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COMPARISON OF SANGER SEQUENCING AND NEXT GENERATION SEQUENCING METHODS FOR INVESTIGATION OF JAK2 EXON 12 MUTATIONS IN FOLLOW-UP OF PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISEASE AND JAK2 V617F NON-MUTATION

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ABSTRACT Myeloproliferative neoplasms (MPNs) are clonal disorders of hematopoietic stem cells with increased proliferation and efficient maturation of myeloid cells, leading to peripheral blood leukocytosis and excess erythrocytes or platelets. Mutations of the JAK2 V617F, CALR and MPL genes confirm the diagnosis of myeloproliferative neoplasm (MPN). Mutations in JAK2 have been identified in the majority of patients with PV, ET and PM, highlighting the importance of constitutive activation of JAK2 signaling induced by mutations. In our study, Sanger Sequencing and Next Generation Sequencing methods were used to search for JAK2 Exon 12 mutations in 100 individuals who suffered from Chronic Myeloproliferative Disease and did not have JAK2 V617F mutation by Real-Time PCR method, and the results were examined comparatively. The examination was made with DNA material isolated from peripheral blood samples taken from patients who were referred to Ankara Numune Training and Research Hospital (ANEAH) Genetic Diseases Diagnosis Center. First of all, individuals who have with negative JAK2 V617F RT-PCR test results were selected. PCR was performed by adjusting sufficient amounts and concentrations from the DNA samples obtained from the peripheral blood of these patients. After the PCR process, the JAK2 Exon 12 regions were sequenced and examined using the Sanger sequencing method. A Next Generation Sequencing (NGS) study was performed by creating libraries from the DNA of the patients whose JAK2 Exon 12 region was negative, and the results were analyzed using the database. Some of the studies were conducted at the ANEAH Genetic Diseases Diagnostic Center, and the other 46 patients were performed at the Intergen Genetic Diseases Diagnosis Center within the scope of NGS study service procurement. According to the analyzes made, the results of Sanger Sequencing and Next Generation Sequencing studies showed similarity. Despite the deep bottom readings, a different result could not be obtained from the Sanger Sequencing method in the NGS study.

Keywords Chronic myeloproliferative disease, sanger sequencing, next generation sequencing, Janus Kinas 2, Real-Time PCR

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1. INTRODUCTION

Myeloproliferative disorders consist of a set of chronic hematological diseases arising from a mutant multipotent hematopoietic stem cell. According to the World Health Organization (WHO) 2016 classification system, Myeloproliferative diseases (MPD); Chronic myeloid leukemia (CML) is divided into seven subcategories: chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, and unclassified MPH [1].

JAK2 (Januskinase 2) gene has tyrosine kinase activity and is a gene located in the JAK-STAT pathway and has an important role in cell proliferation. JAK2 V617F mutation occurs by substituting the amino acid valine to phenylalanine at position 617 of the 14th exon of the gene. It causes increased cell proliferation and sensitivity to cytokines and is frequently seen in PV, ET and PMF diseases classified as Philadelphia negative MPN [1,2]. However, mutations of the JAK2 V617F, CALR and MPL genes confirm the diagnosis of MPN [2]. Although rarer mutations in the 12th exon of the JAK2 gene can also be seen in patients with normal JAK2 V617F [2]. In these clonally proliferating diseases, the same mutation is not expected to be seen in all lymphocytes in the peripheral blood. In a review published by Tefferi and Vardiman in the journal Nature in 2008 [3], it was emphasized that current testing systems are not standardized for JAK2 mutations, there are no allele-specific tests in peripheral blood samples, and false negative test results are inevitable due to low mutant allele densities. For this reason, a search has been made for a method to be used to detect low mutations. New generation testing systems and informatics technologies that develop with technology allow us to eliminate such doubts [3].

Next generation sequencing; It has the potential to significantly accelerate biological research as it makes comprehensive analysis of the genome, transcriptome, and DNA-protein interactions cheap, routine, and widespread [4].

In addition, since it shows mutation rates for clonal diseases, it provides highly sensitive results in cancer diagnosis and treatment follow-up. PV accompanied by JAK2 mutations in 95% of cases; It is an MPH characterized by polycythemia (thrombocytosis, leukocytosis, erythrocytosis) and splenomegaly [5].

If the JAK2 V617F mutation is detected as positive at the time of diagnosis in these patients, it is tested quantitatively at regular intervals because it is an important marker in the selection of treatment and evaluation of response to treatment. In patients with negative JAK2 V617F mutation, JAK2 exon 12 mutations are investigated by Sanger sequencing method [6, 7].

However, although the clinical findings were clear, the fact that no mutation was found in this study suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons due to the low mutation load. Our aim in this study is to make a methodological evaluation by comparing the results of JAK2 exon 12 mutations in patients whose results were found to be normal and those whose PV diagnosis could not

be ruled out clinically and hematologically, and those who had the same test repeated using the new generation sequencing method the JAK2 V617F mutation is detected as positive at the time of diagnosis in these patients, it is tested quantitatively at regular intervals because it is an important marker in the selection of treatment and evaluation of response to treatment. In patients with negative JAK2 V617F mutation, JAK2 exon 12 mutations are investigated by Sanger sequencing method [8, 9].

However, although the clinical findings were clear, the fact that no mutation was found in this study suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons due to the low mutation burden. Our aim in this study is to make a methodological evaluation by comparing the results of JAK2 exon 12 mutations in patients whose results were found to be normal and those whose PV diagnosis could not be ruled out clinically and hematologically, and those who had the same test repeated using the new generation sequencing method [10, 11].

JAK2 gene

It is a gene that makes a protein that sends signals to cells to promote cell growth and helps control the number of red blood cells, white blood cells, and platelets made in the bone marrow. Mutated (changed) forms of the JAK *gene* have been found in some blood conditions, including PV, ET and PMF [10]. This gene encodes a non-receptor tyrosine kinase that plays a central role in cytokine and growth factor signaling. The primary isoform of this protein has an N-terminal FERM domain that is required for erythropoietin receptor association, an SH2 domain that binds STAT transcription factors, a pseudokinase domain, and a C-terminal tyrosine kinase domain. Cytokine binding induces autophosphorylation and activation of this kinase. This kinase then recruits and phosphorylates signal transducer and activator of transcription (STAT) proteins [11].

JAK-STAT signaling pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is considered one of the central communication nodes in cell function. More than 50 cytokines and growth factors have been identified in the JAK/STAT signaling pathway, such as hormones, interferons (IFNs), interleukins (ILs), and colony-stimulating factors. JAK/STAT-mediated downstream events vary and include hematopoiesis, immune fitness, tissue repair, inflammation, apoptosis, and adipogenesis. Loss or mutation of JAK/STAT components is associated with many diseases in humans [12]. The JAK/STAT signaling pathway has profoundly influenced recent understanding of human health and disease. Many studies have been published reporting the importance of this pathway in malignancies and autoimmune diseases. The JAK/STAT signaling pathway was first discovered while studying how IFNs lead to activation of a transcription factor. The JAK/STAT pathway is a highly conserved signal transduction pathway. It regulates multiple cellular mechanisms associated with various disease development [13]. Dysregulation of

the JAK/STAT pathway is associated with various diseases. For example, the JAK2 V617F mutation frequently occurs in MPH [12, 13, 14]. The JAK-STAT pathway is important for functional hematopoiesis, and several activating mutations in JAK proteins have recently been identified as underlying causes of blood disorders [15-16]. One of the best studied examples is the JAK2 V617F mutant, which is found in 95% of patients with polycythemia vera and 50% of patients affected by essential thrombocythemia and primary myelofibrosis [17]. Much work has been done to understand how JAK2 V617F affects hematopoietic stem cell (HSC) renewal and lineage differentiation [18]. Because convincing evidence has been found supporting the idea that the mutation is acquired at the HSC level [12-19].

2. MATERIALS AND METHODS

Included 100 patients who were sent from the Hematology outpatient clinic of Ankara Numune Training and Research Hospital to the Genetic Diseases Diagnosis such as genetic testing between January 2016 and March 2018. In this study; Patients diagnosed with MPH, without JAK2 V617F mutation, without any mutation detected by JAK2 *exon 12* Sanger sequencing, and who agreed to participate in this study, were investigated for JAK2 exon 12 mutations by next-generation sequencing method. This study was found ethically appropriate by Ministry of Health Turkish Public Hospitals Institution; Ankara Province 1st Regional Public Hospitals Association General Secretariat SBÜ Ankara Sample was found ethically appropriate by the SUAM Clinical Research Ethics Committee with the number E.Board-E-18-1956.

DNA Isolation

After the blood in EDTA tubes taken from the patients is recorded in the sample acceptance unit, with the help of commercial kits were used for DNA isolation and it was shown during the our study. Invitrogen™ PureLink™ Genomic DNA Mini Kit was used for DNA isolation. High quality DNAs with an A260/A280 ratio between 1.7 and 1.9 were included in this study [20, 21].

Polymerase Chain Reaction

It is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on exploiting the ability of DNA polymerase to synthesize a new DNA strand that is complementary to the presented template strand. Because DNA polymerase can only add a nucleotide to a pre-existing 3'-OH group, it needs a primer to which it can add the first nucleotide [22]. This requirement makes it possible to delineate a specific region of template sequence that the researcher wishes to expand. At the end of the PCR reaction, the specific sequence will be collected in billions of copies (amplicons) [23, 24]. In our study, the Polymerase Chain reaction was performed using the GML® SeqFinder Sequencing System JAK-2 Kit.

Polymerase Chain Reaction

Gel electrophoresis is a laboratory method used to separate DNA, RNA or protein mixtures based on molecular size. In gel electrophoresis, the molecules to be separated are pushed through a gel containing small porous inserts with the help of an electric field. Molecules move through the pores in the gel at a speed inversely proportional to their length [21, 22]. Agarose gel using a microwave oven, 1% agarose was placed in 1X TAE- and boiled until the agarose was completely dissolved. After the gel was left to cool for a while, it was poured into the gel plate and allowed to polymerize [23] (Figure 1).

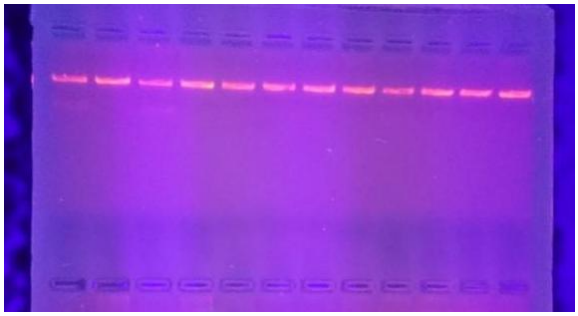

FIGURE 1. Appearance of amplicons containing JAK2 gene included Exon 12 regions on agarose gel

Sanger Sequencing

The method was developed in 1975 by Frederick Sanger, who was later awarded the Nobel Prize in Chemistry in 1980. By using this method it has to understanding which DNA sequences occurs. Sanger sequencing uses the SBS approach, in which a DNA polymerase produces DNA reads from a template to be analyzed of the DNA. The nature of the nucleotide at a particular position is now determined using special dyes. Although Sanger sequencing is too laborious and expensive for WGS, it continues to be used routinely when specific genes or gene fragments need to be sequenced, for example for viral or bacterial genotyping or for resistance testing. Modern Sanger sequencing typically uses fluorescently labeled dideoxynucleotides detected by a laser after capillary electrophoresis to generate an array chromatogram with fluorescence peaks corresponding to the inclusion of four different fluorescent dyes bound to ddATP, ddCTP, ddGTP and ddTTP [24, 25] (Figure 2).

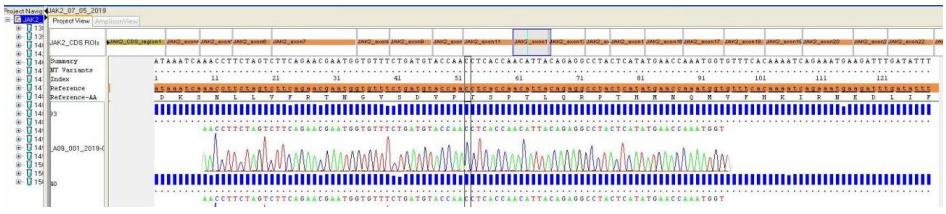

FIGURE 2. Patient example with Sanger Sequencing of the JAK2 Exon 12 region

Next Generation Sequencing

Next-generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology that is revolutionizing genomic research. Using NGS, the entire human genome can be sequenced in a single day [26]. Millions to billions of sequences from individual strands of DNA are analyzed separately but simultaneously. This technology allows the entire human genome to be fully analyzed in a few days for a few thousand dollars. Undoubtedly, as technology advances, speed will increase and cost will decrease; such that it will become practical to obtain full sequence analysis of cancers and matching germline for each patient. Hybridization, and real-time product detection with Sanger sequencing are being replaced by a new technology (NGS) [27].

Statistical Tests Used

Data analysis was done with SPSS 26.0 and worked with a 95% confidence level. Chi-square test, Mann Whitney and Spearman correlation were used in this study. Mann Whitney; It is a testing technique used to compare two independent groups in terms of a quantitative variable. Chi-square test; It is used to determine the relationship between two categorical variables. Spearman correlation; it is a testing technique used to determine the direction and strength of the relationship between two quantitative variables. Mann Whitney analysis was used to compare measurements according to groups, Chi-square analysis was used to compare the reference categories of measurements with grouped variables, and Spearman correlation analysis was used to compare the measurements [28].

3. RESULTS AND DISCUSSION

Sanger Sequencing

As a result of our studies at Ankara Numune Training and Research Hospital Genetic Diseases Diagnosis Center, Sanger Sequencing results of 100 errors were observed to be normal in terms of the JAK2 Exon 12 Gene region.

Next Generation Sequencing

55 of 100 patients with negative Sanger Sequencing results were studied at ANEAH Genetic Diseases Diagnosis Center and evaluated as negative by Dr. Büşranur ÇAVDARLI and Dr. Vehap TOPÇU. The remaining 45 patients were studied by the Intergen Genetic Diseases Evaluation Center within the scope of service procurement and the results were found to be negative. It has been confirmed by quality control methods that the DNAs have sufficient quality and concentration for next-generation sequencing technology. A library was created from DNAs of sufficient quality with the help of IonAmpliSeqTMLibrary Kit 2.0.

The files of the data obtained from this study were taken from the device in BAM (BinaryAlignment/Map) and VCF (VariantCalling Format) formats. The

accuracy of the variants in the VCF file was confirmed by visually evaluating the BAM files in the IGV (BroadInstitute) program. The ANNOVAR program was used to determine the frequency information, in silico prediction tools and other information of the detected variants. When evaluating whether the variants were disease-causing, the mutation evaluation guide published by the American College of Medical Genetics and Genomics (ACMG) in 2015 was taken as reference. Based on this reference, variants are grouped as "pathogenic," "possibly pathogenic," "unknown significance," "possible benign," and "benign." To evaluate the impact of the detected variants, disease databases and in silico prediction tools such as HGMD, ExAC, Pubmed, Provean, SIFT, Polyhen-2, ClinVAR, MutationTaster, Varsome, Uniprot, GERP, PhyloP, Human SplicingFinder were used (Figure 3).

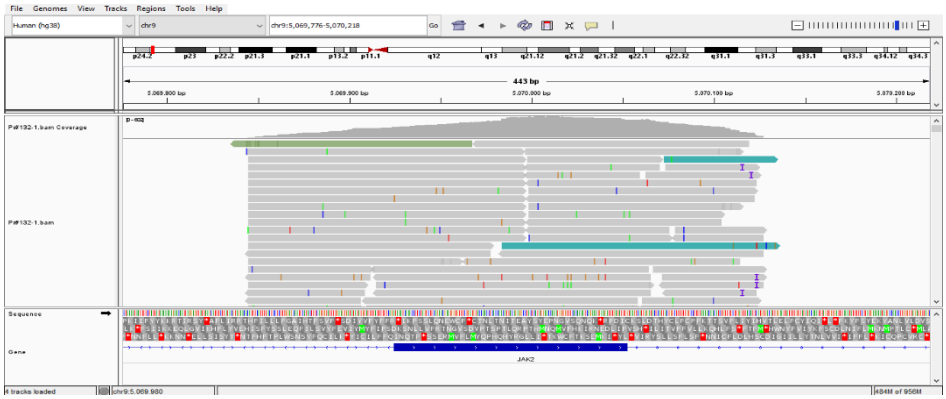

FIGURE 3. Patient example with Next Generation Sequencing of the JAK2 Exon 12 region

Statistical Data

TABLE 1. Testing normality of measurements and descriptive statistics

	Max-Min	Median	Mean± sd	Kolmogorov Smirnov	p
WBC	65.6-4	8.70	9.7±6.31	0.210	0.000
RBC	7.75-4.06	6.03	5.95±0.58	0.116	0.002
PLT	1487-124	241.00	265.46±151.77	0.240	0.000
HGB	22.5-11.1	17.30	17.15±1.66	0.106	0.007
MCV	99-28.3	87.35	85.73±9.34	0.233	0.000
MCH	34-21.3	29.00	28.74±2.07	0.127	0.000
HCT	70.4-38	52.00	51.47±4.81	0.113	0.003

(*p<0.05 normal distribution (-), p>0.05 normal distribution (+), KS test)

Descriptive statistics of the measurements are given. Kolmogorov Smirnov was used to test the normality of measurements. According to the results, WBC, RBC, PLT, HGB, MCV, MCH, HCT measurements do not show normal

distribution ($p<0.05$). Accordingly, non-parametric methods were used in the analyses (Table 1).

TABLE 2. Indication, gender distribution

		n (%)
Indication	Polycythemia Vera	37 (37)
	Secondary Polycythemia	63 (63)
Gender	Woman	24 (24)
	Male	76 (76)

The indication of 63.0% of the patients is Secondary Polycythemia, 76.0% of them are male (Table 2).

TABLE 3. Indication, gender distribution

		n (%)
WBC	not within normal range	26 (26)
	within normal range	74 (74)
RBC	not within normal range	68 (68)
	within normal range	32 (32)
PLT	not within normal range	8 (8)
	within normal range	92 (92)
HGB	not within normal range	51 (51)
	within normal range	49 (49)
MCV	not within normal range	7 (7)
	within normal range	93 (93)
MCH	not within normal range	9 (9)
	within normal range	91 (91)
HCT	not within normal range	56 (56)
	within normal range	44 (44)

(WBC: White Blood Cell, RBC: Red Blood Cell, PLT: Platelet, HGB: Hemoglobin, MCV: Mean red blood cell volume, MCH: Mean cell hemoglobin, HCT: Hematocrit)

WBC value of 74.0% of the patients, RBC value of 32.0%, PLT value of 92.0%, HGB value of 49.0%, MCV value of 93.0%, 91.0% The MCH value of 44.0% and the HCT value of 44.0% are within the normal range (Table 3).

TABLE 4. Relationship of reference groups of measurements to gender

		Woman	Male	P.
WBC	not within normal range	8 (33.3)	18 (23.7)	0.501
	within normal range	16 (66.7)	58 (76.3)	
RBC	not within normal range	13 (54.2)	55 (72.4)	0.157
	within normal range	11 (45.8)	21 (27.6)	
PLT	not within normal range	4 (16.7)	4 (5.3)	0.092
	within normal range	20 (83.3)	72 (94.7)	
HGB	not within normal range	12 (50)	39 (51.3)	0.999
	within normal range	12 (50)	37 (48.7)	
MCV	not within normal range	2 (8.3)	5 (6.6)	0.999
	within normal range	22 (91.7)	71 (93.4)	
MCH	not within normal range	2 (8.3)	7 (9.2)	0.999
	within normal range	22 (91.7)	69 (90.8)	
HCT	not within normal range	20 (83.3)	36 (47.4)	0.004*
	within normal range	4 (16.7)	40 (52.6)	

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Chi-square test)

There is no statistically significant relationship between the gender of the patients and the reference groups of WBC, RBC, PLT, HGB, MCV, MCH measurements (p>0.05). The relationship with HCT measurement is statistically significant (p<0.05). The rate of HCT measurement being within the reference range is higher in men (52.6%) (Table 4).

TABLE 5. Relationship of reference groups of measurements to indication

		Polycythemia Vera	Secondary Polycythemia	P.
WBC	not within normal range	12 (32.4)	14 (22.2)	0.375
	within normal range	25 (67.6)	49 (77.8)	
RBC	not within normal range	24 (64.9)	44 (69.8)	0.769
	within normal range	13 (35.1)	19 (30.2)	
PLT	not within normal range	4 (10.8)	4 (6.3)	0.463
	within normal range	33 (89.2)	59 (93.7)	
HGB	not within normal range	16 (43.2)	35 (55.6)	0.326
	within normal range	21 (56.8)	28 (44.4)	
MCV	not within normal range	6 (16.2)	1 (1.6)	0.010*
	within normal range	31 (83.8)	62 (98.4)	
MCH	not within normal range	7 (18.9)	2 (3,2)	0.012*
	within normal range	30 (81.1)	61 (96.8)	
HCT	not within normal range	18 (48.6)	38 (60.3)	0.354
	within normal range	19 (51.4)	25 (39.7)	

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Chi-square test)

There is no statistically significant relationship between the indication type of the patients and the reference groups of WBC, RBC, PLT, HGB, HCT measurements (p>0.05). The relationship with MCV and MCH measurements is statistically significant (p<0.05). In patients with Secondary Polycythemia indication type, MCV (98.4%) and MCH (96.8%) measurements are more likely to be within the reference range (Table 5).

TABLE 6. Comparison of measurements by gender

	Woman		Male		P.
	Median	Mean± sd	Median	Mean± sd	
WBC	8.75	11.8±11.88	8.65	9.03±2.71	0.495
RBC	5.58	5.51±0.58	6,10	6.1±0.51	0.000*
PLT	257.50	325.75±276.88	240.00	246.42±73.23	0.147
HGB	16.00	15.85±1.46	17.60	17.56±1.51	0.000*
MCV	87.40	85.25±13.1	87.30	85.88±7.91	0.446
MCH	29.45	28.91±1.99	28.90	28.69±2.11	0.493
HCT	47.70	48.13±4.18	52.70	52.52±4.53	0.000*

There is no statistically significant difference between female patients and male patients in terms of WBC, PLT, MCV, MCH measurements (p>0.05). The difference for RBC, HGB, HCT is statistically significant (p<0.05). RBC (6.1), HGB (17.60), HCT (52.70) measurements are higher in men (Table 6).

TABLE 7. Comparison of measurements by indication

	Polycythemia Vera		Secondary Polycythemia		P.
	Median	Mean± sd	Median	Mean± sd	
WBC	9.60	10.06±3.07	8.40	9.48±7.61	0.018*
RBC	6.03	5.92±0.54	6.03	5.98±0.61	0.999
PLT	254.00	297.81±219.1	234.00	246.46±88.95	0.078
HGB	17.00	16.64±1.75	17.50	17.45±1.54	0.039*
MCV	85.10	83.51±10.42	87.80	87.03±8.47	0.010*
MCH	28.40	27.87±2.35	29.40	29.26±1.71	0.002*
HCT	50,70	50.39±4.57	52.60	52.1±4.87	0.156

(*p<0.05 there is a relationship, p>0.05 there is no relationship, Mann Whitney test)

There is no statistically significant difference in RBC, PLT, HCT measurements between patients with indication type Polycythemia Vera and patients with Secondary Polycythemia (p>0.05). The difference for WBC, HGB, MCV, MCH is statistically significant (p<0.05). While WBC (9.6) measurement is higher in those with indication type Polycythemia Ver, HGB (17.5), MCV (87.8), MCH (29.4) measurements are higher in those with Secondary Polycythemia (Table 7).

TABLE 8. Relationship of measurements

		WBC	RBC	PLT	HGB	MCV	MCH	HCT
WBC	r	one	-0.083	.257 **	0.018	0.001	0.046	0.081
	p		0.413	0.010	0.860	0.992	0.651	0.420
RBC	r		one	-0.179	.628 **	-.372 **	-.454 **	.699 **
	p			0.074	0.000	0.000	0.000	0.000
PLT	r			one	-.380 **	-.290 **	-.238 *	-.341 **
	p				0.000	0.003	0.017	0.001
HGB	r				one	0.170	.223 *	.904 **
	p					0.092	0.026	0.000
MCV	r					one	.726 **	.197 *
	p						0.000	0.050
MCH	r						one	0.079
	p							0.434
HCT	r							one
	p							

(*p<0.05 there is a significant relationship, p >0.05 there is no significant relationship, 0<r<0.299 is weak, 0.300<r<0.599 is moderate, 0.600<r<0.799 is strong, 0.800<r<0.999 is very strong; sperman correlation test)

There is no statistically significant relationship between the patients' WBC measurements and RBC, PLT, HGB, MCV, MCH, HCT measurements (p>0.05). There is a positive relationship between RBC measurement and HGB (r=0.628), HCT (r=0.699) measurements, and a negative, statistically significant relationship between MCV (r=-0.372), MCH (r=-0.454) measurements (p<0.05). There is a negative, statistically significant relationship between PLT measurement and HGB (r=-0.380), MCV (r=-0.290), MCH (r=-0.238), HCT (r=-0.341) measurements (p<0.05).

There is a positive, statistically significant relationship between HGB measurement and MCH (r=0.223), HCT (r=0.904) measurements (p<0.05). There is a positive, statistically significant relationship between MCV measurement and MCH (r=0.726), HCT (r=0.197) measurements (p<0.05). Other relationships are not significant (p>0.05) (Table 8).

Retrospective study aimed at conducting a confidence test. JAK2 mutations have become a target in Polycythemia vera patients diagnosed with chronic myeloproliferative diseases. Genus kinases have been associated with CALR and MPL genes in the literature; however, current testing systems have not been updated for JAK2 mutations. In this study, the characteristics of the disease-causing variants were evaluated in detail through a retrospective analysis. As a result of the literature research, it was seen that intronic mutations can also be associated with Myeloproliferative diseases. In fact, in some publications, possible mutations in the JAK2 Exon 13 region have been emphasized. Its relationship with patient hematological data was examined. JAK2 vary in size

between 120-140 kDa and contain seven regions called Janus homology domain 1-7 (JH 1-7) [17]. There are publications showing that JAK2 Exon 12 mutations are more common in women than in men [28]. Since these mutations are associated with splenomegaly, if ignored, they will negatively affect the patient's quality of life. Since making a molecular diagnosis will enable a more careful hematological follow-up, elucidating the underlying mechanism is very important in preventing malformations that may develop in the patient's future periods.

Both testing systems are reliable in themselves. However, they have advantages over each other. Sanger Sequencing produces cost-effective solutions for low numbers of samples and the workflow is easy. NGS, on the other hand, provides higher sequencing depth and higher sensitivity. All the procedure working is complicated by winter Sanger sequencing. NGS offers higher mutation resolution. It is more focused on discovery and sample yield high in NGS. It offers obtaining more data with the same amount of sample [15].

Although the clinical findings were clear, the fact that no mutation was found in this study. It suggested that the clonal nature of the disease and the possibility of a false negative result being missed due to technical reasons to get the low mutation load. There are publications showing that JAK2 Exon 12 mutation is more common in women than in men [16]. No such finding was found in our study. During the literature review, it was emphasized that possible intronic mutations in the JAK2 gene region and possible mutations in the Exon 13 region should also be investigated.

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DECIPHERING LATE EMBRYOGENESIS ABUNDANT (*LEA*) GENES IN *PHASEOLUS VULGARIS* L. THROUGH BIOINFORMATICS

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ABSTRACT The Late Embryogenesis Abundant (*LEA*) gene family is considered vital for plant's ability to survive freezing and desiccation, affecting important developmental and growth processes. These proteins possess notable hydrophilicity and thermal stability, which are essential for preserving cell membrane integrity, forming molecular barriers, aiding in ionic binding, and mitigating oxidative damage during extended periods of exposure to abiotic stress conditions. Although LEA proteins have been extensively studied in numerous plant species, this study represents the initial comprehensive exploration and characterization of LEA proteins in *Phaseolus vulgaris* L. In this context, the biochemical/physicochemical properties of the LEA family at both the gene and protein level have been deeply characterized and defined using various bioinformatics tools. Through comprehensive bioinformatics analyzes, we identified 80 *LEA* genes in common bean and phylogenetically categorized their proteins into eight major groups. Investigating gene duplications, we uncovered 28 events, including 24 segmental and 4 tandem duplications, significantly influencing the evolutionary trajectory of this gene family. In silico micro-RNA (miRNA) target analyzes revealed that 21 *PvLEA* genes were targeted by various miRNAs, with miRN2588 and miR164 being the most prevalent. *PvLEA-63* emerged as the most highly expressed gene across tissues, followed by *PvLEA-27*, *PvLEA-35*, *PvLEA-41*, *PvLEA-49* and *PvLEA-52* genes, demonstrating their ubiquitous expression patterns. Moreover, using publicly available RNAseq data, a comparative expression study of *PvLEA* genes was carried out, and expression alterations in *PvLEA-02*, *-08*, *-20*, *-21*, *-40*, *-42*, *-50* and *-51* genes were detected under both salt and drought stress conditions. These results constitute a substantial resource for future researchers interested in unravelling the functional intricacies of *PvLEA* genes.

