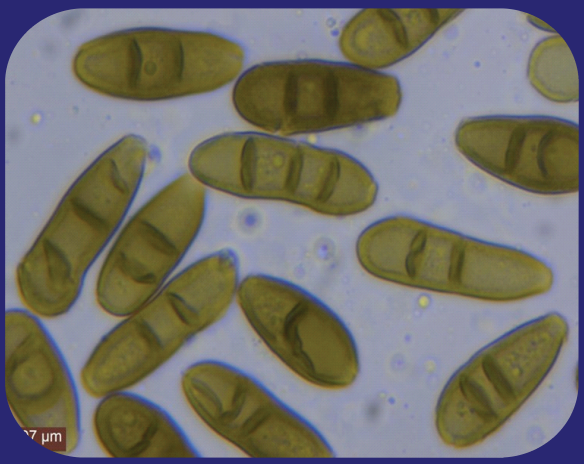
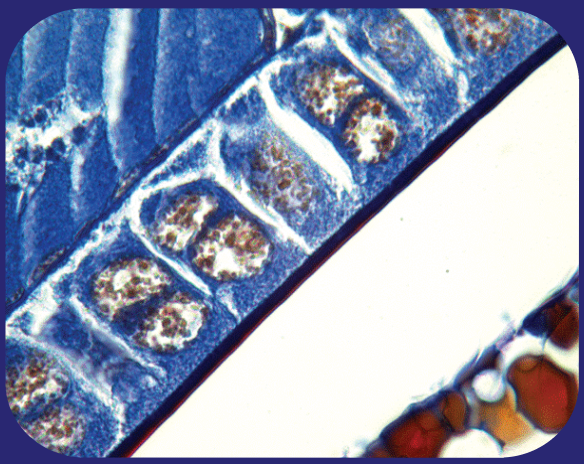
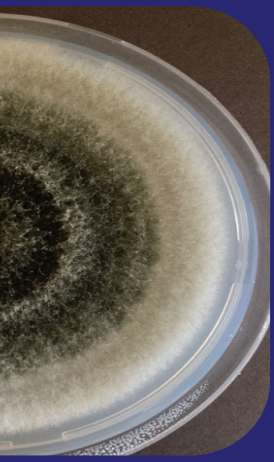
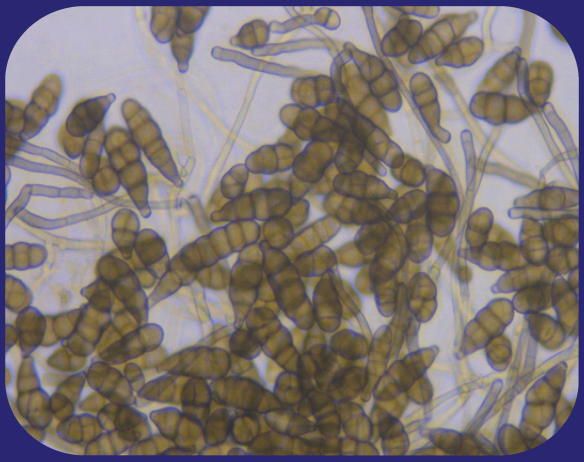
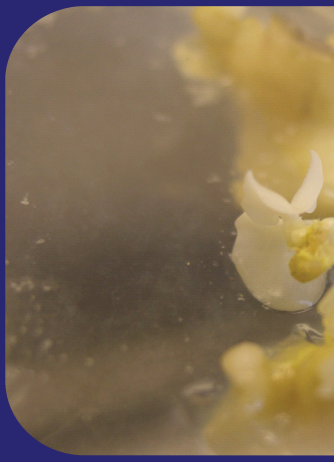

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CONTENTS

Dear Readers

Natural sciences are concerned with natural phenomena. Its subject is natural reality, and physics, chemistry, biology, astronomy and earth science are the main fields of natural sciences. While natural science research and theories can be regarded as an interpretation of nature, its aim is not only to understand but also to explain.

Since their existence in nature, humans have benefited from nature, processed nature, and tried to dominate nature with knowledge and technical advances. For centuries, nature has been used by people without worrying about the future, and the riches of nature have been utilized as if they would never end. However, it is now known by all societies that these resources are not unlimited and they are taking international steps in this regard.

Current Advances in Natural Sciences provides an academic platform for researchers working in these fields. It aims to present articles with an interdisciplinary approach to the scientific literature. Researchers actively share their latest scientific contributions with readers, thus making their discoveries accessible for future research. We hope that these studies will shed light on future studies.

We are honored to share these academic studies with you and would like to express our gratitude to all the authors who contributed to this publication.

EDITORS

CHAPTER 1

SPATIOTEMPORAL DISTRIBUTIONS OF AIRBORNE POLLEN IN TÜRKİYE

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INTRODUCTION

Aerobiology is the study of the sources of biological materials called "airborne" in the atmosphere, such as pollen, spores, fungi, fungal hyphae, algae, protozoa, plant and insect particles, bacteria, viruses, mites, their distribution, their accumulation in different environments or organisms, and all these processes. It is a branch of science that examines the effects of environmental factors on humans (Lacey and West, 2006).

Some of the airborne particles in the air can trigger allergic reactions. These specific types of airborne are called aeroallergens. Aeroallergens are known to be significant contributors to respiratory allergies. At times, they can also act as triggers for other types of allergies due to cross-reactivity between allergens. One of the most well-known phenomena related to this is the Oral Allergy Syndrome, which is caused by cross-reactivity between allergens found in pollen and certain fruits or vegetables. If a patient has become sensitized to pollen through the respiratory system and experiences an allergic reaction to a food antigen that shares structural similarities with the pollen (Kondo and Urisu, 2009). Considering the high diversity of plant species in our country, knowing the spatiotemporal distribution of aeroallergens is important not only for the diagnosis and treatment processes related to respiratory allergies but also for shaping local and regional health policies and making significant contributions to improving allergy management.

The aim of this study is to investigate the spatiotemporal distribution of airborne pollen in Türkiye, with a focus on identifying seasonal variations and geographic patterns to enhance our understanding of allergen exposure and support effective allergen management strategies.

The Significance of Aeroallergens

Bioaerosols can be composed of intact microstructures, such as viruses, intact bacterial cells and spores, protozoa and their cysts, fungal cells and spores, plant pollen grains and spores, and cell fragments (animal hair, epithelium, feathers and secretions) (Harley, 2012). Air is a transportation medium for these bioaerosols and a living medium for other airborne particles. Pollen and mold spores are the first ones that come to mind as aeroallergens. Besides that, house dust mites, animal fur, and dander are also important allergen sources in the atmosphere. There is a report that bacteria can even trigger allergic reactions (Bowers et al., 2011). Thus, the analysis of each of these requires different specialties. Therefore in this chapter, we focused mainly on the spatiotemporal distribution of pollen grains.

The term "aeroallergen" is used to describe allergenic substances found in both indoor and outdoor air, including pollen, mold spores, as well as mites and animal dander as mentioned above. Aeroallergens could result in symptoms such as sneezing, coughing, runny nose, and eye irritation, which are known symptoms of allergies. In particular, pollen grains can hang in the air for a long time, and can cross-react with other aeroallergens, so they are effective all year round (Singh and Mathur, 2012). When compared to other allergens, aeroallergens are the most challenging to avoid because they exist in both indoor and outdoor environments, and it is not always possible to filter the air at all times. In rare situations, it has been reported that certain dander can even lead to an anaphylactic reaction (Amrol et al., 2000).

Allergies are complex conditions caused by the interaction of various genetic and environmental factors. (Cecchi et al., 2018). Due to increasing factors that are causing changes in the composition and quantity of aeroallergens in the air, such as global climate changes, it has a dramatic effect on the prevalence of allergies caused by aeroallergens (Katelaris and Beggs, 2018).

For many years, pollen grains were believed to only trigger upper respiratory system issues. However, this perception has undergone a significant shift with the discovery of particles released from aeroallergens, such as pollen, which could also be responsible for lower respiratory system diseases like asthma (D'Amato et al., 2007). It has been shown that these particles could alter the atmospheric radiative balance and could have a direct climatic impact (Xiao et al., 2013). This is also an interesting finding as it demonstrates that aeroallergens may undergo changes in specific environmental conditions. This is one of the reasons that makes the treatment of allergic diseases difficult.

POLLEN AS AEROALLERGENS

Pollen is the unit containing sperm cells produced by plants for reproductive purposes. After production in the anthers, pollen is released from the anther and migrates to the stigmas of the pistil. Due to this function, pollen has a specialized structure. The average size of pollen grains, depending on the plant species, ranges from 7 to 100 μm (Whitby et al., 2022).

Pollen grains would be exposed to harsh conditions during their transportation from the anther to the stigma. Considering the size of the pollen grains, their structure must be strong enough to withstand these conditions. Therefore, pollen grains have a more complex wall structure than a regular plant cell wall (Blackmore et al., 2007). When a pollen grain is stained by a dye such as safranin, two distinctive wall layers can be distinguished: the outer layer called exine and the inner layer called intine. The differences in staining between these two layers reflect the chemical compositions of the wall layers. Exine is composed of sporopollenin which is defined as "the most resistant organic material known". Oxidative degradation of natural sporopollenins using ozone and synthetic carotenoid polymers reveals that sporopollenins have nearly identical chemical structures and share the same structure as synthetic carotenoid polymers (Brooks and Shaw, 1978). Beyond the

tough resistance of exine to physical and chemical factors, it plays an important role in giving aerodynamic properties to pollen grains and provides them with morphological characteristics that allow for the discrimination of pollen grains from different plant species.

Exine does not have a uniform structure. When examined under Transmission electron microscopy (TEM), it appears to be composed of different layers, which are termed differently by various researchers (Table 1). According to Faegri and Iversen, (1964), exine is composed of two distinct layers, ektexine and endexine. While they evaluated endexine as homogeneous, they classified ektexine into three sub-layers as Tectum, Columella, and Foot Layer, respectively, from outer to inner.

Table 1. Pollen wall layers classification by two different researchers.

<table>
<tr><td colspan="4">Erdtman (Erdtman, 1952)</td><td colspan="3">Faegri & Iversen (Faegri and Iversen, 1964)</td></tr>
<tr><td rowspan="5">Sporoderm</td><td rowspan="4">Exine</td><td>Ektosexine</td><td rowspan="2">Sexine</td><td rowspan="3">Ektexine</td><td>Tectum</td><td rowspan="4">Exine</td></tr>
<tr><td>Endosexine</td><td>Columella</td></tr>
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<tr><td>Endonexine</td><td colspan="2">Endexine</td></tr>
<tr><td colspan="3">Intine</td><td colspan="3">Intine</td><td>Sporoderm</td></tr>
</table>

The inner layer of the pollen wall, known as the intine, is mainly composed of cellulose, hemicellulose, pectin, and structural proteins. It plays an important role in the growth of pollen tubes (Ma et al., 2021).

Some surface features or structures, which is called ornamentation, may be found on the outer layer of pollen grains. These characteristics can vary among different plant species and are used to identify and classify pollen grains. Pollen ornamentation may include various structures such as spines, ridges, grooves, or other distinctive

patterns that can be observed under a microscope. Ornamentation in pollen grains refers to the diverse surface features that serve various ecological and biological functions in the reproductive and survival strategies of plants. Analyzing pollen ornamentation can provide valuable information for identifying plant species and understanding their evolutionary relationships. Pollen ornamentation may affect the aerodynamic properties of the pollen grain. Generally, much simpler types of ornamentation are observed in the pollen grains in the air.

There are different approaches to deliver pollen grains from the anther to stigma depending on the plant species. While some of these approaches include living (biotic) vectors such as bees and bats, others use wind or water streams as vectors (abiotic). Biotic and abiotic vectors that are used by plants for pollination are summarized in Table 2.

Table 2. Classification of Plants According to Their Pollinators.

	Term		Pollinator
Abiotic	Anemophily		Wind
	Hydrophily		Water
Biotic	Entomophily	Melittophily	Bee
		Psycophily	Butterfly
		Phalaenophily	Moth
		Miyofil or sapromyophily	Fly
	Ornitophily		Bird
	Chiropterphily		Bat

There is a direct correlation between the number of pollen grains that are released from a plant and its pollination strategies. Anemophilous plants, which use wind as a vector, tend to produce much more pollen grains than those using other pollination approaches. For example, one billion pollen grains can be released from a single

ragweed plant in a season ("Ragweed Pollen Allergy," 2023). This strategy is aimed at increasing the chance of pollination, but it also leads to an increased chance of these pollen grains reaching the airways of humans. Therefore, most allergenic plants are the ones that release their pollen into the air. Pollen is one of the important seasonal allergy causes. Many proteins or glycoproteins expressed in pollen grains have been determined as allergen.

Allergens are substances that are detected by the immune system after being eaten or inhaled and cause allergic reactions. Allergens are small particles in protein or glycoprotein structure that are mostly harmless to the body and generally have a molecular weight of 5-50 kDa. In addition, substances such as drug active ingredients, haptens and glycans can also cause allergies. The allergy-causing substance may contain many antigens in protein or glycoprotein structure, which may or may not have allergenic properties. Many allergens are proteins with biological activity such as enzymes, enzyme inhibitors, carrier proteins. The three-dimensional structure of allergens is very important in terms of reflecting their allergic properties. There is a region on the allergen with a specific amino acid sequence to which the IgE molecule can bind. The amino acids in this region may be consecutive, or they may be distant from each other, coming together as a result of protein folding. This region is called "epitope" or "antigenic determinant". An allergen may contain more than one antigenic determinant region. This condition is under the control of MHC-Class II genes. For this reason, different individuals may produce IgE specific to different regions of the same allergen, and different IgE may be produced for different epitopes by the same individual. Allergens are named depending on the allergen source. This naming is done in accordance with the principles determined by the Allergen Nomenclature Subcommittee of the World Health Organization, International Union of Immunological Societies (WHO/IUIS). In this classification, the allergen is expressed with a kind of abbreviation consisting of the first 3 letters of the taxonomically

accepted genus name of its source, the first letter of the species name and a number indicating the order in which the allergen was discovered. For example, the abbreviation Bet v 1 refers to the first identified allergen from the *Betula pendula* L. (syn. *Betula verrucosa*) plant. Allergens can be classified in different ways depending on the frequency of sensitization in people, their source, chemical structure, the duration of exposure to the body and the way they are taken into the body. If the allergen causes an allergic reaction in more than 50% of sensitive patients, it is defined as a major allergen, and if it causes a reaction in less than 50% of the patients, it is defined as a minor allergen. The most commonly used of these is the classification of the allergen according to the way it is taken into the body. According to this classification, allergens are primarily divided into three groups: allergens inhaled, allergens ingested orally, and allergens ingested through the skin (parenteral).

Main factors affecting the spatiotemporal distribution of pollen in Türkiye

Türkiye is situated at the crossroads of the continents of Europe and Asia. Therefore, the climatic and vegetation properties of Türkiye also reflect this geographical situation.

Climate

Climate can be simply determined by temperature and precipitation characteristics of a region over time. Therefore, it can be said that factors altering temperature and precipitation characteristics are also defining the type of climate. The most important natural factors are (“Factors affecting climate | UK Environmental Change Network,” 2023):

- distance from the sea
- ocean currents
- direction of prevailing winds
- shape of the land (known as 'relief' or 'topography')

- distance from the equator
- the El Niño phenomenon.

Although Türkiye is located within the expansive Mediterranean geographical region, which generally has moderate climatic conditions, the diverse nature of its landscape, and the presence of mountain ranges that run parallel to its coasts, lead to significant variations in climate from one region to another. Coastal areas benefit from milder climates, while the inland Anatolian plateau encounters extreme weather patterns with hot summers, cold winters, and limited rainfall (Sensoy, 2023). Türkiye experiences three primary climate types: Mediterranean, Continental, and Oceanic. The majority of the country, encompassing a significant portion of the mountainous regions, falls under the influence of various Mediterranean climate variations (Akman and Ketenoğlu, 1986). On the other hand, different types of Mediterranean climates have been observed in central Anatolia due to mountains that run parallel to the coast in the north and south parts of Türkiye, which prevent the penetration of rain clouds into the interior part of Türkiye. A continental climate, characterized by long and extremely cold winters, is prevalent in two distinct areas in Northern and Northeastern Anatolia (Akman and Ketenoğlu, 1986). While Türkiye's coastal areas adjacent to the Black Sea experience a moderate Oceanic climate characterized by mild, rainy winters and cool to cold, humid summers, the coastal regions of Türkiye along the Aegean Sea and the Mediterranean Sea experience a Mediterranean climate with hot, dry summers and mild to cool, wet winters (Toros et al., 2019). The flora of Türkiye has been shaped by the influence of these various climates, resulting in the formation of diverse vegetation types.

Vegetation

Five main vegetation types are dominant in Türkiye. These are Forest, Maki, Garig, Steppe and Alpine vegetation types. Forest vegetation with different structures and characteristics constitutes

approximately 27% of the country's area. Forest vegetation shows significant diversity according to the European, Siberian, Mediterranean and Iran-Turan flora areas. The plant community consisting of evergreen shrubs 2 m or more tall with hard leaves and dense branches, adapted to the climatic conditions and growing environment in the Mediterranean region, is called maquis. Common maquis types in Türkiye: *Quercus coccifera* L. maquis (300 to 1200 m), *Olea europea* L. maquis (700-1000 m), *Arbutus andrachne* L. maquis (300-900 m), *Ceratonia siliqua* L. -*Laurus nobilis* L. maquis (50-850 m). Garig vegetation consists of dwarf shrubs, 0.5-1 m tall, in the Mediterranean flora area, which replaces maquis vegetation with anthropogenic effects in very arid areas where the soil is very stony and shallow. Step vegetation, in other words Iran-Turan flora area; It is the most important vegetation type of Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions. Perennial, deep woody-rooted, cushion-forming plants are common in steppe vegetation. Alpine vegetation consists of cushion shrubs and herbaceous plants after 1700-1800 m at the forest and tree line in high mountainous areas. It spreads from the forest border to the highest levels of the mountains in the Aegean, Mediterranean, Eastern Anatolia and especially in the high mountains of the Eastern Black Sea region (Anşin, 1983; Davis et al., 1971; Yaltırık, 1974).

Urban Landscape

The influence of urban landscapes on allergic pollen distribution is a topic of growing concern and study. As cities continue to expand and urbanization intensifies, the composition of vegetation within these areas undergoes significant changes. Urban areas, with their distinct mix of vegetation, infrastructure, and microclimates, can significantly impact the presence and concentration of allergenic pollen. Urban environments often feature a higher prevalence of certain plant species that are more adapted to city conditions. These urban-adapted plants,

such as certain trees and ornamental shrubs, may release pollen that differs from the natural vegetation found in rural or wild areas. The expansion of concrete surfaces and reduced green spaces in cities can lead to a decrease in tree and grass pollen, but an increase in weed pollen, as hardy and resilient weed species thrive in urban environments. Moreover, the heat island effect in cities can trigger earlier flowering and pollen release in some plants. The planting choices in urban green spaces, such as parks and gardens, also play a pivotal role in shaping local pollen profiles, as specific plant species are chosen for their aesthetic qualities, which may or may not be allergenic. Consequently, this altered composition of allergenic pollen sources in urban landscapes can have a notable impact on allergic pollen distribution. In some cases, urban areas may experience a higher concentration of allergenic pollen, potentially exacerbating allergic reactions among sensitive individuals. Furthermore, factors like temperature and air pollution in cities can influence the timing and amount of pollen released, further complicating the distribution of allergenic pollen in urban environments. Understanding the complex interplay between urbanization and allergic pollen distribution is essential for mitigating allergy-related health issues and guiding urban planning decisions that balance greenery with public health concerns (Cariñanos et al., 2016; Cariñanos and Casares-Porcel, 2011; Cirino et al., 2022; Pinar, 2018).

POLLEN MONITORING TECHNIQUES IN AEROBIOLOGY

Although many different sampling approaches have been developed since 40 years, main approaches that have been used extensively are more or less still the same. These can be classified as passive and active sampling.

In passive sampling, airborne particles are collected mainly with the help of gravity onto a certain surface. Although the surface varies depending on the method, the surfaces generally used are petri plate with agar medium or slide coated with glycerine-jelly or similar substances. The technique using a Petri plate with agar medium is called the settle plate method and is mainly used for microbiological purposes. Another extensively used passive sampling method is the one that uses a device called the Durham Sampler. This sampler simply consists of a microscope slide with glycerine-jelly and two round plates aligned vertically, each about 8 cm apart from the other (Figure 1).

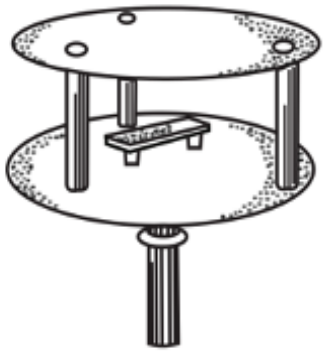

Figure 1. Diagram of the Durham instrument.

Although the Durham instrument has many advantages, such as being simple and having low acquisition and maintenance costs, there are also crucial disadvantages, such as the volume of air sampled and efficiency being unknown. While this device was widely used in studies in the past, it has now been replaced by more advanced active samplers.

Active samplers are generally defined as devices that use a pump or a blower to draw in air for collecting airborne particles. These particles can be collected onto an impactor plate or into a tube. Although many different devices are available in the market, Hirst-type devices are the most common devices used for the purpose of aerobiological monitoring all

Figure 2. A hirst-type device manufactured by Burkard Co, UK.

over the world (**Figure** 2). Hirst-type devices operate on the principle of impaction. They draw in a known volume (10 l/min) of air through a sampling orifice at a constant rate, typically using a motor-driven suction pump. The air containing fungal spores and pollen is directed onto a rotating drum or disc coated with a sticky, transparent adhesive surface. This adhesive surface traps the airborne particles as they impact it. The drum or disc rotates slowly, allowing for continuous sampling over time. As it turns, the particles impact the adhesive surface and adhere to it. The sampling duration can be adjusted, typically ranging from 24 hours to a week, depending on the research requirements.

SPATIOTEMPORAL DISTRIBUTION OF AIRBORNE POLLEN GRAINS

Plants release millions of pollen into the atmosphere during pollination periods. Seasonal changes resulting from global climate change can lead to changes in the vegetation periods and pollen production of plants. In many aeropalynological studies, the total amount of pollen in the air varies depending on the years (D. Gioulekas et al., 2004; Dimitrios Gioulekas et al., 2004; Kobzar, 1999). Meteorological factors such as temperature, humidity, wind speed and precipitation, which are effective in the distribution of pollen into the atmosphere, also vary from year to year. Municipalities' planting of imported plant species in their landscape areas and the arrival of invasive species to the region also affect the type and density of pollen in the atmosphere. *Ambrosia artemisifolia* L. plant, known for producing a lot of seeds and allergenic pollen, is also an invasive species that comes to our country with uncontrolled transport and increases atmospheric pollen density (Bicakci and Tosunoglu, 2015; Kaplan et al., 2003; Pinar, 2018; Zemmer et al., 2012).

Seasonal Variation

The misconception about pollen and the allergy induced by it is that pollen-induced allergy is limited to spring. However, pollen grains can be observed in the air throughout the year. Moreover, different seasons can represent some pollen types depending on a region and its vegetation. While tree pollen tends to be observed earlier than other pollen types, the pollen season of some pollen such as Cupressaceae and *Pinus* L. can extend throughout the whole year. Pollen that belongs to the Poaceae family (sometimes regarded as grass pollen) can be monitored from spring to fall. However, the seasonal dynamics of these pollen grains show great variation because Poaceae pollen is composed of many different species that have different pollen seasons. Weed pollens, which are composed of many pollen grains from numerous species belonging to different plant families, are mainly monitored in summer in many aerobiological studies.

Although the number of stations conducting aerobiological monitoring has increased over the years, the number of stations conducting long-term monitoring is very limited (Figure 3). Additionally, in some stations, the collection method has changed over the years. Therefore, making data comparisons between data collected with old and new methods is challenging. The results for the provinces where aerobiological studies have been conducted for more than two years are summarized below.

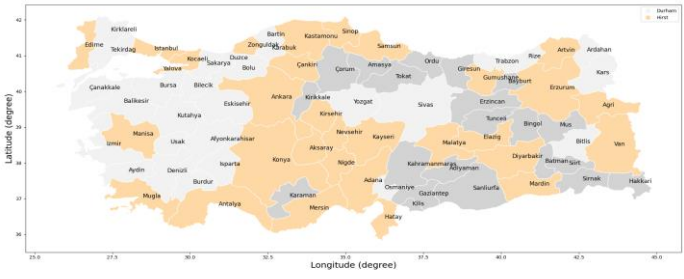

Figure 3. Provinces in Türkiye where aerobiological studies were carried out using Hirst and Durham traps.

Many aeropalinological studies have been carried out in the atmosphere of Ankara province using volumetric and gravimetric methods. The first study was conducted by Karamanoglu and Ozkaragöz (1967) using Durham samplers On the other hand, the first study conducted with the volumetric method was by İnceoğlu et al. (1994)'s study. In the study where Ankara atmosphere pollen was determined between 1990 and 1992; 76% of the pollen is woody, 10% is herbaceous and 14% is Poaceae. Dominant taxa were found as Pinaceae, Poaceae, Cupressaceae / Taxaceae, *Populus* L., *Platanus orientalis* L., Moraceae, *Quercus* L., Betulaceae and Chenopodiaceae / Amaranthaceae. The total pollen concentration in 1991 was higher than in other years and it was associated with the very high wind speed in the spring months of 1991. In another study, Pinar et al. (1999), stated that the highest pollen concentration was in June in 1993. They reported that the reason for this was the high wind speed and low rainfall recorded in June. Kaplan et al. (2003)found that pollens of Pinaceae, Cupressaceae / Taxaceae, *Populus*, Poaceae, *Platanus* L., Moraceae, Chenopodiaceae / Amaranthaceae, *Acer* L., *Robinia* L., *Quercus* L., Betulaceae, *Salix* L., Oleaceae, *Artemisia* L., *Plantago* L. and Urticaceae were dominant in the atmosphere based on a ten-year study (1990-1998). They also come across *Ambrosia* L. pollen. They put forward the view that *Ambrosia* pollen was carried into the Ankara atmosphere from long distances by air currents. Pınar et al. (2004) monitored the effects of meteorological factors on Poaceae pollen during three years. According to this study, the wind speed and direction are the most related meteorological factors on Poaceae pollen concentration. It has been stated that the amount of precipitation has a secondary and negative effect on the pollen density in the atmosphere. Kizilpinar et al. (2011) examined the relationship between pollen counts and meteorological factors in the study they conducted in the atmosphere of Hacettepe University Sıhhıye Campus between 2005 and 2008. In the study, researchers determined that 72.12% of the pollen

belonged to trees and woody taxa, 12.80% of the pollen belonged to Poaceae and 15.07% belonged to other herbaceous taxa. In the study, wind speed influenced on the pollen of tree and woody taxa, temperature and sunshine duration had an effect on the pollen of other herbaceous taxa, and only sunshine duration had an effect on the pollen of other herbaceous taxa. Özmen (2012), in her thesis, collected pollen data from two different stations, Ankara University Tandoğan Campus and Hacettepe University Sıhhıye Campus, for 2 years between January 1, 2009 and December 31, 2010. According to this study, on the Ankara University campus in 2009, it was stated that 10.9% of the pollen belonged to Poaceae, 0.52% belonged to Betulaceae, and in 2010, 6.9% of the pollen belonged to Poaceae and 1.47% belonged to Betulaceae. In the atmosphere of Hacettepe Sıhhıye Campus, in 2009, 20.8% of the pollen belonged to Poaceae, 1.2% to Betulaceae, and in 2010, 8.2% belonged to Poaceae and it has been stated that 0.5% belongs to Betulaceae. It was found that there was a difference in pollen amounts between the two stations. It has been stated that the reason for this difference is due to the location of the stations and the vegetation around them. Since urbanization is especially high around Hacettepe University Sıhhıye Campus, green areas in and around the campus are relatively more limited compared to other campuses. Acar et al. (2017) examined the change in pollen concentrations in the atmosphere of Ankara between 1990 and 2011. According to this study, it was determined that *Betula* pollen concentration and Poaceae pollen concentration tended to decrease over the years. It has been emphasized that the decrease in the amount of Poaceae pollen is linked to urbanization. It was observed that *Ambrosia* pollen concentration increased between the mentioned years. Especially in the last 10 years, there has been a slight increase in the amount of Betulaceae pollen. The increase in average temperatures due to global climate change will lead to early start of pollination and increase in pollen production, especially for *Betula*. Poaceae pollen concentration change is not consistent over

the years. When the effect of environmental factors on the pollen season is taken into account, it is very difficult to obtain a meaningful trend (increase/decrease change in pollen concentration).

Long-term aeropalynological surveys have also been conducted in Bursa. When comparing an earlier study that used the Durham instrument with a more recent study using the Hirst-type instrument, it becomes evident that there was a significant increase in the percentages of pollen from arboreal taxa. These differences may be attributed to the use of different sampling approaches. On the other hand, the percentages of tree pollen in the total pollen showed consistency in a study conducted in the Osmangazi district of Bursa compared to an earlier study conducted in the city center of Bursa .

Aerobiological studies in Zonguldak province began with the monitoring of atmospheric pollen using the Durham instrument (Alan and Kaplan, 2018; Kaplan, 2004). Subsequently, the atmosphere of Zonguldak was analyzed with Hirst-type traps. When considering the general composition of atmospheric pollen, it can be observed that the percentage of tree pollen in the Zonguldak atmosphere tended to be lower from 2004 to 2009 (Özdoğan, 2011). It appears that there is no significant difference in Poaceae pollen percentages over the years. However, the contribution of long-distance transportation of *Ambrosia* pollen to the total pollen load in Zonguldak is inevitable (Alan et al., 2020). Back trajectory analysis also showed that Western Black-Sea region is also placed in the route of transportation of this pollen type to inner Anatolia.

Regional Varieties

In the atmosphere of the Mediterranean Region, Cupressaceae/Taxaceae pollens are dominant in the atmosphere of the coastal provinces (Altintaş et al., 2004; Tosunoglu et al., 2015a), except Kahramanmaraş, while Pinaceae pollens are in the highest amount in the inland areas (Alaca, 2018; Biçakçi et al., 2000; Tosunoglu et al.,

2016). Poaceae pollen concentration (32.6%) is very high in Mersin province (Çakir and Doğan, 2020). Although *Olea* pollen concentration varies, it is highest in the Mersin atmosphere. The highest *Quercus* and *Platanus* pollen concentration was observed in Kahramanmaraş province ("Atmospheric Pollen Profile of Kahramanmaraş - S Turkey," 2016). *Morus* pollen concentration was highest in Hatay (Tosunoglu et al., 2018a), and Chenopodiaceae/Amaranthaceae pollen concentration was highest in Mersin.

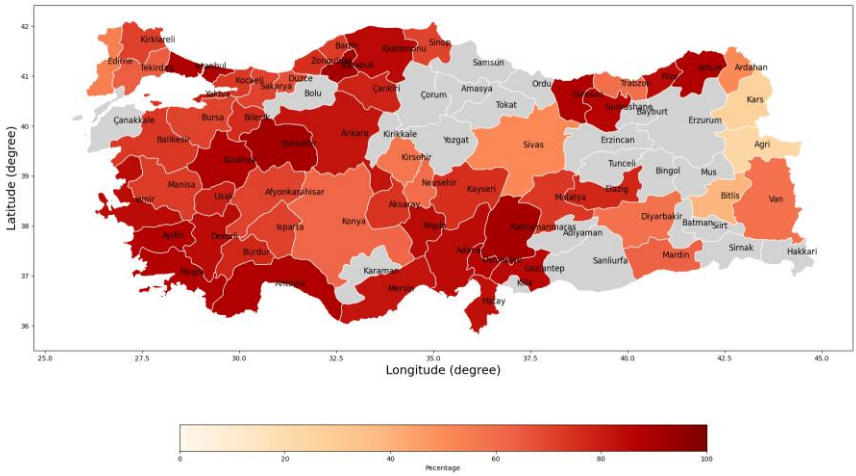

Figure 4. Spatial Distribution of Tree Pollen Percentage in Türkiye Provinces.

In the aeropalynological studies conducted for the Eastern Anatolia region, Pinaceae pollen is dominantly seen in Elazığ and Ardahan provinces, while the highest pollen concentration in all other provinces studied belongs to Poaceae (Çetin, 2015; Kilic et al., 2019; Yalcin et al., 2017). Approximately half (43.96%) of the total pollen concentration of Erzurum atmosphere consists of Poaceae pollens (Saatcioglu, 2016). The most important reasons for this are that the region has high altitude mountain steppe vegetation and dense meadow and pasture areas. While Cupressaceae and *Fraxinus* pollens are dominant in the atmospheres of Elazığ (Kilic et al., 2019) and Van

(Bicakci et al., 2017), Chenopodiaceae/Amaranthaceae pollens are in the highest amount in Kars and Iğdır (Figure 6) (Akpınar, 2018) provinces. In Bitlis, Urticaceae pollens constitute 12.31% of the total pollen concentration. *Quercus* pollen concentration varies, and it was mostly detected in Elazığ and Bitlis provinces (Celenk and Bicakci, 2005).

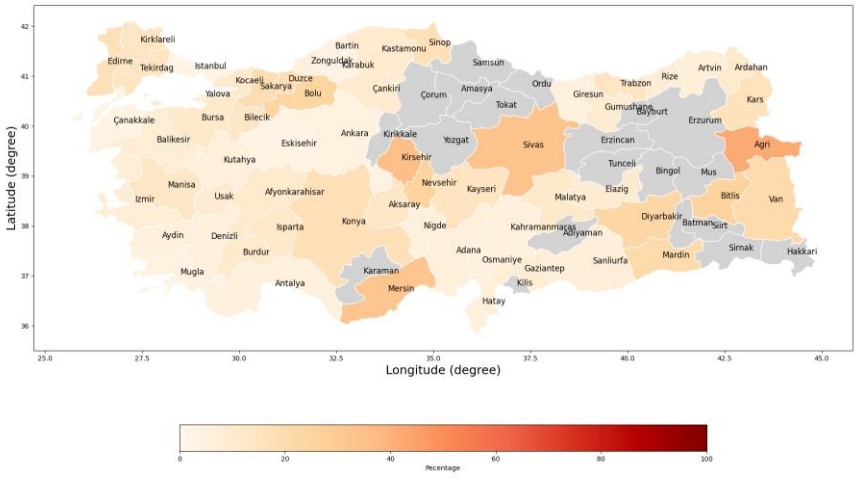

Figure 5. Spatial Distribution of Grass Pollen Percentage in Türkiye Provinces.

While Cupressaceae pollen is dominant in the atmosphere of the coastal provinces in the Aegean region (Bilisik et al., 2008; Tosunoglu and Bicakci, 2015; Uguz et al., 2017), *Pinus* L. pollen is in the highest amount in the inland areas (Bicakci, 2002; Bicakci et al., 1999a; Güvensen et al., 2013). *Olea europeae* pollens constitute approximately 10% of the total pollen concentration in the atmosphere of the provinces in the Aegean region. Although *Quercus* pollen concentration varies, it was mostly detected in Manisa and Uşak provinces. The highest Poaceae pollen concentration was observed in Manisa province (Guvensen et al., 2018).

In the aeropalynological studies conducted for the Southeastern Anatolia region, the highest amount of Cupressaceae pollen was

observed in the provinces of Gaziantep (Tosunoglu et al., 2016), Mardin and Diyarbakır, while the highest pollen concentration in Şanlıurfa province belonged to the *Morus* genus. In the study conducted with the Durham instrument in the Kızıltepe (Potoglu Erkara et al., 2016) district of Mardin (Tosunoglu et al., 2018b), the highest concentration of *Olea* pollen was observed in the atmosphere. Only in the Diyarbakır atmosphere study, *Betula* pollen was found in high amounts in the atmosphere. Poaceae pollen concentration is at the highest level especially in Mardin province and is similar to other provinces (Figure 5) (Saatcioglu et al., 2016). Although *Pinus* pollen concentration varies, it was most detected in Diyarbakır province (Bursali et al., 2006). The highest *Quercus* pollen concentration was observed in Gaziantep province.

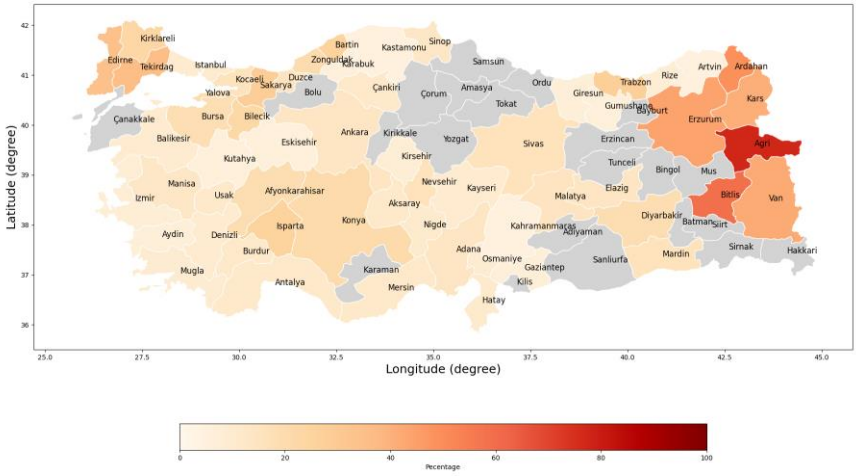

Figure 6. Spatial Distribution of Weed Pollen Percentage in Türkiye Provinces.

In the Central Anatolia region, the highest concentration of Pinaceae pollen was observed in all provinces except Niğde (Secil, 2018) and Kırşehir, while the highest pollen concentration in Niğde belongs to Cupressaceae/Taxaxaceae (Aydan, 2015; Erkara, 2008; Safak, 2016). Poaceae pollens constituted approximately one third

(32.81%) of the total pollen concentration of Kırşehir atmosphere (Erdogan, 2017). The most important reason for this is the dense meadow and pasture areas in the region. Although Chenopodiaceae/Amaranthaceae pollen concentration varies, it was mostly detected in Nevşehir and Konya provinces (Temizer Kizilpinar et al., 2012). The highest *Platanus* pollen concentration was recorded in the Ankara atmosphere (Pinar et al., 1999). One of the reasons for this is the widespread use of *Platanus orientalis* trees in parks, gardens and along roadsides. *Quercus* pollen was found in the highest concentration in the atmospheres of Çankırı (Ceter et al., 2012b) and Nevşehir (Unver, 2012). *Betula* pollen concentration was highest in Nevşehir.

In aerobiological studies conducted for the Black Sea region, the highest amount of Pinaceae pollen is observed in Karabük (Kaplan and Özdoğan, 2015) and Kastamonu (Ceter et al., 2012a) provinces, while the highest pollen concentration in Bartın province belongs to Poaceae (Kaya and Aras, 2004). *Betula* pollen was mostly seen in the atmosphere in Trabzon (Fisne and Pehlivan, 2022) and Artvin. The most important reasons for this are the dense abundance of naturally occurring trees of the *Betula* genus in the region. Although Cupressaceae pollen concentration varies, it was mostly detected in Kastamonu province. The highest amount of *Alnus* pollen was detected in the atmosphere in Giresun and Rize (Bicakci et al., 2002). It was reported that more than 50% of total pollen loads in the atmosphere consists of tree pollen in majority of studies conducted in the Black Sea region (Figure 4) (Ceter et al., 2012c; Kaplan and Serbes, 2014). Considering distribution of this pollen based on genus level, some differences stand out between the coastal lines and inner parts of the region (Turkmen et al., 2018). *Corylus* pollen replaced *Betula* pollen in the inner parts of the region (Turkmen et al., 2018). The Black Sea region records high concentrations of *Corylus* pollen due to extensive hazelnut cultivation in the coastal region (Kaplan and Serbes, 2014). *Corylus* pollen was seen in the highest amount in Giresun province,

which ranks first in hazelnut production in our country. *Quercus* pollen concentration varies, but the highest amount was observed in Artvin province. Despite reports of ragweed invasion in the Black Sea region, the highest concentration of *Ambrosia* pollen has been reported in Zonguldak, where the existence of ragweed has not been reported (Alan et al., 2020).

In the Marmara Region, while Cupressaceae pollen is dominant in the atmosphere of the coastal provinces, *Pinus* pollen is in the highest amount in the inner regions. *Olea europaea* pollen constitutes approximately 10% of the total pollen concentration in the atmosphere of the provinces located in the Southern Marmara region. Although *Quercus* pollen concentration does not vary very much, it was determined at a high rate (10.68%) in the Ergene Region, based on the average of the provinces (Erkan et al., 2010). It was also detected at high rates in Sakarya (10.5%) (Bicakci, 2006), Çanakkale (9.28%) (Guvensen et al., 2005) and Bilecik (8.67%) (Türe and Böcük, 2009). This is because the *Quercus* plant has many species that can grow in both moist and dry forests in the mountainous parts of the region (Erkan et al., 2011). Additionally, Poaceae pollens were mostly found in the atmospheres of Sakarya and Edirne (Bicakci et al., 2004). Wheat and corn cultivation is most intense in these regions. Although *Platanus* pollen concentration varies by province, it was detected at the highest rate in the atmospheres of Istanbul (Europe) and Yalova (Altunoglu et al., 2008; Celenk et al., 2010).

Durham samplers were frequently used for aeropalynological samplings for many years in Bursa. In the center of Bursa, 70.1% of a total pollens belong to woody plants and 27% to herbaceous plants (Bicakci et al., 1996); In Mudanya, 82% of pollens belong to woody plants and 15% to herbaceous plants (Bıçakçı et al., 1995); In Görükle campus, 58.63% of the pollens belonged to woody plants and 37.64% to herbaceous plants (Bicakci et al., 1997); In İnegöl, 60.92% of total pollens belong to woody plants and 36.28% to herbaceous plants

(Bicakci et al., 1999b); In Iznik district, 67.45% of pollens are from woody plants and 16.86% are from herbaceous plants (Bicakci et al., 1999c); In Mustafakemalpaşa district, 63.46% of pollens were from woody plants and 32.01% were from herbaceous plants (Bıçakçı et al., 1999); In Keles district, 82.09% of a total pollens over two years were collected from woody plants and 15.78% from herbaceous plants (Bicakci et al., 2000); In Gemlik district, 82.39% of pollens belong to woody plants and 17.15% to herbaceous plants(Saatcioglu et al., 2011); In Büyükorhan district, it was reported that 87.46% of pollens for two years belonged to woody plants and 12.20% belonged to herbaceous plants (Tosunoglu et al., 2015b).

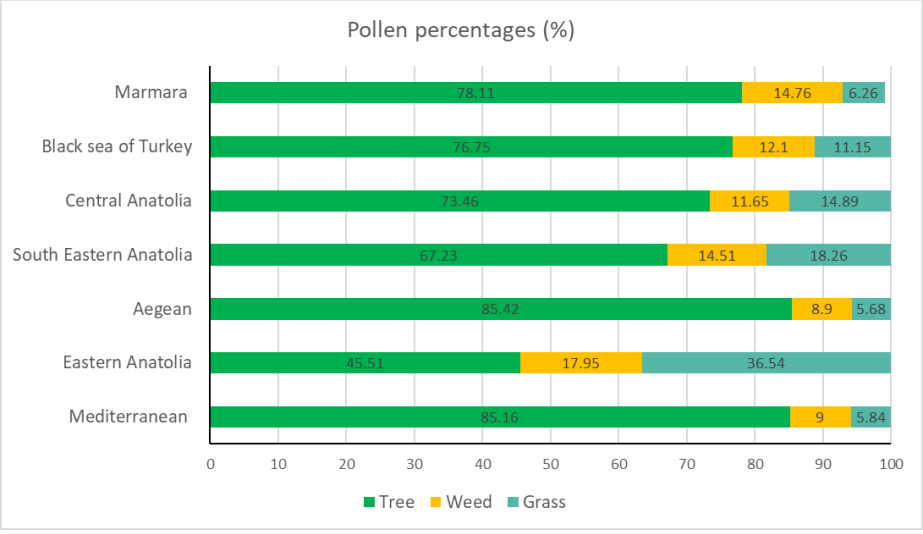

Figure 7. The distribution of tree, weed, and grass pollen percentages across seven regions in Türkiye.

It is observed that there are serious differences between pollen percentages in the districts depending on the vegetation (**Figure 7**). Considering the aerobiological studies conducted in seven geographical regions, while the percentage of tree pollen is dominant in all regions except the Eastern Anatolia Region, the percentage of total herbaceous pollen, including Poaceae, is higher in the Eastern Anatolia Region.

This is due to the dominance of high mountain steppe vegetation in the region. The highest percentage of tree pollen (85%) was recorded in the Aegean and Mediterranean Regions. The clustering of the regions based on the percentages of the dominant pollen types in air was summarized in Figure 8. Cupressaceae and Pinaceae were the highest pollen types in most of the regions except Eastern Anatolia Region. *Olea* was one of the important pollen sources for individuals who live in Aegean and Mediterranean Regions.

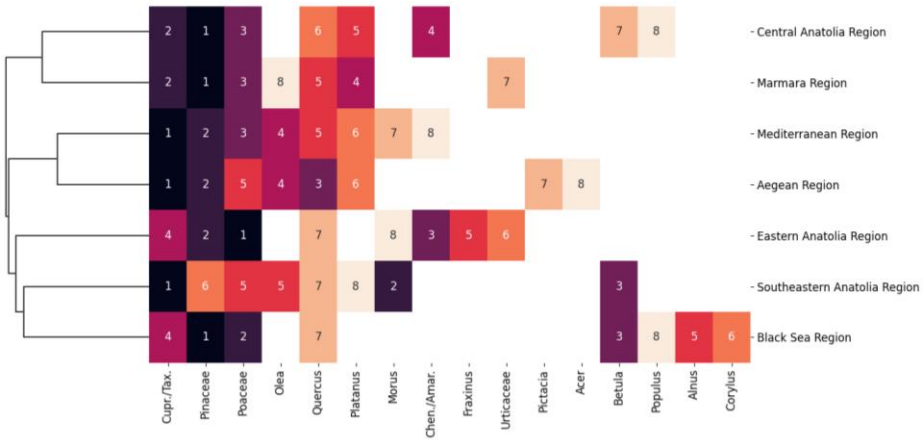

Figure 8. A heatmap depicting the distribution of the most common 8 pollen types across Türkiye's 7 regions, along with a dendrogram resulting from clustering analysis based on regional pollen profiles.

