stability is the plant genomic immune system. These transcriptional and post-transcriptional gene-silencing mechanisms protect genome integrity by preventing uncontrolled proliferation of transposons, viruses, and viroids (Marenkova et al., 2026). However, the same defense mechanisms may also target transgene sequences perceived as foreign by the genome. In particular, multicopy or repetitive transgene integrations can stimulate small RNA production and DNA methylation, leading to reduced transgene expression or complete silencing. Although this does not directly alter chromosome number or structure, it may affect the genetic and phenotypic stability of the transgenic line. The chromosomal location into which a new gene integrates is also a key determinant of its stability. Because transgene integration in plant genomes is often random, the same transgene cassette may show different expression levels in independent transformants. This is known as the position effect and is closely linked to the chromatin environment of the insertion site, neighboring genes, regulatory sequences, repetitive DNA regions, and the local epigenetic state (Matzke and Matzke, 1998). For instance, integration into gene-rich and transcriptionally active subtelomeric regions may favor expression, whereas genes inserted into pericentromeric regions, heterochromatic regions, or near repetitive sequences may show unstable expression because of induced *de novo* DNA methylation (Marenkova et al., 2026). Structural rearrangements arising during integration, or complete deletion of the gene, may also lead to loss of stability (Schouten et al., 2017). In addition, integration of vector-backbone sequences together with the transgene may promote *de novo* methylation and loss of expression (Rajeevkumar et al., 2015; Philips et al., 2019). Unexpected integration of vector-backbone sequences not only complicates the molecular characterization of a transgenic event but may also result in the presence of undesirable DNA fragments in the plant genome, such as antibiotic resistance genes or bacterial regulatory sequences. For this reason, the presence of out-of-border vector sequences should be checked as part of both genetic stability and biosafety assessment.

Agrobacterium tumefaciens-mediated methods are generally reported to result in single- or low-copy transgene integrations and therefore carry a lower risk of structural genomic disturbance (Thomson et al., 2024). By contrast, physical DNA delivery methods such as biolistics introduce

DNA particles directly into cells and may therefore carry a greater potential for chromosomal damage and rearrangement. A genomic study showed that, after biolistic transformation, serious genomic changes such as chromosome breaks, large deletions, partial trisomy, and chromosome fragmentation may occur; some of these changes can be corrected by cellular repair mechanisms, whereas others may lead to stable chromosomal rearrangements. This is related to the nature of the transformation method, which can generate double-strand breaks and lead to random integration events (Liu et al., 2019).

In addition to the preference for Agrobacterium-mediated transformation, the use of matrix attachment regions (MARs) and insulators may improve stability by protecting the gene. These sequences can partially isolate the transgene cassette from the local chromatin environment, reduce position effects, and limit expression differences among independent transgenic lines. However, the effectiveness of MARs and insulators can vary depending on the plant species, the sequence used, and the integration locus. Therefore, these approaches alone cannot guarantee chromosomal stability. Targeted insertion of a gene into stable genomic regions, often called safe harbors, using systems such as CRISPR/Cas9 is another strategy that may improve stability by avoiding disruption of the plant's endogenous genes (Marenkova et al., 2026). Genomic safe-harbor regions are defined as sites where a target gene can be integrated without disrupting endogenous gene function, regulatory sequences, or chromosome organization, while still allowing stable transgene expression. For a region to be considered a safe harbor, it should not only support high transgene expression but also avoid negative effects on plant growth, development, fertility, and stress responses. Therefore, candidate safe-harbor regions should be tested across generations, environmental conditions, and genetic backgrounds.

Prolonged callus culture, high concentrations of growth regulators, repeated subculturing, and extended regeneration periods can induce somaclonal variation *in vitro* and cause changes at both genetic and epigenetic levels. These changes may include alterations in chromosome number, structural rearrangements, and other forms of genomic instability (Duta-Cornescu et al., 2023). In some plant species, *in vitro* culture conditions may promote progression of the cell cycle toward endocycling rather than mitosis, thereby increasing endoreduplication. Repeated DNA replication

without cell division can produce cells with different DNA contents within the same tissue, resulting in endopolyploid or mixoploid structures and ploidy heterogeneity in the cultured material (Weber et al., 2008; Sliwinska et al., 2022). For this reason, the stability of lines obtained after transformation should be evaluated not only by molecular characterization but also by classical karyological and cytogenetic methods.

3. Karyological Methods and Cytogenetic Techniques

3.1. Metaphase Chromosome Analysis

Comparation number and morphology of the chromosome between control and transformed lines through metaphase chromosome analysis is one of the most fundamental methods for checking chromosomal stability after transgenesis (Jha and Halder, 2026). This technique allows direct observation of chromosome number, length, centromere position, and obvious structural abnormalities. Metaphase chromosome analysis also makes it possible to detect numerical and structural changes such as aneuploidy, polyploidy, chromosome loss, chromosome fragmentation, large deletions, duplications, and evident translocations. However, small deletions, short inversions, and rearrangements that do not cause visible changes in chromosome morphology may be missed by classical karyotype analysis. Therefore, these changes should be further examined using FISH or sequencing-based methods. Because chromosomes are condensed and clearly separated at metaphase, structural abnormalities such as aneuploidy, chromosome breaks, and translocations can be detected more readily (Kirov et al., 2014). The analysis generally involves arresting cells at metaphase, preparing high-quality chromosome spreads, and identifying chromosomes using techniques such as FISH (El-Nagish et al., 2025; Sun et al., 2026). For metaphase analysis, tissues with high mitotic activity, such as root tip meristems, young leaves, shoot tips, or callus tissues, are commonly preferred. The developmental stage and mitotic activity of the selected tissue directly affect the number and quality of metaphase plates obtained (Islam et al., 2022; Yan et al., 2024; Jedličková et al., 2024). Because mitotic activity in plants can vary with the time of day, light–dark cycle, temperature, and species, collecting root tips at the right time may improve the metaphase index. Sampling time should therefore be optimized for each plant species through preliminary trials.

Several pretreatment steps are required before chromosomes can be analyzed effectively. For example, ice-water treatment is a pretreatment method applied to root tips in cytogenetics. Its purpose is to accumulate dividing cells, especially at metaphase, sufficiently condense chromosomes, and improve chromosome spreading on the slide. When root tips are kept in very cold water, the low temperature slows spindle function and causes some cells to pause at metaphase. In addition to ice water, pretreatment agents such as hydroxyurea-colchicine, nitrous oxide, 8-hydroxyquinoline, colchicine, alpha-bromonaphthalene, and p-dichlorobenzene are also used to increase metaphase cells, shorten and condense chromosomes, and improve the quality of chromosome spreads (El-Nagish et al., 2025). Fan et al. (2025) stated that the main aim of pretreatment is to arrest cell division at metaphase by inhibiting spindle formation or disrupting existing spindles, and that low-temperature pretreatment with an ice-water mixture is a safer option than many other methods. After pretreatment, root tips are fixed to preserve chromosomal structure and stop cellular enzymatic activity. Mixture of ethanol or methanol and glacial acetic acid, usually prepared at a 3:1 ratio is one of the most commonly used fixatives in plant cytogenetics (Kalefetoğlu Macar et al., 2026).

Enzymatic maceration of root tips with enzymes such as cellulase and pectolyase softens the tissue and helps chromosomes spread more evenly and clearly on the slide. The concentration of the enzyme mixture and the incubation time should be adjusted according to the cell wall structure of the plant and the age of the tissue. Insufficient digestion makes it difficult to separate cells, while excessive digestion may cause chromosomes to disperse, break, or lose their morphology. The macerated tissue is dissected with a needle in acetic acid and dried on a warm surface at approximately 55°C to allow chromosomes to spread well (El-Nagish et al., 2025). This step facilitates the breakdown of cell wall components and the separation of meristematic cells, helping metaphase chromosomes spread individually without overlapping. In plant species with small chromosomes or chromosomes with similar morphology, quality of slide is particularly important for reliable assessment of chromosome changes. DAPI staining is a commonly used fluorescent DNA staining method for checking the quality of metaphase chromosome preparations. Since it binds preferentially to AT-rich DNA regions, it helps assess chromosome morphology, condensation level, spreading quality, and overlap under the microscope.

In well-prepared and properly spread metaphase plates, DAPI staining can make chromosome boundaries clearer, distinguish heterochromatic regions, and, in some species, make centromeric or pericentromeric regions more visible (Bolsheva et al., 2012; El-Nagish et al., 2025). Thus, DAPI staining is an important step for checking preparation quality, selecting suitable metaphase plates, and reliably evaluating chromosomal morphology before karyotype analysis and FISH. After these steps, chromosomes are classified on the basis of chromosome length, arm ratio (long arm/short arm), and centromere position (median, submedian, subterminal, terminal), or they are identified using FISH (Sun et al., 2026). Karyotype asymmetry indices may also be used to quantitatively evaluate structural differences in the chromosome complement. Differences in chromosome lengths and the distribution of centromere positions can help identify large-scale chromosomal rearrangements or deviations in chromosome morphology in transgenic lines.

In transgenic plants, metaphase chromosome analysis allows direct comparison of chromosome number and morphology between the parental genotype and transgenic lines. However, to determine whether an observed change results from transgene integration, tissue culture, or natural chromosomal variation, independent transformation lines, tissue culture controls, and, whenever possible, successive generations should be examined together. Therefore, although metaphase analysis is a fundamental method for the initial evaluation of chromosomal stability, it cannot, by itself, reveal the structure of the integration locus or the molecular integrity of the transgene.

3.2. Evaluation of Mitotic Index and Chromosomal Aberrations

The mitotic index shows the ratio of cells undergoing division in a given cell population and serves as a basic cytological marker of cell proliferation in meristematic tissues. It is calculated by dividing the number of cells in prophase, metaphase, anaphase, and telophase by the total number of cells examined (Macar et al., 2026). In transgenic plants, changes in the mitotic index compared with the parental genotype may reflect the effects of transgene integration, selection pressure, in vitro culture conditions, or regeneration on the cell cycle. A marked decrease in the mitotic index may indicate slowed cell-cycle progression, suppression of mitotic activity, or accumulation of cells in interphase, while an increase may reflect stimulation

of cell division or accumulation of cells at a particular mitotic stage. Nevertheless, the mitotic index alone is not direct evidence of chromosomal instability and should be interpreted together with findings on chromosome structure and segregation (Torres-Bugarín et al., 2026).

Assessment of chromosomal aberrations helps determine how transformation and regeneration processes affect mitotic chromosome behavior. Errors observed at metaphase, such as irregular chromosome alignment, chromosome stickiness, scattered metaphase, C-mitosis, and chromosome fragments, may indicate effects on chromosome structure, condensation, or the spindle system. At anaphase and telophase, chromosome bridges, laggard chromosomes, vagrant chromosomes, multipolar divisions, and irregular chromosome segregation can be evaluated. These abnormalities may lead to chromosome loss, aneuploidy, polyploidy, or micronucleus formation in subsequent cell generations (Yılmaz et al., 2023). Examining the behavior of plant chromosomes during mitotic stages complements the assessment of chromosome number and morphology and provides cellular-level information about genome integrity (Jha and Halder, 2026; Torres-Bugarín et al., 2026).

Chromosomal aberrations can be interpreted as indicators of clastogenic or aneugenic effects according to their mechanisms of formation (Türkoğlu, 2026). Chromosome breaks, acentric fragments, and chromosome bridges are generally considered clastogenic abnormalities associated with DNA breakage or faulty repair. By contrast, C-mitosis, lagging chromosomes, multipolar anaphases, and irregular chromosome distribution may indicate aneugenic effects related to disruption of the mitotic spindle or centromere-kinetochore function.

In transgenic plants, the frequency of chromosomal aberrations can be assessed by calculating the proportion of abnormal mitotic cells among total dividing cells. In addition, each aberration type should be recorded separately to distinguish effects related to chromosome breakage, chromosome loss, and spindle disturbance. Analyses should not be carried out with a single root tip or slide. It is also important to ensure that mechanical breaks, over-digestion during enzymatic treatment, or poor chromosome spreading are not misinterpreted as genuine chromosomal aberrations.

3.3. Micronucleus Test

The production of transgenic plants often includes callus formation, extended in vitro culture, hormone treatments, selective-agent pressure, and

regeneration. These steps can stress the genome and may result in DNA loss, chromosomal errors, and disruptions in the mitotic cycle. Although the micronucleus test cannot directly identify the transgene locus, it can serve as a cytogenetic indicator for monitoring chromosome breaks, chromosome loss, and mitotic segregation errors that may arise from transformation and tissue culture. In plants, micronuclei originate from chromosome fragments or whole chromosomes that fail to be incorporated into the main nucleus; therefore, an increase in micronucleus frequency is considered a cellular reflection of chromosomal instability and genotoxic damage (Yılmaz et al., 2025). The presence of micronuclei is commonly associated with chromosome breaks, missegregation, or chromosomes that fail to attach to spindle fibers. For this reason, increased micronucleus frequency is regarded as an important indicator of chromosomal instability (Fenech et al., 2020). Although the micronucleus test cannot replace molecular approaches such as PCR, Southern blotting, qPCR, sequencing, or FISH, which reveal the presence, copy number, and genomic location of the transgene, it provides cellular-level information on chromosomal damage that these molecular methods cannot supply (Privalle et al., 2020).

3.4. Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a molecular cytogenetic method that uses fluorescently labeled DNA probes to determine the physical position of specific nucleotide sequences on chromosomes (El-Nagish et al., 2025; Sun et al., 2026). In transgenic plants, this technique enables direct visualization of the chromosomal integration sites of introduced genes. In addition, when suitable chromosome- or region-specific probes are used, structural changes in targeted regions, such as deletions, duplications, translocations, and chromosomal rearrangements, can be detected. In metaphase FISH, cells are accumulated at metaphase and condensed chromosome preparations are prepared, whereas in interphase FISH, target chromatin regions can be analyzed directly in non-dividing nuclei (Cheng et al., 2025). Metaphase FISH is advantageous for determining the number of transgene integration sites and their physical location on chromosomes, while interphase FISH allows evaluation of the location and dynamic organization of transgene loci within the three-dimensional nucleus. Because gene and transgene expression largely occur during interphase, the chromatin condensation state of the transgene locus and its position

within nuclear organization may provide complementary information on transcriptional competence and expression stability (Santos et al., 2006).

Preparation is followed by probe development and labeling. Probes can be derived from repetitive sequences, rDNA regions, satellite sequences, or single-copy genes (Li et al., 2023). In in situ hybridization analyses, probes are directly or indirectly labeled DNA or oligonucleotide sequences that are complementary to specific chromosomal target sequences in the cell nucleus. Accurate detection and high binding specificity are achieved by designing oligonucleotide probes whose length and affinity are adjusted to the size and base composition of the target DNA sequence (Cheng et al., 2025). Chromosomal preparations and probe DNA are then denatured so that double-stranded DNA becomes single stranded, allowing the probe to hybridize with the target chromosomal DNA through complementary base pairing (Sun et al., 2026). After hybridization, unbound or nonspecifically bound probes are removed by washing under appropriate temperature and salt-concentration conditions. These washing steps preserve probes that are stably bound to the target sequence while removing free or weakly bound probes. As a result, background signal is reduced and the quality of fluorescent images is improved. In general, the purpose of post-hybridization steps is to strengthen the true target signal and minimize nonspecific background signal, thereby increasing the reliability of FISH analysis (Ling et al., 2025). Preparations are then usually counterstained with dyes such as DAPI to make overall chromosome morphology visible, and fluorescent signals corresponding to target sequences are evaluated under a fluorescence microscope (Sun et al., 2026). In this respect, FISH is a key molecular cytogenetic method for determining the physical positions of DNA sequences on chromosomes (Li et al., 2023). In transgenic plants specifically, FISH is a powerful method for visualizing transgene integration sites, determining the physical localization of the transgene on chromosomes, and better understanding transgene behavior (Santos et al., 2006). For example, Kharb et al. (2001) effectively used FISH to determine the chromosomal position of an introduced T-DNA and the number of integration loci on metaphase chromosomes of rice (*Oryza sativa* L.). Dickinson et al. (2023) also performed DNA-FISH analysis using probes specific for ALS and NPTII sequences in *Arabidopsis thaliana* plants transformed with T-DNA constructs containing retrotransposon-derived sequences. The bright and overlapping fluorescent signals observed in transgenic nuclei showed that the increased T-DNA copy number resulted

not from many independent integration sites distributed throughout the genome, but from a high number of tandemly arranged T-DNA copies at the same locus. Thus, FISH was used as a visual method complementing qPCR and whole-genome sequencing analyses in explaining the chromosomal organization of increased transgene copy number. Similarly, Krispil et al. (2020) investigated complex chromosomal rearrangements associated with T-DNA integration sites in transgenic A. thaliana lines. In that study, translocations between chromosome 1 and chromosome 5 predicted by 4SEE and sequencing analyses were confirmed cytologically using chromosome-specific DNA-FISH probes. In another study, the structural stability of CRISPR/Cas-mediated chromosomal inversions was monitored at the chromosomal level using single-copy oligo-FISH probes (Khosravi et al., 2025). Yan et al. (2017) investigated telomere-mediated chromosome truncation in *Brassica napus* plants transformed with constructs containing Arabidopsis telomeric repeats. In T1 transgenic plants, shortened chromosomes were identified using FISH, and the behavior and inheritance stability of these chromosomes were evaluated in subsequent generations. This example shows that FISH can also be used in transgenic plants to monitor artificial chromosome formation, chromosome engineering, and large-scale chromosomal changes resulting from transformation.

3.5. Meiotic Chromosome Analysis and Reproductive Stability

Meiosis is a specialized cell division that produces haploid gametes through homologous chromosome pairing, recombination, and orderly segregation. Chromosomal rearrangements may be maintained in somatic cells without causing an obvious phenotype or a change in chromosome number, yet they can disrupt homologous pairing, recombination, and regular chromosome distribution to the poles during meiosis. Therefore, analysis of meiotic chromosome behavior can be considered an important complementary approach for evaluating chromosomal stability and intergenerational transmission in transgenic plants (Lambing and Heckmann, 2018; Wang et al., 2021).

In meiotic analyses, the pairing and synapsis of homologous chromosomes during prophase I, the formation of bivalents, univalents, and multivalents at metaphase I, chromosome segregation during anaphase I and II, and tetrad formation can be evaluated (Gutiérrez Pinzón et al., 2021; Park et al., 2020). In regular meiosis, homologous chromosomes are expected to

pair mostly as bivalents and segregate evenly to the two poles. By contrast, univalents, multivalents, chromosome bridges, laggard chromosomes, fragments, irregular tetrads, and unequal chromosome distribution may indicate structural rearrangements or defects in chromosomal pairing. Structural changes in chromosome such as translocations and inversions can cause ring, chain, or irregular chromosomes during meiotic pairing. Such changes can disrupt normal crossing-over patterns, produce unbalanced gametes, and eventually reduce pollen viability, seed set, and overall fertility. Thus, meiotic chromosome analysis makes it possible to evaluate not only chromosomal structure but also the functional consequences of chromosomal changes for reproductive success.

Evaluating meiotic stability in transgenic lines is also important for determining whether the transgene is transmitted regularly to subsequent generations. In addition, the use of chromosome-specific FISH probes on meiotic preparations can help directly monitor homologous pairing defects, translocations, and rearranged chromosomes. Interpreting meiotic findings together with pollen viability, seed set, and transgene segregation ratios in later generations provides a more reliable assessment of the consequences of chromosomal changes for reproductive success. Deviations from expected Mendelian ratios, in particular, may be associated with irregular segregation of the transgene-bearing chromosome or reduced gamete viability (Yin et al., 2004; Privalle et al., 2020).

3.6. Determination of Ploidy and Nuclear DNA Content by Flow Cytometry

Flow cytometry, which is widely used to evaluate genome size, ploidy level, the cell cycle, and endoreduplication (Sliwinska et al., 2022), can contribute to the rapid screening of polyploidy, mixoploidy, and DNA-content changes that may occur during transformation and regeneration in transgenic plants. A flow cytometer consists of several main components: a fluidic system that controls the flow of cells or particles through the instrument; an optical system made up of one or more lasers for excitation and multiple filters, mirrors, and detectors for capturing emitted fluorescence and scattered light; and an electronic and data-acquisition system that is usually integrated with data-analysis software (Robinson et al., 2023).

Because flow cytometry does not directly visualize chromosomes, it cannot reliably detect small aneuploidies, balanced translocations,

inversions, or limited deletions and duplications. Therefore, DNA-content and ploidy differences detected by flow cytometry should be interpreted together with chromosome counting and, when necessary, cytogenetic methods such as FISH (Sliwinska et al., 2022).

4. Discussion

The assessment of chromosomal stability in transgenic plants should not made with a single method. Metaphase analysis helps identify chromosome number and major structural changes, while mitotic-aberration analysis and the micronucleus test provide insight into genome instability at the cellular level. FISH makes it possible to directly visualize the chromosomal location of the transgene and any possible rearrangements, whereas flow cytometry offers a rapid way to screen for changes in ploidy level and nuclear DNA content.