Keywords *LEA* gene family, *Phaseolus vulgaris* L., bioinformatics, RNAseq

1. INTRODUCTION

Common bean is a plant classified under the genus *Phaseolus vulgaris* L. that formed seven thousand years ago in separate regions of the South and North American continents and is known as “new global crop” [1]. The common bean (*Phaseolus vulgaris*, 2n = 22) is an essential component of vegetable protein for millions of people throughout the world and is the most consumed legume in both developing and developed countries [1, 2]. It is sometimes called as "poor man’s meat” due to its considerable protein, minerals, soluble fiber, starch, phytochemicals and vitamin content [3]. Furthermore, because of the high

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protein content, people who ate a diet high in common bean had a decreased frequency of cancer, cardiovascular disease (CVD) and diabetes [4]. Several biotic factors, such as bacteria, viruses and fungal infections, as well as abiotic challenges like low temperature, drought, and salt, significantly impact the yield and quality of common bean [5]. The significance of drought and salt resistance in bean cultivation cannot be ignored, as they are essential to produce food for human consumption [6].

In the 21st century, food problems have arisen due to global climate change, drought, population growth and reduction of agricultural lands. Salinity and drought are environmental stress conditions that induce major modifications in controlling how genes are expressed, including the activation or suppression of genes, as well as the modulation of signal transduction pathways, and these changes have serious effects on the life cycle of plants [6, 7, 8]. In common bean production, cultivating plants tolerant to abiotic stress is crucial [6]. However, developing plants resilient to stress requires further investigation into identifying genes that contribute to the way plants react to stress conditions [9]. Furthermore, two fundamental stress-inducing gene sets that are assumed to be crucial for stress response were found in a study on Arabidopsis [10]. The initial category involves regulatory proteins, protein kinases and transcription factors that regulate other signaling molecules. The next category consist of chaperones that regulate osmotin, abiotic stress tolerance, mRNA binding proteins, antifreeze proteins, water channel proteins, and late abundant embryogenesis (LEA) proteins [10].

Activating the *LEA* gene family confers resistance to abiotic stressors, including drought, cold, and salinity, which are critical for plant development and growth [6, 8, 11-13]. LEA proteins show a strong affinity to water and possess a remarkable capacity to resist extreme temperatures. Moreover, they play a vital role in plant cell membrane stabilization, anti-oxidation, establishing molecular barriers and ionic binding under prolonged abiotic stress [14]. During the late phases of embryonic development, LEA proteins stored in seeds were found to respond to diverse abiotic stresses especially drought stress in higher plants [15]. Previous studies also indicated that the upregulation of *LEA* genes in various plant species, including wheat, lettuce, rice, tobacco, and Arabidopsis, enhanced their ability to withstand abiotic stresses [16].

LEA proteins were initially discovered in the latter stages of cotton seed embryogenesis [17]. Subsequent research has revealed the presence of LEA proteins in various plant tissues, including stems, leaves, flowers, and roots, across a diverse array of plant species such as rice, wheat, potato, maize, barley, rye, sunflower, bean, grape, carrot, apple, Arabidopsis, soybean, tomato and canola [18, 19, 20]. These proteins are mostly located in intracellular organelles like mitochondria, cytoplasm, and chloroplasts [13, 21]. Their presence extends to various ecosystem of prokaryotes, invertebrates, and plants ranging from algae to angiosperms [7]. Different types of higher plants including *P. trichocarpa* L., *O. sativa* L., and *A. thaliana* (L.) Heynh have different members of LEA protein

family due to differences in their amino acid sequences, evolutionary relationships and conserved domains.

In current plant biology, it is significant to be able to examine and evaluate massive, complicated datasets [22]. Thanks to technological strides in genome-wide sequencing, the LEA proteins have now been identified in numerous plant species [19, 20, 23, 24]. The genome-wide characterization and identification studies of commercially important plants like *P. vulgaris* are expected to be valuable. This study aims to identify genes that could enhance plant resistance and reduce crop losses caused by environmental and biological factors in plants. It provides a comprehensive genome-wide analysis along with detailed categorization and identification of *P. vulgaris LEA* genes, a novel contribution to the existing literature. Also, the identification of 80 *PvLEA* genes from the *P. vulgaris* genome was validated by analyzing publicly available RNAseq data which shows transcriptomic changes in response to drought and salt stress in common bean. We anticipate that the information we have collected may contribute to the future studies of the *LEA* gene family in *P. vulgaris*.

2. MATERIALS AND METHODS

2.1 Identification of *PvLEA* gene family members

LEA protein sequences of *P. vulgaris* were obtained from Phytozome v13 database through keyword search with default parameters using following Pfam codes: [(LEA_1 (PF03760), LEA_2 (PF03168), LEA_3 (PF03242), LEA_4 (PF02987), LEA_5 (PF00477), LEA_6 (PF10714), DHN (PF00257), and SMP (PF04927)] (http://pfam.xfam.org/) [25, 26]. To identify the putative proteins, redundant sequences were manually eliminated, and the remaining PvLEA proteins were then subjected to blastp analysis in NCBI database. Physicochemical properties such as amino acid composition, molecular weights (MWs), isoelectric points (pIs) and grand average hydropathicity values (GRAVY) were determined using the ProtParam tool (https://web.expasy.org/protparam/), while protein domains were identified using HMMER (www.ebi.ac.uk/Tools/hmmer/) [27]. Subcellular localization of the PvLEA proteins in *P.vulgaris* was predicted using the WoLF PSORT: Protein Subcellular Localization Prediction Tool [28].

2.2 Phylogenetic analysis and classification of PvLEA members

The alignment of *Arabidopsis thaliana* and *Phaseolus vulgaris* LEA proteins was performed using ClustalW [29]. The phylogenetic tree was created utilizing the neighbor-joining (NJ) method with 1000 bootstrap replicates in MAFFT (https://mafft.cbrc.jp/alignment/software/). The resulting tree was visualized using the iTOL online tool (https://itol.embl.de/) [30, 31].

2.3 Chromosomal localization, gene structure, and conserved motif analyses

P. vulgaris general feature format (GFF3) was downloaded from JGI Data Portal (https://data.jgi.doe.gov/). The chromosomal distribution of *PvLEA* genes was illustrated using the GFF3 file in TBtools software [32]. MEME tool (Multiple Expectation Maximization for Motif Elicitation) (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) was employed to identify conserved motifs in PvLEA proteins using specified parameters including maximum number of motifs '10'; site distribution, any number of repetitions for site distribution, a minimum motif width of '2' and a maximum motif width '50' [33]. TBtools software was utilized to visualize the exon-intron structure of *PvLEA* genes via GFF3 file [32].

2.4 Gene duplication and synteny analysis

GFF3 and genome sequence files of *G.max*, *A.thaliana*, and *P.vulgaris* species were downloaded through the JGI Data Portal (https://data.jgi.doe.gov/). Gene duplication events were analyzed in the TBtools program using the Multiple Collinearity Scan toolkit (MScanX). Gene duplication analyses were conducted using the Multiple Collinearity Scan toolkit (MScanX) in TBtools program. Synteny maps were generated using the Dual Synteny Plotter software based on orthologous genes between *P. vulgaris* and other species (*A.thaliana* and *G.max* L.) [32]. Collinearity analysis validated paralogous relationships which were then visually represented using the Circos tool in TBtools software [32]. Duplicated gene pairs identified through synteny analysis were examined for selective pressure using the ratio of nonsynonymous (Ka) and synonymous (Ks) substitutions (Ka/Ks) calculated with KaKs_Calculator [32]. The duplication period (MYA-million years ago) and divergence of each *PvLEA* gene were calculated using $T = Ks/2\lambda$ ($\lambda = 6.56E^{-9}$) formula [34].

2.5 Cis-Regulatory element analyses in promoter regions of *PvLEA* genes and protein structure homology modeling

To examine the cis-regulatory elements within the promoter region of *PvLEA* genes, the 1500 bp genomic sequence before the initiation codon (ATG) of each gene was obtained from the Phytozome database. Potential cis-elements in the promoter sequence were assessed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [35]. Additionally, all PvLEA proteins were searched against the Protein Data Bank (PDB) using BLASTP (default settings) to recognize templates with similar sequences and three-dimensional structures [36]. Subsequently, Phyre2 (Protein Homology/AnalogY Recognition Engine; http://www.sbg.bio.ic.ac.uk/phyre2) was employed for protein homology modeling in "intensive" mode to forecast protein configurations [37].

2.6 miRNA analysis in the *PvLEA* gene family

The PmiREN database (https://www.pmiren.com/) was utilized to download all known *P.vulgaris* miRNA sequences [38]. The psRNATarget Server was then used to determine the miRNAs targeting *PvLEA* genes (https://www.zhaolab.org/psRNATarget/) [39]. The miRNAs and targeted *PvLEA* genes were displayed using the Cytoscape software [40].

2.7 Tissue-specific mRNA levels of *PvLEA* genes

The Phytozome v13 Database was utilized to evaluate the expression levels of *PvLEA* genes across various plant tissues during different developmental stages, including root 10, nodules, root 19, young buds, stem 10, stem 19, green mature buds, leaves, young trifoliates, flower buds, and flowers [26]. In-silico expression levels were quantified using FPKM (fragments per kilobase of transcript sequence per million base pairs sequenced) values, which were log2 transformed for analysis. A heatmap illustrating the expression patterns was created utilizing the TBtools program [32].

2.8 Identification of *PvLEA* expression levels in response to salt and drought stresses through RNAseq analysis

The expression levels of *PvLEA* genes were quantified using Illumina RNA-seq data obtained from the Sequence Read Archive (SRA) during exposure to salt and drought stressors. The data accession numbers SRR957667 (control leaf for salt experiment), SRR957668 (salt-treated leaf), SRR8284481 (drought-treated leaf), and SRR8284480 (control leaf for drought experiment) were utilized as previously specified by Büyük et al. (2016) [9, 41, 42]. Expression data underwent conversion to Log2 format, followed by the creation of a heatmap using the TBtools software to visualize the results [32].

3. RESULTS AND DISCUSSION

3.1 An extensive *LEA* gene family in *P. vulgaris*

In this study, 80 *LEA* genes have been identified in *P. vulgaris* genome (Supplementary Table 1). These genes were named from *PvLEA-01* to *PvLEA-80* according to their chromosomal order. The *PvLEA* genes were found to be classified into eight subfamilies [(LEA_1 (PF03760), LEA_2 (PF03168), LEA_3 (PF03242), LEA_4 (PF02987), LEA_5 (PF00477), LEA_6 (PF10714), SMP (PF04927), and DHN (PF00257)] based on sequence homology. The number of identified *PvLEA* genes in *P. vulgaris* genome was found to be higher than those previously identified in *Solanum tuberosum* L. (n = 74) [7], *Zea mays* L. (n = 32) [43], *Oryza sativa* L. (n = 34) [44] and *Cucumis sativus* L. (n = 79) [45]. However, *Triticum aestivum* L. (n = 179) [46], *Nicotiana tabacum* L. (n = 123) [8], and *Arachis hypogaea* L. (n = 126) genomes were found to have more *LEA* genes than *Phaseolus vulgaris* L. (n = 80) [21].

PvLEA genes were mostly found to be clustered in LEA_2 subfamily according to the phylogenetic analysis. Similarly, the majority of *LEA* genes from *Camellia sinensis* L. [23], *Sorghum bicolor* (L.) Moench [47], and *Triticum aestivum* [48] were also found to be clustered in LEA_2 subfamily. LEA_6 subfamily was the cluster with lowest number of genes and contained only single *PvLEA* gene in this study. In previous studies on *Camellia sinensis* [23] and *Solanum lycopersicum* [49], LEA_6 subfamily could not even be detected. This absence might be attributed to the LEA_6 subfamily being considered the most recent addition to the LEA family in plants, emerging only in early angiosperms [50].

The lengths and molecular weights of identified PvLEA proteins ranged from 82 to 468 amino acids and from 8.76 to 50.61 kDa, respectively. The pI (the theoretical isoelectric point) values of PvLEA proteins were between 4.7 to 10.3. It was determined that 80% (64 members) of PvLEA proteins showed basic properties. Calculated grand average of hydropathy index (GRAVY) values of all PvLEAs were between −1.558 and 0.411. A significant number, constituting 71% of PvLEAs (57 members), demonstrated GRAVY values below zero suggesting the hydrophilic nature of most PvLEAs. Similarly, previous studies conducted on *Salvia miltiorrhiza* Bunge [14], *Brassica campestris* L. [51] and *Panax notoginseng* L. [52] also showed that LEA proteins were mostly hydrophilic. The aliphatic index, which shows the amount of aliphatic side chains (leucine, isoleucine, valine, and alanine) in a protein, is known to increase its overall thermostability [53]. The aliphatic index values of PvLEA proteins were between 23.3 and 121.3, indicating the high level of protein thermostability. The instability index values were found to range between -3.16 and 68.04 and it was determined that instability index values of 48 members (60%) were below 40, signifying the stability of the majority of PvLEA proteins. Notably, PvLEA-26 stands out as the protein with the lowest instability index value, suggesting the most stable protein structure. In a previous study on tomato, more than %50 of SlLEA proteins were also found to be stable according to the instability index values [54].

The subcellular localization analysis of PvLEA proteins showed that approximately 27.5% and 32.5% of PvLEA proteins were located in cytoplasm and chloroplast, respectively (Supplementary Table 1). In previous studies, LEA proteins were mostly shown to localize in cytoplasm, mitochondria, and chloroplasts [7, 13, 55]. A study by [43] showed that 56.3% of ZmLEA proteins from maize were specifically localized in the nucleus while the remaining part was predominantly distributed in chloroplast, mitochondria, and cytoplasm. The presence of LEA proteins across various cellular compartments and tissues strongly implies their essential role in cellular activities during stress [14].

3.2 Phylogeny, conserved motif and gene structure analysis of PvLEA members

To date, the *LEA* gene family has been identified in across diverse plant species [16]. However, the specific number of genes encoding LEA proteins in *P. vulgaris* remains unknown. Thus, we conducted a phylogenetic analysis to characterize the LEA protein family in *P. vulgaris.* Accordingly, 80 LEA proteins were identified which were found to be categorized into eight main clades. Phylogenetic studies revealed that eight clades could be distinguished based on different structural domains of PvLEA proteins [15]. Among these clades, the LEA_2 subfamily was notable for having the most members with 101 (63.5%). Following the LEA_2 subfamily LEA_1, LEA_3, LEA_4, LEA_5, LEA_6, SMP and DHN, subfamilies contained 8 (5%), 13 (8.1%), 7 (4.4%), 4 (2.5%), 4 (2.5%), 10 (6.2%) and 12 (7.5%) proteins, respectively. Similar to the findings obtained in this study, LEA_2 group was also found to have the most LEA members in *Solanum lycopersicum*, *Camellia sinensis* and *Nicotiana tabacum* genomes in previous studies [8, 12, 54]. The group with fewest PvLEA proteins was LEA_6 with only single member (*PvLEA-20*).

In addition to phylogenetic analysis, motif structure analysis was carried out to assess the distribution and characteristics of motifs found in PvLEA proteins (Figure 2).

In general, the distribution of motifs was found to be different in different PvLEA subgroups shown in phylogenetic tree, while proteins in the same subgroups were found to have similar motif distribution pattern [56]. The number of motifs present in each LEA_2 subgroup member was different. For example, the majority of proteins in LEA_2 subgroup contained 5 motifs however PvLEA-13, PvLEA-15, and PvLEA-37 proteins restrict the content to a singular motif. The LEA_4 subgroup was found to have proteins including all 10 conserved motifs (PvLEA-03 and PvLEA-50 proteins). Due to the presence of diverse conserved motifs in various subgroups, it is probable that these subgroups might be originated from independent ancestors that possessed different conserved motifs [49]. Furthermore, this analysis highlighted that the closely related proteins displayed a characteristic motif composition and showed greater structural similarity, while the genes encoding them having significantly different exon and intron lengths (Figure 2 and 3).

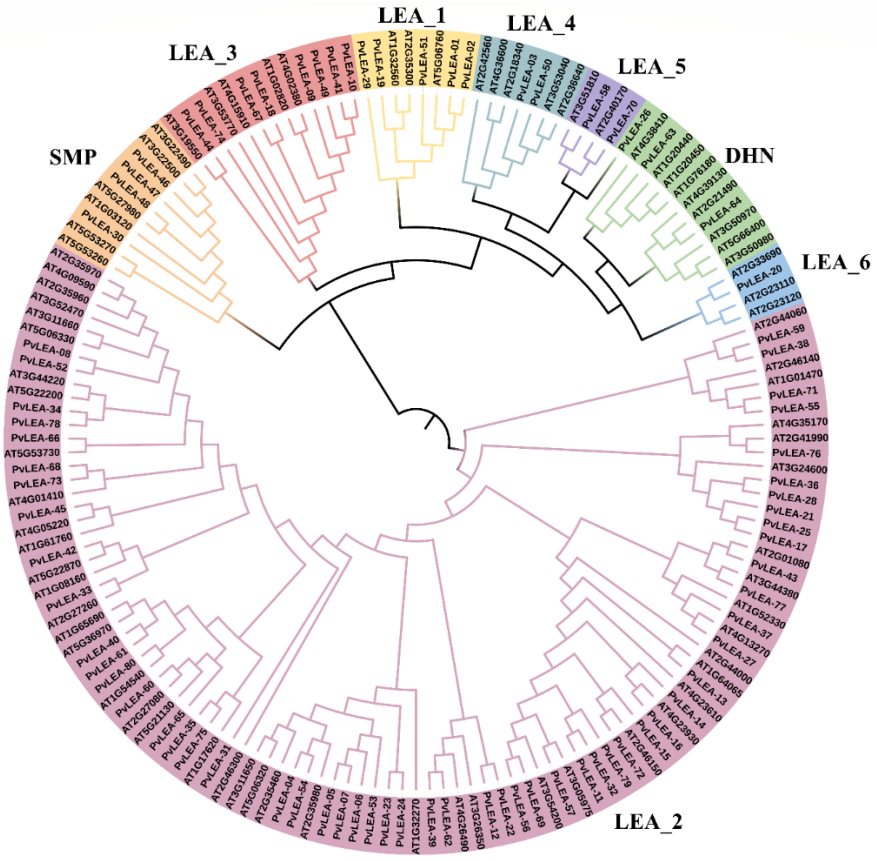

FIGURE 1. Phylogenetic analysis of LEA proteins from *P. vulgaris* and *A. thaliana.*

Further investigation was carried out on the gene structure of the *PvLEA* gene family (Figure 3). According to the findings of gene structure and chromosomal distribution analyses, it is emphasized that tandem duplication events can increase the number of genes in a gene family and duplications are also important for increasing adaptation of plants to different environmental conditions [57, 58]. In this perspective, the organization of exon and intron structures in *PvLEA* genes reflected the diversity in gene structures which could contribute to the functional variations observed in homologous genes. Homologous genes in a group typically have a similar structure, involving the length and number of exons of the genes (Figure 3). Analyses of the 80 *PvLEA* genes demonstrated that 56.25% (45) of these genes lacked introns (intronless), while 43.75% (35) of them had a range of 1 to 3 introns (intron-poor) (Figure 3). In a study on *Nicotiana tabacum*, it was also revealed that among the 123 *NtLEA* genes, two were characterized as intron-rich, while the remaining 121 were identified as intron-poor genes [8]. *PvLEA-48* was found to have the most exons and introns in *PvLEA* gene family bearing 4 exons and 3 introns.

FIGURE 2. Motifs of PvLEA proteins in *P. vulgaris*. Each motif category was shown with a unique color and the numbers (1-10).

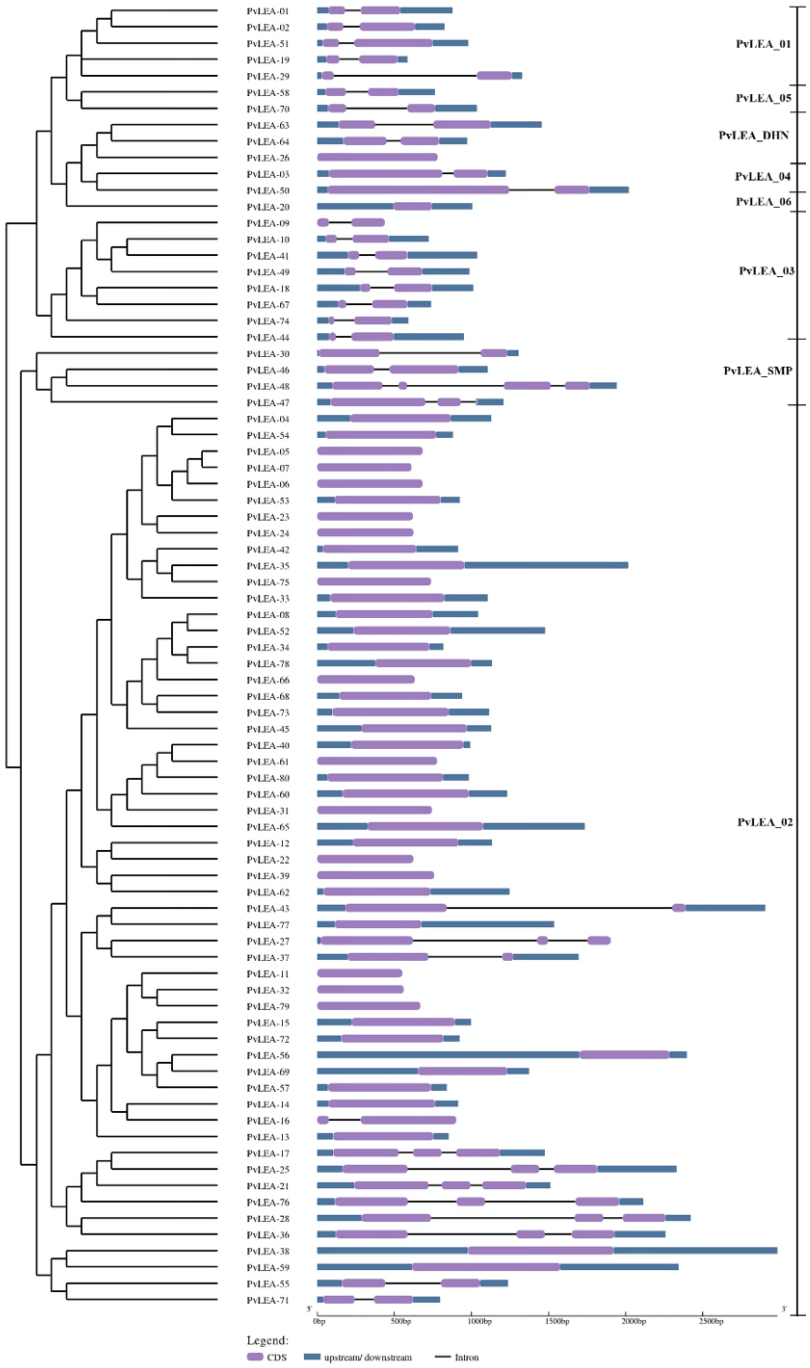

FIGURE 3. The exon and intron arrangement of *PvLEA* genes in *P. vulgaris.* Blue boxes in the diagram denote untranslated regions (UTR), purple boxes signify coding regions (CDS), and lines are used to represent introns.

PvLEA-17, *-21*, *-25*, *-27*, *-28*, *-36*, *-47* and *-76* genes are intron and exon (3 exons and 2 introns) rich members after *PvLEA-48*. The *PvLEA-38* gene, which belongs to the LEA_2 subfamily, was found to be longer than all other genes, having a gene structure of around 3000 bp, as well as intronless (0 intron) and including 1 exon. There was also a significant relationship within each phylogenetic group in terms of intron-exon structures (Figures 1 and 3).

3.3 Chromosomal localization, gene duplication and syntenic analysis of the *PvLEA* genes

The genomic localization analysis showed the localization of *PvLEA* througout 11 common bean chromosomes. According to Figure 4, the highest number of *PvLEA* genes was found on chromosome 7 (11 genes) and the lowest on chromosome 11 (4 genes). Furthermore, five *PvLEA* genes were found to be located on chromosome 3, with six genes on each of chromosomes 4, 6, and 9. Chromosomes 2 and 10 housed seven *PvLEA* genes each, while chromosome 8 contained eight, chromosome 5 held nine, and chromosome 1 accommodated 10 genes. The *PvLEAs* were distributed unevenly among the 11 chromosomes in *Phaseolus vulgaris*. Moreover, just one gene (*PvLEA-80*) was identified on the unassembled scaffolds (Figure 5). Interestingly, all genes on chromosomes 11 and 2 were from LEA_2 subfamily, and LEA_2 members were detected to be present on all chromosomes. This pattern aligns with the findings obtained from a previous study on *Arachis hypogaea* L., which reported the presence of 126 *AhLEA* genes spreaded out across 20 chromosomes. In their study, LEA_2 members were also found to be present on all chromosomes of *Arachis hypogaea* similar to our findings [21]. Moreover, our analysis identified four sets of tandem duplication events, encompassing eight genes found on chromosomes 1, 2, and 7 (Figure 4).

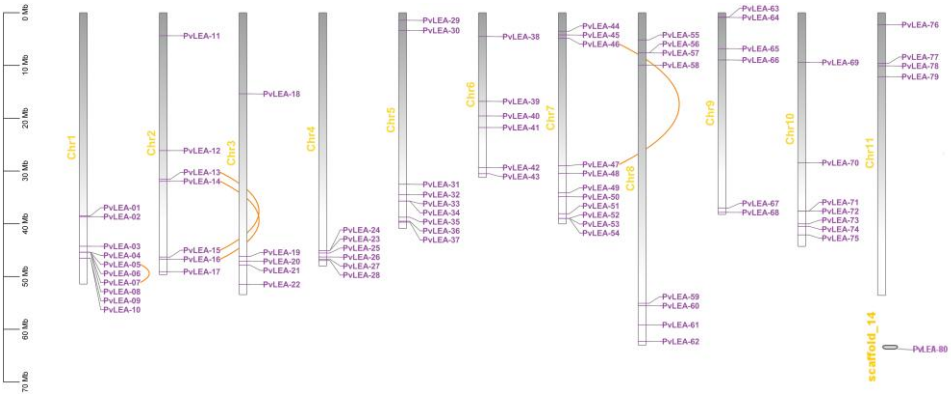

FIGURE 4. The chromosomal positioning of *PvLEA* genes and gene duplication events. The *PvLEA* genes were mapped onto the chromosomes of *P. vulgaris*, and tandemly repeated gene pairs were indicated by orange lines.

Genomic duplications, tandem and segmental duplications, are important events which contribute to the expansion of gene families [59]. In this study, four pairs of tandem duplication and 24 pairs of segmental duplication were detected (Figure 5). In previous studies, different numbers of tandem duplications were also found in *LEA* genes from different plant species including *P. trichocarpa, S. lycopersicum* and *S. pimpinellifolium* [54, 60] [24, 54]. In this study, the origin of tandem duplication events in *PvLEA* genes was found to date back to 0.41 to 25.8 million years ago (MYA) while segmental duplication events were recorded approximately 3.58 to 23.38 MYA. Three of four tandem duplication events were in between *PvLEA* genes from LEA_2 group and the other one was in between *PvLEA* genes from SMP group. The majority (83.3%) of segmentally duplicated genes was from LEA_2 group, followed by LEA_3 (8.3%), LEA_1 (4.1%) and LEA_4 (4.1%) groups. Similarly, in a study on *Arachis hypogaea*, it was discovered that 79.5% of the segmentally duplicated genes belonged to the LEA_2 group [21].