CLIMATE CHANGE AND AIR POLLUTANTS IMPACT ON DISTRIBUTION OF AEROALLERGENS

The number of outdoor aeroallergens including pollen grains and spores and their emission periods and patterns in the air are affected by many environmental factors, such as the increase of greenhouse gases, global climate change, urbanization pressure and air pollution. Studies have shown that air pollution affects the morphology and allergenicity of pollen and spores by changing the protein profile (Cecchi et al., 2018; Haddrell and Thomas, 2017; Majd et al., 2004; Sousa et al., 2008). In addition, anthropogenic effects also lead to the formation of

new allergens and changes to existing allergens (Ziska and Beggs, 2012). Clinical findings indicate that the increased incidence and prevalence of diseases such as allergic rhinitis and asthma are due to the significant concentration increase in allergic pollen rather than just air pollution and changes in living conditions (Beggs and Bambrick, 2005; D'Amato and Cecchi, 2008; Reid and Gamble, 2009).

The quality of ambient air has been greatly affected by climate change over the past few decades. In fact, climate change significantly affects atmospheric processes, including the dynamics of air pollution systems and the dynamics of biogenic emissions, including trees, grass pollen, and fungal spores. In addition, the worldwide prevalence of allergic airway disease (AAD) is increasing, leading to an increasing number of emergency room visits and hospitalizations. Clinical studies have shown that the synergy of air allergens (such as pollen and spores) and air pollutants (such as ozone) can exacerbate AAD. Many studies have produced data on the direct and indirect effects of air pollution on pollen allergens. Air pollutants can alter allergenic proteins and thus it can affect their interactions. Contaminant particles can change protein stability and extend the time to bind to antigen presenting cells. In addition, there may be effects on epitopes and adjuvants in the form of creating new ones or changing existing ones(Sedghy et al., 2018).

CONCLUSION

Spatiotemporal variations of atmospheric pollen in Türkiye exhibit a complex structure. The similarity in tree, herbaceous, and grass pollen percentages between the Aegean and Mediterranean Regions can be attributed to the similarity in vegetation. However, although it is generally stated that tree pollen is the most dominant type in the atmosphere across regions, the clustering of regions with dissimilar climates and vegetation may have been influenced by global climate change, plant selection in landscape design for parks, gardens, and roadsides, and the long-distance transport of pollen. Therefore, this

complex structure should be taken into account, when designing effective allergen management strategies.

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CHAPTER 2

SENSORY BIOLOGY OF OPILIONES

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INTRODUCTION

Opiliones are arachnids that are known for their long walking legs. They are evolutionarily classified between ticks and spiders. Some species are also called "harvestmen" because they experience a seasonal population explosion during farm harvests every autumn. Today, approx. 6,550 Opiliones species have been identified worldwide. Opiliones consist of four suborders that are named Cyphophthalmi, Laniatores, Dyspnoi, and Eupnoi. Most species are tropical, but there are also species that are distributed in colder, semi-arctic, and alpine regions. Opiliones are widely found in moist forest grounds that are less woody. Short-legged species, however, live in loose leaf debris (Edgar, 1990). Some are cavernicolous and have lost their eyes and pigmentation (Goodnight and Goodnight 1960).

Short Notes on the Biology of Opiliones

In these organisms, the body is in one piece and the prosoma and opisthosoma are fused. Six pairs of extremities emerge from the prosoma. The first pair consists of chelicerae, the second pair is pedipalps, and the remaining four pairs form the legs. Opiliones have small bodies but their walking legs are usually long, which allows them to adapt better to open areas. Species living in habitats dominated by dense vegetation have shorter legs in comparison to those living in open areas. Opiliones are very sensitive to water loss and, therefore, they prefer moist areas in their habitats. There are species that are active during daylight, night, and twilight. For instance, species living in hot regions like deserts are generally nocturnal.

Opiliones are not dangerous to humans because they have no venom glands (Blum and Edgar, 1971; Resh and Carde, 2009). Previous research studies showed that Opiliones primarily feed on small and soft-bodied arthropods and other invertebrates. Some species are also known to feed on fruit residues, fungi, and pollen (Edgar, 1971; Adams, 1984).

The sensory biology of an organism is associated with various factors such as mate and predator detection and the exploration and recognition of microclimate conditions. Navigation during food search seems exceptional among arachnids and it might be related to the consumption of immobile prey. Therefore, the dietary data, food-search behavior, and sensory structures seem to be functionally related.

These factors might be useful in explaining why Opiliones have become omnivorous despite belonging to a plesiomorphic predatory group (Riechert and Luczak, 1982; Willemart and Chelini, 2007).

In Opiliones, the detection of prey or predators involves not only direct contact but also mechanical cues such as vibrations and air currents provided by specialized structures on the body surface (Weygoldt, 2000; Brownell, 2001; Barth, 2002a). It was reported that some Opilione species wander during the search for food, which probably helps with their interaction with prey. Unlike scorpions and spiders, they are not typical sit-and-wait predators (Comstock, 1940; Edgar, 1971; Acosta and Machado, 2007; Macias-Ordonez, 2000; Elpino-Campos et al., 2001; Willemart and Gnaspini, 2004; Punzo, 1998).

Sensory structures

The sensory structures in Opiliones comprise eyes, tubercles with spines, slit sensillae, glandular organs, ozopores, and specialized structures on the body surface. The prosomal spines in opilionids significantly vary from the aspect of the structure. These spines, which fulfill different functions, are specific to each species.

Since most Opiliones are nocturnal, it is thought that these spines play an active role in tasks including orientation, finding food, and escaping predators. Furthermore, certain species have glandular organs (other than prosomal spines) that secrete body fluids and fulfill different functions in each species (Figure 1A-B).

For example, species belonging to the Dicranolasmatidae family live in the soil. Members of this group secrete a sticky substance from their bodies to better adapt to the soil. Thus, soil particles adhere to their bodies, enabling camouflage and maintaining the moisture of their bodies (Figure 1C).

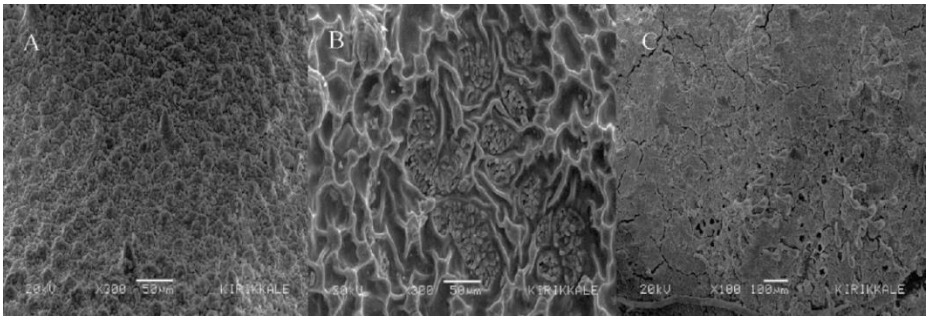

Figure 1. Dorsal integument A. *Egenaus* sp. (Avşar, 2016) B. *Odiellus* sp. (Avşar, 2016), C. *Dicranolasma* sp. (Photo taken by Nazife Yiğit Kayhan)

Eyes

Opiliones have a pair of eyes and they are located on the lateral edges of a protrusion that is called the "ocular area" located in the anterior middle part of the prosoma (Figure 2A).

However, in the Trogulidae family, the eyes are positioned anteriorly to the prosoma and extend forward on a pair of protrusions known as "cucullus." The spines and teeth found on the ocular protrusion serve as distinguishing characteristics in taxonomy (Figure 2B). The eyes have disappeared during the evolutionary process in certain species inhabiting caves.

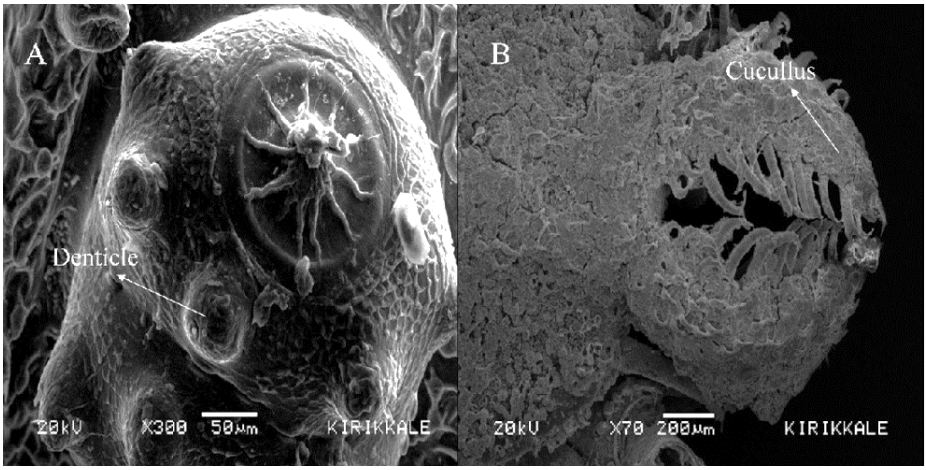

Figure 2. A. Ocular area in Phalangiidae, B. Ocular area in Trugilidae (Photo taken by Nazife Yiğit Kayhan)

Pedipalp

Pedipalps in Opiliones consist of six segments: coxa, trochanter, femur, patella, tibia, and tarsus. Their main function is sensory. All segments of the pedipalps are covered with sensory hairs and they fulfill tasks such as touching, tasting, smelling, exploration, and defense. The segments of the pedipalps are covered with spines, tubercles, small teeth, and stiff hairs (Spicer, 1987).

The pedipalps of Opiliones are generally cylindrical, but there might be differences in size and shape. In some species, the pedipalps can be sexually dimorphic (Wijnhoven, 2013; Buzatto and Machado, 2014). The pedipalps of Opiliones are generally involved in tasks such as hunting, finding food and water, and capturing prey. They can also be used in male-male fights and for sexual interactions to grasp females (Willemart et al., 2006; Requena and Machado, 2014). Recently, it was reported that there are ontogenetic differences in the morphology of the pedipalps (Townsend and Enzmann, 2018; Pagoti et al., 2019).

In addition to the morphological differences, the presence and distribution of sensory hairs, variations in the number of tubercles, and

differences in pores were reported (Figure 3). These differences actually indicate the active role of the pedipalps in sensory functions.

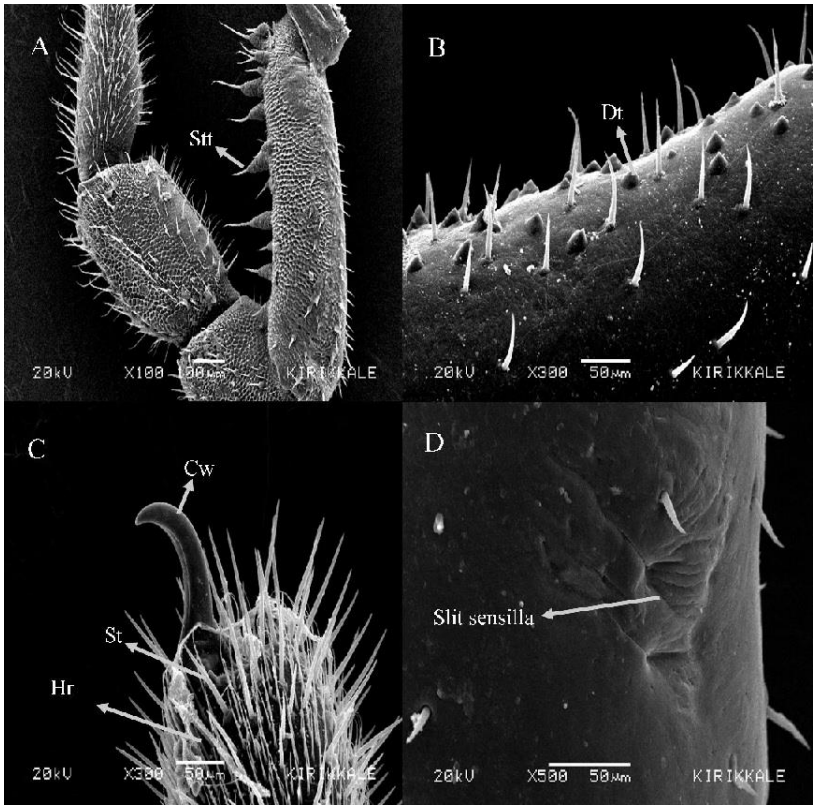

Figure 3. A. spine-tipped tubercule (Stt), B. Denticle (Dt), C. Claw (Cw) Setae (St), Hairs (Hr) D. Slit sensilla (Photo taken by Nazife Yiğit Kayhan)

Ovipositor

Opiliones have a genital opening that is named operculum on the ventral side. Below this operculum, females have an ovipositor, while males have a penis. The ovipositor has a nested, ring-like structure. These rings can shorten by overlapping or extend by adding segments.

As a result, the ovipositor becomes a mobile egg-laying tube. This structure is not present in other arachnid orders, except for mites. The ovipositor has stiff hair groups that serve as sensory organs. These

hair structures are thought to be used in assessing soil quality before depositing eggs (Resh and Carde 2009).

Autotomy

The Opiliones use their first, third, and fourth pairs of legs for walking. The second and longest legs are called "sensory legs" because they constantly wave them in a remarkable way. The second pair of legs carries gripping and sensory hairs and performs a detection function.

When choosing their habitat, organisms use the second pair of legs. For example, they find water using the probing of these legs. Opiliones use their second pair of legs as sensory organs to detect vibrations from their prey and predators. The second pair of legs is the longest leg in all opilionid species. Many opilionids defend themselves by spreading their legs against attacks. If a predator catches an opilionid's leg, the opilionid can detach it from the joint between the femur and trochanter. However, they cannot regenerate.

In a study carried out on two species in Louisiana, it was observed that seven-legged opilionids were able to survive and continue their normal lives as well as intact ones do. The fallen leg continued to shake and twitch significantly, allowing the opilionids to escape from its predator. Some researchers reported that Opiliones are significantly affected when they lose one of their second legs (Resh and Carde, 2009; Cloudsley and Thompson, 1958; Sankey and Savory 1974). In addition, there are opening called "pores" on the legs that assist in respiratory function (Figure 4A-D).

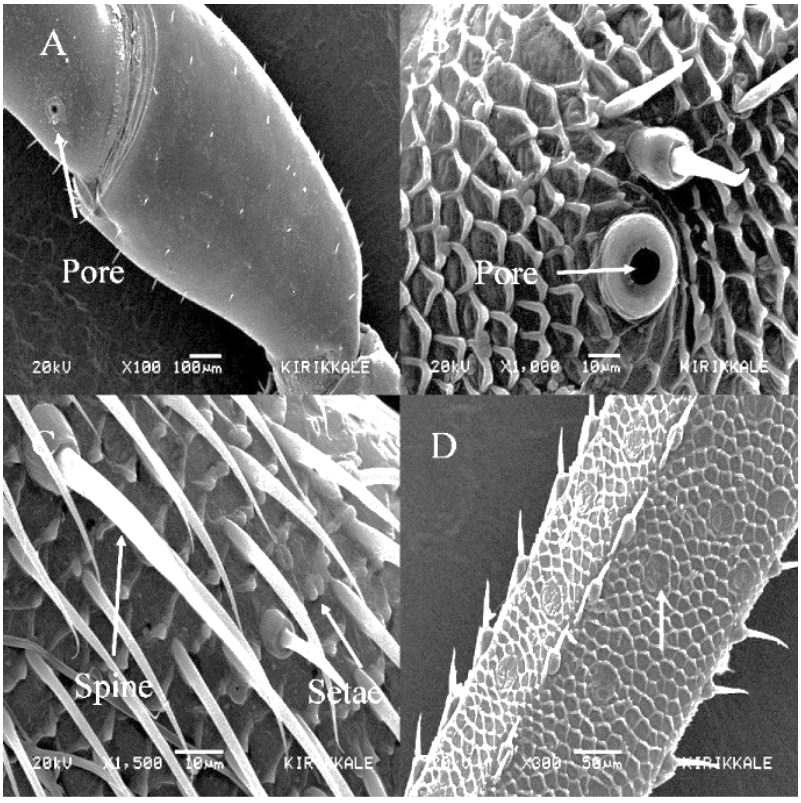

Figure 4. A-B. In a part of the tibia nearby the patella, there is an opening called pore and helping with respiratory function. B. Pore C. D. Structures fulfilling excretory or sensory functions (Photo taken by Nazife Yiğit Kayhan)

Ozopores

In Opiliones, there are a pair of ozopore located on the anterior and lateral sides of the prosoma, which function in connection with the excretory system. The ozopore secrete both foul and pleasant odors (Figure 5A-B). When confronted with a predator, the harvestman releases a malodorous substance from the ozopore, either deterring the predator or buying time to escape.

This is the most effective defense mechanism used by Opiliones and was observed to be successful, especially in active predators such as wolf spiders (Lycosidae). It was also found that these glands release pleasant odors to attract mates during copulation. Moreover, this

secretion may help in the clustering of species to find one another. It exhibits beneficial antibiotic and antifungal activity for species living in the soil (Hillyard and Sankey, 1989).

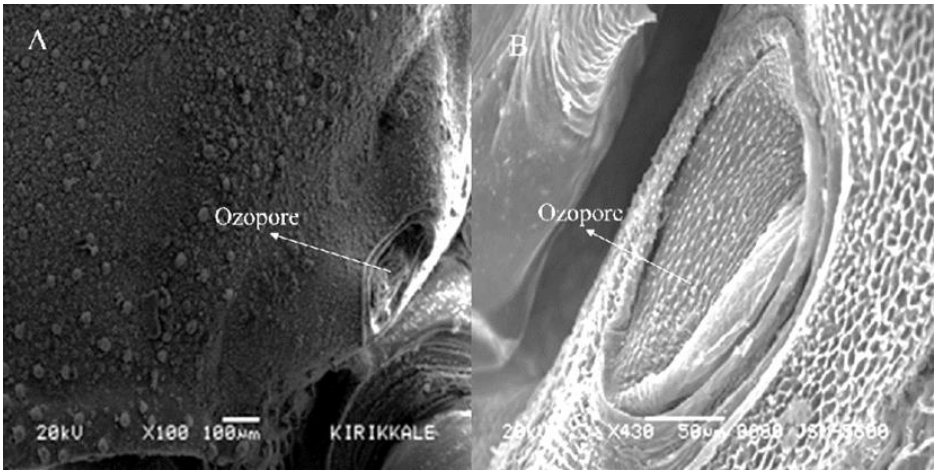

Figure 5. Ozopore in *Egaenus convexus* (Avşar, 2016) and *Metaplatybunus hypanicus* (Corak 2021)

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CHAPTER 3

NON-CODING RNAs

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INTRODUCTION

Since the introduction of the detailed structure of DNA by James Watson and Francis Crick in 1953, rapid developments have been made in the field of molecular biology over the past period. With the development of molecular biology techniques, detailed information about both coding and non-coding sequences of the genome has been obtained in whole genome sequencing. Although it was initially thought that the non-coding sequences of the genetic information had no function, it has been understood that these regions actually have much more important functions than previously thought. According to these studies, contrary to the idea that there may be more genes in higher-structured creatures, it has been found that humans have as many protein-coding genes as *Caenorhabditis elegans*, a nematode. Scientists initially thought that as living things became more complex; their genomes would also become larger and more complex. However, as the full genome maps of many living things were obtained through genome projects, a rather surprising fact emerged; while the genome size of a puffer fish whose genes have been sequenced is about 0.5 Gb, it has been noticed that the genome of a human is about 3 Gb and the genome of a salamander is 50 Gb. In plant genomes, this number have increased to 100 Gb. Researchers have defined this interesting situation as the c value paradox (Hidalgo et al., 2017).

Although a significant part of the genome is selectively copied in humans, only 1-2% of it is coded and translated into protein (Djebali et al., 2012). Non-coding RNAs (ncRNAs), which are RNA transcripts that do not code for proteins, were initially described as "non-functional parts" or "junk DNA" or "transcriptional noise" of the human genome (Uchida and Dimmeler, 2015). Non-coding RNAs are RNA molecules that have biological importance but are not translated into protein (Costa, 2007). Non-coding RNAs are examined in two groups according to their size: Non-coding RNAs, which control genetic regulation of many genes at the post-transcriptional level and are an

important class of microRNAs, are short non-coding RNAs with a length of 18-25 nucleotides. RNAs with a length of more than 200 nucleotides are called long non-coding RNA lncRNA (Majidinia et al., 2016) (Figure 1).

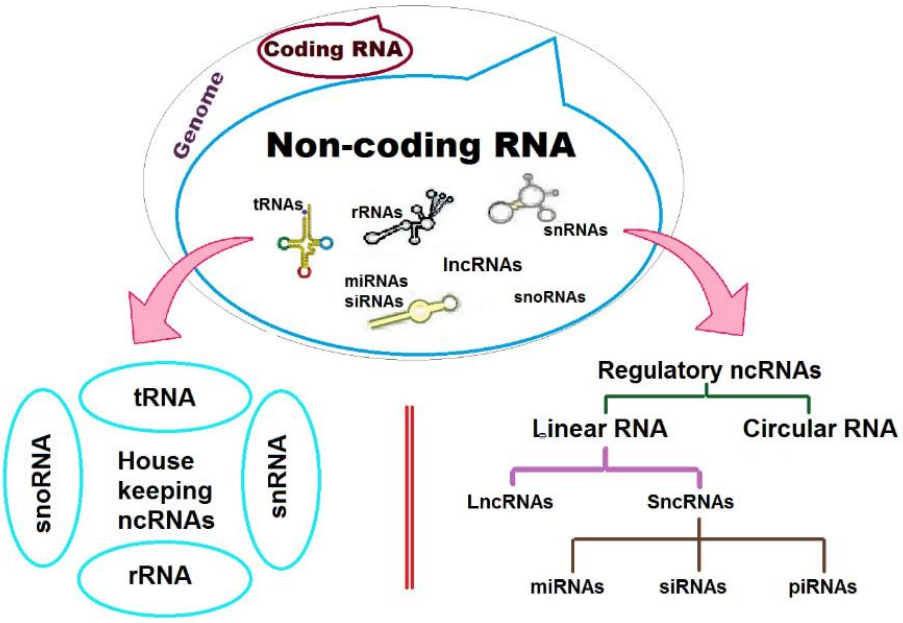

Figure 1. Classification of Noncoding RNAs

Non-coding RNAs are classified as essential for basic cellular functions and RNAs regulator molecules according to their functions. Housekeeping ncRNAs consist of carrier RNA and ribosomal RNA. On the other hand, crucial for fine-tuning gene expression and maintaining cellular processes RNAs include non-coding short RNAs (sncRNAs) and non-coding long RNAs (lncRNAs) (Wei and Zhou large, 2016).

Non-coding RNAs have critical functions in cellular processes. Therefore, abnormal regulation of non-coding RNAs is known to be associated with the pathophysiology of various diseases. Non-coding RNAs; they have been shown to take part in cell-mediated immunity,

maturational processes, specialization of cell, DNA replication, RNA synthesis and after transcriptional processes. Defects in ncRNAs cause many diseases.

Long Non-Coding RNAs

It is known that the genome is expressed as highly non-coding RNAs. The groups of RNAs in this category of non-coding RNAs that have been expressed with a nucleotide content greater than 200 nucleotides are called long non-coding RNAs. The first mammalian lncRNA, H19, was discovered in 1989, followed by the lncRNA Xist, which plays an important role in X chromosome inactivation in mammals (Brown et al., 1991). Long non-coding RNAs also have exon regions, but the number of these exon regions is quite small compared to what mRNA contains. Large non-coding RNAs are transcribed by RNA polymerase II like other mRNAs. Large non-coding RNAs can undergo various post-transcriptional modifications. These modifications include the splicing process, 3' polyadenylation and the addition of 5' cap (Derrien et al., 2012).

In the NONCODE database, which contains information about long non-coding RNAs, there are 173112 long non-coding RNA transcripts and 96411 long non-coding RNA genes identified in humans so far. A total of 549813 long non-coding RNA transcripts and 355074 long non-coding RNA genes have been identified in animals, and 94697 long non-coding RNA transcripts and 68808 long non-coding RNA genes have been identified in plants. Additionally, species-based data on other animals and certain plants are summarized in Table 1 and Table 2.

Table 1. Number of long non coding RNA transcripts and Number of long non coding RNA genes in animals.

Species of animal	Number of long ncRNA transcripts	Number of long ncRNA genes
Human	173,112	96,411
Mouse	131,974	87,890
Cow	23,515	22,227
Rat	24,879	22,127
Chimp	18,004	12,790
Gorilla	18,539	15,095
Orangutan	15,178	13,106
Rhesus	9,128	6,010
Opossum	27,167	17,795
Platypus	11,210	9,163
Chicken	12,850	9,527
Zebrafish	4,852	3,503
Fruitfly	42,848	15,543
Caenorhabditis elegans	3,154	2,552
Yeast	55	52
Pig	29,585	17,811
Total	**549,813**	**355,074**

The long non-coding RNA class is very diverse and its classification is made according to different properties. Depending on the genomic location, there may be intergenic, intronic, opposite-directional antisense, same-directional sense, bidirectional, overlapping, upstream enhancer lnc RNAs. Intergenic lnc RNAs are located in the genomic region between two interveninng sequences and undergo Description in the concurrent direction as these genes. Intronic lncRNAs are located within the intronic segments of a protein-coding gene. Antisense lncRNAs are recorded from the opposite chain of the chain around which protein-coding genes are located. Sense lncRNAs, on the other hand, transcribe from the same chain and in the same direction as the protein-coding genes around them. Bidirectional lnc RNAs are located approximately 1 kilobase within the transcription

start site region of translatable gene, but are transcribed from the antisense strand (Hermans-Beijnsberger et al., 2018; Bhan and Mandal 2014).

Table 2. Number of long non coding RNA transcripts and Number of long non coding RNA genes in plants

Species of plant	Number of long ncRNA transcripts	Number of long ncRNA genes
Arabidopsis thaliana	4,046	3,796
Brassica napus	8,212	6,283
Brassica rapa	6,457	5,657
Quinoa	9,928	7,678
Chlamydomonas reinhardtii	783	760
Cucumber	2,550	2,244
Soybean	2,242	1,937
Gossypium raimondii	1,247	1,164
Banana	1,809	1,546
Apple	1,843	1,545
Cassava	5,601	4,239
Medicago truncatula	2,258	1,856
Oryza rufipogon	7,616	6,352
Oryza sativa	1,190	1,123
Populus trichocarpa	2,248	1,971
Physcomitrella patens	471	435
Tomato	3,822	2,910
Potato	3,069	2,663
Wheat	12,427	427
Cacao	3,532	2,667
Trefoil	5,278	4,588
Grape	3,351	2,688
Maize	4,717	4,279
Total	**94,697**	**68,808**

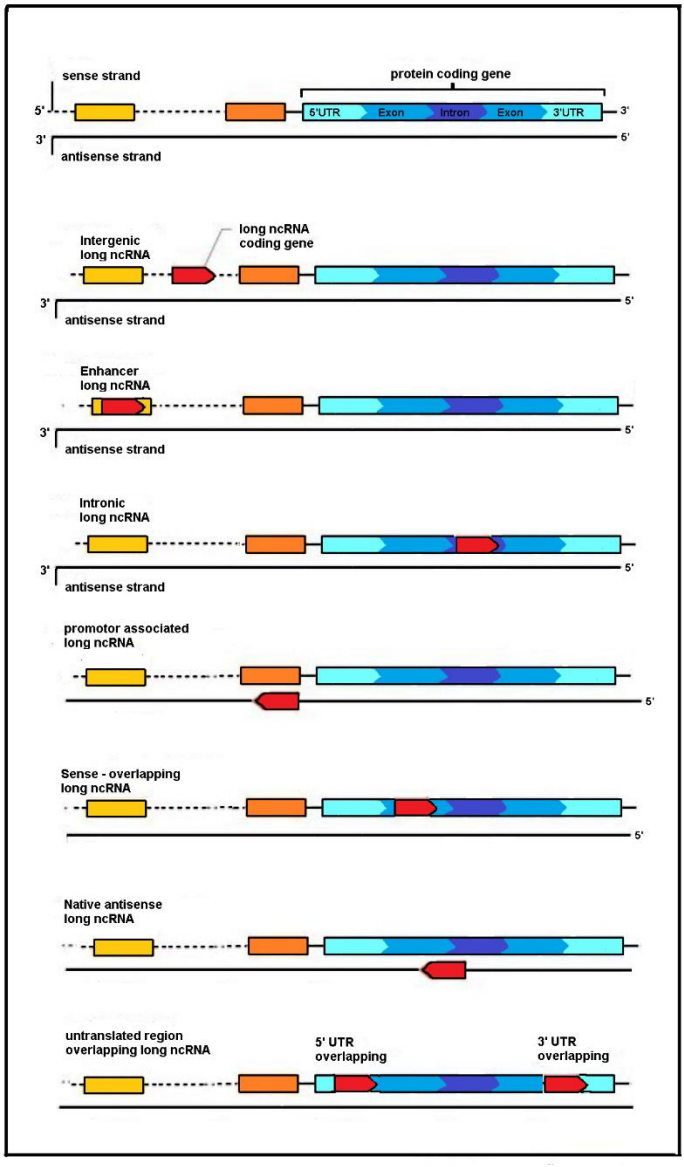

Figure 2. Classification of lncRNA according to genomic location

LncRNAs are classified as linear and circular according to their RNA structure. Circular RNA (circRNA) is formed by reverse splicing in more than 10% of genes and has a major role as a regulator of miRNA activity. Circular RNAs (circRNA) are synthesized from both

coding genes and non-coding regions. circRNAs are formed by a covalent bond between the downstream 3' region and the upstream 5' region through the reverse splicing process. Additionally, circRNAs do not contain poly-A tails and may contain one or more exons along with introns (Szabo and Salzman, 2016). Circular RNAs can be found intronic (ciRNA contains a single intron), exonic (circRNA contains 1-3 exons) or in the neighborhood of an exon intron (ccRNA contains an intron and an exon) (Amin et al., 2019).

It has been reported that between a overall of 5.8% and 23% of human genes undergoing transcription produce circRNAs (Barrett and Salzman, 2016). These circRNAs are dynamically Decedent between tissues and cell types (Du et al., 2017).

circRNA expression is conserved among mammals, some even in the evolutionarily distant Drosophila (Barrett and Salzman, 2016; Rybak-Wolf et al., 2015). In fairly near related species such as humans and mice, 4% of homologous sequences can produce circRNAs, and roughly 5–30% of these circRNAs are absolutely conserved (Memczak et al., 2013). Furthermore, approximately 5-10% of circRNAs in the human brain are expressed in the pig brain, and 23% of circRNAs are sustained between mouse and rat (Venø et al., 2015; You et al., 2015). Presently, more than one million circRNAs have been ascertained in humans and hundreds of maintained circRNAs in different species, indicating that the expression of circRNAs is more than a product of splicing (Vromman et al., 2021). In the formation of circ RNAs, firstly, a closed circular RNA structure is formed by covalently combining the 5'-3' splice regions, and exonic circ RNAs are formed from pre-mRNA through a process called "back-splicing". Another class of circ RNAs containing both intronic and exonic sequences by this mechanism is defined as Eicirc RNA (Eger et al., 2018). Circular RNAs can be observed in different localizations in the cell. These locations can be arranged with exons that form a circular structure or with nuclear localization sequences or nuclear export sequences in introns that are

not removed during splicing. It is determined that ciRNA and EIciRNAs are mostly located in the nucleus. The majority of ecircRNAs are concentrated in the cytoplasmic part (Li et al., 2015). Circular RNAs located in the nucleus regulate gene expression by modulating transcription or alternative splicing (Conn et al., 2017).

Long non-coding RNAs are categorized according to their functions as trap, signal, guide, enhancer and scaffold long non-coding RNAs. Decoy lncRNAs bind to various regulatory elements such as transcription factors, RNA-binding proteins, chromatin modifiers and micro RNAs (miRNA), inhibiting them or changing their biological activities. LncRNAs act as decoys for miRNAs. They can act as sponges for miRNAs and proteins, preventing them from binding to their targets (Wang et al., 2014; Spitale et al., 2011; Devaux et al., 2015). P21-associated non-coding RNA (PANDA) activated by DNA damage prevents p-53-mediated apoptosis by interacting with nuclear transcription factor subunit alpha (NF-YA), which activates various genes associated with apoptosis and cell aging in the event of DNA damage. PANDA RNA binds to NF-YA, removing it from the target chromatin region, and as a result, the expression of genes associated with apoptotic and cellular aging decreases (Hermans-Beijnsberger et al., 2018; Spitale et al., 2011; Statello et al., 2021). lncRNAs with signaling function determine the time and location of gene editing and provide gene-specific expression. These lncRNAs are participated in the gene control and allele-specific expression, and also provide transcriptional silencing of genes by chromatin-modifying enzymes. This gene silencing can occur in cis for nearby genes or in trans for distant genes. Cis regulation relates to lncRNAs located near their target genes, while trans regulation pertains to lncRNAs spotted far from their ambition genes (Devaux et al., 2015). Guide lnc RNAs play supporting roles in helping certain proteins reach their target regions and fulfill their biological functions, and in this way they can act as guide molecules. They mediate the suppression or activation of gene

expression (Gao et al., 2020). Scaffold LncRNAs not only aid in the gathering of multiribonucleoprotein complexes but may themselves serve as efficient units of these formations. These act as molecular scaffolds. Telomerase RNA component (TERC), one of the classic examples of the scaffold lncRNA class, allows the formation of heterochromatin in telomeres by enabling the association of telomere-targeting proteins with reverse transcriptase activity (Balas and Johnson, 2018; Statello et al., 2021). Enhancer lncRNAs are synthesized from the enhancer region, while promoter-related lncRNAs are synthesized in the region close to the promoter and in the counter direction to the protein-coding transcript. They affect the activation of target genes and, together with chromatin regulatory proteins, can modify chromatin structure and topology (Hermans-Beijnsberger et al., 2018). Long non-coding RNAs do not have any biochemical disparities from mRNAs, other than that they do not comprise an Open Reading Frame = ORF) and are shorter than mRNAs. It is able to demonstrate the mechanism of action by cis effective regulation. The number of exons is less, but much longer. They are also expressed at relatively minor levels compared to mrna and have a less robust profile in the preservation of principal sequences (Quinn and Chang, 2016).

More than 11,000 lncRNAs were evaluated with the lncSLdb database and the locations of lncRNAs in the cell are divided into three basic locations as nucleus, cytoplasm, nucleus/cytoplasm and three subdivisons as ribosome, chromosome and nucleoplasm, according to their accumulation (Wen et al., 2018). LncRNAs are especially concentrated in the chromatin regions of the nucleus (Derrien et al., 2012). If the nuclear expression level is more than 2 times the cytoplasm expression, it is considered to be nuclear-accumulated, otherwise, it is considered to be cytoplasm-accumulated, and in other cases, it is considered to be accumulated in both compartments (Wen et al., 2018). More than 80% of lncRNAs are localized in the nucleus (Kapranov et al., 2007). For lncRNAs, their most well-defined function

in the nucleus is their role in regulating gene and genetic behavior at different levels (Schmitz et al., 2016). They participate in many processes, including chromatin remodeling, histone modifications, modification of genes by alternative splicing, and regulation of gene expression (Zhang et al., 2017). Together with chromatin modifying compounds and various transcription regulators, they regulate the expression of genes in the nucleus by interacting directly with DNA. Cytoplasmic non-coding lncRNAs can act as sponges for other transcripts or proteins such as miRNAs, serve as patterns for the synthesis of small peptides, promote mRNA deterioration or regulate translation (Beermann et al., 2016). It also affects mRNA expression by interacting with miRNA. Linc-MD1 has been reported to regulate myoblast differentiation as competitive endogenous RNA targeting miR-133 and miR-135 and is reduced in Duchenne Muscular Dystrophy (DMD) (Cesana et al, 2011). lncRNAs cause gene activation or repression through DNA, RNA and proteins. It can also function as a molecular scaffold to influence gene expression by interacting with proteins such as TFs and chromatin-modifying assemblies. As a result of complementary interactions with DNA, it can direct proteins to specific regions such as promoters or prevent specific proteins from binding to domain of DNA. It is suggested that lncRNA functions are associated with the secondary structure, which is more protected and functional than the primary sequence (Archer et al., 2015).

lncRNAs can regulate gene expression and chromatin structure in 3 different ways:

Trap effect: They can bind to RNA and proteins and change their functions

Skeletal effect: They can form signal connections by binding to chromatin-modifying proteins and DNA regions,

Post-transcriptional effect: They can form RNA dimers with mRNA sequences to block transcription-associated regions, then

regulate the stability, division and translation of protein-coding genes (Zhang et al., 2016).

Although most ncRNAs have an important role in biological processes such as cellular differentiation, dosage compensation, genomic imprinting, they are strongly associated with a large number of complex diseases such as cancer, Alzheimer's disease and cardiovascular disease. It has been reported that more than 90% of disease-associated single nucleotide polymorphisms (SNPs) are found in non-coding regions of the genome or in non-coding RNA genes. Therefore, it is necessary to distinguish the types of ncRNA in order to understand the underlying causes of diseases, develop better health outcomes and more effective treatments (Amin et al., 2019; Hrdlickova et al., 2014).

In addition to all this information, ncRNAs in the genome are mainly classified as housekeeping and regulatory ncRNAs. Housekeeping ncRNAs are constantly expressed and function as key regulators of many cellular processes. This group includes ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA) (Losko et al., 2016). Regulatory ncRNAs are divided into three classes: small, medium and long ncRNA. Short ncRNAs include miRNA, siRNA and piRNA with a length of 20-50 nt. Medium ncRNAs are 50-200 nt in length and long ncRNAs are larger than 200 nucleotides and are a large class of RNAs with maximum regulatory potential (Dahariya et al., 2019).

lncRNAs control genetic activity through transcriptional, post-transcriptional and epigenetic modifications in different biological processes. lncRNAs have many different mechanisms that regulate gene expression through cis, trans and antisense interactions.

lncRNAs manage genomic activity at the transcriptional level by activating or inhibiting it. In order to inhibit transcription, DNA-RNA binds to regulatory sequences to form stable ternary complexes. When lncRNAs are close to the upstream region of the coding gene, they can

positively or negatively regulate the expression of neighboring genes by affecting the binding of their TFS to the promoter region. It also works as a coactivator regulating the activity of TFS and can directly bind TF, promoter and polymerase II enzyme (Li et al., 2019).

As a result of the interaction of lncRNAs with DNA, RNA and other transcriptional molecules, they have a versatile function in different biological processes and diseases. They have been shown to have important roles in numerous biological events such as regulation of gene expression, gene silencing, heat shock response, imprinting, and embryogenesis. In addition, lncRNA mutations are associated with a large number of diseases, including viral infections, cancer, neurodegenerative diseases. Cell proliferation, apoptosis resistance of any irregularities in lncRNAs, it affects normal cellular processes such as escaping tumor suppressors and triggering angiogenesis (Dhanoa et al., 2018) (Figure 3).

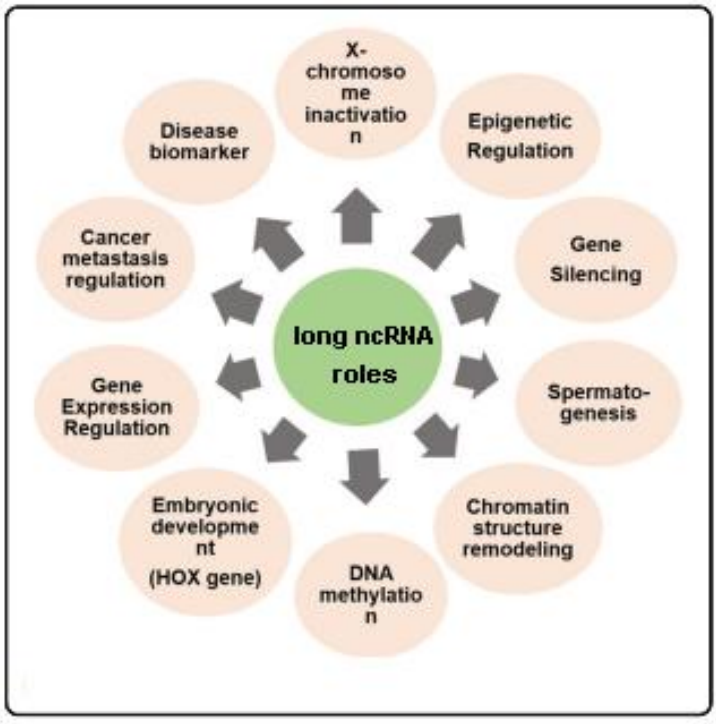

Figure 3. Several functions of long ncRNA

The roles of non-coding long RNAs in diseases

lncRNAs regulate gene regulation at various levels, and disruption of this regulatory role contributes to the development and progression of many complex diseases. ncRNAs are involved in many physiological and pathological processes. It has been determined that abnormalities, especially in the sequence and three-dimensional structures, the interaction between DNA and protein, and abnormalities in expression levels are closely related to the formation of many diseases. Therefore, lncRNAs are recommended as therapeutic targets and biomarkers for many diseases (Zhang et al., 2019).

Tumor formation develops as a result of many biological processes such as genomic mutation, DNA damage, and immune escape. lncRNAs serve as translational regulators in these processes. It has also been reported that lncRNAs regulate proliferation, apoptosis, angiogenesis and metastasis processes at transcriptional and post-transcriptional levels (Jiang et al., 2019).

lncRNAs take on an significant role in cancer development and can display tumor suppressor or oncogenic functions. MEG3 is an anti-tumor factor lncRNA that is abundandly expressed in normal brain tissue and downregulated in gliomas. PCAT-1 (prostate cancer-associated ncRNA transcript 1) is an oncogenic lncRNA that is excessively expressed in metastatic prostate cancer and induces cell proliferation by downregulating BRCA2 gene expression. Genome-wide association studies have identified a numerous lncRNAs linked to different types of cancer. It has been determined that abnormal expression of lncRNAs is closely related to tumor formation, metastasis and cancer stage (Bhan et al., 2017).

Small Non-Coding RNAs

Short ncRNAs include miRNA, siRNA, piRNA with a length of 20-50 nucleotides and can manage the expression of many genes at the post transcriptional uniform (Dahariya et al., 2019). miRNAs

(microRNAs), approximately 21-23 nucleotides long, are the most studied type of short non-coding RNA (Lee et al., 1993). Micro-RNAs perform the expression of protein-coding genes by suppressing translation. Since the discovery of the first miRNA (lin-4) in *Caenorhabditis* elegans, hundreds of miRNAs have been determined in plants, animals and viruses. Currently, there are a total of 4719 human miRNA sequences in the database, 1917 of which are precursor miRNAs and 2693 of which are mature miRNAs (Kozomara et al., 2019) MicroRNA Database Website. Available at: http://www.mirbase.org/ (Accessed on: February, 20. Due to the broad regulatory functions of miRNAs, they can serve as both oncogenes and tumor suppressors (Carninci et al., 2005). Explicit examples of miRNAs acting as oncogenes are miR-17-92 cluster, miR-155 and miR-21, and examples of tumor suppressor miRNAs are miR-15a/16-1, let-7 family and miR-34 family miRNAs (Lujambio and Lowe, 2012).

Although there are differences in their mechanisms of action, siRNA and miRNA have many common features. Both are small RNA molecules that have gene suppression effects at the post-transcriptional level by targeting messenger RNA (mRNA). The significant distinction between siRNAs and miRNAs is that siRNAs are extremely specific to only one mRNA objective, whereas miRNA has numerous targets (Lam et al., 2015).

PIWI interacting RNAs (piRNAs) are short non-coding RNAs, approximately 24-32 nucleotides long, expressed primarily in germline cells (Aravin et al., 2006). piRNAs have two main features that define their class. These are, first, specific interactions of argonate proteins with the PIWI subfamily, and second, 'dicer' (endoribonuclease) independence during their biogenesis. piRNAs are produced from genome regions containing repetitive elements and transposing elements. The primary purpose of piRNA is to prevent retrotransposons and other genetic elements from acting in germline cells, especially in spermatogenesis, by both epigenetic mechanisms (DNA methylation)

and post-transcriptional gene silencing (Lu et el., 2010; Cichocki et al., 2010; Yan et al., 2011). The specific roles of these short non-coding RNAs in carcinogenesis are not yet known. As examples of piRNAs, PIWIL1 and PIWIL2 expression levels have been increased in various types of cancer, and their increased expression has been found to be associated with cellular life cycle detain, anti-apoptotic signaling, and cell proliferation (Liu et al., 2006; Lee et al., 2006).

tRNA-derived stress-induced RNAs (tiRNAs), 30-40 nt long, were first found in human fetal hepatic tissue and human osteosarcoma cells (U2OS) under physiological stress conditions (Fu et al., 2009, Yamasaki et al., 2009). Biogenesis of tiRNAs begins with a truncation of mature tRNAs near the anticodon position, producing 5'-tiRNAs and 3'-tiRNAs. This cleavage is accomplished by angiogenin (ANG), a pancreatic RNase enzyme that has previously been associated with cancer due to its potent blood vessel formation activity (Etoh et al., 2000). Phosphorylation of the eukaryotic transcription initiation factor 2 (eIF2) α subunit under various stress conditions is known to suppress protein synthesis (Clemens, 2001). The primary function of tiRNAs is to suppress protein synthesis in answer to cellular tension in a phospho-eIF2α-independent manner (Emara et al., 2010). Although the mechanism of cancer growth and progression of tiRNAs still remains unclear, a recent study found that the level of 5'-tiRNA is quite high in colorectal cancer patients (Li et al., 2019). siRNAs are short double-stranded RNAs. After double-chain siRNAs are cut by Dicer, an RNase III enzyme, and become single-chain, they are included in the RNA-induced silencing complex (RISC) and pair with the target lncRNA with their complement bases, which causes the destruction of the target lncRNA by the argonate complex (Zhang et al., 2020; Fathi, 2020). Double-stranded RNAs to be used therapeutically must be subjected to some chemical modifications to protect them from nuclease degradation. These include modification of the sugar at the 2' position (2'-O methyl modification) and phosphorothioate bond modifications at

the 3' end. These modifications have improved the pharmacological properties of siRNA-based therapeutics (Parasramka et al., 2016).