Each method has own specific limitations. Some analyses may fail to detect small deletions, balanced translocations, or multiple transgene copies inserted at the same locus. In addition, to determine whether observed changes arise from transgene integration, tissue culture, or regeneration, the parental genotype, tissue-culture controls, independent transformation lines, and different generations should be evaluated together.

Meiotic chromosome analysis is particularly valuable because it helps detect the effects of rearrangements that may remain unnoticed in somatic tissues, including their impact on homologous pairing, chromosome segregation, pollen viability, and transgene transmission.

5. Conclusion

Assessing chromosomal stability in transgenic plants requires more than a single method. Classical and molecular cytogenetic approaches should be used together to understand the picture. Metaphase analysis, the micronucleus test, meiotic examination, FISH, and flow cytometry each provide different but complementary information, helping to detect changes in chromosome number, chromosome structure, segregation patterns, and ploidy level.

Overall, combining cytogenetic findings with molecular characterization and inheritance data is essential for identifying stable transgenic lines and for making biosafety assessments more robust

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CHAPTER III

SECONDARY METABOLITES AND TERPENES: A BIOLOGICAL PERSPECTIVE FROM BENEFICIAL EFFECTS TO TOXICITY

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1. Introduction

Plants, which are among the indispensable sources of food and oxygen necessary for life, have also been used by humans for therapeutic purposes against various diseases since ancient times. Albert Szent-Györgyi emphasized the significance of photosynthesis and the production of oxygen and nutrients essential for living organisms by stating, “Life is driven by a little current caught by sunlight.” Through these processes, plants produce vital compounds that are utilized in many areas such as textile production, papermaking, food, and spices. However, spices are preferred not only for their flavor-enhancing properties but also for their antimicrobial compounds that help protect food against microbial spoilage (Tapsell et al., 2006). Furthermore, humans are not the only

organisms known to benefit from plants therapeutically; animals have also been observed to selectively consume certain plants when ill (Sumner, 2000). For example, the bezoar goat, a species of wild goat, consumes the Euphorbia plant normally avoided during grazing after snake bites and uses the compound "euphorbone" found in the plant as an antidote.

The origins of herbal treatment methods date back to ancient civilizations. It is well known that cultures from Ancient China, India, Tibet, the Middle East, Mesopotamia, Native American societies, Africa, and Ancient Rome used endemic plants in different ways according to their historical and cultural development (Eichhorn and Evert, 2016). Clay tablets from the Sumerian period reveal that myrrh obtained from *Commiphora myrrha* and opium derived from *Papaver somniferum* L. were used medicinally. Similarly, records from Ancient Egypt indicate that more than 800 plants, including papyrus, aloe, cannabis, castor oil plant, garlic, and juniper, were utilized in medicine production (Sumner, 2000). In the 11th century, Ibn Sina (Avicenna) comprehensively explained methods for obtaining plant compounds and their therapeutic applications in various diseases in his work The Canon of Medicine, which was translated into several languages and widely used for educational purposes.

Hippocrates, known as the father of medicine, also emphasized the importance of incorporating plants into healthy living and suggested that they could be beneficial in massage therapies. Likewise, the French philosopher Montaigne stated, "Doctors should make greater use of scents, for I have observed that they influence one's state of mind," indicating that plants were also employed therapeutically during the 16th century. Research conducted during the 19th and 20th centuries demonstrates an even greater interest in medicinal plants. For instance, Gattefossé (2012) reported that the antiseptic and healing properties of lavender oil contributed to the growing popularity of herbal treatment methods in the early 20th century. French physician Dr. Valnet, regarded as the father of phytotherapy, used essential oils from thyme, rosemary, and other plants for antiseptic and therapeutic purposes in wounded soldiers during wartime (Gattefossé, 2012).

Today, plants are widely utilized in fields such as strengthening the immune system, agricultural pest control, vaccine production, and pharmaceutical manufacturing (Eichhorn and Evert, 2016). Medicinal

plants, in particular, continue to maintain an undeniable importance despite technological advances in the pharmaceutical industry. At the same time, increasing public interest in alternative medicine and natural products under the slogan "return to nature" has also drawn attention. Those seeking to commercialize this interest often promote exaggerated claims such as "healing is in nature" or "the miraculous healing power of nature," arguing that medicinal plants have no side effects (Erdem and Eren, 2009). However, plants used therapeutically in alternative medicine without sufficient knowledge of their effects may produce unintended and harmful outcomes (Valerio and Gonzales, 2005).

Studies have demonstrated that plant compounds may exhibit various bioactivities, including anti-inflammatory, antioxidant, antimutagenic, toxic, and mutagenic effects (Clark, 2009). These studies also provide information regarding the appropriate doses required to obtain beneficial effects. Nevertheless, such information is frequently ignored, and many individuals mistakenly believe that "using more will produce stronger effects." Despite the famous statements of the 16th-century physician and alchemist Paracelsus "Everything is poison; the dose makes the poison" and Ibn Sina's assertion that "every plant is both medicine and poison," improper use continues to cause serious health problems. For example, compounds such as morphine, digitalin, and curare may produce therapeutic effects at recommended doses, whereas higher doses can result in toxic or even fatal outcomes (Moon et al., 2011).

Pharmaceutical companies have traditionally identified and isolated therapeutic compounds from widely used medicinal plants, thereby establishing reliable dose standardization and predicting their effects on the human body. However, it should not be forgotten that even licensed drugs released after extensive safety testing may still produce potential side effects (Calixto, 2000). Since medicinal plants contain numerous compounds, other unidentified constituents may also exert harmful effects on health. For instance, researchers have reported that some unidentified compounds found in weight-loss teas may increase blood pressure, reduce renin and aldosterone levels in the blood, and even lead to fatalities (Van Breemen et al., 2008). Similar concerns also apply to mixtures marketed as antioxidant or anticancer remedies claimed to treat cancer and other serious diseases; adverse effects have likewise been observed following their use (Bush et al., 2007).

As a result of studies aiming to identify plants responsible for such adverse outcomes and to determine the functions of all their compounds, it has been discovered that plants contain various organic compounds or metabolites not directly related to their primary life-sustaining functions. Metabolites synthesized by some animals, plants, and bacteria that do not directly participate in vital activities are called "secondary metabolites," whereas the main products of metabolic reactions involved in growth, development, and reproduction are referred to as "primary metabolites". With advances in science and technology, these plant metabolites have found applications in a wide range of industrial sectors, including the food, pharmaceutical, and cosmetic industries, as well as in alternative medicine and aromatherapy. In recent years, the identification of antimutagenic, antioxidant, and anticarcinogenic activities in certain secondary metabolites has led researchers to suggest that these compounds may have potential applications in the prevention and treatment of many diseases, including cancer, and their consumption through foods has been recommended. However, the widespread use of secondary metabolites as additives, flavoring agents, food colorants, pesticides, and fragrance compounds does not imply that they are entirely harmless.

Humans and other living organisms are continuously exposed to secondary metabolites primarily of plant origin through many products used in daily life, either directly, orally, or through environmental contact. Therefore, in addition to their various bioactive effects, the potential harmful effects of secondary metabolites must also be considered, and both the duration and dosage of use should be carefully regulated. Furthermore, studies have demonstrated that some secondary metabolites and their components exhibit genotoxic activity in a dose-dependent manner (Shah et al., 1996). Consequently, extensive *in vivo* and *in vitro* laboratory studies using model organisms have increasingly focused on investigating the effects of secondary metabolites and their subgroups under different doses and conditions of acute or chronic exposure.

2. Secondary Metabolites

Organic compounds that are catalyzed by various enzymes within the cell and serve as intermediates in metabolic reactions are classified into two groups: primary and secondary metabolites. Primary metabolites are molecules found in both prokaryotic and eukaryotic organisms and are directly involved in essential biological functions such as normal growth,

development, and reproduction. Amino acids, proteins, simple sugars, nucleic acids, and lipids are examples of primary metabolites (Eichhorn and Evert, 2016).

Secondary metabolites, first defined by Kossel in 1891 as compounds distinct from primary metabolites, are substances produced by plants that are not direct products of photosynthesis. Instead, they are formed through various physiological mechanisms and are not absolutely essential for vital physiological processes (Theis and Lerdau, 2003). Rather than participating directly in fundamental life functions, secondary metabolites play important roles in helping plants compete against other species, adapt to environmental stress conditions such as drought, salinity, and ultraviolet radiation, attract insects for pollination, establish symbiotic relationships, and defend against bacterial and fungal pathogens. Some of these metabolites are synthesized specifically by certain species, whereas others are produced in broader or more limited quantities depending on environmental and physiological conditions (Sökmen and Gürel, 2001).

Due to their diverse bioactivities, these compounds are widely used in various industrial sectors, including the food, pharmaceutical, and cosmetic industries, and are also preferred in alternative medicine for the treatment of certain diseases (Kırbağ and Bağcı, 2000). For example, salicylic acid obtained from *Salix* species is used as an analgesic; codeine derived from *Papaver somniferum* is used as a cough suppressant and pain reliever; ephedrine from *Ephedra sinica* functions as a bronchodilator; and digitoxin obtained from *Digitalis purpurea* is used in the treatment of cardiovascular diseases.

In the food industry, growing awareness of the mutagenic, carcinogenic, and teratogenic effects of synthetic additives has increased interest in natural products, leading to the widespread use of secondary metabolites as flavoring, coloring, and aromatic agents. Thaumatin, obtained from *Thaumatococcus daniellii*, is used as a low-calorie sweetener because it is approximately 2,000 times sweeter than sucrose. Crocetin, derived from *Crocus sativus*, is used as a coloring agent, while vanillin, obtained from *Vanilla planifolia*, is used as a flavoring and aromatic compound in the food industry.

Because they are natural and generally considered less harmful to the environment than synthetic pesticides, secondary metabolites are also widely preferred in agricultural pest control. The harmful effects of synthetic agrochemicals on soil, vegetation, air quality, and non-target

organisms have become increasingly significant, and growing environmental awareness among farmers has accelerated the use of natural alternatives. For example, nicotine obtained from *Nicotiana tabacum* and pyrethrin, cinerin, and jasmolin derived from *Chrysanthemum cinerariaefolium* are used as insecticides, whereas anacardic acid from *Anacardium occidentale* is used as a molluscicide.

Secondary metabolites are also extensively utilized in the cosmetics industry. These compounds are found in creams with softening and healing properties, as well as in perfumes, deodorants, lotions, soaps, and shampoos. Various plant-derived mixtures prepared even at home, such as almond oil and garlic-based formulations used for healthier and shinier hair, may contain different secondary metabolites. Such metabolites are also present in lavender oil obtained from *Lavandula officinalis*, rose oil from *Rosa damascena*, jasmine oil from *Jasminum* species, and numerous other plant-derived cosmetic products.

Secondary metabolites are broadly classified into two main groups according to their molecular size: large-molecule secondary metabolites and small-molecule secondary metabolites. Large-molecule secondary metabolites include polyketides, fatty acids, and non-ribosomal peptides, whereas small-molecule secondary metabolites include alkaloids, flavonoids, glycosides, natural phenols, phenazines, biphenyls, and terpenes.

2.1. Large-Molecule Secondary Metabolites

2.1.1 Polyketides

Polyketides are large secondary metabolites containing repeating carbonyl and methylene groups (-CO-CH_2-) and are natural products synthesized by bacteria, fungi, plants, and animals. They possess various biological activities and pharmacological properties. Mycotoxins produced by fungi are examples of polyketides. Many polyketides, such as geldanamycin, doxycycline, and erythromycin, are widely used in different sectors because of their antimicrobial, antibiotic, immunosuppressive, antifungal, antiparasitic, and natural insecticidal properties (Katz, 1997).

2.1.2 Fatty Acids

Fatty acids are secondary metabolites generally found in organisms in the form of triglycerides, phospholipids, and cholesterol, which are the

three major classes of esters. These metabolites serve as both important nutrient sources for animals and essential structural components of cells. Fatty acids are used in soap production as lubricants, emulsifiers, texturizing agents, antifoaming agents, and stabilizers. In addition, esters of fatty acids containing more complex alcohols such as sorbitol and ethylene glycol are commonly consumed in foods and used in personal care products (Moss et al., 1997).

2.1.3 Non-Ribosomal Peptides

Non-ribosomal peptides (NRPs) are secondary metabolites produced by bacteria and fungi and synthesized by multifunctional non-ribosomal peptide synthetases (NRPSs). In this process, amino acids are assembled without the involvement of ribosomes. NRPs are natural products that may occur as toxins, siderophores (iron-chelating compounds), or pigments and possess diverse biological and pharmacological activities such as antimicrobial and anti-inflammatory effects. Some NRPs are commercially marketed as antibiotics, cytostatic agents, and immunosuppressive drugs (Ding and Dai, 2012).

2.2. Small-Molecule Secondary Metabolites

2.2.1 Alkaloids

Alkaloids are powerful metabolites produced by a wide range of organisms, including bacteria, fungi, plants, and animals, and are known for both their addictive and therapeutic properties (Robert, 2013). Due to their diverse biological effects, alkaloids have great medical importance and are used in the treatment of various diseases. Morphine obtained from opium poppy is used in painkillers and cough suppressants; ephedrine is included in medications for colds, hay fever, bronchial asthma, and cardiac arrhythmias (Raymond et al., 2010); atropine is used in ophthalmology; and cocaine has historically been used as a local anesthetic in eye and dental surgeries (Eichhorn and Evert, 2016). In addition, alkaloids such as chelerythrine and homoharringtonine are preferred in alternative medicine because of their antibacterial and anticancer properties. Beyond medicinal uses, some alkaloids, including nicotine and anabasine salts, were historically used as insecticides before the development of synthetic pesticides (Robert, 2013).

2.2.2 Flavonoids

Flavonoids are pigment compounds found in plants that function as chemical messengers, physiological regulators, or inhibitors of the cell cycle within cells (Galeotti et al., 2008). Foods particularly rich in flavonoids include parsley, onions, blueberries, black and green tea, bananas, citrus fruits, buckwheat, and dark chocolate. These compounds exhibit antiallergic, antioxidant, anti-inflammatory, antimicrobial, anticancer, and antidiarrheal activities. Manner et al. (2013) reported that flavonoid deficiency may contribute to cancer, aging, arteriosclerosis, ischemic injury, inflammation, and neurodegenerative diseases such as Parkinson's and Alzheimer's disease.

2.2.3 Glycosides

Glycosides are secondary metabolites in which a sugar molecule is attached to another functional group via a glycosidic bond. Many colorful pigments of flowers, naturally occurring dyes, and aromatic compounds exist in glycoside form. Glycosides are found in plant leaves, bark, and roots, as well as in the seeds of fruits such as bitter almonds, apricots, and peaches. Glycosides present in licorice root are particularly valuable medicinally and are used in vaccines, expectorants, anti-inflammatory drugs, and the production of steroid hormones such as progesterone (Patra and Saxena, 2009).

2.2.4 Natural Phenols

Phenolic compounds produced by plants and microorganisms play important roles in biological defense mechanisms. Plants are known to synthesize phenolic compounds in response to ecological stresses such as ultraviolet radiation, injury, and adverse environmental conditions (Robert, 2013). Foods rich in phenols include green and black tea, red wine, coffee, chocolate, olives and olive oil, spices, medicinal herbs, and nuts. Phenols are used in foods to provide flavor and characteristic color, as well as to protect plants against pests and microorganisms (Perumalsamy et al., 2015). They are also utilized in medicines for treating diarrhea and in alternative medicine because of their antimutagenic, anticarcinogenic, antimicrobial, and antioxidant properties (Lee et al., 2005). Additionally, phenols are used in the manufacture of explosives, plastics, paper, dyes, pharmaceuticals, and pesticides.

2.2.5 Phenazines

The best-known biological sources of phenazines are bacteria belonging to the genera *Pseudomonas*, *Streptomyces*, and *Pantoea*. These compounds enhance environmental adaptation and competitive fitness in the organisms that produce them. For example, according to Dietrich et al. (2013), phenazine-1-carboxylic acid produced by *Pseudomonas* species increases their chances of survival.

2.2.6 Biphenyls

Biphenyls are aromatic organic compounds isolated through the distillation of coal tar, crude oil, and natural gas, and are characterized by their distinctive pleasant odor. They are used industrially in the production of polychlorinated biphenyls (PCBs), in the preservation of citrus fruits due to their antifungal properties, in pharmaceuticals as active components of antibiotics used for skin infections and analgesics used in respiratory, muscular, skeletal, and cardiovascular disorders, and as intermediates in the production of optical brighteners, plant protection products, and plastics (Mouquinho et al., 2011).

2.2.7 Terpenes

As a result of numerous studies identifying their diverse chemical properties and biological activities, terpenes have become widely preferred in various sectors such as food, cleaning products, cosmetics, medicine, and pharmaceuticals. These metabolites, which are present in many products used in daily life, can enter living systems either through direct contact or oral intake depending on the mode of use. In recent years, several plant-derived terpenes have also been recommended as part of alternative therapeutic approaches and are increasingly consumed together with food products. According to Kırca et al. (2006), terpenes exhibiting antioxidant, antimutagenic, and anticarcinogenic properties serve as important sources of biologically active compounds. Dorman et al. (2004) reported that terpenes obtained from various plants such as *Monarda citriodora*, *Myristica fragrans*, *Thymus vulgaris*, *Pelargonium* sp., *Origanum vulgare* ssp., coniferous trees.

Terpenes constitute the largest and most widespread group among the more than 2,000 chemical compounds identified within secondary metabolites. They are a class of hydrocarbons primarily obtained from

plants such as *Pinus*, *Abies*, *Juniperus*, and *Picea* (Başer et al., 2005), and they are major constituents of natural products such as essential oils, resins, waxes, and rubber. However, these compounds are also known to be secreted by butterflies belonging to the family Papilionidae. Terpenes are formed by the sequential arrangement of isoprene units and possess the general chemical formula $(C_5H_8)_n$ and its multiples (Figure 1). Depending on the number of isoprene units they contain, terpenes are classified as hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, and polyterpenes.

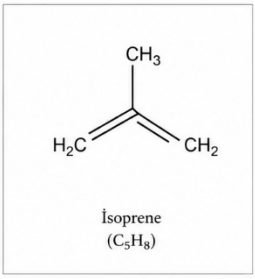

Figure 1. Isoprene Unit

2.2.7. a Monoterpenes

Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. More than 150 different monoterpenes have been identified. These compounds are found in pheromones of some marine organisms and in chemical defense systems secreted by higher plants against insects. Various studies have also reported that monoterpenes possess antiseptic, antibacterial, and antifungal properties (Nieto, 2017). They are widely used in foods as aroma and fragrance and in agriculture and livestock production because of their insecticidal and pesticidal effects additives (Seifried et al., 2007).

Well-known examples of monoterpenes include thymol obtained from *Thymus vulgaris*, menthol from *Mentha arvensis*, carvacrol from *Origanum vulgare*, geraniol from *Pelargonium hybrida* L., citral from *Litsea cubeba*, limonene from lemon peel, and linalool from plants belonging to the Lamiaceae family.

Thymol

Thymol, known as a member of the monoterpene family, is a metabolite obtained from plants such as mint, cumin, eucalyptus, bay laurel, and thyme.

It consists of two isoprene rings and has the molecular formula $C_{10}H_{14}O$ (Figure 2). Due to its antiseptic and antioxidant properties, thymol is used in pharmaceuticals and cosmetics, as a non-persistent pesticide in agriculture, and as an antioxidant and antifungal agent in the food industry (Hammer et al., 1999).

In vitro studies have demonstrated that thymol may have applications in various fields. For example, it is used to prevent food contamination and exhibits contact toxicity against *Drosophila suzukii* (Pavela, 2008). Thymol obtained from *Thymus vulgaris* and *Thymbra spicata* is incorporated into many products such as soaps, toothpaste, shampoos, deodorants, and mouthwashes because of its antimicrobial properties. Owing to its antioxidant effects, it is also recommended for respiratory disorders, as an expectorant, and as a muscle relaxant (Manou et al., 1988). However, several researchers have also reported that thymol may cause mutagenic, genotoxic, and cytotoxic effects in organisms (Franzios et al., 1997). For instance, Azirak and Rencüzoğulları (2008) investigated the *in vivo* genotoxic effects of carvacrol and thymol in rat bone marrow cells at different doses and found that both metabolites significantly induced chromosomal abnormalities, while the mitotic index decreased in a dose-dependent manner across all treatment groups.