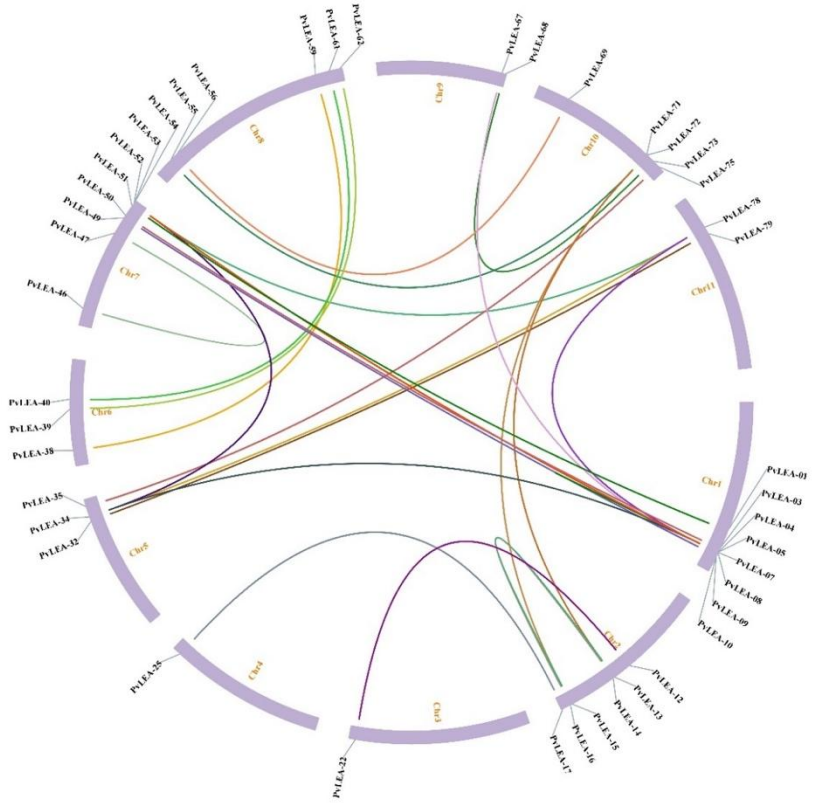

FIGURE 5. Synteny analysis of *PvLEA* genes.

The expansion of the common bean *LEA* gene family might primarily be attributed to the occurrence of segmental duplication events, which outnumber tandem duplication pairs and therefore serve as the main contributing factor [61].

The rate of nonsynonymous substitutions per nonsynonymous site (Ka) and the rate of synonymous substitutions per synonymous site (Ks) commonly utilized to find out how fast protein-coding genes are changing [62]. The Ka/Ks ratio serves as an indicator of the selection pressure and evolutionary rate on a gene. In our analysis for duplicated *PvLEA* genes, the Ka/Ks ratio ranged from 0.649 to 0.114, all falling below 1 (refer to Supplementary Table 2). A Ka/Ks ratio less than 1 signifies purifying selection, where natural selection acts to eliminate deleterious variations, maintaining the functionality of the gene [63, 64]. The constant pattern seen in the ratios indicates that the *LEA* gene family in these species has mostly undergone purifying selection throughout evolution. This observation aligns with findings from the studies on *Cucumis sativus, Populus trichocarpa,* and *Arachis hypogaea L.* species, where genes were similarly found to be affected by purifying selection [21, 45, 65]. It was estimated that tandem duplications occurred 13.81 MYA and segmental duplications occurred 9.10 MYA in this study. These observations indicated that segmental duplications played a more prominent role than tandem duplications for the expansion of *PvLEA* genes. This highlights the essential function of segmental duplication events in stimulating the duplication of *LEA* genes.

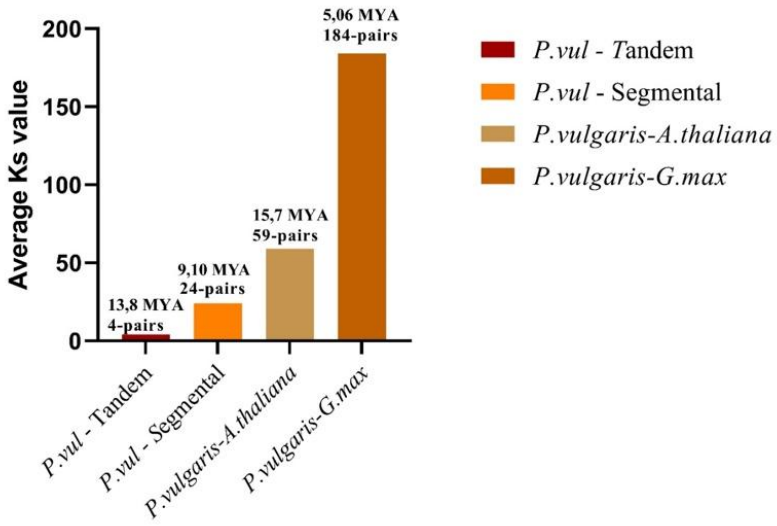

FIGURE 6. The number of ortholog gene pairs (*P. vulgaris* L.-*A. thaliana; P. vulgaris* L.-*G. max*) as well as the tandem and segmental duplication events in *P. vulgaris* L., along with their respective estimated duplication times in million years ago (MYA)

In this study, we explored the orthologous relationships in *LEA* genes among the genomes of *P. vulgaris, A. thaliana*, and *G. max*. The orthologous genes, derived from a shared ancestral gene and exhibiting similar functions in different species, were examined. The genomic comparisons revealed that *P. vulgaris* shares 59 orthologous gene pairs with *A. thaliana* and 184 orthologous gene pairs with *G. max* (Figure 6 and 7, Supplementary Table 2). Notably, an orthologous relationship was identified between *LEA* genes from *G. max* and the *PvLEA-80* gene located on an unassembled scaffold, stemming from the orthologous relationships among *P. vulgaris* and *G. max*. The estimated timeline for these orthologous relationships indicates the occurrence of duplications 5.06 MYA for *P. vulgaris-G. max* and 15.7 MYA for *P. vulgaris-A. thaliana* (Figure 6 and 7, Supplementary Table 2). Our findings revealed that the highest number of *LEA* orthologous gene pairs were observed between *P. vulgaris* and *G. max.* This highest similarity for the *LEA* genes from *P. vulgaris*, and *G. max* is unsurprising given the known genetic resemblance between the two genomes.

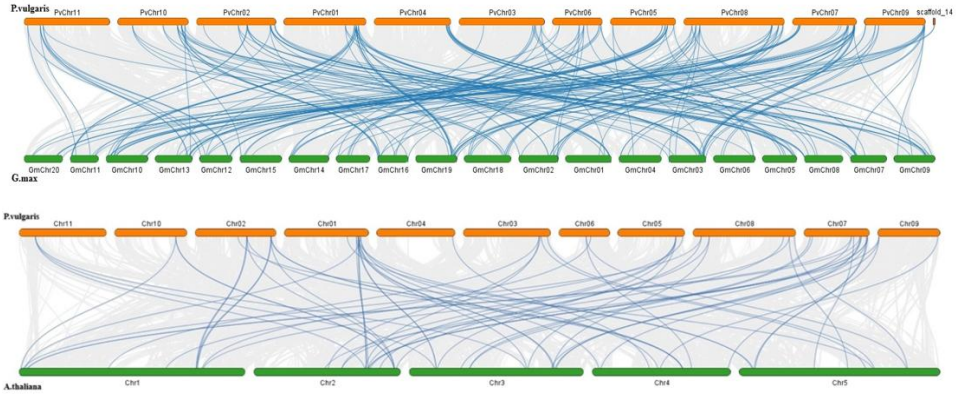

FIGURE 7. The collinearity map illustrates the relationships among *LEA* genes in common bean and two representative plant species, *Glycine max and Arabidopsis thaliana*. In the diagram, blue lines represent collinearity, specifically highlighting synthetic orthologous gene pairs. The background gray lines indicate the collinearity existing among the common bean and the other two species.

3.4. Analysis of cis-regulatory elements and homology modeling of *PvLEA* gene family

The presence of a diverse array of cis-regulatory elements (CREs) in gene promoters suggests a range of functions for these genes, encompassing aspects such as plant growth, responses to abiotic stress, and adaptation to environmental factors [66]. Consequently, the cis-acting elements present in *PvLEA* genes were investigated and 110 cis elements were found accordingly (Figure 8, Supplementary Table 3). These predicted CREs were classified into eight classes rely on their functions and these classes were named as CREs associated with (1) responses to stimuli, including environmental stress, (2) light, (3) development,

(4) hormone, (5) promoter, (6) site binding, (7) biotic stress, and (8) CREs with unknown functions (Supplementary Table 3).

The highest number of CRE was in the LEA_2 (66.1%) subfamily, followed by LEA_3 (10.6%), LEA_1 (6.7%), SMP (5.1%), DHN (4.6%), LEA_4 (2.9%), LEA_5 (2.4%) and LEA_6 (1.3%) subfamilies. When CREs were evaluated excluding promoter, site binding, and unknown function groups, the group with the highest CRE was found to be light with 36.01%, followed by hormone (26.54%), environmental stress (19.15%), biotic stress (9.9%) and, development (8.38%) respectively (Figure 8).

Phytohormones are essential for regulating growth and coordinating responses to environmental and biological stresses [67]. In the promoters of *PvLEA* genes, researchers have identified eight CRE groups and some of them were found to be associated with plant hormone responses. These plant hormone related elements were ABREs (26.8%, abscisic acid- related), TGA-element (3.5%, auxin-related), GARE-motif (1.3%, gibberellin-related), P-box (2.1%, gibberellin- related), CGTCA-motif (9.4%, methyl jasmonate-related), TGACG-motif (9.1%, methyl jasmonate- related), TCA element (6.3%, salicylic acid-related) and ERE (28.1%, ethylene-related). Moreover, many elements, including G-Box, ACE, Box-4, Sp1, TCT-Motif and GATA motifs, have previously been associated with drought, salinity, cold and heat-sensitive genes, and were found to have an important role in modifying transcriptional activity of these genes in different plant species [68, 69].

Furthermore, the promoter regions of *PvLEA* genes were found to contain numerous stress- and phytohormone-responsive CREs. These CREs were MYC (37.2%, linked to drought stress), ARE (26.1%, ABA responsive element), WUN-motif (8.5%, sensitive to wound), MBS (7.9%, associated with drought stress) and LTR (2.9%, responsive to low temperature) elements. In a previous study on LEA promoter regions of *A. thaliana*, the presence of ABRE (82%) or LTRE (69%) CREs was already reported similar to our findings in this study [19]. The diversity of CREs in *PvLEA* genes implies potential involvement of these genes in a range of developmental processes in plants. Therefore, our results offer valuable insights into how the *PvLEA* gene family might be regulated, revealing its role in stress tolerance, response to phytohormones, defense mechanisms, and developmental processes.

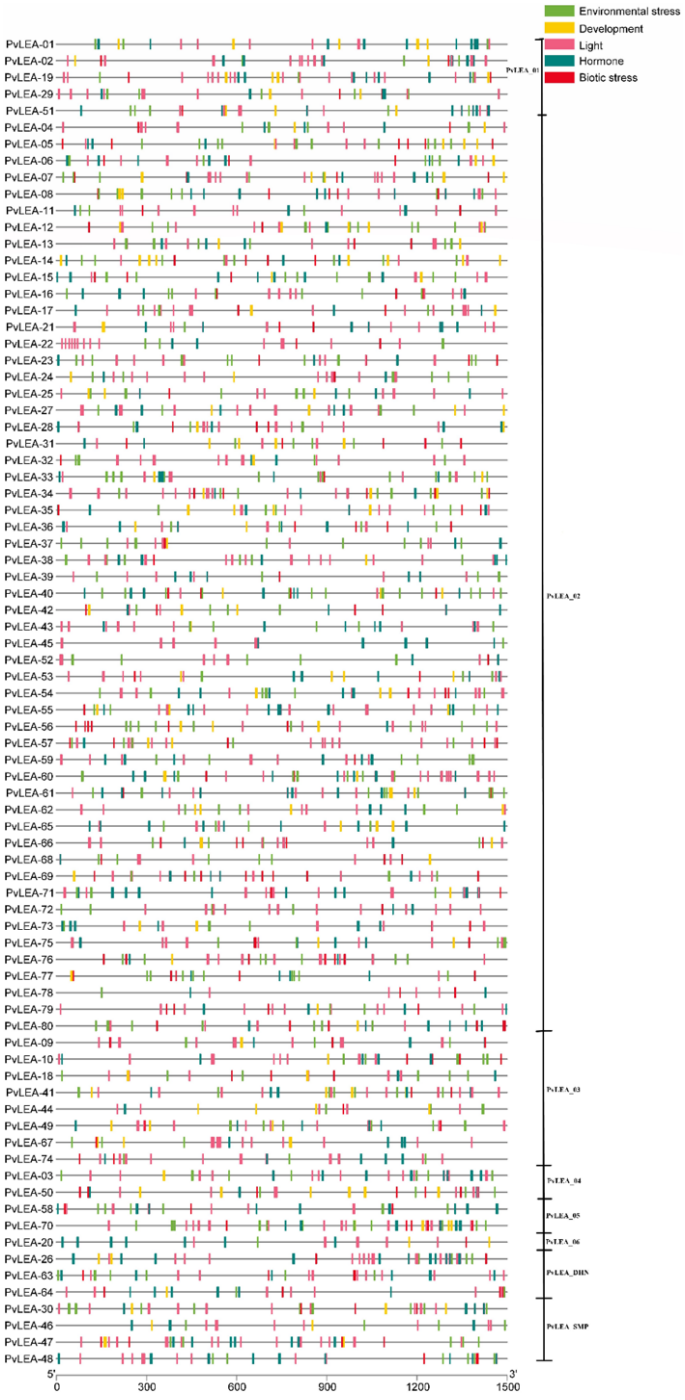

FIGURE 8. The cis-regulatory elements in the promoters of *PvLEA* genes. Different cis-acting elements are represented by colored rectangles.

The 3D structure prediction and homology modeling of 80 PvLEA proteins were conducted using BLASTP against the Protein Data Bank (PDB). Creating 3D models provides valuable insights into protein structure, localization, function, and interaction networks [69]. Differences in 3D topologies were observed in the structure of PvLEA proteins (Figure 9).

Protein models demonstrating high confidence and identity levels surpassing 90% were chosen. A total of four PvLEA proteins [PvLEA-38 (96%), PvLEA-59 (93%), PvLEA-71(99%), and PvLEA-78 (90%)] exhibited high homology with a confidence level higher than 90% in intensive analysis mode (Figure 9). β-sheets were found to be dominant in the secondary structure of PvLEA protein models. Some amino acids are known to disrupt hydrogen bonds during folding due to conflicts among the conformational energy of the side chain and maximal hydrogen bonding [70]. PvLEA-71 (%99) contained the most β sheet structure. Previous studies already showed that LEA_2 group proteins mostly contained β-sheets formation and also some amount of α-helix formation [43, 71]. This structure, like fibronectin Type III domains seen on animal cell surfaces, could function in reducing fluid loss to alleviate the impact of stress or damage on plant tissues [43, 71]. These predicted protein structures are thought to be useful for future molecular biotechnology studies.

FIGURE 9. The three-dimensional structures and binding locations of the PvLEA proteins.

3.5. miRNA analysis of *PvLEA* gene family

MiRNAs affect target gene expression in plants under abiotic and biotic stress conditions, thereby contribute to the determination of the role of the targeted genes [70, 72]. Therefore, using data obtained from the PmiREN and psRNATarget databases, the interactions between miRNAs and *PvLEA* genes were depicted in Figure 10. A total of 21 *PvLEA* gene (*PvLEA-07*, *-09*, *-13*, *-14*, *-23*, *-24*, *-31*, *-35*, *-42*, *-43*, *-46*, *-49*, *-50*, *-53*, *-58*, *-62*, *-63*, *-64*, *-66*, *-69*, *-79*) were found to be targeted by miRNAs (Figure 10 and Supplementary Table 4). Among the *PvLEA* genes, specifically *PvLEA-14*, *PvLEA-63*, and *PvLEA-64*, were the primary targets of miRNAs, with miRN2588 and miR164 being the most prevalent. Kavas et al. (2022) investigated the role of R2R3-MYBs, and genes associated with anthocyanin biosynthesis in the development of seed color

in *P. vulgaris*. Interestingly, they discovered that the R2R3-MYB gene was likewise a target of Pvu-miRN2588 [73]. According to the previous findings in the literature, the primary role of miR164 in plants is to regulate cell division, root development, and stress tolerance [74, 75]. Some other studies also demostrated that miR164 expression was strongly affected by mechanical stresses including tension and compression, as well as by drought stress in various plant species including poplar, *Medicago truncatula* L. and *Populus trichocarpa* [76, 77]. Understanding the structure of miRNAs may facilitate the discovery of miRNAs that are particular to certain tissues, hence enhancing our overall comprehension of gene regulatory networks in plants.

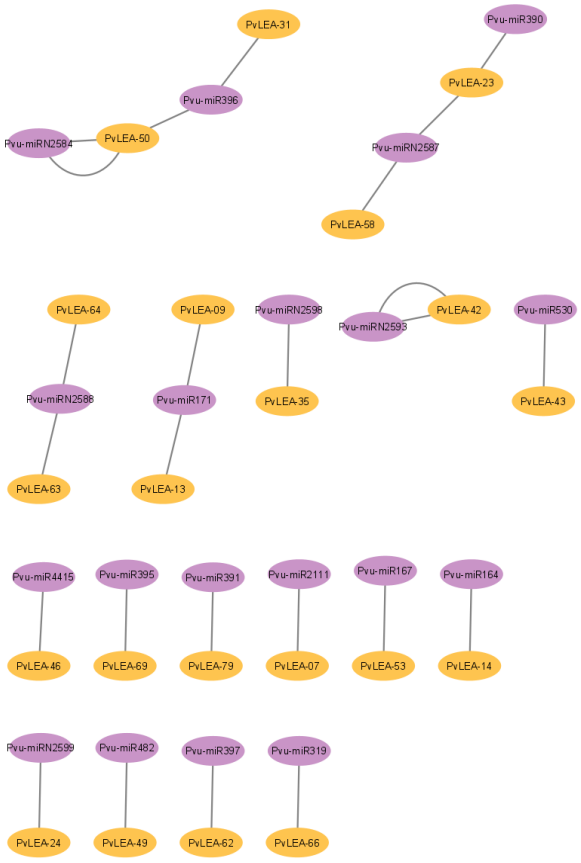

FIGURE 10. The miRNAs targeting the *PvLEA* genes.

3.6 Tissue-specific mRNA levels of *PvLEA* genes

Expression profiles provide important insights into the gene activities [78]. To understand the likely functions and expression patterns of *PvLEA* genes throughout various developmental stages, we investigated their tissue-specific expression under normal conditions. Expression data from eleven distinct tissues including flower buds, flowers, green mature pods, leaves, nodules, root 10, root 19, stem 10, stem 19, young pods, and young trifoliates were analyzed. The resulting expression heatmap visually represents the tissue-specific expression patterns of *PvLEA* genes, aiding in our understanding of their roles in various parts of the common bean (Figure 11). Notably, *PvLEA-63* exhibited the highest expression level, followed by *PvLEA-27*, *PvLEA-35*, *PvLEA-41*, *PvLEA-49*, and *PvLEA-52* genes which are also highly expressed across most tissue types. All *PvLEA* genes, except for *PvLEA-11*, were shown to be expressed in at least one tissue analyzed. Conversely, eight genes (*PvLEA-9*, *PvLEA-23*, *PvLEA-24*, *PvLEA-31*, *PvLEA-32*, *PvLEA-46*, *PvLEA-70*, and *PvLEA-71*) exhibited low expression levels across most tissue types. These findings suggest that *PvLEA* genes exhibit diverse expression patterns across tissues and are implicated in growth and development processes (Figure 11).

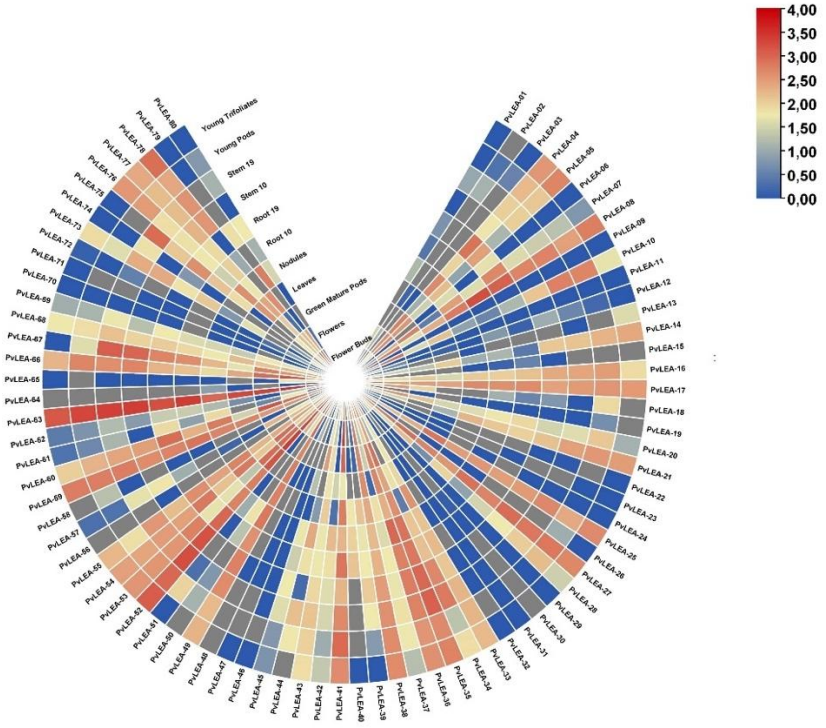

FIGURE 11. Tissue-specific expression level of *PvLEA* genes. Genes exhibiting increased expression levels are shown in red, whereas genes displaying reduced expression levels are represented in blue.

3.7. RNAseq analysis of *PvLEA* genes under salt and drought stresses

The expression patterns of *PvLEA* genes under salt and drought stresses were analyzed using RPKM values, revealing differential expression compared to the control (Figure 12). Notably, when comparing the expression values of *PvLEA* genes under salt and drought stresses, a clear contrast emerged: while salt stress led to decreased expression levels of the affected members, drought stress resulted in increased expression levels compared to the control (Figure 12). Notably, *PvLEA-11* and *PvLEA-29* exhibited no change in expression levels under either stress condition compared to the control. Furthermore, a closer examination based on severe expression changes revealed nuanced responses: some genes, such as *PvLEA-08, -21, -40, -42,* and *-75*, showed decreased expression levels under salt stress, whereas others, including *PvLEA-01, -02, -26, -50, -51,* and *-57*, exhibited increased expression levels. Similarly, under drought stress, while certain genes like *PvLEA-02, -20, -50, -51,* and *-79* showed decreased expression, others such as *PvLEA-44* and *-53* displayed increased expression levels. Overall, when evaluating the cumulative expression changes under both salt and drought conditions, the LEA_2 subfamily emerged as particularly responsive, exhibiting the most significant expression changes compared to the control post-stress. In their study, Khodajou-Masouleh et al. (2021) emphasized the presence of late embryogenesis proteins throughout normal plant growth and development. These proteins play a significant role in responding to abiotic challenges, namely drought tolerance [79]. In summary, these findings provide valuable insights for future research endeavors aimed at understanding plant growth, development, and stress tolerance in the context of both abiotic and biotic stresses.

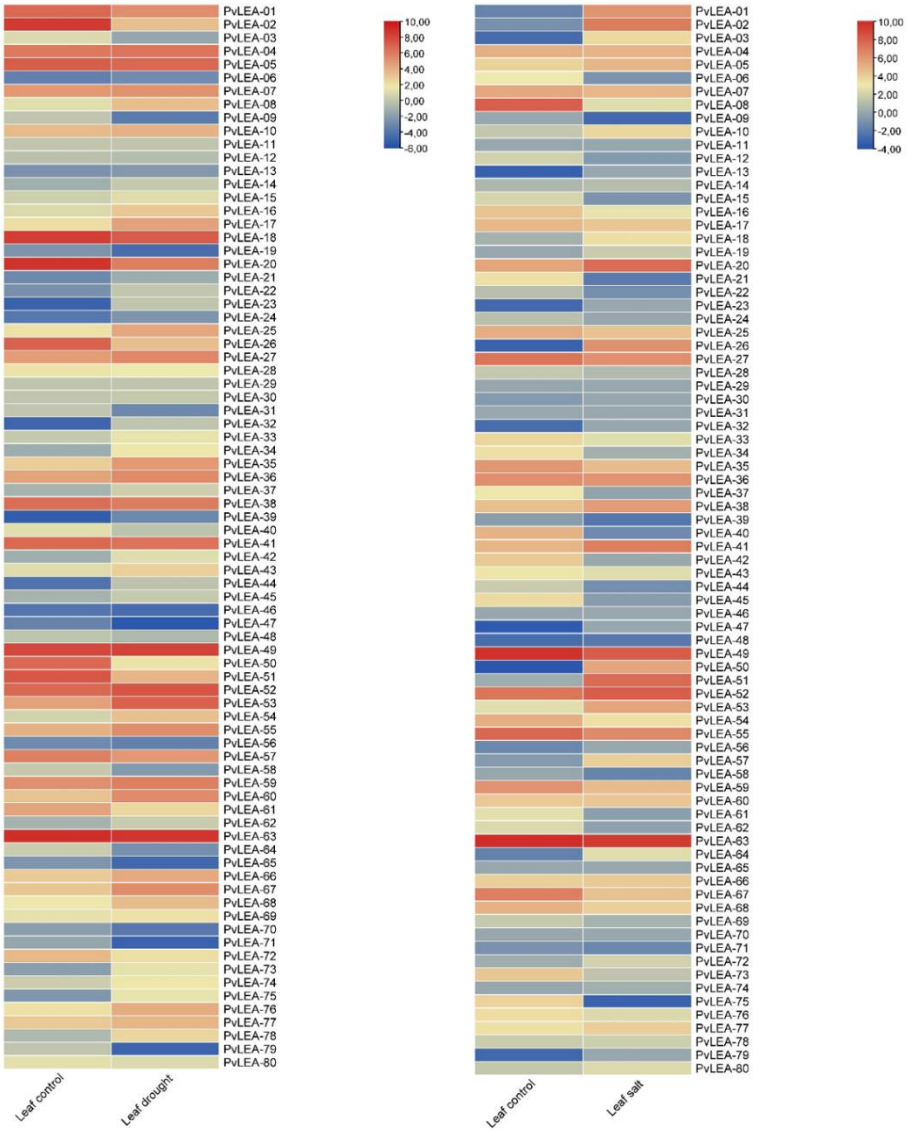

FIGURE 12. A heatmap illustrating the differential expression of *PvLEA* genes in response to drought and salt stress conditions.

4. CONCLUSIONS

In this study, we identified 80 *LEA* genes which were classified into eight subfamilies according to the phylogenetic analyzes. The diversed expression patterns across different tissues and the regulation by both cis-regulatory elements and miRNAs highlight the complexity and importance of *LEA* genes in *P. vulgaris*. The functional significance of gene duplication events and the preservation of these genes via purifying selection highlights their importance in evolutionary insights. The LEA_2 subfamily is particularly noteworthy because to its high prevalence and substantial alterations in gene expression in response to salt and drought stress. It might play a crucial role for enhancing resistance to abiotic stress. These findings offer a valuable genetic resource for further functional characterization studies, which could pave the way for genetic improvements in crop resilience to environmental stresses. Overall, this work establishes a robust framework for future investigations into the roles of LEA proteins in *Phaseolus vulgaris* and potentially other legume crops.