Results

It is known that DNA is expressed as non-coding RNAs at a high rate. The RNAs in this class of non-coding RNAs that have been expressed with a nucleotide content greater than 200 nucleotides are long non-coding RNAs (lncRNAs) (Derrien et al., 2012). lncRNAs constitute an important class of ncRNAs; They are transcripts lacking ORFs, ranging in length from 200 nt to 100 kb, with lower expression than protein-coding genes, and are generally tissue specific (Bhat et al., 2016).

lncRNAs; It has a regulatory role in many mechanisms such as transcription, splicing and epigenetics; Accordingly, it is effective in important biological processes such as cell cycle, cell differentiation, development and pluripotency, as well as various pathological processes such as carcinogenesis (Huarte, 2016; Liu et al., 2020). In recent years, many lncRNAs have been identified as a new factor affecting drug resistance in cancer, and their relationship with drug resistance in various types of cancer has been revealed. It has been determined that non-coding RNAs contribute significantly to the formation of cancer by regulating the expression of many gene groups that pave the way for cancer formation and negatively affect the prognosis of patients diagnosed with cancer (Prensner and Arul, 2011; Huarte, 2015)

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CHAPTER 4

RECENT MOLECULAR STUDIES INVOLVED IN GUT MICROBIOTA ON FISH FARMING

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INTRODUCTION

Gut Microbiota

Microbiome refers to the term for microbial communities that live in clearly defined habitats and describes the microorganisms and their environment. The microbiota is made up of microscopic members of the bacterial, protozoal, fungal, and algal kingdoms (Berg et al., 2020). The ingredients of ingested food, which is crucial for host fitness, is facilitated in part by the fish gut microbiomes.

The intestinal epithelium in animals is home to a vast and diverse collection of microorganisms, known as the intestinal microbiota, which plays a crucial role in the absorption of nutrients. The microbiota consists of various microorganisms, including bacteria, archaea, eukaryotes, and viruses, and has a significant impact on the health and immunity of the host. These microorganisms maintain a symbiotic balance within the system (Yukgehnaish et al., 2020; Vargas-Albores et al., 2021).

The gut microbiota plays a crucial role in supporting various bodily functions, including nutrition, immunity, physiology, and pathogen defense. It also contributes to the development of the intestinal epithelium, provides essential nutrients, and stimulates the innate immune system. Similarly to land mammals, aquatic organisms rely on the gut microbiota for their proper development and survival. Therefore, it is a crucial element that cannot be overlooked when considering the well-being of fishes (Larsen et al., 2014; Xiong et al., 2019; Vargas-Albores et al., 2021).

Fish are constantly exposed to a diverse and ever-changing population of microorganisms in the water. Consequently, the microbiota in the gut of fish is highly susceptible to the influence of environmental factors. However, the strong bond between fish and gut flora can be impared by various factors, including diseases (Xiong et al., 2019).

The global aquaculture industry is expanding rapidly, with parallel growth in the fish products sector for food. However, inadequate disease control remains a significant barrier to this progress (Larsen et al., 2014; Xiong et al., 2019). The growth and intensification

of the fish farming industry have led to increased fish and feed production per unit area. However, this has caused a significant rise in organic contaminants caused by aquaculture practices. Additionally, the use of antibiotics to treat and control bacterial diseases has resulted in drug residues and the gradual spread of antibiotic resistance. As a result, it is necessary to develop new strategies that can control organic pollutants and infections for the sustainable development of the aquaculture industry (Zhou et al., 2018). Modulating the gut microbiota is a viable strategy for mitigating emerging diseases in aquaculture, given its functional importance in various aspects of host physiology (Xiong et al., 2019).

In this context, in recent years, intestinal microbiota has attracted the attention of scientists worldwide. Many scientific studies have been conducted to examine the intestinal microbiota of animals and the relationship between microbiota and health (Yukgehnaish et al., 2020). In order to comprehend how microorganisms contribute to the host's homeostasis, it is first necessary to understand the taxonomic composition of the host.

This section summarizes the microbiota composition in various fish species with an emphasis on the compilation of data published for the most often seen bacterial genera in the gut microbiota with a molecular approach.

Gut microbiota and immune system

Diet unquestionably has a significant role in influencing the gut flora. Intestinal health is regulated by the gut flora, and intestinal health impacts the host's physical processes and health. The intestines are a crucial organ for both the digestion and absorption of food as well as an immunological organ. Fish have a distinctive intestinal flora, which affects how the intestinal microecological system is structured and operated. Along with helping the body digest and absorb nutrients, the gut flora also plays a role in immunological defense (Kononova et al., 2019). An alternate technique involves adding immunostimulants to fish diets, particularly fungi and herbal substances that are currently legal for human consumption and therefore pose no risks to the

environment or public health (Vallejos-Vidal et al., 2016). Mougin at al., (2023) investigated the immunostimulatory effects of two fungi, *Trametes versicolor* and *Ganoderma lucidum*, one herbal supplement, capsaicin from Espelette pepper (*Capsicum annuum*), mixture of these fungi and herbal additives on rainbow trout (*Oncorhynchus mykiss*). Without causing an inflammatory reaction, the ingestion of fungus and herbal components affected gene expressions relevant to the immune system (Mougin et al., 2023). Type I membrane proteins called toll-like receptors (TLRs) that are involved in pathogen detection and are crucial for immune cell activation in response to pathogen invasion, are responsible for triggering the immune system (Rebl et al., 2010). Although Mougin at al., (2023) did not conduct this research as experimentally infected, it has been demonstrated that the products under study influence the defense mechanism, particularly in connection to expression of the tlr2 gene in the spleen.

Gene expression and relationships between microbiome alpha diversity are still in under research. Research results show strong correlations between the expression of host immune-related genes and microbial diversity. Siddik at al., (2021) researched on *Lates calcarifer* to invesitgate the synergistic effects of probiotic yeast, *Saccharomyces cerevisiae*, and lactic acid bacteria affecting performance of growth, biochemical reaction, gut histomorphology, expression of cytokine gene, and young barramundi's microbiological composition. According to results, along with increasing the quantity of goblet cells in the intestines and the length of the microvilli, probiotic treatment also caused up-regulation of immune response and inflammation-related cytokine genes (*TNF-α* and *IL-10*). Sequence analysis of the distal intestine revealed a probiotic diet had a significant favorable impact on alpha-beta bacteria diversity and pathogenic *Corynebacterium* and *Staphylococcus* was diminished. These findings support the notion that co-supplementing *S. cerevisiae* and *L. casei* enhanced juvenile barramundi development, immunological response, and gut health by broadening their microbiota.

For many fish species, fishmeal is typically regarded as the ideal dietary protein source. Plant proteins have traditionally been thought of

as the most obvious fish meal substitute, yet high doses of replacement can produce numerous symptoms such as enteritis, such as mucosal folds shortening, enlargement of the lamina propia and submucosa, and inflammatory cells invading the distal intestine (Egerton et al., 2020). Dawood et al., (2022) investigated for the first time how modulating β-mannanase as plant-based diet enhance *C. carpio* of intestinal microbiota and the activation of genes involved in immune system and digesting. It is found that the beneficial effects of dietary β-mannanase supplementation on *Cyprinus carpio,* increased relative expression of immune related genes such as *Def, IL1-β, NK-lys, Lys* and *SOD.*

Certain immune-response cells and elements in mucous membrane of intestine are also competent of the mucosal immune system in fish. tumor Necrosis factors (*TNFs*), interferon regulatory factors (*IRFs*) and interleukins (*IL*s) are the primary immune-relevant factors associated with inflammation in the distal gut of fish (Marjara et al., 2012). Different dietary substitutes such as soybean meal significantly altered the makeup of the gut microbiom. Miao et al., (2018) applied 0%, 25%, 50%, and 75% of the defatted fishmeal to northern snakehead to assess intestinal microbiota proliferation on the impact of food on fish intestinal health. *Firmicutes* had the lowest phylum-level abundance in the highest group fed with soybean, in contrast to *Proteobacteria, Bacteroidetes*, and *Planctomycetes*. In the distal intestine, the relative expression of *IL-1*, *IL-10,* and *IL-17F* was considerably elevated with increasing dietary soybean meal, although microvillus height, muscularis thickness and fold height all reduced.

Fuess at al., (2021) looked at associations between the diversity of microbial taxa in Stickleback and expression of the host gene. One negatively and ten positively correlated biological process gene ontology (GO) terms were considerably enriched for in the genes significantly associated with microbiome community. Positive regulation of common myeloid progenitor cell proliferation and *interleukin-12* production and are two terms connected to immune system that were favorably associated with microbiota. Interferon Regulatory Factor 8, Tumor Necrosis Factor Receptor Superfamily Member 5, Toll-like receptor 9, Peregrin and Receptor-type tyrosine-

protein kinase FLT3 were among the genes that significantly enriched for these two terms. Overall, microbial diversity had a positive correlation with the expression of genes involved in immune cell formation and *IL-12* regulation but a negative correlation with the expression of genes involved in inflammatory processes (Fuess, et al., 2021).

Gut microbiota, environment and feeding

A poor microbiome in wild fish might be improved by moving to a better environment and food supply or by altering feeding behavior. According on feeding patterns and environmental factors, researchers expect to identify a unique microbial structure (Egerton et al., 2018). Morshed et al., (2023) carried out researched on Asian sea bass (*Lates calcarifer*) juveniles to understand effects of migration from seawater (SW) to freshwater (FW) on the microbiota and cytokine gene expression. According to Schirmer et al. (2016), cytokines have a significant role in the regulation of the microbiota. It is resulted that microbiota of Asian sea bass linked with the mucosa and microbiota were both impacted by FW transfer and the expression of cytokine genes was associated either positively or negatively with particular bacterial species.

In the research on atlantic salmon how the shifting microbiota and an epidemic of tenacibaculosis affect DNA methylation in the stomach of Atlantic salmon, a possible homolog of the mammalian *ramp1* gene, LOC106590732 was analysed as hypermethylated at the very beginning of the gene (Hansen, et al., 2023). The pathogenic marine bacteria *Tenacibaculum maritimum* is responsible for tenacibaculosis and all around the world, many different marine fish species suffer from this ulcerative condition (Mabrok et al., 2023). *Ramp1* is associated with dysbiosis of the microbiota in mice and is implicated in mucus release in intestinal goblet cells that affects mucosal thickness (Yang et al., 2022). While a thinner mucus layer in ill fish and probable dysbiosis may result from hypermethylation of LOC106590732 and additionally *ramp1* function in fish is need for further research (Hansen, et al., 2023).

In order to determine nutrition's influence on fermented rice protein (FRP) on growth, digestion and absorption abilities, intestinal microbiota in juvenile hybrid groupers (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) feeding and inflammatory-related gene expression, the research was conducted (He et al., 2021). Fish fed FRP showed down-regulated immune-related gene expression levels, including *TGF-1, IL-8, IL-6, IL-2, TNF, MyD88* and *TLR22* (He et al., 2021). The effects of substituting fermented soybean meal (FSBM) protein with different concentrations (0%, 20% and 40%) for fish meal protein were also investigated on the growth performance, intestinal morphology, immunity and microbiota of the pearl gentian grouper (*Epinephelus fuscoguttatus* x *E. lanceolatus*) (Pang et al., 2023). Compared to the gene expression levels of *TGF-1, IL10, IL5* and *IL4* which were noticeably downregulated, the levels of *TNF, IL17*, *IL12* and *IL1* were noticeably elevated on fish given the 40% diet including fermented soybean. Overall, intestinal inflammation in pearl gentian grouper is brought on by high amounts of FSBM substitution for fish meal. The alteration of the gut flora is probably responsible for this. The harmful impact of dietary FSBM on gut flora has to be more fully understood (Pang et al., 2023).

Probiotics are described as live or dead microorganisms given through feed or the environment of raising that promote the wellbeing of their host(Merrifield at al., 2010). Sadeghi et al., (2023) conducted the research on Chinook salmon (*Oncorhynchus tshawytscha*) treated with control diets, probiotic and antibiotic to see effects on gut microbiome composition by examining the bacterial populations in the host intestine and rearing water using 16S rRNA gene metabarcoding. In comparison to healthy controls, administering antibiotics and probiotics on a daily basis cause substantial alteration in aquatic microbiota and the fish microbiom along with more than 100 differentially expressed genes in the treated fish. As anticipated, the probiotic group in this study had greater levels of the bacteria with possible probiotic qualities (*Lactobacillaceae, Bifidobacteriaceae,* and *Streptococcaceae*) than the other treatment groups. According to this study, antibiotic treatment caused the upregulation of genes linked to

cell death. The research revealed that certain microbial taxa had an impact on the regulation of a number of host genes. For instance, the prevalence of *Lactobacillaceae* and *Bifidobacteriaceae* was inversely correlated with the transcription of the *prmt3* and *manf* host genes. It was demonstrated that the use of antibiotics and probiotics not only altered the microbiome (composition) of Chinook salmon, but also caused significant alterations in the fish's gut tissue's gene expression.

Lactobacillus and Bacillus are utilized as probiotics in aquaculture because they enhance the function of the fish gut barrier and the fish immune system's capacity to combat pathogens. The probiotic *Bacillus amyloliquefaciens* R8, which heterologously expresses xylanase from rumen fungus, was used in the study to assess the effects of food supplementation on zebrafish (Lin et al., 2019). As a probiotic in aquaculture, *Bacillus amyloliquefaciens* has gained popularity because of its immunostimulatory action against pathogenic infection (Saputra et al., 2016). To improve nutrient absorption and speed up the growth of different animals, xylanases are often employed in animal feed. In the research, the livers of the treated fish displayed increased expression of the anti-apoptotic gene bcl-2 and decreased the expression of genes associated with oxidative stress *Hsp70, NOS2, Gpx* and *SOD.* Expression of genes associated to the innate immune system (*TLR-4, TLR-3, TLR-1, TNF-4, TLR 3, TLR-1, IL-21, IL-6* and *IL-1*) was elevated in the fish treated with probiotics. As a result, the findings demonstrated that the injection of xylanase-expressing *B. amyloliquefaciens* R8 can potentially promote disease resistance and immunity against *S. agalactiae* and *A. hydrophila* in zebrafish, as well as nutritional metabolism and hepatic stress tolerance (Lin et al., 2019).

Energy balance and well health depend on an understanding of how the gut microbiota affects energy homeostasis and lipid metabolism. To determine how the intestinal microbiota affects lipid metabolism, two zebrafish models—germ-free and antibiotic-treated—were employed (Sheng et al., 2018). Egg yolk was used to feed conventional, germ-free zebrafish larvae. According to Dandekar et al. (2016), LPS controlled the *apoa4* expression and hence encouraged the formation of high-density lipoprotein. It was confirmed that *apoa4* and intestinal bacteria

have a close link because conventional zebrafish expressed *apoa4* at a higher level than germ-free or antibiotic-treated fish. Additionally, it is discovered that conventional zebrafish had higher hormone sensitive lipase (*hsl*) expression levels than germ-free or antibiotic-treated fish (Sheng et al., 2018).

Fish that are adapted to hyperosmotic habitats consume water continually to replace water lost, which can change the physiology of the gut and affect parameters including bile salts, gastrointestinal enzymes, and acidity (Usher et al. 1990). As a result, the resident and transient intestinal microorganisms may be severely impacted by changes in internal pH and osmolality (Ray and Ring 2014). As a result, the resident and transient intestinal microorganisms may be severely impacted by changes in internal pH and osmolality (Ray and Ring 2014). Prior studies on euryhaline fish and anadromous species have demonstrated that salinity can alter the makeup of the intestinal microbial population by reducing its richness and evenness (Zhang et al. 2016; Dehler et al. 2017a). It is showed that 3 weeks after being exposed to saltwater environment, the variety of gut flora in Atlantic salmon drastically decreased (Lokesh et al. 2019). Hieu et al., (2022) resulted in the research on young striped catfish that the probiotic known as *Akkermansia* was less prevalent at the greater salinities and extended exposures, *Vibrio* and *Sulfurospirillium* were the most prevalent in high salinity treatments, demonstrating a significant relationship between environmental factors and fish intestine bacterial populations. Additionally, it is concluded that *tlr1* and *tlr2* expression significantly decreased in a highly salt environment of 20 (practical salinity unit), which was followed by decrease in the expression of inflammatory genes such as *il1*, *tnf*, *il10*, and *tgf* at 20 experimental days. Rakoof-Nahoum et al., (2016) reported that under normal conditions, Toll-like receptors (TLRs) could detect commensal microorganisms and such interactions between commensal bacterial products and microbial pattern recognition receptors were crucial in the ability to withstand epithelium damage and intestinal homeostasis.

It is yet unknown how lower and higher vertebrates- fish and mammals- conserve the effects of particular gut bacteria on bile acid

metabolism. It was added to the fish and mouse meal as *Citrobacter freundii* GC01, which was obtained from a grass carp's gut (*Ctenopharyngodon idella*) (Xiong et al., 2022). The *CYP7A1* gene was considerably enhanced in both mice and grass carp, according to analysis of hepatic transcriptome sequencing data and validation by RT-qPCR. It is concluded that *HNF4B* may be a key regulator of bile acid metabolism in fish, and some intestinal bacteria may control the production of bile salts via *CYP7A1*.

Complex carbohydrate hydrolysis can produce monosaccharide molecules that can go on to be fermented and produced into short chain fatty acids (SCFAs) and glycolysis, which are both used to provide energy. In the preservation of the gut environment and the host's general physiology, SCFAs have been demonstrated to be crucial (Tyagi et al., 2019). It is important that SCFA-producing pathways and microbial genera (*Bifidobacterium, Prevotella, Megasphaera, Streptococcus Escherichia, Clostridium* and *Enterococcus*) are present in the intestines of fish because this provides chance to control the production of SCFAs in the fish intestine in vivo for positive impacts on growth and health. Few researches have evaluated the role of the intestinal microbiota in the lipid deposition brought on by diet or genetics, which limits our understanding of how gut bacteria are involved in metabolic disorders. During the four-week feeding trial, the amount of lipids accumulated in wild-type zebrafish given either a control diet or a high-fat diet as well as two zebrafish lines with knockout genes (*pparab-/-* or *cpt1b-/-*) fed a control diet were assessed with 16S rRNA gene sequencing and 16S rRNA sequencing to examine the makeup of gut microbiota in these groups. These results indicated that the bacterial composition of the intestine that correlated with lipid accumulation, was more strongly influenced by a high-fat diet than by the deletion of *pparab* or *cpt1b* (Qiao et al., 2021).

It was widely known that bile acids were essential for the elimination of cholesterol, stimulation of biliary secretion and bile flow, reversibly inhibiting the production of BA and cholesterol and enhancement of digestive process and absorption of fat-soluble elements and dietary lipids (de Aguiar Vallim et al., 2013; Olsen et al.,

2005). To examine the bile acids' actions on intestinal barriers in *Micropterus salmoides*, four type of diets were created with BAs supplemented at 450, 300, 150 and 0 mg/kg designated as control, BA450, BA300 and BA150 (Xia et al., 2023). The findings of experiment showed that the gut microbiota transferred from the BA300 group was more likely to upregulate gut barrier-related genes than the control group was, including immunoglobulin *IL-10, IL-1, IL-6* and Z/T (*IgZ/T*). *IL-10, IL-6, occludin-2, lysozyme, IgZ/T* and *IgM* expression was increased in GF zebrafish when the BA300 meal was fed directly and resulted that BAs can enhance fish gut barriers that are mediated by the intestinal microbiota both indirectly and directly (Xia et al., 2023).

Intestinal microbial composition may be considerably impacted by antibiotic exposure and accumulation. This situation results in compromised anti-infection immune responses, compromised metabolism and generation of useful metabolites, and unoccupied niches for pathogenic bacteria to cross intestinal epithelial cells barrier (Zimmermann & Curtis, 2019; Hagan et al., 2019 and Duan et al., 2022). The effects of ciprofloxacin (CIP) misuse on intestinal homeostasis and immunological response was investigated on ayu (*Plecoglossus altivelis*) after infection of subsequent *Pseudomonas plecoglossicida*. According to immunohistochemistry research, CIP exposure dramatically reduced the amount of goblet cells in the intestine, which may compromise the *P. plecoglossicida*-infected ayu's ability to fight infection. In most immunological tissues (including head kidney, spleen and gill) (CIP group), CIP exposure significantly reduced the expression of *IL-1β*, *TNF-α*, and *IL-10* and increased the production of *TGF- β* and additionally increase *TNF-α* and decrease *TGF- β* in spleen (Wu et al., 2022). Additionally, the proportions of Actinobacteria and Verrucomicrobia did, however, rise after *P. plecoglossicida* infection. This finding is consistent with rats suffering from intestinal dysbiosis and inflammatory illness, which have been shown to have higher *Verrucomicrobia* and lower *Tenericute* counts (Lakshmanan et al., 2021). Antibiotics are used in aquaculture to combat diseases however they can have significant and lasting impacts on fish microbiomes, which reduces production. After using antibiotics,

the effects of interventions on the microbiome can be followed by observing changes in the gut microbiome.

In conclusion, fish health could be assessed applying the gut microbiome. Understanding fish health and the characteristics of gut microbiome homeostasis can help with providing guidelines for controlling the intestinal microbiota to enhance fish performance and health. Over host taxonomy and trophic level, host habitat (freshwater vs. ocean) particularly shapes the microbial communities found in the guts of wild fish. To fully investigate the potential of gut microbiota analysis as a biomarker or a general indication of fish health, more research is required.

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CHAPTER 5

MICROBIOLOGICALLY INVESTIGATION OF TRADITIONAL CHEESES AND COMMERCIAL CHEESES

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INTRODUCTION

Cheese production began with the aim of extending the shelf life of milk and increasing its usability. Later on, cheese became an important source of nutrition worldwide, with different flavors and aromas. Various groups of microorganisms play a role in cheese production and are important for its quality. Since milk provides a suitable environment for the growth of microorganisms, it is also highly susceptible to microbial spoilage (Kongo, 2013; Rakhmanova et al., 2018). Although heat treatments are applied during cheese production to eliminate microorganisms from milk, some microorganisms play an important role in the differentiation of cheese in terms of taste, aroma, and texture (Yaygın and Kılıç, 1993; Yerlikaya, 2014). For example, the famous Roquefort cheese is caused by a fungus called *Penicillium roqueforti*, which is a green-colored mold found in caves in France. The cheese is kept in these caves to allow it to be contaminated with this fungus. However, similar cheeses can be produced in different parts of the world by intentionally introducing this contamination (Oğuz and Andiç, 2019). In addition to beneficial microorganisms, as mentioned in cheese, there are also microorganisms that can cause spoilage and reduce the quality of the cheese. Some fungi that readily develop in cheese can also produce mycotoxins, which are a food safety concern.

There are numerous types of cheese produced for commercial purposes, as well as those produced in small businesses or homes for consumption at local markets or within households. Many traditional varieties of cheese that were once forgotten are still being made today. Cheese made in homes is generally made from raw milk or heated to temperatures slightly above the temperature of fermentation, then cooled and allowed to ferment. As a result, it is known that the microorganisms present in the natural flora of the milk or those that have contaminated the milk are transferred to the cheese and grow

during fermentation (Karabıyıklı and Erdoğmuş, 2019; Sert and Özdemir, 1989).

In addition to causing changes in the cheese structure, the microorganisms present in cheese can also lead to food poisoning when consumed. Numerous studies in Turkey have shown that adherence to hygiene conditions at all stages of cheese production, from manufacturing to consumption, is critical to the microbiological quality of the cheese (Kaynar, 2011).

Fungi responsible for problems in cheese production belong to several genera including *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Eurotium*, *Exophiala*, *Fusarium*, *Gliocladium*, *Lecanicillium*, *Mucor*, *Penicillium*, *Rhizopus*, and *Wallemia*. However, the genus most commonly isolated from spoiled cheese is *Penicillium*, followed by *Aspergillus*. Due to the various characteristics of different cheese types, it is not surprising that a wide range of different fungal genera can spoil cheese. Nevertheless, many studies have shown that although many different genera and species can occasionally be isolated from a particular type of cheese, only a few dominant species are regularly present. Each food product has its own associated mycobiota, so different cheese types may have a very specific mycobiota on their visible mold (Kure and Skaar, 2019). A study was conducted in cheese ripening rooms in milk farms in Korea to evaluate contamination sources at production sites in terms of fungal formation and diversity on walls, floors, cheese boards, air, and ripened cheese. The fungal contamination was found to be outside acceptable ranges in 8 out of 10 milk farms. A total of 986 fungal isolates were identified, which were classified into Ascomycota (14 genera) and Basidiomycota (3 genera). *Penicillium*, *Aspergillus*, and *Cladosporium* were the most commonly and dominantly detected. *Penicillium* was observed in cheese ripening rooms on 9 farms, while *Aspergillus* was only detected on one farm. Among the 39 highlighted members, *P. commune*, *P. oxalicum*, *P. echinulatum*, and *A. versicolor*

stood out. Most of the mold species detected on surfaces were found to be the same fungi as those present in the indoor air of the cheese ripening rooms (Kandasamy et al., 2020).

A microflora study was conducted on a type of cheese matured with surface mold in caves in southern Italy. 148 fungi were isolated from 22 different cave cheese samples collected from 13 geographical regions of Italy, mostly from the Apulian region. They detected the presence of twenty-four fungal species on the outer surface of the cave-matured cheese. *A. westerdijkiae* and *P. biforme* were the most frequently isolated species, followed by *P. roqueforti* and *P. solitum*. 86% of the cheese samples presented at least one toxigenic species, and 45% showed the presence of ochratoxigenic species, A. *westerdijkiae* and *A. steynii*. The fact that 36% of the samples' rinds contained ochratoxin A (OTA) confirms the potential mycotoxin risk during the ripening stage in the caves (Anelli et al., 2019).

In southern Spain, a study was conducted on 52 commercial cheeses made from different types of milk (cow, sheep, goat, and mixed), including 10 fresh, 17 semi-mature, and 25 mature cheeses, from which fungi were isolated in 41 (79%) of the samples. *Penicillium* was found in 63% of the samples, *Mucor* spp. in 27%, *Geotrichum candidum* in 17%, and eleven other fungal genera ranging from 2% to 4% incidence were also detected. The incidence of *Penicillium* species was higher in more mature cheeses, with 20% in fresh cheese, 71% in semi-mature cheese, and 76% in mature cheese (Barrios et al., 2007).

In a study conducted by Alzamily (2022) to detect contamination in dairy products, *P. expansum* was detected in Ravan cheese from Turkey, and it was reported that this fungus produces mycotoxins, although in low amounts.

Various types of tulum cheese samples were collected from production and sales locations as well as homes in the Erzincan province, and 14 species belonging to 5 genera of *Aspergillus*, *Fusarium*, *Geotrichum*, *Penicillium*, and *Cladosporium* fungi were

detected (Çolakoğlu and Erkol, 2018). As seen, in various studies on the microbiological quality of different cheeses in our country (Brine white cheese, semi-hard cheese, salted or unsalted cheese, Caucasian cheese, Cheddar cheese, Tulum cheese, moldy cheese), the most commonly identified fungi were *Penicillium* sp., followed by *Mucor* sp., *Aspergillus* sp., *Geotrichum* sp., *Rhizopus* sp., *Alternaria* sp., *Cladosporium* sp., *Trichoderma* sp., and *Scopulariopsis* sp. genera (Özdemir and Demirci, 2006; Kamber, 2008; Kıvanç, 1990; Özçakmak and Dervişoğlu, 2011). The studies conducted on the microbiological quality of cheeses produced and consumed in Turkey have shown that many of them are inadequate in terms of microbiological quality, and therefore pose a risk to public health. It is important to ensure that proper food safety measures are in place during the production and distribution of cheese to prevent the spread of harmful microorganisms and ensure that the cheese is safe for consumption.

This study investigated the microfungi that develop in commercially sold cheeses in markets and locally produced cheeses sold in rural markets in Çankırı province. The isolates were identified and diagnosed to determine which fungus species were present and dominant in the samples. According to the results of the study, many of the cheeses had insufficient microbiological quality and were therefore considered risky for public health.

MATERIALS AND METHODS

Fungi isolations

The thirty-four cheese samples, 14 homemade and 20 commercially sold, were collected from Çankırı province's village markets and stores in 2020-2021. There was no visible fungal growth in the collected samples. The cheese samples were stored in sterile containers. Preparation of the samples for analysis; 10 g of cheese was weighed from each sample and crushed using a sterile spatula and hand in 90 mL of sterile water until it reached a yogurt-like consistency, and

diluted in a 10^{-1} dilution. For fungal analysis, Potato Dextrose Agar (PDA; Difco) culture media were used for plating the dilutions. The plates were then incubated at 23-25°C for 24-48 hours. Additionally, a 10-fold serial dilution was performed on the 10^{-1} solution, and 100 µL of the diluted solution was plated on Plate Count Agar (PCA) culture media. The plates were then incubated at 37°C for 24-48 hours. All experiments were performed in duplicate.

Determination of the internal transcribed spacer region (ITS) of Ribosomal RNA

DNA isolation and PCR

The fungi isolated from cheese samples were incubated in PDA culture medium for 7 days, and the fungal hyphae were transferred to sterile Eppendorf tubes using a sterile scalpel for DNA isolation. The DArT DNA isolation method (http://www.diversityarrays.com) was used with "fresh buffer" for DNA isolation. The same method was used for bacterial isolations as well. The obtained DNAs were measured using a spectrophotometer. For the identification of fungi, ITS-1/ITS-4 primers (White et al., 1990) were used, while 27F/1492R (De Lillo et al., 2006) universal primers were used for the detection of bacterial species. The PCR amplifications were carried out with the selected primers in a 25 µL reaction mix containing 40 ng template DNA, 1× FirePol PCR Buffer BD [0.8 M Tris-HCl, 0.2 M (NH_4)], 10 µM of each primer, 200 µM dNTPs, and 1 unit of FIREPol Taq DNA polymerase.

The PCR conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 52°C and 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 5 min, for a total of 30 cycles. The presence of PCR products was confirmed by running the products on a 1.4% agarose gel using 1× TAE buffer and staining with ethidium bromide. Gel images were captured using a gel documentation system under UV light. Raw sequence data were processed using the MEGAX program (Kumar et

al., 2018) and the closest species were identified by performing a BLAST search against the entire National Center for Biotechnology Information (NCBI) nucleotide collection (http://blast.ncbi.nlm.nih.gov/).

RESULTS

Microorganisms isolated from cheeses

Dilution method was used to inoculate the cheese samples onto the agar medium, and growth was observed starting from the 5th day. Pure cultures obtained from the growths were first separated by genus for diagnosis. Then, grouping was made at the genus level using various diagnostic books and some websites. *P. commune* and *G. candidum* were the most commonly detected fungi in homemade cheeses (Figure 1 a, b). *P. commune* was the most frequently detected species in commercial cheeses (Supplementary data).

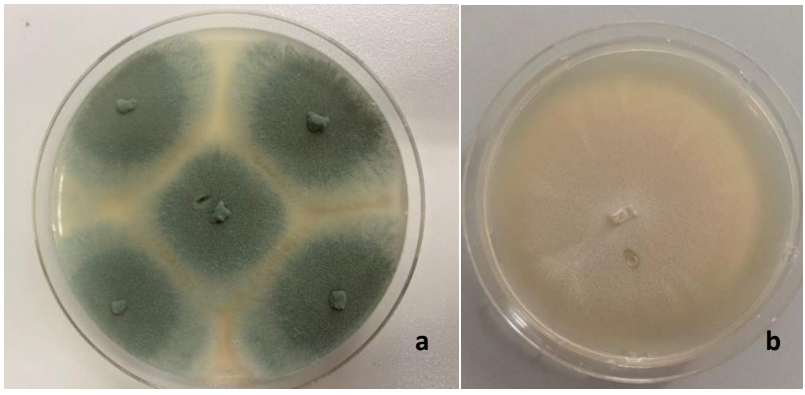

Figure 1. Isolation of *Penicillium commune* (a) and *Geotrichum candidum* (b) from cheese samples and their growth on PDA medium

Trichoderma sp., *R. stolonifer* species were also detected in homemade cheeses. In commercial cheeses, only one sample was found to contain *C.* aff. *radians*.

In another commercial cheese sample, *C. herbarum* (Figure 2 a-b) and *C. cladosporiodes* were detected in one cheese sample each.

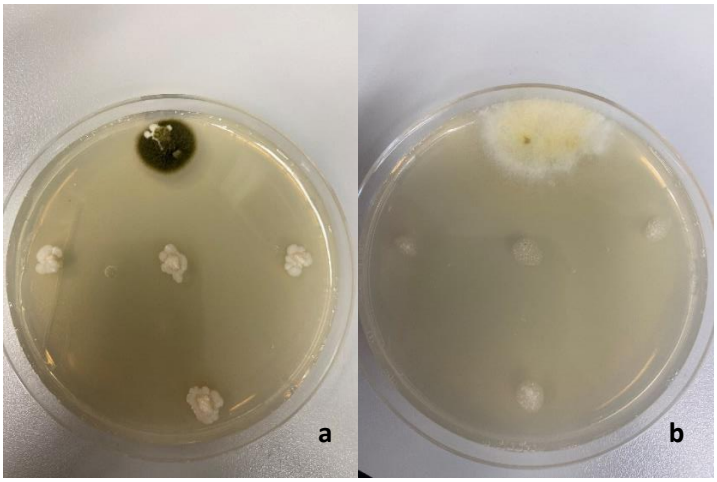

Figure 2. *Cladosporium herbarum* (a), *Coprinellus* aff. *radians* (b) isolated from some cheeses

In the study, 13 out of 20 commercial cheese samples showed no fungal growth, while 7 samples showed the presence of only one fungal species. No mixed fungal contamination was detected. Of the 14 homemade cheese samples examined, only 2 showed no fungal growth, while the other 12 samples showed contamination with various fungal agents. Two bacterial colonies were also observed in the cultures and separated based on their shape and color and stored for further analysis. Two bacterial species were frequently isolated from the cheeses (Figure 3). *Staphylococcus aureus* was commonly found in homemade cheeses (5), while in commercially sold cheeses, *S. aureus* (1) and *Serratia* sp. (1) were detected (Figure 3, Table 1). In the study, 10 of the homemade cheeses were found to be contaminated with bacteria, and some also had fungal contamination.

Figure 3. Different bacterial colonies growing on Plate Count Agar media

Table 1. Cheese samples showing bacterial growth

Isolates ID	Cheeses type
1,2,3,4,5,6,7,8,9,10	Homemade
11,12,13,14,15,16,17,21,22,23,24,25,26,27,29,30,31,32,33,34	Commercial

Molecular confirmation of the internal transcribed spacer region of ribosomal RNA and bacterial 16S rDNA region

A total of 9 fungal isolates were identified at the species level based on the ITS region, including 3 isolates of *G. candidum*, 3 isolates of *P. commune*, 1 isolate of *C. herbarum*, and 1 isolate of *C. cladosporiodes*, and 1 isolate of *C.* aff. *radians* (GenBank accession nos. OQ372945, OQ372946, OQ372947, OQ372955, OQ372953, OQ372954, OQ372948, OQ372949, OQ372950). Additionally, a total of 2 isolates were identified at the bacterial species level based on the 16S rDNA region, including 1 isolate of *S. aureus* and 1 isolate of *Serratia* sp. (GenBank accession nos. OQ372948 and OQ372944).

DISCUSSION

According to the study, 7 different fungal species and 2 different bacterial species were detected in 34 cheese samples. The identified fungi and bacteria were consistent with previous studies. However, a fungus called *C.* aff. *radians* was isolated from only one cheese sample and it was reported for the first time in a cheese product. *Penicillium commune*, which is detected in both types of cheese production, is one of the most common fungi that causes mold in commune cheeses. In this study, it was also found to be the most prevalent and widespread fungus. It is known that this fungus can produce mycotoxins, and researchers have reported differences in mycotoxin production among different strains of this fungus. Therefore, it is important to determine whether this fungus is producing mycotoxins in locations where it is detected. The growth of *P. commune* on cheese causes the surface color to fade and the bad taste to be eaten (Lund et al., 1995; Garnier et al., 2017). In an 8-year study on the diversity and distribution of *P. commune* contaminants in two different cheese factories, swab and air samples were taken from production facilities, processing environments, and contaminated cheeses. It was observed that some isolates of *P. commune* contaminants in the cheese factories had different colony appearances and conidial colors on agar medium. Similarly, it was found that some *P. commune* isolates did not produce three characteristic secondary metabolites, cyclopiazonic acid, rugulovasin A&B, and cyclopaldic acid (Lund et al., 1995) while some isolates only produced one of these secondary metabolites, indicating differences between isolates (Lund et al., 1995). Cyclopiazonic acid and regulovasin A and B are important mycotoxins produced by *P. commune* (Boutrou and Gueguen, 2005). According to Lund et al. (1995) in a study conducted on hard, semi-hard, and semi-soft cheeses from Denmark, France, Greece, the United Kingdom, and other countries, *P. commune* was the most common and most frequently identified fungus, with a prevalence rate of up to 42%.

Another fungal agent identified in the study, *G. candidum*, is important in cheese ripening and much is known about its direct contribution to the maturation and flavor development of cheese. *G. candidum* can play an important role in the ripening of many soft and semi-hard cheeses and can contribute positively to taste and aroma development. At the same time, it can affect the growth of both beneficial and harmful microorganisms. The importance of the presence of *G. candidum* depends on the specific production type of the cheese and the presence of biotypes that include specific metabolic types. However, the in situ metabolic pathways involved in cheese ripening and their regulation are not well understood (Boutrou and Gueguen, 2005). The sixty-four isolates were obtained from milk, curd and cheese collected in seven major cheese producing regions of France and reported to have high genetic diversity. The diversity of French cheeses ripened with fungi is partly due to the succession of fungi that colonize the cheese during ripening. In soft cheeses such as *G. candidum*, Camembert and St. It occurs in the early stages of ripening in semi-hard cheeses such as Nectaire and Reblochon reveals an enormous variety of *G. candidum* that has been empirically selected by French cheese makers over the centuries (Marcellino et al., 2001).

Trichoderma sp., *R. stolonifer* species were also detected in homemade cheeses. *R. stolonifer* was also detected from the Italian cheese (Fossa cheese) produced by traditional production and the Iranian commercial cheese (Caspian cheese) produced for commercial purposes (Santi et al., 2010; Ando et al., 2012).

In commercial cheeses, only one sample was found to contain *C.* aff. *radians*. The presence of this fungus, which is a wood-loving mushroom that can live in manure and soil (Badalyan et al., 2011), suggests that it may have contaminated the cheese from some wooden material. This is the first report of this particular fungus as a contaminant in cheese.

In another commercial cheese sample, *C. herbarum* and *C. cladosporiodes* were detected in one cheese sample each Hocking and Faedo (1992) reported the presence of *Cladosporium* (*C. cladosporioides* and *C. herbarum*) species in cheeses and suggested that they could pose a threat during vacuum packaging and ripening stages.

In addition to all these fungi, different fungal agents were not detected. The determination of these factors also shows that the conditions in cheese production are far from hygiene. The fact that the variety of fungal agents is more common in house cheeses suggests that there are problems in the milk processing stage.

Cheese is a nutrient-rich food. It can also serve as a source of foodborne infection in humans due to the presence of pathogenic bacteria in milk and dairy products (Koïche et al., 2013). Therefore, it is a suitable environment for the growth of *S. aureus*. The presence of *S. aureus* in cheese may be due to various reasons such as the use of unpasteurized milk with a high level of *S. aureus* for cheese production, insufficient activity of the starter culture, use of contaminated milk after pasteurization, and inadequate storage conditions (Baran et al., 2017). *Serratia* spp. are ubiquitous environmental bacteria that can cause opportunistic infections that can cause outbreaks of mastitis in dairy cattle herds. These organisms have been isolated from water, soil, different plant species and insects, farm environments such as litter and parlor, and dairy cow feces and even tank milk samples (Friman et al., 2019). The use of unpasteurized milk is particularly possible in homemade cheeses.

In the cheeses examined in the study, home-produced cheeses were more contaminated in terms of fungal flora, but it was an interesting result that there was not much difference between the two production types in terms of bacteria.

The study indicates that adequate measures for controlling microbiological spoilage in milk, dairy products, and production environments were not taken or applied. It is believed that this data can contribute to raising awareness and informing producers if relevant authorities and responsible individuals take necessary actions.

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SUPPLEMENTARY DATA: Fungi species isolated from cheeses

Sample Code	Cheeses type	Fungi species isolated from both cheese						
		Penicillium commune	*Geotrichum candidum*	*Cladosporium herbarum*	*Cladisporium cladosporiodes*	*Coprinellus* aff. *radians*	*Trichoderma sp.*	*Rhizopus stolonifer*
1	Homemade	-	+	-	-	-	-	+
2	Homemade	+	+	-	-	-	-	-
3	Homemade	-	-	-	-	-	+	+
4	Homemade	+	-	-	-	-	-	
5	Homemade	+	-	-	-	-	-	+
6	Homemade	+	+	-	-	-	-	-
7	Homemade	+	+	-	-	-	-	-
8	Homemade	+	-	-	-	-	-	-
9	Homemade	+	-	-	-	-	-	-
10	Homemade	+	-	-	-	-	-	-
11	Homemade	-	-	-	-	-	-	-
12	Homemade	+	-	-	-	-	-	-
13	Homemade	-	-	-	-	-	-	-
14	Homemade	+	-	-	-	-	-	-
15	Commercial	-	-	-	-	-	-	-
16	Commercial	-	-	-	-	-	-	-
17	Commercial	-	-	-	-	+	-	-
18	Commercial	-	-	-	-	-	-	-
19	Commercial	-	-	+	-	-	-	-

20	Commercial	-	-	-	-	-	-	-
21	Commercial	-	-	-	-	-	-	-
22	Commercial	-	-	-	-	-	-	-
23	Commercial	+	-	-	-	-	-	+
24	Commercial	-	-	-	-	-	-	-
25	Commercial	+	-	-	-	-	-	-
26	Commercial	-	-	-	-	-	-	-
27	Commercial	-	-	-	-	-	-	-
28	Commercial	-	-	-	-	-	-	-
29	Commercial	-	-	-	-	-	-	-
30	Commercial	-	-	-	+	-	+	-
31	Commercial	+	-	-	-	-	-	-
32	Commercial	+	-	-	-	-	-	-
33	Commercial	-	-	-	-	-	-	-
34	Commercial	-	-	-	-	-	-	-

CHAPTER 6

IN VITRO MICROPROPAGATION OF *ALYSSUM NEZAKETIAE* AYTAÇ & H. DUMAN

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INTRODUCTION

Anatolia has a very important potential in terms of biodiversity due to its different geographical characteristics and diversity. In fact, while 2,750 of a total of 12,000 plant species are endemic in Europe, Anatolia hosts approximately 11,000 plant taxa, 1/3 of which is endemic (Ekim et al., 2000). Serious studies are carried out to protect endemic species in the world and to ensure the continuation of their generation. Studies on this subject in Turkey began to gain value between the years 1992-1997. Endemic species, which continue their lives in a very limited area, are faced with some threats in order to survive. In particular, factors such as urbanization, industrialization, tourism, overgrazing, unconscious expansion of agricultural areas, fires, and the use of herbicides pose a great danger to plants.

The soil structure of the region where the provincial center of Çankırı has located consists of the gypsum (gypsum) series (Figure 1). According to the records of the first 10 volumes of the Flora of Turkey, a total of 357 species, 119 of which are endemic, live in this region, where many endemic plants are found (Davis, 1965-1988). It is estimated that the number of species is over 1000 with various flora studies carried out later.

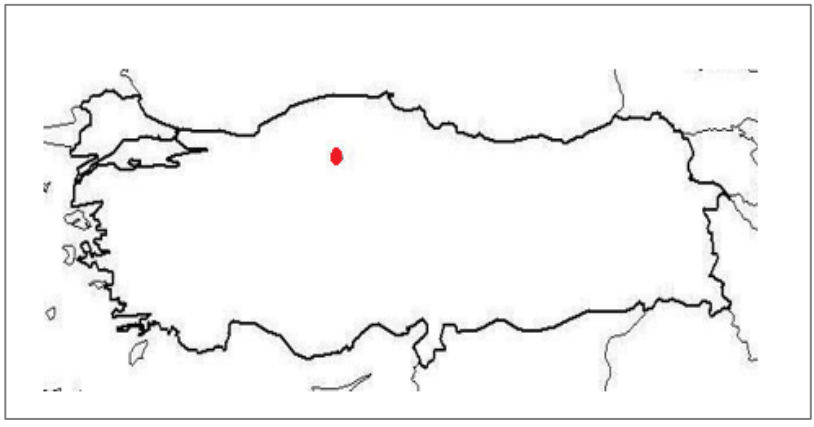

Figure 1. Distribution area of *A. nezaketiae*, Çankırı/TÜRKİYE (Bakis *et al.* 2011)

It has a local and endemic distribution in the arid and semi-arid climate of Çankırı and is not yet in the danger category according to the International Union for Conservation of Nature (IUCN) data. However, *Alyssum nezaketiae* AYTAÇ & H. DUMAN (Aytaç and Duman, 2000), which is known to be a candidate for the CR (Critical Endangered to Endangered Extinction) category, is very important due to its habitat size of around 100 km^2 and the minimal exact boundaries of its habitat (Figure 2).

These individuals, which belong to the Brassicaceae and are usually annual or perennial in small and herbaceous forms, spread over a wide area worldwide, excluding the Antarctic continent (Koch and Kiefer, 2006). Today, Brassicaceae has approximately 320 genera and 3,700 species belonging to these genera (Anonymous, 2016).