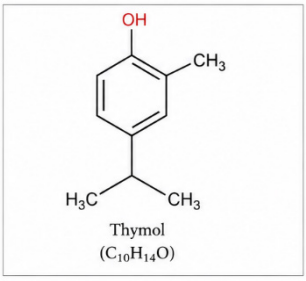

Figure 2. Chemical Structure of Thymol

2.2.7. b Sesquiterpenes

Sesquiterpenes, the largest class of terpenes, consist of three isoprene units and are represented by the molecular formula $C_{15}H_{24}$. Important sesquiterpenes include bisabolol found in *Matricaria recutita*, chamazulene in *Artemisia absinthium*, farnesol in *Vachellia farnesiana*, and guaiazulene in *Cuminum cyminum* (Fiori et al., 2011). Members of the Compositae family, which includes approximately 133 genera and more than 1,156 species in Türkiye as well as certain *Juniperus* species (Tümen and Hafızoğlu, 2003),

Salvia species (Bağcı and Koçak, 2008), *Centaurea* species (Arif et al., 2004), *Prunella* species (Ahmed and Ezer, 2008), *Satureja* species (Azaz et al., 2002), and *Seseli* species within the Umbelliferae family (Tosun and Özkal, 2003), contain high amounts of sesquiterpene compounds.

Guaiazulene

Guaiazulene, belonging to the sesquiterpene group, is found as a pigment in *Guaiacum officinale, G. sanctum*, *G. coulteri*, and in soft corals (Fiori et al., 2011). Composed of three isoprene units, guaiazulene has the molecular formula $C_{15}H_{18}$ (Figure 3). Researchers have reported that, like other terpenes, guaiazulene possesses antioxidant, antimicrobial, and anti-inflammatory properties (Sleha et al., 2014). Due to these characteristics, it is widely used in cosmetics today, particularly in hair and skin care products, bath and cleaning products, and hair dyes. Nevertheless, studies have shown that guaiazulene may also exhibit mutagenic effects at high doses. Wang et al. (2003) observed that guaiazulene, used as a skin-healing agent in cosmetics, caused mutations in *Salmonella typhimurium* TA98 and TA100 strains when applied at high concentrations.

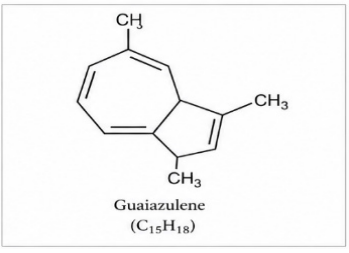

Figure 3. Chemical Structure of Guaiazulene

2.2.7. c Diterpenes

Diterpenes consist of four isoprene units and are represented by the molecular formula $C_{20}H_{32}$. They are widely distributed in nature and are known for their various pharmacological effects. For example, diterpenes obtained from plants belonging to the genus *Taxus* are currently used in the treatment of prostate, ovarian, lung, and breast cancers (Di Costanzo et al., 2009). Common examples of diterpenes include retinol found in fish, red meat, and dairy products; retinal found in carrots, spinach, and fish; and abietic acid present in the resins of coniferous trees.

Abietic Acid

Abietic acid, also known as abietinic acid or sylvic acid, has the molecular formula $C_{20}H_{30}O_2$ and is especially abundant in the resins of coniferous trees. It consists of four isoprene units (Figure 4). This metabolite is used in soap production, varnishes, polishes, pharmaceuticals, and cosmetics. According to Fallarero et al. (2013), abietic acid is a strong healing agent against bacterial infections, while Fernandez et al. (2001) reported that it exhibits anti-inflammatory effects against ear and paw edema in mice and rats.

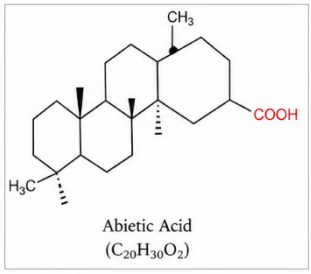

Figure 4. Chemical Structure of Abietic Acid

However, both dehydroabietic acid and abietic acid resin metabolites have been shown to produce toxic effects. Kamaya et al. (2005) observed that increasing concentrations of these compounds shortened survival time and reduced total body size in *Daphnia magna* following both acute and chronic exposure.

2.2.7. d Triterpenes

Triterpenes ($C_{30}H_{48}$), produced by animals, plants, and fungi, consist of six isoprene units. Examples include squalene found in shark liver oil and stigmasterol, oleanane, and ursane found in soybeans, legumes, and nuts. Because of their antioxidant properties, triterpenes are widely used in the food, cosmetic, and pharmaceutical industries (Huang et al., 2009).

Squalene

Squalene, also known as supraene or spinesene, is a triterpene metabolite composed of six isoprene units and has the molecular formula $C_{30}H_{50}$ (Figure 5). It is found in various products such as amaranth flower, olive oil, wheat germ oil, rice bran oil, and shark liver oil. Like phytol, stigmasterol, geraniol, abietic acid, and oleanane terpenes, squalene is

known to possess antioxidant, anticarcinogenic, anti-inflammatory, and neuroprotective effects (Rani et al., 2012). Triterpenes obtained from *Bougainvillea glabra* leaves have also been reported to exhibit antibacterial (Gupta et al., 2009), antihyperglycemic, antifungal and analgesic activities (Elumalai et al., 2012).

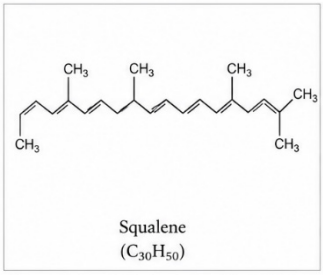

Figure 5. Chemical Structure of Squalene

2.2.7. e Tetraterpenes (Carotenoids)

Tetraterpenes are represented by the molecular formula $C_{40}H_{56}$ and consist of eight isoprene units. Carotenoids found in peaches, carrots, apricots, spinach, and peppers are examples of tetraterpenes. Carotenes play important biological roles in light harvesting, antioxidant activity, protection against free radicals, synthesis of plant hormones, and maintenance of membrane structure. Because of their antioxidant and anti-inflammatory properties, carotenoids are highly valuable compounds in the food and pharmaceutical industries and can be synthesized by both photosynthetic and non-photosynthetic organisms. Xanthophylls represent another widespread group of tetraterpene pigments in nature (Domonkos et al., 2013). Phytoene and phytofluene, which are present in high amounts in tomatoes, are also tetraterpenes and have demonstrated anticarcinogenic properties against various cancers, including liver, lung, breast, prostate, colon, and skin cancer (Khachik et al., 2009).

Astaxanthin

Astaxanthin, a member of the tetraterpene family, consists of eight isoprene rings and has the molecular structure $C_{40}H_{52}O_4$. It is a red-colored pigment (Figure 6). Found in algae known as rhodophytes, this pigment is responsible for the pink or red coloration observed in salmon, trout, lobster, shrimp, and other marine organisms.

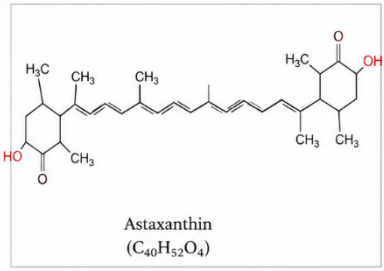

Figure 6. Chemical Structure of Astaxanthin

Like other terpenes, astaxanthin exhibits antioxidant, anti-inflammatory, antitumor, and anti-aging activities (Jyonouchi et al., 2000). Li et al. (2004) demonstrated that alpha-tocopherol and astaxanthin reduced apoptosis in rabbit atheromas. Astaxanthin is also frequently used in drugs designed for the treatment of hypertension and cardiovascular diseases (Hussein et al., 2006). Despite their economic importance and widespread use across many industries, terpenes may also exert toxic effects. Since their applications in cosmetics, cleaning products, pharmaceuticals, food production, and phytotherapy involve direct human exposure, researchers agree that potential genotoxic effects must be carefully considered. Studies have demonstrated that, in addition to their beneficial properties, terpenes may also produce adverse effects, and therefore both the intended application and dosage should be carefully regulated. Furthermore, the genotoxic activity of some metabolites has been shown to increase in a dose-dependent manner (Stammati et al., 1999).

Metabolites such as eugenol, eugenol acetate, and caryophyllene found in *Eugenia caryophyllata*, used in dentistry as an antiseptic and analgesic, and *Cinnamomum cassia*, commonly used in Southeast China, were found to be mutagenic in the *Bacillus subtilis* DNA repair test (Sekizawa and Shibamoto, 1982). *Anethum graveolens*, commonly used in alternative medicine to aid digestion, relieve gas, reduce spasms, and increase breast milk production, contains the terpenes d-limonene and cymene. Phillips et al. (1984) observed that intraperitoneal administration of these terpenes at four different doses to newborn mice caused DNA binding in liver cells and led to tumor formation. The same researchers also reported that different doses of *Petroselinum crispum*, which contains metabolites such as apiol, pinene, and phellandrene, induced liver tumors in newborn mice when administered intraperitoneally. Howes et al. (1990) further reported that terpenes such as methyleugenol and caryophyllene found in *Juglans regia*, *Cinnamomum*

verum, *Piper nigrum*, *Rosmarinus officinalis*, *Eugenia caryophyllata*, and *Cymbopogon citratus* produced similar effects.

Toxicological studies conducted on various diterpene metabolites have also demonstrated that undesirable effects may occur depending on increasing concentrations. For example, Bushnell et al. (1985) observed that dehydroabietic acid, known for its antioxidant and antibacterial properties, caused liver dysfunction, hemolysis, and jaundice in fish. Terpenes such as menthol and menthone, widely used in medicine, pharmaceuticals, cosmetics, food products, teas, and spices, are naturally obtained, particularly from *Mentha piperita*. In one toxicological study, Haseman et al. (1986) administered high doses of menthol to both male and female mice and observed increased liver cancer formation, especially in male mice at higher doses. Franzios et al. (1997) also reported that these mint-derived terpenes exerted cytotoxic and genotoxic effects in *Drosophila melanogaster*.

Lazutka et al. (2001) also reported that monoterpenes such as menthone, limonene, and thymol obtained from mint increased chromosomal aberrations and sister chromatid exchanges in human lymphocytes in a dose-dependent manner. In addition, *in vivo* SMART tests conducted on *D. melanogaster* demonstrated that these compounds caused somatic mutations. Acute oral administration of menthol, menthone, pulegone, and limonene terpenes found in mint to rats revealed low-level toxic effects, while subchronic oral studies showed that these compounds caused cystic lesions in the brain (Nair, 2001). Pavlidou et al. (2004) further reported that menthol, menthone, pulegone, and limonene produced severe intradermal reactions in rabbits, that pulegone exhibited hepatotoxic effects, and that menthone showed genotoxicity in *Bactrocera oleae*. The diterpene dehydroabietic acid, a resin metabolite, has been shown to induce both acute and chronic toxicity in various aquatic organisms, including invertebrates such as *Daphnia magna* and *D. pulex* (Liss et al., 1997). The same metabolite also caused jaundice in *Salmo gairdneri* due to liver dysfunction (Mattsoff and Oikari, 1987). In another study investigating the toxicity of dehydroabietic acid and isopimaric acid terpenes, *Melanotaenia trifasciata* were used as the model organism, and increasing terpene concentrations were found to induce cellular damage and various physiological disorders (Rabergh et al., 1999).

Numerous studies have investigated the biochemical and pharmacological properties of monoterpenes used in the cosmetic, food, and pharmaceutical industries. In particular, thymol and carvacrol obtained from

Origanum onites and *Thymbra spicata* were shown to increase superoxide dismutase (SOD) activity in leukocyte cells of chimpanzees, baboons, and humans (Suzuki et al., 1987). However, the same terpenes were also observed to act as acetylcholinesterase inhibitors in *D. melanogaster,* leading to toxic effects (Quinn, 1987). Hirono et al. (1987) determined that ptaquiloside, a sesquiterpene derived from *Pteridium aquilinum*, caused esophageal and gastric cancers in humans and induced bladder tumors in rats following oral administration. Similarly, Shahin et al. (1998) demonstrated that intravenous administration of ptaquiloside as a DNA-alkylating agent caused mammary gland tumors in rats. Gil da Costa et al. (2014) also reported that this sesquiterpene exhibited genotoxic effects in human lymphocytes by inducing sister chromatid exchange. Gossypol, a metabolite obtained from *Gossypium* species, induced genotoxicity by stimulating sister chromatid exchange in peripheral lymphocyte cells (Best and McKenzie, 1988). *Soja hispida*, which contains terpenes such as phytosterols and stigmasterol, was similarly found to induce sister chromatid exchange and micronucleus formation in mouse bone marrow cells, thereby exhibiting genotoxic effects (Qu et al., 1992).

Metabolites such as limonene, α-terpinene, aromadendrene, terpinen-4-ol, and p-cymene found in *Melaleuca* species, as well as pinene, cineole, and borneol present in *Lavandula*, were reported to cause allergic skin reactions (Knight and Hausen, 1994). Moreover, Hard and Whysner (1994) demonstrated that high doses of limonene induced kidney tumors in male rats. Gardner et al. (1997) noted that methyleugenol, found in *Cinnamomum verum*, *Eugenia caryophyllata*, and *Cymbopogon citratus*, may pose risks to human health because of its carcinogenic effects in rodents at high doses. Somatic mutation and recombination tests conducted with carvacrol, thymol, terpinen, and cymene metabolites obtained from *Coridothymus capitatus* and *Satureja thymbra* demonstrated that carvacrol and thymol, in particular, exhibited genotoxic effects and stimulated somatic mutations in *D. melanogaster* (Stammati et al., 1999). Nogueira et al. (1995) also reported that citral and 1,8-cineole terpenes, widely used in medicine, pharmacy, and the food industry, exhibited cytotoxic and genotoxic effects in human lymphocytes. Sugiura et al. (2000) demonstrated that pinene, cineole, and borneol metabolites present in lavender caused allergic skin reactions in humans. Another monoterpene, methyleugenol, was also reported to pose health risks due to its carcinogenic activity in rodents at high concentrations

(Burkey et al., 2000). Furthermore, Dudai et al. (2000) showed that exposure of germinating wheat to citral inhibited germination, suggesting that some monoterpenes may function as growth inhibitors. According to Al-Bataina et al. (2003), metabolites such as pinene, phellandrene, and cymene obtained from *Cuminum cyminum*, widely used for its calming and balancing effects, exert oxidative activity on *S. typhimurium* strains.

Guaiazulene and azulene sesquiterpenes are frequently used in the cosmetic and pharmaceutical industries, and Wakabayashi et al. (2003) reported that these metabolites exhibited cytotoxicity in human cells. Similarly, Çelik and Uysal (2020) demonstrated through an *in vitro* micronucleus (MN) assay using human peripheral blood cultures that guaiazulene and abietic acid terpenes produced both genotoxic and cytotoxic effects depending on dose and exposure duration. The genotoxic effects of squalene were also investigated using *D. melanogaster* mutant strains carrying the multiple wing hair (*mwh*) and flare (*flr*^{3}) mutations. Administration of squalene at different concentrations stimulated somatic mutations, indicating that this terpene also possesses genotoxic potential (Çelik and Uysal, 2023). Other studies further demonstrated that various terpenes shortened lifespan in both male and female populations of *D. melanogaster*, thereby contributing to population aging (Çelik and Uysal, 2019; Uysal and Çelik, 2021; Uysal, 2022). Terpenes such as limonene, pinene, citral, and eucalyptol found in *Myrtus communis*, an important plant in the food and perfumery industries, are traditionally used as laxatives and antiseptics. However, Hayder et al. (2004) determined that these plant components exhibited mutagenic effects in *Escherichia coli* strains. Applications performed on *Saccharomyces cerevisiae* to evaluate the toxic activity of *Helichrysum italicum*, commonly used for skin diseases, revealed cytotoxic effects. Likewise, metabolites such as thujone, phellandrene, thymol, pinene, and caryophyllene from *Artemisia absinthium*, which is widely used medicinally and pharmacologically, were found to exert mutagenic and toxic effects in the *S. cerevisiae*. Terpenes such as camphor, limonene, and thymol obtained from *Cinnamomum camphora*, preferred in the treatment of respiratory diseases, were also shown to induce genotoxicity (Bakkali et al., 2008). Neral, geranial, citral, and limonene metabolites present in *Litsea cubeba*, widely used in the food, cosmetic, and alternative medicine sectors, caused micronucleus formation and chromosomal aberrations when administered to mice orally, intraperitoneally, or through inhalation (Luo et al., 2005).

The possible genotoxic activities of thymol and carvacrol derived from thyme, commonly consumed as a tea for antibacterial and anti-inflammatory purposes and used against whooping cough, rheumatism, and intestinal disorders, were examined in human lymphocytes using the single-cell gel electrophoresis assay. Significant structural DNA damage was observed at high concentrations (Aydın et al., 2005).

Korkmaz (2005) determined that menthone obtained from *Mentha spicata* and pinene metabolites derived from *Pinus sylvestris* exhibited genotoxic effects in both *D. melanogaster* and human lymphocyte cells. Hassan et al. (2006) reported that different doses of β-sitosterol and stigmasterol from *Trigonella foenum-graecum* altered DNA and RNA contents in mouse liver and ovarian tissues, leading to histopathological changes. When eugenol, a terpene obtained from *Syzygium aromaticum* was applied to rat liver cells under *in vitro* conditions, it induced chromosomal abnormalities (Maralhas et al., 2006). In another study, Silva and Fernandes (2010) observed that monoterpenes such as limonene, pinene, myrcene, linalool, and terpinolene reduced cell viability in human prostate and kidney cells as well as monkey kidney cells. Linalool, a monoterpene, and its ester-derived terpene linalyl acetate are found in *Lavandula angustifolia*, *Ocimum basilicum*, and many other members of the Lamiaceae family. Di Sotto et al. (2008) demonstrated that these metabolites exerted mutagenic effects in *S. typhimurium* TA98 and TA100 strains and in E. *coli* WP2uvrA strains. Acute and subacute effects of neral, myrcene, estragole, linalool, eugenol, and thymol terpenes derived from *Cymbopogon citratus*, *Ocimum gratissimum*, and *Ocimum basilicum*—widely used in the food and cosmetic industries—were also investigated in rats. These terpenes were found to cause functional damage in the stomach and liver of the animals (Fandohan et al., 2008). Fiori et al. (2011) conducted photodegradation studies on azulene and guaiazulene sesquiterpenes, commonly used as natural ingredients in health products, and identified guaiazulene as a photoreactive compound. Azirak and Rencüzoğulları (2008) further demonstrated that thymol and carvacrol monoterpenes significantly induced chromosomal abnormalities in rat bone marrow cells and reduced the mitotic index in a dose-dependent manner. Similarly, Büyükleyla and Rencüzoğulları (2009) reported that thymol stimulated structural chromosomal abnormalities, sister chromatid exchanges, and micronucleus formation while simultaneously decreasing replication and mitotic indices, thereby causing cytotoxicity. The effects of

terpenes such as cineole, thujone, camphor, pulegone, and menthone found in *Salvia fruticosa* and *Mentha pulegium* were investigated in *B. oleae* and *D. melanogaster*. Somatic mutation and recombination tests revealed that camphor, pulegone, and menthone induced mutagenic effects in *B. oleae,* whereas cineole showed weak toxic effects in both organisms (Kounatidis et al., 2009). The toxic effects of squalene, commonly used in cosmetic products, were also observed in mice during studies conducted by Huang et al. (2009), who reported low acute toxicity at high concentrations. Eugenol, another monoterpene, was similarly found to exert toxic effects in Caco-2 cells, a human epithelial colorectal adenocarcinoma cell line (Slamenova et al., 2009). Zerumbone, a sesquiterpene obtained from *Zingiber zerumbet*, is frequently used in the pharmaceutical industry. However, studies evaluating its potential genotoxic effects demonstrated that increasing concentrations inhibited the mitotic index, while micronucleus and cell proliferation assays confirmed significant genotoxic activity (Al-Zubairi et al., 2010). Several studies have shown that monoterpenes such as camphor, eucalyptol, and thujone can induce apoptosis, that terpinen can trigger DNA strand breaks in monkey cells, and that exposure to methyleugenol results in DNA damage in the liver, bone marrow, and kidneys of rats (Ding and Dai, 2012).

