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Utilization of Steel Slag as a Soil Amendment and Mineral Fertilizer in Agriculture: A Review

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ABSTRACT
The gradual increase in industrial wastes allowed the utilization of these wastes in different areas. Steel slag, one of the industrial wastes, is formed during the reduction of molten crude iron to molten crude steel in basic oxygen furnaces or scrap to molten crude steel in electric arc furnaces and induction furnaces. Removal, storage, or disposal of steel slag is an important environmental and economic problem. Steel slag offers opportunities to be used as an alternative material in various areas and contributes to the national economies through recycling. This research provides information about the studies and application examples on the use of steel slag as a soil amendment and mineral fertilizer in the world. This usage allows reducing the consumption of natural resources and providing great agricultural, environmental, and economic gain by minimizing the negative environmental effects of steel slag.

Keywords: Steel slag, Basic oxygen furnace (BOF), Electric arc furnace (EAF), Induction furnace (IF), Soil amendment, Mineral fertilizer

1. Introduction

The use of steel slag in the remediation of acidic soils and the improvement of plant nutrient availability of infertile soils is a cost-effective and environmentally friendly approach that helps reduce waste issues. Therefore, the use of steel slag as agricultural lime material and/or mineral fertilizer is of great importance (Hemalatha 2013; Wen et al. 2020). The slag formed as a result of the steelmaking process can be successfully used as a soil amendment for the neutralization of soil pH in the reclamation of acidic soils as an alternative to natural agricultural lime material (limestone or dolomite) (Manso et al. 2013; Deus et al. 2018; Mamatha et al. 2018). On the other hand, since steel slag contains essential plant nutrients such as P, S, Mn, Fe, and Mo at various concentrations, it is possible to contribute to soil fertility as mineral fertilizer. Steel slag, with its calcium silicate content, can be used as an important source of nutrients for some plants sensitive to silicon, such as wheat, rice, and sugar cane, and as a material to improve disease resistance in many cultivated plants (Ito 2015; Yang et al. 2018; Das et al. 2019; O'Connor et al. 2021).

There are many studies and successful applications in different parts of the world which explicitly demonstrate that steel slag can be effectively used in the reclamation of acidic soils as an alternative liming material and is an economical soil amendment material (Proctor et al. 2000; Hemalatha 2013; Mamatha et al. 2018). On the other hand, steel slag has the potential to contribute to soil fertility and thus to crop yield as it has a significant amount and variety of essential plants. Steel slag can be used directly as a fertilizer, or it can be used more effectively by mixing and preparing compost with vegetable and animal wastes (Reuter et al. 2004; Winkler 2011; Manso et al. 2013).

This review has been prepared to pay attention on use of steel slag produced as a by-product of steel making industry, which has very high production value for Turkey in terms of plant nutrition and soil reclamation purposes.

2. Steel Slag Formation

Basic oxygen furnace slags (BOFS), electric arc furnace slags (EAFS), and induction furnace slags (IFS) are referred to as steel slags (TSPA 2015). Since the amount of production in IF is low, the amount of slag is also negligible. Steel slags, which are the by-products resulting from the oxidation of impurities in molten steel during steelmaking, primarily contain CaO, SiO_2, Al_2O_3,

MgO, MnO, Fe_2O_3 and P_2O_5 (Proctor et al. 2000; Reuter et al. 2004; Yi et al. 2012; Guo et al. 2018; Fisher & Barron 2019; Song et al. 2021). The general chemical compositions of BOFS and EAFS is given in Table 1.

Table 1 - Chemical compositions of BOFS and EAFS (wt, %) (Yi et al. (2012))

Slag type	*CaO*	*SiO_2*	*Al_2O_3*	*MgO*	*MnO*	*Fe_2O_3*	*P_2O_5*
BOFS	45-60	10-15	1-5	3-13	2-6	3-9	1-4
EAFS	30-50	11-20	10-18	8-13	5-10	5-6	2-5

The pH of steel slag generally changes between 8 to 10 but depending on the limestone content, it increases up to 12 (NSA 2021). Steel is classified as high, medium, and low carbon-based on its carbon content. During production, the amount of oxygen blown must be changed in order to change the carbon content (FHWA 2017). Wang et al. (2021) found that carbon content of steel slag was 0.87%. After the blast furnace melting process in integrated plants, the scrap is added to the molten iron and processed in the BOF. At this stage, 10-15% of BOFS is obtained per ton of crude steel produced as a by-product (Cebeci & Sonverdi 2012). EAFS is produced as a by-product in steelmaking from scrap. Because the resulting molten steel is not of sufficient quality for direct use, the molten steel is taken into the crucible and subjected to additional processes. Undesirable elements in the molten steel are removed in the form of oxides by the oxidation process (Yonar 2017). IF uses alternating current flowing through a coil to create a magnetic field within the metal. The induced current provides rapid heating and melting. Since the metal is not in contact with the heating elements, the setting must be well controlled to produce molten metals of high quality and purity. Despite the lower capacity than other furnaces, the greatest advantage of IF is their lower investment cost compared to other melting furnaces. Since it operates at a low capacity, the amount of IFS is relatively less (GDH 2017).