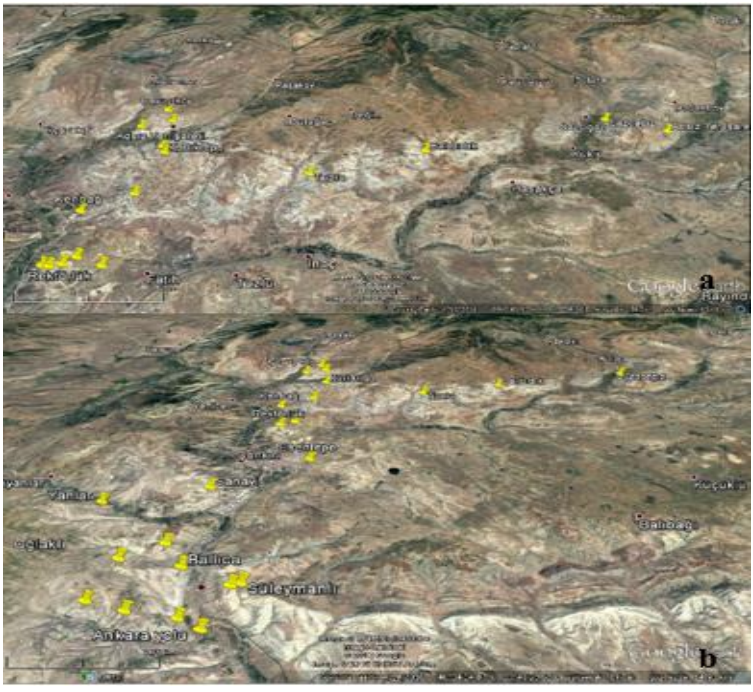

Figure 2. Satellite image of the points where the species was detected (Anonymous, 2016)

While classified as vascular plants as a group, the Brassicaceae taxonomy classification, which is also a member of the flowering plants family, is as follows (Mabberley, 1987);

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Brassicales

Family: Brassicaceae

While there are about 210 valid species belonging to the genus *Alyssum* in this family (Figure 3), 110 of them are located in Turkey. About 50 of these plants are endemic and are generally seen in mountainous and hilly areas. Although it is mostly seen in the Mediterranean environment throughout the world, it is known that a few species are also found in North Africa, Central Asia, Siberia and North America (Dudley, 1964; Dudley, 1965).

It is very important both to protect the habitats of endemic species with limited natural habitats and to ensure species continuity. For this purpose, it is very important to carry out studies on culturing these plants.

Alyssum nezaketiae, which is locally endemic in the province of Çankırı, is unique to gypsum areas (Aytaç and Duman, 2000). Individuals of this species have been found even in very extreme conditions where erosion is severe, organic matter in the soil is low, and the bedrock dissolves on the surface.

Figure 3. *Alyssum nezaketiae* a) flowers b) habitat

Factors such as overgrazing, field clearing, road construction works, and urbanization due to the rapidly increasing population, which spread around a residential area and therefore are likely to be exposed to human influence, endanger the extinction of *A. nezaketiae*. In addition to all these, although it spreads in extreme areas threatened by erosion, it is possible to say that such rapid erosion limits the areas where the plant can live day by day. Considering all these, it is very important to propagate this species vegetatively in a short time using in vitro techniques.

In this study, shoot tips and nodes of the *A. nezaketiae* plant were used as explant sources, and they were propagated rapidly and clonally by tissue culture micropropagation technique. *A. nezaketiae* explants were cultured in vitro, and shoot regeneration was performed by rooting with 36 different combinations of gelrite as a solidifier, 2 different basal media, and plant growth regulators (PGR). The plants showing root and shoot development were taken to the acclimatization stage, but the plants could not adapt to the external conditions. This study was the first to study the reproduction of *A. nezaketiae* in vitro conditions.

In vitro Propagation Studies of Different Species in Brassicaceae

Ceylan (2015), examined the effects of NAA and BAP at different doses in order to perform micropropagation in *Crambe orientalis* L. in his study. *C. orientalis* seeds were cultured in PGR-free Murashige and Skoog (MS) medium. Node and shoot were used as explant sources in seedlings that developed after 14 days. The explants were cultured in an MS medium containing 12 different combinations of BAP and NAA. Thus, the percentage of callus and shoot formation rates, the number of explants/mean shoots, and their effects on shoot length were determined. Callus formation was observed at all BDM concentrations by culturing shoot tips. The maximum average shoot length was reached in MS-BAP (2 mg/L). Rooted the regenerated shoots in MS basal medium containing different concentrations of NAA, but it was observed that the rooting of the regenerated shoots was difficult in general.

Özdemir and Yıldırım (2016), in their study, aimed to micropropagation *Crambe maritima* L., which they used as an explant source from hypocotyl parts of plants grown in vitro. Explants were cultured in MS basic nutrient medium formed with 9 different combinations of BAP (0.50-1-2 mg/L) and NAA (0-0.25-0.5 mg/L). The average percentage of callus formation was in MS medium containing the most BAP (0.5 mg/L) + NAA (0.5 mg/L), and the average percentage of regenerated shoots was MS-BAP (2 mg/L) + NAA (0.25 mg/L) average shoot number produced by each explant in maximum MS-BAP (1 mg/L) + NAA (0.5 mg/L) basal medium, average shoot length maximum MS-BAP (2 mg/L) in the basal medium they have recorded. Shoots with good growth were rooted in a medium containing MS-NAA (1 mg/L).

Memon and Memon (2021), in their study, created an effective plant regeneration protocol for *Brassica nigra*, which is known to be a Cu accumulator. Nodes taken from 10-day-old mature plants were used

as explants. The highest percentage of callus formation (100%) was observed in MS media containing 0.1 mg/L BAP + 0.5 mg/L NAA, 0.6 mg/L + BAP 0.2 mg/L NAA. The highest shoot formation percentage (100%) was obtained in 0.6 mg/L BAP + 0.05 mg/L IBA, 0.2 mg/L IBA + 0.2 mg/L NAA media. The highest number of shoots per explant (3.25) was obtained in MS medium supplemented with 0.6 mg/L BAP + 0.05 mg/L IBA. Plants growing in MS were transferred to soil and grown in the plant growth chamber for one month.

Yıldızhan (2007), aimed to in vitro micropropagation of the *Brassica nigra*. In the study, a low-cost and efficient regeneration protocol was developed with intensive production of *B. nigra* for phytoremediation purposes. Sterilized *B. nigra* seeds were cultured in a PGR-free MS medium for 30 days; transferred the plants in this period to MS media containing different doses of BAP and NAA. High callus formation was detected in hypocotyl, apex, and stem explants, while it detected both direct and indirect shoot regenerations in apex explants. While it provided direct shoot formation in all combinations of NAA and BAP, indirect shoot formation from the apex was detected in media where NAA and BAP growth regulators were in appropriate proportions. Indirect and direct shoots were transferred to 5 different MS media for root regeneration and root development was observed within 7-20 days. The plants that completed the regeneration were transferred to the soil.

Demir (2020), aimed to reveal an effective in vitro clonal production protocol by providing regeneration of endemic *R. carnosula* by somatic embryogenesis or organogenesis. Hypocotyl and cotyledons of plantlets obtained from embryos were used as explant sources. Explants were planted in 73 different regeneration media with various concentrations of auxin group IAA, NAA, and 2,4-D, cytokinin group BAP, TDZ, and Zeatin. Thus, the interaction of explant types and regeneration media alone and with each other; investigated the effects of explant response, amount of callus, type of callus (embryogenic or

organogenic), anthocyanin formations, direct or indirect somatic embryogenesis and organogenesis including shoot-root. At the end of the study, somatic embryo, shoot, and root structures were observed in control environments.

Various Studies on *Alyssum* sp.

Oturgan (2007), investigated 11 species of plants belonging to 5 genera in terms of their biological activities. In his study on plants, it was stated that two of them were potentially cytotoxic, one was DPPH, and all of them had reducing properties and were antioxidants according to their water purification status, and the microbiological activities of eight plants were significant. Hamedi et al., (2015) and Erdogan et al., (2015) stated in their studies that some Alyssum species are used as natural stabilizers in the food industry, as well as have some important biological activities in some species. Bayramoglu et al., (2012) found that the chemically modified biomass of *Alyssum discolor* T.R. Dudley & Hub.-Mor. can be used as a biological solvent for the separation of reactive yellow textile dye that can be found in aqueous media. Akdemir et al., (2019) investigated the mass reduction coefficients and molar extinction coefficients in the root, aerial and flower parts of *A. pateri* subsp. *prostratum* plant belonging to the genus *Alyssum*. They determined that the above-ground and flower structures had a higher radiation absorption ability than the root structure. Babaoğlu (2008), emphasized that *A. corsicum* has a very important role in the bioremediation and recovery of heavy metals, especially nickel, as a candidate plant for use in herbal treatment studies. It was observed that the plant accumulated more nickel metal in its stem than in its roots, and with the increase in nickel metal concentration, changes occurred in leaf hair density and structure. In addition, Adıgüzel and Reeves (2012), reported that *Alyssum pterocarpum* T.R. Dudley, *Alyssum cypricum* Nyár, *Alyssum discolor* T.R. Dudley & Hub-Mor. *Alyssum huber-morathii* T.R. Dudley, *Alyssum masmenaeum* Boiss.,

Alyssum caricum T.R. Dudley, *Alyssum davisianum* T.R. Dudley, *Alyssum pinifolium* (Nyár.) T.R. Dudley, *Alyssum peltarioides* Boiss. sub sp. *peltarioides*, *Alyssum callichroum* Boiss. & Balansa, *Alyssum crenulatum* Boiss., *Alyssum samariferum* Boiss. & Hausskn species are the prominent Ni-accumulator *Alyssum* species. In the study of Tozyılmaz (2019), the antibacterial and antifungal activities of the extracts obtained from Alyssum discolor Dudley & Hub-Mor., which grows endemic in Turkey, against eighteen bacterial and two fungal strains by disc diffusion method. *Alyssum discolor* Dudley & Hub-Mor. while it showed the lowest effect in the antimicrobial study, it could not show any effect against fungi. It also showed the lowest activity in the anti-biofilm study. Tajbakhsh et al., (2012) investigated the phenolic content, antioxidant properties and activities in gram positive and negative bacteria in methanol extracts of *Alyssum*. Kürşat et al., (2008) reported that *Alyssum* species, especially the perennial ones, can be used as a pioneer plant in erosion studies because they are drought resistant and content with soil requirements. Ertuğ (2014), reports that the leaves of the *Alyssum constellatum* Boiss. species called Goramaz are consumed as food in Turkey. Amiri et al., (2012; 2013) and Parsaei et al., (2016), *Alyssum minus* Rothm. seeds as a relaxant and laxative; *Alyssum campestre* L. leaves are used as a relaxant and cough suppressant; *Alyssum alyssoides* L. stated that it is used as an antipyretic, relaxing, anti-pharyngitis, cough suppressant and voice suppression.

In vitro Propagation Studies in *Alyssum* sp.

Winterhalter et al., (2008) studied with *Alyssum murale* and revealed that the ideal medium for shoot formation is MS medium containing 0.46 µM Kinetin. Root formation was observed on the explants after approximately one month. Roots were isolated and propagated in PGR-free medium. While they observed rapid growth in some explants, they observed spontaneous shoot formation.

In the study of Bayat (2017), micropropagation of *Alyssum pinifolium* (Nyar.) TR. Dudley species, which is distributed in Çanakkale, was carried out. As an explant source, healthy nodes, internodes, and leaves of in vitro-grown plants were used and cultured in an MS medium. BAP and IAA were added to the culture medium in different concentrations and combinations. The best results in callus formation were achieved in the medium of MS-BAP (2 mg/L) and IAA (0.1 mg/L). Indirect organogenesis was obtained as a result of subcultures.

Material Supply of *Alyssum nezaketiae* in vitro Propagation

Alyssum nezaketiae was first collected by Prof.Dr.Hayri Duman in 1995 from Kenbağ Mevkii on the Çankırı-Kastamonu road. In later studies, the plant was also found on the Çankırı-Ankara road, in Ballıca (Vural and Şahin, 2013). In Turkey, only around the provincial center of Çankırı; It is a small and pleasant-looking plant, about 10 cm tall, with yellow flowers, which is known to be a candidate for the Critically Endangered (CR) category, growing on gypsum soils on sloping hills near Ankara road, Kastamonu road, and Yapraklı road.

A.nezaketiae, which started to appear in the field in spring (April-May) with the beginning of the vegetation period, usually begins to bloom in mid-May. Plants collected from the area with coordinates 40°28'54"N-33°38'48"E around Çankırı Karatekin University Campus were brought to the laboratory in a way that their natural habitat and reproduction would not be adversely affected (Figure 4).

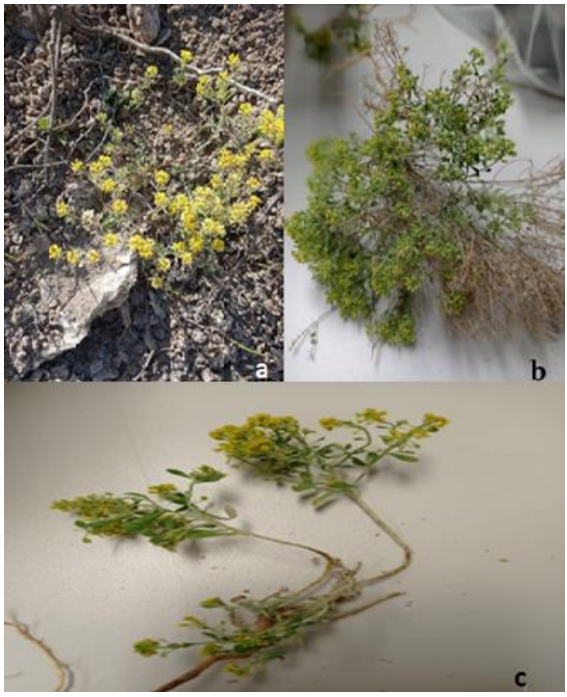

Figure 4. a) Full flowering view of *A.nezaketiae* b) *A.nezaketiae* collected from the field and brought to the laboratory c) flowers, leaves and shoots of *A.nezaketiae*

Sterilization of Plants After the Field Study

Alyssum nezaketiae, collected from the growing area, was brought to the laboratory, and the required surface sterilization protocol of the plant was applied as follows before planting in vitro conditions (Sezgin and Dumanoğlu, 2014) (Figure 5):

- First, the plant was washed under tap water for 20 min.
- It was treated in ethanol (70%) for 3 min.
- Tween 20 (8-10 mg/L) was added to a 20% solution of commercial sodium hypochlorite (NaOCl containing 15% chlorine) to increase the effectiveness and treated for 10 min.
- In order to remove NaOCl from the tissues, it was rinsed with sterile distilled water for 5 min under aseptic conditions and this process was repeated 3 times.

Basal Media and Plant Growth Regulators

The shoot tip and the nodes on the shoot of the *A.nezaketiae* plant, on which surface sterilization was performed, were used as explant sources. For this purpose, different basic nutrient media and combinations of auxin and cytokinin group plant growth regulators (PGR) with different doses were used in the initial stage (Şan et al., 2007; Yıldızhan, 2007; Sezgin and Dumanoğlu, 2014). At this stage, the initial effects of combinations of media and PGR were tested (Table 1).

1) Initial stage media
   a. MS (Murashige and Skoog, 1962)
   b. Woody Plant Medium (WPM) (Lloyd and McCown, 1981)
2) Plant growth regulators and doses
   a. 6-benzylaminopurine (BAP)-(0 mg/L, 0.5 mg/L, 1 mg/L)
   b. Kinetin (KIN)-(0 mg/L, 0.5 mg/L, 1 mg/L)
   c. Indole-3-acetic acid (IBA)-(0 mg/L, 0.25 mg/L, 0.5 mg/L)
   d. α-naphthaleneacetic acid (NAA)-(0 mg/L, 0.25 mg/L, 0.5 mg/L)

Sucrose (30 g/L) as a carbon source and gelrite (2.1 g/L) as a solidifier were added to the basal media prepared in 36 different combinations with 2 basal media, 6 cytokinin, and 6 auxin doses for *A.nezaketiae*. The pH of the media was adjusted to 5.7 (Babaoğlu et al., 2001).

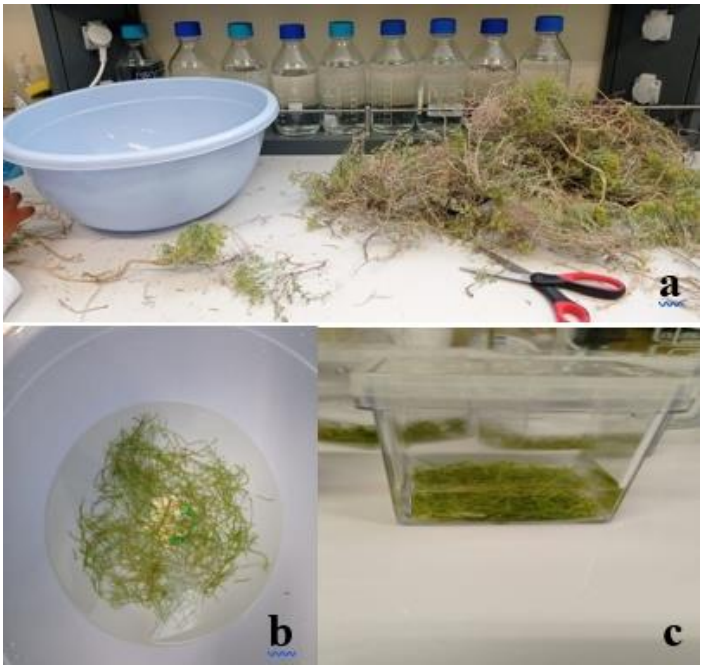

Figure 5. Sterilization of *A.nezaketiae* a) separation of shoots of the plant b) EtOH application c) explants ready for planting

All combinations were prepared in ten repetitions. In order to prevent possible contamination after planting, Plant Preservative Mixture (PPM-Duchefa®) (1 ml/L) was added to the media. The media containing appropriate amounts of PGR and solidifier were divided into the erlenmeyers into equal amounts (120 ml) and autoclaved in order to ensure sterilization. In the autoclave, the media was sterilized at 121°C and 1.2 atm pressure for 20 min (Sezgin and Dumanoğlu, 2014). After sterilization, the media was divided into sterile tubes of 130x10 mm size, approximately 10 mL each, before the gelrite solidified in a laminar airflow cabinet.

Table1. PGR combinations

1	BAP (0mg/L) + IBA (0mg/L)	**19**	KIN (0mg/L) + IBA (0mg/L)
2	BAP (0mg/L) + IBA (0.25mg/L)	**20**	KIN (0mg/L) + IBA (0.25mg/L)
3	BAP (0mg/L) + IBA (0,5mg/L)	**21**	KIN (0mg/L) + IBA (0.5mg/L)
4	BAP (0.5mg/L) + IBA (0mg/L)	**22**	KIN (0.5mg/L) + IBA (0mg/L)
5	BAP (0.5mg/L) + IBA (0.25mg/L	**23**	KIN (0.5mg/L) + IBA (0.25mg/L)
6	BAP (0.5mg/L) + IBA (0.5mg/)	**24**	KIN (0.5mg/L) + IBA (0.5mg/L)
7	BAP (1mg/L) + IBA (0mg/L)	**25**	KIN (1mg/L) + IBA (0mg/L)
8	BAP (1mg/L) + IBA (0.25mg/L)	**26**	KIN (1mg/L) + IBA (0.25mg/L)
9	BAP (1mg/L) + IBA (0.5mg/L)	**27**	KIN (1mg/L) + IBA (0.5mg/L)
10	BAP (0mg/L) + NAA (0mg/L)	**28**	KIN (0mg/L) + NAA (0mg/L)
11	BAP (0mg/L) + NAA (0.25mg/L)	**29**	KIN (0mg/L) + NAA (0.25mg/L)
12	BAP (0mg/L) + NAA (0.5mg/L)	**30**	KIN (0mg/L) + NAA (0.5mg/L)
13	BAP (0.5mg/L) + NAA (0mg/L)	**31**	KIN (0.5mg/L) + NAA (0mg/L)
14	BAP (0.5mg/L) + NAA (0.25mg/L)	**32**	KIN (0.5mg/L) + NAA (0.25mg/L)
15	BAP (0.5mg/L) + NAA (0.5mg/L)	**33**	KIN (0.5mg/L) + NAA (0.5mg/L)
16	BAP (1mg/L) + NAA (0mg/L)	**34**	KIN (1mg/L) + NAA (0mg/L)
17	BAP (1mg/L) + NAA (0.25mg/L)	**35**	KIN (1mg/L) + NAA (0.25mg/L)
18	BAP (1mg/L) + NAA (0.5mg/L)	**36**	KIN (1mg/L) + NAA (0.5mg/L)

Planting and Incubation of the Explants

After the sterilization stage, the node and shoot tips of *A.nezaketiae* plant were used as explant sources and these explants were planted in tubes. One explant was planted in each tube. From this stage onwards, all cultures were taken into the climate cabinet at 25±1ºC, 16/8 hours of light/dark (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) conditions, and their incubation was ensured by being checked regularly for one month (Figure 6).

Subculture

The explants of *A.nezaketiae*, which were cleaned from the leaves, sterilized under appropriate conditions, and then planted, were incubated in the climate room and controlled every other day. Explants, media, and PGR combinations were subcultured, adhering to the initial combinations, and their growth and development were followed.

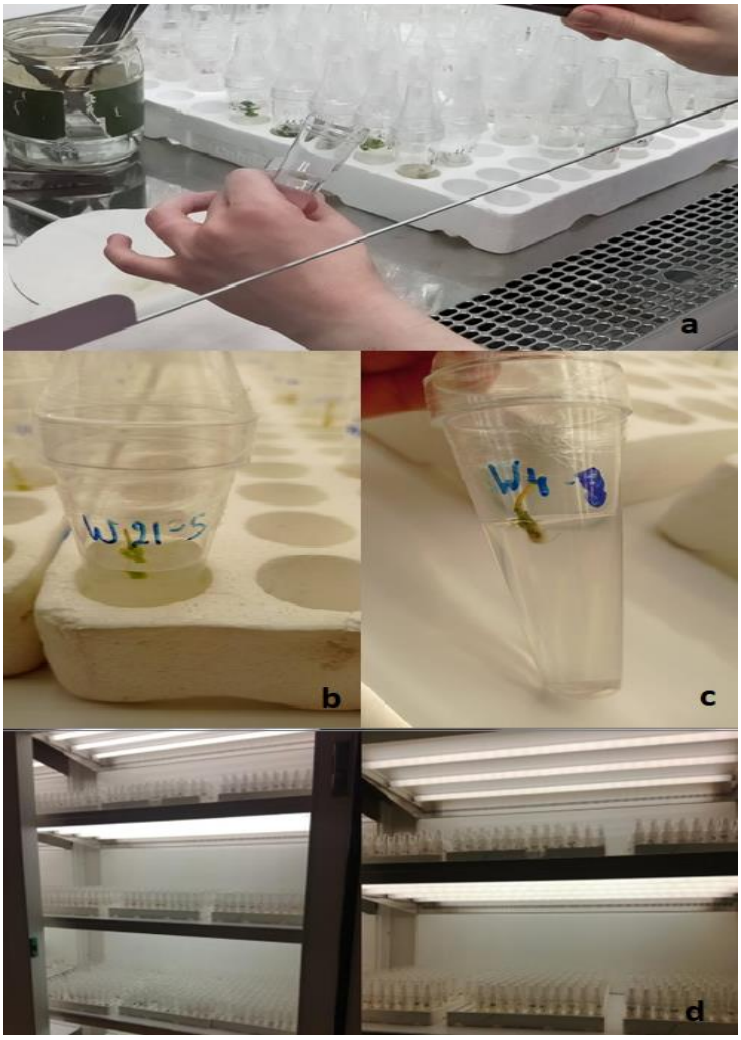

Figure 6. Planting of *A.nezaketiae* a) planting explants into tubes in a laminar air flow cabinet b-c) explants after planting d) incubated explants (Growth chamber)

The explants were subcultured 4 times at 28-day intervals in order to enable them to develop shoots and roots after callus formation. The subculture studies of the explants were continued in the ongoing process, with the medium MS and solidifier. Some explants showed shoot and root development. At this stage, data on root number and length and shoot number and length were recorded. Explants showing

direct shoot growth were transferred to rooting media for root development (Figure 7).

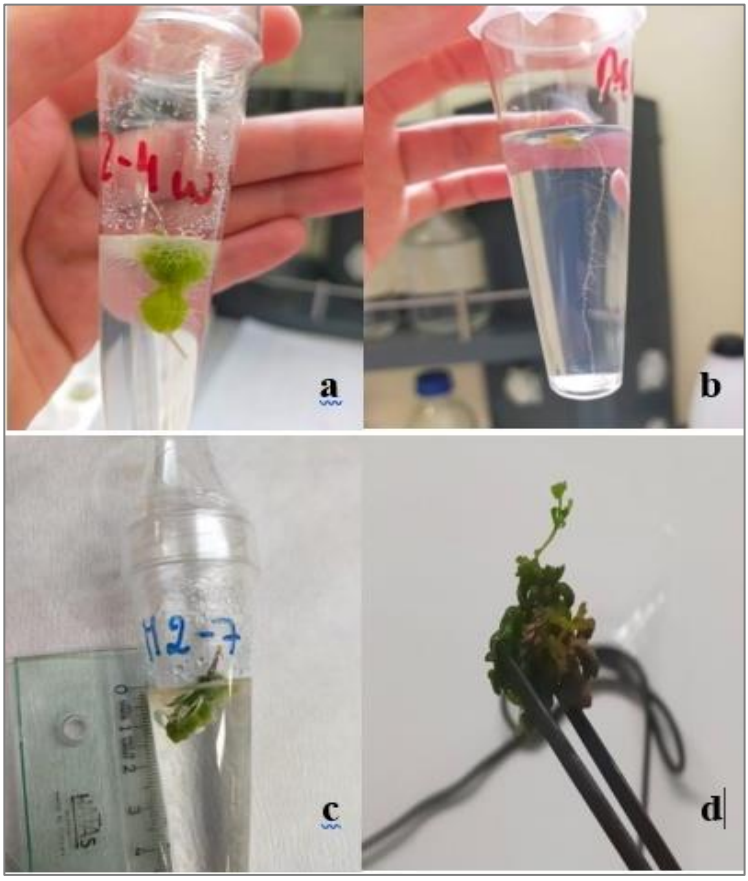

Figure 7. Growth of explants a) root growth in WPM-BAP (0 mg/L) + IBA (0.25 mg/L) b) root formation in MS-BAP (0 mg/L) + NAA (0.5 mg/L) c) callus formation in MS-BAP (0 mg/L) + IBA (0.25 mg/L) d) shoot formation in WPM-KIN (0.5 mg/L) + IBA (0 mg/L)

Rooting

Explants, which also showed shoot development during the rooting stage, were added to the MS and WPM media with different doses of KIN and IBA as a solidifier and planted. At this stage, the explants were planted in 125x65x80 mm sterile tubes containing 120 mL of basal medium (Figure 7). Rooting stage was carried out in

twenty-eight days periods. At this stage, the plants planted for the rooting stage were incubated at in the climate cabinet (Mandal et al., 2013). In explants showing root formation, the number of roots and the lengths of the roots (cm) and the number of shoots and shoot lengths were recorded as data (Figure 8).

Acclimatization

The shoots and roots of *A.nezaketiae* explants that started to develop were taken to the acclimatization stage. The plants, which were carefully taken from their media, were thoroughly washed with distilled water in order to clean the media residue on them. Growing plants were planted in a mixture of flower soil, sand and perlite (3:1:1 v/v). This mixture was first sterilized in an autoclave at 121^{o}C and 1.2 atm pressures for 90 minutes to prevent possible contamination from soil. After the plants were planted in the small plastic pots, they were watered with approximately 50 mL of distilled water every other day.

Figure 8. Regenerated *A.nezaketiae* a) Root and shoot formation in MS-KIN (1 mg/L) + IBA (1 mg/L) medium b) Acclimatization of regenerated plants

Statistical Analysis

Analysis of variance was performed using SPSS program and Game-Howell non-parametric Post-Hoc test as the mean of repetitions according to the data of the study ($P<0.05$, 0.01, 0.001). In order to determine the statistically significant differences, the Duncan Multiple Comparison Test was applied according to the 5% error limit and the differences were expressed with the help of letters.

The shoot tips and nodes of *A.nezaketiae* were used as explants, and the regeneration of these explants was observed depending on the media they were in and the PGR combinations they contained. For this purpose, numerical data were recorded by observing the callus, shoot, and root structures that developed after each subculture.

Shoot Formation in Subcultures

In vitro propagation of *A.nezaketiae*, the number of shoots formed by the regeneration of callus in media was observed and the data were evaluated statistically. In this sense, the interaction between 'basal medium x PGR combinations' is statistically significant (P≤0.05). In the explants, the values of the shoot formation formed from the callus after the culture start stages are given in Table 2. In the interaction between callus shoot formation "basal medium x PGR combinations" in *A.nezaketiae*, MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium together with WPM-KIN (0.5 mg/L), the best values for shoot formation were obtained in L) + NAA (0.5 mg/L) medium. In addition, regeneration of shoots in MS-KIN (1 mg/L) + IBA (0.5 mg/L) medium and WPM-BAP (0.5 mg/L) + IBA (0.25 mg/L) medium was found to be statistically significant.

Table 2 Interaction of "basal medium x PGR combination" of shoot regeneration from callus in *A.nezaketiae* (P≤0.05)

	Basal Medium			**Basal Medium**	
PGR	**MS**	**WPM**	**PGR**	**MS**	**WPM**
1	0±0c	0.3±0.6cd	19	0.8±1.8 cd	0±0d
2	1.4±3.4 cd	1±2.3cde	20	1.3±2.2 cd	2.7±3.7bcde
3	2.8±4.3 abcd	5.3±8.4abcd	21	2.7±3.6 abcd	5.1±13.1abcde
4	0±0c	0±0d	22	0.7±1.6 cd	2.6±4.5bcde
5	2.9±3.6 abcd	6.3±8.1ab	23	1.3±1.6 cd	4.6±6.6abcde
6	1.9±3.10abcd	2±3.6bcde	24	3.4±4.9 abcd	5.5±7.6abc
7	1.6±4.3bcd	0±0d	25	0.1±0.3 c	0.1±0.3cd
8	1.7±3.6 abcd	1.3±3.7bcde	26	**4.9±3.7 a***	1.3±2.7bcde
9	1.3±3.7cd	1.5±1.7bcde	27	4.7±4.7 ab	2±5bcde
10	1.1±2.5 cd	0.8±2.2cde	28	1±2.5 cd	0±0d
11	2.6±4.2 abcd	2.4±7.5bcde	29	1.2±3.7 cd	0.1±0.3cd
12	0.5±1.2 c	0.3±0.9cd	30	1.6±2.2 bcd	2.9±6.2bcde
13	1.3±2.0 cd	1.8±5.6bcde	31	0.2±0.4 c	0±0d
14	3.9±5.2 abc	1.1±2.6cde	32	1.8±3.5 abcd	1.5±4.4bcde
15	2.1±2.4 abcd	1.5±1.9bcde	33	2.7±4.1 abcd	8±8.5a*
16	2.1±3.9 abcd	0.1±0.3cd	34	1.9±2.0 abcd	1.6±2.9bcde
17	0.2±0.4 c	0.2±0.4cd	35	0.8±1.2 cd	2.7±4bcde
18	0.1±0.3 c	0.3±0.6cd	36	0±0 c	1.8±1.9bcde

* Different letters indicate the difference between applications.

Number of Shoots in Subcultures

In vitro propagation of *A.nezaketiae*, the data on the number of shoots formed in the media of the explants were taken and statistically evaluated. In this sense, the interaction of 'basal medium x PGR combinations' turned out to be statistically significant (P≤0.05). The statistical averages of the data on the number of shoots occurring in the explants are given below (Table 3). The interaction of "basal medium x PGR combinations" was found to be significant in the number of shoots in explants. In addition to MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium, WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium, in terms of shoot number, the best data has been obtained. In addition, shoot numbers in MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium and WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium, were statistically significant.

Table 3. Interaction of "basal medium x PGR combinations" of shoot numbers in *A.nezaketiae* explants (P≤0.05)

Basal Medium			Basal Medium		
PGR	**MS**	**WPM**	**PGR**	**MS**	**WPM**
1	0±0c	0±0c	19	0±0c	0±0c
2	0.2±0.6c	2.2±4.6abc	20	0.4±1.2c	4.4±7.6abc
3	0.7±1.4bc	2.6±3.6abc	21	1.2±1.9abc	2.7±5.1abc
4	0.1±0.3c	0±0c	22	0.8±2.5bc	0.7±1.6c
5	0.5±1.2c	1.1±2bc	23	0.2±0.6c	0.2±0.6c
6	0.7±1.4bc	3.3±5.9abc	24	1.2±2.5abc	**5.8±5.8a***
7	0.3±0.94c	0±0c	25	0±0c	0.1±0.3c
8	0.4±1.2c	1.4±3.7abc	26	2.4±3.5ab	1.8±5.6abc
9	0.3±0.9c	4.2±6.9abc	27	2.6±3.8a*	2.5±5abc
10	0±0c	1.2±3.1bc	28	0±0c	0±0c
11	0.7±1.6bc	3.8±8.6abc	29	0.4±1.2c	0±0c
12	0±0c	1.4±4.4abc	30	1.1±2.3abc	2.1±4.3abc
13	0.8±2.5bc	1.5±3.8abc	31	0±0c	0±0c
14	0.4±0.6c	5.6±7.3ab	32	1.4±2.6abc	1.3±4.1abc
15	1.6±2.7abc	3.5±5.2abc	33	0.7±2.2bc	1.8±2.1abc
16	0.9±1.9abc	2.8±5.1abc	34	1.4±1.7abc	3±5.5abc
17	0±0c	0±0c	35	0±0c	0.6±1c
18	0±0c	0±0c	36	0±0c	0.1±0.3c

* Different letters indicate the difference between applications.

Length of Shoot in Subcultures

The data on the shoot lengths occurred in the explants during the subculture stage of the in vitro propagation of *A.nezaketiae* were taken and after statistical evaluation, the interaction of "basal medium x PGR combinations" emerged as statistically significant (P≤0.05) (Table 4).

Table 4. Interaction of "basal medium x PGR combinations" of shoot length in *A.nezaketiae* explants (P≤0.05)

Basal Medium			Basal Medium		
PGR	**MS**	**WPM**	**PGR**	**MS**	**WPM**
1	0±0b	0±0e	19	0±0b	0±0e
2	0.14±0.4b	0.29±0.6abcde	20	0.2±0.6ab	0.53±0.8abcde
3	0.16±0.3b	0.6±0.5abcde	21	0.25±0.5ab	0.43±0.7abcde
4	0.04±0.1b	0±0e	22	0.08±0.2b	0.13±0.2de
5	0.1±0.2b	0.24±0.4bcde	23	0.05±0.1b	0.04±0.1e
6	0.47±1ab	0.41±0.6abcde	24	0.27±0.4ab	0.86±0.7a*
7	0.03±0b	0±0e	25	0±0b	0.1±0.3e
8	0.45±1.4ab	0.22±0.4bcde	26	0.24±0.3ab	0.15±0.4cde
9	0.06±0.1b	0.6±0.9abcde	27	0.34±0.4	0.46±0.8abcde
10	0±0b	0.16±0.3cde	28	0±0b	0±0e
11	0.26±0.6ab	0.32±0.6abcde	29	0.06±0.1b	0±0e
12	0±0b	0.22±0.6bcde	30	0.16±0.3b	0.28±0.4abcde
13	0.16±0.5b	0.19±0.4bcde	31	0±0b	0±0e
14	0.22±0.3ab	0.81±1ab	32	0.15±0.2b	0.12±0.3e
15	0.37±0.7ab	0.77±0.9abc	33	0.1±0.3b	0.64±0.76abcde
16	0.2±0.4ab	0.36±0.6abcde	34	0.65±0.7a*	0.75±1.2abcd
17	0±0b	0±0e	35	0±0b	0.17±0.3cde
18	0±0b	0±0e	36	0±0b	0.04±0.1e

* Different letters indicate the difference between applications.

Shoot length data in explants "basal medium x PGR combinations" interaction, best shoot on MS-KIN (1 mg/L) versus WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium length is reached. In addition, shoot length in media with a combination of MS-BAP (0.5

mg/L) + NAA (0.25 mg/L) and BAP (0.5 mg/L) + NAA (0.5 mg/L) BDM was statistically significant.

Root Number in Subcultures

In vitro propagation of *A.nezaketiae*, the numbers of roots formed by explants in subcultures were taken as data and in the statistical evaluation, the interaction of "basal medium x PGR combinations" did not reveal a difference that could be considered statistically significant (P≤0.05) (Table 5).

Table 5. Interaction of "basal medium x PGR combinations" of root number in *A.nezaketiae* explants (P≤0.05)

Basal Medium			Basal Medium		
PGR	**MS**	**WPM**	**PGR**	**MS**	**WPM**
1	0±0a	0.5±1.5a	19	0.1±0.3a	0±0a
2	1.4±3.5a	6.5±13.6a	20	0.2±0.6a	7.9±15.3a
3	2.7±5a	4±10.2a	21	0.8±1.1a	4.4±7.5a
4	0.1±0.3a	0±0a	22	0.7±2.2a	0.7±1.4a
5	0±0a	0±0a	23	0.1±0.3a	0.1±0.3a
6	1.3±2.6a	4.9±12.3	24	2.2±6.2a	3.3±5.7a
7	0.3±0.9a	0±0a	25	0.1±0.3a	0.5±1.5a
8	2±6.3a	4±9.2a	26	1.1±2.8a	6.6±20.8a
9	2±6.3	4.4±8.4a	27	0.8±1.6a	5±15.12a
10	0±0a	1.2±3.7a	28	0±0a	0±0a
11	3.2±7.5a	7.4±15.9a	29	0±0a	0±0a
12	0.2±0.6a	0.6±1.8a	30	0.1±+0.3a	5.9±13.2a
13	0.9±2.8a	2.9±9.1a	31	0.1±0.3a	0±0a
14	0.9±2.2a	6.5±10.4a	32	0.4±1.2a	0.4±1.2a
15	2.2±4.5a	6.8±14.6a	33	0.4±0.8a	0.7±1.4a
16	0±0a	2.5±7.9a	34	2.1±3.5a	3.3±8.5a
17	0.2±0.6a	0±0a	35	0±0a	0.4±0.6a
18	0±0a	0±0a	36	0±0a	0.6±1.8a

* Different letters indicate the difference between applications.

There was no statistically significant difference in the interaction of root numbers in explants between "basal medium x PGR combinations". The obtained data revealed that all combinations and media had the same effect on root numbers.

Root Length in Subcultures

In vitro propagation of *A.nezaketiae*, the length data of the roots formed in the subcultures were taken and statistically evaluated, and the interaction between the "basal medium x PGR combination" was statistically significant (P≤0.05) (Table 6).

Table 6. Interaction of "basal medium x PGR combinations" of root length in *A.nezaketiae* explants (P≤0.05)

Basal Medium				Basal Medium	
PGR	**MS**	**WPM**	**PGR**	**MS**	**WPM**
1	0±0a	0.1±0.3bcd	19	0.5±1.5a	0±0d
2	0.3±0.6a	0.65±1.1abcd	20	0.15±0.47a	0.8±1.1abcd
3	0.7±1.1a	0.37±0.6abcd	21	0.49±0.9a	0.58±0.9abcd
4	0.05±0.1a	0±0d	22	0.17±0.5a	0.3±0.6abcd
5	0±0a	0±0d	23	0.002±0a	0.08±0.2bcd
6	0.27±0.4a	0.28±0.7abcd	24	0.19±0.4a	0.96±1.3ab
7	0.15±0.4a	0±0d	25	0.03±0a	0.1±0.3bcd
8	0.2±0.6a	0.62±1.3abcd	26	0.31±0.6a	0.3±0.9abcd
9	0.35±1.1a	0.62±1.1abcd	27	0.2±0.4a	0.45±1.1abcd
10	0±0a	0.04±0.1cd	28	0±0a	0±0d
11	0.39±0.8a	0.47±0.9abcd	29	0±0a	0±0d
12	0.5±1.5a	0.15±0.4bcd	30	0.02±0a	0.4±0.8abcd
13	0.15±0.4	0.24±0.7abcd	31	0.03±0	0±0d
14	0.2±0.4a	1.14±1.2a*	32	0.1±0.3a	0.28±0.8abcd
15	0.385±0.6a	0.45±0.9abcd	33	0.06±0.1a	0.5±1.2abcd
16	0±0a	0.23±0.5bcd	34	0.57±0.8a	0.95±2abc
17	0.2±0.6a	0±0d	35	0±0a	0.29±0.5abcd
18	0±0a	0±0d	36	0±0a	0.04±0.1cd

* Different letters indicate the difference between applications.

In the "basal medium x PGR combinations" interaction of root length data in explants, the best data were obtained in terms of root length in WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium. In addition, WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium is also statistically significant.

Rooting Stage

In vitro propagation of *A.nezaketiae*, the explants were taken into rooting media following the subculture step. At this stage, the effects of auxin and cytokinin group PGR and their different combinations, which gave the best results in the subculture stage, were examined and the effects of media on plant growth were examined (Table 7 and 8). The data on the number and length of shoots, root numbers and lengths formed by the explants were recorded and statistically evaluated.

Table 7. Application table of PGR doses used in rooting stage

PGR DOSES	
1	KIN (0 mg/L) + IBA (0 mg/L)
2	KIN (0.25 mg/L) + IBA (0.25 mg/L)
3	KIN (0.5mg/L) + IBA (0.5mg/L)
4	KIN (1mg/L) + IBA (1 mg/L)

Table 8. The effect of "PGR doses" on root number and length and shoot number and length during rooting stage in *A.nezaketiae* explants (P≤0.05)

	PGR DOSES	Shoot number	Shoot length	Root Number	Root length
1	KIN (0 mg/L) + IBA (0 mg/L)	0.2±0.5c	0.05±0.15b	0.05±1.3b	0.05±0.15c
2	KIN (0.25 mg/L) + IBA (0.25 mg/L)	0.9±1.2ab	0.82±1.16a*	13.75±25.17ab	0.48±0.71ab
3	KIN (0.5mg/L) + IBA (0.5mg/L)	1.4±1.18a*	0.87±0.89a*	21.75±35.62a*	0.65±0.87a*
4	KIN (1mg/L) + IBA (1 mg/L)	0.70±0.9bc	0.66±0.92a*	4.25±13.69b	0.15±0.47bc

When the effect of PGR doses on root numbers-lengths and shoot numbers-lengths in rooting stage of *A.nezaketiae* explants was examined, the best root number (21.75) and the maximum root length (0.65) and the best shoot number (1.4) were examined. It was reached in the presence of KIN (0.5 mg/L) + IBA (0.5 mg/L) BDM dose (Figure 9). There was no statistically significant difference between the

PGR doses regarding the lengths of the shoots. The effect of media on shoot numbers, lengths, root numbers and lengths during the rooting phase is given in Table 9.

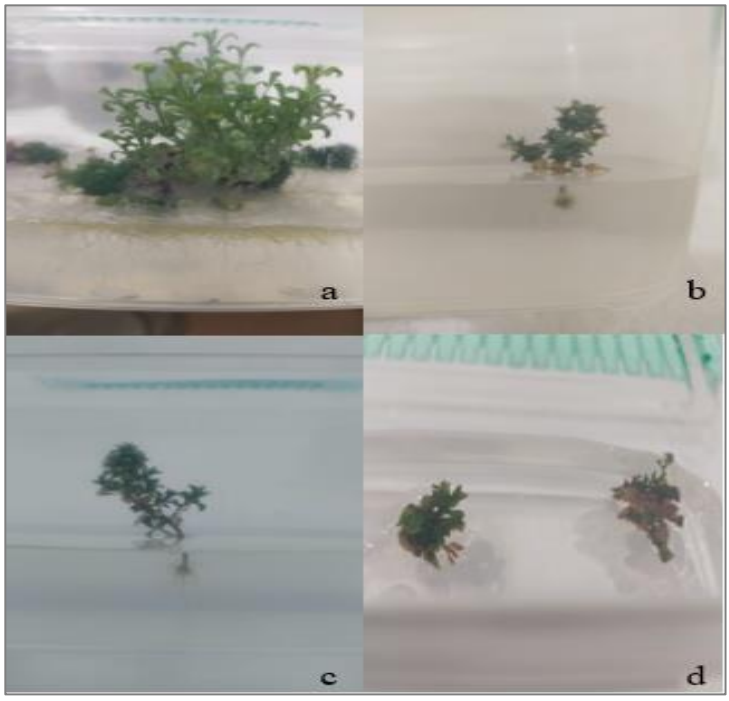

Figure 9. Root and shoot growth in rooting medium a)MS-KIN (0.5 mg/L) + IBA (0.5 mg/L) b) MS-KIN (0.25 mg/L) + IBA (0 ,25 mg/L) c) MS-KIN (1 mg/L) + IBA (1 mg/L) d) WPM-KIN (0.5 mg/L) + Shoot and root development in IBA (0.5 mg/L)

Table 9. The effect of "media" on root number and length, shoot number and length in rooting stage of *A. nezaketiae* explants (P≤0.05)

	Media	**N**	**Mean**
Shoot number	MS	40	1.125±1.20
	WPM	40	0.475±0.81
Shoot length	MS	40	0.62±0.88
	WPM	40	0.585±0.95
Root number	MS	40	16.52±31.38
	WPM	40	3.62±9.47
Root length	MS	40	0.43±0.69
	WPM	40	0.2425±0.6

When the effects of "media" on root number and length and shoot number and length were examined in the rooting stage of *A. nezaketiae* explants, all 4 dependent variables (root number 16.52; root length 0.43; shoot number 1.125; shoot length 0.62) for MS medium was found to be statistically significant.

Acclimatization Stage

Eight specimens of *A. nezaketiae* showing the best root and shoot development were selected. It was observed that the plants incubated in the climate chamber could not develop under external conditions.

Result and Discussion

The basal media containing PGR in different concentrations, in which shoot tips and nodes are used as explant sources in the *A. nezaketiae* plant belonging to the genus *Alyssum*, which is known to be a candidate for the critically endangered (CR) category and is endemic to Çankırı, micropropagation is the aim of the study.

As in many plant species, shoot tip, node, hypocotyl, and cotyledons have been used as explant sources in many studies for in vitro propagation of plants belonging to the Brassicaceae family under aseptic conditions (Yıldızhan, 2007; Ceylan, 2015; Özdemir and Yıldırım, 2016; Demir, 2020; Memon and Memon, 2021).

A. nezaketiae, which is locally endemic in Çankırı province, started to be seen in the field at the beginning of the vegetation period of 2021. It was found in full flowering from the middle of May. The flowering plants, which have completed their physiological development, were taken from the field towards the end of May for in vitro propagation and brought to the laboratory. The simple sterilization protocol applied was effective, and infection was rare during the study. After the sterilization step, MS and WPM were added to the basal media. It was ensured that the media were solidified with gelrite.

In the study, plant regeneration was achieved from the shoot tip and node explants of *A. nezaketiae*, but it was observed that the

obtained shoots and roots developed very slowly. As a result of each subculture applied after the initial stage, it was observed that some explants carried out root and shoot development, but the development continued in a limited way. In addition, it is thought that infections that are thought to develop during the planting of explants make the process difficult.