Carotenoids, another class of terpenes widely preferred because of their antioxidant activities, have also been shown to cause DNA damage in human peripheral cell cultures and in mice, rats, beagle dogs, and gerbils when used at high concentrations. This effect has been attributed to the pro-oxidant activity that carotenoids may exhibit at elevated doses (Azqueta and Collins, 2012). Metabolites such as methyleugenol and caryophyllene derived from lemongrass also displayed cytotoxic effects in human colon and neuronal cells at high concentrations (Sharma et al., 2009) and caused DNA damage in human lymphocytes (Sinha et al., 2014). Chang et al. (2014) reported that thymol found in *Thymus vulgaris* oil increased calcium release from the endoplasmic reticulum and induced apoptosis by enhancing reactive oxygen species production. According to Koci et al. (2015), gingerol terpene obtained from *Zingiber officinale*, widely used as a flavoring agent in food and as a fragrance component in cosmetics, exhibited cytotoxic effects in canine proximal renal tubule cells. Excessive consumption of retinol diterpene has been associated with liver infections and tumor formation, cellular cytotoxicity, and even congenital malformations in infants (Rajoshok and Sumathi, 2015). Astaxanthin, a red

pigment found in algae known as rhodophytes, is a tetraterpene. Its toxic effects were investigated using *in vivo* lifespan assays in the Oregon-R wild-type strain of *D. melanogaster*, while its genotoxic effects were evaluated through somatic mutation and recombination tests in the same organism (Uysal and Çelik, 2021). To evaluate the safety of terpenes used in foods derived from plants such as *Dianthus caryophyllus*, *Thymus vulgaris*, *Cinnamomum verum*, and *Zingiber officinale*, researchers used canine bone marrow mesenchymal and enterocyte cells. Various terpenes, including thymol, gingerol, eugenol, myrcene, and methyleugenol, exhibited toxic effects in both cell types (Ortega et al., 2016). Finally, Baldissera et al. (2016) investigated terpinen obtained from *Elettaria cardamomum* and *Origanum majorana*. Administration of terpinen at doses of 0.5, 0.75, and 1.0 mg/mL to mice reduced cell viability at all concentrations, induced DNA damage, altered sodium metabolism, and produced cytotoxic as well as genotoxic effects in brain cells.

In conclusion, secondary metabolites and terpenes are highly complex and functional compounds that can be regarded as the chemical language of nature. They play critical roles in interactions between living organisms and their environments and exhibit remarkable chemical diversity. These compounds are involved not only in plant defense mechanisms but also in ecological balance, interspecies communication, and adaptive processes. Growing interest in pharmacology, agriculture, and biotechnology continues to encourage more comprehensive investigations into the biological activities of terpenes. Future studies are expected to provide deeper insights into the molecular mechanisms of these natural compounds, contribute to sustainable applications, and facilitate the development of next-generation biological products. The biological potential offered by these compounds represents not only an opportunity to better understand nature but also a promising avenue for developing innovative solutions for humanity.

3. Conclusion

Secondary metabolites and terpenes represent chemically diverse compounds with important biological and ecological functions. Evidence from the literature demonstrates that these metabolites exhibit beneficial properties, including antioxidant, antimicrobial, anti-inflammatory, and therapeutic activities. However, many studies also indicate that their effects

may become toxic, mutagenic, or genotoxic depending on dose, exposure duration, and biological conditions. Therefore, although terpenes possess significant industrial and pharmacological potential, careful evaluation of safety profiles and dosage parameters remains essential. Future studies focusing on molecular mechanisms and long-term biological effects may contribute to safer and more sustainable applications.

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CHAPTER IV

NEONICOTINOID PESTICIDES: AGRICULTURAL USE, ENVIRONMENTAL PERSISTENCE AND EFFECTS ON HUMAN HEALTH

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1. INTRODUCTION

Neonicotinoids (NEOs) are systemic insecticides first developed in the 1990s and widely commercialized as alternatives to classical organophosphates and carbamates, which are associated with high mammalian toxicity and environmental persistence (Tomizawa & Casida, 2005; Jeschke et al., 2011). These compounds exert their effects on the nervous system by targeting nicotinic acetylcholine receptors (nAChRs), leading to neurotoxicity in insects. By binding to insect nAChRs, neonicotinoids induce continuous stimulation of the postsynaptic membrane, ultimately resulting in paralysis and death (Tomizawa & Casida, 2011; Insecticide

Resistance Action Committee [IRAC], 2023; Solomon, Benoit & Thompson, 2013).

The molecular structures of neonicotinoids, characterized by the presence of nitro or cyano groups, heterocyclic rings, and bridging chains, play a critical role in determining receptor-binding affinity and biological activity (Tomizawa & Casida, 2005). These structural variations also influence their systemic distribution within plants and their selective toxicity toward insects, while maintaining relatively low acute toxicity in mammals (Casida, 2018). Common commercial representatives include imidacloprid, acetamiprid, clothianidin, thiamethoxam, dinotefuran, and thiacloprid (Pisa et al., 2015).

Neonicotinoids are widely applied in agriculture to protect crops such as maize, cotton, soybeans, and sugar beet from sap-sucking pests, including aphids, whiteflies, and thrips. In veterinary medicine, they are used to control ectoparasites such as fleas and lice in domestic animals, and in public health, they are employed for vector control against disease-carrying insects (Jeschke et al., 2011; Pisa et al., 2015). While their use contributes to increased agricultural productivity, these compounds may also exert toxic effects on non-target organisms, particularly pollinators and aquatic invertebrates, thereby raising concerns regarding ecosystem health and biodiversity (Simon-Delso et al., 2015).

2. USES AND TYPES OF NEONICOTINOIDS

Neonicotinoids were first synthesized in 1977 and became widely used following their commercial introduction in the 1990s (Tomizawa & Casida, 2005). Based on their chemical structures and modes of action, these compounds are categorized into four generations, each exhibiting distinct efficacy profiles and environmental impacts (Table 1).

2.1. First-Generation Neonicotinoids

First-generation neonicotinoids, including imidacloprid, acetamiprid, nitenpyram, and thiacloprid, were developed in the 1990s as systemic insecticides with broad-spectrum activity against sap-sucking pests such as aphids and whiteflies. These compounds have been extensively applied in agricultural systems through seed treatments, foliar sprays, and soil applications to protect major crops, including

maize, soybean, sugar beet, and cotton (Tomizawa & Casida, 2011b; Jeschke et al., 2011).

However, their environmental persistence and potential adverse effects on non-target organisms, particularly pollinators, have raised significant ecological concerns. Accumulating evidence indicates that even low, sublethal concentrations of first-generation neonicotinoids can negatively affect a wide range of free-living organisms, including pollinators and aquatic invertebrates (Wood et al., 2017). For example, studies on acetamiprid have demonstrated that it can disrupt redox homeostasis in the central nervous system of honey bees, leading to increased oxidative stress and potential impairment of bee health (Mackei et al., 2024).

Similarly, imidacloprid has been reported to exert toxic effects on avian species. Ingestion of imidacloprid-treated seeds has been shown to cause mortality in adult partridges, as well as to reduce reproductive investment and impair offspring immunity (Lopez-Antia et al., 2015). Furthermore, exposure to imidacloprid in the passerine species *Agelaioides badius* has been associated with behavioral and physiological alterations, including changes in activity patterns and reductions in body weight (Sánchez-Bayo et al., 2023).

Collectively, these findings highlight the broader ecological risks associated with first-generation neonicotinoids.

2.2. Second-Generation Neonicotinoids

Second-generation neonicotinoids, particularly thiamethoxam and clothianidin, were developed with structural modifications aimed at reducing environmental accumulation and increasing selectivity. Thiamethoxam exhibits both systemic and contact activity and is capable of rapidly translocating within plants, thereby providing effective protection. Clothianidin, on the other hand, is known for its long-lasting effects and is especially preferred in seed treatment applications.

Despite these advantages, concerns remain regarding their environmental impact. For instance, studies on clothianidin have highlighted its persistence in soil and its long-term effects in aquatic ecosystems. The reported half-life of clothianidin in soil ranges from 57 to 95 days, increasing the risk of leaching into groundwater, particularly during periods of heavy rainfall. Although clothianidin can degrade rapidly via photolysis,

its persistence in soil raises concerns about long-term ecological effects (NaturePest, 2025).

2.3. Third-Generation Neonicotinoids

Dinotefuran, a third-generation neonicotinoid, represents an advancement in neonicotinoid chemistry, characterized by its unique furan-group structure. This modification distinguishes it from earlier compounds such as imidacloprid and acetamiprid, which contain nitroguanidine or cyanoamidine functional groups. The presence of the furan moiety contributes to increased water solubility, faster environmental degradation, and reduced bioaccumulation, thereby enhancing environmental compatibility while maintaining strong insecticidal efficacy (Mori et al., 2002; Ihara & Matsuda, 2018).

Dinotefuran acts as an agonist of nicotinic acetylcholine receptors (nAChRs), leading to overstimulation of the insect nervous system and subsequent paralysis. Its receptor-binding profile demonstrates greater selectivity for insect nAChR subunits compared to those in vertebrates, which underlies its relatively lower toxicity in mammals and birds (Tomizawa & Casida, 2011).

Despite its improved safety profile, recent ecotoxicological studies indicate that dinotefuran is not entirely benign. It can induce both acute and chronic effects in aquatic organisms, particularly invertebrates such as *Daphnia magna* and early developmental stages of fish. Experimental findings suggest that exposure to dinotefuran can lead to neurological dysfunction, oxidative stress, and mitochondrial impairment, likely mediated by calcium ion imbalance and excessive production of reactive oxygen species (ROS) (PMC, 2021a; PMC, 2021b; Ma et al., 2023). These disruptions may result in impaired swimming behavior, reduced growth, and increased mortality, even at sublethal concentrations.

Furthermore, residues of dinotefuran have been detected in surface waters and sediments in agricultural regions, indicating its potential for temporary bioaccumulation and adverse effects on aquatic biodiversity (Ghosh et al., 2014; Taira et al., 2020). Although designed to minimize persistence, environmental factors such as temperature, pH, and organic matter content can prolong its half-life, emphasizing the need for careful monitoring in sensitive ecosystems such as rice paddies and wetlands.

2.4. Fourth-Generation Neonicotinoids

The fourth generation of neonicotinoids represents a novel structural class designed to retain insecticidal potency while addressing the environmental and ecotoxicological limitations of earlier compounds. The most prominent example, flupyradifuron, belongs to the butenolide group and acts as an agonist of nicotinic acetylcholine receptors (nAChRs), similar to classical neonicotinoids (Bass et al., 2015; Pisa et al., 2015).

Flupyradifuron is classified under IRAC Group 4D, a subgroup of nicotinic receptor agonists, and was developed to achieve enhanced selectivity for insect nAChR subtypes, resulting in lower toxicity to vertebrates and pollinators. Compared with compounds such as imidacloprid and clothianidin, it demonstrates faster degradation rates, reduced soil persistence, and minimal bioaccumulation, thereby offering improved environmental compatibility (European Commission, 2023b; IRAC, 2025).

Toxicological assessments indicate that flupyradifuron exhibits low acute and chronic toxicity to non-target organisms, including bees, birds, and aquatic species (Pisa et al., 2015). However, laboratory and semi-field studies have reported potential sublethal effects, such as altered foraging behavior and reduced motor coordination in pollinators following prolonged exposure or exposure to high concentrations. These findings highlight that, despite its advantages, continuous ecological risk assessment remains essential to ensure pollinator protection and maintain long-term ecosystem stability.

In addition to agricultural applications, flupyradifuron and related compounds have also been used in veterinary medicine for the control of ectoparasites such as fleas and lice in domestic animals (Xerces Society, 2025). Nevertheless, increasing awareness of the ecological impacts of neonicotinoids has led to regulatory restrictions and bans in several regions, including the European Union, in order to protect biodiversity and pollinator health (European Commission, 2023a; Xerces Society, 2025).

Overall, fourth-generation neonicotinoids, such as flupyradifuron, represent an important step toward safer and more sustainable pest control strategies. However, their continued use underscores the need for integrated pest management approaches that minimize non-target risks and support environmental conservation.

Table 1. Generational Classification of Neonicotinoid Compounds.

Generation	Representative Active Compounds	Chemical Properties and Applications	Year of Commercial Introduction	Notes / References
1st Generation	*Imidacloprid, Acetamiprid, Nitenpyram, Thiacloprid*	Chloropyridinyl structure, broad-spectrum activity	1990s	First commercial products. Highly effective against various pests; however, environmental persistence and adverse effects on non-target organisms have raised concerns (Tomizawa & Casida, 2005; Bass et al., 2015).
2nd Generation	*Thiamethoxam, Clothianidin, Thiacloprid*	Thiazolyl structure, low environmental accumulation	2000s	Developed to reduce environmental persistence and minimize adverse effects on non-target species (Jeschke et al., 2011; Bass et al., 2015).
3rd Generation	*Dinotefuran*	Furfuryl group structure, specific mode of action	2002	Designed for more specific mechanisms of action and enhanced environmental safety (Simon-Delso et al., 2015; Bass et al., 2015).
4th Generation	*Flupyradifurone*	Butenolide group; neonicotinoid-like mode of action	2014 (Bayer launch)	Classified by IRAC as Group 4D. Provides advantages in environmental safety and target selectivity (IRAC, 2025a; European Commission, 2023).

3. MECHANISM OF ACTION OF NEONICOTINOIDS

Neonicotinoids primarily act on the insect nervous system by binding with high affinity to nicotinic acetylcholine receptors (nAChRs). These receptors normally respond to acetylcholine, a key neurotransmitter responsible for synaptic transmission. As agonists or partial agonists, neonicotinoids induce persistent activation of nAChRs, resulting in prolonged neuronal depolarization, disruption of normal synaptic signaling, and ultimately paralysis and death in target insect pests (Tomizawa & Casida, 2011b; Casida, 2018).

The structural differences between insect and mammalian nAChRs underlie the selective toxicity of neonicotinoids toward insects. However, this selectivity is not absolute. Variations in receptor subtypes, density, and expression patterns among species, together with prolonged or high-dose exposure, may lead to neurophysiological disturbances in non-target organisms. Reported effects include behavioral alterations, cognitive impairment, and endocrine disruption, indicating the potential for sublethal and chronic toxicity beyond intended targets (Gibbons, Morrissey & Mineau, 2015).

Moreover, the chemical stability and environmental persistence of neonicotinoids increase the likelihood of exposure in non-target organisms. Residues in plant tissues, pollen, nectar, and soil, along with contamination of aquatic environments, facilitate indirect effects on beneficial insects, birds, and mammals (Bonmatin et al., 2015; Simon-Delso et al., 2015; Morrissey et al., 2015). Although these effects are often sublethal, they can impair foraging behavior, navigation, reproduction, and immune function, thereby influencing pollination services, food web dynamics, and overall ecosystem biodiversity (Gibbons, Morrissey & Mineau, 2015).

This mechanistic framework complements the preceding sections on environmental distribution and ecological impacts, establishing a critical link between molecular interactions and ecosystem-level consequences. A detailed understanding of nAChR-mediated mechanisms enables more accurate risk–benefit assessments of neonicotinoid use and supports the development of mitigation strategies aimed at minimizing adverse effects on non-target organisms (Casida, 2018).

4. TOXIC EFFECTS OF NEONICOTINOIDS

The toxic effects of neonicotinoid pesticides across diverse organism groups are summarized in Table 1, highlighting their neurotoxic, physiological, and behavioral impacts over a broad biological spectrum, ranging from pollinators to mammals. Owing to their systemic properties and high water solubility, neonicotinoids can persist in environmental compartments for extended periods. For instance, the half-life of imidacloprid in soil ranges from 28 to 1,250 days, reflecting substantial environmental persistence. Their mobility facilitates transport into surface and groundwater systems, where they exhibit pronounced toxicity to aquatic organisms, including fish and invertebrates (Goulson, 2013; Morrissey et al., 2015).

Neonicotinoids remain relatively stable under mildly acidic to neutral conditions (pH 5–7), although they degrade more rapidly in alkaline environments. Residues detected in pollen and nectar constitute a continuous exposure source for pollinators, while their persistence in soil and water promotes bioaccumulation and trophic transfer, thereby increasing risks to non-target organisms across different ecological levels (Tomizawa & Casida, 2005; Simon-Delso et al., 2015).

At the cellular and molecular level, neonicotinoids have been shown to elevate reactive oxygen species (ROS) production, leading to oxidative stress, DNA damage, and apoptosis in various tissues, including hepatocytes and erythrocytes (El-Garawani et al., 2022; Huang et al., 2021). In addition to these biochemical effects, significant behavioral and neurophysiological alterations have been documented. In insects, neonicotinoids impair essential behaviors such as foraging and navigation, whereas in fish, exposure has been associated with abnormal swimming patterns and reduced locomotor activity (Whitehorn et al., 2012; Azadikhah et al., 2023).

Chronic exposure further contributes to developmental toxicity. Experimental studies in mammalian models have demonstrated outcomes such as reduced birth weight, structural abnormalities, and delayed organogenesis (Morrissey et al., 2015). Species-specific sensitivity to neonicotinoids is largely influenced by differences in metabolic capacity and variability in nicotinic acetylcholine receptor subtypes, which explains the generally higher susceptibility observed in insects compared with vertebrates such as fish and mammals (Huang et al., 2021; Casida, 2018).

In humans, acute exposure to neonicotinoids may induce symptoms including headache, dizziness, nausea, and, in rare cases, tremors. Chronic

exposure has been associated with neurocognitive impairments, particularly affecting memory and attention, as well as broader neurophysiological disturbances (Casida, 2018; Gibbons et al., 2015). Biochemical alterations, such as impaired hepatic and renal function and increased oxidative stress, have also been reported.

Overall, current evidence indicates that neonicotinoids exert multi-level toxic effects, spanning molecular disruptions, cellular damage, behavioral alterations, developmental toxicity, and long-term ecological consequences. The data summarized in Table 2 further emphasize the diversity and severity of these effects across different taxa.

5. SOME STUDIES ON NEONICOTINOIDS

Whitehorn et al. (2012) reported that exposure to low doses of imidacloprid significantly reduced both colony growth and queen production in bumblebees, whereas Henry et al. (2012) demonstrated that acute neonicotinoid exposure impaired the navigation ability of honeybees. These findings provided experimental evidence of the ecological risks posed to pollinators and subsequently served as a foundation for institutional risk assessments.

The European Food Safety Authority (EFSA, 2018), in its comprehensive risk assessment of clothianidin, imidacloprid, and thiamethoxam, concluded that these compounds pose a high risk to both honeybees and wild bee populations. Following this evaluation, the European Union implemented a ban on the open-field use of these neonicotinoids. This regulatory decision confirmed the risks identified in experimental studies and established a scientific basis for policy measures aimed at safeguarding pollinator health.

Straub et al. (2019) demonstrated that the combined effects of neonicotinoids and the ectoparasite *Varroa destructor* significantly reduced the survival of long-lived winter honeybees. Although neonicotinoids alone did not produce a significant effect, their interaction with mite infestation resulted in pronounced negative impacts on colony health. The authors emphasized that future mitigation strategies should prioritize reducing neonicotinoid exposure and developing sustainable control methods for *V. destructor*. These findings further reinforce the scientific basis for the regulatory measures proposed by EFSA and broader ecosystem protection strategies.

Although the effects of imidacloprid (IMI) and its metabolites on terrestrial organisms have been extensively investigated, the underlying

toxicity mechanisms in aquatic arthropods remain insufficiently understood. Huang et al. (2021) examined the acute toxic effects of IMI and four of its metabolites on *Cloeon dipterum* and *Gammarus pulex*. The results indicated that only the IMI-ole metabolite exhibited toxicity in *C. dipterum* comparable to that of the parent compound. While *C. dipterum* showed limited capacity to eliminate IMI-ole, *G. pulex* was able to eliminate it more efficiently. These findings suggest that both biotransformation capacity and species-specific differences influence IMI toxicity, which appears to be dependent on both exposure duration and concentration.

Akbulut (2021) investigated the histopathological effects of imidacloprid (IMI) on the gonadal tissues of zebrafish (*Danio rerio*). The fish were exposed to 9.5, 19, and 38 mg/L IMI for five days, based on LC_{50} values. Histological analyses revealed marked alterations in the experimental groups. In testicular tissue, degeneration of seminiferous tubules, hypertrophy of spermatogenic and Leydig cells, and interstitial fibrosis were observed. In ovarian tissue, structural disruptions in oocytes, an increased number of atretic oocytes, thickening of the zona radiata, and separations within the perifollicular layers were detected (Figure 1).

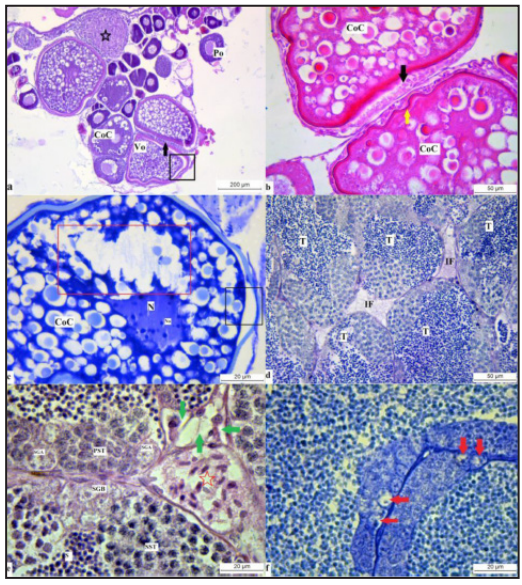

Figure 1. As a result of the experiment, normal gonadal histology was observed in the control group, whereas histopathological alterations were detected in the testis and ovarian tissues of the experimental groups. (Akbulut, 2021).