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Author Contribution Statements DF- data collection, management and manuscript writing. SEA- project development, manuscript editing. IB- project development, data analysis, manuscript writing and manuscript editing. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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INVESTIGATION OF LECTIN BINDING ON RABBIT SPLEEN CELL MEMBRANE INFECTED WITH *Proteus vulgaris*

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ABSTRACT This study investigated the effects of *Proteus vulgaris* OX19 infection on the carbohydrate composition of spleen cell membranes in New Zealand adult male rabbits. Rabbits were injected with increasing doses of *P. vulgaris* OX19 (0.5 ml, 1 ml, 2 ml, 4 ml, 5 ml) at five-day intervals over the course of one month. Following the treatment period, spleen tissues were collected from both the control and infected groups. Tissue sections were stained using the Avidin-Biotin-Peroxidase method with five different lectins: *Concavalia ensiformis* (Con A), *Arachis hypogaea* agglutinin (PNA), *Bauhinia purpurea* agglutinin (BPA), *Griffonia simplicifolia* I (GS-I), and *Ulex europaeus* agglutinin I (UEA-I). The stained sections were examined by light microscopy to evaluate lectin binding. Among the lectins used, Con A showed strong binding (+++) to spleen cell membranes of the *Proteus*-infected group, while moderate binding (++) was observed in the control group. UEA-I exhibited weak binding in the control group but demonstrated moderate binding in the *Proteus*-infected group. In contrast, PNA, BPA, and GS-I exhibited strong binding (+++) to spleen cell membranes in the control group and moderate binding (++) in the infected group. These findings suggest that *P. vulgaris* OX19 infection induces alterations in the carbohydrate moieties of glycoproteins and glycolipids in the spleen cell membranes of infected rabbits. It is hypothesized that *P. vulgaris* modifies the terminal carbohydrates of glycoproteins and/or glycolipids in spleen cell membranes, contributing to the observed changes in lectin binding patterns.

Keywords *Proteus vulgaris*, rabbit, spleen, lectins, histochemical staining, light microscopy

1. INTRODUCTION

Proteus species are part of the Enterobacteriaceae family of gram-negative bacilli. *Proteus* organisms are implicated as serious causes of infections in humans, along with *Escherichia*, *Klebsiella*, *Enterobacter*, and *Serratia* species. Enterobacter strains are among the leading causes of nosocomial infections. They cause a wide variety of infections in humans, especially in the lungs, urinary systems and surgical wounds [1, 2]. *Proteus* is a normal flora element in the human intestine and is therefore frequently found in sewage. Urinary tract infections caused by *Proteus* bacteria are long-term infections and can cause kidney stone formation [3]. Frequent urinary tract infections in humans are probably due to the ability of *Proteus* to rapidly break down urea [4]. *Proteus* genus bacteria can be isolated in meningitis, sepsis and organ abscesses. It

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usually causes co-infections with other bacteria in hospitals [5]. Also, *Proteus* bacteria can cause umbilical cord infections in newborns and sepsis and meningitis, which can occur as epidemics resulting from these infections [6-8].

Lectins are proteins/glycoprotein molecules in the extracellular matrix and cell membranes that bind complex sugar chains with high affinity but do not have an enzymatic activity against the bound sugar residues [9-13]. It has been determined that lectins, obtained from plants and able to bind to sugar residues in the surface glycoproteins and organelles of cells, stimulate the immune system cells and increase their cell number and activity [14-16]. Many metabolic events and structural features occurring in living things are determined by commercial lectins obtained from various sources [17]. Lectin histochemistry can therefore be used to visualize different cellular glycosylation patterns which is helpful elucidating the structure, physiology and pathology of tissues [18]. For example, species identification and detection of diseased cells are made using labeled lectin [19].

As a laboratory animal, the rabbit has an immune system that is immunologically similar to humans. The spleen, as an immune system organ, is where macrophages and B and T lymphocytes are located and where both cellular and humoral immune responses occur in infections [20, 21].

2. MATERIALS AND METHODS

2.1 Bacterial culture

Bacterial culture of *Proteus vulgaris* OX19 (Pasteur Institute No:54160) was obtained from Refik Saydam Hıfzıssıhha Center. Bacteria were maintained on Nutrient Broth including %1 glucose and incubated at 37°C.

a. Experimental Animals

In the study, the care and animal experiments of New Zealand adult male rabbits were carried out at the Refik Saydam Hygiene Directorate Experimental Animal Production Center. Each animal was growth in separate cages under laboratory conditions with an appropriate photoperiod (14:10 hours of light/darkness) and a temperature of 20±2ºC [22]. In the experiments, 5 of a total of 10 rabbits weighing 2.5±0.4 kg were used as the control group and 5 as the *Proteus* treated group.

b. Bacteria Injection

At the end of the logarithmic phase, the bacteria were centrifuged and diluted with physiological saline solution (0.9% NaCl) at Mc Ferland density, that is, 2.109 bacteria/ml. Bacteria were injected into rabbits at five-day intervals, with the first dose subcutaneously (0.5 ml) and other doses (0.5 ml, 1 ml, 2 ml, 4 ml, 4 ml) intravenously. Control group animals were injected with the same amount of physiological saline solution [23].

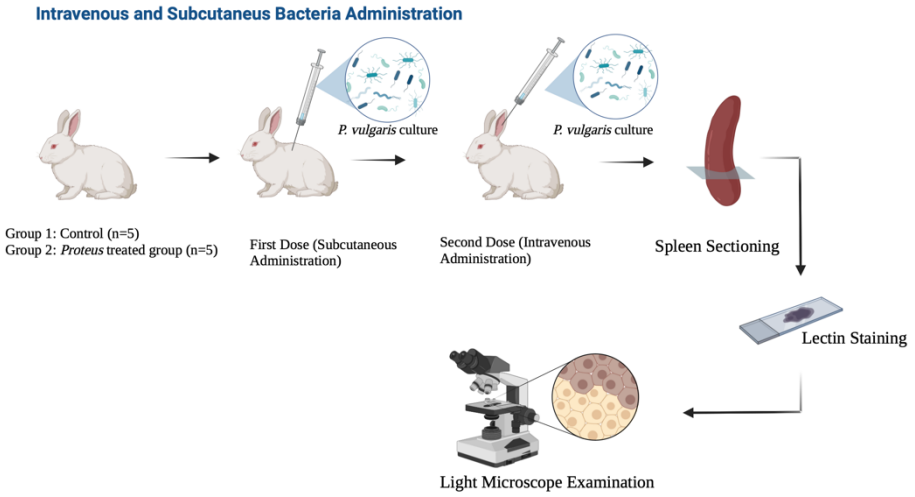

FIGURE 1. Schematic representation of the implementation stages of the experiment (Created with BioRender.com)

c. Lectin Staining Method

Spleen samples taken from control group and *Proteus*-treated rabbits were fixed in 10% formaldehyde solution for 24 hours. Then, the samples were washed with distilled water and dehydrated by passing through the Alcohol series (70%, 80%, 90%, 100%, 100% alcohol) and placed in Paraffin embedding medium. 4µm thick sections were taken from the blocks in the paraffin embedding medium with a microtome and placed on a polylysine slide. The sections taken on a polylysine slide were kept in a 70°C oven for 30 minutes to melt the paraffin. Paraffin residues in the sections were removed by keeping them first in pure xylene and then in 96% alcohol for 30 minutes [24, 25]. In the experiments, Phosphate Buffer Saline (PBS) (pH:7.4) was used both in the preparation of the solutions and in washing the sections during the staining process [26]. The sections were first kept in H_2O_2 for 10 minutes to block endogenous peroxidases, and then kept in lectin solutions prepared at certain concentrations for an hour (Table 1).

TABLE 1. List and concentrations of lectins used in the experiment.

Lectins	**Specific carbohydrates they bind to Lectins**	**Lectin (µg/ml)**
Canovalia ensiformis (Con-A)	α-D- Mannose > α-D-Glucose	2.5
Arachis hypogaea (PNA)	β-D galactose, β-D N-Acetyl galactosamine	5.0
Ulex europaeus (UEA-1)	α-L-Fucose	5.0
Griffonia simplicifolia (GS-I)	α- D- Galactose	5.0
Bauhinia purpurea (BPA)	β-D- Galactose, β-D N-Acetyl galactosamine	5.0

After this process, the sections were washed 3 times with PBC. The sections were incubated with Avidin-Biotin Peroxidase enzyme complex for 45 minutes and then washed three times with PBS. Then, the sections were incubated in 0.6 mg/ml Diaminobenzidine (DAB) prepared with 3µl H_2O_2 for 5 minutes [27]. Finally, the sections were stained with Harris Hematoxylin dye (for counterstaining) for 1 minute and were examined under a light microscope (Nikon Eclips 50i).

3. RESULTS

In this study, the structural changes caused by *Proteus vulgaris* OX19 strain in the carbohydrates of glycoproteins and glycolipids in rabbit spleen cell membranes were examined under light microscope with histochemical method. *P. vulgaris* OX19 strain, known as an infectious agent, was injected into rabbits. One month after the bacterial injection, spleen samples taken from the control group and *Proteus* group rabbits were embedded in paraffin and sectioned. Paraffin sections were stained with five types of lectins according to the Avidin-Biotin Peroxidase enzyme complex method. The staining intensity of cell membranes with lectins was evaluated under light microscopy. As a result of the observations, Con A, which specifically binds to mannose and glucose, was bound to the spleen cell membranes of the control group rabbits with moderate strong (++) (Figure 1), and unlike the control group, it was strongly (+++) bound to the spleen cell membranes of the rabbits injected with *P. vulgaris* OX19 bacteria (Figure 2).

The binding status of the lectins used in the study to cell membranes is briefly shown in Table 2 below.

TABLE 2. Lectin binding of *Proteus* and control groups

Lectin	**The carbohydrate group it binds to**	**Control Group**	***Proteus* Group**
Con A	α-D- Mannose > α-D-Glucose	(++)	(+++)
PNA	β-D Galactose and β-D- N-Acetyl Galactoseamine	(+++)	(++)
BPA	β-D Galactose ve β-D D- N-Acetyl Galactoseamine	(+++)	(++)
GS-I	α- D- Galactose	(+++)	(++)
UEA-I	α-L-Fucose	(+)	(++)

+++ Strong, ++ Medium strong, + Less strong

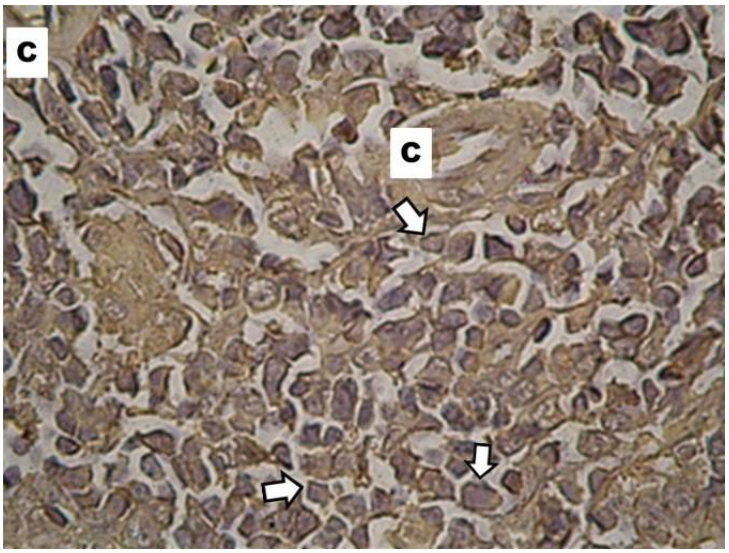

FIGURE 2. Moderate strong (++) staining of control group rabbit spleen cell membranes with Con A. (→) Cell membrane stained with lectin, (C) Capillary. x1000

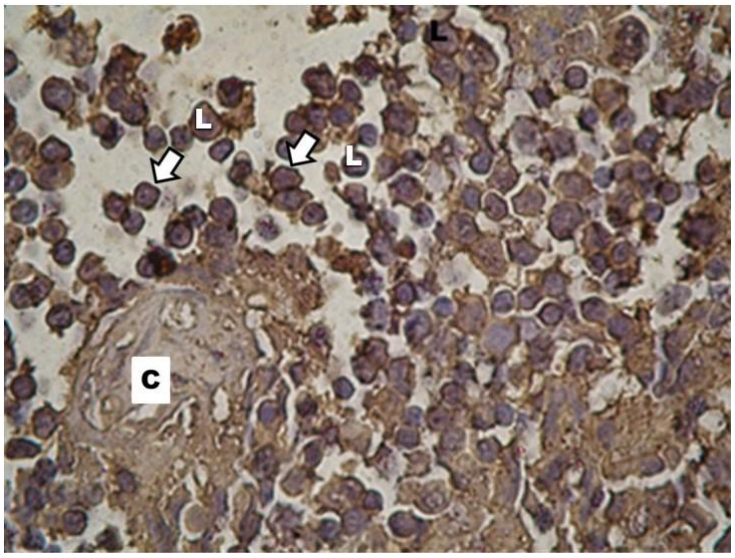

FIGURE 3. Strongly (+++) staining of *Proteus*-treated rabbit spleen cell membranes with Con A. (→) Lectin stained cell membrane, (L) Lymphocyte, (C) Capillary. x1000

PNA, which was used in the study and specifically bound to β-D galactose and β-D N-Acetylgalactosamine, was strongly (+++) bound to the spleen cell membranes of control group rabbits (Figure 3), and to the spleen cell membranes of *Proteus* rabbits. It was observed that it bonded with moderate strong (++) (Figure 4).

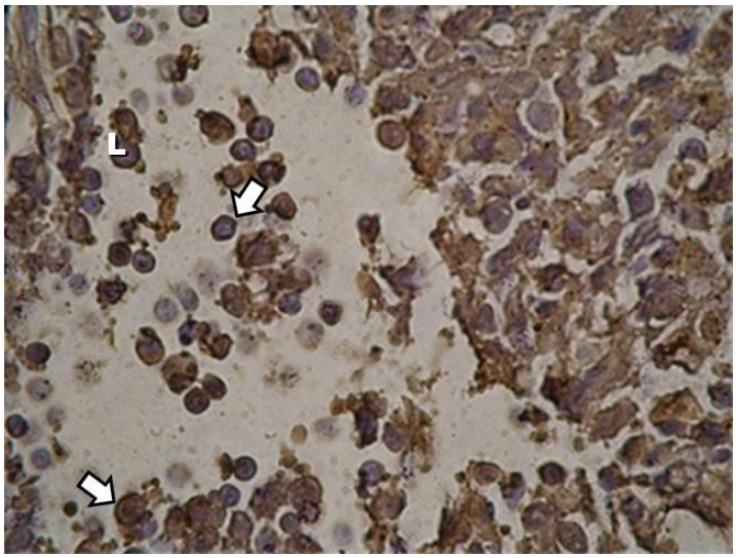

FIGURE 4. Strong (+++) staining of control group rabbit spleen cell membranes with PNA. (→) Lectin stained cell membrane, (L) Lymphocyte. x1000

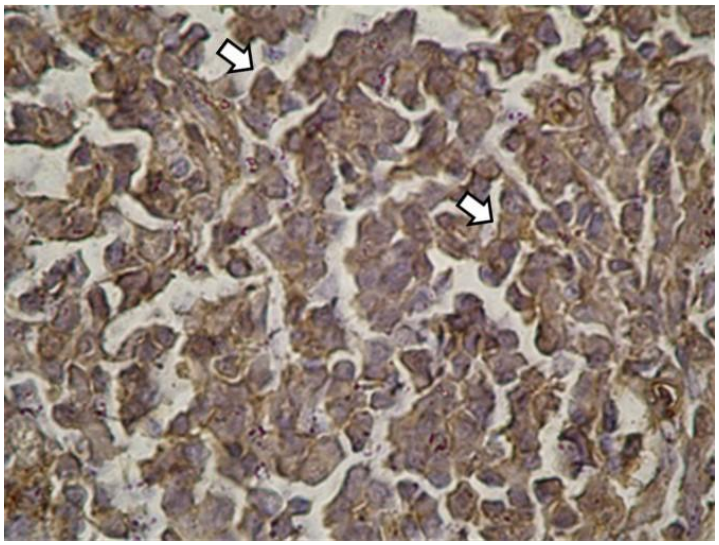

FIGURE 5. Medium strong (++) staining with PNA of rabbit spleen cell membranes given *Proteus vulgaris* OX19 strain. (→) Cell membrane stained with lectin. x1000

BPA, one of the lectins used in the experiments, showed a similar situation to PNA binding. While BPA bound strongly (+++) to the spleen cell membranes of rabbits in the control group (Figure 5), it bound to the spleen cell membranes of rabbits injected with *Proteus* bacteria with moderate strong (++) (Figure 6).

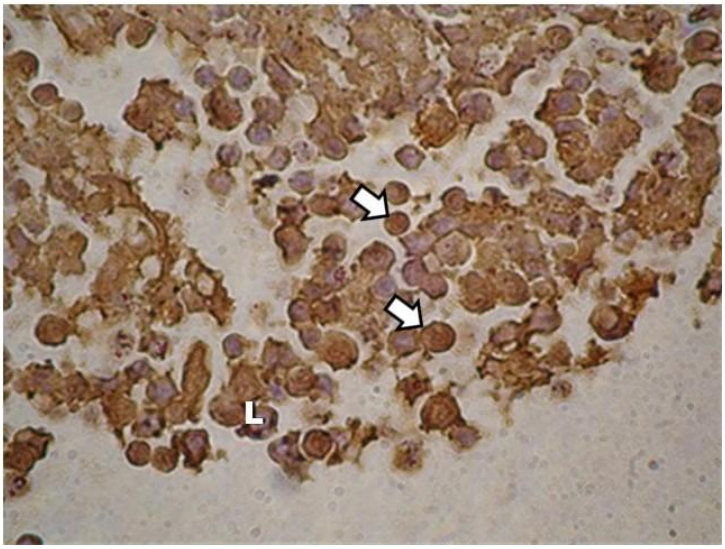

FIGURE 6. Strongly (+++) staining of control group rabbit spleen cell membranes with BPA. (→) Lectin stained cell membrane, (L) Lymphocyte. x1000

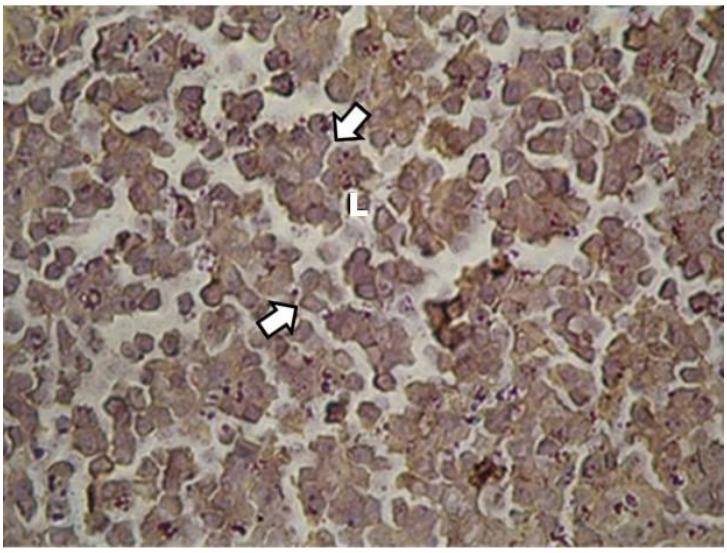

FIGURE 7. Moderate strong (++) staining of rabbit spleen cell membranes in the *Proteus* treated group with BPA. (→) Lectin stained cell membrane, (L) Lymphocyte. x1000

It was observed that BS-I lectin, which specifically binds to α-D-Galactose, bound strongly (+++) to the spleen cell membranes in the control group (Figure 7), and bound to the spleen cell membranes of rabbits in the *Proteus*-injected group with moderate strong (++). (Figure 8).

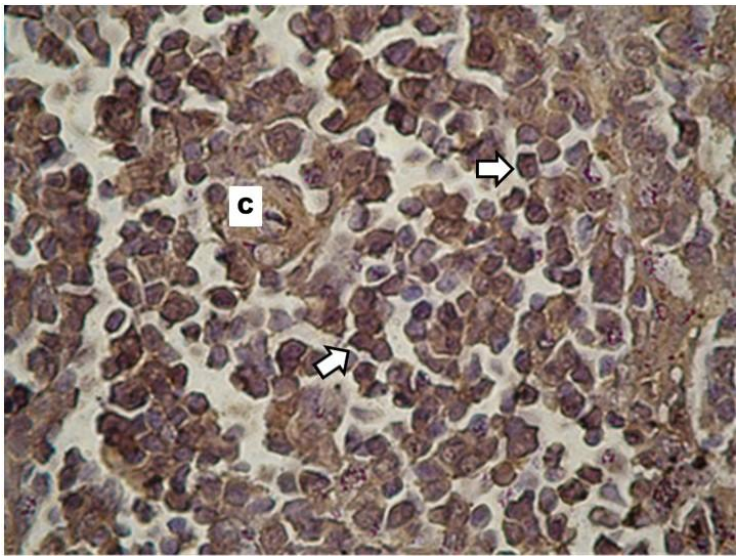

FIGURE 8. Strongly (+++) staining of control group rabbit spleen cell membranes with BS-I. (→) Lectin stained cell membrane, (C) Capillary. x1000

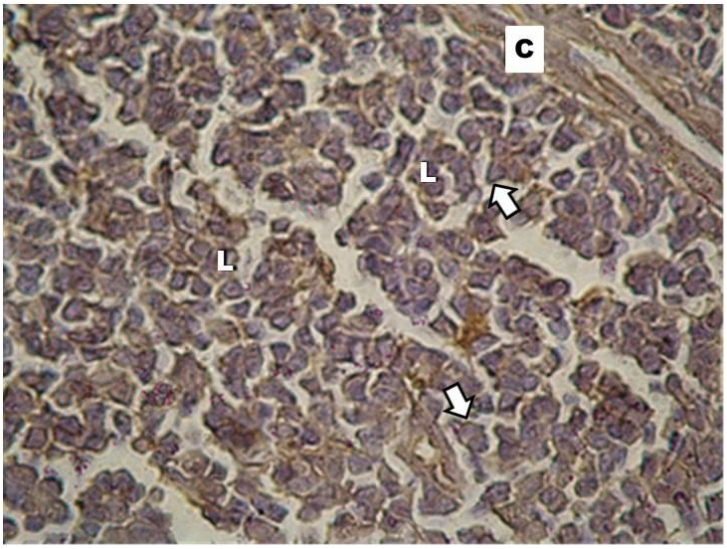

FIGURE 9. Moderate strong (++) staining of Proteus group rabbit spleen cell membranes with BS-I. (→) Cell membrane stained with lectin, (C) Capillary, (L) Lymphocyte. x1000

UEA-I specifically binds to α-L-Fucose (Table 2). It was observed that this lectin bound to the spleen cell membranes in the control group with a weakly strong (+) (Figure 9), and to the *Proteus* cell membranes with a moderate strong (++) (Figure 10).

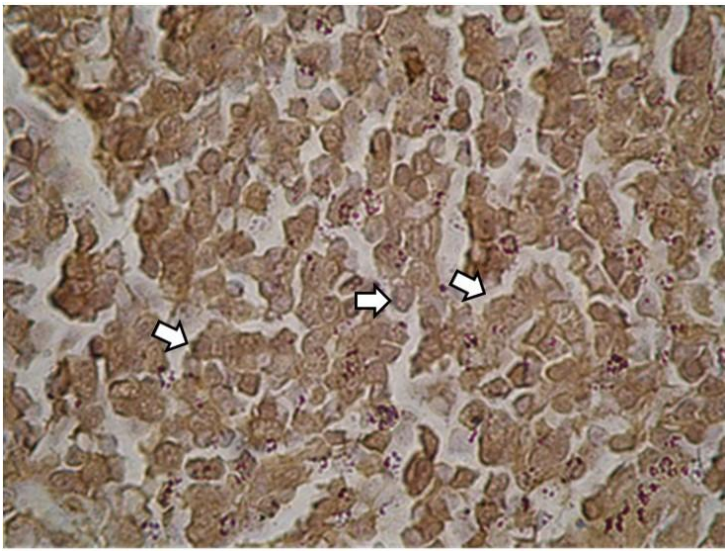

FIGURE 10. Weakly strong (+) staining of control group rabbit spleen cell membranes with UEA. (→) Cell membrane stained with lectin. x1000

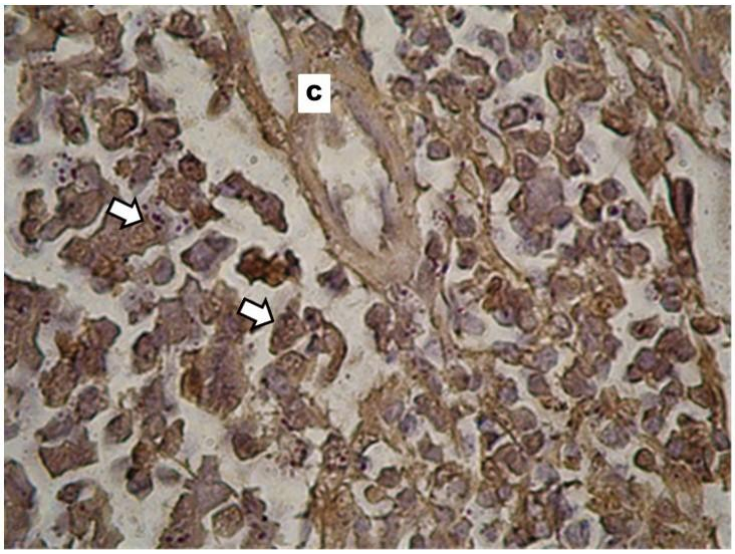

FIGURE 11. Moderate strong (++) staining of rabbit spleen cell membranes injected with *Proteus vulgaris* OX19 strain with UEA-I. (→) Cell membrane stained with lectin, (C) Capillary. x1000

4. DISCUSSION

Considering the binding of other lectins used, it was observed that the rabbit spleen cell membranes in the *Proteus* group bound lectins (BPA, PNA and GS-I) less than the control group. BPA, PNA and GS-I lectins bind to D-Galactose units. At the same time, BPA and PNA lectins also bind to β-D N-Acetyl galactosamine [28]. Infected rabbit spleen cell membranes bind less lectins than the control group, suggesting that D-Galactose and β-D N-Acetylgalactosamine units are likely reduced in cell membranes. It was thought that the increase or decrease in carbohydrate units in infected rabbits may have affected the synthesis of glycoproteins containing carbohydrates in the membrane or the enzyme synthesis that binds/separates carbohydrates.

Changes in the carbohydrate expression and glycosylation of cells during the development, diseases and embryogenesis of living things are detected by the binding of lectins to the cells [29, 30]. Sobral et al. [30] reported that changes in cell adhesion, changes in the tumor cell surface and changes in the carbohydrate composition on the surface of the cells occur in metastasis and abnormal cell development. The same researchers examined Con A and UEA-I lectin binding to parotid gland cell membranes with mucoepidermal carcinoma and normal parotid gland cell membranes. Researchers have suggested that Con A binds weakly to normal parotid gland myoepithelial cells and vascular endothelium, but strongly binds to parotid gland duct cells with moderate and high levels of carcinoma. In our study, Con A bound more strongly to the cell membranes of *Proteus* rabbits than the control group. Accordingly, the increase in the binding

of Con A to cancer cell membranes and *Proteus* cells shows that there is an increase in the expression of D-Mannose and D-Glucose in the cell membranes. According to the observations made by some researchers, it has been understood that Mannose units increase in the cell membranes of organisms against urinary tract infection of bacteria [31-33]. It has been reported that mannose found in cell membranes binds to Escherichia coli bacteria, which causes urinary tract infection, and thus is excreted along with the bacteria adhering to D-Mannose during urination [32]. *Proteus* and *E.coli* are in the same family (Enterobacteriaceae), in rabbit spleen cell membranes treated with this bacteria (*Proteus vulgaris* OX19), Con A (which specifically binds mannose) increased due to infection, thus mannose on the cell surfaces may have increased. It is estimated.

It has been reported that parasitic organisms cause changes in the cell membranes of the host organism. Melo-Júnior et al. [34] observed under light microscopy the binding of lectins to cells in hepatic-egg granuloma caused by Schistoma mansoni in humans. It has been reported that Con A and PNA lectins bind moderately strongly (++) to normal hepatic cells, while PNA binds strongly (+++) to cells in hepatic-egg granuloma and Con A binds weakly. Lectin binding to cell membranes in *Proteus* rabbits was different from the lectin binding detected by Melo-Júnior et al. For example, although Con A bound strongly to the cells of *Proteus* rabbits compared to the control group, Con A bound weakly to the cells in the hepatic-egg granuloma. PNA binding also showed a different situation. Although PNA binds moderately to *proteus* cells, it has been reported that PNA binds very strongly to cells in hepatic-egg granuloma. Tritrichomonas foetus was inoculated into the genital organs of mice, and genital tissue was taken from the mice at intervals from the 3rd day to 60 days. It has been reported that the binding of PNA and SBA lectins to uterine endometrial cells is significantly different compared to the infected group. It was reported that 16 days after parasite application, UEA-I bound strongly to the vaginal epithelium, while PNA and SBA lectins bound strongly to uterine cells [35]. In our study, although PNA and UEA-I were bound to a moderate degree in *Proteus* spleen samples, PNA was bound to a high degree and UEA-I to a low degree in the control group.