'Basal medium x PGR combinations' of shoot regeneration consisting of callus obtained at the beginning of reproduction in *A. nezaketiae* explants, in interaction (Table 2), MS-KIN (1 mg/L) + IBA (0.25 mg/L) and WPM-KIN (0.5 mg/L) + NAA (0.5 mg/L) shoot formation data were the best results. In addition, shoot regeneration was statistically significant in MS-KIN (1 mg/L) + IBA (0.5 mg/ L) and WPM-BAP (0.5 mg/L) + IBA (0.25 mg/L) was found to be significant. The most successful results were achieved at different BDM doses in WPM. Bayat (2017), added BAP and IAA in different amounts and combinations to the MS medium in his study. It performed shoot formation from healthy callus by subcultures, and determined that the best shoot formation was in MS medium supplemented with BAP (2.0 mg/L) and IAA (0.1 mg/L) from callus obtained from node explants. In our study, WPM was used together with MS.

At the beginning of propagation in explants, the data on the number of shoots in the "basal medium x PGR combinations" interaction (Table 3), MS-KIN (1 mg/L) + IBA (0.25 mg/L) in medium (shoot number 2.6) and WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium (shoot number 5.8), the best values for shoot number were reached. At the same time, MS-KIN (1 mg/L) + IBA (0.25 mg/L) (shoot number 2.4) and WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) (shoot number 5.6) data obtained from shoot numbers were evaluated as statistically significant. Ceylan (2015), examined the effects of only BAP and NAA concentrations in his study with *Crambe orientalis* L., unlike our study. PGR-free MS was used as a source of cotyledon node and hypocotyl explant of plants obtained from seeds cultured in basal

medium. When hypocotyl explants were cultured in MS, the maximum number of explants/mean shoots was 27.34±0.69 in MS-BAP (1.0 mg/L) + NAA (0.25 mg/L). When cotyledon explants were cultured in MS, the maximum number of explants/mean shoots was determined in MS-BAP (2 mg/L) + NAA (0.25 mg/L) (18.14%).

There was no statistically significant difference in the interaction of root numbers between "basal medium x PGR combinations" in *A. nezaketiae* explants. The obtained data revealed that all combinations and media had the same effect on root numbers. Although the data obtained after subculture was meticulously collected, the consistency between the data was not found statistically significant. The best data were obtained in terms of root length in WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium, in the interaction of the lengths of the roots at the beginning of reproduction, "basal medium x PGR combinations". In addition, root length was found to be statistically significant in WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium. Ceylan (2015), *Crambe orientalis* L. var. *orientalis* rooted the regenerated shoots in MS medium containing different concentrations of NAA, but observed that the rooting of the regenerated shoots was difficult in general.

In our study, data on root number and length, shoot number and length that occurred during the rooting stage after subculture applications were obtained and statistically evaluated. At this stage, the effects of KIN and IBA used in different doses and the medium were examined. The effects of media and PGR doses during the rooting stage were found to be statistically significant. In all trials, data on the best root number (root number 21.75) and maximum root length (root length 0.65), and the best shoot number (shoot number 1.4) were obtained in a medium with 1 mg/L dose of KIN and IBA while obtaining; the data obtained did not reveal a significant difference in the effects of BDM doses on shoot length. However, the effect of media on the rooting stage was found to be statistically significant. MS medium was found

to be successful in all trials (root number 16.52; root length 0.43; shoot number 1.125; shoot length 0.62).

For the acclimatization stage, the most successful plants in terms of shoot and root development were selected and transferred to external conditions. Root and shoot development occurred in plants obtained by in vitro propagation of *A. nezaketiae*, but the developments remained within certain limits. Therefore, the plants transferred to the external conditions kept at the optimum level could not develop within the framework of these conditions, so abiotic stress factors could not be applied to the plants.

The goal of "Developing technologies for the characterization of gene resources, conservation and protection of biological diversity" prepared by TUBITAK (The Scientific and Technological Research Council of Türkiye) between 2003 and 2023 and in line with the sustainability of development goals, is also an important issue in the National Science and Technology Policies Strategy Document (Anonymous, 2011). In this study, *Alyssum nezaketiae*, which is known to be a candidate for the CR (Critical Endangered to Endangered) category in terms of distribution criteria, due to its habitat size of around 100 km^2 and the very small habitat boundaries, although it is not yet in the endangered category according to IUCN data, in addition to its aromatic properties in terms of in vitro reproduction (Aytaç and Duman, 2000). This thesis topic, which is a comprehensive optimization study, can be used as a source for in vitro propagation of *Alyssum nezaketiae* and other endemic plants.

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CHAPTER 7

MAREK'S DISEASE (MD)

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INTRODUCTION

Marek's disease (MD) is a lymphoproliferative and highly contagious disease of domestic birds, manifested by demyelination and mononuclear infiltrates in peripheral nerves. The disease was first described in 1907 (Marek, 1907). The causative agent of the disease is Marek's disease virus (MDV) which is a lymphotropic and oncogenic herpesvirus (Biggs, 1975; Buckmaster et al., 1988). MDV, located in the *Mardivirus* genus that separated from the mammalian herpesviruses 131 million years ago, does not cause infection in human beings (Payne, 1999).

The most important feature of MDV is the formation of lymphomas in T cells (Biggs, 1975). In addition, it suppresses immunity and makes animals susceptible to other pathogens (Miles, 2015).

MDV replicates in feather follicle epithelium and is separated horizontally by the dust in the coop (Rispens et al., 1972). A chicken infected with MDV carries MDV throughout its life, sporadically clustering and shedding virus into the coop and its environment (Miles, 2015). Although Marek's disease can be observed in chickens of all ages, it is more common in chickens between 12 and 30 weeks of age (WOAH, 2023). Marek's disease is found in all poultry farming countries (Schat and Nair, 2013). This disease was first detected in Turkey in the 1960s (Atılgan and Yeşilada, 1961; Başkaya and Minbay, 197).

Marek's disease (MD), which has no effective treatment and is tried to be controlled through vaccinations, costs the worldwide poultry industry between one and two billion dollars a year (Hildebrandt et al., 2014). Therefore, control of MD is economically important.

Since its description, MDV has evolved every 10-15 years, the clinical-pathological findings and severity of the disease have changed, and due to increases in its virulence, it has remains important as a

serious problem for the poultry industry, which grows every year. (Witter, 1997; Osterrieder et al., 2006).

HISTORY

Marek's disease has been observed sporadically among chickens and turkeys for many years (Pappenheimer et al., 1929). In 1907, Dr. Joseph Marek who was a Veterinary Clinician and Pathologist at the Royal Hungarian Veterinary School in Budapest described a disease in the wings and legs of 4 roosters as multiple polyneuritis. When he examined the roosters in detail, he noticed that the sacral plexuses were thickened and the spinal tract was infiltrated by mononuclear cells. He later described the disease as an "inflammation of nerves" or a "polyneuritis" (Biggs, 2001). In 1908, a year after Dr. Joseph Marek's report, two researchers named Ellerman and Bang succeeded in reproducing the disease again. Later it was understood that this disease began to spread all over the world after the disease was described in 1921 by Kaupp (who was the first to diagnose eye blindness in sick animals) in the United States of America, in 1924 by Van der Walle and Winkler-Jenius in Netherlands, and in 1929 by Galloway in England (Başkaya and Minbay, 1974; Pastoret, 2004). In these early studies, it was reported that it was a disease condition affecting the nerves, and the disease was defined as polyneuritis, chicken paralysis and neuromyelitis gallinarum. However, since Marek's disease showed similar symptoms to lymphoid leukosis in those years, the two diseases were defined together as the Winged Lymphoid Leukoid Complex (Biggs, 2004).

One of the important turning points in the history of Marek's disease was reported by Pappenheimer et al. (1929). In a study they conducted on 60 chickens, they reported that, in addition to the lesions seen in the nerves, there were lymphoid tumors in the visceral organs such as spleen and ovary of 6 chickens, and they defined this disease as visceral lymphomatosis (Pappenheimer et al., 1929).

Significant increases in mortality were observed in commercial flocks between the 1930s and 1940s. Evaluating these clinical findings that cause an increase in mortality, to eliminate the confusion in terminology, Dr. Biggs suggested at the World Veterinary Poultry Association Conference held in Utrecht in 1960, that this disease be named after Marek, who first described the disease. Although this name was accepted, some researchers insisted on calling it Avian Leukosis Complex, based on the pathological symptoms and lesions observed (Biggs and Payne, 1964). Later, by Churchill and Biggs (1967) and Nazerian et al. (1968), the etiological agent of the disease, herpesvirus, was isolated and named "Marek's disease virus" (MDV). With the isolation of the agent, it was accepted that Marek's disease was a different disease from lymphoid leukosis and interestingly 60 years after the disease was first described, the confusion in terminology has been resolved (Biggs, 2004). Figure 1 describes a summary of four decades of Marek's disease research.

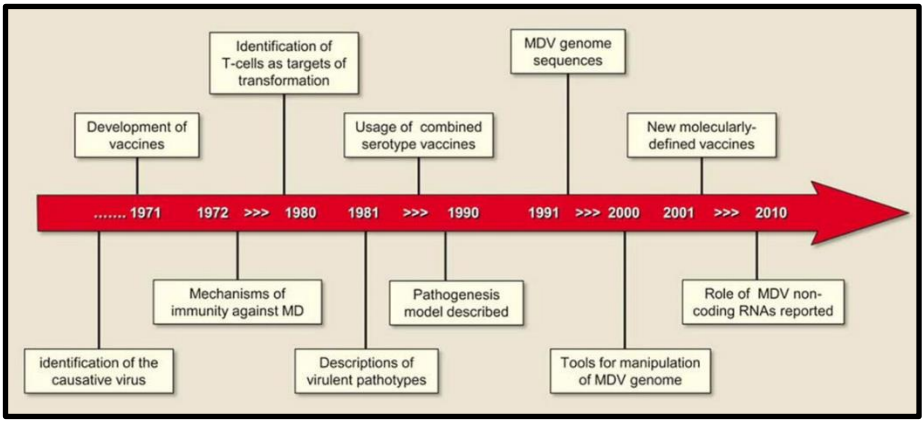

Figure 1. Summary of 40 years of MD research (Biggs and Venugopal, 2012)

ETIOLOGY

According to the latest classification made by the International Committee on Taxonomy of Viruses (ICTV, 2023), MDV is included in the *Herpesvirales* order, *Herpesviridae* family, *Alphaherpesvirinae*

subfamily and *Mardivirus* genus (Marek's disease-like viruses) (Witter et al., 2005).

While MDV was initially included in *Gammaherpesvirinae* due to its biological properties, it was reclassified in the *Mardivirus* genus after whole genome analyzes were completed in 2002 (Tulman et al., 2000). To date, this genus consisted of 4 species: Gallid herpesvirus 3 (GaAHV3), Meleagrid alphaherpesvirus 1 (MeAHV1) [commonly known as turkey herpesvirus (HVT)], Anatid alphaherpesvirus 1 (AnAHV1), and Columbid alphaherpesvirus 1 (CoAHV1) is. Today, GaHV-2 (Gallid herpesvirus-2) is called serotype 1, GaHV-3 (Gallid herpesvirus-3) is called serotype 2, and MeHV-1 (Meleagrid herpesvirus-1) is called serotype 3 (Osterrieder et al., 2006; Pandey et al., 2016; ICTV, 2023). They are divided into serotype 1 oncogenic, serotype 2 and serotype 3 non-oncogenic (Witter and Schat, 2003). These three serotypes of MDV are classified as follows in The World Organisation for Animal Health according to their antigenic properties (WOAH, 2023):

1. Serotype 1: All pathogenic strains of MDV; virulent plus (e.g., 648A), very virulent (e.g., Ala-8, Md/5, Md/11, RB-1B), virulent (e.g., JM GA, HPRS-16), slightly virulent (e.g., Conn A, HPRS-B14), and finally weakly virulent (e.g., CVI-988, CU-2) are in this serotype.

2. Serotype 2: Naturally avirulent MDV strains (e.g., HN-1, SB-1, 301B/1, HPRS-24,) are in this serotype and many of them have been shown to protect against virulent strains.

3. Serotype 3: Naturally avirulent HVT strains (e.g., FC126, PB1) are in this serotype and generally used as a monovalent vaccine or polyvalent vaccine as combinations with serotype 1 and 2 strains (WOAH, 2023).

By DNA sequencing, it was determined that the gene contents of these three serotypes were close. It was determined that the MDV serotype 1 had 103 genes, the MDV serotype 2 had 102 genes, and the MDV-3 serotype had 99 genes. But the ratios of guanine and cytosine

(%G/C) between these serotypes vary. MDV serotypes 1, 2 and 3 contain 44.1%, 53.2% and 47.2% G/C, respectively (Lee et al., 2008).

MDV is a virus with a linear, double-stranded DNA (dsDNA) of 160-180 kbp in size (Witter et al., 2005). Virions are observed usually within the nucleus and sometimes in the cytoplasm (Schat and Nair, 2013). Virus particles with hexagonal nucleocapsid have a diameter of 85-100 nm, and enveloped virus particles have a diameter of 150-160 nm (Calnek et al., 1970). MDV consists of an inner core, outermost envelope (containing 15 glycoproteins) and a capsid with 256 capsomeres. It has a layer of protein called tegument between the envelope and the capsid (Figure 2) (Poonam et al., 2017).

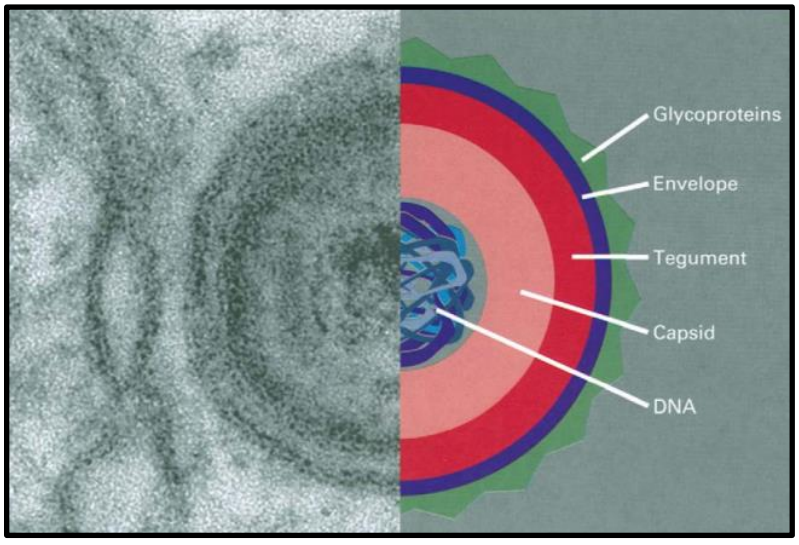

Figure 2. Electron microscope image of an enveloped MD virus (Baigent and Davison, 2004)

Three different gene groups have been identified in the genome of MDV. The first group of genes are responsible for oncogenicity and pathogenicity, and these are named as *meq* (EcoRI-Q), phosphoprotein pp38 and 132 bp repeat gene region (Gimeno et al., 2004; Nair and Kung, 2004; Lee et al., 2008). The *meq* gene encodes a protein consisting of 339 amino acids (Tulman et al., 2000). Additionally,

MDV has vIL8 (viral interleukin 8), which plays a role in pathogenesis (Gimeno et al., 2004; Lee et al., 2008). The vIL8 gene is absent in serotype 2 and HVT (Parcells et al., 2001). The second group of genes encodes glycoproteins. The third group encodes all other genes (Nair and Kung, 2004).

The DNA of MDV is similar to the DNA of herpes simplex virus (HSV) and includes two distinct regions called U_L (unique long region) and U_S (unique short region) which are formed by long inverted repeat regions (TR_L and IR_L) and short inverted regions (TR_S and IR_S). By comparing the fragments of pathogenic and nonpathogenic MDV DNAs when cut with restriction enzymes, the region where the viral DNA is different between these two types of MDV was identified as the region that may be responsible for tumor formation in chickens. It has been reported that in nonpathogenic viral DNAs, a particular expansion is observed in the long inverted TRL and IRL regions localized to BamHI-D and -H. (Fukuchi et al., 1985). Insertion of retroviral long terminal repeats (LTR) into the host genome results in altered host responses to MDV leading to carcinogenesis and metastasis (Cui et al., 2016). The entire genome of MDV is shown schematically in Figure 3.

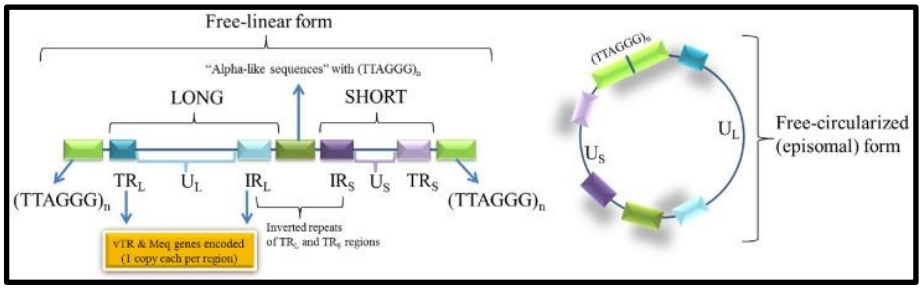

Figure 3. Schematic view of the complete genome of MDV (Pherson and Delany, 2016)

EPIZOOTOLOGY

Hosts of Marek's disease

Chickens have been identified as the most important natural host for Marek's disease. Quail, turkey, pheasant, duck and some geese species have also been reported to be susceptible to the disease. Experimental inoculations in various mammalian species, including primates, have shown disease resistance (Nair et al., 2008).

Natural outbreaks of MD are reported to be common in commercial Japanese quail flocks. Lymphomas may develop in the visceral organs in affected animals. However, peripheral nerves are rarely affected. The mortality rate can reach 10-20%, but deaths occur relatively late (Kobayashi et al., 1986; Imai et al., 1990; Pennycott et al., 2003).

Serious outbreaks of MD were reported in commercial turkey flocks in Europe (Coudert et al., 1997; Voelckel et al., 1999; Pennycott and Venugopal, 2002) and in Israel (Davidson et al., 2002) between 1997 and 2002. In some cases, affected turkey flocks were found to be raised in close proximity to broiler flocks. Paralysis has been observed in some animals, and peripheral nerves have occasionally been infiltrated with lymphocytes. Mortality caused by tumors reached 40-80% between the ages of 8-30 weeks (Coudert et al., 1997; Davidson et al., 2002).

The disease is not yet endemic within commercial turkey populations. In experimental studies, MDV infections resulting in mortality of up to 70% were encountered (Sandelini and Estola, 1974; Witter et al., 1974).

In experimental studies, no clinical disease occurred in ducks inoculated with MDV. However, the presence of antibodies has been observed (Baxendale, 1969). Recently, MD cases in white-fronted geese has been reported in Japan (Murata et al., 2007). Subsequent analysis revealed the presence of the *meq* gene in feather tips in the

majority of this species and in several different duck species (Murata et al., 2012).

Spread of Marek's disease, carriers, vectors

Marek's disease virus (serotype 1) is transmitted directly or indirectly between chickens through the air (Biggs, 1985). Feather follicle epitheliums can carry and produce infectious virus (Calnek et al., 1970). Chickens in the coop constitute the source of infection for the spread of these epithelial cells carrying MDV throughout the chicken coop. Contaminated poultry dust can be a source of infection for at least several months at 20°C-25°C and for years at 4°C (Calnek, 1986). Once MDV enters a chicken flock, vaccination and genetic resistance are not effective and the infection is quickly transferred from animal to animal (Kenzy and Biggs, 1967; Nair et al., 2008).

It has been determined that MDV is not transmitted vertically through the egg. However, it has been reported that day-old chicks and broilers can be infected horizontally by the tools, equipment and personnel within the shelter production systems.

Migratory birds, ducks and white-footed geese have been reported to be carriers of MDV. Chicken red mites have also been identified as carriers of poultry pathogens, but it is unclear whether they carry MDV. The transmission model of MD is shown in Figure 4 (Boodho, 2016). It has been reported that litter mites, flies and coccidial oocysts do not transmit MDV, but black beetles transmit it passively (Brewer et al., 1969; Beasley and Lancaster, 1971).

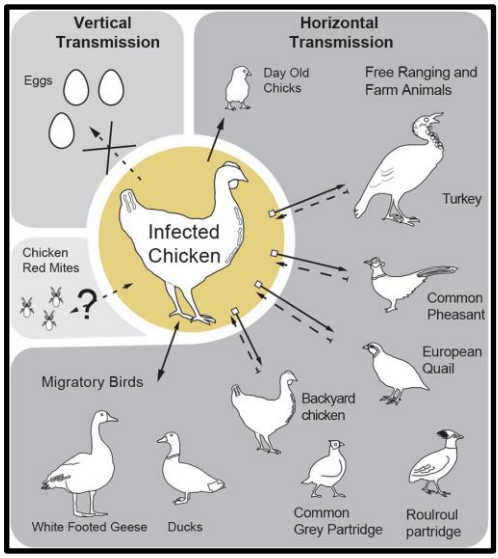

Figure 4. Vertical and horizontal transmission pattern among poultry species (Boodho, 2016).

Studies have shown that horizontal transmission occurs between turkeys and chickens (Coudert et al., 1997). Another study reported the transfer of MDV from chickens to quails and from quails to chickens (Dutton et al., 1973; Witter and Schat, 2003). Marek's disease spreads unevenly, being more common in some areas and less common in others and it has been reported that vaccination applications are effective in preventing this as well (Biggs and Payne, 1967).

Incubation period and incidence of Marek's disease

The determination of the incubation period in Marek's disease occurring under natural conditions has not yet been determined precisely because the time of infection with the virus is not known exactly. In experimental studies, it has been determined that incubation period is between 21-28 days, although it varies from individual to individual. However, it is reported that in some individuals the process may take up to 2-3 months (Biggs and Payne, 1967). It has been found that clinical findings and pathological lesions generally appear 3-4 weeks after experimental inoculation (Schat and Nair, 2013). MD

outbreaks sometimes occur in unvaccinated 3- to 4-week-old laying hens in the field and most serious cases occur after 8-9 weeks of age (Morrow and Fehler, 2004).

It has been determined that adult chickens are more resistant to Marek's disease than young ones (Witter et al., 1974). This disease is encountered at all ages, at the age of 3-4 weeks or later, most commonly at the age of 12-30 weeks (WOAH, 2023). The onset of MD in broilers is especially between 4-6 weeks of age (Okwor and Eze, 2011). In layer flocks, it usually occurs at 10-12 weeks of age. This is also an indication that the tumor rate will reach its highest level at the age of 30 weeks, when the ovulation period reaches its peak (Zhuang et al., 2015).

Many factors affect the incidence of MDV. These are age at exposure to MD virus, genetic makeup, level of maternal antibodies, virulence of the virus strain, gender of the host, and complicating factors (e.g., infection with other immunosuppressive agents) (Miles, 2015). Generally, females are more prone to tumor formation. The presence of maternal antibodies delays and reduces paralysis and mortality but cannot prevent the spread of MDV in tissues several days after exposure (Calnek, 1972; Payne and Rennie, 1973).

It has been reported that morbidity in MD varies between 10-50%, and mortality may initially reach 80% and then decrease (Frederick et al., 1999). In the classical form of the disease, mortality rarely exceeds 10-15% and can sometimes occur after a few weeks or months. In acute form, the incidence of MD is between 10-30%, sometimes this rate can reach 70%. Mortality may increase rapidly over a few weeks and then stop, or it may continue at a steady or slowly decreasing rate for several months. In the acute form, poultry often become severely depressed and some of the animals may die without showing clinical signs of disease (WOAH, 2023).

Since it is not a notifiable disease, reporting systems of the countries differ and it is difficult to determine the true incidence of MD

(Schat and Nair, 2013). According to WOAH data, it is common all over the world (Couteaudier and Denesvre, 2014). According to current data from the WOAH, cases of MDV infection have been reported in approximately half of the world's countries and are schematized in Figure 5 (Boodhoo et al., 2016).

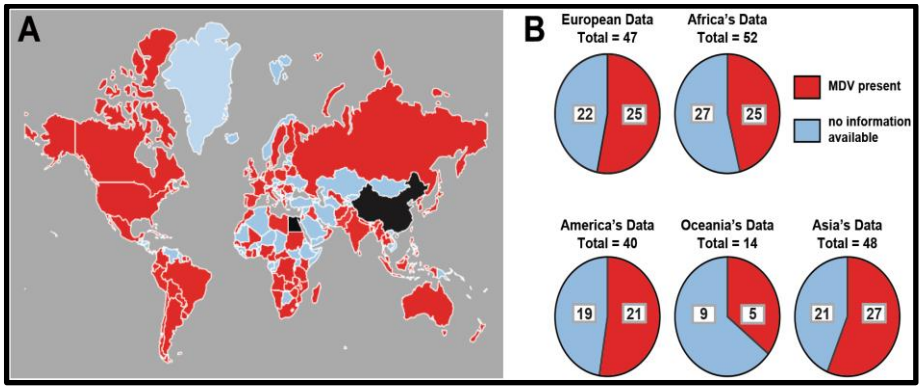

Figure 5. Distribution map of MD virus worldwide (Boodhoo et al., 2016). The map was created based on information shared through the World Animal Health Information Database (WAHIS). Red areas show areas where MDV was reported before 2009 and between 2009-2016, and blue areas show areas where MDV was not reported. A) World map depicting MDV positive countries. Both China and Egypt (black) are endemic regions with annual MDV outbreaks. B) Circular graphs show the number of countries reporting MDV cases to the WOAH according to their continents

Various studies have shown that Marek's disease is common in Turkey (Köküuslu and Özkul, 1975; Aydın et al., 1991). In the study conducted by Alkan and Bayraktar in Ankara, Istanbul, Izmir, Konya and Manisa provinces in 1994, it was reported that Marek's disease was the second most common viral disease in poultry and was most frequently encountered in Ankara and Konya provinces (Alkan and Bayraktar, 1995).

PATHOGENESIS

The pathogenesis of Marek's disease has very different distinguishing features (Venugopal, 2018). Four phases have been defined in infections caused by MDV that lead to lymphoma formation:

a) early cytolytic phase; b) latent phase; c) second cytolytic phase and cytolytic infection in feather follicle epithelium; d) transformation and development of B lymphomas (Schat and Nair, 2013). Although they are sequential, these stages do not have to occur separately from each other. While the first two phases are sharply separated from each other, both latent and transformation phases can exist mixed with cytolytic infections in different cell populations, and lymphomas occur in the later stage (Calnek, 2001).

Serotype 1 (MDV) enters the body through breathing. The virus first begins to multiply in the epithelial cells of the respiratory tract, undergoes replication in the lungs, and passes from the lungs to lymphoid tissues such as bursa Fabricius, thymus and spleen with the help of macrophages (Nair, 2005). With the involvement of macrophages, cell-dependent viremia begins to occur (Venugopal and Payne, 1995; Witter and Schat, 2003; Nair et al., 2008). Viral interleukin-8 (IL-8) is effective in the transfer of the virus from cell to cell (Cortes and Cardone, 2004; Engel et al., 2012). Cytolysis occurs in these organs after 3-6 days and thus the early cytolytic infection period begins (Schat, 1981; Venugopal and Payne, 1995). Although it has been reported that the main target of cytolytic infection is B lymphocytes, T lymphocytes are activated and an inflammatory response is formed as a result of the immune reaction (Delecluse et al., 1993; Venugopal and Payne, 1995). At this stage, it is stated that immunosuppression occurs, in which immunity is suppressed as a result of atrophy in the thymus and bursa Fabricius (Payne and Venugopal, 2000; Witter and Schat, 2003). Near the end of the cytolytic phase, MDV is transported to the feather follicle epithelium by infected lymphocytes, and virus shedding begins after an average of 10 days (Calnek et al., 1970; Nair, 2005).

After the cytolytic phase, MDV enters the latent period in infected lymphocytes. This stage is sometimes seen in CD4+ T cells and sometimes in CD8+ T cells and B lymphocytes. It develops in

approximately 6-7 days. Apoptosis occurs in T cells during the latent phase. Tumor formation does not occur (Morimura et al., 1995; Osterrieder et al., 2006). Although the virus is present in the cells, virus-related antigens are not seen by immunohistochemical staining. It has been reported that the latent phase is also formed in infection with non-oncogenic serotypes (Shek et al., 1983).

After the latent phase, the late cytolytic period begins in the 2nd-3rd weeks of the disease (Witter and Schat, 2003). This phase is not very detailed in studies and does not always occur. The duration and development of this phase depends on the virulence of MDV and the genetic resistance of the host (Schat and Nair, 2013). Lymphoid organs, including the kidney, pancreas, glandular stomach, and feather follicle epithelium are affected, and mononuclear lymphocytic cell infiltrates and atrophy are observed in these organs (Witter and Schat, 2003). Permanent immunosuppression occurs simultaneously with cytolytic infections at this stage (Calnek, 2001).

The transformation phase occurs in infections with serotype 1 (Miles, 2015). At this stage, the main targets are CD4 + and CD8 + T lymphocytes (Schat et al., 1991; Burgess and Davison, 2002). It has been reported that 75% of lymphomas seen in internal organs originate from T lymphocytes, while 15% originate from B lymphocytes (Rouse et al., 1973; Payne and Rennie 1976). At this stage, active T lymphocytes in the lymphoid organs (thymus, spleen, bursa Fabricius) undergo degeneration. As a result of the degeneration of B lymphocytes, the activation of T lymphocytes results in the transformation of helper T lymphocytes as a result of the immune response that develops against the immature virus particles found in B lymphocytes (Witter and Schat, 2003; Baigent and Davison, 2004; Keleş, 2007). Transformed T cells proliferate in peripheral nerves, internal organs and tissues, and this continues until tumor formation (Calnek and Witter, 1997). The pathogenesis of MD is schematized in Figure 6.

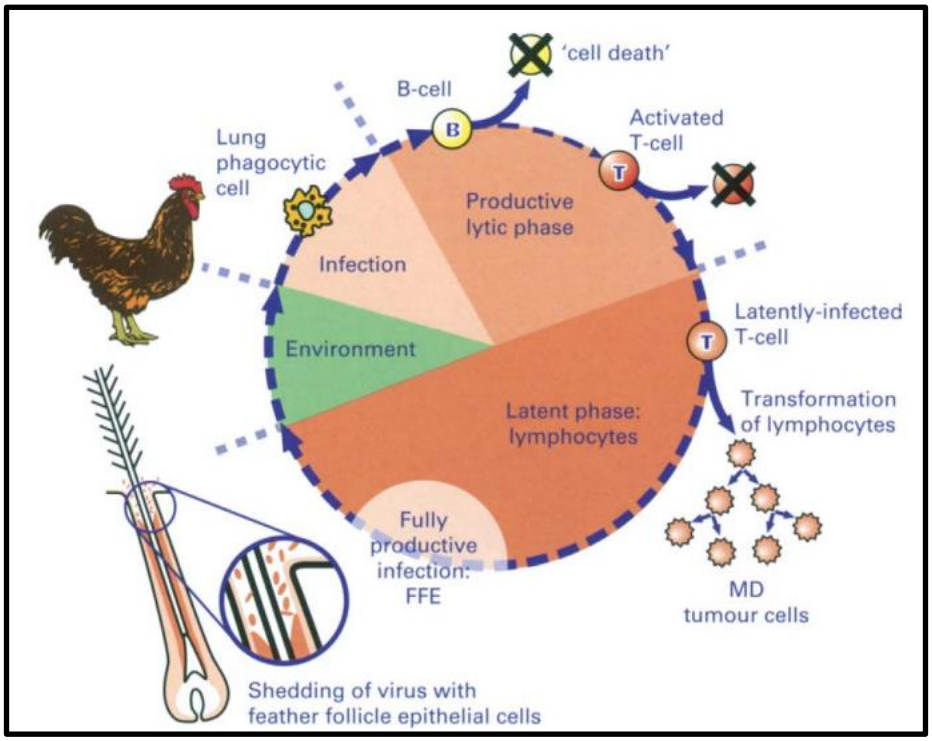

Figure 6. Schematic representation of the pathogenesis stages of Marek's disease (Baigent and Davison, 2004)

It has been observed that in those who underwent embryonal bursectomy (removal of all B cells) before MDV infection, cytolytic infection did not occur, but latent infection and lymphoma formation occurred (Schat, 1981). It has been reported that nitric oxide may play an important role in the control of MDV replication (Jarosinski et al., 2002).

CLINICAL FINDINGS

In poultry coops infected with serotype 1 (MDV), it has been observed that there are generally four clinical forms of the disease, and it has been reported that the clinical symptoms specific to each form vary (Payne and Venugopal, 2000):

a) Classical or nervous form of MD: It is mostly characterized by paralysis of nerves and the most common clinical manifestation of MD is partial or complete paralysis of the legs and wings. The characteristic finding is enlargement of one or more peripheral nerves. The vagus, brachial and sciatic nerve plexuses are the most affected ones. Affected nerves often enlarge two or three times their normal thickness and have a normal, cross-striated and shiny appearance. The nerves may also appear gray or yellowish, and sometimes they may be edematous. Legs and wings become paralyzed because of the expansion of peripheral nerves, but nerve involvement is sometimes not seen in adult birds (WOAH, 2023).

Chickens whose leg nerves (*Nervus ischiadicus*) are affected extend one leg forward and the other back, which is specific to Marek's disease, and called "*fowl paralysis*" or "*range paralysis*" and its Turkish equivalent is "*ballerina sitting*", due to paralysis (Atılgan and Yeşilada, 1971; Calnek and Witter, 1997; Witter and Schat, 2003). It is usually seen in chickens between 2 and 12 weeks of age (Payne and Venugopal, 2000).

As a result of lymphocytic infiltration into the iris, blindness and anomalies occur in the eyes, and this condition is defined as "*grey eye*". Mostly, these anomalies are seen in adult chickens aged 16-18 weeks (Ficken et al., 1991; WOAH, 2023).

b) Acute form of MD: Typical lesions in visceral organs appear as lymphomatous lesions in the ovaries, liver, spleen, kidneys, lungs, heart, proventriculus and skin depending on the strain of MDV. Tumors produced by MDV may also resemble and are important to differentiate from tumors induced by retroviral pathogens such as avian leukosis virus and reticuloendoteliosis virus (WOAH, 2023).

c) Temporary paralysis form of MD: It is a rare condition. It usually occurs between the ages of 5 and 18 weeks. It is a temporary paralysis that begins suddenly with brain inflammation and ends after

24-48 hours. In some cases, death may occur (Payne and Venugopal, 2000).

d) Acute mortality form of MD: This form is thought to be caused by very highly virulent strains and death occurs in affected animals before the onset of lymphomas. Atrophy of the bursa Fabricius and thymus is characteristic in affected animals (Payne and Venugopal, 2000).

DIAGNOSTIC METHODS

The main diagnostic methods used to determine the presence of MDV are; the isolation of the virus, the demonstration of viral nucleic acid or antigens in the tissue, and the detection of the antibodies (Schat and Venugopal, 2013; WOAH, 2023). Immunoperoxidase and immunofluorescence tests were used in various studies conducted for immunohistochemical analysis (Bülow and Biggs, 1975; Özbilgin et al., 2001). Nowadays, molecular methods (PCR, Real Time PCR, etc.) are used for diagnosis (Davidson et al., 1995; Afonso et al., 2001; Handberg et al., 2001; Baca, 2002; Islam et al., 2006; Baigent et al., 2011; Gimeno et al., 2014; Baigent et al., 2016).

MDV isolation

Marek's disease virus (serotype 1) can be isolated from the tissues of infected animals (WOAH, 2023). When cells are infected with MDV, their morphology changes and this change is called cytopathic effect (CPE). In-vitro culturing of serotype 1 was first performed by Churchill and his colleagues (Churchill and Biggs, 1968). Generally, spleen cells, lymphoma suspensions, and buffy coat cells from heparinized blood are used as sources for isolation. MDV is usually produced and tested in young chickens, embryonated eggs and tissue cultures. Chicken kidney cells or duck embryo fibroblasts cell cultures are the best options for isolation and propagation of new strains. It is reported that CPE as plaque formation, appears 3-5 days

after the infected inoculum is transferred to cell culture, but these plaques can be counted within approximately in 7-10 days. CPE effects that occur during virus isolation can sometimes be due to contamination or nutritional deficiency, and evaluation of cell culture requires expertise (WOAH, 2023).

Serological tests

Detection of the MDV antibodies in approximately 4 weeks old chickens can be considered as an indicator of infection. Before this age, such antibodies may represent maternal transmission of the antibody via the egg yolk and cannot be considered as an active infection (WOAH, 2023).

Due to the presence of common antigenic determinants between vaccine virus strains and the glycoproteins of oncogenic MDVs (serotype 1), it is not possible to distinguish whether the source of infection is vaccine or field type strains by displaying antibodies determined using conventional serological methods. Although serotype discrimination can be made in an infection by restriction endonuclease, in situ hybridization analyzes and serological analyses, it is not possible to distinguish between field (pathogenic) serotype 1 and attenuated serotype 1 used as a vaccine (Endoh et al., 1994; Fadly et al., 1996; Garcia et al., 1996). Serological tests such as ELISA, Agar Gel Immunodiffusion, Direct and Indirect Fluorescent Antibody are used in the diagnosis of MD (Kutsal, 1989; WOAH, 2023).

Hispathological methods

In the diagnosis of Marek's disease clinical, macroscopic and histopathological findings, as well as immunohistochemical (immunoperoxidase and immunofluorescence test) tests have been widely used for diagnosis from the time the disease was identified until today. Although histopathology test is still used as the "gold standard" test, it is a time-consuming method and requires expertise (Helmboldt,

1972; Bülow and Biggs, 1975; Özbilgin et al., 2001; Keleş, 2007; Çiftçi et al., 2011).

In MD mononuclear infiltration is observed in the skin, muscles, gonads, visceral organs, iris and peripheral nerves (Schat and Venugopal, 2013). These infiltrated organs of dead animals are used for diagnostic purposes. In histopathology, heterogeneous lymphoid cell populations within MD lymphomas are observed in Hematoxylin-Eosin stained sections or by May-Grünwald-Giemsa staining of smear preparations made from lymphomas. In MD-induced lymphomas, the presence of T cell antigens (MD tumourassociated surface antigen-MATSA) on MD tumour cells has been detected (Çarlı, 2018).

Two types of lymphotropic lesions, called type A and type B, are seen in peripheral nerves. Type A lesions are neoplastic and mostly contain CD30+ and CD4+ T cells. Inflammation and edema are seen in type B (Burgess et al., 2001; Witter and Schat, 2003). In some cases, although there is not much edema, an increase in the number of Schwann cells and fibroblasts is observed. Macrophages are also encountered (Biggs and Payne, 1967).

Molecular techniques

Serological techniques are not sufficient to detect the effectiveness of vaccination or the presence of different strains in poultry houses. Therefore, other methods that provide the necessary viral detection and quantification should be used.

Molecular tests enable the detection of Mardiviruses in feather and blood samples and make it possible to distinguish serotypes (Baigent et al., 2011) and can even detect the pathotype of serotype 1 strains (Becker et al., 1992; Davidson and Borenshtain, 2003). These molecular techniques include quantitative polymerase chain reaction techniques (qPCR) which are highly sensitive for absolute quantification of GaHV-2 (Baigent et al., 2005), GaHV-3 (Renz et al., 2006) and MeHV-1 (Islam et al., 2006). Through this technique, it is

possible to determine the average number of viral genomes per cell by serotype. Only the distinction and presence of three serotypes can be determined with techniques such as conventional PCR (Becker et al., 1992).

In their experimental studies Zhu et al. (1992) obtained 2-3 copies of the target region in the DNAs obtained from tissue samples of animals which they infected with oncogenic strains, while they obtained many copies of the target region in the DNAs of animals which they infected with non-oncogenic strains.

The gene region of MDV responsible for oncogenicity can be detected by molecular methods, and oncogenic and non-oncogenic serotypes can be distinguished from each other (Zhu et al., 1992; Lee et al., 2000; Afonso et al., 2001). While 1-3 copies of the target region are determined in oncogenic MDV strains, 4-9 copies are detected in the viruses used as vaccine strains (Davidson et al., 1995).

PREVENTION AND CONTROL STRATEGIES

Various methods have been developed to prevent Marek's disease. Vaccination represents the main strategy for the prevention and control of MD today and in the near future. It is an important achievement that effective vaccines have been developed and put into use in controlling MD (Churchill et al., 1969; Rispens et al., 1972; Nair and Kung, 2004). However, genetic resistance and biosecurity are important supports of properly implemented vaccination procedures (Miles, 2015).

Genetic resistance

Chickens' MD sensitivities vary depending on their innate characteristics. Studies have been carried out to develop disease-resistant chicken lines by identifying the genes that cause the change in susceptibility. An example of these studies is the study conducted by Hutt and Cole in 1935 (Hutt and Cole, 1947). It has been determined that resistance to MD is encoded by major histocompatibility complex

(MHC) genes (Kaufman et al., 1999). It has been determined that these genes are based on three MHC loci. These are B-F (class I), B-L (class II) and B-G (class IV) (Briles et al., 1983). In recent studies, a new region known as Rfp-Y, which is non-MHC and associated with resistance to MD, has been identified (Lakshmanan et al., 1998).

Vaccination

A vaccine against Marek's disease was first developed by Churchill and colleagues shortly after the description of the disease in 1969 (Churchill et al., 1969). It is administered at one-day-old age or by in-ovo vaccination to provide protection against Marek's disease (Rispens et al., 1972; Powell, 1975). Different MD vaccines are in common use, both alone and in various combinations.

Three types of vaccines have been developed for use against MD. The first of these, turkey herpes virus (HVT) was produced in the 1970s. In the early seventies, the spread of MDV which was considered to be responsible for mortality and morbidity in poultry coops was prevented by HVT vaccinations against MDV (Pastoret, 2004). More effective vaccines were needed because the continuous evolution of MDV strains towards higher virulence caused a concomitant decrease in vaccine efficacy (Witter, 1997; Osterrieder et al., 2006). Therefore, bivalent vaccines (HVT+serotype 2) were used secondly. Finally, the CVI988 (Rispens) attenuated serotype 1 (MDV) vaccine was developed (Gimeno et al., 2014). CVI988/Rispens, which provides superior protection against high virulence strains, is used all over the world.

In Turkey, vaccines licensed from the Ministry of Agriculture and Forestry [Vectormune HVT NDV Rispens, Cevac MD HVT Rispens, Innovax ND HVT, Nobilis Rismavac (CVI988) + CA (HVT) 126, Marek's disease Vaccine Serotype 3, Marek's disease Vaccine Serotype 1 and 3, Poulvac Marek CVI, Poulvac Marek CVI + HVT] are used in the fight against Marek's disease and layer flocks are vaccinated with vaccines in the hatchery when they are day-old chicks.

It has been reported that mortality rates reach 25-30% and sometimes 60% in cases where vaccination is not performed, and that it decreases to less than 5% with vaccination (Calnek and Witter, 1997; Witter and Schat, 2003; Meydan, 2012) but still, MD epidemics cannot be completely prevented.

MDV vaccine strains provide highly effective protection against tumors and mortality, providing lifelong immunity and preventing persistent infections. But the vaccine cannot completely prevent virulent viruses, superinfections, replication and spread of the disease (Davison and Nair, 2005; Gimeno, 2008), so chickens can potentially be infected with both vaccine and virulent MDV strains. Distinguishing between oncogenic virus (serotype 1) and vaccine virus is important for evaluating the protection of the vaccine and diagnosing the disease (Gimeno et al., 2014).

Detecting and measuring vaccine and virulent virus in the same chicken and distinguishing between them is important to provide and monitor effective protection. It is necessary to confirm the effective management and replication of the vaccine virus and to identify the causes of poor vaccine uptake. Additionally, detecting the presence or absence of virulent virus in a poultry house helps determine the frequency of Marek's disease in the poultry house and surrounding animals (Baigent et al., 2016).

If chickens are vaccinated with HVT, they can be easily distinguished from virulent MDV-1 strains by real-time quantitative PCR test due to the genetic difference between them. But if chickens have been vaccinated with the CVI988/Rispens vaccine, it is very difficult to tell the difference. Because the vaccine strain is 98% genetically similar to the virulent MDV-1 (Spatz et al., 2007).

This chapter is extracted from master thesis of Dr. Özlem KARDOĞAN entitled "Multiplex Quantitative Real-Time PCR developing rapid diagnostic kit for the detection of discrimination and Marek's Disease vaccine used in Turkey" (Master Thesis, Ankara University, Ankara, 2019).

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CHAPTER 8

THE CONCEPT OF 'ECOTONE' IN BIOLOGY

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INTRODUCTION

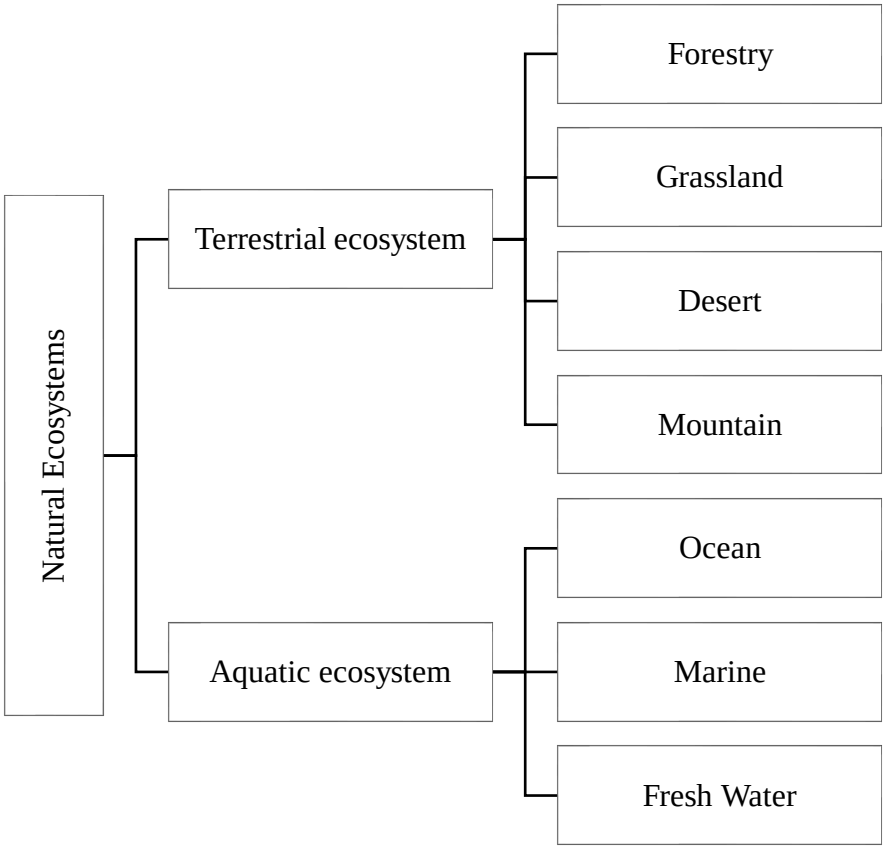
The ecosystem includes all the organisms living in the community and the environmental conditions in which they exist, namely abiotic factors. Ecosystems can vary in size, such as deserts, lakes, and forests. Ecosystems are composed of non-living components including producers, consumers, decomposers, and abiotic factors. Producers are the most important among the living elements of the ecosystem. they are also commonly referred to as primary producers. Consumers, mostly animals, are divided into primary and secondary consumers. decomposers are composed of bacteria and fungi and are important components of every ecosystem.