Studies investigating the effects of neonicotinoids on aquatic organisms have revealed significant genotoxic and histopathological alterations,

particularly in the *Oreochromis niloticus* model. El-Garawani et al. (2022) demonstrated that exposure to low doses of acetamiprid and imidacloprid for 21 days significantly increased the frequency of micronuclei and other nuclear abnormalities in erythrocytes (Figure 2a). In addition, both insecticides induced pronounced degenerative structural changes in gill, liver, and muscle tissues.

Similarly, several studies have reported that acetamiprid and imidacloprid exposure leads to oxidative stress, biochemical disturbances, and tissue damage in tilapia (El-Garawani et al., 2022; Guedegba et al., 2021; Almeida et al., 2021) **(Figure 2b).** Collectively, these findings suggest that neonicotinoids pose a substantial risk to fish health and may have broader implications for aquatic ecosystem integrity.

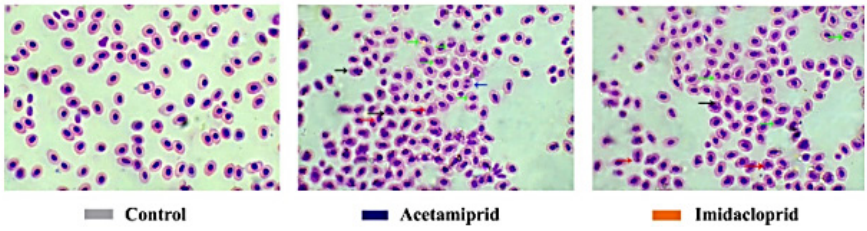

Figure 2a. Erythrocytic nuclear abnormalities and micronuclei induced by 21-day exposure to acetamiprid (19.5 ppm) and imidacloprid (15 ppm) in *Oreochromis niloticus*.

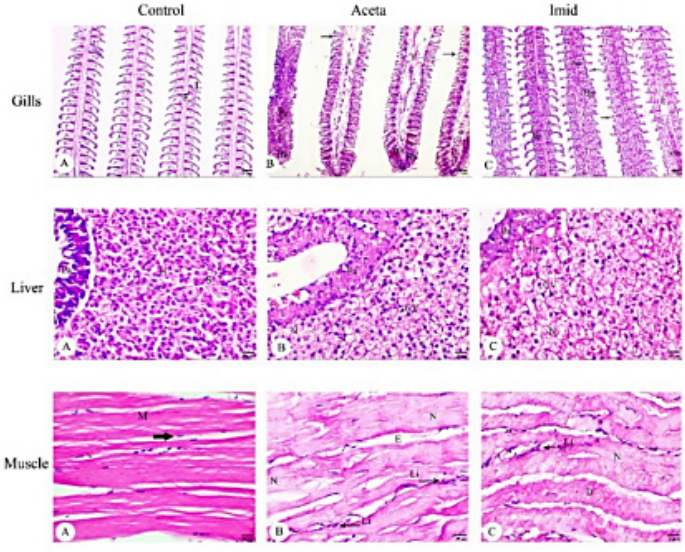

Figure 2b. Light micrographs of the gill, liver, and muscle tissues of *Oreochromis niloticus* showing the effects of acetamiprid (Aceta, 19.5 ppm) and imidacloprid (Imid, 15 ppm) exposure. (El-Garawani et al., 2022).

Azadikhah et al. (2023) investigated the acute toxic effects of acetamiprid on *Ctenopharyngodon idella*, reporting increased mortality rates, pronounced tissue damage, and significant alterations in hematological parameters at higher concentrations. Histopathological examination revealed hypertrophy, hyperplasia, and epithelial necrosis in the gill filaments **(Figure 3).** In the liver, hepatocytes exhibited marked degenerative changes, including pyknosis, karyorrhexis, and hemorrhagic lesions. In addition to structural damage, acetamiprid exposure induced notable behavioral alterations in fish, such as rapid surface swimming, irregular opercular movements, and mortality associated with persistent open-mouth symptoms. Hematological analyses further demonstrated significant increases ($P < 0.01$) in stress-related biomarkers, including glucose, total protein, albumin, and cholesterol levels.

Collectively, these findings provide clear evidence of the toxic impact of acetamiprid on fish physiology and behavior, underscoring its potential environmental risks, particularly in aquatic ecosystems.

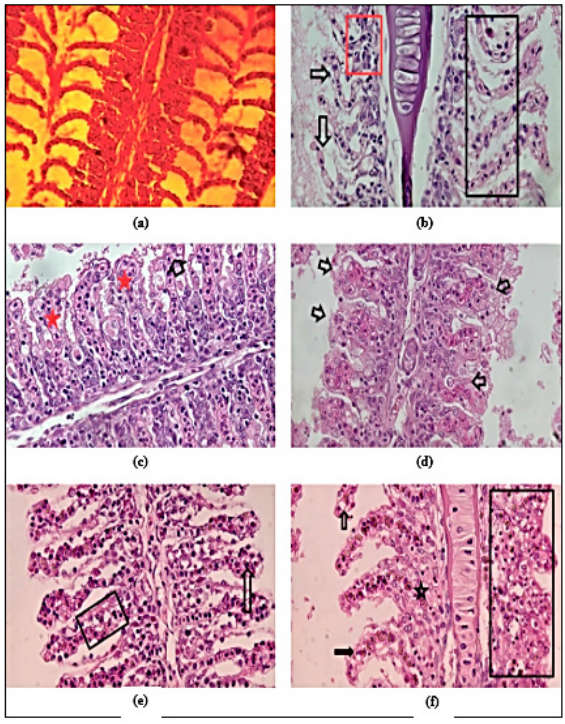

Figure 3. Histopathological examination revealed hypertrophy, hyperplasia, and necrosis of gill epithelial cells in the filaments. (Azadikhah et al., 2023).

Thiacloprid (THD) has been reported to exert potential toxic effects on vertebrates. Initially regarded as a neonicotinoid with minimal impact on non-target organisms, particularly invertebrates, emerging evidence has challenged this assumption. Recent findings by Flach et al. (2023) indicate that THD may exhibit carcinogenic and reproductive toxicity, suggesting its potential to induce adverse environmental effects. These observations highlight the necessity for a more comprehensive evaluation of THD, particularly with respect to its impact on amphibian embryogenesis. In this context, Flach et al. (2023) investigated the effects of THD on early developmental stages by exposing stage 2 embryos of the African clawed frog (*Xenopus laevis*) to a range of concentrations (0.1–100 mg/L) for 14 days. The results demonstrated that THD exposure led to significant developmental impairments. Notable findings included reductions in body length and locomotor activity, as well as marked morphological abnormalities such as shrinkage of cranial cartilages, eyes, and brain structures, shortening of cranial nerves, and defects in cardiogenesis **(Figure 4).**

At the molecular level, THD exposure resulted in decreased expression of key developmental markers, including the brain-specific gene *emx1* and the cardiac marker *mhcα*. Collectively, these findings indicate that THD can interfere with both morphological and molecular processes during embryonic development. Consequently, these results emphasize the importance of stringent monitoring of THD application levels and regulatory frameworks to mitigate potential environmental risks.

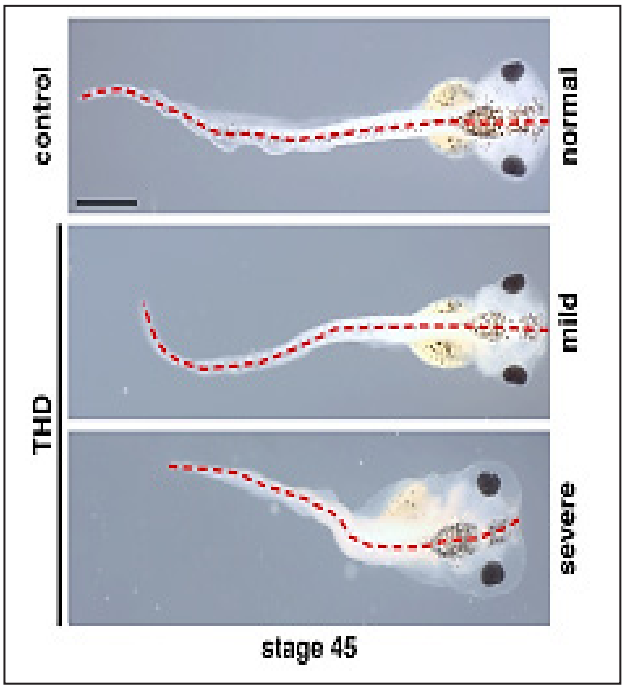

Figure 4. Effects of THD exposure on stage 45 *Xenopus laevis* embryos. Normal development was observed in the control group, whereas THD-exposed groups showed progressively increasing body curvature and developmental retardation (mild → severe). The red dashed line indicates the embryonic spinal axis. (Flach et al., 2023).

Salvaggio et al. (2018) reported that high-dose exposure to thiacloprid (THD) induces pronounced teratogenic effects in *Gallus gallus domesticus* embryos. The study demonstrated significant growth retardation, characterized by marked reductions in both body length and body weight. In addition, various structural abnormalities were observed, including limb malformations and ectopic organ positioning.

Histological analyses further revealed substantial cellular alterations in both liver and lung tissues. These changes included hepatocellular degeneration, dilation of hepatic sinusoids, and disruptions in alveolar architecture. Collectively, these findings indicate that high-dose THD exposure can adversely affect embryonic development at both morphological and histological levels, thereby posing significant teratogenic risks **(Figures 5a,b).**

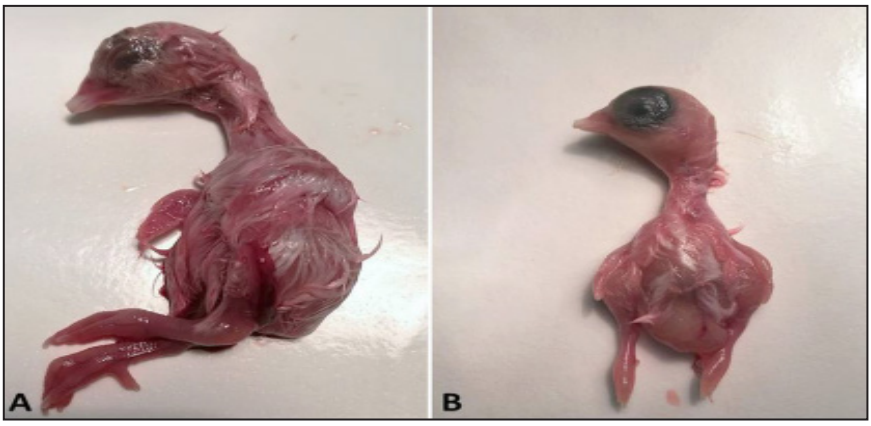

Figure 5a. Comparison between the control group and chicks exposed to the lowest dose of thiacloprid. (Salvaggio, A., 2018).

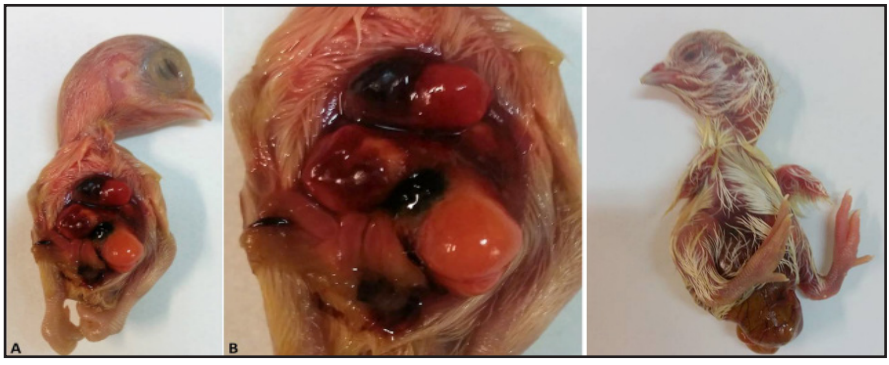

Figure 5b. Total visceral ectopy, unabsorbed yolk sac, developmental abnormalities, growth retardation, and limb defects. (Salvaggio, A., 2018).

Toor et al. (2013) investigated the effects of repeated oral administration of imidacloprid (IMI) on the liver of female albino rats (Figure 6). The study reported marked dilatation of the central veins and hepatic sinusoids following high-dose IMI exposure, indicating a disruption of normal hepatic architecture. Furthermore, plasma biochemical analyses revealed significant elevations in AST (aspartate aminotransferase), ALT (alanine aminotransferase), and ALP (alkaline phosphatase) levels, reflecting hepatocellular injury and impaired liver function.

Collectively, these findings suggest that repeated high-dose exposure to IMI induces both histopathological alterations and functional impairment in the liver, underscoring the potential risks associated with chronic exposure.

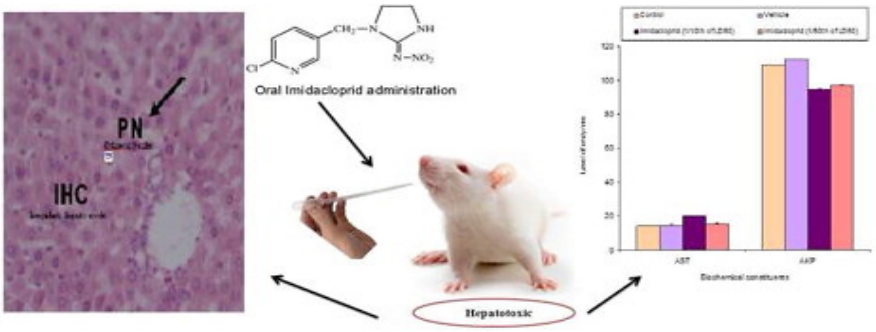

Figure 6. Study on neonicotinoids conducted by Toor et al. (2013).

In recent years, the potential impacts of neonicotinoids on human health have attracted increasing scientific attention. Tu et al. (2023) conducted a comprehensive review of human biomonitoring studies published between 2010 and 2022, analyzing a total of 57 articles encompassing 64 individual studies. Their findings demonstrated that neonicotinoid (NEO) insecticides and their metabolites have been detected in a wide range of human biological matrices, including urine, blood, plasma, serum, and hair.

The review further indicated that the most commonly employed analytical techniques were liquid chromatography–tandem mass spectrometry (LC–MS/MS), often combined with solid-phase extraction (SPE) and liquid–liquid extraction (LLE). Across the analyzed studies, a total of 20 different NEO compounds and their metabolites were identified in human samples. Moreover, the authors reported regional variations in exposure levels, evidence of potential transplacental transfer in certain cases, and emphasized the need for more comprehensive investigations focusing on vulnerable populations, particularly children and pregnant women **(Figure 7).**

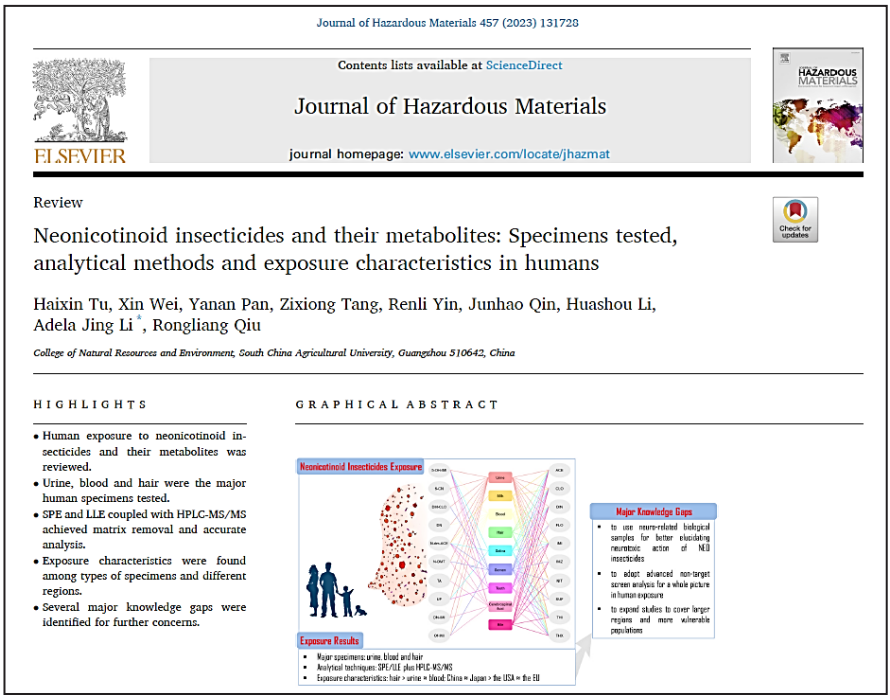

Figure 7. Study on neonicotinoids conducted by Tu et al. (2023).

In addition, Tang et al. (2021) conducted a large-scale modeling study to evaluate the global risk of pesticide pollution. This research mapped the potential contamination risks of pesticides in surface waters and terrestrial ecosystems worldwide by integrating variables such as pesticide type, application intensity, environmental conditions, and soil characteristics.

The results indicated that approximately one-third of global croplands are exposed to a high risk of pesticide pollution, with intensively cultivated regions being particularly susceptible. These findings underscore the urgent need for the development of sustainable agricultural policies and the implementation of strategies aimed at reducing the environmental impacts of pesticide use **(Figure 8).**

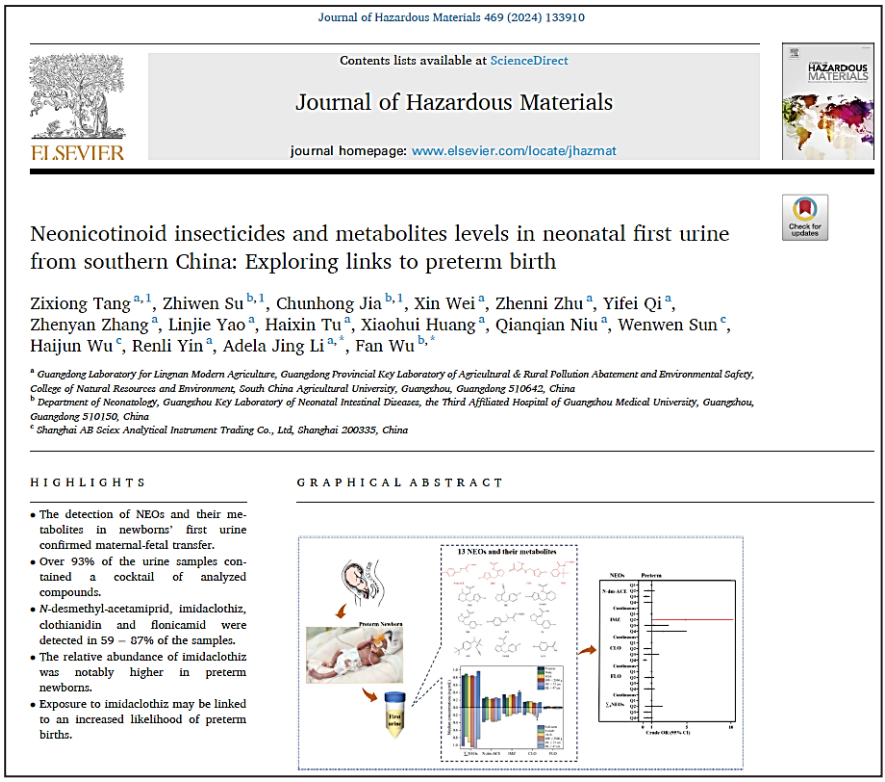

Figure 8. Study on neonicotinoids conducted by Tang et al. (2023).

6. CONCLUSION

In conclusion, neonicotinoid insecticides, which have been extensively used in agricultural production over the past three decades, can be applied effectively and relatively safely when regulatory guidelines, recommended application rates, and appropriate usage protocols are strictly followed. However, deviation from these conditions through overuse, misuse, or prolonged application may result in significant adverse effects on environmental compartments, including soil, water, and the atmosphere, similar to those associated with other classes of pesticides.

Notably, neonicotinoids have been shown to exert toxic effects on non-target organisms, particularly pollinators such as bees, thereby posing a substantial threat to biodiversity, ecosystem functions, and ecological stability. In addition, repeated and improper use may promote the development of resistance in both target and non-target species, leading

to ecological imbalances, reduced pest control efficacy, and considerable economic losses in agricultural systems.

Beyond their environmental impacts, increasing scientific evidence suggests that the widespread use and environmental persistence of neonicotinoids may also pose risks to human health. Although these insecticides generally exhibit lower acute toxicity in humans than many conventional pesticides, continuous exposure through contaminated food, drinking water, occupational activities, or environmental residues has become an issue of growing concern. Experimental and epidemiological studies indicate that long-term exposure may be associated with adverse effects on the nervous, reproductive, and immune systems, as well as potential developmental and endocrine-related disorders. Nevertheless, the current evidence remains insufficient to establish definitive causal relationships, highlighting the need for further long-term epidemiological and toxicological studies to better understand human exposure pathways and associated health risks.

Therefore, the responsible and controlled application of neonicotinoids within the framework of integrated pest management (IPM) and sustainable agricultural practices is of critical importance. Strengthening regulatory oversight, promoting the rational use of pesticides, and encouraging the development of environmentally friendly alternatives are essential to preserving the effectiveness of neonicotinoids while minimizing ecological and human health risks. Such an approach will contribute to sustainable agricultural production, environmental protection, and the safeguarding of public health for future generations.