Alroy et al. [36] investigated the binding of Con A, UEA-I and PNA lectins to spleen cells in the diagnosis of carbohydrate storage diseases. It has been observed that in mannose storage disease (mannosidosis), Con A binds very strongly to spleen cells, while UEA-I and PNA do not bind at all. It has been reported that in fucose storage disease (fucosidosis), Con A binds weakly, UEA-I binds moderately, and PNA does not bind at all. Spleen cells in mannosidosis showed strong binding of Con A and low binding of PNA, similar to spleen cells with *Proteus*. It is thought that lectins bind with different strengths to spleen cells in carbohydrate storage disease and to spleen cells infected with *Proteus*, and disease and infection may affect the structural and functional properties of enzymes that bind or degrade carbohydrates.

Considering the histochemical studies conducted with lectins, it is thought that in infectious conditions and cancer, a change occurs in the form of an increase

or decrease in the carbohydrates at the ends of the glycoproteins or glycolipids in the cell membranes, compared to normal or control group experimental animals.

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Author Contribution Statements MD and MNA: designed the study, performed the wet-lab work and analyzed the data, wrote and reviewed the manuscript. HE and NG: writing–review & editing.

Declaration of Competing Interests The authors declare no conflict of interest.

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ANALYSIS OF THE TOP-DOWN AND BOTTOM-UP EFFECTS ON ZOOPLANKTON BIOMASS IN EUTROPHIC LAKE YENİÇAĞA

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ABSTRACT Several aquatic ecological studies have focused on the contrasting effects of top-down and bottom-up interactions on zooplankton communities. It is essential to comprehend the relative strength of these interactions to evaluate the trophic interactions of pelagic food webs, an area that is still extensively researched due to its complexity. Therefore, we examined the biomass of zooplankton over a one-year period in a freshwater lake that is subject to multiple stressors such as anthropogenic activities, eutrophication. Top-down effects, namely fish biomass, and bottom-up effects, including total phosphorus, total nitrogen, and chlorophyll a concentrations were considered. Structural equation modelling (SEM) was employed to evaluate the relative impact of top-down and bottom-up effects on zooplankton. The SEM analysis revealed that zooplankton is influenced by both top-down and bottom-up effects in Lake Yeniçağa. The biomass of cladocerans was found to have a negative correlation with increasing chlorophyll a, while the Calanoida group was negatively affected by both fish biomass and chlorophyll a from top-down and bottom-up controls. The fish biomass had a positive effect on both Cyclopoida and Rotifera, but only Rotifera showed a negative interaction with chlorophyll a. Direct bottom-up effects of total phosphorus and total nitrogen on chlorophyll a were found, with total nitrogen having a stronger interaction than total phosphorus.

Keywords Lake Yeniçağa, zooplankton, trophic interaction, structural equation modeling

1. INTRODUCTION

Trophic interactions are essential aspects of limnology and have been the focus of much scientific investigation within lakes. Trophic interactions occur when one organism feeds on another, involving the flow of energy within a community. The question of whether food webs are resource- (bottom-up) or predation- (top-down) controlled is one of the most fundamental research questions in ecology [1]. In the past, most scientists believed that the structure of the food web was primarily regulated by available resources, a concept known as bottom-up control [2]. This implies that phytoplankton is regulated by abiotic factors such as light and temperature, and the flow of nutrients, which in turn regulate zooplankton [3]. However, it is now evident that food webs can also be significantly regulated by top-down effects through consumers. For instance, zooplankton are regulated by fish, and phytoplankton are regulated by

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zooplankton [4, 5]. In addition to biotic factors, nutrients such as total nitrogen and total phosphorus can also play a crucial role in freshwater ecosystems by affecting phytoplankton growth rates [6, 7]. Numerous scientific studies have focused on the contrasting impacts of fish feeding (top-down) and phytoplankton biomass (bottom-up) on zooplankton communities [4, 8, 9]. Recent studies have also acknowledged the significance of the conditions under which top-down and bottom-up effects prevail [10].

Zooplankton play a crucial role in transferring matter and energy from phytoplankton to fish. They act as primary consumers and regulate phytoplankton community structure in food webs. To make informed decisions about environmental management, such as biomanipulation, it is important to understand how this link between phytoplankton and zooplankton varies along trophic gradients [11, 12]. Identifying and comprehending the distinct effects exerted by both top-down and bottom-up forces on zooplankton can be challenging due to the diverse nature of zooplankton communities, which consist of various taxonomic and functional groups. Investigating the top-down and bottom-up effects on zooplankton is critical for food web studies [13].

Excessive nutrient inputs can cause eutrophication, which is a critical environmental problem that significantly degrades water quality. This phenomenon leads to changes in biodiversity that pose serious threats to aquatic ecosystems. The consequences include the decline of economically valuable fisheries, a shift in community composition from benthic periphyton and epiphytes to phytoplankton, and a limitation of zooplankton [14, 15, 16]. Shallow eutrophic lakes are characterized by high nutrient content, which can lead to serious ecological problems such as Cyanobacteria blooms. Although cyanobacteria are not normally a desired food source for zooplankton due to their low nutritional values, difficulty in digestion, and toxicity, several studies have shown that large zooplankton can remove small cyanobacteria in nutrient-enriched shallow lakes [17, 18, 19]. However, in such lakes, the fish community is frequently dominated by planktivorous species, which may prevent the development of large zooplankton that could control phytoplankton populations [20].

Several studies have investigated trophic interactions, specifically the top-down and bottom-up effects of organisms, in the food webs of shallow lakes in Turkey with varying nutrient levels and predation pressures [21-24]. The results of these studies revealed that food web dynamics can vary spatially and temporally across trophic gradients.

Lake Yeniçağa is a freshwater lake that has been significantly impacted by various human activities including the excessive expansion of residential areas and the drainage of agricultural areas. The main threats to the lake are the discharge of domestic, urban, and industrial waste, agricultural activities, and eutrophication. It is classified as a habitat of high biodiversity and a threatened natural habitat according to the criteria of Important Plant Areas in Turkey. The entire shoreline of the lake is surrounded by reeds (*Typha minima*, *Typha*

shuttleworthii), some of which are over 200 meters wide, providing resting and wintering areas for migratory bird populations. Furthermore, the catchment area connected to the lake has one of the largest peatlands in Turkey, with a depth of 2 meters. It is classified as a rich calcareous peatland under the Bern Convention [25, 26, 27].

This study aims to test the relative importance of top-down and bottom-up forces on zooplankton in a eutrophic shallow lake affected by massive cyanobacterial blooms using structural equation modeling. It was hypothesized that top-down interactions would have a greater impact on the Crustacea group compared to the Rotifera group because of their larger body size, particularly in the case of fish predation [28, 29]. Simultaneously, we proposed a second hypothesis that takes into account the trophic level of the lake. According to this hypothesis, phytoplankton would have a positive effect on the Cyclopoida and Rotifera groups, which typically thrive in eutrophic conditions. Conversely, the Cladocera and Calanoida groups, which exhibit higher biomass in oligotrophic waters, would be negatively impacted [29, 30]. This study is expected to be a valuable contribution to the existing literature on zooplankton food web interactions in shallow freshwater lakes with high levels of eutrophication.

2. MATERIALS AND METHODS

2.1. Study Area

The study was conducted in Lake Yeniçağa (40°47'N, 32°01'E), located in the interior of Western Black Sea, within the borders of Bolu city and north of Yeniçağa town. This shallow eutrophic lake covers approximately 1800 hectares, with mean and maximum water depths of 3.87±0.87 and 6.0 m, respectively. Commercial fishing activities take place in the lake. The lake is inhabited by a total of four fish species feeding omnivorously, but the pelagic fish community is dominated by *Cyprinus carpio* and *Squalius cephalus*. The contribution of these species to total fish biomass is 96%. Since 2014, varying numbers of *Cyprinus carpio* larvae have been stocked in the lake each year, which can potentially control zooplankton through predation and indirectly affect phytoplankton [26, 31]. Over the past 30 years, urban and agricultural settlement in the basin has led to a significant increase in nutrient loading, resulting in accelerated eutrophication. This has caused water quality deterioration and increasingly severe cyanobacterial blooms. The phytoplankton flora of the limnetic zone of Lake Yeniçağa consists mainly of Chlorophyta, Bacillariophyta, Cyanobacteria, and Dinoflagellata. *Anabaena circinalis* and *Aphanizomenon flos-aquae* have been the most abundant species in the lake [25, 31, 32].

2.2. Sampling

The study was carried out in the limnetic zone of Lake Yeniçağa, which does not contain any vegetation. Monthly samples were collected from Lake Yeniçağa between December 2021 and November 2022, excluding January and February

due to a completely ice-covered lake surface. A total of three sampling stations were determined: the first site (1) was selected near the 100 m of the stream mouth (mean depth: 371±53 cm), another site (2) was in the middle of the lake (mean depth: 455±93 cm), a third (3) was located near the 100 m of the sewage outfall (mean depth: 335±70 cm) (Figure 1).

FIGURE 1. Locations of sampling stations where field studies are carried out in Lake Yeniçağa. Station 1: 40°46'59.952"N - 32°1'11.496"E; Station 2: 40°46'50.664"N - 32°1'32.987"E; Station 3: 40°46'37.848"N - 32°2'2.184"E.

Water samples were collected from each site at the 0, 2, and deepest point of the water column (ca. 4m) using a 2 L Hydro-bios Ruttner water sampler. The samples were then combined in a bucket for analysis of physicochemical parameters. Turbidity was measured using an ORION AQ3010 turbidimeter. The transparency of the water was determined by monitoring Secchi depth (cm) with a white Hydro-bios Secchi disc (20 cm in diameter) in situ. Total nitrogen (TN) and total phosphorus (TP) concentrations in each sample were measured in the laboratory according to standard methods 4500 NO2-B, 4500-Norg-B, 4500P-B, and 4500PE [33]. The concentration of chlorophyll a was determined using spectrophotometry at 665 nm after extracting the samples collected on GF/C filters with a hot methanol solution [34].

Zooplankton samples were taken monthly at three sampling stations. The samples were obtained by filtering 100 L of water samples and concentrated to 50 mL through a plankton net with a mesh size of 30 μm (HYDRO-BIOS, Althengolz, Germany). Surface sampling was made at a depth of 15-20 cm and vertical sampling was made by vertical tow from a few cm above sediment to surface. Concentrated zooplankton samples were gently preserved with a 4% (v/v) formaldehyde solution. Organisms from the Cladocera, Calanoida, Cyclopoida, and Rotifera groups were examined in a Sedgewick Rafter counting

cell with a volume of 1 ml and under a Leica DMR microscope (Wetzlar, Germany). The examination identified all samples, which were then counted and measured for total length under the microscope. At least 30 individual bodies of each species were measured under the microscope. The dry weight of species in the Cladocera, Calanoida, and Cyclopoida groups was calculated using the length-weight formulas provided by Dumont et al. [35] and Bottrell et al. [36]. To determine the biomass of the Rotifera group, we first calculated the biovolume of the samples using the formulas provided by Ejsmont-Karabin [37]. We then converted the data to dry weight values using the formulas given in Bottrell et al. [36].

Monthly fish sampling was conducted at three sampling stations with the assistance of Yeniçağa Fisheries Cooperative (Figure 1). Two nets were used at each sampling station. We performed fish sampling with multi-mesh gill nets (length 50 m; height 4 m) with mesh sizes knot to knot of 10, 32, 40, 60, 80, and 90 mm. The nets were set at dusk in areas where the water depth was less than 5 m and removed at dawn, with the duration of exposure recorded. Fish were counted, measured (total length), and weighed (fresh mass).

Catch Per Unit Effort (wCPUE) estimates of relative weight for all fish species were obtained using gillnetting and the following formula:

$$wCPUE = (CN \times (AS/AN))/t \quad (1)$$

where CN is the nominal catch (kg), AS is the area of the standard net (100 m2), AN is the area of the used net (m2) and t is the time of exposure (t) . The results obtained from the formula were converted to kg/ha/h [38].

Carlson's trophic state indices (TSI) were calculated according to the provided equations [39].

$$TSI(\text{Chlorophyll a}) = 9.81 * \ln(\text{Chlorophyll a}) + 30 \quad (2)$$

$$TSI(\text{Total Phosphorus}) = 14.42 * \ln(\text{Total Phosphorus}) + 4.15 \quad (3)$$

$$TSI(\text{Secchi Depth}) = 60 - 14.41 * \ln(\text{Secchi Depth}) \quad (4)$$

2.3. Data Analysis

In this study, Structural Equation Modeling (SEM) was used to analyze the role of top-down and bottom-up controls in the food web, specifically their effects on the zooplankton community. SEM is a statistical analysis that estimates the relationships between variables. These variables can be either dependent or independent, factors, or measured variables. In comparison to other statistical tools such as factor analysis and multivariate regression, SEM has the ability to measure the errors of observed variables while simultaneously predicting causal relationships between both latent and manifest variables [40, 41]. Since 2000,

ecologists have been actively using SEM to study the complex interactions found in ecosystems [42]. SEM is distinguished from other data modeling methods by it is the ability to examine path relationships. It is increasingly popular among biologists as it helps to understand direct and indirect interactions within data [43]. Standardized path coefficients between two variables represent the relative strength of a relationship. In SEM, R2 values indicate the proportion of variance explained by each variable. SEM assumes a predefined set of predictor variables that impact other variables, establishes the direction of these effects, and subsequently tests them using empirical data.

All data were log10(x+1) transformed prior to analyses to ensure the assumptions of normality, homogeneity of variance, and linearity of the analysis. During the SEM analysis, Comparative Fit Index (CFI) and Standardised Root Mean Square Residual (srmr) values were calculated to test the validity of the path diagram, and R2 and standartized estimates were determined [44]. In the literature, a CFI value greater than 0.95 and an srmr value less than 0.08 indicate a successful model [45, 46]. In our SEM, we obtained CFI values of 0.950 and srmr values of 0.058, confirming that the constructed model adequately explained the dataset. All path coefficients used in the analysis were standardized.

SEM was carried out using the R Statistical Software (v4.3.2) and the 'lavaan' and 'tidySEM' packages [47, 48].

3. RESULTS

In this study, Calanoid copepods were found to be the group that made the largest contribution to the zooplankton biomass in Lake Yeniçağa, while the group that contributed the least was the Cyclopoid copepods. Zooplankton biomass exhibited significant monthly changes, with the lowest values recorded as 8114 µg/m3 during the summer period, and the highest values observed as 130779 µg/m3 during the winter period. The biomass of Calanoid copepods ranged between 1125-91123 µg/m3, with maximum values observed in the winter and minimum values in the spring. The biomass of the cladocerans ranged between 43-38794 µg/m3, with the lowest biomass values observed in the summer, and the highest biomass values in the spring. Rotifera biomass also displayed significant monthly variation, with the lowest values recorded as 502 µg/m3 during the winter and the highest values as 39416 µg/m3 in the spring (Table 1).

TABLE 1. Mean, minimum, maximum, and standard deviation values of zooplankton biomass and fish wCPUE measured monthly in Lake Yeniçağa.

	Mean	**Minimum**	**Maximum**	**SD**
Cladocera Biomass (µg/m^3)	25073	43	38794	17228
Calanoida Biomass (µg/m^3)	25887	1125	91123	43559
Cyclopoida Biomass (µg/m^3)	5188	122	14178	6576
Rotifera Biomass (µg/m^3)	17542	502	39416	19896
Zooplankton Biomass (µg/m^3)	73690	8114	130779	51900
Cyprinus carpio (kg/ha/h)	43.43	16.91	57.61	22.98
Squalius cephalus (kg/ha/h)	13.54	5.32	19.03	7.25
Tinca tinca (kg/ha/h)	1.63	0.28	2.75	1.25
Carassius gibelio (kg/ha/h)	0.34	0.34	0.34	-
Total Fish (kg/ha/h)	58.71	22.51	79.39	31.45

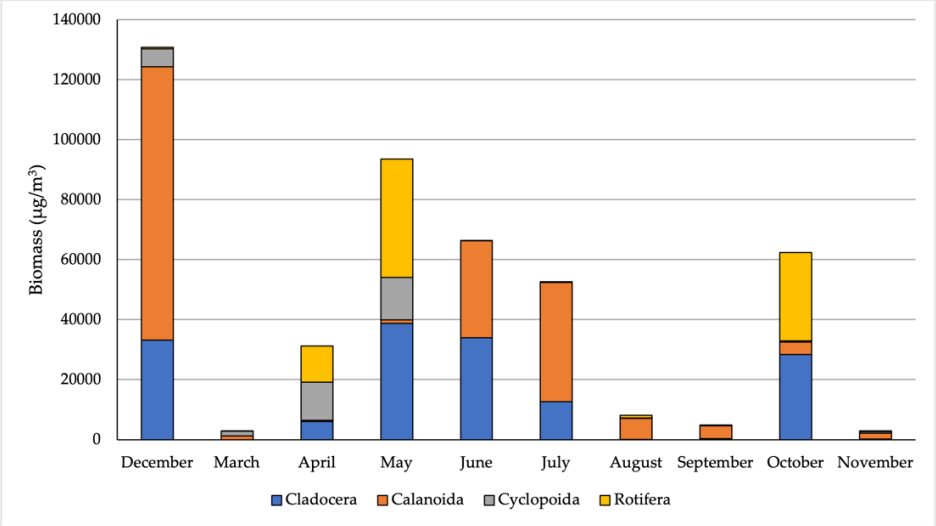

FIGURE 2. Monthly zooplankton biomass changes in Lake Yeniçağa.

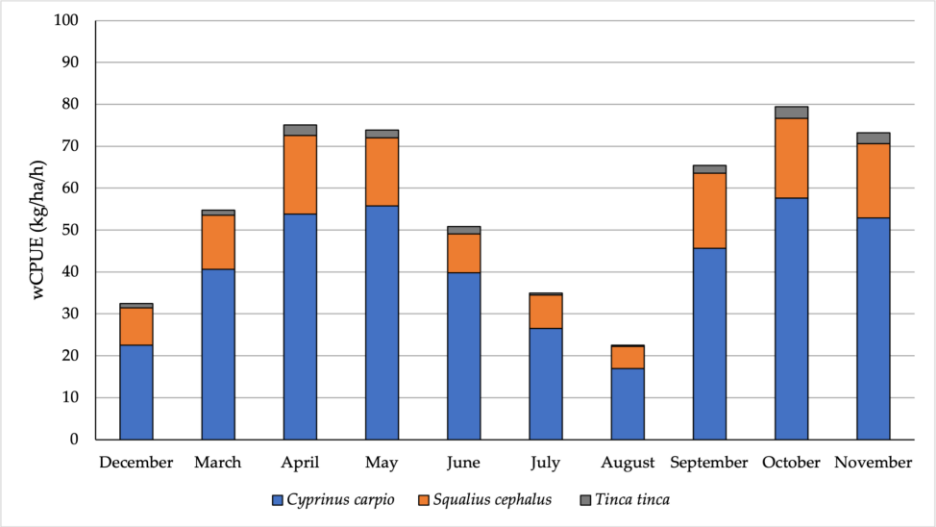

FIGURE 3. Monthly fish wCPUE changes in Lake Yeniçağa.

Nineteen taxa of zooplankton were identified in the lake, including 12 Rotifera, 4 Cladocera, and 3 Copepoda. The most significant contributors to the zooplankton community in terms of biomass were *Acanthodiaptomus denticornis* (38%) from the Calanoida group, *Daphnia pulex* (31%), from the Cladocera group, and *Cyclops strenuus* (7%) from the Cyclopoida group. The biomass values of Rotifera were lower than those of other groups due to their small size, with *Asplanchna priodonta* (6%) making a significant contribution to rotifer biomass (Figure 2).

Among the fish species sampled in the lake, four fish species were identified: *Cyprinus carpio*, *Squalius cephalus*, *Tinca tinca*, and *Carassius gibelio*. *Cyprinus carpio* contributed the most to the fish biomass, while *Carassius gibelio* contributed the least. The total fish wCPUE values in Lake Yeniçağa were calculated between 22.51-79.39 kg/ha/h (Table 1). The wCPUE values of *Cyprinus carpio* varied between 16.91-57.61 kg/ha/h (Figure 3). The range of total lengths of the fishes was between 165-500 mm (mean 369 mm) for *C. carpio*, 150-330 mm (mean 241 mm) for *S. cephalus*, 182-316 mm (mean 212 mm) for *T. tinca*. During the studies, only one *C. gibelio* specimen was sampled, and the total length of the specimen was measured as 275 mm. Based on the literature data, we categorized the fish as omnivorous (bentho-planktivorous) depending on the measured body length [49, 50, 51].

During the study, chlorophyll a values in the lake ranged from 15.1 to 114.4 mg/m^3, total nitrogen from 88 to 1987 µm/L, total phosphorus from 141 to 310 µm/L, Secchi depth from 60 to 300 cm, and turbidity from 1.17 to 41.53 ntu, as shown in Table 2. TN:TP ratios in Lake Yeniçağa were calculated to be between 0.60 and 9.24.

This study evaluated monthly chlorophyll a, total phosphorus, and Secchi depth values in Lake Yeniçağa using Carlson TSI Indices. Carlson TSI(TP) Index varied between 75.48 and 86.87 throughout the year with an annual average of 78.91. The Carlson TSI(CHLa) Index, was calculated between 57.23 and 77.10 throughout the year, with an annual average of 65.65. Carlson TSI(SD) Index ranged from 44.16 to 67.36 and the annual average of this value was determined to be 55.19 (Table 2).

TABLE 2. Mean, minimum, maximum, and standard deviation values of zooplankton biomass and fish wCPUE measured monthly in Lake Yeniçağa.

	Mean	**Minimum**	**Maximum**	**SD**
Chlorophyll a (mg/m^3)	42.06	15.10	114.44	28.70
Total Phosphorus (µg/L)	184	141	310	52.55
Total Nitrogen (µg/L)	986	88	1987	587.77
Secchi Depth (cm)	163.5	60	300	93.77
Carlson TSI (CHLa)	65.65	57.23	77.10	5.78
Carlson TSI (TP)	78.91	75.48	86.87	3.59
Carlson TSI (SD)	55.19	44.17	67.36	8.69
Turbidity (ntu)	8.19	1.17	41.53	2.59
TN:TP	5.36	0.60	9.24	3.18

The calculated Carlson TSI Index values based on the total phosphorus results indicated that the lake was at a hypertrophic level. The results obtained from chlorophyll a showed that the lake had a hypertrophic tendency but at a eutrophic level, while the Secchi depth values indicated that the lake was at a eutrophic level. Based on these results, the lake can be considered to be at the eutrophic level with a hypertrophic tendency.

Data on how much each variable can be explained by other variables in the SEM is presented in Table 3. The largest R^2 value was found to be 0.854 for chlorophyll a, while the smallest R^2 value was 0.426 for Cladocera. The results of the SEM, using fish, zooplankton, chlorophyll a, total nitrogen, and total phosphorus values in Lake Yeniçağa, are schematized in Figure 4. It was

observed that total nitrogen (r = 0.62, p < 0.001) and total phosphorus (r = 0.57, p < 0.001) values exhibited a positive interaction with chlorophyll a values. The standardized estimation values of the variables examined in the dataset are also presented in Table 4. It was understood that the effect of total nitrogen on chlorophyll a was higher than that of total phosphorus. The effect of chlorophyll a parameter on Rotifera (r = -0.48, p < 0.001), Cladocera (r = -0.48, p < 0.001), and Calanoida (r = -0.71, p < 0.001) was found to be negative. However, no significant relationship was found between chlorophyll a and Cyclopoida (p = 0.15). According to the statistically significant standardized estimation values, the zooplankton group most affected by chlorophyll a was the calanoids. The analysis results for fish-zooplankton interactions in the food web were statistically insignificant for the Cladocera group (p = 0.30). The effect of fish on the zooplankton community was found to have a negative effect on Calanoida (r = -0.74, p = 0.002) and a positive effect on Cyclopoida (r = 0.82, p < 0.001) and Rotifera (r = 0.42, p < 0.001). According to the standardized estimation values, the effect of fish on the Cyclopoida group was higher compared to other groups (Table 4).

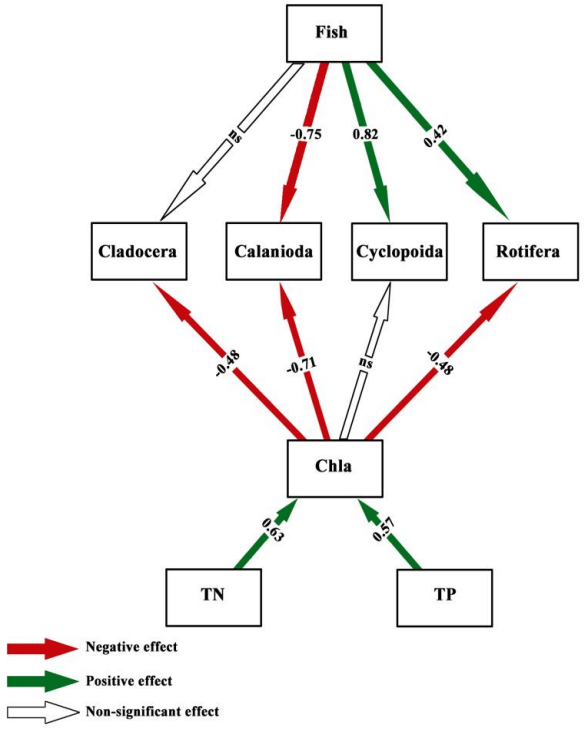

FIGURE 4. Path diagram obtained by using SEM of monthly determined fish, zooplankton, chlorophyll a, total nitrogen, and total phosphorus values in Lake Yeniçağa. Boxes represent observed variables. (χ^2 = 19.616, *df* = 9, p = 0.020).

TABLE 3. R^2 values of variables used in SEM.

	Estimate
Chlorophyll a	0.854
Rotifera	0.633
Calanoida	0.481
Cyclopoida	0.546
Cladocera	0.426

TABLE 4. Standardized estimates, standard errors, z-values, and p-values in SEM (**: p < 0.01, ***: p< 0.001).

			Standardized Estimate	**S.E.**	**z-value**	**p**
Cladocera	<---	Fish	0.249	0.242	1.027	0.305
Cladocera	<---	Chlorophyll a	-0.482	0.130	-3.698	***
Calanoida	<---	Fish	-0.747	0.241	-3.096	**
Calanoida	<---	Chlorophyll a	-0.715	0.160	-4.458	***
Cyclopoida	<---	Fish	0.820	0.115	7.126	***
Cyclopoida	<---	Chlorophyll a	0.174	0.122	1.423	0.155
Rotifera	<---	Fish	0.421	0.120	3.509	***
Rotifera	<---	Chlorophyll a	-0.482	0.099	-4.875	***
Chlorophyll a	<---	Total Nitrogen	0.626	0.065	9.704	***
Chlorophyll a	<---	Total Phosporus	0.573	0.073	7.848	***

4. DISCUSSION

The main reason for eutrophication, one of the most important problems affecting freshwater ecosystems, is the excessive presence of nitrogen and phosphorus elements in the water column that are essential for phytoplankton growth [52]. Secchi depth, an indicator of water transparency and trophic level, is inversely proportional to the density of phytoplankton populations in the water as suspended matter scatters and weakens incoming light [53].