In ecosystems, living and non-living elements connect with each other with energy flow, chemical substance cycles and population controls. The boundaries of ecosystems are never fixed and sharp in nature, so they are called open systems. ecosystems change in a progressive or regressive direction. Every ecosystem has a dynamic, and these dynamics are changed by activities such as birth, development, death, decomposition events and matter flow. The human factor is also very effective on ecosystems. Especially ecosystems where human influence is high are called anthropogenic ecosystems. Ecosystems on Earth can extend from a very small area to very large areas, from the oceans.

The ecosystems that make up ecotones are diverse. While natural ecosystems belonging to the habitats in water are called aquatic ecosystems, ecosystems in terrestrial areas are called terrestrial ecosystems (Table 1). Terrestrial ecosystems are also called under the title of biome. biomes on Earth from the poles to the equator; They are tundras, coniferous forests of northern regions, broadleaf forests of temperate regions, evergreen forests, temperate-zone grasslands, savannas, deserts, chaparral, and tropical rainforests. aquatic ecosystems are divided into groups as marine ecosystems, lake ecosystems and oceanic ecosystems. Since aquatic ecosystems are

divided into sub-units such as fresh water, salt water, river, and lake ecosystems, they show quite a variety of features. Ecosystems are generally referred to as terrestrial and aquatic, but there are ecosystems that have been modified by human influence. Also called agroecosystems, agricultural ecosystems, agro-forest systems, plantation forests, urban-industrial technoecosystems are also ecosystems modified by human factor.

Table 1. Classification of natural ecosystems



Special areas located in the border region of two or more habitats or ecosystems that are distinctly different from each other are called ecotones (Figure 1). Ecotones are important transition zones as they are the meeting point of different ecosystems and new features not found in neighboring ecosystems are seen in these areas (Odum and Barrett,

2008). The strength of the interactions between time, space and adjacent region is important in ecotones that are transitional regions (Shea et al., 2022). In the literature, the terms ‘community boundary’, ‘edge’ and ‘transition zone’ are also used synonymously with ecotone (Lloyd et al., 2000).

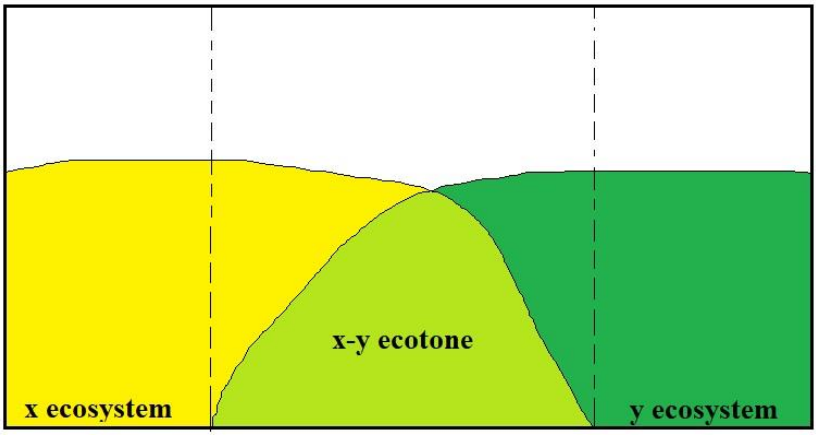


Figure 1. Conceptual model of ecotone

Ecotones are special areas of interest to ecologists. Research on ecotones in aquatic and terrestrial life began to gain momentum in the 1990s (Kolasa and Zalewski, 1995). Two different concepts, *ecotone* and *ecocline*, are used in the analysis of boundaries between plant communities (Attrill and Rundle, 2002), and the differences between them have been widely discussed by some researchers (Van der Maarel, 1990). Ecotone is the gradient shift in physicochemical properties between two ecosystems, while ecocline represents a new region formed by the merger of two identical ecosystems. The differences between these concepts are given in Table 2.

Table 2. Differences between ecotone and ecocline concepts (Attrill and Rundle, 2002)

Ecotone	Ecocline
defined earlier	defined more recently
contains homogeneous communities	contains heterogeneous communities
boundaries between ecosystems are clearer	boundaries between ecosystems are less clear
an area of relatively rapid change	more gradual, progressive change
highly dynamic	less dynamic than ecotone
usually unstable	more stable
well defined in terrestrial systems	rarely applied outside of the terrestrial area
transitions are more distinctive	transitions are less distinct

The unique environments of the ecotone allow for the formation of specialist ecotonal species not found in adjacent environments (Odum 1983; Neilson et al. 1992). In addition, ecotones show greater species diversity than adjacent areas, and these areas provide more opportunities for the invasion of exotic species due to tensions between neighboring communities (Lloyd et al., 2000). The presence of dispersal input from neighboring communities is also very important in the high plant species richness and abundance in ecotones (Kark, 2013). The species richness seen in ecotones is called the *edge effect*. The edge effect, an important ecological process, was first studied in 1942 (Beecher, 1942). Narrow and definite habitat transition areas are called *edge*, and species that prefer to breed and survive on the margins of the community are called *edge species*.

Some species living in an ecotone can use two neighboring communities while surviving. For example, a bird living in the X-Y ecotone can use the X ecosystem for nesting and the Y ecosystem for feeding. The best habitat for this bird species is the X-Y ecotone. Also, some game animals are often referred to as edge species. In the habitats of these creatures, vital activities such as finding food and reproduction are supported by shaving in the forests. Although it is generally

mentioned that the diversity of species and the number of individuals increase in ecotones, this is not always and everywhere true. The best example of this is the diversity of tree species and the number of trees in forest edge ecotones are less than in forest areas (Odum and Barrett, 2008).

Various examples can be given of ecotones, which are the meeting point of different ecosystems (Figure 2):

Lake-terrestrial ecotones are one of them, and they are in the transition area between aquatic and terrestrial ecosystems, which is very important for pollutants to enter natural lake water systems (Zhang et al., 2012). Water-terrestrial ecotones in lakes capture and degrade some pollutants (N, P, organic pollutants) in the water (Syversen, 2002). In addition, ecotones are very important in maintaining the water quality of lakes and basins. In addition to these positive features of lacustrine-terrestrial ecotones, there are also various negative features. This ecotone area is the most vulnerable parts of lakes, as it is exposed to human activities and natural processes on the coast (Zhu et al., 2023).

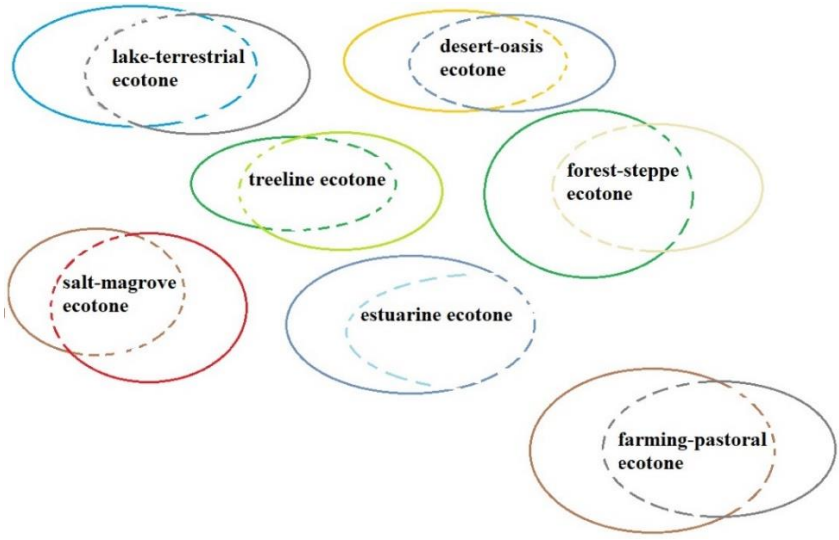


Figure 2. The types of ecotones

The treeline ecotone represents the transition zone between forest and treeless areas (Kaplan and New, 2006). Climate changes, especially in the transition zones of Alpine treeline ecotone, affect the formation, growth or extinction of trees and other woody vegetation (Mukhopadhyay et al., 2023). Moderate temperature increases or precipitation changes encourage the growth of existing trees in Alpine treeline ecotone, promoting the formation of pioneer trees in treeless areas (Mukhopadhyay et al., 2023). In addition to climate changes, herbivory is another cause of changes in treeline ecotone (Mienna et al., 2022).

One of the most important transition zones between terrestrial ecosystems is the **desert-oasis ecotones** (Zhou et al., 2020). Due to the fragile ecological environment in desert-oasis ecotones, short-lived plants play an important role in maintaining the ecological balance of the ecotone (Peng et al., 2022). Desert-oasis ecotones are considered as critical ecological barriers and buffers that prevent deserts from turning into oases (Ullah et al., 2023). Since oases are the source of human life and the basis of economic development in arid stress environments, they are highly sensitive areas against land use and human factors (Zhou et al., 2020). As the increase in human presence causes intense occupation of desert-oasis ecotones, it can cause a permanent extinction in ecosystems (Yonaba et al., 2021).

The **forest-steppe ecotone** is one of the most complex ecosystems in terms of composition, structure and function, and the transition between these two biomes is accompanied by a continuous macroclimatic gradient (Du et al., 2022; Chytrý et al., 2022). Forest-steppe ecotones, which consist of natural or near-nature ecosystems, are distributed in mosaic patterns mostly in temperate regions and are faced with land uses such as grazing /mowing (Schmidt et al., 2017; Li et al., 2022). Habitat heterogeneity and different edge effects are observed in these ecotones consisting of woody and herbaceous communities (Erdős et al., 2014; Erdős et al., 2018; Walker et al., 2003). In recent

years, the forest-steppe ecotone has started to deteriorate with the human factor together with global climate changes, and as a result, drying of the lakes, severe drought, and a decrease in water yield are observed (Bai et al., 2021; Sun et al., 2021; Ma et al., 2022; Wei et al., 2022).

Playing an important ecological role as a maintenance and feeding area for many living things, estuaries are highly productive ecosystems that also take part in carbon fixation (Innal and Giannetto, 2000; Ostermann et al., 2021). In ***estuarine ecotones*** lying between coastal and terrestrial environments, food webs are supported by a wide variety of estuarine primary producers (Selleslagh et al., 2015). These ecotones also contain important ecosystems of commercial importance for fish migrating along coastal and marine habitats (Pelage et al., 2022).

Salt marshes and mangroves coexist in the transitional zones of temperate-tropical continents and form the **salt-mangrove ecotone**. Wetlands such as salt marshes and mangroves also can bury vast amounts of organic carbon in sediments (Xiao et al., 2023). Highly productive mangroves dominate tropical and subtropical coastlines (Wang et al., 2022). These ecotones, which play an important role in buffering pollutants such as dissolved metals and metalloids from land to coastal waters, reduce metal(loid)s pollution in surface waters by increasing the pollutant storage capacity in sediments and changing plant-soil interactions (Xiao et al., 2023).

The areas that are a transition zone between farming and animal husbandry, where agriculture and pastures are intertwined, are called **farming-pastoral ecotones** (Cheng et al., 2022). These ecotones are very important with efficient productivity, industrial structure, and socio-economic characteristics of countries such as agriculture/livestock. At the same time, it is one of the most sensitive ecotones that are rapidly affected by socio-economic developments and climate changes (Jian et al., 2022).

There is a lot of ecosystem diversity, soil diversity, climatic diversity, and biological diversity in Turkey. If the studies specific to the important ecotone areas of our country are summarized; Ilgaz Mountain is one of the most important of these ecotone areas. Ilgaz Mountain is an ecotone region extending from the Central Anatolian steppes to the Black Sea forests (Oğul et al., 2022). Dedegöl Mountain, located in the east of Isparta, is a part of the western Taurus Mountains and Beyşehir Lake is in the east. One of the best examples of the alpine tree border ecotone, located at the intersection of forest and herbaceous vegetation, is the treeline ecotone in the Dedegöl mountains (Bilgin, 2021). The Acıçay riparian zone in the province of Çankırı is the fluvial coastal regions and is important areas as migration routes and stopping points among the habitats (Ediş et al., 2022). Yeşilırmak Delta (Samsun province), located in the north of Turkey, contains ecotone areas where wetland and terrestrial systems intersect, including sand dune coastal areas, salt marshes, irrigated alluvial plains, lakes of different sizes, lagoons and terrestrial areas. (Mumcu and Korkmaz, 2021; Mumcu et al., 2023). The habitat of *Onosma aksoyii*, which grows on serpentine rocks between 1350 and 1650 m in the *Pinus nigra* subsp. *nigra* var. *caramanica* forest in Southern Anatolia, is an ecotone between the Mediterranean and Iran-Turan regions (Aytaç and Türkmen, 2011). In the paleovejatation study carried out in the Çankırı-Çorum Basin (Central Anatolia), it was found that this basin has an ecotone between mixed mesophytic forests and broad-leaved evergreen forests (Kayseri-Özer et al., 2019). *Hesperis hamzaoglui*, which is a good example of the forest-steppe ecotone, grows in granitic rocky areas in the forest-steppe ecotone of the Zorkun Yayla (Osmaniye province) and is an endemic whose habitat is limited to Southern Anatolia (Duran, 2008). Considering its contribution to reptile fauna, Amasya province, which hosts 16.4% of all reptile species in Turkey, is an ecotone between Mediterranean, Caucasian, and European ecosystems (Şahin and Afşar, 2018). Manavgat, Göksu, Seyhan and Ceyhan River estuaries in the

Mediterranean region of Turkey are important ecotone areas (Innal and Giannetto, 2020). Imrahor Valley, which is an ecotone area with ecologically vital importance, is one of the valuable areas under threat formed between rural and urban ecosystems in the southeast of Ankara province (Karadeniz et al., 2020). Turkey's lagoons ((Milic, Bafa, Güllük and Köyceğiz Lakes) are also important ecotone areas (Ustaoğlu et al., 2012).

As a result, an ecosystem is a popular depiction of collaboration that transcends the boundaries of an organization (Cobben et al., 2022). Ecotones are the meeting point of different ecosystems and there is an intense interaction between ecosystems. In these transition areas, new features are formed that are not found in neighboring ecosystems. Thanks to these new features, the diversity of species is also quite high. Plant diversity is quite high, especially as terrestrial ecotones contain rare and endemic species. There are also various living species in ecotones that include coastal zones in the seas. The tidal events that occur in the coastal zones also allow the formation of many different species. Dalyans, steppe-forest zones, where rivers flow into the sea, are also very valuable with their unique nature. Conservation of ecotones is important for sustaining biodiversity.

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CHAPTER 9

STATISTICAL ANALYSIS OF THE INHIBITORY EFFECTS OF LACTOFERRIN AGAINST MULTI-DRUG RESISTANT PATHOGENS

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INTRODUCTION

The transferrin family of proteins includes lactoferrins, which bind iron. Since their initial isolation from both bovine and human milk, lactoferrins have undergone extensive structural and functional investigations. This is particularly true given their wide range of functions, characteristics, and uses in the food and pharmaceutical industries (Johansson et al. 1960, Groves et al. 1960).

Although lactoferrins have been found in other mammalian species, bovine and human lactoferrins have received the most attention thus far. Colostrum, milk, tears, nasal and bronchial secretions, saliva, bile and pancreatic secretions (and thus in gastric and intestinal fluids), urine, and seminal and vaginal fluids are just a few exocrine secretions that contain lactoferrins because they are produced by epithelial cells of various body organs (Masson et al. 1971, Heremans et al. 1971). Additionally, the hematopoietic tissue of the bone marrow also produces lactoferrins, which are present in the granules of polymorphonuclear neutrophils (Berlov et al. 2007).

The most plentiful sources of lactoferrins are colostrum and milk, whose quantities vary depending on the type of mammal and lactation stage. Other exocrine secretions have lower lactoferrin amounts. Additionally, different mammalian species have different quantities of lactoferrin in neutrophils (Barton et al. 1988). Despite the fact that lactoferrin from neutrophils in humans has the same structure as lactoferrin from milk (Moguilevsky et al. 1985).

With a molecular weight of about 80 kDa, lactoferrins are monomeric glycoproteins with well-known three-dimensional geometries and amino acid sequences. There have been reports on the primary amino-acid sequences of lactoferrins from a variety of mammalian species, including those for the human (Moore et al. 1997, Cutone et al. 2020), cow (Pierce et al. 1991), goat (Lee et al. 1997), sheep, camel (Khan et al. 2001), buffalo (Karthikeyan et al. 1999), horse (Ashwan et al.1998). Human lactoferrin has two extra amino

acids (691 total), compared to bovine lactoferrins single polypeptide chain of 689 amino acids (Lepanto et al. 2019).

Since lactoferrins have a lot of promise as a natural defense agent, antibacterial activity was the first of these to be attributed to them (Zarzosa et al. 2020). In addition to having a wide range of antibacterial activities against Gram-positive and Gram-negative bacteria and having the potential to be used as natural antibiotics in human and veterinary medicine, lactoferrin and lactoferrin-derived peptides also have a wide range of antiviral activities against enveloped and naked viruses (Redwan et al. 2014), fungi, yeast, and parasites (Fernandes et al. 2017, Leboffe et al. 2009, Giansanti et al. 2009).

Additionally, lactoferrin has been touted as an effective medication for the current COVID-19 pandemic brought on by the severe acute respiratory syndrome coronavirus 2. (SARS-CoV2). Although lactoferrin also exhibits antimicrobial activity in the iron-independent route through direct interaction with microbes, its antimicrobial activity is tied to its capacity to chelate iron and so deprive germs of these vital nutrients (Wang et al. 2020, Elnagdy et al. 2020, Zimecki et al. 2021).

Production

Different ion exchangers were investigated by (Stanic et al. 2012) for the segregation of whey proteins. In this work, numerous biochemical techniques to separate lactoferrin from milk of diverse species have been addressed. There have been attempts to isolate lactoferrin from goat, elephant, sheep, alpaca, camel, gray seal, and human milk. An SP-Sepharose is used to purify lactoferrin utilizing cation exchange chromatography. SDS-PAGE with 10% polyacrylamide gel is used to identify the lactoferrin that was isolated by chromatography. It is then freeze-dried and kept at 20°C for storage. Use of the optimal wavelength, 280 nm, for ion exchange chromatography using a monolithic column attached with a UV-VIS

detector is possible. The flow rates of the retention and elution solutions are 0.25 ml/min and 0.75 ml/min, respectively, and the ionic strength of the elution solution is 1.5 M NaCl. Under ideal circumstances, the detection limit of lactoferrin was 0.1 g/ml.

Pseudomonas aeruginosa

The Gram-negative bacteria *Pseudomonas aeruginosa*, which is typically prevalent in hospitals, is essential for nosocomial infection as well as acute and chronic infection. *P. aeruginosa* is widely distributed in nature and exhibits a high resistance to many antibiotic classes (Kerckhoffs et al. 2011). The bacteria quickly proliferate, colonize surfaces with water, and perform all of their metabolic processes for growth and development, forming an association of complex matrix called a biofilm (Murga et al. 2001).

According to the study, a biofilm renders bacteria more vulnerable to factors like antibiotic use, UV radiation exposure, and salt (Johani et al. 2018). A wide range of research is being done to better understand the causes and mechanisms of resistance. *Pseudomonas* species exhibit high resistance to many kinds of antibiotics that are employed to persistently eradicate the microbial infection because of the complicated biofilm-forming capabilities. The presence of Pseudomonas species in hospitals aids in the formation of biofilms on medical devices, including implants in patients and other similar devices with exposed surfaces (De Silva et al. 2017).

For the study of the biochemical pathways underlying the pathovars' susceptibility to numerous antibiotics groups, including amikacin, gentamicin, carbapenem, ofloxacin, ciprofloxacin, tigecycline, tobramycin, and norfloxacin, *Pseudomonas* species are employed as a model organism (Mesaros et al. 2007, Moore et al. 2016).

By modifying the genome sequences and the expression of proteins that ultimately strengthen the resistance of the pathovars, the

pathogenic *Pseudomonas* species create a significant problem in the diversity of bacteria (Overhage et al. 2008). Due to the bacteria's resistance, numerous metabolic processes and channel protein functions are impacted (Tenover 2006, Miko et al. 2019).

Materials and methods of work

In the current study, high-efficiency devices such as a pressure oven, a digital camera, a sensitive balance, a deep freeze, and an optical microscope are used from international origins.

Sample Collection

In the period from February to May 2022, children and adults (outpatient and inpatient) at Azadi Hospital in Kirkuk city participated in the study. A total of (71) patient agreed to participate, and an interview was conducted for each patient under the supervision of a physician. Various clinical samples (wound, blood, urine, sputum) will be produced.

Effect of lactoferrin on bacterial growth

100 L of 24-hour-old cultures will be transferred to 96-microtitre plates with MIC contraction of lactoferrin or lactoferrin with a chosen antibiotic after 24-hour-old cultures are diluted to an initial OD600nm 0.1. Afterward, the culture plate will incubate for 24 hours at 37°C. Spectrophotometric growth monitoring (OD600nm) will be done after 2, 4, 6, 8 and 24 hours of incubation at 37°C. There will also be a bad control. For each test, three separate experiments will be conducted.

Identification

The shape, diameter and shapes of the bacterial isolates were determined on blood agar, mannitol agar, chocolate agar and McConkey agar. Microscopic and biochemical examinations were used, which included qualitative tests for each type, as well as the System Compact Vitek-2 device, to confirm the results of the biochemical determination. The results of Vitek-2 were identical to the

biochemical tests and the proportions of bacteria isolated from the clinical sources were determined as follows:

Table 1. Numbers and percentage of *Pseudomonas aeruginosa* isolated from clinical source

Bacteria isolates	**Urine**	**Wound**	**Blood**	**Sputum**	**Total**	**C.S. (*) *P-value***
P. aeruginosa	15(22.4%)	19(27.9%)	5(45.5%)	3(11.5%)	42(24.4%)	C.C.=0.071 P=0.973 (NS)
A. baumonii	1(1.5%)	3(4.4%)	3(27.3%)	6(23.1%)	13(7.6%)	C.C.=0.251 P=0.150 (NS)
S. aureus	40(59.7%)	25(36.8%)	1(9.1%)	12(46.2%)	78(45.3%)	C.C.=0.184 P=0.172 (NS)
S.pyogenus	11(16.4%)	21(30.9%)	2(18.1%)	5(19.2%)	39(22.7%)	C.C.=0.067 P=0.797 (NS)
Total	67(38.9%)	68(39.6%)	11(6.4%)	26(15.1%)	172(100%)	C.C.=0.000 P=1.000 (NS)

Identification of *Pseudomonas aeruginosa*

Colony morphology of *Pseudomonas aeruginosa* in MacConkey agar showing 2-3 mm, flat, smooth, non-lactose fermenting colonies with regular margin and Alligator skin like from top view (Figure 1).

Figure 1. *P. aeruginosa* colonies on MacConkey agar

The colorless colonies on MacConkey agar and the huge flat colonies that developed zones of beta-haemolysis with a grape-like odor on the latter media were grown onto Cetramide agar. Colonies had a yellow-blue/green pigmentation on this medium. Therefore, based on the data, we can infer that these isolates may be Pseudomonas species. When grown on cetramide agar, which serves as a selective differential medium for the identification of *P. aeruginosa* and functions as a detergent and inhibits the growth of other microbes, the ability to create green-blue/yellow pigments is demonstrated. Pyocyanin and a brilliant yellow-green pigment are produced by this organism in response to the iron level of the media.

Table 2. Biochemical tests of gram negative isolated.

Biochemical tests Types		Catalase	Kliguler test	Motility	H_2S	Simmons citrate	V-P	M-R	Indol	Oxidase
P. aeruginosa	S1	+	-	+	-	-	+	-	-	+
	S2	+	-	+	-	-	+	-	-	+
	S3	+	-	+	-	-	+	-	-	+
	S4	+	-	+	-	-	+	-	-	+
	S5	+	-	+	-	-	+	-	-	+
A. baumannii	S1	+	-	-	-	+	-	+	-	-
	S2	+	-	-	-	+	-	+	-	-
	S3	+	-	-	-	+	-	+	-	-
	S4	+	-	-	-	+	-	+	-	-
	S5	+	-	-	-	+	-	+	-	-

Table 3: Biochemical tests of gram positive isolated.

Biochemical tests / Types		Motility	Hemolysis	Urease test	Mannitol salt agar	Novobiocin antibiotic	Coagulase	Oxidase	Catalase	Gram stain
S.aureus	S1	-	B	+	+	/	+	-	+	+
	S2	-	B	+	+	/	+	-	+	+
	S3	-	B	+	+	/	+	-	+	+
	S4	-	B	+	+	/	+	-	+	+
	S5	-	B	+	+	/	+	-	+	+
S.pyogenes	S1	-	B	-	-	-	-	-	-	+
	S2	-	B	-	-	-	-	-	-	+
	S3	-	B	-	-	-	-	-	-	+
	S4	-	B	-	-	-	-	-	-	+
	S5	-	B	-	-	-	-	-	-	+

Antibiotic susceptibility test for *Pseudomonas aeruginosa*

Amoxicillin/clavulanic acid, ampicillin, tetracycline, cefoxitin, ceftriaxone, chloramphenicol, and trimethoprim were all completely ineffective against *P. aeruginosa*. 50% resistance to Amikacin, 75% resistance to Nitrofurantoin and Nalidixic Acid. 25% had Doxycycline, Azithromycin, and Ciprofloxacin resistance. Tobramycin, Cefepime, and Imipenem all demonstrated complete sensitivity to P. aeruginosa, respectively. Contrarily, 75% are susceptible to azithromycin and gentamicin. According to the table, 50% of people are susceptible to ciprofloxacin, doxycycline, and amikacin (Table 4.5).

The new findings are in line with Al-Jendy and Al-Ofairi's (2019) claim that *P. aeruginosa* was 100% resistant to Amoxicillin and Penicillin in terms of antibiotic resistance. Other than that, Ciprofloxacin has little effect on *Pseudomonas aeruginosa*. Prior reports indicated that Ciprofloxacin was highly effective against UTIs.

Table 4. Antibiotic susceptibility test of *P. aeruginosa*.

Antibiotics	Resistant		Sensitive		Intermediate	
	No.	%	No.	%	No.	%
AMP (10mg)	4	100	0	0	0	0
AMC (10/20mg)	4	100	0	0	0	0
CX (30mg)	4	100	0	0	0	0
CRO (30mg)	4	100	0	0	0	0
CPM (30mg)	0	0	4	100	0	0
IMI (10mg)	0	0	4	100	0	0
CN (10mg)	0	0	3	75	1	25
AK (30mg)	2	50	2	50	0	0
TE (30mg)	4	100	0	0	0	0
DXT (30mg)	1	25	2	50	1	25
C (30mg)	4	100	0	0	0	0
AZM (15mg)	1	25	3	75	0	0
NA (30mg)	3	75	1	25	0	0
CIP (5mg)	1	25	2	50	1	25
TMP (5mg)	4	100	0	0	0	0
F (300mg)	3	75	1	25	0	0
TOB (10mg)	0	0	4	100	0	0

R=Resistant, S=Sensitive, I=Intermediate, AMP= Ampicillin, AMC= (Amoxicillin and Clavulanic acid (Augmentin)), CRO=Ceftriaxone, CPM= Cefepime, IMI= Imipenem, CN=Gentamicin, AK=Amikacin, TE=Tetracycline, DXT= Doxycyclin, C=Chloramphenicol, AZM=Azithromycin, NA=Nalidixic acid, CIP=Ciprofloxacin, TMP=Trimethoprim, F=Nitrofurantoin, TOB= Tobramycin, CX=Cefoxitin

According to a study by (Al-Salamy et al, 2012. Emad et al, 2012), the susceptibility of 181 clinical isolates of *P. aeruginosa* showed that ampicillin had the highest resistance (100%) in accordance with the current findings. Cephalothin (92.81%), Cefotaxem (84.53%), Gentamycin (69.61%), and Trimethoprim (67.99%) were the next most resistant antibiotics. Tobramycin (14.91%) and Amikacin (14.3%) are two of the antibiotics with the lowest resistance rates that are most effective against *P. aeruginosa*.

They found that the majority of P. aeruginosa isolates were susceptible to amikacin (79%), tobramycin (70%) and piperacillin (65%), in contrast to the current findings (Anjum et al. 2010, Mir et al.

2010). There are many factors that contribute to the spread of *P. aeruginosa* resistance to the majority of antibiotics in humans and animals. Reducing unnecessary antibiotic use, treating with narrow spectrum agents, improving adherence to therapy, reducing antibiotic use in animals and agriculture, and improving infection control are all important ways to address this issue. Additionally, vaccination can lessen the effects of resistance by reducing the risk of infection and transmission.

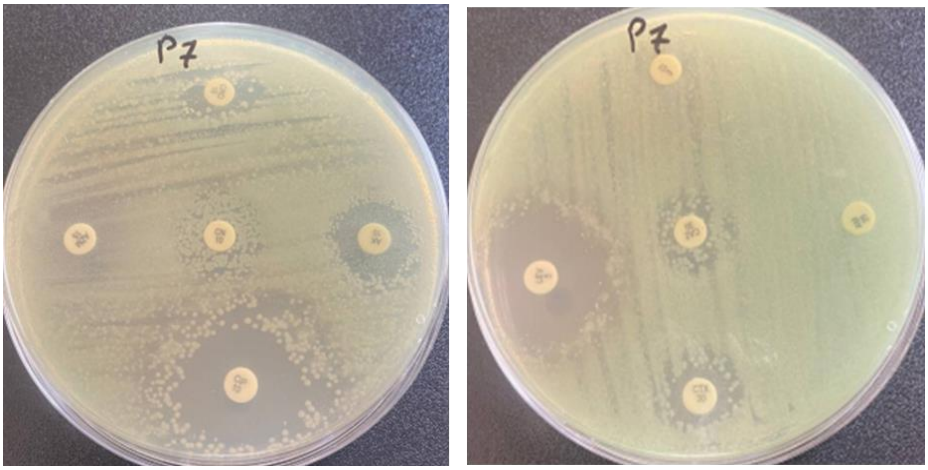

Figure 2. Antibiotic susceptibility test of *P. aeruginosa*

The antimicrobial activity of lactoferrin against the studied pathogenic bacteria was investigated, and the preliminary examination showed that lactoferrin showed more antibacterial activity compared to the antibiotics, where the diameter of the inhibition zone for *P. aeruginosa* was 13 mm.

Table 5. Antimicrobial activity of lactoferrin against representative human pathogenic bacterial strains.

Bacterial strains	Diameter of inhibition zone (mm)
P. aerugenosae	13
A. baumannii	11
S.aureus	14
S.pyogenes	16
C.S. (*) *P-value*	C.C.=0.067 *P*=0.505 (NS)

As bacterial growth can be reinstated by the simple addition of exogenous iron in excess of the lactoferrin's chelating capacity, lactoferrins' bacteriostatic action results from their ability to bind to iron and thereby deprive bacteria of this vital nutrient (Arnold et al. 1982). Were the first to describe the bactericidal mechanism of lactoferrins (1977). They showed that human lactoferrin reduced the growth of *Streptococcus* mutans and *Vibrio cholerae* but not *Escherichia coli* in an iron-rich media, and that this antibacterial action was not reversed in the presence of more iron. They hypothesized that human lactoferrin attaches to the surface of bacterial cells based on immunofluorescence investigations.

More research has revealed that human and bovine lactoferrins can directly interact with bacterial cell membranes and bind and release lipopolysaccharides (LPS) from enteric Gram-negative bacilli (Yamauchi et al. 1993, Ellison et al. 1991). Additionally, LPS binds to both human and bovine lactoferrin at the same location (Elass Rochard et al. 1995).

Several mechanisms could account for Lf's antimicrobial effect against Gram-negative bacteria. The first mechanism is that Lf is an iron-binding protein that reduces in the context of microorganisms and scavenges free iron. Thus, a lack of iron hinders *Pseudomonas* from forming biofilms. Patients with cystic fibrosis have a high incidence of biofilm formation, which was suggested as a colonial organization adhesion technique for *Pseudomonas aeruginosa*. Bacteria develop a

strong resistance to the host cell defense system and antibiotic therapy as a result of biofilm development (Caraher et al. 2007).

The need for high levels of iron for the formation of biofilms by various bacteria stains is well established. Therefore, it has been proposed that Lf's role as an iron chelator efficiently inhibits biofilm formation through iron sequestration (Weinberg et al. 2004). According to the second process, when lactoferrin binds to lipid A, it disrupts the gram-negative bacteria's outer membrane, causing the release of lipopolysaccharide (LPS), which can eventually cause alterations in the membrane's permeability (Brink et al. 2002). There have been reports of N-terminal Lf receptors being found on several bacteria' surfaces. Due to cell wall breakage caused by the binding of Lf to these receptors, Gram negative bacteria experience cell death (Arnold et al. 1977).

Synergistic effect of lactoferrin with antibiotics by discoid diffusion methods

Synergistic effect of lactoferrin and antibiotics resulting in enhanced antibacterial activity; therefore, the development of resistant pathogenic bacteria could be addressed by the simultaneous action of lactoferrin and antibiotics. In this study the synergistic effects of AgNPs with 5 antibiotics against *P. aeruginosa* genes were studied using the disc diffusion method.

Table 6. The inhibition zone lactoferrin with and without different types of antibiotics against multidrug resistance bacteria.

S. pyogenes			*S. aureus*		*A. baumannii*		*P. aerugenosae*			Bacterial strains	
40	20	10	20	10	20	10	40	20	10	The concentration of L.f	
Nil			Nil		Nil		Nil			Control	
17			17		17		17			TOB	Standard
23	22	25	14	11	19	22	21	17	20	Sample	
18			18		18		18			Nitro	Standard
25	23	21	15	18	21	19	24	23	19	Sample	
16			16		16		16			Nalid	Standard
24	24	22	21	25	30	29	29	31	28	Sample	
17			17		17		17			Chloram	Standard
21	18	20	16	21	14	13	15	24	23	Sample	
15			15		15		15			Ampic	Standard
21	24	25	26	28	26	23	22	24	25	Sample	
C.C.=0.213 P=0.096 (NS)			C.C.=0.057 P=0.663 (NS)		C.C.=0.059 P=0.558 (NS)		C.C.=0.102 P=0.789 (NS)			**C.S.** (*) ***P-value***	

Statistical Analysis

Table 6 shows the normality scores of the dependent variables. Since the significance scores of the Shapiro-Wilk test is less than $p<0.05$, the variables are not normally distributed. Therefore, non-parametric test should be used. Kruskal Wallis H test is used for the group comparison and Mann-Whitney U test is used to understand the difference within the groups.

Table 7. Descriptive Statistics of the Variables.

	Gender	Age	Sample Type	Resistant	Sensitive	Diameter	Bacteria Type
N-Valid	150	150	150	150	150	150	150
Missing	0	0	0	0	0	0	0
Std. Deviation	.45	18.94	1.08	.086	.055	1.22	1.60
Minimum	1.00	16.00	1.00	.30	.37	11.00	1.00
Maximum	2.00	90.00	4.00	.57	.55	16.00	
Percentiles	1.00	28.00	1.00	.55	.38	13.00	2.00
25							
20	1.00	43.50	2.00	.55	.38	13.00	4.00
75	2.00	58.25	2.25	.57	.40	14.00	4.00

Table 8 shows the normality scores of the dependent variables. Since the significance scores of the Shapiro-Wilk test is less than $p<0.05$, the variables are not normally distributed. Therefore, non-parametric test should be used. Kruskal Wallis H test is used for the group comparison and Mann-Whitney U test is used to understand the difference within the groups.

Table 8. Post-hoc results of the variables based on the resistant scores.

Sample1 Sample2	Test statistic	Std. Error	Std. Test statistic	Sig	Adj.Sig,
Sp-sa	47.50	10.418	4.560	.000	.000
sp-sa+sp	47.50	15.134	3.139	.002	.025
sp-ab	29.00	1.378	6.399	.000	.000
sp-pa	119.5	11.57	10.32	.000	.000
sp-pa+sp	199.5	15.61	7.65	.000	.000
sa-sa+sp	.000	13.03	.000	1.00	1.00
sa-ab	44.5	12.13	3.66	.000	.004
sa-pa	72.00	8.626	8.34	.000	.000
sa-pa+sp	72.00	13.57	5.30	.000	.000
sa+sp-ab	44.50	16.36	2.72	.007	.098
Sa+sp-pa	72.50	13.96	5.16	.000	.000
Sa+sp- pa+sp	72.00	17.45	4.12	.000	.001
ab-pa	27.50	13.14	2.09	.036	.545
ab_pa+sp	27.50	16.80	1.63	.102	1.00
pa-pa+sp	.000	14.47	.000	1.00	1.00

The resistant, sensitive and diameter are used as a dependent variable separately and Kruskal Wallis test for each variable showed that there is a statistically significant difference between the bacterium types. There is the pairwise comparison of the bacteria type considering resistant scores. The significance scores less than 0.05 shows the statistically significant differences.

Table 9. Post-hoc results of the variables based on the sensitive scores.

Sample1 Sample2	Test statistic	Std. Error	Std. Test statistic	Sig	Adj.Sig
Ap-sa	-44.50	12.14	-3.664	.000	.004
Ap-sa+sp	-44.50	16.36	-2.791	.007	.098
Ap-ba	103.50	13.14	7.878	.000	.000
Ap-ba+sp	103.50	16.80	6.160	.000	.000
ab-sp	-134.00	14.38	-9.320	.000	.000
sa-sa+sp	.000	13.03	.000	1.00	1.00
sa-ab	59.50	8.62	6.840	.000	.000
sa-pa+sp	59.00	13.57	4.348	.000	.000
Sa-sp	-89.50	10.42	-8.591	.000	.000
sa+sp-pa	59.00	13.96	4.226	.000	.000
Sa+sp-pa+sp	59.00	17.45	3,380	.001	.011
Sa+sp-sp	-89.50	15.13	-5.914	.000	.000
Pa-pa+sp	000	14.47	.000	1.00	1.00
Pa-sp	-30.50	11.57	-2.636	.008	.126
pa-pa+sp	-30.50	15.60	-1.954	.051	.760

Conclusion

According to the study's findings, 172 (68.8%) of the samples had bacterial growth that had been grown at an ideal level. The findings revealed that 67 samples of urine yielded 15 isolates of *P. aeruginosa*. Out of 68 wound isolates, there were 19 *P. aeruginosa* isolates, yielding a rate of 27.9%. Moreover, in the current investigation, 5 isolates of *P. aeruginosa* were isolated from 11 blood samples with a proportion of 45.5%.

The findings of the current study indicated that 67 urine samples yielded 1 isolate of *A. baumonii*. 3 *A. baumonii* isolates were found in 68 wound samples. 3 *A. baumonii* isolates were found in 11 blood samples. 26 samples of sputum yielded 6 *A. baumonii* isolates. The findings revealed that 40 *S. aureus* isolates were discovered from 67 urine samples. 25 *S. aureus* isolates were found in 68 wound samples. *S. aureus* was isolated from 1 of 11 blood samples. 26 samples of

sputum yielded 12 *S. aureus* isolates. The findings revealed that 11 isolates of *S. pyogenus* were found from 67 urine samples. *S.pyogenus* was isolated from 21 out of 68 wound samples. *Streptococcus pyogenus* was isolated from 2 of 11 blood samples. 26 samples of sputum contained 5 isolates of *S.pyogenus*. *P.aeruginosa* showed total resistance to Amoxicillin/clavulanic acid, Ampicillin, Tetracycline, Cefoxitin, Ceftriaxone, Chloramphenicol and Trimethoprim. A. baumannii showed total resistance to Amoxicillin/clavulanic acid, Ampicillin, Gentamicin, Amikacin, Chloramphenicol, Nalidixic acid, Ciprofloxacin, Trimethoprim and Tobramycin. *S. aureus* showed total sensitive to Nitrofurantoin and Refampicin respectively. *S. pyogenes* showed total resistance to Ampicillin, Tetracycline and Ceftriaxone. Otherwise, *S. pyogenes* showed total sensitive to Penicillin, Cefepime, Vancomycin and Clindamycin. The results of lactoferrin showed that the inhibition zone diameter was (13, 11, 14, 16 mm) for *P. aeruginosa*, *A. baumannii*, *S. aureus* and *S. pyogenes* respectively.

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CHAPTER 10

STATISTICAL INVESTIGATION OF miRNA ALTERATION IN HYPERTHYROIDISM PATIENTS IN AL-ANBAR PROVINCE

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INTRODUCTION

Recently, there has been a great deal of interest in microRNAs, often referred to as miRNAs. MicroRNAs are non-protein-coding RNA molecules that are found in certain organs and are responsible for controlling gene expression in eukaryotic animals (Bavelloni et al. 2017). These microRNA molecules are tiny and their altered levels are diagnostic or causative in a number of pathological conditions, such as cancer or cardiovascular disease. miRNAs are an example of a promising new class of biomarkers that have the potential to be used in the diagnosis of life-threatening diseases as well as in tracking disease progression in patients. A significant number of the therapeutically targetable microRNAs are involved in the process of immune response control. Since their reduction has an impact on the overactive response to infection, this suggests that control of the immune response, which is also important in the regulation of genes, is crucial. This highlights the importance of regulating gene expression (Brosnan and Mitter, 2021).

The thyroid is a butterfly-shaped gland located in the centre of the neck, above the collarbones and below the larynx (voice box). Triiodothyronine (T3) and thyroxine (T4), two hormones produced by the thyroid, control how the body uses and stores energy (Beynon and Pinneri, 2016). In people with hyperthyroidism, the immune system produces an antibody similar to TSH, which causes the thyroid to overproduce thyroid hormone. This can happen to men and women of any age, although it most commonly affects women between the ages of 20 and 40. The symptoms of hyperthyroidism are caused by the thyroid gland becoming enlarged and producing excessive amounts of thyroid hormone (Stathatos, 2012). For therapeutic management of hyperthyroidism, early and accurate biomarkers to predict recurrence of hyperthyroidism are therefore of paramount importance. The role of microRNAs (miRNAs), a group of endogenous non-coding short RNAs, has recently received increased attention. In humans, miRNAs are located in the intron region of coding genes. These miRNAs can affect gene expression by acting on the promoter region of the target gene, preventing the binding of suppressor genes and inhibitory factors, thereby increasing gene expression. The hypermetabolic condition of

thyrotoxicosis is the end result of a group of illnesses known collectively as hyperthyroidism (Qadir and Faheem 2017).These conditions are characterised by abnormally high levels of thyroid hormones (T3 and T4) produced and released by the thyroid gland (Wilczynska and Bushell, 2015).

Both underactive and overactive thyroids may have a negative impact on fertility. However, there is little evidence to support the hypothesis that hyperthyroidism is associated with infertility, and this data is sometimes contradictory. Thyroid hormone has a number of effects on human reproduction, both at central and peripheral levels, and these effects are caused by different processes. It is possible for men and women with hyperthyroidism to experience infertility; however, this condition is often reversible once euthyroidism is achieved (Paraskevopoulou and Hatzigeorgiou, 2016). Certain microRNAs, which can be targeted therapeutically, play a crucial role in modulating the immune response. Their depletion has an effect on the hyperactive response to infection, demonstrating that regulation of the immune response, which also plays an important role in regulating gene expression, is essential (Ludwig et al. 2016).In the light of this information, the aim of this study is to investigate the relationship between thyroid antibodies (antithyroid peroxidase and antithyroid) and thyroid hormones and microRNA (miRNA) gene expression with hyperthyroidism in a specific group of women with hyperthyroidism.

MicroRNAs

MicroRNAs, also known as miRNAs, are short sequences of endogenous RNA that act as regulatory molecules but do not code for proteins. They have been found to play an important role in the regulation of gene expression in the majority of eukaryotic organisms. These naturally occurring RNA sequences are the focus of a considerable amount of research in a wide range of model species, from plants to mammals (Turchinovich et al. 2016). MicroRNAs are involved in a wide range of key biological activities in plants, including the control of growth and development and the response to abiotic stress. There is increasing evidence that microRNAs, which are found

in a wide range of animals, influence gene expression at the post-transcriptional level.

In the nucleus, stem-loop structures of about 70 nucleotides in length, called pre-miRNAs, are processed into mature microRNAs of 21 to 24 nucleotides in length. MicroRNAs are 21-24 nucleotides in length. Base pairings determined by sequence homologies are responsible for the formation of the stem and loop structures. Since the Pri-miRNA is thought to be longer than the pre-conserved miRNA stem-loop structures, the subsequent processing step involves some modification. In addition, microRNAs have been shown to stimulate gene expression under certain circumstances. Recent research has led scientists to hypothesize that microRNAs are transported from one subcellular compartment to another to modulate the rate of translation and perhaps even transcription. MicroRNAs are critical for proper animal development and play important roles in a variety of biological processes. In humans, abnormal expression of miRNAs has been linked to a wide range of diseases. In addition, microRNAs can be found in extracellular fluids, where they are released. Extracellular miRNAs act as signalling molecules that mediate interactions between cells (McGuire et al. 2015).