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CHAPTER V

CLIMATE CHANGE AND INSECT-PLANT INTERACTIONS: MOLECULAR ECOREGULATION, THERMAL PLASTICITY, AND MULTITROPHIC DYNAMICS

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1. Introduction to Climatic Forces and Insect Ecology

Global climate change represents one of the most transformative anthropogenically driven pressures on global terrestrial ecosystems. Ectothermic organisms, particularly insects, are acutely sensitive to shifts in ambient temperature, altered precipitation regimes, and elevated atmospheric carbon dioxide. Because their internal body temperature, metabolic rate, developmental timeline, and reproductive output are bound to external environmental parameters, understanding these shifts is vital. Over the past several decades, environmental perturbations have accelerated, pushing species toward critical thermal limits and disrupting evolutionary partnerships between insects and their host plants (Bale et al., 2002).

The field of molecular ecology and insect physiology has integrated high-throughput transcriptomics, metabolomics, and field-based multi-trophic manipulation experiments to explore the mechanisms underlying insect responses to environmental stress. These responses operate across scales from cellular signal transduction pathways to massive shifts in regional biodiversity.

The historical stability of macroclimatic networks has allowed the coevolution of complex trophic webs where plant volatile emissions, chemical defense synthesis, and insect nutritional requirements exist in structural balance. However, the current pace of global warming threatens to decouple these interactions. High-temperature anomalies alter the expression of physiological cascades in both phytophagous insects and their hosts, which changes the structural stability of agricultural and natural ecosystems. Zvereva and Kozlov (2006) indicated that these changes are not linear but represent a web of direct thermal impacts on insect survival and indirect modifications mediated through host-plant tissue quality. This chapter provides an advanced synthesis of the molecular, biochemical, and ecological factors governing insect-plant interactions in a warming world, highlighting the current paradigms driving modern entomology and global change biology.

2. Microclimatic Refugia and Behavioral Ecoregulation

This behavioral ecoregulation fundamentally alters the spatial and temporal dynamics of how insects interact with their host plants. During extreme heat events, macroclimatic ambient temperatures frequently surpass the physiological optima of herbivorous insects, turning open microenvironments into lethal zones. To survive, herbivores actively abandon highly exposed, sunlit upper canopy leaves and migrate vertically down the plant architecture to settle in the lower, shaded portions of the plant or relocate horizontally to the abaxial (underside) leaf surfaces (Bale et al., 2002; Caillon et al., 2014). This non-random behavioral shift drastically alters the localized feeding pressure on the host plant. Instead of distributing damage evenly across the canopy, herbivores concentrate their feeding activity on specific, localized tissues. This creates a critical ecological bottleneck because abaxial and lower-canopy tissues often exhibit vastly different chemical defense profiles, higher concentrations of secondary metabolites, tougher structural cell walls, or significantly lower baseline nutritional quality compared to the primary upper leaves (Hunter, 2001; Pincebourde & Casas, 2019). Consequently, the insect is forced to consume suboptimal diet profiles simply as a consequence of prioritizing thermal safety.

Furthermore, the continuous behavioral avoidance of thermal stress imposes severe, hidden fitness costs that ripple through the insect's life

history. Time spent actively seeking or resting within microclimatic refugia directly subtracts from the organism's total diurnal time budget available for essential behaviors such as active foraging, mate locating, and strategic oviposition (Caillon et al., 2014). For instance, females spending excessive hours hiding from lethal leaf boundary layers experience a marked reduction in their real-time oviposition window, which can drastically lower their net lifetime reproductive output.

3. Key Ecological Insight

The availability and structural complexity of host-plant microclimates act as a primary buffer against macroclimatic warming, enabling vulnerable insect life stages to survive extreme regional heat waves and directly influencing spatial herbivory patterns.

4. Thermal Plasticity and Molecular Chaperones

When behavioral ecoregulation is insufficient or environmental shifts exceed an organism's behavioral capacity, insects must rely on physiological and molecular mechanisms to survive. Thermal plasticity includes both rapid heat hardening and long-term acclimation, and it is regulated by specific transcriptional networks that stabilize cellular proteostasis. Feder and Hofmann (1999) established that the cellular response to heat stress is characterized by the down-regulation of standard housekeeping genes and the immediate, massive up-regulation of molecular chaperones known collectively as heat shock proteins. These proteins prevent the irreversible aggregation of denatured cellular proteins, stabilize polypeptide chains, and assist in refolding damaged enzymes during recovery periods.

The heat shock response is highly conserved across insect orders, yet significant variation exists in the induction thresholds and expression kinetics among different species and populations. The molecular cascade begins when thermal stress causes cellular proteins to unfold, releasing heat shock factor 1 from its inactive cytoplasmic complex (Feder & Hofmann, 1999). Heat shock factor 1 then trimerizes, translocates to the nucleus, and binds to highly specific heat shock elements within the promoter regions of genes encoding molecular chaperones. The synthesized molecular chaperones are categorized into distinct families based on molecular weight, including HSP90, HSP70, HSP60, and small heat shock proteins.

Members of the HSP70 family are particularly critical for acute thermal survival because they interact directly with exposed hydrophobic regions of denatured proteins to prevent aggregation, whereas small heat shock proteins act as ATP-independent holdases (Feder & Hofmann, 1999).

5. Biochemical Trade-offs and Energetic Costs

While the induction of molecular chaperones is an indispensable physiological strategy for surviving transient thermal extremes, this cellular defense mechanism carries substantial biochemical, metabolic, and evolutionary costs. The intensive transcription, translation, and deployment of heat shock proteins (HSPs) require considerable allocations of adenosine triphosphate (ATP), which drastically diverts essential energy reserves away from somatic growth, larval development, and reproductive systems (Feder & Hofmann, 1999). Under optimal conditions, an organism's energetic budget is finely balanced between maintenance and fitness-limiting traits. However, when thermal anomalies occur, cellular machinery shifts its priority toward immediate cytoprotection. Sorenson et al. (2003) demonstrated that the hyper-expression of genes such as *Hsp70* leads to an immediate reduction in the synthesis of standard housekeeping proteins, effectively halting normal physiological growth. Consequently, prolonged or frequent activation of the heat shock response yields severe fitness penalties, including reduced lifetime fecundity, slower developmental rates, prolonged pupal stages, and significantly shortened lifespans. This creates a stark and unavoidable physiological trade-off between immediate somatic survival and long-term evolutionary fitness (Feder & Hofmann, 1999; Sorenson et al., 2003). (Table 1).

Beyond the energetic depletion caused by protein misfolding management, elevated ambient temperatures trigger secondary cellular crises by inducing severe oxidative stress within insect tissues. Thermal stress accelerates mitochondrial respiration and disrupts the electron transport chain, leading to the excessive generation and intracellular accumulation of reactive oxygen species (ROS), such as superoxide radicals (), hydrogen peroxide (), and hydroxyl radicals () (Monaghan et al., 2009). When the production of these pro-oxidants outpaces the basal scavenging capacity of the cell, ROS cause irreversible damage to macromolecular structures, manifesting as lipid peroxidation of cellular and organelle membranes,

carbonylation of essential metabolic enzymes, and single- or double-strand DNA breaks (Monaghan et al., 2009; Storey, 1996).

To combat and mitigate this pervasive oxidative damage, insects must simultaneously activate an array of enzymatic and non-enzymatic antioxidant defense systems (Storey, 1996). The primary line of defense involves a tightly coordinated enzymatic cascade beginning with superoxide dismutase (SOD), which catalyzes the dismutation of highly reactive superoxide radicals into less toxic hydrogen peroxide molecules. Subsequently, this hydrogen peroxide must be rapidly detoxified into benign water and molecular oxygen; this critical step is carried out by catalase (CAT) and various peroxidases, including glutathione peroxidase (GPx) and phospholipid hydroperoxide glutathione peroxidase (Monaghan et al., 2009; Storey, 1996). Furthermore, accessory pathways such as thioredoxin-linked systems and glutathione S-transferases (GSTs) are heavily recruited to maintain the internal cellular redox state and repair oxidized cellular components.

The simultaneous and massive induction of both the heat shock response and these diverse antioxidant enzyme networks places an unprecedented dual metabolic burden on the insect's fat body. The insect fat body serves as the functional analogue of the vertebrate liver and adipose tissue, functioning as the primary organ for metabolic integration, vitellogenesis, and macronutrient storage (Arrese & Soulages, 2010). It is within this organ that glycogen, triacylglycerols (lipids), and storage proteins are synthesized and mobilized. Under compounding thermal and oxidative stress, the fat body must rapidly catabolize its stored triacylglycerols via lipolysis and mobilize glycogen reserves to generate the massive pools of ATP required to sustain chaperone synthesis and antioxidant enzyme production (Arrese & Soulages, 2010; Storey, 1996).

This intense metabolic reallocation creates severe ecological vulnerabilities. If the combined energetic cost of synthesizing these defensive macro-molecules and maintaining proteostasis exceeds the net caloric and nutritional intake obtained from host-plant consumption—which is often simultaneously compromised by heat-induced reductions in plant tissue quality (Hunter, 2001)—the insect enters a state of negative energy balance. Consequently, the organism will experience a rapid, catastrophic depletion of its endogenous glycogen, lipid, and protein stores within the fat body (Arrese & Soulages, 2010). Without adequate energy reserves to fuel basal

metabolic processes or maintain ion homeostasis, this resource exhaustion culminates in severe larval starvation, developmental arrest, systemic organ failure, or absolute metabolic collapse.

Table 1. Key Molecular Chaperones and Antioxidant Systems Involved in Insect Thermal Stress Response

Component	Functional Class	Primary Molecular Mechanism	Ecological & Fitness Consequences
HSP70 Family	ATP-Dependent Chaperone	Binds to exposed hydrophobic residues on unfolded proteins to prevent aggregation and assist refolding (Feder & Hofmann, 1999).	Sustains cellular survival during acute heat shocks; high metabolic cost reduces lifetime egg production.
Small HSPs	ATP-Independent Holdase	Binds and stabilizes denatured substrates in an unaggregated state until refolding resources become available (Feder & Hofmann, 1999).	Provides immediate protection against sudden thermal spikes; delays growth and extends larval developmental periods.
Superoxide Dismutase	Antioxidant Enzyme	Catalyzes the dismutation of highly reactive superoxide radicals into hydrogen peroxide and molecular oxygen.	Prevents lipid peroxidation of cellular membranes during heat-induced oxidative stress events.
Catalase	Antioxidant Enzyme	Converts harmful hydrogen peroxide molecules into benign water and molecular oxygen, preventing cellular toxicity.	Works with superoxide dismutase to maintain cellular redox state; requires structural allocation of nitrogen resources.

6. Altered Host-Plant Phenology and Trophic Uncoupling

Climate change significantly influences seasonal timing, leading to alterations in ecological interactions. In temperate regions, spring warming serves as a primary cue for plant budburst and insect egg hatching. Because different species rely on varying environmental cues, these responses can

become desynchronized; for instance, woody plants often track photoperiod which remains constant across years, whereas phytophagous insects track microclimatic temperatures (Bale et al., 2002). When spring temperatures rise rapidly, insects may emerge before their host plants produce young foliage, creating a spatial and temporal mismatch that leaves neonate larvae without adequate food resources.

Conversely, if host plants flush their leaves early due to unseasonably warm winter conditions, the foliage may age and toughen before insect herbivores emerge. As leaves mature, their nutritional profile declines, characterized by decreased nitrogen content and increased structural polymers such as lignin and cellulose (Robinson et al., 2012). This forced shift in diet forces insects to feed on lower-quality tissues, which slows larval growth and increases vulnerability to natural enemies. Research across multiple ecosystems confirms that even minor shifts in seasonal timing can reduce population viability for specialized herbivores, causing cascading effects throughout multi-trophic food webs (Bale et al., 2002; Robinson et al., 2012).

7. Conceptual Model of Trophic Synchronization

To visualize how rising temperatures alter ecological timing, we can examine the alignment of plant development and insect emergence. Under historic climate conditions, the peak abundance of high-quality young leaves matches the peak energetic demands of emerging larvae (Bale et al., 2002). Under accelerated warming, the larval emergence curve shifts earlier at a faster rate than plant budburst, leading to a substantial reduction in shared temporal space. This reduction decreases larval survival and impacts overall population growth.

8. Phytochemical Shifts and Plant Defensive Cascades

Elevated atmospheric carbon dioxide () and thermal stress modify plant primary and secondary metabolism. Hunter (2001) demonstrated that under elevated levels, plants increase photosynthetic carbon assimilation while nitrogen uptake lags behind, increasing the carbon-to-nitrogen ratio within plant tissues and reducing overall nutritional value for herbivores. To obtain sufficient nitrogen, insects must increase their consumption rates. This compensatory feeding behavior can accelerate absolute defoliation rates in both agricultural and forest ecosystems (Robinson et al., 2012).

Simultaneously, the balance of secondary metabolites shifts under climate stress. The carbon-nutrient balance hypothesis suggests that excess carbon is directed toward carbon-based defenses such as tannins, phenolics, and flavonoids. Conversely, nitrogen-based defenses, including alkaloids and cyanogenic glycosides, often decrease under elevated (Bidart-Bouzat & Imeh-Nathaniel, 2008). However, when high temperatures accompany elevated , these defensive pathways can change unpredictably. Severe thermal stress can inhibit the synthesis of jasmonic acid and salicylic acid signaling pathways, leaving plants vulnerable to specialized pests that thrive in warmer environments (Zvereva & Kozlov, 2006). (Table 2).

Table 2. Impact of Elevated Carbon Dioxide and Thermal Stress on Plant Primary and Secondary Metabolite

Environmental Driver	Phytochemical Shift	Impact on Herbivore Physiology	Ecological Consequences
Elevated CO_2	Increased Carbon-to-Nitrogen ratio; reduction in foliar protein concentration (Hunter, 2001).	Triggers compensatory feeding; insects eat more tissue to meet metabolic nitrogen demands.	Accelerated leaf area damage; prolonged larval exposure to canopy predators (Robinson et al., 2012).
Thermal Stress	Volatilization of monoterpenes; suppressed jasmonic acid induction pathways.	Reduces exposure to effective repellent compounds; lowers physiological detox burdens.	Increases plant vulnerability to specialized herbivores and wood-boring pests (Zvereva & Kozlov, 2006).
Combined Stress	Variable induction of complex polyphenols and structural lignin polymers.	Increases wear on mandibles; decreases digestion efficiency and assimilation rates.	Alters decomposition dynamics of leaf litter; impacts soil macro-invertebrate food webs.

9. Multitrophic Dynamics and Community Structure

The ecological impacts of climate change extend far beyond simple dyadic plant-herbivore pairs, cascading upward to encompass and destabilize

entire multi-trophic networks. Parasitoids and invertebrate predators rely heavily on highly specific, complex bouquets of volatile organic compounds (VOCs) released by plants in response to feeding damage to locate their herbivorous hosts or prey. These Herbivore-Induced Plant Volatiles (HIPVs)—which primarily consist of lipoxygenase-pathway green leaf volatiles, volatile terpenoids, and shikimate-pathway phenylpropanoids—can change significantly in their synthesis, emission rates, and chemical ratios under elevated temperatures and levels (Bidart-Bouzat & Imeh-Nathaniel, 2008).

For instance, elevated atmospheric can suppress the transcription of genes in the jasmonic acid pathway, which directly governs a plant's induced defensive response, leading to a quantitative reduction or structural distortion of critical volatile blends (Bidart-Bouzat & Imeh-Nathaniel, 2008). At the same time, elevated temperatures can accelerate the volatilization of highly volatile monoterpenes while suppressing the slower-emitting sesquiterpenes (Zvereva & Kozlov, 2006). These climate-driven changes in volatile blend composition can disrupt the host-seeking, olfactory-guided behaviors of beneficial insects. Because parasitoids rely on precise chemical ratios to distinguish hosts from background noise, a skewed volatile profile can completely mask the signal of an infested plant, decoupling natural biological control mechanisms and removing vital regulatory pressures within the community (Bidart-Bouzat & Imeh-Nathaniel, 2008).

Furthermore, higher-trophic-level organisms, such as parasitoids, are often significantly more sensitive to thermal stress than their herbivorous hosts (Bale et al., 2002; Hance et al., 2007). This disparity stems from their typically smaller body sizes, higher surface-area-to-volume ratios, lower baseline thermal tolerances (), and strict energetic constraints (Hance et al., 2007). Higher temperatures can dramatically reduce the searching efficiency of foraging parasitoids, limit adult longevity by accelerating metabolic exhaustion, and cause structural damage to reproductive organs (Bale et al., 2002; Hance et al., 2007). In many Hymenopteran parasitoids, extreme heat events also skew sex ratios toward a male-biased population by altering maternal oviposition choices or causing sperm mortality within the female's spermatheca (Hance et al., 2007).

This asymmetric vulnerability across trophic levels creates a structural mismatch (Bale et al., 2002). While the herbivore host may survive a heat wave by exploiting microclimatic refugia or activating robust heat shock proteins, its primary parasitoids may face localized extinction or severe population crashes (Bale et al., 2002; Feder & Hofmann,1999).

This imbalance allows herbivore populations to escape natural top-down regulation. When freed from the suppressive pressures of natural enemies, herbivore populations can experience rapid, exponential growth, leading to frequent, severe, and prolonged pest outbreaks in both agricultural and natural forest ecosystems (Bale et al., 2002; Hance et al., 2007).

As a consequence of these decoupled interactions, entire community structures are shifting away from highly diverse,complex webs toward simplified, fragile food webs dominated by a few climate-tolerant, opportunistic species (Bale et al., 2002). This erosion of vertical and horizontal biodiversity diminishes the redundant ecological links that buffer communities against environmental shocks, ultimately reducing overall ecosystem resilience and leaving habitats highly vulnerable to secondary collapse under the ongoing pressures of the Anthropocene (Bale et al., 2002; Hance et al., 2007).

10. Conclusion

The intricate, compounding impacts of anthropogenically driven climate change on insect-plant interactions underscore the critical need for integrated ecological research across spatial, temporal, and biological scales. Ectothermic insects utilize behavioral thermoregulation to exploit localized microclimatic refugia, successfully buffering themselves against immediate macroclimatic extremes, while simultaneously activating complex molecular networks when critical physiological thresholds are exceeded (Bale et al., 2002; Feder & Hofmann, 1999). However, as environmental perturbations accelerate, the production of molecular chaperones, heat shock proteins, and antioxidant networks introduces heavy energetic costs that significantly deplete metabolic reserves and compromise long-term reproductive fitness. Concurrently, climate-driven alterations in plant primary and secondary metabolism profoundly alter nutritional food quality, forcing behavioral counter-adaptations such as compensatory feeding, which inadvertently amplifies absolute defoliation rates in both managed agricultural and natural forest ecosystems (Hunter, 2001; Robinson et al., 2012). When these immediate metabolic strains combine with regional phenological shifts, they disrupt historical, coevolved multi-trophic connections, triggering cascading uncoupling events that threaten entire ecosystem stabilities.

To address these escalating ecological crises, future entomological and macroecological research must move beyond short-term, single-species

climate simulations and actively prioritize multi-generational experiments designed to capture true evolutionary adaptability in a changing world. A major limitation of current global change biology is its reliance on acute, single-generation thermal assays, which fail to account for the mechanisms of evolutionary rescue—the process by which a population recovers from a climate-induced decline via genetic adaptation (Gonzalez et al., 2013). By executing multi-generational selection experiments across prolonged timelines, researchers can determine whether vulnerable insect populations possess sufficient standing genetic variation to adaptively shift their upper thermal limits () or whether structural trade-offs will drive specialized lineages toward localized extinction.

Furthermore, future paradigms must integrate the rapidly expanding field of ecological epigenetics. Recent advances demonstrate that transgenerational plasticity, mediated via epigenetic modifications such as DNA methylation, histone acetylation, and non-coding RNA inheritance, can pre-adapt subsequent generations to environmental stressors without altering the underlying genetic sequence (Jeremias et al., 2018). Investigating how parental exposure to heat waves or elevated shapes the defensive cascades and metabolic efficiencies of offspring will clarify the latent resilient capabilities of insect communities.

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CHAPTER VI

FUNCTIONAL AND BIOACTIVE PROPERTIES OF INSECT PROTEINS

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1. Introduction

Insects are the most numerous animal group on Earth, belonging to the Hexapoda subphylum, which is the group of six-legged animals with the most members. Thanks to their high species diversity, insects constitute the largest and richest group within arthropods (Nongonierma & FitzGerald, 2017). Insects exhibit a high level of biodiversity, including species diversity as well as extremely rich genetic and ecosystem diversity, and constitute a vast majority, approaching 95%, of the total mass of living organisms in the animal kingdom (de Castro et al., 2018).