According to Carlson and Simpson [54], some interpretations can be made about ecosystem functioning based on the relationships between TSI indices calculated from chlorophyll a, total phosphorus, and Secchi depth values. In this study, to better understand the deviations in TSI values, the Trophic Index deviation graph, which is Carlson's two-dimensional approach has been used (Figure 5) [54, 55, 56]. Values below the horizontal axis indicate that chlorophyll a is not limited by phosphorus, while values above the horizontal axis indicate that it is limited by phosphorus. On the vertical axis, the values to the right of the line represent cases where the light transmittance is higher than the expected chlorophyll index. In this case, it shows that zooplankton use small-sized phytoplankton as food, and therefore large organisms such as filamentous cyanobacteria become dominant. Values to the left of the vertical axis indicate that water transparency is controlled by factors not dependent on phytoplankton [54]. According to the trophic index deviation graph given in Figure 5, almost all values for Lake Yeniçağa are gathered below the vertical axis and to the right of the horizontal axis. This suggests that phosphorus is not the limiting nutrient for chlorophyll a in the lake, and phytoplankton is dominated by larger-sized species, with zooplankton exerting grazing pressure on small-sized phytoplankton organisms.

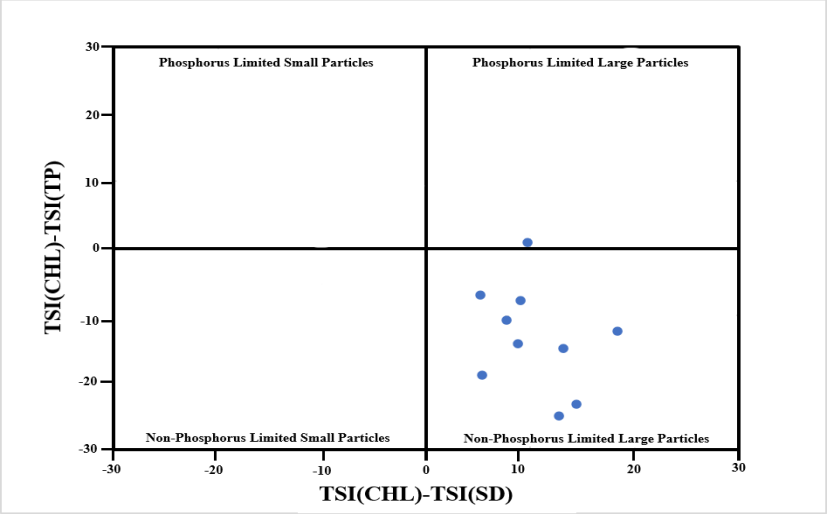

FIGURE 5. Deviations in the trophic state index.

TN:TP ratio provides information about the limiting element for photosynthetic organisms in an ecosystem. When the TN:TP ratio is lower than 10, nitrogen is considered the limiting nutrient, and when it is higher than 17, phosphorus is considered the limiting nutrient. Ratios between 10-17 indicate that both nitrogen and phosphorus are limiting nutrients [57]. The average TN:TP ratio (9.24 ± 3.18) in Lake Yeniçağa indicates that nitrogen is the limiting element for photosynthetic organisms. In lakes characterized by a low TN:TP ratio, the predominant phytoplankton are typically cyanobacteria, and these low ratios promote the growth of nitrogen-fixing cyanobacteria [58-60]. These results are consistent with our findings in the Carlson Trophic Index deviation graph. Additionally, the effect of total nitrogen and total phosphorus on chlorophyll a was evaluated using SEM. The analysis results showed that bottom-up controls had a positive effect on chlorophyll a. This finding is consistent with what is commonly observed in freshwater lakes and reservoirs [61]. The SEM analysis also revealed that the effect of total nitrogen on chlorophyll a is stronger than the effect of total phosphorus in Lake Yeniçağa.

The analyses conducted for Lake Yeniçağa revealed that zooplankton was influenced by both top-down and bottom-up controls, and different groups within the zooplankton community responded differently to these effects.

It is considered that the Cladocera group in Lake Yeniçağa is not controlled by top-down effects as no significant relationship was found between the biomass of fish and this group. It has been reported in the literature that omnivorous fish species exert feeding pressure on large-bodied Cladocera species [28, 62]. While there are studies that support this relationship, there are also some studies where no significance could be detected [63, 64]. Thus, our analysis results for cladocerans are partially contradictory to the literature. Fish species in Lake Yeniçağa, such as *Cyprinus carpio*, *Squalius cephalus*, and *Tinca tinca*, are not exclusively dependent on zooplankton, but are also omnivorous organisms that can feed on phytoplankton, insect larvae, and benthic macrovertebrates [65, 66]. Consequently, fish may have reduced the top-down pressure on cladocerans by shifting their food preference towards calanoids. On the other hand, the feeding habits of fish may also change depending on the turbidity level of the water they are in, and the effect of size-related predation may decrease due to turbidity [67]. Lake Yeniçağa can be classified as a lake with medium turbidity based on its turbidity values (mean: 8.19±2.59). Studies have shown that the pigmentation rate of zooplankton affects the food selectivity of fish in turbid lakes. Therefore, transparent organisms with weak pigmentation can avoid predation pressure from fish in turbid environments [68, 69]. In the aquatic environment, cladocerans exhibit daily vertical or horizontal migration behaviours to escape predator pressure from fish. Experimental studies have shown that fish signals affect this migration behaviour [70, 71]. The SEM analysis results also indicated a negative bottom-up effect due to chlorophyll a on the cladocerans (r = -0.48). As the level of eutrophication increases in freshwater ecosystems, there is a decrease in the growth and fertility rates of cladocerans [72, 73]. Additionally, the increase in eutrophication in lake ecosystems triggers the overgrowth of

cyanobacteria, which provide poor nutrition for cladocerans and limit their energy for growth and reproduction, especially in the case of large-sized cladocerans [74,75,76]. Similarly, there are studies in which cladocerans are negatively affected by phytoplankton through bottom-up control. Elser et al. [77] stated that the grazing of *Daphnia* over phytoplankton in a lake at a high eutrophic level decreased considerably compared to a mesotrophic lake, and the increase in the trophic level turned the bottom-up effects on *Daphnia* into negative. This could explain why the Cladocera group in eutrophic Lake Yeniçağa was negatively affected by chlorophyll a.

The top-down effects on the Copepoda group in Lake Yeniçağa exhibited opposite patterns for the two different orders studied within this group. The biomass of calanoid copepods was negatively affected by the presence of fish ($r = -0.75$), while the biomass of cyclopoid copepods was positively affected ($r = 0.82$). According to Soto and Hurlbert [29], calanoid copepods are more sensitive to top-down effects than cyclopoid copepods. Calanoid copepods may enter into a food competition with cyclopoid copepods by consuming common food groups such as rotifers and protozoa with cyclopoid copepods, leading to a numerical decrease in the Cyclopoida group [29, 78]. The top-down control of calanoid copepods by fish in Lake Yeniçağa may indirectly affect the food competition between calanoid and cyclopoid copepods. This situation likely explains the positive association between fish CPUE and both Cyclopoida and Rotifera biomass. Matsuzaki et al. [63] found a positive relationship between Cyclopoida abundance and the CPUE of fish, while Li et al. [79] found a negative relationship between the increase in fish and calanoid biomass. The results obtained for copepods in Lake Yeniçağa are consistent with the literature.

The bottom-up effect, another effect seen in the Copepoda group, was clearly detected for the order Calanoida ($r = -0.71$), but no significant relationship was found for the order Cyclopoida ($p > 0.05$). It is known that calanoids are much more sensitive to eutrophication than cyclopoids and have lower biomass in eutrophic water systems [80-85]. In our study, SEM results revealed that both top-down ($r = -0.75$) and bottom-up ($r = -0.71$) controls acted together on the Calanoida group, and it was understood that these effects were close to each other. The only significant effect in cyclopoid copepods was a positive top-down interaction. It is thought that the reason for this positive effect is the food competition with the calanoids, which decreases as a result of the negative effect exerted by the fish. As an omnivorous species, *Acanthodiaptomus denticornis*, the only Calanoida species identified in the study area, can feed on rotifers and ciliates [86]. At the same time, *Cyclops strenuus*, quantitatively the most dominant Cyclopoid Copepod species in the lake, has an omnivorous feeding characteristic and feeds on rotifers, other crustaceans, and phytoplankton [87]. For these reasons, it can be assumed that these two species entered into significant food competition.

Increasing the top-down effect of fish predation on Crustacea in the aquatic food web may reduce predation pressure and trigger an increase in both the abundance

and biomass of rotifers [88, 89, 90]. The results of SEM analysis in Lake Yeniçağa showed a positive top-down control on rotifers from fish (r = 0.42). An increase in fish biomass also indirectly increased Rotifera biomass, and there are studies consistent with our results [63, 91, 92]. Predation pressure from fish, which negatively controls the calanoids from the top-down, may have indirectly contributed to this increase. Since *Acanthodiaptomus denticornis*, the only Calanoid species detected in Lake Yeniçağa, is a species that feeds on rotifers [86], the decrease in their abundance may have caused a decrease in the predation pressure on the Rotifera. On the other hand, studies in freshwater ecosystems reveal that rotifers are also controlled from the bottom-up, and this effect is higher than top-down control [30, 93]. In bottom-up controls, besides phytoplankton, there is also the effect of food sources such as bacteria, detritus, and Protista at a much higher rate. Due to the organism size of the filamentous algae, the lack of suitable nutrients for the rotifers reduces the filtration rate and creates negative effects on their nutrition and life cycles [75, 94-96]. In this respect, it can be thought that the negative interactions we detected between Rotifera and chlorophyll a are caused by dominant phytoplankton species (*Anabaena circinalis*, *Aulacoseira granulata*, *Aphanizomenon flos-aquae*) in the phytoplankton of Lake Yeniçağa [32]. According to the SEM analysis results in Lake Yeniçağa, the standardized path coefficient value was calculated as r = -0.48 in the bottom-up interaction of chlorophyll a. Rotifers can interact positively with phytoplankton with bottom-up control in the food web, but these effects are weaker than interactions with other food sources such as bacteria and Protista [30, 93, 97]. In the zooplankton community, organisms with small body sizes, such as rotifers, may consume food sources like bacteria and detritus in the absence of suitable phytoplankton nutrients [94, 98, 99]. This situation may also be applicable for the Rotifera group in Lake Yeniçağa. However, it is not possible to reach a definite judgment since we do not have data on supplementary food sources.

5. CONCLUSION

In this study, we examined top-down and bottom-up effects on zooplankton in Lake Yeniçağa, which shows a eutrophic level close to hypertrophic, using SEM. According to the results of the standardized path coefficients, the summary of bottom-up and top-down controls in the zooplankton community is as follows: a) The Cladocera group was controlled from the bottom up by phytoplankton, b) The Cyclopoida group was controlled from the top-down by omnivorous fish, c) Both top-down and bottom-up effects observed on the Calanoida and Rotifera groups were quite close to each other (r = -0.75 and -0.71 for Calanoida; r = 0.42 and -0.48 for Rotifera). For this reason, it was determined that both top-down and bottom-up controls acted together on these two groups.

We believe the data from this study can set an example for future research on the interactions between zooplankton, fish, and phytoplankton in Lake Yeniçağa, making it valuable for potential biomanipulation studies in the lake. To conduct the biomanipulation study, it is necessary to reduce the number of omnivorous

fish species that exert predation pressure on *Acanthodiaptomus denticornis*, which is known to be an omnivorous species and can feed on phytoplankton in the lake [86]. Also, reducing fish biomass may indirectly affect the trophic level of the lake by decreasing internal loading [100]. We propose that a piscivorous species that can feed on these fish species should be introduced into the lake. However, it should be noted that any manipulation of the fish community in the lake will not be effective unless the untreated wastewater entering the lake is prevented.

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DENTAL STEM CELL BANKING: A PROMISING FUTURE FOR REGENERATIVE MEDICINE APPLICATIONS

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ABSTRACT Dental stem cells originating from different oral tissues in and around dental structures have recently gained attention as a potential alternative for regenerative medicine applications. To date, many dental stem cells are identified specific to the tissue from which they originate. They exhibit many valuable advantages including high proliferation ability, self-renewal capacity, and multiple differentiation potentials that make them an important candidate for clinical applications, especially in treating degenerative and inflammatory diseases. The fact that they can be easily obtained from an individual's waste tooth without any ethical concern provides them an excellent opportunity for autologous treatment with a low risk of immune rejection. Nowadays, the storage of autologous dental stem cells isolated from wisdom teeth or healthy extracted teeth in biobanks without ethical concerns has become a very important approach for the regeneration of damaged and diseased tissue and for the treatment of life-threatening diseases that may be encountered in the future life of the donor. This study provides a comprehensive overview of dental stem cells, recent advances in their clinical use, long-term preservation processes, and the latest advances in Dental Stem Cell Banking.

Keywords Dentistry, biobanks, stem cells, regeneration

1. INTRODUCTION

Stem cells are cell populations comprised of unspecialized cells that have a remarkable capacity for proliferation, clonality, and differentiation into various cell types [1]. Considering their regenerative capacity, stem cells are classified as totipotent, pluripotent, and multipotent. Cells that can differentiate into any cell type and possess the potential to form an entire organism are defined as totipotent. Although pluripotent stem cells display differentiation ability into any cell type, they differ from totipotent stem cells in that they cannot form an entire living organism on their own. On the other hand, multipotent stem cells only exhibit the potential to differentiate into a limited number of specific cells in the body [2, 3].

Regarding their origin, stem cells are classified as embryonic stem cells which are isolated from the inner cell mass of the blastocyst of the embryo. Besides,

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adult stem cells can be isolated from diversified tissues. While embryonic stem cells are distinguished by their pluripotency, adult stem cells possess limited differentiation capacity which is the general barrier to their regenerative medicine applications. Nevertheless, the use of embryonic stem cells has societal limitations and ethical issues due to the derivation of cells from early human embryos that inhibits embryonic development. Scientists aiming not only to leave ethical concerns behind but also to obtain cells with high differentiation capacity discovered the induced pluripotent stem cells which exhibit simulant proliferation capacity and gene expression characteristics compared to embryonic stem cells. Unlike embryonic stem cells, these cells were developed from individual somatic cells with certain gene modifications but no ethical limitations. With this scientific discovery, Shinya Yamanaka and John Gurdon earned the Nobel Prize in Physiology or Medicine in 2012 [4, 5].

In addition to different types of stem cells, dental stem cells are also considered excellent candidates for stem cells that can be easily obtained from wisdom teeth or healthy extracted teeth and used with less ethical concerns [6]. To date, several types of dental stem cells have been isolated from different parts of tooth and tooth-related tissues, which possess remarkable regenerative potential. Therefore, they have recently gained great attention as a potent platform for developing regenerative medicine applications, in the future [7].

Personalized stem cell-based therapy is going to be the face of the future of medicine which holds hope for many previously incurable cases such as type 1 spinal cord injuries, neurodegenerative diseases, diabetes, and many others. As a source of stem cell therapy, cord blood banking which involves invasive and expensive procedures, is now being widespread all over the world through different government agencies and private companies [8]. If patient lost the opportunity to store their stem cells from the umbilical cord at the time of birth, they have now another opportunity to preserve their stem cells originating from their extracted teeth which are generally thought to be medical waste. Therefore, instead of discarding teeth, storing their cells in a professional dental stem cell bank will be a better option for their future life. Preserving stem cells derived from tooth or tooth-related tissues which includes the same procedures as storing the umbilical cord stem cells in biobanks, is called “Tooth Banking” or “Dental Stem Cell Banking”. For this process, baby teeth, wisdom teeth, or healthy extracted teeth are considered a precious source of highly potent stem cells. When a child’s or an adult’s tooth is extracted by dental professionals, stem cells can be isolated from different tissues, cryogenically frozen following their characterization, and then preserved. Banking these stem cells provides a reliable source for medical treatments in the future [3].

In the last decade, the number of stem cell banks that store the stem cells obtained from bone marrow and placenta cord blood has been increasing rapidly all over the world. Despite this trend, the number of banks specializing in dental stem cells is still very small worldwide. Moreover, in many countries, there is no awareness of this issue yet. This review focuses on the recent advances in dental

stem cells, and procedures related to dental stem cells and serves as an introduction to their “banking”.

2. DENTAL STEM CELLS

Dental stem cells are defined as multipotential mesenchymal stem cells that can be isolated from oral tissues [9]. Similar to mesenchymal stem cells, dental stem cells can undergo self-renewal and have multipotent differentiation ability. In recent years, they have been considered a relatively non-invasive source of autologous stem cell therapy. Besides, they have many remarkable advantages such as being nonimmunogenic, easily accessible, having a good match for the entire family, displaying a higher capacity for proliferation and differentiation, and having the potential to remedy organ shortage which is an expected future necessity [10]. Additionally, they do not pose any ethical problems compared to other stem cell sources.

To date, dental stem cells have been identified from different parts of oral tissues in and around the tooth, and named according to their origin such as dental pulp stem cells from dental pulp (DPSCs), stem cells from human primary exfoliated deciduous (SHEDs) teeth (primary teeth of children), periodontal ligament stem cells (PDLSCs) from periodontium, dental follicle stem cells (DFSCs) from human third molars, gingival mesenchymal stem cells (GMSCs) from gingiva, stem cells from alveolar bone (ABSCs), and stem cells from the apical papilla (SCAPs) (Figure 1).

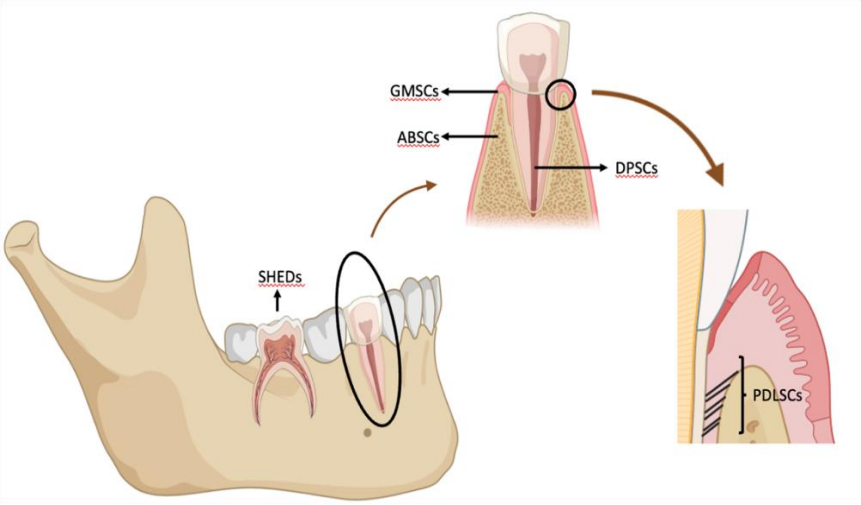

FIGURE 1. Dental tissue sources for therapeutically relevant stem cells. SHEDs, Stem Cells from Human Deciduous Teeth; DPSCs, Dental Pulp Stem Cells; PDLSCs, Periodontal Ligament Stem Cells; ABSCs, Alveolar Bone Stem Cells; GMSCs; Gingival Mesenchymal Stem Cells. Figure created with BioRender.com (accessed on 24 January 2024).

2.1 Dental pulp stem cells (DPSCs): Dental pulp stem cells (DPSCs) were first isolated and identified from the dental pulp of adult 3rd molar (19-29 years old) teeth without caries or infections by Gronthos et al. in 2000 [11]. Since their identification, DPSCs have been the subject of an increasing number of scientific studies in the field of tissue engineering, especially in dental tissue regeneration. In addition to their easy accessibility, these cells exhibit mesenchymal stem cell-like properties with their self-renewal potential and multilineage differentiation capacity. Besides, they have positive surface markers such as CD105, CD90, and CD70, similar to mesenchymal stem cells. Recent studies have shown that DPSCs have a higher proliferation capacity than bone marrow mesenchymal stem cells. Besides, the ability of these cells to differentiate into neuronal and adipose cells, odontoblast and osteoblast cells, and chondrocytes, has been shown by different studies [12, 13]. Therefore, DPSCs are now considered a potential candidate for autologous regenerative therapy.

2.2 Human primary exfoliated deciduous stem cells (SHEDs): In 2003, Miura et al. [14] identified the stem cells from the pulp tissue of human exfoliated deciduous teeth and named the cells as human primary exfoliated deciduous stem cells (SHEDs). Similar to DPSCs, SHEDs express the surface markers including CD13, CD44, CD73, CD90, CD146, and CD166. As a novel and non-invasive source of mesenchymal stem cells, SHEDs exhibit higher proliferation capacity than DPSCs and can differentiate into neurons, adipocytes, odontoblast, and hormone-secreting cells. Other advantages exhibited by SHEDs include painless cell collection without major ethical concerns and minimal risk of invasion. All these valuable properties make them important candidates to be used in tissue engineering-based treatments [15].

2.3 Periodontal ligament stem cells (PDLSCs): Periodontal ligament stem cells (PDLSCs) were first identified from the periodontal ligament of third molars by Seo et al. (2004) [16]. With the exhibition of important characteristics similar to mesenchymal stem cells, they include positivity for mesenchymal stem cell surface markers such as CD90, CD73, and CD105. Recent studies have also proven that PDLSCs have a significant capability of differentiating into cementoblast-like cells, adipocytes, glial and neuron-like cells, and collagen-forming cells when stimulated with appropriate growth factors. They are considered a source of multipotent stem cells that play important roles, especially not only in periodontal tissue regeneration but also in other dental and non-dental tissues [16].

2.4 Dental follicle stem cells (DFSCs): Stem cells isolated from the dental follicle tissue of third molars are defined as dental follicle stem cells (DFSCs). Within a group of dental mesenchymal stem cells, they display multipotential differentiation ability into periodontium, alveolar osteoblast, periodontal ligament, fibroblast, neuronal, adipogenic, chondrogenic, and cementoblast cells in specifically induced culture media. They express a series of classic cell surface

markers specific to mesenchymal stem cells, including CD44, CD73, CD90, and CD105 [17].

2.5 Alveolar bone stem cells (ABSCs): Alveolar bone stem cells (ABSCs) were first isolated from the alveolar bone of the jaw by Matsubara et al. (2005). With their multipotential ability, they exhibit chondroblast, osteoblasts, and adipocyte differentiation. Therefore, ABSCs are considered a potential candidate for the regeneration of cranial bones especially the alveolar bone [18].

2.6 Apical papilla stem cells (SCAPs): Apical papilla stem cells (SCAPs) were initially isolated from the apical papilla of immature permanent teeth of swine by Sonoyama et al. (2006). SCAPs express the cell surface markers including CD24, CD29, CD73, CD90, CD105, CD106, CD146, and CD166. They can differentiate into osteoblasts, adipocytes, and odontoblasts. Besides, SCAPs have higher proliferation and mineralization capacity than DPSCs [19].

2.7 Gingival mesenchymal stem cells (GMSCs): In 2009, Zhang et al. [20] defined the human gingival tissue as a source of mesenchymal stem cells (GMSCs). They are characterized by the markers of Stro-1, Oct-4, and SSEA-4. Like mesenchymal stem cells isolated from human bone marrow or umbilical cord, GMSCs have shown excellent self-renewal capacity, multipotent differentiation ability and exhibit successful differentiation into neural cell lineages [21].

Especially their differentiation properties and being easy to obtain without ethical concerns paved the way for dental stem cells to be used in regenerative and therapeutic applications for human disorders.

3. REGENERATIVE MEDICINE APPLICATIONS

Regenerative medicine, based on the principles of stem cell and tissue engineering, is an important and rapidly developing field of application that has attracted attention among scientific innovations in recent years. The main aim of regenerative medicine is to repair, replace, or renew the injured, diseased, or dysfunctional tissues or organs to restore their normal functionalities [22]. Remarkable progress in regenerative medicine applications has recently been made using stem cells derived from dental tissues due to their glorious properties such as easy accessibility and high proliferative ability. Recent clinical experiments and trials have shown their potential in regeneration and various therapeutic applications, particularly in inflammatory, metabolic, or neurodegenerative diseases. Besides, dental stem cells can serve as a valuable platform for oral and maxillofacial tissue homeostasis, regeneration, and repair.

3.1 Regenerative treatments of dental stem cells in dentistry: Generally, dental stem cells such as DPSCs, SHEDs, and PDLSCs are considered as trustworthy and easily accessible cell sources for the regeneration of oral tissues [23]. In dentistry, they are commonly used for the regeneration of periodontal

tissue, pulp-dental complex, entire-tooth, and salivary gland with or without scaffolds. PDLSCs are frequently applied in periodontal tissue (periodontal ligament, cementum, and alveolar bone) regeneration procedures, especially in the case of periodontitis which is characterized by the loss of the alveolar bone that supports teeth [23]. The first application of human PDLSCs resulted in the successful reconstitution of the cementum and periodontal ligament in an animal model and opened up new scientific research including different experimental models and scaffold-cell combinations [16]. Current studies have shown promising results that induce periodontal regeneration using allogeneic or autologous grafts seeded with PDLSCs [24]. In a clinical trial, 3 patients with periodontitis were treated with autologous PDLSCs on a membrane. After using autologous PDLSCs cell membrane, the cementum and periodontal ligament formation was improved [25]. For long-term treatment of autologous PDLSCs cell membranes also confirmed their safety and efficiency [26]. In addition to PDLSCs, GMSCs are commonly used for repairing jaw-bone defects and regenerating the periodontal ligament as well as dentin. Besides, SHEDs have potential roles in promoting the repair of periodontium including cementum, alveolar bone, dentin, and the periodontal ligament [27].

Dental pulp tissue is located in the center of the tooth and has important functions in maintaining vitality and the neural network of teeth. Therefore, neurogenic and angiogenic potential is essential for the dental pulp regeneration. DPSCs with their neurovascular differentiation characteristics are the best candidates for the regeneration of dental pulp tissue. Numerous preclinical and clinical studies have shown their critical roles in dentin-pulp tissue repair via stimulating the proliferation and migration of progenitor cells, inhibiting apoptosis and inflammation, and enhancing angiogenesis [11, 12]. These important properties make DPSCs a potential alternative to traditional endodontic treatments for some cases. One of the clinical studies carried out by Nakashima et al. (2017) declared that the transplantation of DPSCs to patients with irreversible pulpitis resulted in positive responses for the neurologic reactions following the 24-week application [28]. Likewise, Xuan et al. (2019) proved that autologous implantation of SHEDs highly regenerated and vascularized the dental pulp with tooth root development [29].

3.2 Application of dental stem cells for the regeneration of non-dental tissues: Apart from oral practices, dental stem cells are valuable candidates for use in other tissue engineering applications due to their multipotent differentiation capacity to non-dental tissues [30]. With their versatility, they can be applied in neuronal [31, 32], bone [33, 34], muscle [35], corneal [35], renal [36], and hepatic [37] regeneration.

Owing to the neurodifferentiation potential, DPSCs are considered attractive candidates for the therapy of various systemic diseases. Recent studies have shown their neurotrophic effects on neurodegenerative diseases including Alzheimer's and Parkinson's [23]. The potential effects of DPSCs for neural regeneration have been also shown under *in vitro* [38], and *in vivo* [39]

conditions for spinal cord injury. Besides, the angiogenic potential of DPSCs has been defined in muscular dystrophy. The results exhibited their integration in muscle fibers and the improvement in angiogenesis [40]. The implementation of DPSCs in a rat myocardial infarction model increased the number of vessels and decreased the size of the infarction [41]. Recent studies have shown that the main effects of DPSCs on cardiac repair effect after myocardial infarction can be through the growth factors and cytokines they secret [42]. Studies providing strong evidence about the differentiation capacity of the DPSCs into hepatocyte-like cells indicate that these cells may be potential candidates for the treatment of liver diseases [43]. Besides, DPSCs exhibited a suppressive effect on the colon cancer cells via mitogen-activated protein kinase pathways [44]. Recent studies also suggest that DPSCs and SHEDs with their differentiation ability into pancreatic cell lineages could be effective in the treatment of diabetes [45, 46, 47]. A clinical report demonstrated that intravenous application of SHEDs decreased the rate of unified Huntington's disease [48]. It has also been reported that SHEDs display chondrogenic differentiation ability which makes them potential candidates for cartilage regeneration [49]. Besides, Nishino et al. (2011) have shown that SHEDs promote re-epithelialization and extracellular matrix formation that enhances wound healing [50]. In another study, SHEDs were used for limbal stem cell deficiency. Following the transplantation into the rabbit eye, corneal regeneration was observed [51]. When the DPSCs were transferred to the damaged cornea of the human eye by the contact lens, they inhibited the conjunctival diseases [52].