3. Disposal Methods and Usage Areas of Steel Slag

More than 400 million tons of steel slag is produced in the world every year (Carvalho et al. 2017). The World Steel Association aims to achieve zero waste in the steel industry by reusing and recycling steel slag. Using the slag in other areas reduces the amount of waste, disposal costs, and energy use. In the notice published by the American National Slag Association (NSA 2003), it is stated that steel slag is a suitable aggregate material that can be used in residential, agricultural, industrial, and construction applications. Application areas and rates of steel slag in Europe in 2012 are given in Table 2. According Euroslag (2021) 68% of the steel slag was used in various application areas in 2012, while 19% was stored temporarily for later use, and only 13% was finally stored. The primary area of application of steel slag is aggregate production in road construction with a rate of 43%. While the rate of internal use for metallurgical purposes is 11%, it is used in cement production, hydraulic structures, and fertilizer production by 3 to 5% (Table 2).

Table 2 - Application areas and rates of steel slag in Europe in 2012 (Euroslag (2021))

Application areas	*Application rates (%)*
Road construction	43
Temporary storage	19
Final storage	13
Metallurgical usage	11
Cement production	5
Hydraulic structures	3
Fertilizer production	3
Others	3

Steel slag is a suitable aggregate material that can be used in residential, agricultural, industrial, and construction applications (NSA 2003; He et al. 2012; Mengxiao et al. 2015; Xue et al. 2016; Poulikakos et al. 2017). The slags have been tested following the USEPA and ASTM procedures, and it has been stated that slag applications are an environmentally friendly approach. Since slags are formed at very high temperatures, they do not contain organic, semi-organic, or volatile compounds but contain calcium, iron, silicon, aluminum, magnesium oxides and calcium silicate, aluminosilicate, and aluminoferrite compounds. According to the risk assessment analyses and the Human Health and Ecological Risk Assessment, the use of steel slag in residential, agricultural, industrial, and construction applications do not pose a threat to human health and the environment. A study conducted in Sweden investigated the suitability of ladle slags, by-product of secondary steel treatment, and EAFS for landfill cover, considering that large amounts of mineral materials were required by the landfills to be closed, and it was very costly to fulfill this need from natural resources. The opportunities to use EAFS and ladle slags from 4 different sources were investigated. Accordingly, regarding these slags with different particle sizes, it was determined that slags in the range of 16-32 mm could be used in the drainage layers, and finer slags could be used in the impermeable layer (Andreas et al. 2005).

Uibu et al. (2011) stated that EAFS could be used for CO_2-sorbent. Their study indicated that the EAFS are suitable for mineral carbonization in the liquid phase due to their Mg and Ca contents and that they can be used for CO_2-sorbent to reduce

CO_2 emissions. Moreover, it has been determined that steel slag has 95-100% removal efficiency in arsenic removal in aqueous systems (Yi et al. 2012). Kim et al. (2008) investigated the removal mechanisms of copper using steel slag. Many studies show that steel slag can be an effective material in phosphate adsorption from wastewater. When used as filter material in constructed wetlands, BOFS is found to be effective in the removal of phosphate (Park et al. 2017). Regarding the use of slags in wastewater treatment, there are phosphate removal applications, especially in domestic wastewater, by passing the wastewater through a tank filled with slag after biological treatment (Okochi & Mcmartin 2011). It has also been proven that steel slag can be used as ballast material on roads and railways as well as being used as backfill material in coastal structures (Unal et al. 2014; GDH 2017; Tozsin et al. 2023).

In Turkey, as of 2018, there are 34 established crude steelmaking facilities, 3 of which are BOF, 25 EAF, and 6 IF. The total crude steel production amount of these plants in 2018 was 37.311.733 tons, and the amount of slag formed was 5.562.018 tons (Table 3) (TSPA 2018).

Table 3 - The amounts of crude steel production and steel slag formed in Turkey in 2018 (TSPA (2018))

Slag type	*Crude steel*	*Steel slag*
	(tons)	
BOF	11.513.194	1.692.237
EAF	25.079.946	3.761.992
IF	718.593	107.789
TOTAL	37.311.733	5.562.018

For each ton of steel produced, 120-150 kg of slag is obtained. It is known that 100-140 million tons of slag have been obtained from iron and steel production in Turkey until now, most of this by-product has been disposed of or kept in storage areas in an inactive state, and around 4-5 million tons of slag is added to this idle pile every year (GDH 2017). The use of steel slag in different areas allows savings from natural resource consumption while minimizing the adverse effect on the environment by safely removing a material occurring in large masses under normal conditions and being kept in storage areas (Uysal & Bahar 2018). In Turkey, it is seen that steel slag is;

- regularly/irregularly stored in the fields,
- sold in the market as road filling material after being recycled in recycling facilities (iron in the slag is removed using magnetic separators, then processed in the crushing-screening facilities and sold as aggregate),
- used as raw material in cement and sinter plants.