The segmented body structure, divided into three main body parts—head, thorax, and abdomen—their articulated limbs, and their chitinous exoskeleton are among the basic morphological characteristics of insects (Chapman, 1998; Grimaldi & Engel, 2005). The main reasons why insects are so successful from an evolutionary and ecological perspective include their ability to adapt quickly to environmental conditions, their ability to fly, their high reproductive capacity, and their short generation times (Grimaldi & Engel, 2005). Insects, thanks to the characteristics mentioned above, are a group of organisms with a wide range of habitats; they commonly live in terrestrial ecosystems and freshwater habitats, and have a wide geographical distribution extending from tropical regions to polar regions (Danks, 2007).

Insects play central ecological roles in fundamental processes such as maintaining and regulating food webs, decomposing organic matter, pollination, and preserving the natural balance of ecosystems (Schowalter et al., 2018; Yang & Gratton, 2014; Weisser & Siemann, 2004). It is emphasized that insect species, especially those contributing to pollination, provide crucial ecosystem services for agricultural production and food security worldwide (Potts et al., 2010). The involvement of insects in these critical roles demonstrates the complex and multifaceted nature of insect-human interaction (Noriega et al., 2018).

According to population projections by the United Nations Food and Agriculture Organization (FAO), the human population on Earth is expected to approach a significant level of 9 billion in approximately 40 years (FAO, 2009). This demographic increase shows that meeting the demand for high biological value protein-rich food sources, especially for human nutrition and animal feed, is becoming an increasingly critical issue (Xinyan et al., 2025). The increasing need for sustainable and protein-rich food sources has led to increased scientific interest in considering edible insects as alternatives to traditional plant and animal proteins, attracting international attention (Xinyan et al., 2025). Furthermore, insect farming, which has lower greenhouse gas and ammonia emission values compared to traditional animal husbandry practices, is generally considered a sustainable production approach. Its more limited environmental impact, especially compared to cattle, poultry, fish, and other seafood farming, makes insect-based production a prominent option among environmentally friendly alternatives (Nongonierma & FitzGerald, 2017). In this context, insects have become an important research subject for food, feed, and biotechnological

applications due to their rich content of fatty acids, essential amino acids, various bioactive components, and high-quality proteins (Rumpold & Schlüter, 2013).

Throughout human history, the widespread practice of consuming insects has not been limited to an ecological context, but has also acquired cultural, economic, and social dimensions (Sánchez-Estrada et al., 2024). Various data from archaeological sites and research on modern indigenous societies show that they have been consumed as food for thousands of years, particularly by groups in Latin America, Africa, and Asia, a practice known as entomophagy (Jantzen da Silva Lucas et al., 2020). The protein content in the nutritional content of many edible insects can range from 20–70% on a dry weight basis, and units are reported to be comparable to or greater than traditional protein sources (Xinyan et al., 2025). Due to their short life cycles, high feeding rates, and moderate temperature ranges, they can be an effective solution to significant protein formation and food security problems for sustainable protein production (Halloran et al., 2016; Miglietta et al., 2015). The chemical composition of many insect types used for food has shown they have a lot of protein throughout their life cycles, including eggs, larvae, pupae and adults. In addition, scientific evidence indicates that the protein in insects can be digested well in a laboratory setting, and that protein from insects will be broken down into amino acids during digestion processes involving human-like conditions. The results are currently being evaluated to determine how well the protein from insects compares to those obtained from sources such as animal and soy proteins (Xinyan et al., 2025).

Insects offer a variety of potential solutions in terms of their numerous macronutrient bases as well as a number of other biologically active substances, according to recent studies (Sánchez-Estrada et al., 2024). Bioactive peptides from insect protein sources are known to show a number of important properties such as being antimicrobial, antioxidant, and metabolic regulators and thus are a major area of functional food and biomedical research (Hall et al., 2017; Di Mattia et al., 2019). In the context of biotics, insects are also viewed as both a basic component of biodiversity and a complex biological resource with broad implications for health and sustainable food systems for humans (Cardoso et al., 2020; van Huis, 2020). The increase in the world's population, the rising demand for protein, and the growing interest in sustainable development goals have increased research into alternative protein sources that are

highly nutritious and sustainably produced. In this context, insect proteins are being addressed within the scope of research focusing on production technologies, new product development, and consumer acceptance. It is reported that scientific and technological advancements in these areas are being examined in the context of issues related to food supply, environmental indicators, and public health (Xinyan et al., 2025). Promoting insects as food sources, as a potential future development, will require prioritizing controlled breeding and production systems. Furthermore, large-scale production of active amino acid sequences derived from insects is considered an important and promising area of biotechnology. Achieving these goals requires an interdisciplinary approach (da Silva et al., 2020; de Castro et al., 2018).

Insects can be consumed at different stages of their life cycle, referred to as eggs, larvae, pupae, and adults. the majority of recorded species are primarily valued during their larval and pupal stages. Indirect consumption occurs through products produced or secreted by insects. These products include honey, beeswax, pollen, various oils, dyes, medicinal preparations, teas and infusions, and products in powder form; these products can be used alone or in combination with other ingredients. (da Silva et al., 2020). The most prominent food groups in terms of consumption, in order of importance, are reported as follows: Hemiptera (aphids and leafhoppers), Coleoptera (beetles), Orthoptera (grasshoppers), Lepidoptera (caterpillars), Isoptera (termites), Hymenoptera (ants, bees, and wasps), and Diptera (flies) (Stamer, 2015).

2. Nutritional Components in Edible Insects

Insects are attracting increasing attention today as an alternative, sustainable, and highly useful food reserve due to their high nutritional value and are considered a promising food group (da Silva et al., 2020; Van Huis, 2016). Despite the increasing diversity of insect species and research in this area, data on their nutritional composition remains limited. However, insects that can be consumed as food are generally considered a nutritious food source containing quality protein, fat, minerals, and vitamins. Studies have shown that consuming 100 grams of caterpillar food has the potential to meet approximately 75% of the human body's 24-hour protein requirements and significantly exceed reference vitamin needs (da Silva et al., 2020; Agbidye et al., 2009). It is reported that three silkworm pupae are

equivalent in nutritional value to one chicken egg; these pupae consist of approximately 30% lipids and 50% protein (Mitsuhashi, 2010).

The nutritional composition of insects that can be consumed as food varies according to their species and developmental stage; however, they generally contain high levels of crude protein and lipids (Tekiner et al., 2022). Furthermore, the amino acid profile determined in many species is sufficient to meet the requirements of a balanced diet, especially in young individuals (Jansson & Berggren, 2015). The nutritional content and quality of edible insects vary considerably, particularly due to the wide range of species diversity (de Castro et al., 2018; Finke & Oonincx, 2018). The nutritional components of insects primarily consist of high protein, but also contain sufficient amounts of various essential nutrients for the organism, such as minerals, vitamins, lipids, and beneficial fatty acids (Bukkens, 2005; Rumpold & Schlüter, 2013; Nowak et al., 2016; Sun-Waterhouse et al., 2016; de Castro et al., 2018).

When compared in terms of protein quality from plant and other animal sources, insect proteins are reported to be high-quality protein sources in terms of both total protein content and essential amino acid composition, with protein percentages generally ranging between 50-80% (Bukkens, 2005; Rumpold & Schlüter, 2013; de Castro et al., 2018). In the nutritional composition of edible insects, fat is the second largest component after protein, and its quantity is particularly higher during the larval life cycle compared to other life cycles. In edible insects, approximately 80% of the total lipid content consists of triacylglycerols, while the remaining 20% is made up of phospholipids, which vary depending on the life stage (Ekpo et al., 2009; Tzompa-Sosa et al., 2014; de Castro et al., 2018;). The fatty acid composition varies depending on the insect species and their dietary preferences. This compound contains a high proportion of straight-chain fatty acids with 18 carbon atoms, particularly linolenic, oleic, and linoleic acids, and also includes significant amounts of palmitic acid (Bukkens, 2005; Tzompa-Sosa et al., 2014; de Castro et al., 2018). Research data shows that the lipid content in insect diets contains a high proportion of unsaturated fatty acids compared to saturated fatty acids (Finke, 2002; Rumpold & Schlüter, 2013; Tzompa-Sosa et al., 2014; de Castro et al., 2018;).

Insects contain a variety of minerals, including sodium, potassium, magnesium, calcium, manganese, copper, iron, zinc, and phosphorus. They

also contain various lipophilic vitamins, as well as vitamins important for metabolism such as riboflavin, pantothenic acid, biotin, and in some species, folic acid (Finke, 2002; Xiaoming et al., 2010; Van Huis et al., 2013; Rumpold & Schlüter, 2013; de Castro et al., 2018). The literature reports that insects have the potential to meet a significant portion of daily mineral requirements, and are considered a particularly good source of highly bioavailable iron. Depending on the method and quantity of consumption, grasshoppers, crickets, and mealworms can provide rich sources of zinc (Zn), calcium (Ca), iron (Fe), magnesium (Mg), copper (Cu), and manganese (Mn) in the human diet (de Castro et al., 2018). Further detailed research is needed to improve the existing data source on this important topic (de Castro et al., 2018). According to the results obtained from studies conducted on this subject, data on the nutritional content of various insect species are shown in Table 1.

Table 1. Nutritional content and values of insects (based on dry matter weight) (Ramos-Elorduy et al., 1997; Bukkens 1997; Rothman et al., 2014; Payne et al., 2016; Cammack & Tomberlin 2017; de Castro et al., 2018).

Nutritional Value of Insects (Based on Dry Matter)

Insect Order	Protein (g/100 g)	Fat (g/100 g)	Minerals (g/100 g)	Carbohydrates (g/100 g)	Energy (kcal/100 g)
Coleoptera	3.7–54	3.7–52	1–3	12–34	126–574
Diptera	17.5–67	4.2–31	1.24–8	8.38–23	199–460
Hemiptera	33–65	7–54	1–19	7–19	329–622
Hymenoptera	1–81	1.3–62	0–6	5–94	234–593
Lepidoptera	13.2–69.6	7–77	2–8	3–41	126–762
Orthoptera	13–77	2.4–25.14	2–27	16–30	117–436

When the current data obtained from the studies are examined (Table 2), the protein content of edible insects as food components varies considerably, and the reasons for this variability are taxonomic group differences and developmental stages. (Rumpold & Schlüter, 2013). The fact that the protein content of most teams with a defined protein composition is generally above 20%, and in certain groups it reached 70%, proves the potential of insects as an alternative protein source (Rumpold & Schlüter, 2013).

Table 2. Protein content in the most commonly consumed insect taxa (Xiaoming et al., 2010; de Castro et al., 2018).

Protein Content of Common Edible Insects by Order anda Life Stage

Common Name	Insect Order	Life Stage	Protein Content (%)
 Beetles	Coleoptera	Adults and Larvae	23–66
 Butterflies and Moths	Lepidoptera	Pupaeand Larvae	14–68
 Cicadas, Aphids, Scale Insects, Leafhoppers and True Bugs	Hemiptera	Adults and Larvae	42–74
 Wasps, Bees and Ants	Hymenoptera	Adults, Pupae, Larvae and Eggs	13–77
 Dragonflies	Odonata	Adults and Nymphs	46–65
 Grasshoppers, Crickets and Locusts	Orthoptera	Adults and Nymphs	23–65

Edible insect proteins are reported to be quite competitive in terms of nutritional value compared to protein sources from other organisms such as plants and animals. Many insect species with protein values ranging from 20% to 70% are said to be comparable to classic animal protein sources such as fish, beef, and chicken (Zhou et al., 2022). Furthermore, insect proteins exhibit a balanced profile in terms of essential amino acids, and some groups have been reported to have higher leucine content than soy protein (D'Antonio et al., 2023). As a result of these properties, it is likely that insect protein will supplement the protein provided from a plant source because they contain amino acids that tend to be less available in a plant protein source.

It is reported that the in vitro digestibility of insect proteins ranges from about 78 to 98%. The differences in digestibility are largely due to the species of insect selected and how the protein is processed prior to being digested (Zhou et al., 2022; Rodríguez-Rodríguez et al., 2022). One factor affecting digestibility is chitin and there are specific processing methods that can be used to reduce the impact of chitin on digestibility (D'Antonio et al., 2023). Therefore, the evidence suggests that insect proteins are a very good alternative to both plant proteins and traditional animal proteins in terms of nutrition and functionality.

3. Amino Acid Profile and Protein Quality

The examination of the amino acid profiles of edible insect proteins shows that many species contain sufficient amounts of all essential amino

acids required for the human diet (van Huis, 2013; Payne et al., 2016) In particular, insects have been reported to have consistent levels of certain amino acids, including leucine, isoleucine, valine, and lysine (Payne et al., 2016) Which helps explain why insect proteins score highly on protein quality assessments (van Huis, 2013) In fact, numerous studies have shown that the amino acid profiles of edible insects are comparable to those of commonly used animal protein sources such as chicken and fish (Payne et al., 2016).

From the perspective of essential amino acid balance, insect proteins are reported to play a complementary role, particularly in terms of lysine, which is limiting in grain-based diets (Belluco et al., 2013). However, protein quality depends not only on amino acid content but also on digestibility and bioavailability (van Huis, 2013). It has been suggested that the chitin fraction found in edible insects may partially reduce protein digestibility (Belluco et al., 2013). However, it has been reported that appropriate processing techniques such as grinding, defatting, and enzymatic hydrolysis can significantly increase digestibility (Mishyna et al., 2020). In general, the proteins contained in insects are considered to be high-quality and beneficial alternative protein sources in terms of both amino acid balance and digestibility (Payne et al., 2016; Mishyna et al., 2020). The vast majority of edible insect species are reported to contain all the essential amino acids necessary for human nutrition (Table 3) (Sánchez-Estrada et al., 2024).

Table 3. Essential amino acid content of edible insects (Sánchez-Estrada et al., 2024).

Amino Acid Profiles and Evaluation of Different Insect Orders

Insect Order	Main Amino Acids Identified	Overall Evaluation
 Coleoptera	Leucine, isoleucine, valine, lysine, methionine, threonine, phenylalanine	Rich in essential amino acids; high protein quality
 Lepidoptera	Leucine, lysine, valine, threonine, histidine, phenylalanine	Balanced amino acid profile; potential suitability for child nutrition
 Orthoptera	Leucine, isoleucine, valine, lysine, tryptophan, threonine	Amino acid composition similar to animal proteins
 Hymenoptera	Leucine, lysine, methionine, phenylalanine, threonine	Protein structure with high biological value
 Hemiptera	Leucine, valine, lysine, histidine, phenylalanine	May vary depending on species and diet

The presence of sufficient levels of limiting amino acids, particularly leucine, lysine, and valine, in many insect orders suggests that insect proteins may have high biological value (Sánchez-Estrada et al., 2024). However, the amino acid composition may vary depending on species, life cycle, diet, and processing methods (Sánchez-Estrada et al., 2024).

4. Structural Characteristics of Insect Proteins

The characteristics of proteins in terms of quality, composition, and structure are directly influenced by variables such as species, the developmental stage of the organism, diet, and post-harvest processing methods (Yi et al., 2013; Ma et al., 2023). The literature emphasizes that larval stages, in particular, are richer in terms of protein and lipid ratios, and it has been reported that applied thermal or mechanical treatments can alter the functional behavior of proteins (Yi et al., 2013; Kim et al., 2022). Therefore, this variability is considered one of the most fundamental parameters determining the potential use of insect proteins in industrial food applications and requiring standardization (Ma et al., 2023). Research data showing that edible insects have high protein content and different protein fractions has led to them attracting scientific attention as alternative protein sources, especially in recent years, within the scope of sustainability policies (Yi et al., 2013). However, since most proteins in edible insects have not yet been fully identified, this potential has not been fully realized. In this context, based on information obtained from the literature, insect proteins should be considered as a complex organization consisting of different fractions such as muscle proteins, hemolymph (insect blood) proteins, storage proteins, and cuticle structural proteins (Ma et al., 2023). It has also been reported that chitin, the main component of the exoskeleton in insects, is a factor that can negatively affect the digestibility of insect proteins and that this can limit their bioavailability (Ma et al., 2023). The structural organization characteristics of insect proteins are directly influenced by many factors, such as species, the current developmental stage of the organism, the insect's diet, and the processing methods applied to the proteins present in insects (Yi et al., 2013; Ma et al., 2023). Research has shown that edible insects, particularly their larvae, are richer in protein and lipid content, and that thermal or mechanical processing can alter the functional behavior of proteins (Yi et al., 2013; Kim et al., 2022). Therefore, this variability is

considered one of the key parameters affecting the potential use of insect proteins in food applications (Ma et al., 2023).

5. Functional Properties of Insect Proteins

The functional properties of their proteins are critically important for their suitability as food additives and for providing alternatives to traditional ingredients currently in use (Tarahi et al., 2024). When this situation is evaluated in terms of edible insect proteins, it is reported that the literature on the subject is more limited compared to traditional sources such as plant proteins due to differences in the methods used in research and the aims of studies in this field (Tarahi et al., 2024). Recent studies on the functional characterization of insect proteins reveal that the most frequently investigated functional properties they exhibit are solubility, water and fat retention capacity, emulsifying and foaming capacity, and gelling potential (Mishyna et al., 2021; Villaseñor et al., 2022; Xinyan et al., 2025).

5.1. Resolution Characteristics

The solubility properties of proteins are one of the key parameters determining the other functional behaviors of insect proteins. The solubility of edible insect proteins is generally pH-dependent and decreases to a minimum near the isoelectric point (Yi et al., 2017; Kim et al., 2019). Studies have shown that *Tenebrio molitor, Acheta domesticus,* and *Hermetia illucens* proteins exhibit higher solubility under alkaline pH conditions (Stone et al., 2019; Ndiritu et al., 2019). This issue is particularly concerned with how proteins are distributed (charge) and how they are unfolded (degree) (Vihinen, 2020); recent studies indicate that the solubility of insect proteins is significantly affected by chitin content, fat percentage, and pretreatment (Xinyan et al. 2025; Ma et al. 2023). Protein solubility is designated as one of the major factors that have an effect on all other functional characteristics of insect proteins (Williams et al. 2016). Protein solubility varies based on the amino acid structure, size of molecules, and conformational characteristics, along with environmental conditions such as pH levels, temperature levels, and ionic strength (Kramer et al., 2012; Sathe et al. 2018). Solubility will increase due to the additional amount of polar or charged amino acids contained within the protein. This increase in solubility then creates a corresponding increase in protein stability (Kramer et al., 2012).

Enzymatic hydrolysis of proteins into small peptides has shown to increase solubility. Research on edible insect proteins confirms this, especially for *Locusta migratoria* and *Gryllodes sigillatus* proteins, where solubility improved following hydrolysis (Hall et al., 2017; Purschke et al., 2018). Some characteristics of proteins of interest, such as emulsifying properties and gel forming abilities, may decrease as a result of hydrolysis depending on the amount of hydrolyzation (Wouters et al., 2016). High temperature generally has a positive effect on solubility; however, temperatures beyond a specified limit cause protein denaturing and result in aggregation of proteins due to the production of hydrophobic sites which in turn affects the solubility (Sathe et al., 2018). Another environmental variable, ionic strength, greatly affects solubility. Increasing ionic strength by using low concentrations of sodium chloride increases the solubility of proteins while high concentrations decrease the solubility (Duong-Ly & Gabelli, 2014; Sathe et al., 2018). Similar to other food protein sources, insect-based proteins exhibit low solubility at or near their isoelectric points and display high solubility at alkaline Ph (Bußler et al., 2016; Chatsuwan et al., 2018; Mishyna et al., 2019). Research has proven that edible insect proteins also possess these properties. *Tenebrio molitor, Hermetia illucens, Locusta migratoria*, and some *grasshopper* species exhibit low solubility at a pH value of approximately 4 and high solubility in alkaline conditions. (Bußler et al., 2016; Chatsuwan et al., 2018; Mishyna et al., 2019; Purschke et al., 2018).

5.2. Water and Oil Retention Capacity

Properties such as the water-holding and fat-holding capacities exhibited by insect proteins are some of the most important functional characteristics that directly affect the texture and taste of the food products produced (Villaseñor et al., 2022). Water-holding capacity (WHC) is defined by the activity of proteins in absorbing and retaining water despite external forces when pressure is applied (Chen & Lin, 2002). This property is very important for improving the tenderness, juiciness, taste and total yield of food products like meat and baked goods (Zielińska et al.,2015; Han et al.,2023). Oil holding capacity (OHC) is also a critical property in enhancing or preserving flavour, and improving food service efficiency; likewise, it is strongly associated with the emulsifying properties of food (Aremu et al., 2007; Bußler et al., 2016; Aryee et al., 2018; Sosa et al., 2020). The WHC

and OHC values exhibited by insect proteins vary under the influence of various factors such as insect species, pH, temperature, ionic strength, and denaturation (Dong et al., 2024).