Nowadays, immunomodulatory and anti-inflammatory characteristics of dental stem cells have gained attention for treating diseases related to the immune system or inflammation. Many preclinical and clinical studies in the literature confirm that SHEDs exert immunomodulatory effects by regulating the proliferation of immune cells, suppressing the inflammatory response, and adjusting immune-related mediators [53]. DPSCs have also been demonstrated for cell-based therapy of immune and inflammation-related diseases [54]. Similar to other dental stem cells GMSCs also display immune-modulating and anti-inflammatory properties. They modulate macrophage immunity, significantly reduce the degranulation of mast cells, and control macrophage polarization [55].

Consequently, dental stem cells hold great potential in the field of tissue engineering and regenerative medicine applications. However, further clinical studies are needed to fully understand their regenerative potential and their safety and efficacy.

4. DENTAL STEM CELL BANKING

Generally, human biobanks are defined as organizations that store biological materials with personal information, and perform operations when necessary [56]. Among them, stem cell banks have gained attention as a promising approach for research and clinical applications, worldwide [57]. Recently, dental

stem cells represent a new and potential source for regeneration remarkably. Their collection and storage for therapeutic usage is a new service called “Dental Stem Cell Banking”. Long-term preservation of dental stem cells for the treatment of different diseases such as cancer, autism, and neural degeneration has led to the establishment of dental stem cell banks. After the first establishment of a dental stem cell bank by Hiroshima University (Japan) in 2004 [58], many private Dental Stem Cell Banks have been opened in more than 20 countries, particularly in Norway, the United Kingdom, Germany, India, Singapore, Mexico, and the United States. In our country, dental stem cell storage started in 2016. However, there is still no licensed dental stem cell bank, yet. Therefore, dental stem cells can be stored in special cord blood banks. However, health insurance does not currently cover the long-term preservation of stem cells [3]. The processing fee including laboratory analysis can cost in the range of $500-$2000 and annual maintenance from $100-$250. Some private companies offer a 20-year plan with no annual maintenance costs of $2000 - $3000.

To translate dental stem cells into clinical applications, numerous standards and regulations must be followed. In the United States, the Food and Drug Administration (FDA) is responsible for the regulations and procedures. For Europe, the European Medicines Agency (EMA) is the main unit for the regulatory mechanisms [57]. Although each country has its additional regulatory system, bank facilities must fulfill certain requirements according to ISO 9000 which includes quality management and assurance standards [59]. Besides, guidelines on European Good Manufacturing Practices (GMP) and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) are considered other official international regulatory ways for quality [60, 61]. Additionally, the TRS 878 document provided by the World Health Organization (WHO) and the documents related to ICH, ICHQ7, GMP, ICHQ9, ICHQ10, Quality Risk Management and Pharmaceutical Quality Systems, are recommended as the other relevant guidelines for scientific and technical aspects [61].

Banking of dental stem cells is a critical step for the efficient advancement of clinical translation [57]. A licensed dental stem cell bank usually consists of four main departments including laboratory services, medical services, cryogenic services, and sale services with the purpose of collection, isolation, characterization, preservation, and marketing of the dental stem cells (Figures 2a and 2b).

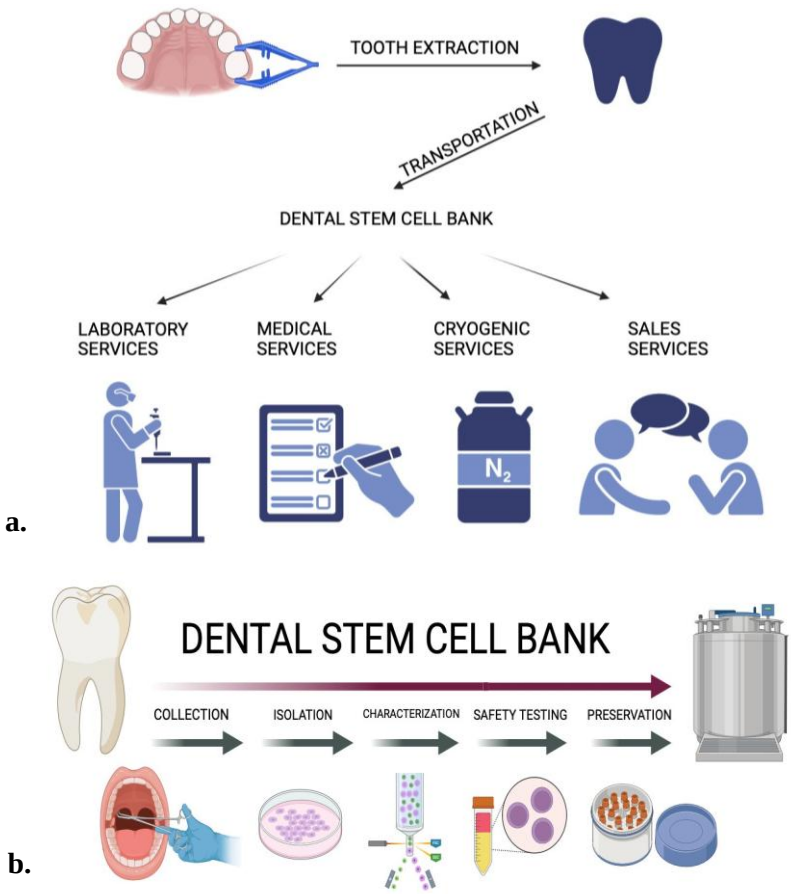

FIGURE 2. Schematic representation of main services (a) and general banking procedures (b) of dental stem cell banks. Figure created with BioRender.com (accessed on 24 January 2024).

4.1 Harvesting and transport: Since the tooth has to meet some requirements, the tooth selection process for dental stem cells is considered as a critical stage in dental stem cell banking. Extracted deciduous teeth with two-thirds of the root with a pulp are preferred over exfoliated teeth. For the adult teeth, only vital teeth without any infection and pathology and with a sufficient amount of pulp have to be harvested. The tooth that will be used as a source for dental stem cell isolation has to be first examined by a dental surgeon to rule out any infection in the targeted area. Extraction and the transfer protocol have to be carried out under aseptic conditions [10]. Transportation is carried out using special collection kits provided by the banking company.

4.2 Stem cell isolation, quality control, and characterization: One of the main critical issues for applying dental stem cells in clinical approaches is collecting a significantly high number of desired cells from different parts of the dental tissues. According to their location on tooth or oral tissue, dental stem cells can be harvested and then isolated using various techniques in the laboratory. Mechanical and enzymatic methods can be used for cell isolation. Following the isolation, the isolated stem cells have to be tested according to the quality and control assessments of the cell bank as well as within the framework of regulation rules of the country [10, 62-65]. According to international guidelines; the characteristics, viability, and purity of the isolated cells have to be checked. Besides, their genetic stability and identity have to be confirmed, and osteogenic, adipogenic, and chondrogenic differentiation capabilities have to be tested. The cells that do not meet these requirements have to be excluded from the cell banks [61].

4.3 Storage (Cryopreservation): Once cells are isolated and characterized, they have to be quickly suspended in a preservation medium containing cryoprotectants and dimethyl sulfoxide (DMSO). Following the transfer of cell suspensions into specialized cryo-vials, they have to be immediately frozen and stored in low-temperature storage containers including liquid nitrogen that maintains the cells below -195,8 °C / -320.5 °F. Considering the entire process, the preservation of the stem cells is still considered the most crucial step of the banks. Therefore, new technological improvements are being developed to optimize a more efficient storage protocol. It has been shown that using magnetic fields in the freezing process increases the viability of frozen cells and reduces DMSO usage in the cryopreservation medium [3]. However, appropriate preservation procedures for dental stem cells are still needed.

4.4 Post-thaw quality control: Post-thaw quality control is another critical step for dental stem cell banks. Optimal past-thaw cell recovery has to be analyzed according to the total cell count, cell viability which is expected to be more than 80%, membrane integrity, metabolic activity, etc. [66, 67].

In particular, the storage of dental stem cells which offer potential applications for the donor and other family members in the future, has gained increasing attention. However, this application still has significant disadvantages. For instance, every extracted tooth cannot be equally suitable for stem cell harvesting. Stem cells should preferably be obtained from extracted teeth with a bleeding pulp and uninfected teeth [68]. In addition, ethical regulations on the use of biospecimens and the slow engraftment rates are also considered other important disadvantages [69]. Recently, dental practitioners reported that cost might be the biggest barrier to hindering a prospective dental stem cell bank [70]. Importantly, the sector still lacking in terms of education and raising awareness about the effects of dental stem cells on different health issues. Patients, dentists, and the government must be aware of the presence, source, and therapeutic effects of dental stem cells. Therefore, educational health campaigns can also provide the public with a great amount of information about the therapeutic benefits of dental stem cell banking for their health in the future [71]. Internet

and social media which are the best sources of information can be utilized to increase public awareness about dental stem cell banking. Furthermore, the shortage of medically trained personnel is considered an important problem, worldwide [69]. There are few adequately trained personnel for sample collection, storage, and processing of dental stem cells to provide banking services and stem cell transplantation. The lack of trained medical laboratory scientists, doctors, nurses, medical laboratory scientists, and other related personnel is one of the major disadvantages in this field. Low-trained personnel to the world population density pose a major challenge to implementing stem cell banking. Therefore, medical personnel qualified to collect stem cells and provide banking services should be trained appropriately. Education of stem cell banking personnel has to be applied through media, workshops, symposiums, etc. Adequate funding should be allocated to this sector and medical research in particular by the federal government, non-governmental organizations, and international agencies [72]. To overcome these disadvantages, finding resources, training the employees, and providing easy access for the citizens to such services have to be the main focus of this sector. Overall, additional efforts are still needed for dental stem cell banking to become a more accessible application for patients in the future.

5. CONCLUSION

Over the last few decades, several preclinical and clinical studies have been performed, and their results make stem cells an attractive candidate in the field of tissue engineering and regenerative medicine applications. As reviewed in this article, dental stem cells have recently received the greatest attention due to their superiorities such as easy accessibility, self-renewal ability, and higher capacity to differentiate into several cell types. Therefore, dental stem cell banking is rapidly finding its place in health strategies similar to umbilical cord blood banking. However, it is necessary to conduct further studies into their cryopreservation. In addition, studies to raise awareness of individuals and dentists about dental stem cell banking need to increase rapidly.

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Author Contribution Statements HBE and FK: designed the study, wrote and reviewed the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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A MINI-REVIEW ON THE MICROPLASTIC-HEAVY METAL INTERACTIONS AND THE FACTORS AFFECTING THEIR FATE IN AQUATIC HABITATS

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ABSTRACT Microplastic particles found in water bodies are recognized a serious environmental concern due to their effects on aquatic biota. Microplastics, with their large surface area, are considered as vectors since they provide suitable surfaces for the adherence of several toxic pollutants, including heavy metals, pesticides, and nanoparticles. Several physico-chemical properties of plastic particles including chemical structure, polymer chain organization, specific surface area, and particle dimensions, and environmental parameters (ambient temperature, pH and salinity of the media and the dissolved organic matter concentration) may reshape the dynamic interactions between heavy metal ions and microplastic surfaces. Microplastic-heavy metal interaction poses a global health threat to aquatic biota and eventually human beings through the food chain since attached metal ions may be transported to aquatic organisms. Therefore, it is critical to clarify the mechanisms responsible for the adherence of metal ions to plastic surfaces. Such an approach will help government departments to promote management strategies and design of treatment practices. In this study, recent reports on the adherence of heavy metal ions to microplastic particles in aquatic habitats, along with the factors that might change the adsorption capacity of microplastics, are reviewed and discussed in detail.

Keywords Pollution, heavy metal, microplastics, adsorption

1. INTRODUCTION

The family Brassicaceae is represented by 321 genera in the world [1] and 96 genera in Türkiye [2]. The genus *Barbarea* is represented by 29 species in the world [3] and 19 taxa belonging to 14 species in Türkiye, 11 of which are endemic [4, 5]. Members of this genus are distributed in the warm regions of Eurasia, Australia, and North America, and in some countries of South America and the eastern parts of Africa. Plastics are strong and light, resistant to water and shock, poor conductors of electricity and heat, and are highly flexible and durable [1]. Furthermore, they can be produced in great masses with low costs [2]. Thus, they are widely favored in packaging, textile, construction, transportation, electronics and automotive industries and in the production of several household goods [3, 4]. Manufacture of several household and industrial plastic goods has increased steadily on a global scale since 1950's and increased

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approximately to 391 million tons in 2021 on a global basis. Furthermore, the estimates reveal that it will reach 33 billion tons by 2050 [5, 6]. The abundance of plastic wastes discharged into environment increased over the last years because of increasing the global demand to plastic materials [7, 8]. Plastic materials constitute 54% of the wastes in nature by mass [4, 9]. Although, there is a great effort to enhance reuse rate of plastic wastes, estimations reveal that only less than 10% of plastic products could be recycled globally [4]. Aquatic habitats function as an environmental sink for several hazardous chemicals such as heavy metals [10], pesticides [11], nanoparticles [12] and plastics [13] released into the environment. Approximately up to 13.10^6 tons of plastic debris are drifted annually from land-based habitats to oceans [14]. Plastic wastes are non-biodegradable. For example, it takes 500 years for a bottle made of high-density polyethylene (HDPE) to degrade completely in soil [15], or quite longer for some other plastic goods such as monofilament fishing line in the marine habitats [16]. Therefore, plastic waste accumulation increases gradually in aquatic habitats [17].

The particle size of plastic waste material released into terrestrial and aquatic ecosystems range from micrometers to meters. Microplastics particles range between 1 μm to 5 mm in diameters [13, 18] which are further divided into 2 categories; primary and secondary particles. The former is directly produced for industrial use such as medical, textile and cosmetics [19, 20]. Secondary microplastic particles originate from the bigger plastic materials which break into fine particles through physical, chemical and biological processes [21, 22]. Microplastics can be found in various shapes including pellets, particle fiber, films, beads, and foam based on the appearance of particles [23, 24]. Their color shows great variability including yellow, cream, white, transparent, orange, blue, purple, green, and opaque [25]. Although, polyethylene is the prevalent man-made polymer type, several other polymer types are also available; polyvinyl chloride (PVC), polyamide (PA), polyethylene terephthalate (PET), polypropylene (PP) and polystyrene (PS) [26].

The prevalence of plastic particles in the seas has increased remarkably soon after their first record in 1970s [27]. For instance, the Northwest Pacific Ocean's surface water contains up to 42,000 units/km^2 of microplastics [28]. Estimations affirm that 80% of land-based plastic wastes are disposed into oceans either directly from wastewater treatment facilities or indirectly through rivers and surface runoffs [29,30]. Microplastics can move great distances within the atmosphere by wind, storm, hail, and precipitation [31, 32]. Microplastic particles are observed even in highly isolated regions on earth including the Arctic Ocean, isolated ocean islands, and even deep ocean waters [33]. In a recent survey, the amount of the microplastic particles in the Arctic Sea (depth ~16 cm) were found as 0-1.31 particles/m^3 [34].

Microplastic particles found in atmosphere, terrestrial and aquatic habitats create a global health concern for several organisms and eventually human beings through consumption of contaminated food items and can be accumulated in the body of several aquatic animals [35-39]. Biochemical, physiological and

behavioral alterations [13, 40] including genotoxicity [41], oxidative stress [21], abnormalities in glycolipid and energy metabolism, disturbance of inflammatory response [42] and death [43] are reported in several aquatic animals after exposure to microplastic particles.

Microplastics may contain various amounts of toxic compounds and endocrine disruptors such as bisphenol A, triclosan, bromine based flame retardants, dyes, pigments, and biphenyl in their chemical structure [44, 45]. Due to their interactions with other toxic compounds, particularly heavy metals (HMs) in aquatic environments, they are referred as vectors [46] or more commonly a Trojan horse [33]. Adsorption of contaminants present in aquatic environments to microplastic surfaces can increase retention time of that contaminants in the pelagic zone [47, 48] and causes the organism to be exposed to a greater concentration when ingested [39, 49, 50].

Heavy metals, generally defined as metal and metalloid groups with densities exceeding 5 g/cm³, pose a significant threat to organisms due to their potential toxicity and ecotoxicological effects [10, 51]. Heavy metals typically exist as sulfates, hydroxides, oxides, phosphates, and silicates in the Earth's crust [52, 53]. Despite their detrimental nature, these elements are ubiquitous in the environment, released through both natural processes (forest fires, rock weathering, volcanic activity, erosion, etc.) and anthropogenic activities (agriculture, mining, industry, etc.) [54]. These elements have several uses across numerous sectors due to their unique properties, including conductivity, catalytic activity, high density, corrosion resistance, magnetism, biocidal activity, and their ability to form versatile alloys [55]. Some of the heavy metals are considered as essential such as iron, copper, cobalt, zinc, chromium, molybdenum, selenium, and manganese. Although essential heavy metals are critical micronutrients for physiological and biological functions and required at moderate or trace amounts, excess levels can trigger adverse effects. For example, copper is essential for a variety of biological functions and is used as a cofactor in many enzymes [56]. But, high levels of copper can lead to Alzheimer's disease, gastrointestinal disorders, and organ damage [57, 58]. On the other hand, some of the heavy metals such as aluminum, antimony, arsenic, barium, beryllium, cadmium, mercury, and lead pose a significant threat to organisms even at quite low concentrations and considered non-essential heavy metals [59]. Their toxic effects are documented in numerous studies, affecting vital organelles like the cell membrane, mitochondria, and nucleus, disrupting DNA, cell cycle, and inducing carcinogenesis and apoptosis [60]. Furthermore, their concentrations are increasing exponentially within the food web due to their persistence and bioaccumulative nature [53]. Thus, there is a great concern on their toxic effects on organisms from an ecological standpoint. Each heavy metals has its own unique physical and chemical characteristics [61]. In addition, their interactions with other elements, toxicity to organisms, deposition, presence in water column, uptake and accumulation in cells are dependent upon several abiotic and biotic parameters. Thus, as mentioned above the presence of

microplastic particles (MPs) creating a suitable surface for adherence of heavy metals may increase their uptake (either voluntarily or involuntarily ingestion of MPs) by living organisms and the residence of heavy metal ions in the water column [46].

For example, Ashton et al. [62] reported that new polyethylene pellets strongly adsorbed the manganese (Mn), lead (Pb), aluminum (Al), cobalt (Co), silver (Ag), iron (Fe), tin (Sn), copper (Cu), zinc (Zn) and molybdenum (Mo) ions when exposed to seawater for eight weeks, in Sutton Harbor. Similarly, adsorption of mercury (Hg) to microplastic particles in marine environment increased when exposed to metal ions for longer periods [63]. Microplastic particles initially have a non-porous structure but undergo significant surface transformations due to weathering, abrasion, and photo-oxidation in aquatic environments. These processes not only enhance their adsorption potential by increasing specific surface area, but also generate negatively charged sites in natural aqueous environments [64]. Consequently, these negatively charged microplastic particles act as potent adsorbents for positively charged heavy metal (HM) ions through electrostatic interactions which is the primary driver of adsorption. In addition, Van der Waals and π–π interactions which are specific to certain polymer type play a complementary role [65]. Furthermore, biofilm and dissolved organic matter mediate the complexation and accumulation of heavy metals on microplastic particles through altering the surface properties. Thus, adsorption of HMs to MPs are enhanced through presence of organic molecule interactions, increased charge, roughness, porosity, and hydrophilicity [64,66]. However, there is a debate on the adsorption dynamics of heavy metal ions to microplastic surfaces. Several factors including chemical composition, polymer chain organization, specific surface area and dimensions of the particles and environmental factors (temperature, salinity, dissolved organic matter, pH, etc.) and treatment methods applied (ball milling, UV radiation, Fenton and hydrogen peroxide aging, etc.) have been found to affect adsorptive capacity of microplastic particles for heavy metal ions [67-71]. The ecological risks associated with heavy metal contamination, are increased by the interaction between microplastic particles and HM ions. Therefore, it is vital to reveal the complex adsorption mechanisms from an environmental standpoint, in order to plan management strategies and design treatment practices. In this study, recent reports on the adherence of HM ions to microplastic particles in aquatic habitats, along with the parameters that might alter the adsorption dynamics, are reviewed and discussed in detail.

Microplastic-Heavy Metal Interactions in Aquatic Habitats

During recent years, the threat raised by the interaction between microplastic particles and HMs in aquatic habitats gained attention from scientists worldwide [72]. The concentration of HM ions adsorbed to microplastic surfaces can be quite higher compared to the HM ion concentration in the aquatic media (Table 1). For example, HM load in the Beijang River sediments were considerably less than the HMs adsorbed by microplastic particles [73]. Likewise, Liu et al. [74]

compared HM ion concentration and microplastics in water samples collected in Hong Kong from 5 meters below the water's surface. They found that Pb^{2+} ion concentration absorbed by microplastics ranged from 0.72-1.742 μg/g, whereas the total Pb concentration in sea water was ranging between 0.09 and 0.51 μg/L. We summarized the data available on the sorption of HM ions to microplastic particles in aquatic environments in Table 1.

Factors Effecting the Microplastic Adsorption Capacities

To accomplish a better insight on the adsorption kinetics of HM ions to plastic particles, simulations were carried out under fully controlled laboratory studies. Table 2 summarizes the adsorption capacity and the factors affecting the adsorptive capacity of common plastic polymers observed in aquatic environments for several heavy metals. In the studies summarized, selected and known amounts of microplastics and HM ions were added to the experimental media and the adsorbed heavy metal concentration was measured after certain periods of time. In general, the physico-chemical properties of microplastics (chemical structure, specific surface area, particle dimensions and polymer chain organization), laboratory simulation methods and environmental factors (dissolved organic matter concentration, ambient temperature, salinity and pH of the media) are shown to have a great effect the adsorption dynamics [68, 87, 88]. Several researchers tried to imitate the environmental weathering of plastic particles by aging them through ball milling, oxidation and UV radiation. Their results confirm that heavy metal ion adsorption rates increase as the particles age [89-91]. Researchers attributed this situation to the alteration of the surface features of particles after mechanical abrasion or oxidation [92]. For instance, the concentration of arsenic ions adsorbed to polystyrene microplastic particles (PSMPs) increased after ball milling which increased the SSA of particles [91]; they also found that arsenic ion concentration adsorbed to particles decreased with increasing PSMP size. Similarly, the Cd^{2+} adsorption ability of microplastic particles exposed to Fenton and H_2O_2 solutions, were found to increase with increasing aging time [69]; they also found that Fenton was a better aging agent compared to H_2O_2. PET particles aged by exposing to UV radiation was also found to have a greater adsorption efficiency with increasing exposure time; therefore, indicating that microplastics exposed to sunlight can adsorb larger amounts of heavy metals [68].

TABLE 1. The comparison of heavy metal concentrations in water and sediment samples and the heavy metal concentration adsorbed to microplastics in aquatic habitats.

Location	Sampled compartment	Sampling Method	Analysis Method	Concentration of microplastic particles	Polymer type	Heavy Metal concentration in water	Heavy metal concentration adsorbed to microplastics	References
Plymouth and Kingsbride Estuary, England	Beach	Plastic tweezers	Ultrasonication (5 min) Washed in aqua reqia SEM-EDS FT-IR ICP-OES ICP-MS	100 particles/m^2	PE plastic pellet	Al: 6.20±3.65 µg/g Fe: 17.98±7.49 µg/g Mn: 2.61±1.97 µg/g Cu: 0.28±0.18 µg/g Pb: 1.72±0.92 µg/g Zn: 0.25±0.18 µg/g Ag: 24.7±9.4 µg/g (heavy metal concentration on new polyethylene pellets suspended in harbor for 8 weeks)	Al: 7.05±0.66 µg/g Fe: 25.85±2.09 µg/g Mn: 1.58±0.57 µg/g Cu: 0.06±0.03 µg/g Pb: 0.15±0.04 µg/g Zn: 0.55±0.27 µg/g Ag: 2.4±0.4 µg/g (heavy metal concentration in pellets collected from the beach)	[62]
Musi River, Indonesia	Surface water	Neuston net (300 µm)	Suspended in NaCl (1.2 g/cm^3) Washed in 95-97% H_2SO_4 and HNO_3 Stereomicroscope ATR-FT-IR AAS	-	PES, PP, PE, PVC, Nylon	Pb: 0.0245-0.0711 mg/L Cu: 0.0099- 0.0173 mg/L	Pb: 0.152-2218 µg/g Cu: 0.012-0.365 µg/g	[72]

Chao Phraya River, Thailand	Surface water	Manta trawl	Organic material was removed with H_2O_2 (%30) and NaI (1.5 g/cm^3) Washed in aqua regia Optic Microscope, Fluorescence Microscope FT-IR µ-FT-IR ICP-OES	80±65 items/m^3	PP, PE, PS	Pb: 0.01±0.00 µg/L Cr: 1.1±0.00 µg/L Cu: 0.05±0.03 µg/L Ni: 0.05±0.06 µg/L	Pb: 17.61±18.26 µg/g Cr: 2.95±2.96 µg/g Cu: 13.02±18.26 µg/g Ni: 0.78±1.11 µg/g	[75]
Freshwater Wetlands, India	Surface water	Steel bucket, Mesh sieve (63 µm)	Density separation method with $ZnCl_2$ (1.80 g/cm^3) Organic matter was removed with H_2O_2 (%30) and %1 HNO_3 Optic Microscope Fluorescence Microscope ATR-FT-IR ICP-MS	7.87-20.39 items/L	PE, PET	As: 0.02-0.24 µg/L Cd: 0.15-1.23 µg/L Cr: 0.85-23.65 µg/L Cu: 0.26-23.14 µg/L Ni: 1.24-42.36 µg/L Pb: 1.24-12.15 µg/L Zn: 15.36-286.35 µg/L	As: 1.56-4.51 µg/g Cd: 0.65-5.78 µg/g Cr: 26.26-342.28 µg/g Cu: 0.29-119.59 µg/g Ni: 12.43-75.77 µg/g Pb: 0.04-104.63 µg/g Zn: 1.88-1191.52 µg/g	[76]
Punnakayal Estuary, India	Surface water	Teflon pump, Stainless steel sieve	Organic matter was removed H_2O_2 (%30) Ultrasonication with %2 HNO_3 Stereomicroscope µ-ATR-FT-IR AFM ICP-OES	7.8 particles/L	PP, PA, PE, PVC, P	As: 20.56 µg/g Pb: 6.26 µg/g Cd: 6.26 µg/g Mn: 9.37 µg/g Cu: 1.02 µg/g Cr: 0.33 µg/g Zn: 16.24 µg/g	As: 0.12-0.96 µg/g Pb: 4.13-4.56 µg/g Cd: 0.24-0.32 µg/g Mn: 8.59-12.35 µg/g Cu: 0.24-6.33 µg/g Cr: 0-0.24 µg/g Zn: 1.24-5.69 µg/g	[77]

Hong Kong, China	Samples of seawater were collected 5 meters below the sea surface.	Manta trawl (330 μm), Stainless steel sieve	Density separation with NaCl Ultrasonication with %2 HNO_3 and H_2O_2 (%30) Stereomicroscope SEM ATR-FT-IR ICP-MS	-	PE, PP, PS	Cd: - As: 3.02-3.83 μg/L Zn: 3.79 μg/L Pb: 0.09-0.51 μg/L Mn: 1.03-11.9 μg/L Ni: 0.94-1.39 μg/L Cu: 2.38-4.65 μg/L	Cd: 676 μg/g As: 0.027-28.1 μg/g Zn: 9.58-1.712 μg/g Pb: 0.72-1.742 μg/g Mn: 0.03-248 μg/g Ni: 0.61-34.8 μg/g Cu: 1.22-124 μg/g	[78]
Trombol Beach, Malaysia	Plastic debris	Sampling quadrat	Rinsed under running warm water Washed in 95-97% H_2SO_4, and %65 HNO_3 ICP-OES	30 items	PE, PP, PS, PET	-	Cd: 0.3584±1.6 ppm Pb: 1.2696±4.6 ppm Ni:0.0408±0.3 ppm Cu: 3.3165±8.3 ppm Zn: 4.5515±9.8 ppm As: 0.0193±0.2 ppm Hg: 0.0004±0.0 ppm	[78]
Lake Garda, Italy	Sediment	20 m transect with 10 sediment cores	Density separation with $ZnCl_2$ (1.6-1.7 kg/L) Organic matter was removed with H_2O_2 (%30), HNO_3 (%65) and H_2SO_4 (%95) Raman microspectroscopy ICP-MS	561 particles/m^2	PE, PA, PET, PS, PVC	-	Cd: 23.64 μg/g Cr: 154.23 μg/g Cu: 19.56 μg/g Ni: 1.12 μg/g Pb: 219.7 μg/g Ti: 1046.01 μg/g	[79]