Biogenesis

The first step in miRNA synthesis involves the processing of RNA polymerase II/III transcripts, either following or simultaneously with transcription. MicroRNAs can occasionally be produced in clusters, which are large single transcripts. Since their seed regions are likely to be relatively similar, the miRNAs are then classified as belonging to the same family. There are two types of miRNA biogenesis pathways: canonical pathways and non-canonical pathways. Among these, the canonical biogenesis pathway is by far the most dominant and important miRNA processing pathway (Lukasik et al. 2016). The RNase III endonuclease Dicer is responsible for processing pre-miRNAs after they have been produced and subsequently exported to the cytoplasm by a complex consisting of exportin 5 (XPO5) and RanGTP. As a result of this processing step, a mature miRNA duplex is

formed, in which the terminal loop is removed. The mature version of the miRNA is named because the miRNA strand can be read in either orientation. These variations can range from almost equal amounts to predominantly one or the other (Mishra et al. 2016).

The degree of thermodynamic stability at the 5'-ends of the miRNA duplex and the presence of a 5'-U at nucleotide position 1 play an important role in determining whether the 5p or 3p strand is used. The passenger strands of the miRNA (Figure 1), which do not contain any mismatches, are selectively cleaved by AGO2 and subsequently degraded by cellular machinery, leading to a potential bias towards one strand. This happens when the miRNA duplexes have a central mismatch or lack AGO2-loaded miRNA (Lekchnov et al. 2016). After processing, the miRNA is incorporated into the RISC complex (RNA-induced silencing complex) to prevent translation of the target mRNA or to cleave the target mRNA. The AGO1 protein, which contains a PAZ domain, an RNA binding domain and an RNaseH-like PIWI domain, is a critical component of the RISC complex responsible for degrading miRNA* strands. The AGO protein family is vital and indispensable for the functionality of the miRNA-RISC complex, as its absence would render the complex ineffective (Militello et al. 2017).

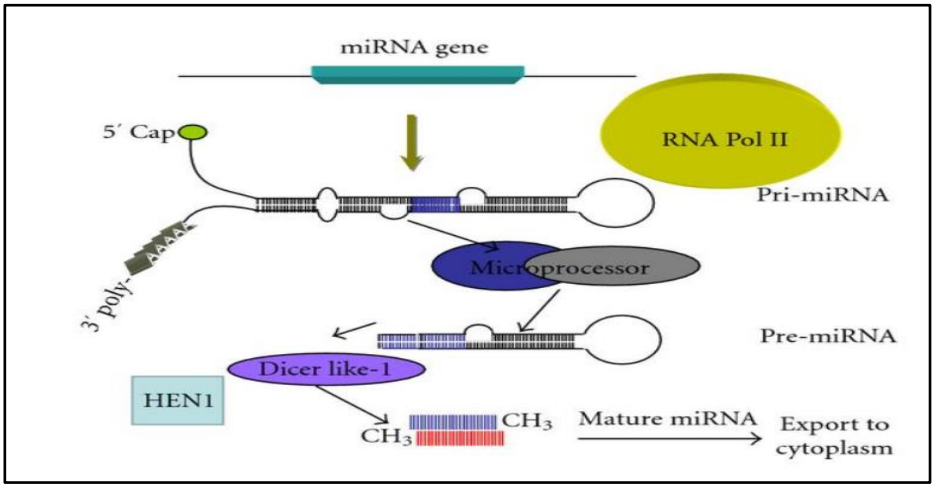

Figure 1. MicroRNA biogenesis (Achkar et al. 2016)

Mechanisms

Upon binding to specific sequences in the 3' untranslated region (UTR) of their target mRNAs, microRNAs initiate translational repression, mRNA deadenylation and decapping (Dori and Bicciato, 2019), as shown in Figure 2. Numerous research studies have consistently demonstrated the validity of this observation. In addition to the 5' ends of miRNA duplexes, miRNA binding sites have also been identified in different regions of the mRNA, including the 5' untranslated region, the coding sequence and even within promoters. However, further research is needed to fully understand the practical significance of this type of interaction (Michlewski and Cáceres, 2019). AGO and the guide strand form the miRISC, which is an acronym for the minimum miRNA-induced silencing complex. The degree of complementarity between MREs and target mRNAs determines whether there is AGO2-dependent slicing of the target mRNA or miRISC-mediated translational inhibition and degradation of the target mRNA. The induction of AGO2 endonuclease activity and mRNA cleavage can be attributed to an interaction between fully complementary miRNA and MRE (Rupaimoole et al. 2016).

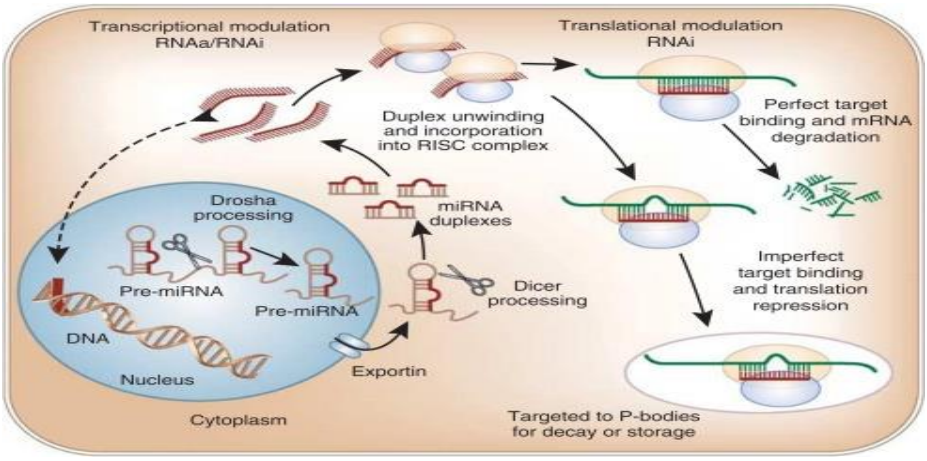

Figure 2. The role of miRNA (Cambiagno *et al.* 2021)

Hyperthyroidism

The condition known as hyperthyroidism, or an overactive thyroid, occurs when the gland responsible for thyroid function produces too much thyroid hormone. Excessive levels of thyroid hormones in the body lead to the acceleration of various physiological processes (Kravets, 2016).The condition known as hyperthyroidism (Figure 3) is characterized by a fast heartbeat, weight loss, increased hunger and feelings of worry. Surgery, anti-thyroid drugs, radioactive iodine and beta blockers are some of the treatments available for hyperthyroidism (Osuna et al. 2017).

In general, the thyroid gland is responsible for producing two primary hormones that affect how your body functions. These are called thyroxine (T4) and triiodothyronine (T3). Things like the rate at which your heart beats and the rate at which you burn calories are both controlled by your thyroid. It does this by releasing hormones that control your metabolism, the process by which your body converts food into energy and maintains its vitality (Srinivasan and Misra, 2015).

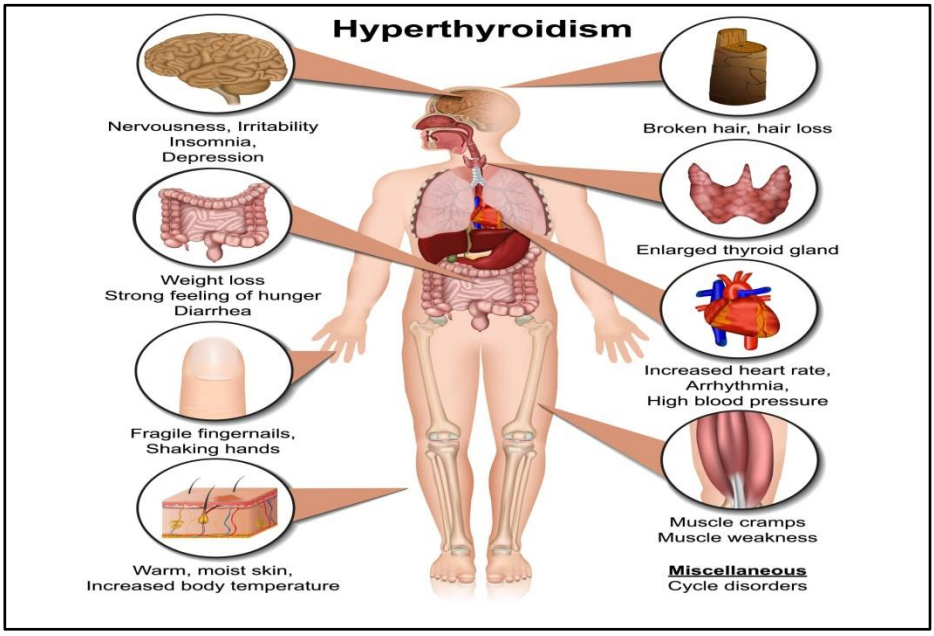

Figure 3. Overview of hyperthyroidism (Sjölin *et al*. 2019)

Hyperthyroidism can be caused by several conditions, including Graves' disease, Plummer's disease and thyroiditis. Thyroid hormones play an important role in regulating every aspect of the metabolic process. The thyroid plays an important role in producing a hormone called calcitonin, which helps maintain a healthy balance of calcium in the blood (Moleti et al. 2019).

The thyroid gland normally produces an appropriate amount of the hormone, but there are cases where it produces excessive amounts of T4. For example, Graves' disease is an autoimmune disease in which antibodies produced by the immune system cause the thyroid to overproduce T4, leading to symptoms of the disease. For unknown reasons, such as an autoimmune condition, your thyroid may become irritated after giving birth. Inflammation can cause excessive amounts of thyroid hormone, which should be kept in the gland, to be released into the bloodstream. Thyroiditis comes in different forms, some of which can be painful and some of which are not completely fatal.

Inflammation of the thyroid is called thyroiditis. Some forms of thyroiditis can cause thyroid hormone to leak from the thyroid gland into the bloodstream. This can be dangerous. As a direct result, you are at risk of developing symptoms of an overactive thyroid. Some forms of thyroiditis are associated with the development of hyperthyroidism. Subacute thyroiditis is characterized by a painful swollen thyroid gland enlarged by inflammation. Postpartum thyroiditis is an inflammation of the thyroid gland that can occur after a woman has given birth (Nguyen et al. 2018). Painless thyroiditis is a type of thyroiditis that can develop at any time and, unlike postpartum thyroiditis, is not caused by pregnancy. It is possible that your thyroid gland is too large. Experts agree that the cause of painless thyroiditis is most likely to be an autoimmune disease. Symptoms of hypothyroidism, commonly known as an underactive thyroid, can also be caused by thyroiditis. If your thyroid gland remains hyperactive for a long time, there is a chance that it will eventually become hypothyroid (Banigé et al. 2018).

Complications

If left untreated, hyperthyroidism can increase the risk of a thyroid storm. A thyroid storm is an extremely rare health condition that can develop if hyperthyroidism is left untreated for a long time. Because there is too much thyroid hormone in your system, your body goes into overdrive. A fast heart rate, increased blood pressure and fever are all symptoms that can occur during a thyroid storm, which is a potentially life-threatening combination of symptoms (Ferrari et al. 2019). Women who already have thyroid problems or who develop hyperthyroidism during pregnancy are both susceptible to developing pregnancy-related problems. Both the pregnant woman and the baby are at risk if there is an excess of thyroid hormone. There is a risk of ectopic pregnancy or premature birth. Having your thyroid hormone levels checked regularly during pregnancy can help detect any abnormalities, and your doctor may recommend treatment with medication (Andersen and Laurberg, 2016). If you have an overactive thyroid, your bones may become brittle and thin, putting you at risk of developing osteoporosis. Bone health can be improved by taking vitamin D and calcium supplements during and after treatment for the condition. Getting the recommended amount of exercise or doing any kind of physical activity regularly can also help prevent osteoporosis (Quérat et al. 2015). People with an overactive thyroid have an increased risk of developing thyroid cancer, often called thyroid cancer. According to a study summary published in 2018, hyperthyroid people with thyroid cancer had a more "aggressive" form of the disease, which was associated with a worse prognosis than euthyroid patients (those with a healthy thyroid). Atrial fibrillation is a potentially fatal arrhythmia (irregular heartbeat) that can be caused by hyperthyroidism. It can also cause congestive heart failure and increase the risk of stroke. Thyroid conditions that aren't properly managed can put a significant strain on your body and, if left untreated, can lead to life-threatening complications. There are a number of simple blood tests that can be used to diagnose hyperthyroidism and other thyroid problems (Bartalena et al. 2016).

Diagnosis

Because many of its symptoms are common to other conditions, hyperthyroidism cannot be diagnosed on the basis of symptoms alone. As well as ordering imaging tests, doctors may also order a range of thyroid blood tests to help confirm the diagnosis and identify the underlying cause. As hyperthyroidism has been linked to various fertility problems, it is advisable for women who are having difficulty conceiving to have their thyroid levels checked (Kaplowitz and Vaidyanathan, 2020).

Hyperthyroidism is characterised by increased levels of the thyroid hormones T3 and T4, and decreased levels of thyroid-stimulating hormone (TSH). The following imaging tests can be used to diagnose hyperthyroidism (Figure 4) and to examine the thyroid gland, a test known as radioactive iodine uptake (RAIU). In this test, you take a small amount of radioactive iodine, also known as a radiotracer, by mouth to determine the percentage of this substance that is absorbed by your thyroid gland (Vaske et al. 2016).

After a period of time, usually between six and twenty-four hours, the healthcare professional will scan your neck with a device called a gamma probe to see how much of the radioactive iodine your thyroid has absorbed. If a significant amount of radioactive iodine has been taken up by your thyroid, this indicates that your thyroid gland is producing excessive amounts of thyroxine (T4). If this describes your condition, you almost certainly have Graves' disease or thyroid nodules (King et al. 2016).

The gamma camera will take these images while you are in the same position as during the RAIU. The radioactive material may cause some or your entire thyroid to appear 'bright' on the screen. Ultrasound is a diagnostic procedure that uses high-frequency sound waves to create images of your thyroid gland. It is a non-invasive procedure that allows your doctor to see your thyroid gland on a screen. This test can be used by your doctor to look for nodules in your thyroid (Goichot et al. 2016).

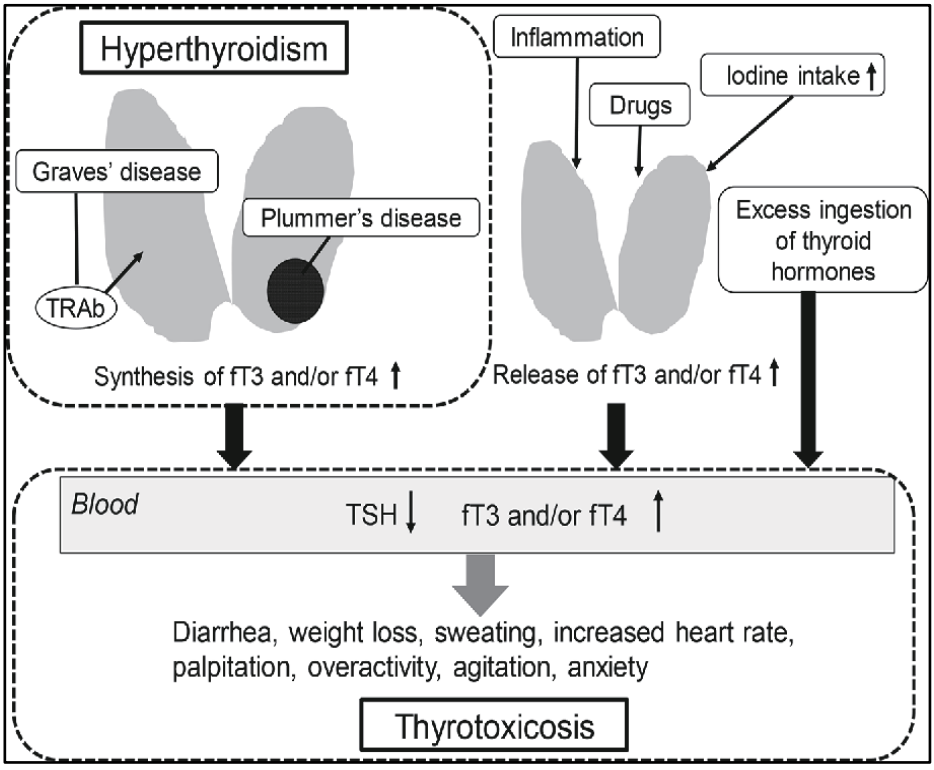

Figure 4. Diagnosis of hyperthyroidism (Watad et al. 2016)

Treatment

There are many different ways to treat hyperthyroidism. Depending on what caused your hyperthyroidism in the first place, some treatments may work better than others. Below are some of the treatments available for hyperthyroidism: Methimazole (Tapazol) or propylthiouracil (PTU), both anti-thyroid medicines: These medications suppress the production of thyroid hormone in the body, allowing for quick control of your thyroid condition (Donangelo and Suh, 2017).

Overactive thyroid cells can be treated with oral radioactive iodine therapy. Over the course of several weeks, radioactive iodine effectively eliminates these cells, resulting in a reduction in the size of your thyroid gland and lower thyroid hormone levels. This treatment usually results in permanent destruction of the thyroid gland and effectively treats hyperthyroidism. Unlike radioactive iodine uptake (RAIU) tests and diagnostic scans, the amount of radiation absorbed by

the body as a result of taking this medication varies (Dekkers et al. 2017). The majority of people who take this medicine will need to take thyroid hormone prescriptions for the rest of their lives to maintain normal hormone levels. These drugs, also called beta blockers, prevent the effects of thyroid hormone in the body. Although they do not change the actual levels of the hormone in your blood, these treatments can help relieve symptoms caused by hyperthyroidism, such as a fast heartbeat, anxiety and tremors. When treating hyperthyroidism over the course of a patient's life, this treatment is almost always combined with one or more other approaches (Turunen et al. 2020). The time taken to treat hyperthyroidism can vary greatly depending on the underlying cause of the condition. It may be treated with high doses of non-radioactive iodine drops. This treatment will restore thyroid levels within seven to ten days. However, this is only a temporary treatment and more permanent treatment, such as surgery, is likely to be needed in the long term. It is widely accepted as a long-term treatment option for hyperthyroidism (Léger and Carel, 2018). Most treatments come with a warning that they may have side effects. These potential risks include Methimazole and propylthiouracil are two drugs that can be used to treat hyperthyroidism (Gronich et al. 2020).

Both treatments have adverse effects (PTU). It is well known that these drugs can cause a wide range of adverse effects. PTU can cause liver damage, but only in a very small number of patients (less than 1% of the population). Agranulocytosis, a severe reduction in the number of white blood cells, is another harmful side effect that occurs in less than 1% of cases. All age groups are susceptible to these adverse effects (Sundaresh et al. 2017).

When taken by a pregnant woman, this medicine has the potential to cross the placenta into the developing foetus. This has the potential to cause hypothyroidism and goiter in the unborn child. Because of this potential adverse effect, pregnant women are closely monitored. There is also the possibility of an adverse reaction to these treatments, which occurs in about 5% of patients (Kobaly and Mandel, 2019).

With radiation, there is always a risk of developing cancer as an adverse effect. There is currently no evidence that treating

hyperthyroidism with radioactive iodine increases the risk of developing cancer. This risk is small and not very likely to occur. There is a known risk that radioactive iodine can be passed from a pregnant or breastfeeding mother to her baby. You should avoid using radioactive iodine while you are pregnant or breastfeeding as it may affect your child's thyroid. Loss of sensation in the mouth may occasionally occur after radioactive iodine (RAI) treatment (Scappaticcio et al. 2021).

Housekeeping Genes

Housekeeping genes are often described as essential, conserved, stably expressed in all cells and conditions, and members of cellular maintenance pathways. Broadly speaking, housekeeping genes are a set of genes that are essential for the maintenance of life. In practical terms, housekeeping genes can be defined as genes that show consistent expression in every cell of an organism, regardless of tissue type, developmental stage, cell cycle state or external signals. They serve as indicators of the biological well-being of an organism. These genes provide valuable information about species-specific and higher taxonomic level genomic features and gene functions that may influence conservation or evolutionary change. Consequently, a complete understanding of housekeeping genes can significantly improve exploratory, basic and translational research. However, despite their fundamental and translational importance, their definition has remained unchanged for more than 40 years (Bartha et al. 2018; Kiat et al. 2019).

Housekeeping genes have often been associated with the following four biological traits: evolutionary conservation, essentiality, involvement in cellular maintenance, and expression stability across samples. However, the link between these traits has not received much attention. The study of housekeeping genes is popular in both basic and translational science. Therefore, it is crucial to demonstrate the experimental basis for these standards (Joshi et al. 2022).

MATERIAL AND METHOD

General laboratory equipment, instruments and chemicals used during the study are as given in Table 1.

Table 1. Equipment and tools that were used

Equipment and instruments	Company	Country
Automatic micro pipette (100-1000µL)	Slamed	German
Automatic micropipette (2-20 µL)	Eppendrof	German
Centrifuge	Hattich	Germany
Refrigerator	Kelon	Turkey
Micro plate Elisa Reader	Rayto	China
Micro plate Elisa washer	Allsheng	China
VIDAS	Bio Merieux	France
Vortex shaker	INNOCOM	China
96-wall ELISA Plate	Bioneer	Korea
Vortex Mixer	TOPSCIEN	China

In Table 2, the kits used in the procedures of the study are given with their respective sources.

Table 2. The kits and their sources

Kit	Company	Origin
Thyroid stimulating hormone ELISA (TSH) kit	Bio merieux	France
VIDAS® FT4 kit	Bio merieux	France
VIDAS® FT3 kit	Bio merieux	France
VIDAS Anti-Tg ki	Bio merieux	France
VIDAS Anti-Tpo kit	Bio merieux	France
Anti-phospholipid ELISA kit	Aeskulisa	Germany

The trial will take place between 1 February 2022 and 1 July 2022. The study was approved by the ethics committees of the Department of Biology. A total of 165 women took part in the study. The experimental group consisted of 120 patient women (women with a history of miscarriage in the first trimester of pregnancy) and the control group consisted of 45 women. The women were aged between 19 and 46 years. Clinical information was collected directly from the patients. The aim of the study was to evaluate thyroid hormone and anti-thyroid hormone levels in the study group compared to the control group. The study also aimed to determine the percentage of thyroid disorders in women with a history of miscarriage in the first trimester

of pregnancy. Secondly, since pregnant women with a history of miscarriage in the first trimester are divided into normal weight and overweight women according to the average results, weight changes were analyzed to make comparisons between the two groups and to find out to what extent the likelihood of miscarriage is increased under the influence of weight changes.

A disposable plastic syringe was used to collect 5 mL of venous blood from pregnant women in three trimesters. The collected blood was then transferred to a gel tube and placed in a water bath where it was allowed to clot for ten minutes at 37°C. After clotting, the samples were centrifuged at 3000g for five minutes. The resulting clear serum was stored frozen in a refrigerator at -10°C. This serum was divided into separate portions for further analysis.

- Hormonal analysis.
- Immunological parameter assay.
- Molecular analysis.

Thyroid Function Tests (TFT) for TSH, FT4, FT3, ANTI-TPO and ANT-TG

Hormonal testing was performed using (VIDAS) from BioMerieux Company, France, by an enzyme linked fluorescence assay (ELFA).

Princible: The enzyme linked fluorescence immunoassay (ELFA) used in the VIDAS assay is performed on automated equipment. The instrument controls the temperature and each step of the assay. In this particular assay, the Solid Phase Receptacle (SPR®) acts not only as a pipetting tool but also as the solid phase, being a disposable alternative to a pipette. During manufacture, mouse anti-TSH, mouse anti-T4 and sheep monoclonal anti-T3 antibodies are coated onto the Solid Phase Receptacle (SPR) used for TSH analysis. If the sample contains Tg antibodies, these antibodies will form a specific bond with the protein coating the inside of the SPR. This binding occurs because the Tg antibodies interact specifically with the protein coating on the inner surface of the SPR.

The reagents required for the assay are contained in sealed reagent strips. A fluorescent substrate called 4-methylumbelliferyl phosphate is passed through the Solid Phase Receptor (SPR). The process that converts the substrate to the fluorescent product 4-methylumbelliferone is catalyzed by enzymes that are not released from the walls of the SPR. The concentration of TSH in the sample can be deduced from the result of the optical scanner's measurement of the intensity of the fluorescence, which is directly proportional to this concentration. The instrument automatically analyses and prints a report for each sample when the VIDAS, TSH, FT4, FT3, ANTI-TPO and ANTI-TG tests are completed. Automated analysis ensures efficient and accurate data processing, providing comprehensive reports that facilitate further evaluation and interpretation.

Determination of Thyroid Stimulating Hormone (TSH)

- Only what was needed was taken out of the package, and what wasn't used was returned to storage at 2-8 degrees Celsius as soon as possible.
- Approximately thirty minutes was allowed for the components to reach room temperature.
- One "TSH" strip and one "TSH" SPR were required for each test sample, control and calibrator. The pouch was then resealed for storage.
- The "TSH" code of the device serves as the test identification. The calibrator must be tested twice and marked 'S1'. The control should be marked C1 when tested.
- Mix the calibrator, control and samples (for serum or plasma separated from the pellet) using a vortex mixer.
- The total volume of the calibrator, control and test samples for the experiment is 200 liters.
- SPRs and "TSH" Reagent Strips were positioned in their usual locations on the instrument. SPRs and reagent strips with the assay code were compared for color consistency of the label.

- All assay steps were set up to be performed automatically by the instrument and the assay procedure was started according to the instructions in the user manual.
- The assay took approximately 40 minutes to complete and when it was finished the vials were resealed and returned to the correct temperature. The SPRs and strips were removed from the instrument after scanning.
- Used SPRs and strips were disposed of in the appropriate container. The normal range for micro international units per milliliter is 0.38 - 4.31 LU/mL.

Determination of the FT3 and FT4

- Once the necessary components had been removed from the kit, any other components that were not required were returned to storage at a temperature between 2 and 8 degrees Celsius.
- Allow approximately thirty minutes for the components to reach room temperature.
- After using one 'FT3' strip (or FT4 strip) and one 'FT3' SPR (or FT4 SPR) for each sample, control or calibrator tested, the storage bag was resealed. This was done to prevent contamination.
- The test was labeled on the instrument with the "FT3" (or FT4) code. "S1" was identified as the calibrator and was evaluated three times. In cases where the control was examined, it was given the designation "C1".
- A vortex type mixer specifically designed for use with serum or plasma after separation from the pellet was used to combine the calibrator, control and samples.
- During testing, the calibrator, control and sample test portions were measured at 100 µL for FT3 and 200 µL for FT4.
- The "FT3" SPRs (FT4 SPR) and "FT3" strips (FT4 strip) were inserted into the device. The color labels on the SPRs and Reagent Strips were checked to ensure they matched the corresponding assay code.

- The test procedure was started by following the instructions in the user manual. The instrument performed the entire procedure without human intervention.
- After pipetting, the vials were securely re-closed and stored at 2-8°C.
- The test was completed in just under half an hour. After analyzing the test results, the SPRs and strip cassettes were removed from the device.
- Normal ranges for FT3 = 2.17 - 3.34 pg/mL (pictograms per milliliter).
- Normal ranges for FT4 = 0.82 - 1.63 ng/dL (nanogram/deciliter).

Determination of anti-thyroglobulin antibody and anti-thyroid peroxidase

- The required reagents were taken from a ready-to-use refrigerator.
- Each specimen, control and calibrator was tested with an "ATG" Strip (ATPO Strip) and an "ATG" SPR® (ATPO SPR). Carefully reseal the storage pouch after removing the required SPRs.
- The "ATG" (ATPO) code on the instrument is used to identify the test being performed. The calibrator is marked "S1" and checked three times. If the control is attempted, it should be marked "C1".
- A vortex type mixer was used to combine the calibrator, control and samples.
- For this test, the calibrator, control and sample test portion is 100 μL for both ATPO and ATG.
- We loaded the instrument with an ATG, some SPRs (ATPO SPRs) and some ATG strips (ATPO strips). The color labels on the SPRs and reagent strips were checked to ensure they matched the assay code.
- The test was started after reading the manual recommendations. The instrument performed the entire assay procedure mechanically.

- After pipetting, the vials were reclosed and stored at 2-8°C.
- The test was completed in just over a quarter of an hour. At the end of the test, the SPRs and strips were removed from the device and the used SPRs and strips were disposed of in the designated container.
- Normal ranges for anti-thyroglobulin antibody (ANTI-TG) = 2.68 - 33.2 ng/mL.
- Normal ranges for anti-thyroid peroxidase (ANTI-TPO) < 8.0 IU/mL.

Determination of immunological parameters

Estimation of anti-phospholipid autoantibodies (APL) screen IgM was performed using ELISA kits according to the manual procedure provided by Aeskulisa Company (Germany). The analysis was performed in human serum using a microplate enzyme-linked immunosorbent assay for quantitative determination.

Principle: Serum samples are diluted 1:101 and incubated in microplates coated with a specific antigen. If the patient's antibodies are present in the sample, they will bind to the antigen. Any unbound fraction is removed by washing in the next step. Anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are then added and incubated with the antigen-antibody complex in the microplates. In the next step, any unbound conjugate is washed away. The addition of TMB substrate then triggers an enzymatic colorimetric reaction characterized by a blue color. The addition of dilute acid stops this process and causes the color to change to yellow. The initial concentration of each antibody in the patient sample is directly proportional to the amount of conjugate bound to the antigen-antibody complex formed as a result of the reaction. This means that the amount of conjugate bound to the antigen-antibody combination is directly related to the degree to which the chromogenic contributes to the color intensity.

Molecular studies

Extraction of genomic RNA; RNA was extracted using a commercial TRIzol extraction kit following the steps of RNA

separation, RNA precipitation, RNA washing and RNA solubilisation. The RNA was reverse transcribed (RT) to complementary DNA (cDNA), which was used as a template in the PCR reaction. AccuPower Rocket Script RT PreMix from Bioneer/Korea was used for the RT reaction. This product is a lyophilised master mix ready for immediate use. This kit contains everything you need to synthesize first-strand cDNA from an RNA template, including all the essential components. The RT-PCR programme for complementary DNA synthesis was as given in Table 3.

Table 3. RT-PCR program to complementary DNA synthesis

Step	**Temperature**				**Time**
	dN6	**dN12**	**dT20**	**Specific primer**	
Primer annealing	15°C	30°C	37°C	Tm of primer	10min
cDNA synthesis			42°C		30min
Heat inactivation			95°C		5min

RT-PCR was used to detect gene expression of the microRNA gene. Species-specific micRNA146a primers were used to develop RT-PCR primers sensitive enough to detect gene expression of the blood micRNA gene. The primers were supplied by Macrogen Company as lyophilised products in different picomole concentrations. The primers were prepared according to Macrogen's protocol. Some of these details are shown in Table 4.

Table 4. RT-PCR master mix used to detect the micRNA146a gene expression

Component	**Concentration**	**Amount (µL)**
Master Mix	-	10
micRNA146a -F primer	10 µM/µL	1
micRNA146a –R primer	10 µM/µL	1
Nuclease free water	-	6
cDNA sample	-	2
Total	-	20

The following RT-PCR protocol was used to determine the expression of the micRNA146a gene (Table 5).

Table 5. RT-PCR program used to detect the micRNA146a genes expression

Step	Temperature	Time	Cycles
RT inactivation/Hot-start activation	95 °C	2 Minutes	1
Step qPCR a. Denaturation b.	95 °C	15 Seconds	40
Annealing and extension	72 °C	1 Minute	
Dissociation	72 °C	30 Seconds	
	95 °C	30 Second	

Statistical Analysis

The Statistical Analysis System (SAS) programme was used for data analysis to determine the effect of various factors on the study parameters. The mean and standard deviation were calculated for all parameters. After the Kruskal-Wallis test to determine whether the data were normally distributed, the Student t test was used to assess the significance of differences between normally distributed variables. A significance level of 0.05 was used for statistical comparisons.

RESULTS AND DISCUSSION

Serum hormone concentrations including FT3, FT4, TSH, ANTI-TPO and ANTI-TG were measured using the Vidas method. A comparison was made between the study and control groups and significance was assessed using analysis of variance (ANOVA) and t-test. The results are shown in Tables 6-7.

Table 6. Comparing the levels of TSH and thyroid hormones between expectant women in their first trimester and their controls

Parameters	First trimister (Mean ± SD) n= 120	Control (Mean ± SD) n= 45	P-value
TSH (µLU/ML)	4.417 ± 0.613	1.889 ± 0.222	0.046*
FT3 (pg/mL)	3.092 ± 0.463	2.945 ± 0.603	0.628
FT4 (ng/dL)	1.735 ± 0.287	1.192 ± 0.296	0.391

The results showed that the TSH level in the first trimester was (4.417 ± 0.613) µIU/mL, which was higher than the mean ± SD (1.889 ± 0.222) µIU/mL observed in the control group. This study shows that there is a significant difference in TSH levels between the control and patient groups ($P<0.05$). FT3 was (3.092 ± 0.463), lower than the mean ± SD (2.945 ± 0.603) pg/mL of the control group. This shows that there is no significant difference ($P = 0.628$) in FT3 levels between controls

and patients. FT4 is (1.735 ± 0.287), which is closer to the mean ± SD (1.192 ± 0.296) ng/dL of the control group. Furthermore, this study shows that there is no significant difference in FT4 levels between controls and patients (P=0.391).

Table 7. A comparison of APL and anti-thyroid hormone levels between first-trimester pregnant women and their control expectant counterparts

Parameters	**First trimester (Mean ± SD) n= 120**	**Control (Mean ± SD) n= 45**	**P-value**
APL (U/mL)	7.087 ± 0.8426	4.762 ± 0.3437	0.045*
ANTI-TPO (IU/mL)	83.665± 17.565	4.595 ± 0.379	0.013 **
ANTI-TG (ng/ml)	19.325 ± 1.9816	16.588 ± 0.9915	0.549

Table 7 shows the mean levels of anti-TPO, anti-TG and anti-phospholipid antibodies in the sera of pregnant women and control group patients. The mean ± SD value of ANTI-TG was (19.325 ± 1.9816) ng/mL, which is higher than the mean ± SD value of the control group (16.588 ± 0.9915) and not significant.

Moreover, the result shows high significance between ANTI-TPO (0.05) and APL (0.01) when compared with the control group. ANTI-TPO (83.665 ± 17.565) IU/mL and anti-phospholipid (7.087 ± 0.842) U/mL values were all higher than the control group.

Our study found that the risk of miscarriage was significantly higher in pregnant women who were diagnosed with autoimmune thyroid disease (AITD) in the first trimester of their pregnancy compared with those who were not diagnosed with AITD. This finding is consistent with the study by (Glinoer et al. 1994). Another research study conducted by (Triggianese et al. 2021) showed that pregnant women who experienced recurrent miscarriage had a prevalence of thyroid autoimmunity of (37.8%) compared to the control group, which was statistically higher (P = 0.01) than the control group.

According to the division of body mass of women in the target group for study (first trimester group) into normal weight (BMI = 18.5-24.9) and overweight (BMI = 25-29.9), there was a significant difference ($p \leq 0.001$) between the body mass index, where the mean ± SD body mass of pregnant women with normal weight is (23.895 ±

2.454) and the mean ± SD body mass of overweight is (26.738 ± 3.964). Also the table shows the mean ± SD of abortion in overweight (1.900 ± 0.834) is high of normal weight that mean ± SD (1.815 ± 0.827) with no a significant as shown in Table 8.

Table 8. Mean and SD of abortion in women of normal weight and overweight

	Groups	N	Mean ± SD	P – value
BMI	Normal weight	85	23.895 ± 2.454	0.000
	Over weight	35	26.738 ± 3.964	
Abortion	Normal weight	85	1.815 ± 0.827	0.524
	Over weight	35	1.900 ± 0.834	

Our research found that women with a higher than normal body mass index (BMI) were more likely to have a spontaneous abortion.

In addition, total RNA extraction was successfully performed from a variety of samples with RNA concentrations ranging from 62 to 288ng/μL. Aseptic procedures are required to obtain high concentrations of total RNA and are highly dependent on the extraction conditions. The use of TRIzol is recommended for total RNA extraction from blood samples.

After RT-PCR analysis and normalization to the expression of a housekeeping gene, we were able to find the MIR146A gene in these samples. The 2-ΔΔCt approach comparing threshold cycles (CT) was used to quantify relative changes in expression levels. Amplification and detection of the MIR146A gene was performed using SYBR Green qRT-PCR.

Positive results in hyperthyroidism showed amplification at CT (threshold cycle) values in the range of 16.38-17.04 for the housekeeping gene and 28.86-29.42 for the MIR146A gene (Figure 5).

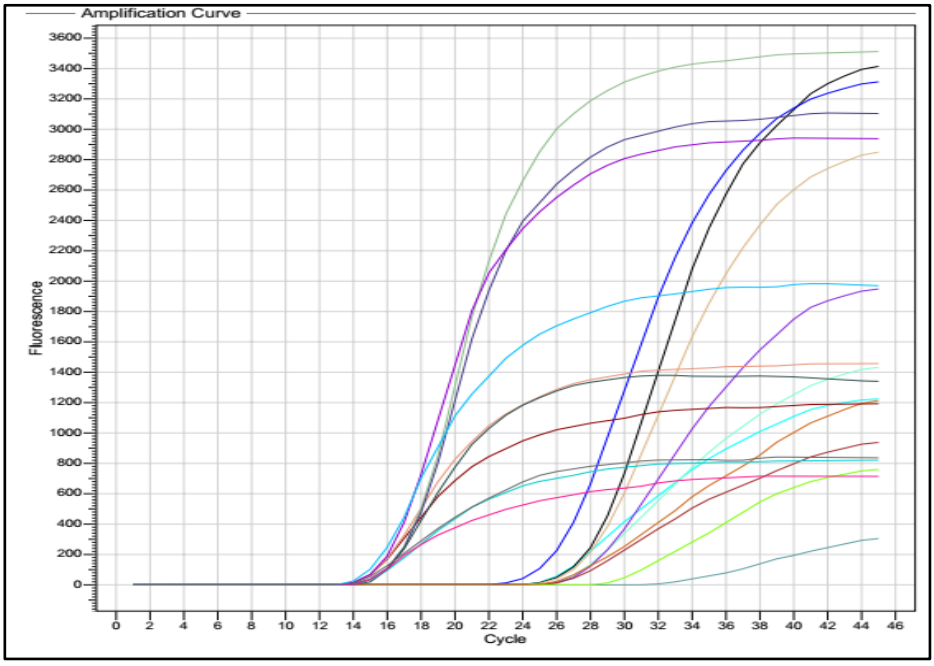

Figure 5. Curve of cycling of *MIR146A* gene and housekeeping gene

The melting temperature (Tm) values obtained for the hyperthyroidism patients and the control sample; the melting temperature was in the range of 81.64, 82.04, 81.94, 81.44, 81.04, 81.34, 82.54, 82.64, 82.34, 82.44 ºC respectively for the housekeeping gene and the melting temperature of MIR146A was in the range of 81.24, 82.44, 54, 81.94, 81.24 ºC (Figure 6).

The expression of microRNA by itself or in conjunction with multi-chaperone complexes acts as a significant repressor of heat shock transcription factor. Research by Zuehlke et al. (2015) elucidates that in some disease cases, increased expression of MIR146A could potentially serve as a prognostic indicator.

Furthermore, Calderwood (2018) reported that a significant upregulation of numerous microRNAs, including MIR146A, MIR146B, MIR148A and miR-146a-5p, was observed in several cancer types, such as breast, prostate, lung and melanoma. This increased expression of microRNAs is strongly associated with poor patient outcomes. MicroRNA itself is overexpressed in thyroid cancer, as observed in the study by Soudry et al. (2017). Furthermore,

increased microRNA expression is associated with poor patient survival. This provides a strong rationale for inhibiting microRNA function as an innovative therapeutic approach in the treatment of certain subtypes of thyroid cancer, as suggested by Lettini et al. (2020).

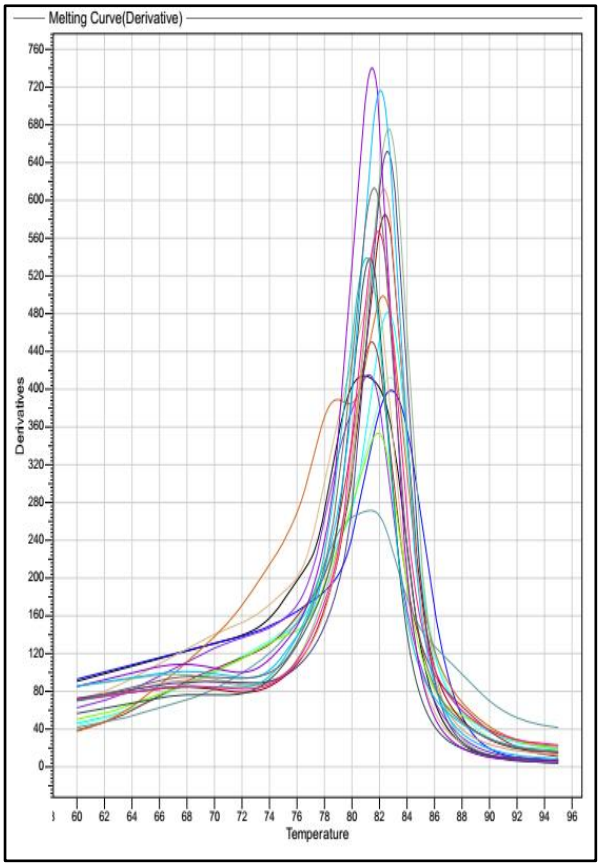

Figure 6. Curve of melting *micRNA146A* gene and housekeeping gene

CONCLUSION AND DISCUSSION

Thyroid hormones (FT3, FT4) and TSH are associated with oxidative stress and antioxidant status in patients with hypo- and hyperthyroidism. The micRNA146A genes can be used as a risk indicator in the diagnosis of thyroid disorders. Real-time PCR is an important tool to determine the gene expression of micRNA146A genes as risk factors, which could be a powerful molecular tool to study the relationship between these genes and thyroid disorders.

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CHAPTER 11

BENEFICIAL HETEROPTERA (HEMIPTERA)

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INTRODUCTION

Turkey according to their climate, flora and fauna, location, agricultural diversities, topography, human habitat, transportation, etc is divided into three zoographical zones. The first region along the coast of the Mediterranean, the Marmara and the Aegean Regions is called the Mediterranean. The second region along the coast of the Black Sea is named the Euro-Siberian. The other region according to its location in Anatolia (Central, Eastern and Southeastern Anatolia Regions) is named Irano-Turanian (Gür, 2016).

Turkey's remarkable ecosystem and habitat diversity have caused the formation of major species diversity in the country. Anatolia has been the motherland of many plants since agriculture began on them. The diversity of Turkey's fauna is even bigger than the diversity of flora. While the overall 60,000 animal species in all of Europe, this figure is more than 80,000 in Turkey and 100,000 passes when incorporated into sub-genres. As with other insect groups, the diversity of insects belonging to the suborder Heteroptera depends on factors such as the extent of Turkey's geological location, climate and vegetation.

The Hemiptera order in the world is ranked fifth with 104,165 species after Coleoptera, Diptera, Lepidoptera and Hymenoptera (Zhang, 2013). While the Heteroptera suborder is represented by 42,377 species and 58,197 genera belonging to 89 families in the world (Henry, 2009), this ratio is 1632 genera and 9365 species in the Palaearctic Region (Aukema et al., 2013), and it is 1349 species belonging to 469 genera belonging to 40 families in Turkey (Önder et al., 2006; Dursun and Fent, 2010, 2013, 2015; Dursun, 2011a, b; 2012; Fent et al., 2011; Dursun and Salur, 2013; Matocq et al., 2014; Çerçi and Koçak, 2016; Konstantinov et al., 2016; Yazıcı, 2020; Yazıcı and Bal, 2022).

Especially in the 19th and 20th centuries, The Heteroptera fauna in Turkey has been studied by both local and international researchers, and important findings were obtained. Its topographical and climatic structure allows it to host a rich and diverse fauna. It is known that Turkey has a very rich fauna of Heteroptera. For this reason, some

faunistic and systematic studies on Heteroptera have been carried out by both local and foreign researchers in Turkey.

Species of the suborder Heteroptera have a four-spined stinging-sucking mouthpart with a long, thin beak that feeds on plant sap (Weber, 1930; Dolling, 1991; Schuh and Slater, 1995). True bugs have front wings called hemielytra that lie flat on the back when resting. However, in some species the wings have become smaller. Hemelitra hardens at the base and turns into a membrane towards the tip. There is a triangular scutellum between the front wings. As part of cobweb symbiosis, cobwebs have adapted to different aquatic and terrestrial habitats such as epilimnetic and hypolimnion and intertidal zones. (Schuh and Slater, 1995; Naranjo et al., 2010). Some of these families, such as Pentatomidae and Miridae, are important pests. They are harmful to their hosts directly or indirectly through the transmission of plant pathogens such as bacteria and viruses. In addition, some predatory species are used as biological control agents (Henry, 2009; Schaefer and Panizzi, 2000).

Although some species belonging to the families Miridae, Lygaeidae, Pentatomidae, Nabidae and Reduviidae are predatory, most of the species feed on plants (Meyer, 2016; 2020). Insects belonging to the family Anthocoridae are predatory species that feed on a wide variety of small arthropods, such as small insects and their eggs (Horton, 2008). Other true bugs, such as predatory Pentatomidae species, may feed on mature larvae or adults. In addition, some parasite species belonging to Heteroptera (from Reduviidae) can transmit some diseases by feeding on human blood (Meyer, 2016). For example, *Triatoma rubida* (Uhler, 1894), a species of Reduviidae, feeds on human blood. Kissing bugs (Reduviidae: Triatominae), which feed on vertebrate blood, spread the trypanosomes that cause Chagas disease and cause more than 7000 deaths per year. They also cause a decrease in the quality of life in affected individuals.

Scientific content
Order: HEMIPTERA
Suborder: HETEROPTERA

Mouthparts

The proboscis emerges from the lower front part of the head and the mouthparts are of the stinging-sucking type (Fig. 1A). The labium forms a long proboscis with a segmented structure. The front part of it is slit all the way through. There are four needles in this tube formed by the labium. Two of the needles are formed from the mandible and two from the maxilla (Fig. 1.B). Essentially, insertion and sucking are done by these needles. Labium has nothing to do with stinging and sucking. It only serves to protect the needles. During feeding, the needles come out of the slit in the front part of the labium and the labium folds and remains outside, only the needles consisting of the maxilla and labium enter the tissue (Fig. 1.C). There are two longitudinal slits (cavities) on the inner surfaces of the needles formed from the maxilla. When the two maxillae come together, these slits become two very thin tubes. The front of these tubes serves as the food channel (fc), and the other serves as the secretion channel (sc). The mandibles are located on the outer part of the maxilla and strengthen the needle by acting as support for them. The labrum has become thinner, and relatively longer, and covers the anterior base of the proboscis. The palps of the maxilla are reduced and sometimes absent (Güçlü, 1999).