5.3. Emulsifying Properties

Emulsification efficiency is assessed by using three separate, interrelated aspects of insect protein: emulsifying effectiveness (the speed and how well an emulsion is formed) or emulsifying activity (EA), emulsifying capacity (the total fat that a protein can hold before separating into layers), and emulsifying stability (the resistance to separation over time) (Xinyan et al., 2023; Amarowicz, 2010). Many of the specific structures and the ratio of hydrophobic and hydrophilic amino acids in the protein give them unique advantages by allowing them to remain attached to aqueous surfaces and be effective natural emulsifiers. (Xinyan et al., 2023; Gravel and Doyen, 2022). Therefore, their excellent surface activity qualifies them to be a possible ingredient for many processed food products (Xinyan et al., 2023; Gould and Wolf, 2017). A number of studies have focused on the use of yellow mealworm larvae (*Tenebrio molitor*) proteins in meat products (Yi et al., 2012), while other studies have shown that the proteins have high temperature, salt concentration, and pH tolerance and can produce highly stable emulsions while maintaining their physical structure (Xinyan et al., 2025; Gould & Wolf, 2018). Nevertheless, studies relating to processing have revealed that to maximise the efficiency with which insect protein is utilised in industrial food products requires specific protein concentration; ionic strength within the medium; and optimisation of processing conditions. If one of these parameters strays outside its desired range of acceptable values, then, for example, too high a temperature, or a very high pH will lead to denaturation of the protein and reduce the emulsification capacity (Stone et al., 2019).

5.4. Foam Formation Capacity

The capacities of insect proteins to foam and form a foam/air bubble matrix are unique functional characteristics that contribute to the mouthfeel and texture of foods, particularly when incorporating air bubbles through whipping in baked goods and foamed desserts (mousse) (Aryee et al., 2018). There are two main criteria used to determine the functional properties of foaming: Foaming Capacity (FC) indicates the volume of foam (air)

produced by the insect protein, while Foam Stability (FS) is the term that describes how long the produced foam (air bubbles) persists. The functional properties of foaming are directly related to the ability of insect proteins to adhere to the interface between air and water. This creates a sticky film around each air bubble that adapts to the situation, i.e., it is flexible and plays a role in maintaining the foam structure (Zielińska et al., 2015). There are two main factors that influence the formation and shaping of the foaming ability of insect proteins: internal and external. Intrinsic factors include surface hydrophobicity of the protein, flexibility of the protein structure and amino acid composition, while extrinsic variables have to do with environmental factors including temperature, ionic strength and method of processing (Dickinson, 2010; Kavle et al., 2023; Ma et al., 2023).

5.5. Gel Forming Property

As a result of its ability to facilitate gelaion (a functional property of protein), insect proteins are able to form cross links that create a three-dimensional flexible network structure capable of capturing water (Yi etapa al., 2013). Insects have shown a substantial variation of gelation and have been seen to gel differently than plants and animals that have been traditionally consumed and the gelation of insect proteins within the literature is mostly studied based on gels produced by heat (Mishyna eta al., 2021). Moreover, the stable gelling potential of insects can also be affected by external factors such as medium pH, protein concentration, temperature condition and ultrasonication (Kim eta al., 2022; Kumar et al., 2022). An Example of this is the unique gelling characteristic of insect proteins during the cooling phase which presents this innovation for use within the food industry (Scholliers et al., 2020). In Fact, while there is an association between the gelling of insect proteins and the improvement of texture and flavor of foods it is not a universal outcome and depends on specific processing conditions and proportionally equal use of the insect protein relative to other ingredients (Choi et al., 2023; Boonarsa et al., 2025).

6. Bioactive Peptides in Insects

Bioactive peptides are short chains of amino acids (typically 2-20) that regulate specific functions in humans, animals, and plants, promoting health and exhibiting positive physiological effects (Quah et al., 2023; Apostolopoulos et al., 2021; Apostolopoulos et al., 2022; Chai et al., 2021).

Bioactive peptides, which can be produced naturally in organisms, can also be obtained through enzymatic breakdown (hydrolysis) of food proteins or through chemical synthesis (Jakubczyk et al., 2020; Chai et al., 2017). The biological functionality of peptides largely depends on the type and sequence of amino acids they contain (Fields et al., 2009). In terms of functionality, these molecules can exhibit multifaceted biological activities such as antioxidant, anticancer, anti-inflammatory, antihypertensive and antimicrobial and effects by undertaking numerous different and critical regulatory roles in the immune, nervous, digestive, and endocrine systems for the regulation and health of the body (Akbarian et al., 2022; Korhonen et al., 2006; Sánchez & Vázquez, 2017; Shahidi & Zhong, 2008; Zaky et al., 2022). Furthermore, their specificity to targeted tissues, effectiveness even at low doses, and low toxicity make bioactive peptides an extremely safe and promising alternative, particularly in the treatment of chronic diseases (Apostolopoulos et al., 202; Quah et al., 2023).

6.1. Obtaining Bioactive Peptides

Bioactive peptides, which are part of protein sequences found in the body, are initially in an inactive form and need to break free from the protein sequence to exhibit their bioactive potential and become available (de Matos et al., 2024; Majid et al., 2022). The release of these peptides through protein hydrolysis can occur naturally in the body during digestion, as well as in a laboratory setting through fermentation, enzymatic hydrolysis, and chemical hydrolysis methods. However, chemical hydrolysis is not among the frequently used methods because it causes non-specific decomposition, insufficient yield, and generates chemical waste (de Matos et al., 2024; Majid et al., 2022; Bechaux et al., 2019). The methods used to obtain bioactive peptides from insect proteins and their general workflow diagram are schematically shown in Figure 1.

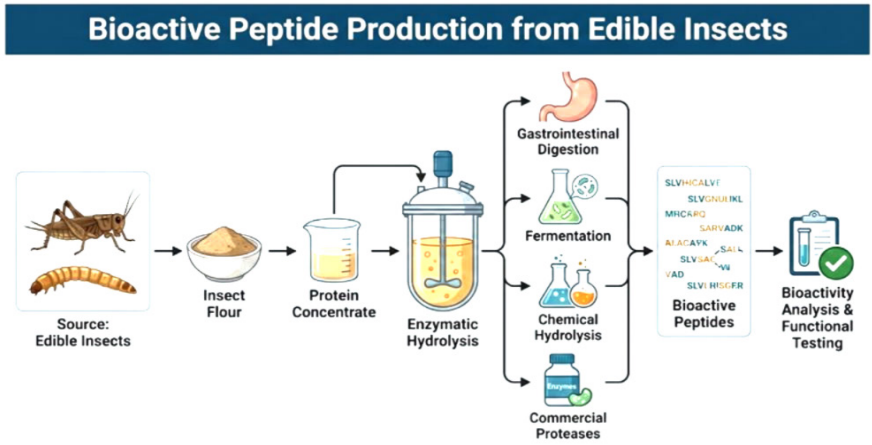

Figure 1. Methods for obtaining bioactive peptides from insect protein sequences (de Matos et al., 2024).

6.1.1. Obtained Through Natural Digestion

The digestion of dietary proteins results in the release of numerous free amino acids and peptides (Aluko, 2012). Some of the peptides released at the end of the digestive process can exhibit bioactive functional properties, interacting with specific receptors and cells involved in the digestive system to exert regional effects. Additionally, they can be actively transported to target tissues and organs via the bloodstream, thus exhibiting systemic effects (Fernández-Tomé & Hernández-Ledesma, 2020). Direct evaluation of bioactive peptides released as a result of the digestion of proteins in living organisms involves methodological difficulties, therefore this approach is not frequently preferred in applications (de Matos et al., 2024).

6.1.2. Obtaining it via Enzymatic Hydrolysis

The biological activities exhibited by proteins in edible insects generally do not have a direct functional effect because they are usually located as a specific code within the protein sequence (Nongonierma & FitzGerald, 2017). The bioactive function hidden within these proteins can be revealed by breaking the peptide bonds that make up the polypeptide chain at specific points (Rumpold & Schlüter, 2013). According to Zhu and colleagues (2019), enzymatic hydrolysis is the most common method to create functional changes in protein while maintaining biological activity.

Enzymatic hydrolysis also has advantages compared to chemical hydrolysis, including not compromising the nutritional content and not producing any toxic byproducts (Rumpold & Schlüter, 2013).

The process of enzymatic hydrolysis typically begins by drying and grinding the insect material, followed by a defatting process to allow the enzymes to have better access to the target proteins (Zielinska & co-workers, 2020). This is then followed by selecting a protease for the enzyme reaction that matches the desired activity, based on the desired end result from the process (Nongonierma & FitzGerald, 2017). Research data have shown that the Alcalase enzyme, with its broad substrate specificity, is effective in breaking down insect proteins and producing highly efficient hydrolysates (Purschke et al., 2018). Following the steps mentioned above, the most active peptide fractions are identified and purified using mass spectrometry or chromatographic methods (Leni et al., 2020). The peptides obtained as a result of all these processes have both much higher solubility and biological activity that can act in targeted tissues compared to the natural protein structure (Zielinska et al., 2018). The steps of the enzymatic hydrolysis method are shown in Figure 2.

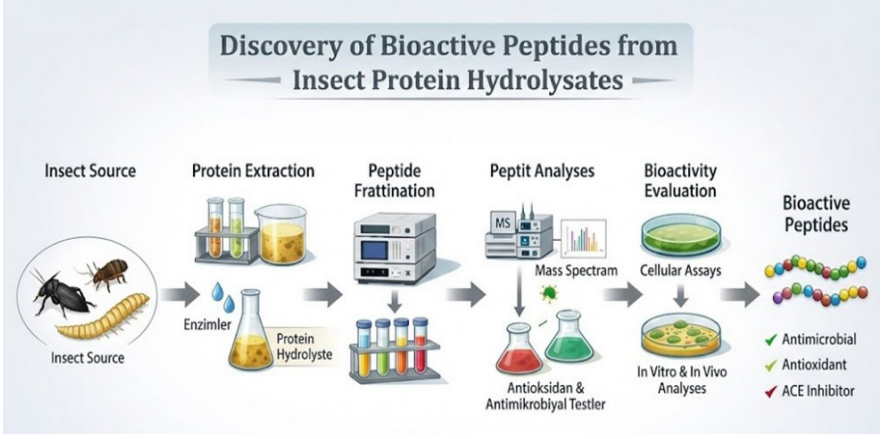

Figure 2. Process of enzymatic hydrolysis, a commonly used method for identifying bioactive peptides from insect protein hydrolysates (de Matos et al., 2024).

6.1.3 Obtaining it through Chemical Hydrolysis

Although chemical hydrolysis is rarely preferred over enzymatic methods, it is still a technique used for separating protein sequences. In

this method, the procedures applied to proteins generally involve prolonged processing under high temperature and pressure with strong acids or bases (Kristinsson & Rasco, 2000). The most important advantages of the chemical hydrolysis process are its low cost and its speed, as it can break down almost all proteins to the amino acid level (Adebiyi et al., 2005). As previously mentioned, this method has serious disadvantages, including extreme conditions during processing, complete destruction of some extremely important amino acids such as tryptophan, and a decrease in the functional quality of the protein (Adebiyi et al., 2005). Furthermore, due to the uncontrolled degradation of proteins in this method, preserving specific bioactive peptide sequences is quite difficult, and neutralization of acid/base residues is absolutely necessary for food applications (Kristinsson & Rasco, 2000; Ma et al., 2023).

6.2 Antioxidant Activity of Bioactive Peptides Derived from Insects

Antioxidants are compounds that provide a vital defense against damage caused by reactive oxygen species (ROS) and free radicals, which are harmful to the body. By preventing the oxidative breakdown of lipids in the cell membrane, structural and functional proteins, and DNA (genetic information), these compounds help to keep the cellular environment stable so that the cell is in equilibrium. Protecting against oxidative damage is crucial to minimizing the risk of developing diseases caused by oxidative stress (Xinyan et al., 2025; Marian et al., 2007).

An experiment was conducted with *Tenebrio molitor* proteins treated with various ethanol levels to evaluate the functionality, structural organization, and antioxidant characterization of the proteins. The research results showed that treatment with ethanol led to significant changes in the properties of *Tenebrio molitor* proteins. Twenty percent ethanol treatment resulted in the greatest antioxidant activity of the concentrations tested (Lee et al., 2024). Various modification and processing approaches aimed at increasing the antioxidant activity of insect proteins have been reported in the literature. In this context, enzymatic hydrolysis, ultrasound-assisted processes, chromatographic fractionation, and glycosylation applications are reported to be effective methods in improving the antioxidant potential of proteins (Xinyan et al., 2025). It has been reported that enzymatic hydrolysis, including ultrasound therapy, can positively alter the antioxidant activity of hydrolysates obtained from protein (HILMP). derived from the larval life

cycle of *Haplia nitidula*. In the study, it was determined that samples treated with ultrasound after enzymatic hydrolysis at 50 °C for 80 minutes showed a 72% level of hydroxyl radical scavenging activity. This obtained value has been reported to be significantly higher than the 58% activity detected in the control group treated only by classically applied enzymatic hydrolysis (Mintah et al., 2019). Enzymatic hydrolysis of the protein contained in *Gryllus assimilis* (black cricket) has been reported to significantly improve its antioxidant properties. In this study, it was determined that hydrolyzing the protein with Neutrase™ 0.8 L, Alcalase™ 2.4 L, and Flavourzyme™ 500 L enzymes, alone or in combination, significantly increased its antioxidant capacity. Furthermore, among the enzymes studied, it was reported that the hydrolysates obtained with Flavourzyme™ 500 L exhibited a highly significant level of antioxidant activity against radicals called DPPH and ABTS. (Xinyan et al., 2025; de Matos et al., 2024). It has been found that heat treatment can increase the antioxidant potential of peptides formed from the simulated gastrointestinal digestion of proteins obtained from the insect *Gryllodes sigillatus* in a laboratory environment. Peptides isolated from these protein preparations were reported to exhibit impressive and clear scavenging activity against free radicals called ABTS and DPPH, and the EC_{50} values, representing the antioxidant property of the peptides, were recorded as 2.75 (ABTS) and 6.91 (DPPH) µg/mL. Additionally, a comparative evaluation reported that peptides obtained from *Tenebrio molitor* protein preparations exhibited the highest level of Fe^{2+} chelation capacity (radical formation inhibition ability) EC_{50} = 2.21 µg/mL and the highest level of reducing power (0.198) in terms of stabilizing free radicals. (Xinyan et al., 2025; Zielińska et al., 2018). Similarly, *Spodoptera littoralis* hydrolysates have been confirmed to exhibit significant antioxidant activity according to DPPH and FRAP tests (Xinyan et al., 2025; Vercruysse et al., 2009). Furthermore, it has been reported that conjugation of *Hermetica illucens* larval proteins with glucose via the Maillard reaction significantly increases their antioxidant potential, particularly with increasing temperature and reaction time; this effect is thought to be linked to changes in protein conformation and the formation of browning products (Xinyan et al., 2025; Mshayisa et al., 2021). When these findings are considered together, it can be seen that various processing and modification approaches can enhance the antioxidant potential of components derived from insect proteins and offer significant opportunities for the use of these proteins as functional food ingredients (Xinyan et al., 2025).

6.3. Antimicrobial Activity of Bioactive Peptides Derived from Insects

Antimicrobial peptides (AMPs) are natural defense molecules that can be synthesized by many organisms, including animals, plants, and fungi. These peptides, typically cationic and short in structure with around 15 to 30 amino acids, play a critical role in defense mechanisms by directly eliminating pathogens, neutralizing endotoxins by chelation, and regulating immunity against infections (Xinyan et al., 2025; Brogden, 2005). The function of AMPs is not limited solely to pathogen suppression; they can also play a role in maintaining host-associated microbiota balance. In this context, some AMPs have been reported to suppress non-symbiotic pathogenic bacteria through a selective mechanism, contributing to the maintenance of a mutually beneficial relationship between host and beneficial microorganisms (Xinyan et al., 2025; Masson et al., 2016).

AMPs are commonly found in eukaryotic organisms, but have been characterized more extensively in insects (Xinyan et al., 2025; Bulet & Stocklin 2005). Approximately 150 AMPs have been identified from numerous species, including *Heterometrus spinifer, Holotrichia diomphalia, Acalolepta luxuriosa, Galleria mellonella, Apis mellifera* and *Bombyx mori* (Xinyan et al., 2025; Yi et al., 2014).

Insect-derived AMPs exhibit high structural diversity and are generally categorized into four main groups: proline and glycine-rich peptides, defensins and cepropins. The broad-spectrum antileishmanial, antibacterial, antitrypanosomal, and antifungal activity potentials displayed by these molecules represent significant potential for the development of next-generation antibiotic alternatives. (Xinyan et al., 2025).

6.4. Antihypertensive Activity of Bioactive Peptides Derived from Insects

Hypertension is considered a critical risk factor for many health problems, including cardiovascular diseases, stroke, and kidney disorders. Angiotensin-converting enzyme (ACE), a key component of the renin-angiotensin system, plays a central role in regulating blood pressure by converting angiotensin I into angiotensin II, a potent vasoconstrictor (Xinyan et al., 2025; Pattarayingsakul et al., 2017). This transformation leads to an increase in blood pressure through mechanisms such as vasoconstriction, stimulation of aldosterone release leading to vascular remodeling, and promotion of fibrosis (Xinyan et al., 2025; Igić, 2018; Vercruysse et al.,

2009). Therefore, ACE inhibition stands out as one of the main therapeutic strategies heavily focused on in the treatment of hypertension. In recent years, research has also focused on the ACE inhibitor potential of insect-derived proteins and peptides in order to identify new components that can contribute to the nutrition-based management of hypertension (Xinyan et al., 2025; Devi et al., 2022).

Studies investigating the angiotensin-converting enzyme inhibitor biological activity of aqueous extracts obtained from cell lines of three different insect species revealed that these samples exhibited significant biological activity with IC_{50} values in the range of approximately 700-900 μg/mL (Staljanssens et al., 2011). Studies on fermented cricket powder fractions have shown that specific fermentation conditions can enhance both antioxidant activity and ACE inhibition ($IC_{50} \approx 970$ μg/mL; inhibition 23.47%) (Mendoza-Salazar et al., 2021). In in vivo experiments conducted with peptides obtained from silkworm pupae, it has been reported that blood pressure is significantly reduced in spontaneously hypertensive rats (Mendoza-Salazar et al., 2012). Lys-His-Val tripeptide, purified by ultrasound and enzymatic hydrolysis, demonstrated strong ACE inhibitor activity ($IC_{50} = 12.82$ μM) and was evaluated as a candidate for a natural antihypertensive component (Jia et al., 2015). Similarly, it was determined that silkworm larva protein hydrolysates digested with gastrointestinal enzymes exhibited ACE inhibition comparable to that of captopril, a standard ACE inhibitor (Wu et al., 2015). Furthermore, it has been reported that *Tenebrio molitor* supplementation exerts regulatory effects on the serum proteome and metabolome in hypertensive rats and can normalize blood pressure-related biological pathways (Stella et al., 2022).

Overall, these findings suggest that insect-derived proteins and peptides have promising potential as natural ACE inhibitors in the development of antihypertensive functional foods or drugs (Xinyan et al., 2025).

7. Conclusion

Edible insects are considered a leading alternative among sustainable protein sources due to their high nutritional value and amino acid profiles with high biological content (Mishyna et al., 2021). Many scientific studies have shown that insect proteins can exhibit functional properties equivalent to many traditional protein sources in terms of solubility, emulsification capacity, water and oil retention properties, foaming, and gelling behavior

(Ma et al., 2023). These functional properties are reported to increase the potential for using insect proteins in the food industry (Ma et al., 2023). In addition, various studies have reported that insect protein hydrolyzates obtained through commonly used enzymatic hydrolysis and other suitable processing methods exhibit antioxidant, antihypertensive, and antimicrobial activities, showing promise for the future and warranting further research (Xinyan et al., 2025).

All findings from studies conducted to date indicate that insect proteins are not only an essential food source but also a very valuable bioresource that can be used in the production of health-protective and supportive foods (Ma et al., 2023).

Insect proteins are hindered and limited in widespread industrial use due to various scientific and technological gaps (Ma et al., 2023). Some of these gaps include differences in the composition of proteins from different species, the effects of chitin on protein digestibility, potential allergens associated with insect proteins, and acceptance by consumers (Ma et al,. 2023). Current studies highlight the need for future studies to develop standardised fractions of protein, create targeted methods to modify proteins via enzymatic means, scale up extraction processes, and demonstrate clinical level bioavailability (Xinyan et al., 2025). Additionally, ensuring compliance with regulatory requirements and completing adequate assessments of the safety of insect proteins while also increasing efforts to improve taste, aroma, and textural properties of products made from insect proteins, are some additional necessary actions that would facilitate the incorporation of insect protein products into the global food system (Ma et al., 2023; Xinyan et al., 2025).

Considering this, it is anticipated that holistic and multidisciplinary approaches to researching insect proteins will be instrumental in advancing the role of insect proteins within sustainable nutrition strategies (Ma et al., 2023; Xinyan et al., 2025).

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ISBN 979-10-7023-078-7

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