Beijang River, China	Sediment	Stainless Steel Shovel	Density separation with NaCl Ultrasonication (5 min) with H_2O_2 (%30), HNO_3 (%65-68), and H_2SO_4 (%95-98) SEM-EDS μ-FT-IR ICP-MS	78±69 - 544±107 items/kg	PP, PE	Cd: 0.07±0.01 μg/g Pb: 2.458±0.019 μg/g Cu: 57.93±4.32 μg/g Ni: - Zn: 79.5±2.5 μg/g	Cd: 2.16-17.56 μg/g Pb: 38.24-13.11 μg/g Cu: 80.9-500.6 μg/g Ni: 0.54-2.39 μg/g Zn: 2414-14815 μg/g	[73]
Adriatic Sea, Croatia	Sediment	Stainless Steel Spoon	MQ water Organic matter was removed with HCl (%37) and HNO_3 (%65) AAS	6-36 particles/dm^3	-	-	Cu: 0.08-0.61 μg/g Mn: 0.19-8.25 μg/g Ni: 0.04-0.27 μg/g Pb: 0.04-0.85 μg/g Fe: 18.2-88.5 μg/g Cr: 0.03-0.14 μg/g	[80]
Persian Gulf, Iran	Sediment	-	Density separation with 360 g/L NaCl Washed in aqua regia Epifluorescence microscope ICP-OES	82.612 items/m^2	-	Cd: 0.81 ± 0.18 μg/g Cr: 5.01 ± 0.73 μg/g Fe: 3045 ± 11.31 μg/g Al: 186 ± 2.82 μg/g Mn: 126.5 ± 3.53 μg/g Ni: 14.5 ± 0.7 μg/g Pb: 48.55 ± 10.81 μg/g Cu: 5.43 ± 0.73 μg/g	Cd: 0.035±0.007 μg/g Cr: 0.915±0.03 μg/g Fe: 531±135.7 μg/g Al: 114.56 ± 5.47 μg/g Mn: 32.2±12.4 μg/g Ni: 2.03±0.16 μg/g Pb: 4.59±0.53 μg/g Cu: 3.6±0.28 μg/g	[81]
Coast of São Paulo, Brazil	Sediment	Samples were collected by hand	Organic matter was removed with H_2O_2 (%30), pure HNO_3 and HCl ICP-OES	300 items pellet	PP, PE	-	Al: 45±9 μg/g Zn: 8±9 μg/g Cu: 1 ±1 μg/g Fe: 228±142 μg/g Mn: 9±6 μg/g	[82]

Coast of Hong Kong, China	Sediment	Stainless Steel Shovel and Sieve	Washed in aqua regia ATR-FT-IR ICP-OES	-	PE, PP, PS	Fe: 799±507 µg/g Mn: 25.3±14.6 µg/g Ni: 0.18±0.14 µg/g Cu: 3.47±2.83 µg/g Zn: 24.2±9.29 µg/g	Fe: 302±224 µg/g Mn: 18.6±12.7 µg/g Ni: 0.15±0.13 µg/g Cu: 0.89±0.89 µg/g Zn: 19.6±11.4 µg/g	[83]
Jinjiang Estuary, China	Sediment	Stainless Shovel	Density separation with NaCl 1.2 g/L Organic matter was removed with H_2O_2 (%30), HNO_3 (%65-68), and H_2SO_4 (%95-98) Raman microspectroscopy SEM-EDS ICP-MS	963±175.4 items/500 g	PE, PP, PET	Cr: 20.52-42.17 µg/g Ni: 14.63-27.10 µg/g Cu: 11.07-41.55 µg/g Zn: 72.41-199.26 µg/g Pb: 41.93-107.52 µg/g As: 5.84-8.68 µg/g Cd: 0.14-0.89 µg/g Hg: 0.65-1.13 µg/g	Cr: 4.79-15.70 µg/g Ni: 2.11-6.00 µg/g Cu: 2.42-23.25 µg/g Zn: 7.16-185.39 µg/g Pb: 13.42-51.58 µg/g As: 0.64-6.53 µg/g Cd: 0.02-0.78 µg/g Hg: 0.00-0.076 µg/g	[84]
Pearl River Estuary, China	Surface Sediment	Stainless Shovel and Sieve	Washed in aqua regia and H_2O_2 (%30) SEM µ-ATR-FT-IR ICP-MS	328-82276 particles/m^2	EPS	-	Cd: 0.27±0.19 µg/g Cr: 14.9±8.25 µg/g Cu:15.0±7.66 µg/g Ni: 17.2±17.6 µg/g Pb: 24.8±7.39 µg/g Mn: 730±797 µg/g Fe: 8340±4760 µg/g	[85]

Freshwater Wetlands, India	Sediment	Grab, Mesh sieve	Density separation method with $ZnCl_2$ (1.80 g/cm^3) Organic matter was removed with H_2O_2 (%30), HNO_3, $HClO_4$ and H_2SO_4 Optic Microscope Fluorescence Microscope ATR-FT-IR ICP-MS	2124.84-6886.76 items/kg	PE, PET	As: 17.42-24.74 µg/g Cd: 0.99-6.87 µg/g Cr: 115.78-273.85 µg/g Cu: 75.10-268.53 µg/g Ni: 72.32-87.62 µg/g Pb: 6.09-11.13 µg/g Zn: 204.26-383.22 µg/g	As: 1.56-4.51 µg/g Cd: 0.65-5.78 µg/g Cr: 26.26-342.28 µg/g Cu: 0.29-119.59 µg/g Ni: 12.43-75.77 µg/g Pb: 0.04-104.63 µg/g Zn: 1.88-1191.52 µg/g	[76]
South China Sea, China	Sediment	Stainless steel shovel	Spectrometer ATR-FT-IR LIBS	450 samples	PE, PP, PET, PS	-	Cr: 60.33-75.73 Cu: 10.25-15.32 Fe: 36.66-60.46 Zn: 55.85-80.91 Pb: 30.43-40.56 Cd: 18.33-40.73 Mn: 15.33- 24.78	[86]

(SEM; Scanning Electron Microscopy, EDS; Energy Dispersive X-ray Spectroscopy, FT-IR; Fourier Transform Infrared Spectroscopy, µ-FT-IR; Micro Fourier Transform Infrared Spectroscopy, ATR-FT-IR; Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy, ICP-OES; Inductively Coupled Plasma - Optical Emission Spectrometry, ICP-MS; Inductively Coupled Plasma Mass Spectrometry, AAS; Atomic Absorption Spectroscopy, AFM; Atomic Force Microscopy, LIBS; Laser-Induced Breakdown Spectroscopy, PE; Polyethylene, PVC; Polyvinyl Chloride, PES; Polyether Sulphone, PP; Polypropylene, PS; Polystyrene, PET; Polyethylene Terephthalate, PA; Polyamide, P; Phenolic, EPS; Expandable Polystyrene, As; Arsenic, Al; Aluminum, Cd; Cadmium, Co; Cobalt, Cr; Chromium, Cu; Copper, Fe; Iron, Hg; Mercury, Mn; Manganese, Ni; Nickel, Pb; Lead, Ti; Titanium, Zn; Zinc, HCl; Hydrochloric Acid, $HClO_4$; Perchloric Acid, HNO_3; Nitric Acid, H_2O_2; Hydrogen Peroxide, H_2SO_4; Sulphuric Acid, NaCl; Sodium Chloride, NaI; Sodium Iodide, $ZnCl_2$; Zinc Chloride).

TABLE 2. Laboratory simulation tests indicating the adsorption capacities of microplastic particles for some heavy metals.

Experimental media	Treatment methods applied to MPs	Analysis method	Specific surface area	Microplastic concentration	Initial heavy metal concentration	Solution pH	Microplastic polymer type	Adsorption Capacity	References
Milli-Q water	Ball milled (4 h)	FT-IR SEM XPS	0.95 m^2/g	0.02 g	10-50 mg/L	-	PTFE	As (III): 1.03 mg/g	[93]
			0.40 m^2/g					As (III): 0.94 mg/g	
			0.32 m^2/g					As (III): 0.83 mg/g	
Milli-Q water	-	FT-IR SEM EDS	1.4 m^2/g	0.1g	0.5-32 ppm	6.3	PE	Cr: 4.70 mg/g Cu: 0.259 mg/g Pb: 2.36 mg/g Zn: 0.505 mg/g	[94]
							PS	Co: 0.813 mg/g Cr: 0.473 mg/g Cu: 0.358 mg/g Pb: 2.94 mg/g	
							PP	Cr: 0.624 mg/g Cu: 2.95 mg/g Pb: 5.55 mg/g	
							PVC	Zn: 0.634 mg/g Cr: 2.44 mg/g Pb: 1.90 mg/g	
							PET	Pb: 4.93 mg/g	
Seawater					4-8 ppm	7.72	PE	Cr: 2.56 mg/g Pb: 3.28 mg/g	
			3.2 m^2/g				PS	Co: 0.4 mg/g Pb: 3.29 mg/g	
							PP	Cu: 2.87 mg/g Pb: 3.15 mg/g	
			0.59 m^2/g				PVC	Cr: 6.14 mg/g Pb: 3.45 mg/g	
			0.35 m^2/g				PET	Pb: 3.73 mg/g	

Distilled water	-	FT-IR FE-SEM	0.231 m^2/g	40 mg	0-10 mg/L	-	PE	Sr: 470±106 μg/g	[95]
			0.842 m^2/g				PVC	Sr: 790±238 μg/g	
			0.162 m^2/g				PET	Sr: 360±79.2 μg/g	
Distilled water	Washed with HCl and water	FT-IR SEM FAAS EDS	-	1 g	5 mg/L	7.6	High degree PE	Cd: 30.5 μg/g	[96]
High-purity Milli-Q water	-	ATR-FT-IR SEM FAAS	2.11 m^2/g	0.5g	0.05-10 mg/L	-	PE	Cu: 8.46±13.4 μg/g	[89]
			<0.001				PS	Cu: 8.28±2.55 μg/g 51.4 μg/g	
Milli-Q water			0.556 m^2/g				PVC	Cu: 6.29±0.948 μg/g	
			0.475 m^2/g				PET	Cu: 8.71±7.74 μg/g; 360±79.2 μg/g	
	Exposed at UV radiation		8.710 m^2/g				PA	Cu: 324±38.2 μg/g and 265±25.7 μg/g	
			0.110 m^2/g				PMMA	Cu: 41.0±1.78 μg/g and 79.4±10.5 μg/g	
Milli-Q water	Ball milled (2 h)	FT-IR SEM XPS	6.77 m^2/g	0.02 g	10-50 mg/L	-	PS	As (III): 0.920 mg/g	[91]
	Ball milled (4 h)		9.25 m^2/g					As (III): 1.047 mg/g	
	Ball milled (8 h)		9.80 m^2/g					As (III): 1.120 mg/g	
-	Natural aged	FT-IR SEM XRD XPS	8.4 m^2/g	0.2-0.6 g/L	2-15 mg/L	5	PE	13.60 mg/g	[97]
-	Washed with HCl and rinsed with water	FT-IR SEM XRD	0.173 m^3/g	-	-	-	PE	Cd: 36.63 μg/g	[98]
			0.508 m^3/g				PS	Cd: 40.49 μg/g	
			0.348 m^3/g				PP	Cd: 36.10 μg/g	
			0.836 m^3/g				PVC	Cd: 53.48 μg/g	

Distilled water	-	SEM	0.584 m^2/g	45 mg	0-3.4 mg/L	4.5	PS	Sr: 51.4 µg/g	[99]
			0.314 m^2/g				PP	Sr: 52.4 µg/g	
			0.48 m^2/g				PA	Sr: 31.8 µg/g	
Milli-Q water	Washed with water and oxidized using O_2, then stirred at reagent water and air dried	ATR-FT-IR ICP-OES SEM-EDS XPS	0.1036 m^2/g	25 g	1000 mg/L	7.5	LDPE	Cu: 1109 µg/m^2 Mn: 199 µg/m^2 Pb: 1038 µg/m^2 Zn: 893 µg/m^2	[100]
-	-	FT-IR XPS	-	0.01 g	0.1-2 ppm	4	PS	Cd: 20.154 µg/g	[69]
Ultrapure water	Fenton aging							Cd: 168.63 µg/g (1 day) Cd: 188.92 µg/g (3 day) Cd: 214.68 µg/g (5 day) Cd: 238.72 µg/g (7 day)	
	H_2O_2 aging							Cd: 44.02 µg/g (1 day) Cd: 54.74 µg/g (3 day) Cd: 67.29 µg/g (5 day) Cd: 76.90 µg/g (7. day)	
Ultrapure water	Rinsed and freeze dried	FT-IR SEM FAAS XRD XPS	-	0.005 - 0.04g	0.1-2 mg/L	-	PS	Pb: 160 µg/g Cu: 210 µg/g Cd: 106 µg/g Ni: 125 µg/g Zn: 78.1 µg/g	[101]

	UV aging in air for 3 months							Pb: 202 μg/g Cu: 173 μg/g Cd: 175 μg/g Ni: 196 μg/g Zn: 183 μg/g	
	UV aging in pure water for 3 months							Pb: 199 μg/g Cu: 176 μg/g Cd: 168 μg/g Ni: 259 μg/g Zn: 312 μg/g	
	UV aging in simulated sea water 3 months							Pb: 199 μg/g Cu: 170 μg/g Cd: 161 μg/g Ni: 211 μg/g Zn: 236 μg/g	
-	Dipped in HNO_3, then aged by UV	FT-IR SEM	-	0.5-2.5 g	2-10 mg/L	5	PET	Cu: 375.53 μg/g Zn: 211.03 μg/g	[68]
-	-	XRD FT-IR ICP-AES GC-MS SEM-EDS	0.235 m^2/g	-	0-5 mg/L	5.8	PE	Cu: 27.57μg/g	[102])
Milli-Q water	Washed and air dried	FT-IR FEI-SEM XPS EDS	4.13±0.4 m^2/g	0.1 g	20-140 mg/L	6	PS	Cd: 0.76±0.02 mg/g	[103]
			5.29±0.38 m^2/g				PVC	Cd: 1.04±0.03 mg/g	
			9.51±0.51 m^2/g				PA	Cd: 1.70±0.04 mg/g	
			1.95±0.29 m^2/g				PET	Cd: 0.25±0.01 mg/g	
Distilled water	-	FT-IR SEM XPS GC-MS	1.3 m^2/g	10-80 mg	0.1-50 mg/L	6.5	Low degree crystallinity PE	Cu: 56±2 μg/g Cd: 345±29 μg/g Pb: 590±21 μg/g	[104]
			3.1 m^2/g				High degree crystallinity PE	Cu: 385±39 μg/g Cd: 242±18 μg/g Pb: 2316±283 μg/g	

			2.3 m^2/g				Chlorinated PE	Cu: 3868±98 µg/g Cd: 7485±1544 µg/g Pb: 45306±1109 µg/g	
			8.9 m^2/g				PVC	Cu: 431±11 µg/g Cd: 1748±505 µg/g Pb: 2518±125 µg/g	
Artificial seawater, distilled water	-	FAAS	-	0.05 g	1-10 mg/L	-	Virgin PS	Zn: 0.043-0.204 µg/g Pb: 0.07-0.23 µg/g Cd: 0.031-0.121 µg/g Cu: 0.049-0.227 µg/g	[105]
Milli-Q water	-	µ-FT-IR	-	0.10 g	0-100 mg/L	-	PE	Pb: 2.01 mg/g	[106]
		XPS	-				PP	Pb: 1.57 mg/g	
		SEM-EDS	794.5 m^2/g				PMMA	Pb: 4.21 mg/g	
Simulated seawater	UV aging for 2 months	µ-FT-IR SEM	-	20 mg	1 mg/L	-	PET	Cu: 0.36 mg/g	[107]
							PA	Cu: 0.30 mg/g	

(SEM; Scanning Electron Microscopy, EDS; Energy Dispersive X-ray Spectroscopy, FT-IR; Fourier Transform Infrared Spectroscopy, µ-FT-IR; Micro Fourier Transform Infrared Spectroscopy, ATR-FT-IR; Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy, FAAS; Flame Atomic Absorption Spectroscopy, ICP-OES; Inductively Coupled Plasma - Optical Emission Spectrometry, ICP-MS; Inductively Coupled Plasma Mass Spectrometry, AAS; Atomic Absorption Spectroscopy, LIBS; Laser-Induced Breakdown Spectroscopy, XPS; X-Ray Photoelectron Spectroscopy, XRD; X-ray Diffraction, GC-MS; Gas Chromatography-Mass Spectrometry, ICP-AES; Inductively coupled plasma atomic emission spectroscopy, FEI-SEM; Field Emission Scanning Electron Microscopy, FE-SEM; Field-Emission Scanning Electron Microscope, LDPE; Low Density Polyethylene, HDPE; High Density Polyethylene, PTFE; Polytetrafluoroethylene, PMMA; Polymethyl Methacrylate, PE; Polyethylene, PS; Polystyrene, PP; Polypropylene, PVC; Polyvinyl Chloride, PET; Polyethylene Terephthalate, PA; Polyamide, PP; Polypropylene, As; Arsenic, Cd; Cadmium, Cr; Chromium, Ni; Nickel, Sr; Strontium, HCl; Hydrochloric Acid, H_2O_2; Hydrogen Peroxide, HNO_3; Nitric Acid).

Polymer Type

Each type of plastic polymers has their own functional groups, specific surface area, polarity and molecular chain organization; leading to differences in affinities for different heavy metal ions [108, 109]. Zou et al. [104] found the affinity of Pb, Cu and Cd for different microplastic polymer types as: CPE > PVC > HDPE > LDPE. They reported that polarity, chemical structure and electronegativity have crucial impact on the adsorption kinetics of HM ions to microplastic surfaces. The high affinity for Cd^{2+} and Cu^{2+} to CPE particles was attributed to the chlorinated polar groups in present in the particles. Yang et al. [89] also stated that polar groups (e.g. -NHCO- and -COO-) are responsible for higher Cu^{2+} adsorption by PA and PMMA. In an experimental study [94], PVC and PE were observed to have a greater affinity for heavy metal ions compared to PET, PP, and PS. PE had the highest adsorptive capability followed by PVC > PS > PP > PET and the researchers attribute this to the rubber-like nature of PE, which probably plays a significant role in its higher capacity to adsorb chemicals.

Specific Surface Area and Particle Size

The specific surface area (SSA) and particle size are considered important parameters for the adsorptive behavior of plastic particles [93]. There are studies showing that smaller microplastics with a larger SSA have a larger adsorptive area for metal ions [74,110]. Although the surface area of microplastics particles with a similar size were found as: PS > PP > PE > PVC > PET, the sorption affinities for metals were found as PE > PVC > PS > PP > PET; probably due to the smaller SSA and pore size [94]. The concentration of lead, copper and cadmium ions adsorbed to PP decreased substantially for larger particles [87]. Cu adsorption to PET and PS were also found to be dependent on particle size; with smaller sized particles (25 μm) having a higher adsorption rate compared to larger ones (180 μm) [111]. They also stated that mass of pollutants adsorbed to MPs were higher for particles with a larger SSA and a smaller particle size.

Crystallinity Structure

Another factor affecting the adsorptive capability of MPs is the crystallinity structure; generally, with a positive correlation between polymer chain organization (i.e. crystallinity) and the adsorptive behavior [88]. For example, the highest cadmium adsorptive potential was recorded for PA which had the highest crystallinity among the polymers with crystallinity with the following order PA > ABS (acrylonitrile butadiene styrene) > PVC > PS > PET; ABS having a lower Cd^{2+} adsorption compared to PVC, PS and PET [103]. Similarly, Zou et al. [104] compared LDPE (which has a lower crystallinity) and HDPE (which has a higher crystallinity) and found that HDPE had a higher affinity for Cd^{2+}, Cu^{2+} and Pb^{2+} ions. But they attributed this difference to the chemical structure and electronegativity of particle surfaces, not to the crystallinity of microplastics. Opposite findings are also available showing that high degree of crystallinity can result in a low adsorptive affinity [112].

pH

Researchers revealed that the pH of the experimental media can affect both heavy metal ionization dynamics and the properties of adsorptive surfaces and eventually adsorptive capacity of microplastic particles [68]. Zou et al. [104] recorded that the affinity of copper, lead and cadmium to microplastic surfaces increased as pH increased. Similarly, Li et al. [113] reported that cadmium concentration adsorbed to microplastic particles increased with increasing pH, but then gradually decreased and the highest adsorption occurred in the pH 6-7 range. They attributed this situation to the increases in anionic surface in that pH range [68]. In addition, Wang et al. [114] indicated that the H^+ ion concentration in the media has a direct effect on the sorption capacity of particles. When the pH is between 3 to 4, there is more H^+ in the solution which competes with the lead and cadmium ions for surface binding sites on microplastics and as a result decreases the adsorptive behavior of microplastic particles. When the pH is between 4 and 7, adsorption capacity increases since the reactive groups on particles can combine with metal ions easily, and being highest at pH=7. Oppositely, Dong et al. [91] found that the sorption of arsenic ions decreases gradually as the pH increases. Park et al. [115] stated that the competition of the ionic compounds for the active sites on the microplastic surfaces at high ambient pH levels may decrease the adsorption rates.

Temperature

Many studies have shown that solution temperature has a significant and controversial effect on adsorptive potential of microplastic particles for HM ions [93,116]. Heavy metal adsorption to microplastic particle surfaces is an endothermic reaction, thus, increasing temperature may lead to increased adsorption capacity [117]. For example, a rise in the ambient temperature accelerated the sorption of zinc and cadmium ions to plastic particles [68]. There are also contradictory findings; Dong et al. [91] reported that higher temperatures may inhibit adsorption of arsenic ions to PSMPs through the breakdown of H bonds between metal ions and carboxyl groups of particles; thus, leading to a gradual decrease in the amount of As (III) adsorbed. Similarly, Li et al. [118] revealed that binding of Cr (VI) ions to microplastic particles gradually increases as the temperature rises (between 278 K–298 K). They attributed this situation to the accelerated movement of the molecules in the media. However, a negative trend was found showing that if the temperature continues to rise, a marked decrease will be observed in the adsorptive affinity of PE and PS particles. They indicated that increased temperature may lead to activation of desorption between microplastics and heavy metals.

Salinity

Several researchers demonstrated that the salinity of the media may lead to changes in the adsorptive properties of pollutants to microplastic surfaces. For example; Alimi et al. [119] recorded a reduction in the adherence of organic pollutants including PAHs and PCBs to microplastic surfaces with decreasing salinity rates. Barus et al. [105] reported that increasing salinity in the

experimental media decelerates the adsorption rates of HM ions. Additionally, according to Wang et al. [114] salinity can significantly change the capacity of microplastic particles to capture metal ions. They also observed that increasing the sodium chloride concentration in the media led to a reduction in the adhered lead and cadmium ion concentration. The decrease in the concentration of adsorbed Pb ions was attributed to the increased Na^+ ions in the media which in turn neutralizing the negative charge of particles and inhibiting the adsorption. On the other hand, the decline in the adsorption of copper ions could be as a consequence of the competition of Na and Cu for available surface binding sites on microplastic surfaces which will eventually prevent the electrostatically interactions between copper and microplastic surfaces. Godoy et al. [94] compared the adsorptive properties in aqueous solution and seawater, and found that salinity led to both increases and decreases in the adsorption rates. They stated that, the increase could be due to the conversion of cations on the microplastic surfaces into molecules that can readily adsorb metals. The decreases in adsorption rates were attributed to the competition between cations in adsorptive sites.

Biofilm

Upon introduction to aquatic ecosystems, microplastics (MPs) promptly emerge as favored environments for microbial colonization, due to their advantageous properties. Compared to natural microbial habitats, MPs offer a larger specific surface area, facilitating greater attachment sites for microorganisms. Moreover, their intrinsic hydrophobic nature promotes the development of biofilms, establishing a favorable habitat for microbial growth and activity. The formation of biofilms, which is one of the most important factors that enable heavy metals to adhere to microplastics, has been studied by many researchers. In both in-situ and ex-situ studies, elements such as As, Cd, Au, V, Zn, Pb, Al, Cr, Hg, Mo, and Ni have been reported to accumulate in the biofilm layer of various microplastics (PLA, LDPE, PET, PP, PVC, HDPE, PE, PP) [49, 92, 120-124]. Prunier et al. [125] showed that a large proportion of the Cd, Ti, V, Zn, and Ni heavy metals accumulated on the surface of PE microplastics were adsorbed by the biofilm. In addition, Wang et al. [126] demonstrated that the presence of biofilm on the surface of PS increases the adsorption ratio of Cu and Pb.

Dissolved Organic Matter (DOM)

The adherence of HM ions to microplastic surfaces can be affected by the class and abundance of DOM in the media [127]. DOM can reduce adsorption rates by interacting with the pollutant and thus reduce the available pollutant amount to adhere to plastic particles or by blocking the adsorptive sites on particles [128]. Zhou et al. [103] found that Cd^{2+} adsorption to microplastic surfaces decreased as a result of increased humic acid (HA) concentration in the experimental media. They attributed this decrease to the high affinity of negatively charged HA to cationic pollutants, which leads to competitive adsorption. However, there are also contradictory findings. Guo et al. [98] pointed out that adsorption of cadmium ions to microplastic surfaces increased with increasing HA

concentration, indicating that HA can be adsorbed on certain types of microplastics and therefore, facilitating the ionic bonding with positively charged HMs. Similarly, Wang et al. [114] found that the amount of lead and copper ions adsorbed to new and artificially aged microplastic particles increased with increasing fulvic acid (FA) concentration. They stated that FA was adsorbed to particle surfaces and complexed with metals, thus increasing the adsorptive capability of microplastic particles. Additionally, Li et al. [118] demonstrated that the adsorption of chromium ions on PE and PS particle surfaces was increasing with increasing of FA concentration, whereas for PA, the adsorption of chromium was increased when FA concentration was 1 to 10 mg/L; but continuous rise of the FA concentration led to decrease in the adsorption.

2. CONCLUSIONS

The amount of microplastic particles in the components of the ecosystem including the atmosphere, soil, and the water bodies gradually increased over the last decades. In aquatic ecosystems, microplastic particles can endure for a significant period of time since they are considered as persistent pollutants. Microplastic particles are found nearly in all aquatic habitats, even in alpine lakes and highly isolated water bodies [113]. Microplastic particles can be accumulated in various tissues of exposed animals and can be transported to higher order animals throughout the food chain. Thus, they considered as a major health concern to human beings [129-131]. Additionally, recent studies indicated that microplastics particles create an additional risk by adsorbing heavy metal ions on their surfaces and therefore acting in a similar manner to a Trojan horse [132, 133]. Besides, microplastic particles can accumulate heavy metal ions on their surfaces at a higher rate compared to ambient heavy metal concentrations [62, 75, 76].

The results of both field and laboratory studies reviewed here clearly indicates that several heavy metals including As, Al, Cr, Cu, Pb, Zn, Mn, Co, Cu, Cd and Ni can be absorbed by the plastic particles at high rates depending on several factors. Several factors including the pH and salinity of the media, dissolved organic matter concentration, ambient temperature, specific properties of microplastics (chemical structure, specific surface area, particle dimensions, and polymer chain organization) and ambient heavy metal concentrations have been found to affect the adherence of metals ions to plastic particles [110]. Therefore, it is pivotal to reveal the parameters effective on sorption and desorption dynamics of HM ions to plastic surfaces in order to develop realistic risk assessments. The techniques used in fully controlled experimental designs to simulate environmental degradation-decomposition processes (such as UV exposure, milling, and oxidation treatments) may potentially have an impact on the results obtained; therefore, should be handled carefully. The studies summarized here indicates that more data both from field and laboratory studies is required to comprehend the adsorptive behavior of microplastic particles and to reveal factors effecting sorption of heavy metals to microplastic surfaces.

Author Contribution Statements NSC, ŞK and DN drafted the manuscript and conducted the literature review. MBE conceived of the presented idea and advised on the overall direction and reviewed the manuscript. All authors have read and approved the article.

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