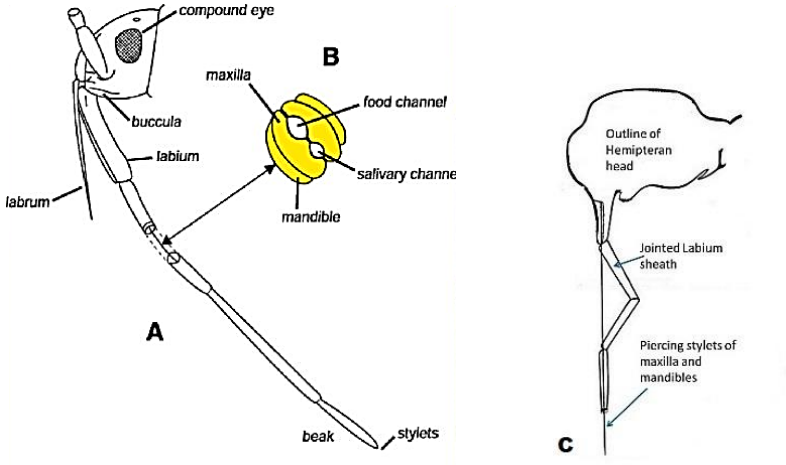

Figure 1. A-Lateral view of the head showing the beak, with the labrum detached from the front of the beak, B-cross-section of the stylets (From Güçlü, 1999); C-insertion of the needle into the tissue (From Unwin, 2001).

Thorax

In species belonging to the suborder Heteroptera, the prothorax part of the thorax is free and larger than the mesothorax and metathorax. Individuals of the species have a triangular-shaped scutellum, which is an extension of the mesothorax, with a blunt or pointed distal end and sometimes upturned. The metasternum has holes for scent glands in many family species. There is a pair of stigmas on the meso and metasternum. Winged and wingless. When winged, the wing is of the "hemielytra" wing type, consisting of skin-shaped part with chitinization and membrane parts (Fig. 2).

Wings

Hemielytra consists of three parts corium, clavus and membrane. Corium consists of two regions exocorium and endocorium. The membrane, whose structure and number of vessels vary according to family, is located at the tip of the wing and has a membranous structure. Sometimes these vessels combine to form closed cells (Fig. 2). The corium and clavus generally have a chitinous structure, which is a characteristic feature of the Heteroptera suborder, and sometimes the intervascular spaces are membrane-shaped and the clavus extends beyond the tip of the scutellum.

Legs

The legs are generally in the form of walking legs and the tarsi is three-segmented (Fig. 2). Coxae articulate with the thorax by a rotation or a hinge joint in Heteroptera. Femurs are highly developed, and smooth, and may have spiny or hairy surfaces. Tibias are generally cylindrical, sometimes triangular, and may be grooved on the outside. In some species, hair and thorn-shaped protrusions may be found on the tibiae. There is usually a pair of nails at the tip of the tarsi. Number of tarsal segments 2 to 3 (Lodos, 1986).

Abdomen

In the Heteroptera suborder, the abdomen consists of 11 segments, as in other members of the order, but since the segments are fused, it often appears as having 7-9 segments, and the last segment is

quite small, where the mating organs are located. The first and sometimes the second abdominal segments are very small and appear to be connected to the metathorax. The abdomen is quite wide, long and cylindrical. Generally, it consists of 11 segments. II. segment and its last piece have become smaller. The first abdominal segment is in the form of a narrow tergum and is often hidden. Therefore, the part that can be seen when viewed from below is the second abdominal segment. IX. segment carries genitalia. In men, this segment is usually a capsule-shaped part called pygophora. The structure of this organ varies according to genus and species, and it plays an important role in distinguishing species. The genital segment in females has a characteristic structure. Pentatomidae species do not have ovipositors because they lay their eggs on plants. VIII. The abdominal segment has often turned into a bracelet shape and become thinner, and it is often not visible from the outside. The pygophore is located at the tip of the abdomen. In the abdomen, terga protrude more or less laterally in many species, except for the genital segment, forming a part called connexivum (paratergite). The part below the paratergite is called parasternite. Stigmas mostly on the underside and on the sides, first VII. or VIII. are found in pairs in each of the segments, IX. and they are not found in the following segments (Lodos, 1986; Önder and Lodos, 1986).

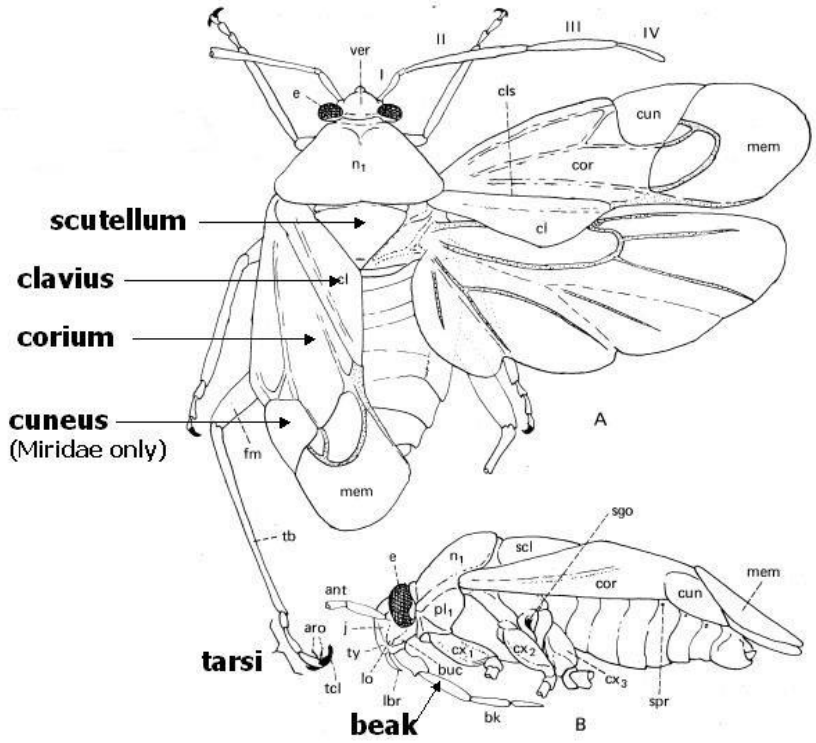

Figure 2. General body parts of Heteroptera (From Anonymous, 1999).

True Bug Life Cycle

Heteroptera family species are hemimetabolous insects. They have five pre-adult stages. There is no rest period. They usually spend the winter as adults. Although it varies depending on the species or environmental factors, after a certain incubation period, the nymphs lift the operculum of the egg either by using the egg breaker or by pushing it with their head and go out (Önder and Lodos 1986). The nymph stage requires 1-2 months or longer. Species of this family tend to live collectively, especially in the 1st and 2nd instar nymphs. Nymphs, which are similar to adults in terms of nutrition, change their first coat after feeding for a while. Feeding and movement stop temporarily during shirt changing. Nymphs become adults after five molts (Önder and Lodos 1986). They usually give one generation per year. However, there are also species that give two or more generations per year.

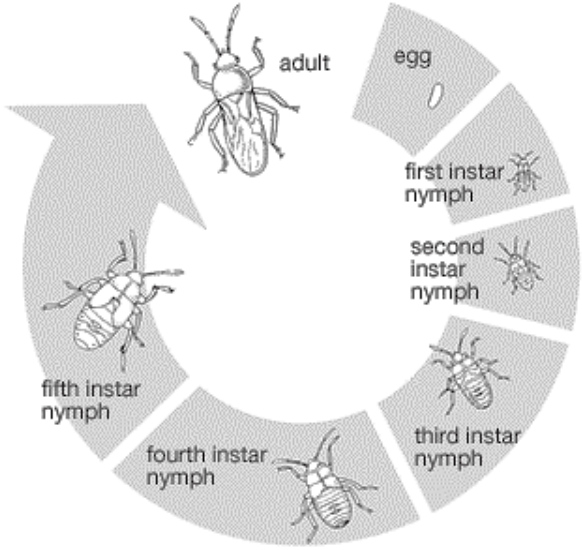

Figure 3. Life cycle of Heteroptera (From Froeschner, 2022).

Reduviidae (Assassin Bugs)

Reduviidae is part of Heteroptera and is a monophyletic group within Hemiptera, with some other predatory families such as Anthocoridae, Cimidae (Yıldırım et al., 2010). Reduviidae are predatorily distributed throughout the world. Body sizes vary between 2 and 50 mm. They are more common in tropical and subtropical regions and form a group of approximately 6900 species belonging to 23 subfamilies (Weirauch and Munro, 2009; Putshkov and Moulet, 2009). The color of Reduviidae is mostly brown and brownish yellow, but some are red, yellow, orange and black. Most assassin bugs have a narrow neck, long legs, and a long head with a distinctive curved piercing beak (Fig. 4) (Hungson and Patterson, 2007). The head often protrudes (Froeschner, 2022). The rostrum is almost always short (3-segmented) and strong in predatory habits. Reduviidae, or assassin bugs, are predatorily distributed throughout the world. Body sizes vary between 2 and 50 mm. They are more common in tropical and subtropical regions and form a group of approximately 6900 species belonging to 23 subfamilies (Weirauch and Munro, 2009; Putshkov and Moulet, 2009). The color of Reduviidae is mostly brown and brownish

yellow, but some are red, yellow, orange and black. The front legs are predatory (Phymatinae, Emesinae), or the front tibiae (and sometimes the middle part) carry a sticky pad (the "*fossula spongiosa*") to hold the prey tightly. Reduviids live in indifferent biotopes (forest, meadows), on the ground (Harpactorinae, Stenopodainae) and in vegetation (Phymatinae, Harpactorinae). The family Reduviidae consists of predatory insects with an extremely voracious lifestyle that allows them to colonize and successfully survive their progeny in extremely hostile environmental conditions (Evagelin et al., 2014).

For example, *Reduvius personatus* Linnaeus, 1758, also lives in human homes or stables. Some (Triatominae) suck are blood and vectors of Chagas disease, but the majority feed on arthropods. Hunting adaptations and trophic habits of Reduviidae are diverse, some are higly specialized feeders (Ectrichodiinae and diplopods; Salyavatinae and termites ...) but the majority are more generalized (one old name of Reduviidae is general-feeders), in some cases real tropic habits are unknown. Some are secondary phytophagous. The biology varies greatly according to the families or genera. The biotopes vary according to the hunting method: on the ground, in or under the vegetation, or in the flowers. The preys vary accordingly. (Yıldırım et al., 2010).

Reduviidae are usually single generation, but in some species development takes more than a year. They form the largest group of predatory terrestrial bugs (Fig. 4) (Putshkov and Moulet, 2009; Hungson and Patterson, 2007).

Figure 4. Adult of *Zelus renardii* (K.) preying on a cotton bollworm (From anonymous, 2023a).

Nabidae

Nabidae (damsel bugs) have worldwide distribution. These small to medium-sized predators (3−15 mm long) live in low vegetation or grasses (Lattin, 1989), where they are lost thanks to their cryptic coloration mostly yellowish or light brown. Ocelli are present. The beak is 4-segmented, long, and usually curved outward from the underside of the head. Front legs are more or less thick for grasping; There is no cuneus in the front wings. It is frequently investigated as a possible biological control agent for insect pests of field crops (Froeschner, 2022). Predatory on soft-bodied insects (Péricart,1987). Carayon (1952, 1955) demonstrated that “traumatic insemination” exists in Nabidae, a phenomenon also known as Anthocoridae. There are 20 genera belong 400 species (Kerzhner, 1981, 1996; Kerzhner and Henry, 2008) distributed in 2 subfamilies worldwide (Schuh and Slater, 1995). The Palaearctic fauna has been revised and keyed by Kerzhner (1981) and Péricart (1987); the catalog was compiled by Kerzhner (1996), and supplements were completed by Aukema et al., (2013).

Nabids are generalists of small invertebrates, mostly arthropods. The Nabis genus, which attacks many insect families, is considered important due to its contribution to the suppression of economically important pests. They feed mostly on crop pests, but they also attack other predators. Nabids immediately kill their prey and suck them dry. Nymphs start eating immediately after they hatch and consume large numbers of individuals to complete their development. Therefore, nabids may be valuable biological control agents against pest pests in agricultural environments. Their general appetite allows them to feed on a variety of insects that are of economic importance in field crops. However, this general feeding habit may make them less effective biocontrol agents than specialist predators that attack certain pest species (Hooks et al., 2016). These predators are preying on (Fig. 5) caterpillars, aphids, leafhoppers, and plant-feeding insects etc. (Hongson and Patterson, 2007).

Figure 5. Adult of Damsel bug preying on *Lygus* sp. (From Berry, 2000).

Anthocoridae

Anthocoridae, also known as tiny pirate bugs, are small representatives of the infraorder Cimicomorpha (1.5−5 mm long) and there are more than 475 species in the world (Henry, 2009). Antennae and rostrum are thin and more or less filamentous. The forewings have

a sticky pad or "fossula spongiosa" on the cuneus and foretibiae. The male genitalia is asymmetrical with only a single paramere developed, and the female ovipositor is of the laciniate type. Men practice “traumatic insemination.” Traumatic insemination is a specialized fertilization method known in some Cimicomorpha such as Anthocoridae, Cumicidae, Prostemmatinae (Schuh and Slater, 1995).

Anthocoridae are generally predatory (Carayon, 1961; Péricart, 1972), but some are known to be phytophagous (at least part of their lives) (e.g. some eat pollen Orius Wolff, 1811 - Salas Aguilar and Ehler, 1977), some prey on birds, rodents or humans They can suck your blood optionally (Stys and Daniel, 1958). Anthocorids are effective predators and play an important role in the natural control of many pests in different parts of the world. Anthocorid predators are recognized as potential biocontrol agents and are represented in all zoogeographic regions of the world. The small lepidopteran larvae (Figure 6) feed on small grubs, psocids, mites, thrips, aphids and storage pests and are commonly known as small flower bugs or small pirate bugs (Ballal and Yamada, 2016).

Figure 6. Life stages of *Blaptostethus pallescens* adult predating on eggs of tobacco caterpillar (From Ballal and Yamada, 2016).

Miridae

The Miridae family is one of the two families within the Miroidea superfamily belonging to the Heteroptera suborder of the Hemiptera order. Species of the Miridae family can be easily distinguished from

other families of the Heteroptera suborder by their four-segmented antennae and proboscis, the absence of ocelli at the beginning, the presence of the cuneus at the tip of the hemielytra, and the presence of two closed cells in the membrane, one large and the other small.

The Deraeocorinae subfamily within the Miridae family includes all predatory species (Fig. 7). They attack soft-bodied insects that are smaller than themselves. They feed on adults and nymphs of aphids and psyllids, small Lepidoptera larvae and mites. Again, *Camylomma diversicornis* feeds on *Tetranychus utricae* adults and nymphs. In addition, it is a larva and egg predator of *Helicoverpa armigera*, which causes significant damage in cotton fields (Yazıcı, 2015). In a study conducted on the effect of *Macrolophus pygmaeus* (Hemiptera: Miridae) on whiteflies, which are a problem in greenhouses, under different light and temperature conditions, it was determined that *M. pygmaeus* could be considered as an alternative to biological control in low temperature and short day conditions (Franco et al., 2011). In a study by Lucas and Alomar (2002), in which they stated that *Macrolophus caliginosus* (Wagner 1951) was commercialized in Europe for the control of whitefly in tomato greenhouses, *Macrolophus caliginosus* and *Dicyphus tamaninii* (Wagner 1951) were used together to control whitefly in greenhouses, and as a result, the heterospecific combination turned into a monospecific combination. They determined that there was more hunting compared to. As a result, it was determined that among these leaves, leaves infected with *Myzus persicae* showed the highest oviposition preference (Yazıcı, 2015).

Figure 7. *Deraeocoris ruber* feeding on *Aphis fabae cirsiiacanthoidis* on thistle (From Anonymous, 2023b).

Pentatomidae

Pentatomidae is a large family of stink bugs, with more than 4,500 species in the world. Their bodies vary between 4-50 mm (Dursun 2004). Although the body size of the Pentatomidae family varies depending on the species, they generally have medium to large bodies and an oval appearance. Most pentatomids are light colored or have distinct markings. While some species have flat bodies, there are also species with almost round bodies. The integument may be hard, bare, rarely hairy or hairy (Lodos 1986).

Insects belonging to the Asopinae subfamily include especially predatory species. Their nymphs and adults feed by stinging and sucking soft-bodied insects. Some species play a very important role in biological control. *Picromerus bidens* (Fig. 8) and *Picromerus conformis* in some butterfly, chrysomelid and saw bee larvae; *Rhacognatus punctatus* feeds on some chrysomelid larvae and *Zicrona coerulea* feeds on various butterfly and chrysomelid larvae (Lodos et al., 1978). Predator species are useful because they feed on pests.

During feeding, these species secrete a liquid from the salivary duct of their proboscis that prevents the blood of its host from clotting. They feed by sucking the internal contents of their hosts (Önder and Lodos, 1986).

Figure 8. Adult *Picromerus bidens* predating on insect (From llavaneras, 2013).

Geocoridae - Bigeyed Bugs

Geocoridae is a small family of predatory insects, with only 275 species of big-eyed insects in the world. Adults are brown or black in color and their length varies between 3-5 mm. Adults have a triangular head and large, prominent eyes that occasionally extend backwards. These predators are often found in weeds, sparse grass, forests and stream banks. Sometimes big-eyed bugs are confused with chinch bugs or other pests (Hongson and Patterson, 2007). It is among the most important natural enemies of cotton. They feed on eggs and small larvae of most lepidopteran pests (boldworm, pink bollworm, tobacco budworm), eggs and nymphs of plant bugs (e.g. lygus), and all life stages of whiteflies, mites and aphids (Hagler and Cohen, 1991).

Figure 9. *Geocoris punctipes* preydating on mites (From Wikipedia, 2023).

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CHAPTER 12

SYMBIOTIC LIFE IN AQUATIC ECOSYSTEMS, THE CASE OF *Rhodeus* sp. and *Unio* sp.

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INTRODUCTION

Aquatic ecosystems are areas rich in biological diversity and have various ecological relationships between organisms. While water is vital for humans, it is also home to many organisms as animal and plant form in macro and micro dimensions. All of the relationships between these organisms living in water with each other and their environment constitute aquatic ecosystems. Outside the water, organisms in water are connected to their environment with vital bonds. Activities such as nutrition, shelter and reproduction provide these bonds.

There are views that symbiotic relationships in aquatic organisms play a critical role in the evolutionary process (Heckman et al., 2001; Lipnicki, 2015). It is known that a species that lives in an ecosystem and is one of the components of the environment increases its resistance to environmental threats, predators and parasites due to vital relationships with other species (Lipnicki, 2015; Stock et al., 2021). These views are supported by the results of molecular studies in lichenised fungi (Lutzoni et al., 2001; Lücking and Grube, 2002). It has been reported that determinations of the biological and ecological characteristics of lichenised fungi and their components play an essential role in symbiotic relationships and transition processes from aquatic to terrestrial ecosystems (Hawksworth, 1988; Grube and Hawksworth, 2007; Peréz-Ortega et al., 2010).

This study aims to contribute to a better understanding of the relationships occurring among aquatic organisms by providing examples of symbiotic relationships in aquatic systems and more information about the relationship, mainly focusing on their reproductive activities between individuals of *Rhodeus sp.* (bitterling) and *Unio sp.* (freshwater mussels).

SYMBIOTIC RELATIONSHIP

Symbiosis can be defined as two or more organisms of different species living together and benefiting or being harmed by that life. Symbiotic relationships are achieved in 3 basic steps.

Mutualism: A type of symbiosis in which both organisms benefit. For example, the relationship between corals and zooxanthellae is mutualistic. Corals host photosynthetic algae called zooxanthellae within their tissues. The zooxanthellae provide organic matter and oxygen to the corals, and the corals provide carbon dioxide and nutrients to the zooxanthellae.

Commensalism: A kind of symbiosis in which one organism benefits and the other is not affected. For example, the relationship between sharks and remora fish is commensal. Remora fish attach to sharks and feed on their waste without harming them.

Parasitism: A type of symbiosis in which one organism benefits and the other is harmed. For example, the flat trematodes Clinostomum species infect some freshwater fish. These parasites use freshwater snails, freshwater fish and finally, birds as hosts through the food chain (Aktop et al., 2021).

Symbiotic Life in Aquatic Ecosystems

Symbiosis is an important ecological phenomenon that increases the diversity and harmony of living organisms. Symbiotic relationships allow organisms to use resources more efficiently, develop better defence mechanisms and have a more complex structure. How symbiotic life arose and how it functions in the evolutionary process is still being studied in biological sciences. Symbiotic life is common among the forms in both terrestrial and aquatic ecosystems. The aquatic organism forms symbiotic relationships in order to survive and benefit from resources in the environment. Some of these relationships are described below.

Microorganisms can sometimes colonise a larger host organism inside the cell, in any tissue of the host, around epithelial tissues and in the gastrointestinal tract, and can mutually sustain their lives as an endo-symbiotic relationship (Zoccarato and Grossart 2019). The host organism benefits physiologically in this relationship, while the microorganisms provide a sheltered habitat and nutrients (Stock et al., 2021).

Chemosynthetic bacteria and some marine invertebrates can live in sunlight-poor deep-sea vents and sediments with high sulphur concentrations. Chemosynthetic bacteria are responsible for converting carbon dioxide and methane gas into organic compounds in the water. Other invertebrate species are also responsible for the distribution of chemosynthetic bacteria and their interactions with other bacterial species. Five different invertebrate families (Mytilidae, Thyasiridae, Lucinidae, Solemyidae and Vesicomyidae) have been reported in symbiotic relationships between chemosynthetic bacteria and aquatic invertebrates (Roesellers and Newton, 2012).

Lichens result from the symbiosis between fungi and algae. The algae provide organic material to the fungi through photosynthesis, while the fungi supply water and minerals required for fundamental vital processes of the algae (Karplus, 2014).

Coral reefs formed in the seas through the photosymbiotic relationship between jellyfish and dinoflagellate algae are known to host more than 25% of all species identified in the oceans, making them significant biodiversity hotspots (Weis, 2019).

A symbiotic association between fungi and plant roots is called mycorrhiza. The fungi help expand the surface area of the roots, facilitating the absorption of water and minerals while simultaneously providing organic matter to the plant. This results in the development of an aerobic sedentary micro-ecosystem stimulated by the oxygen released through the roots. The roots thereby become increasingly efficient, enabling plants to thrive in wetland environments. The

oxygenized zone diminishes the quantities of sulphates and metal ions in the surroundings by corrupting them (Roth, 2008).

It is widely accepted that a photosynthetic symbiotic relationship exists between sponges and zooxanthellae algae, resulting in a characteristic deep green hue for sponges. The algae residing within the sponges' pores can produce oxygen even in low-light conditions, sustaining their survival. In exchange, sponges provide the high levels of carbon dioxide required by the algae. This relationship significantly enhances the population growth rate (Jensen and Pedersen, 1994).

Some microorganisms in the digestive systems of aquatic animals assist in their nutrient digestion and further support their immune systems. In this relationship, aquatic animals also provide a habitat and sustenance for these microorganisms (Karplus, 2014).

Plant organisms, one of the fundamental components of mangrove ecosystems, create sheltered areas for juvenile fish to evade predator pressure, mainly through their submerged spiral root structures. Additionally, it has been reported that these plants meet their nutritional needs from the metabolic waste products of the juvenile fish they harbour (John and Lawson, 1990).

Among the species of fish adapted to live within anemones are *Amphiprion sp.* and *Premnas biaculeatus*. These fish species evolved to inhabit the poisonous tentacles of anemones, minimize predator pressure and provide cleaning and organic waste disposal services to the anemones (Karplus, 2014). Moreover, these fish species also utilize the anaesthetizing substance in the anemone's tentacles for cleaning their epidermis (Fishelson, 1964).

In the symbiotic relationship between corals and sea anemones, *Symbiodinium sp*. flagellates play a role in the growth and sustainability of reefs, particularly in nutrient-poor waters. In contrast, coral reefs provide shelter and habitat for algae (Davy et al., 2012).

A mutualistic symbiotic relationship exists between shrimp and sea anemones based on mutual benefit. Harmful parasites affecting

anemones are consumed as food by shrimp, thus preserving the health of the anemones. In return, shrimp find shelter among the tentacles of anemones, creating a clean and healthy living environment (Karplus, 2014).

Sea stars (*Asterias* sp.) residing in the same ecosystem secrete anaesthetic substances to induce the opening of mussel shells and utilize mussels as a food source. As a result, they significantly contribute to regulating mussel populations within the ecosystem (Lavoie, 1956).

In aquarium fishkeeping, one commonly preferred species is the cleaner wrasse (*Labroides dimidiatus*), which is known to feed on juvenile gnathiid parasites that infest host fish and has a broad distribution in warm tropical regions (Türkmen and Aktuğ, 2011). By consuming these parasites as food, cleaner wrasses effectively clean other fish species of this parasitic influence (Grutter, 1996).

While most bioluminescent fish produce light in their photophores, a few light-producing fish species have formed relationships with specific groups of bacteria hosted in their light organs. Symbiotic bioluminescent bacteria, such as Photobacterium and Aliivibrio, have been observed in flashlightfish, and bacterial genera not yet defined have been found in lanternfish. When studying these symbionts, it becomes evident that light-producing bacteria can only reproduce in the light organs of flashlight fish and ponyfish. This symbiotic relationship serves as a noteworthy example of mutual benefit, providing these bacteria with favourable conditions for nutrition, reproduction, and propagation, while aiding the hunting strategies of the fish (Karplus, 2014).

The symbiotic relationship between sponges and fish was first reported in 1741 off the coast of Indonesia, and in the 1970s, relationships with 39 more sponge species were described (Tyler and Bohlke, 1972). In subsequent years, the relationship with many more species has been identified with sponges. Almost all fish species

involved in this relationship belong to the Gobiidae family (Karplus, 2014). *Evermannichthys* sp. and *Risor* sp. have specialized large and raised ctenoid scales only on the front part of their caudal fins to facilitate movement within sponges (Beebe, 1928; Bohlke and Robins, 1969). Specialized canine teeth of the *Risor ruber* species are used to capture aquatic invertebrates hidden within the sponge (Tyler and Bohlke, 1972), while *Luposicya lupus* possesses a lateral row of outer teeth to scrape particles embedded in the mucus layer while feeding on sponges (Larson, 1990). It has been noted that symbiotic evolution processes, such as colouration, occur in *Pleurosoicya elongata* and *Risor ruber* species (Tyler and Bohlke, 1972). In this relationship, sponges provide fish with a sheltered area for feeding on plankton, nekton, and invertebrate parasites that inhabit the sponges. Sponges benefit from cleaning and removing parasitic invertebrates (Karplus, 2014).

Siphonophores are aquatic invertebrates from the solential plankton group, generally distributed as coastal zone colonies (Karplus, 2014). Little is known about the relationship between siphonophores and fish, including the well-known Portuguese man-of-war species *Physalia physalis* (Purcell and Arai, 2001). Some tentacles of siphonophores are visualized to resemble larvae or copepods (Purcell, 1980). The bioluminescent tentacles glow red in deep water, attracting mid- and deep-water fish. Fish can feed by preying on tiny organisms attracted to the light emitted by the siphonophores. Siphonophores catch their prey more easily, thanks to the water movements caused by the movements of the fish (Haddock et al., 2005).

Scyphoza, a small class of the phylum Cyndaria, contains about 200 planktonic species, mainly marine. Although they are found in two morphological structures, medusa and polyp, the medusa form is the most dominant. The medusa form is a vast, slow-swimming exoskeletal organism with a diameter of about 2 metres. Its defence mechanism against its predators consists of poisonous tentacles. Their relationship

with fish is usually short-lived. Although this relationship with *Merlangius merlangus* was first reported in 1671 (Thiel, 1978), relationships with Carangidae, Stromateidae, Centrolophidae, Nomeidae, Gadidae, Girellidae, Centriscidae, Tetragonuridae and Zoproridae families are also known (Mansueti, 1963). In these relationships, fish species are protected against the harmful effects of nematocysts in the tentacles of jellyfish by the mucus and scale layer on their skin (Scheuring, 1915), the chemical camouflage formed with the mucous layer of jellyfish (Zaan, 1980) and the secretions of nematocysts produced in the bodies of fish (Arai, 1988). Fish live sheltered among the cilia of medusa-shaped jellyfish and can feed on mini-organisms in the environment. In addition, since the fish recognise dangers quickly, they act as an early warning system for jellyfish against hazards.

The most well-known cleaners in aquatic environments are fish, mussels and shrimps. Since these organisms are usually concentrated around sponges and anemones, these areas have been described as cleaning stations (Feder, 1966). The relationship between fish and shrimps has been described as a cleaning symbiosis, and this subject was first recorded by Harry Pederson in 1950 with underwater photographs (Limbaugh et al., 1961). Today, about 40 shrimp species in the families Palaemonidae, Hippolytidae, Stenopodidae, Gnathophyllidae and Alpheidae and a crab species, *Stenorhynchus lanceolatus* have been reported (Van Tassell et al., 1994). Cleaning shrimps and crabs consume ectoparasites, diseased and injured tissues in the mucus layer of fish, as food while the fish are cleaned (Feder, 1966).

The relationship between sea anemones, cleaner shrimps, and fish is also a cleaning symbiosis. Sea anemones provide a safe habitat for cleaning shrimps, while fish easily find cleaning shrimps. Fish that want to be cleaned come close to the anemones at the cleaning stations. The cleaning shrimps show their willingness to clean by performing

some dances (Sargent and Wagenbach, 1975; Becker et al., 2005) and constantly closing and opening one of their pincers. It has also been reported that proximity, contact and colouration are effective in dance movements (Becker et al., 2005). In this relationship, anemones and cleaning shrimps can easily reach and feed with mini-organisms, while the fish are free from harmful ectoparasites, bacteria and various external surface lesions (Kulbicki and Arnal, 1999).

Cephalopoda is a large class that includes squids, squid, and octopuses and many species are reported to be extinct. Octopuses have a pair of gills and ten arms (Ruppert et al., 2004; Pechenik, 2009). With suction cups on these arms and a mouth similar to the beak structure of a parrot, they are one of the best predators of marine ecosystems. Octopuses feed on mussels and other shellfish. They catch the crustaceans with their arms, attach their suction cups to the crustaceans, and then feed by breaking the shell with their strong beak-like jaw structure and separating it with their arms (Karplus, 2014).

It is known that octopuses keep their previous prey's shells, skeletons and meat residues in their nests. These scraps also attract the attention of other scavenger fishes. It is reported that this situation shows the hunting strategy of octopuses. The relationship between octopuses (*Octopus vulgaris*, *O. cyanea* and *O. macropodus*) and various fish species (*Ascelichthys rhodorus*, *Hemilepidotus hemilepidotus*, *Sebates* sp., *Gobiesox maendricus* and *Nautichthys oculofasciatus*) has been reported (Mather, 1991). When octopuses hunt, fish follow the octopus. As the octopus breaks down and eats its prey, the tissues scattered around become bait for the fish (Diamant and Shpigel, 1985; Mather, 1991). It has also been reported that some of these fishes can enter the nests of octopuses (Hartwick and Thorarinsson, 1978). Although octopuses are known to be unresponsive to these fishes, it has been observed that they rarely hunt some of them (High, 1976). The relationship between the scavenging fishes finding

the opportunity to feed and the octopus cleaning the nest can be considered a symbiosis of feeding and cleaning.

It is accepted by all scientific circles that water plays a vital role in the evolutionary processes of life. It is stated that the development of aquatic organisms, from the first known organisms to today's organisms, has developed depending on their adaptations and has become quite complex. It is known that Adaptations to the unique conditions of aquatic environments have differentiated living species (Roth, 2008).

The relationships that the organisms forming ecosystems form among themselves and with their environment bring about differentiation in continuing their vital processes. In the evolutionary process, these relationships and differentiations will provide important information about the future of living organisms. It is known that individuals of each species have a role in the food web and chain. However, when the vital processes of species exposed to environmental pressures face problems, it causes problems for the future of other species with which it is related. Especially in aquatic organisms, an extensive structure is seen in mutual relations. Keeping these environments away from environmental threats is crucial for a sustainable life and evolutionary process.

BITTER FISH AND FRESHWATER MUSSELS

Some genera of *Rhodeus* and *Unio* species are distributed in Turkey (Çiçek et al., 2020; Arslan, 2022; Arslan and Günal, 2023). It is known that there are remarkable and mysterious relationships between these species in terms of both vital and evolutionary processes related to reproductive strategies. Information on the bio-ecological characteristics of *Rhodeus sp*. and *Unio sp.* were given among these essential relationships, and an evaluation was made of the symbiosis between them.

Bitter Fish (*Rhodeus sp*.)

The systematic categories of the genus *Rhodeus* are given below, and a photograph of male and female individuals is given in Figure 1.

Phylum : Chordata
Subphylum : Vertebrata
Class : Actinopterygii
Order : Cypriniformes
Family : Acheilognathidae
Genus : *Rhodeus*

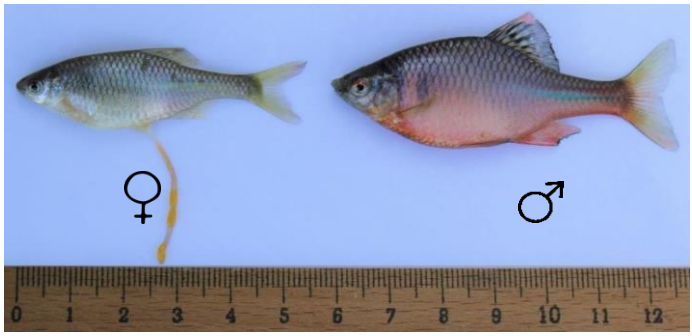

Figure 1. *Rhodeus sp*. (Photographed by: Göktuğ GÜL)

Bitter fish species are large and flattened-bodied species of the carp family and are generally small in size. Male individuals usually have a tubercle structure on the nose. They are small-sized freshwater fishes with an average length of 10-12 cm. It has an oval body structure that shrinks towards the nose and is flattened from the sides. Although it has a large-scale system compared to its body, there is no complete and clear lateral line. It has a small mouth with a partially ventral position. It has large eyes on both sides of the body. The dorsal fin (III/8-10) up to the caudal peduncle starts from the middle of the body. The homocercal caudal fin (0/19-20) with oval ends is attached to the caudal peduncle, which is thin and long. Anal fin (III/8-10) with a very slight curvature and short and pointed pectoral fin (I/10-11). The ventral fin (II/6-7) is near the body's centre (Gül, 2021; Fishbase, 2023).

It shows various colouration characteristics depending on its sex and size. It is tough to distinguish between male and female in individuals with silver-grey colouration except during the breeding period. The blue-green colouration from the lateral to the caudal peduncle is considered an essential species marker. Individuals show profound differentiation when they enter the reproductive period. Male individuals have different and eye-catching colouration during the reproductive period. Purple, red and yellowish colours in other body parts and a red spot on the white of the eye become prominent. In addition, male individuals have intense tubercles on the head during the reproductive period. It is reported that freshwater mussels are found in the same habitats when reproductive characteristics occur (Moreva et al., 2017; Gül, 2021).

Rhodeus sp. is distributed in rivers and canal systems of Central and Eastern Europe and the Baltic, Black Sea, Aegean and Mediterranean basins (Figure 2), where pollution, weed control, anthropogenic sediment disturbance and pressure from invasive/predatory species pose a severe threat to the persistence of the species (Fishbase, 2023). As a habitat, it prefers areas with clean and abundant oxygen, slow flow, sandy bottom, and shaded regions covered with aquatic plants where mussels, especially *Unio sp*. individuals, are found (Freyhof and Kottelat, 2007; Gül, 2021).

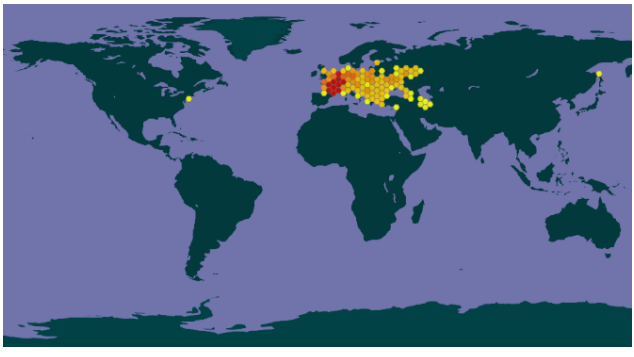

Figure 2. Distribution of *Rhodeus* sp. (Map: Global Biodiversity Information Facility, gbif.org)

They reach sexual maturity after going a length of about 30-35 mm at one year, and their life span is about five years. They reproduce between April and August when the water temperature is 15 °C and above (Freyhof and Kottelat, 2007; Fishbase, 2023).

Freshwater Mussel (*Unio sp*.)

The systematic categories of the Unio genus are given below, and its photograph is in Figure 3.

Phylum : Mollusca
Class : Bivalvia
Subclass : Autobranchia
Order : Unionida
Family : Unionidae
Genus : *Unio*

Figure 3. *Unio* sp. (Photographed by: Göktuğ GÜL)

Unionidae has the wealthiest species diversity, with more than 620 species in the order Unionida (Arslan and Günal, 2023). Freshwater mussels are long-lived organisms distributed in lentic and lotic systems almost everywhere in the world and can reach their first reproductive maturity in the nearly tenth year (Haag, 2009). They live in sandy areas of freshwater systems, usually buried in the sand. In their sedentary life, their distribution is only realised through the hosts of their larvae that make parasitic symbiosis (Graf, 2002). They are widely distributed in many large water basins from west to east in

Turkey (Arslan, 2022). Since their general nutrition is based on filtration, they ensure the removal of many substances, such as phytoplankton, phosphorus, ammonium, and heavy metals from the water systems they live in. In this way, they also improve water quality (Güler et al., 2017; Coşkun et al., 2019).

Reproductive Symbiosis of Bitter Fish (*Rhodeus sp*.) and Freshwater Mussel (*Unio* sp.)

Species belonging to 3 different genera (*Rhodeus, Tanakia* and *Acheilognathus*), known as bitter fish in the literature (Arai, 1988), establish symbiotic relationships with Ableminae, Anodontinae and Unioninae subfamilies of freshwater mussels (Smith et al., 2004; Liu et al., 2006). This study evaluated the association between *Rhodeus* sp. and *Unio* sp., widely distributed in Turkey, with their reproductive strategies.

The first research on the reproductive behaviour of bitter fishes was conducted in 1877 (Wiepkema, 1961). Subsequent studies have shown that a resource-based mating system is a good model for behavioural ecology and population dynamics (Smith et al., 2000a, 2004, 2006) and that *Rhodeus sp*. and *Unio* sp. have a high level of reproductive association in the wild. Bitterfish is triggered to reproduce when the water temperature rises above 15 C in autumn or spring (Brian et al., 2022). During the breeding period, the male individuals prefer areas with a certain number of mussels and defend them against other individuals (Karplus, 2014). It has been reported that mussel odour is more effective than many other factors (ammonium hyaluronic acid in mussel mucus) in the spawning area preference of bitterns (Heschl, 1989). During the breeding period, male bitterns attract females with their vivid colours towards the area where the mussels are located.

Spawning females approach the mussel in an upside-down position and wait in this position for a few seconds. This position aims

to test whether the mussels are suitable for spawning by determining the oxygen level from their siphons (Smith et al., 2001; Mills and Reynolds, 2002). It has been reported that low or high oxygen levels affected the spawning and that the size of the mussel is not considered by the fish during this test (Smith et al., 2000b; Kitamura, 2005). When spawning is decided, an ovipositor with a long oviduct is inserted into the respiratory tract of the freshwater mussel, and the eggs are deposited into the so-called mantle cavity (Maitland, 1977) or gill chamber (Wheeler, 1969). This process is repeated intermittently on multiple freshwater mussels until all eggs are used. Females are reported to lay fewer eggs by reducing ovipositor contact with sick and parasitised mussels in the breeding grounds while laying more eggs on healthy mussels (Smith et al., 2000b).

Male individuals also deposit their sperm on the gill ducts of the mussels in which the eggs are laid. The sperms are taken into the mussel by respiratory water siphon, a natural physiological activity of mussels. Thus, the fertilisation process of the eggs is completed in the mussel gills. At the end of the incubation period of about four weeks (Smith, 2004), the bitter fish hatch and continue their development, lying side by side between the lamellae parallel to the gill lamellae. Up to 60 juvenile bitter fish can continue their growth in a mussel (Aldridge, 1999). Noshino et al. (2023) reported that during the development of bitter fish juveniles, they could be suppressed by a parasite of freshwater mussels, the leech *Hemiclepsis kasmiana*. It has been reported that this parasitic leech feeds on bitter fish larvae in mussels (Noshino et al., 2023). When the bitter fish reach a specific body size, they emerge from the gill canal of the mussel (Smith et al., 2004). There are different studies on the size at which bitter fish leave the mussels. For example, Maitland and Campbell (1992) reported a length of 9-10 mm, Wheeler (1969) 20 mm, Aldridge (1999) 10 mm. The times to reach the specified lengths were reported as 2-3 weeks (Maitland and Campbell, 1992), 3-4 weeks (Wheeler, 1969) and 6-7

weeks (Bresse, 1950). Experimental studies have shown an inverse relationship between the water temperature increase and the time juvenile bitter fish leave the mussels. As the water temperature increases, juvenile bitter fish leave the mussels much earlier (Aldridge, 1999). Juvenile bitter fish that leave the mussel continue their lives in the aquatic environment.

Freshwater mussels are a species at high risk of extinction globally (Lydeard et al., 2004). Certain species have been classified as endangered on the IUCN Red List (IUCN, 2011). Anthropogenic stressors are exacerbating the impact on them continuously (Arslan, 2022; Arslan and Günal, 2023). Studies have found that bitter fish egg-laying adversely impacts freshwater mussels by reducing their reproductive and growth performance, leading to oxygen stress, gill damage, and mortality (Smith, 2017). This phenomenon has led to a decline in the populations of mussels, which directly affect other aquatic organisms, according to previous research (Williams et al., 1993; Haag and Williams, 2014; Lopes-Lima et al., 2017).

Unionid freshwater mussels employ unique reproductive strategies compared to other mussels. They lack planktonic larvae and instead use glochidia larvae. These larvae undergo incubation within the mussel and become encapsulated in modified water tubes in fish's gills, eventually settling as obligate parasites (Tankersley and Dimock, 1993). Glochidia larvae attach to fish gills with an elastic and highly adhesive larval thread lacking cellular structure. The larvae exhibit strong selectivity, attaching themselves securely to the gill lamellae using adductor muscles only upon encountering a suitable host. Additionally, the larval thread slows the larva's vertical movement in the water upon its departure from the fish gill (Wood, 1974; Karplus, 2014).

When the reproductive female *Unio sp*. larvae begin to mature, they transmit their larvae to the host fish in several different steps. In the first step, the mantle's edges thicken and appear curtain-like with

attractive colours. This curtain-like appearance is structured as a fish silhouette and moves like a fish. The image of a juvenile fish, including the eye, fin and lateral line, is rendered in detail. Thanks to the flow of water and mantle movements, the swimming activities of this fake baby fish are also completed (Kraemer, 1970; Haag et al., 1998).

In another step, the mantle edges are transformed into tentacle-like structures that beat rapidly (Haag et al., 1998). Then, a sticky sac containing thousands of glochidia is sprayed into the water through the gill opening of the mussel. This spraying continues continuously. Fish that come to hunt the fish-like mantle are exposed to the glochidia larvae by this spraying, and larval parasitism occurs (Haag and Warren, 1999). All of the steps are a fish trapping system, and this trap was first described by Barnhart et al. (2008). From this moment on, they are fed by the flow of water provided by the movement of the fish. At the same time, the fish transport the larvae to different parts of the water system. The parasitism of the larvae ends after a few weeks when the capsule ruptures and the juvenile mussel falls through the gill to the bottom.

The reproductive symbiosis of bitter fish and freshwater mussels has been the subject of many scientific studies. Tatoj et al. (2017) stated that some species belonging to the Unionidae family are endangered, and ecological studies should continue. It is thought that *Rhodeus* sp. individuals may enter into relationships with other mussel species for reproduction as a result of habitat losses and population declines. It has been reported that gills of other mussel species may offer a better habitat for *Rhodeus* sp. larvae in the evolutionary process (Liu et al., 2006; Smith, (2017). Kujawa and Piech (2021) tried to breed *Rhodeus* sp. and *Unio* sp. under laboratory conditions in order to prevent the possible loss of species in response to the deterioration of aquatic ecosystems. As a result of the experiment, they determined that healthy and productive individuals were obtained. Arslan and Günal (2023) stated that there may be decreases in mussel populations due to direct exposure of mussels to various anthropogenic contaminations in

aquatic natural ecosystems. Lopes-Lima et al. (2017) stated that bitter fish can develop different strategies for reproduction in response to the decrease in mussel populations. Douda et al. (2017) noted that these strategies designed are essential for the continuation of the generations of species and that research in this field should continue. Van Damme (2017) and Reichard et al. (2007) described *Rhodeus* sp. individuals as both invasive and parasites of freshwater mussels and considered them a natural threat to mussel populations. They also stated that ecological conditions can change or expand the distribution areas of bitter fish populations. Reichard et al. (2007) reported that with the distribution of bitter fish to different habitats, they may come together with mussel species with which they have never developed reproductive symbiosis before and that they may interact with other species of mussels by experiencing various changes for reproductive symbiosis in the evolutionary process.

Aquatic ecosystems can be damaged due to human impacts based on various needs. Negative changes in aquatic conditions such as deterioration of water quality of aquatic ecosystem with environmental mixtures, inappropriate reclamation works, change in sediment structure as a result of sand extraction, temperature and flow rate change due to global warming, excessive and unconscious hunting, invasive species propagation may cause the number of individuals of aquatic organisms to decrease or even disappear. Every living organism in ecosystems has duties and relationships with other organisms. The functioning of the biosphere is only possible with the continuity of these relationships. Ecosystem interventions with various effects can turn symbiotic relationships into dysbiosis.

It has been determined that any degradation within freshwater ecosystems could negatively impact the reproduction and population dynamics of mussels and bitter fish, which is this study's focus. As the deterioration of one ecosystem can lead to the decline of others, it is

crucial to maintain continuous environmental protection and monitoring.

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