4. Soil Application of Steel Slag

Steel slag has an important potential to be used as a mineral fertilizer as well as a good soil amendment. Therefore, steel slag can be used as an environmentally friendly application as a fertilizer or soil amendment in order to promote plant growth, regulate the structural properties of the soil, and increase crop yield (Lopez et al. 1995; Pistocchi et al. 2017; Jafer et al. 2018). Steel slag, which is a by-product of an industrial process, offers significant cost advantages over commercially produced limestone. In addition, steel slag is not only a liming material, but also contributes to the structural development of the soil due to its Ca, Mg, and Fe content and significantly reduce fungal infections. Slag is used as a soil amendment due to its high Ca and Mg content, and on the other hand, it is used directly as a raw material for the production of silicon fertilizer or phosphorus fertilizer in order to increase the resistance of plants against diseases and pests (Shi 2004; Branca et al. 2014; Das et al. 2019). Chand et al. (2015) stated in their study on the use of BOFS in agriculture that slag can be successfully used instead of limestone to neutralize soil acidity, and it may be possible to utilize it as a fertilizer material. Likewise, Das et al. (2007) pointed out that slag can be used safely and effectively as fertilizer and soil amendment.

Pinto et al. (1995), investigating the possibilities of using slag, a by-product of the iron and steelmaking industry and containing 29% Ca, 21% Fe, and 5% Mg, as a dolomitic liming material in pasture soils, indicated that soil pH increased significantly and linearly with slag application (0, 1, 1.5, 3, 5, and 7.5 t ha^{-1}), and pasture yield increased by 41% with the application of 3 tons of slag per hectare. Munn (2005) used steel slags as liming material for the improvement of agricultural and acidic mining areas in Ohio State (USA) and examined the effects of slag application on plant growth in these areas with a 3-year greenhouse trial. Steel slag and $CaCO_3$ were applied to acid soil with pH 3.5 in the ratio of 12.5 and 25 g $CaCO_3$ kg^{-1} equivalent. Selected plants, oat (*Avena sativa* L.), wheat (*Triticum aestivum* L.), corn (*Zea mays* L.) and soybean (*Glycine max* (L.) *Merr.*) were grown and harvested at the seedling stage. It was determined that the steel slag and $CaCO_3$ application increased the yield at the $P<0.01$ level compared to the control. Depending on the application, it was determined that the Ca and Mg content of the soil and all studied plants increased. Mihalache et al. (2016) reported that steel slag application at different doses (control, 1 t ha^{-1}, 2 t ha^{-1}, 3 t ha^{-1}, 5 t ha^{-1}) increased the wheat yield.

Steel slag is effectively used as a soil amendment to stabilize rice production, reduce greenhouse gas emissions in paddy fields, and most importantly, increase soil productivity in countries where rice production is vital, such as Korea, Japan, Bangladesh, and China. Wang et al. (2018a) stated that steel slag is very rich in terms of Fe and other nutrients and noted that if

it is applied to the soil, the acidic soil pH can be increased, especially in paddy fields, the methane emission occurring in these areas can be reduced, the soil quality can be improved, and a significant increase in paddy yield can be achieved. Wang et al. (2018b) also determined that depending on the application of steel slag, Ca and Si concentrations in the soil as well as N and P concentrations in leaf and root increased significantly. They stated that the rice yield increased depending on the increase in the P, Ca, Si, N and P concentrations. As a result, it was determined that the application of steel slag to the paddy fields promoted the use of nutrients, increased plant growth and yield, and regulated soil and plant chemistry. In the same study, Wang et al. (2018b) investigated the effects on soil organic carbon stock by applying steel slag and biochar both separately and together as a soil amendment. They found that the amount of organic carbon in the 30 cm topsoil layer increased by 28.7-42.2% depending on the application at a rate of 8 t ha^{-1}. In this study, it was determined that the application of steel slag reduced the soil fungus population by 62.8% compared to the control soil. Makela et al. (2012) stated that the compost obtained by mixing steel slag with pulp and paper industry solid wastes gives very successful results in the improvement of acidic soils, and therefore, it can be substituted with commercial fertilizers.

Agricultural lime material used in the reclamation of acidic soils is generally reapplied every 3-5 years. The low solubility of limestone can ensure that the desired soil pH remains stable during this time. The situation in the application of steel slag is slightly different. The $Ca(OH)_2$ produced when CaO in the slag reacts with soil moisture causes a spike in soil pH, but this is temporary, and the desired pH is created when the poorly soluble calcareous components in the slag react with the soil. Therefore, the frequency of applying steel slag to the soil for liming depends on the time the slag can maintain the desired soil pH, which can be determined by monitoring the soil reaction at intervals of several years. The amount of salt added to the soil together with the steel slag is one of the most important issues to be considered in terms of the slag being used as agricultural lime material. Since agricultural lime has low solubility in water, the amount of soluble salts accumulated in the soil by the use of these materials does not pose a serious risk. However, the water-soluble salt content of steel slag is much higher than that of limestone. CaO and MgO in the slag react with water to form $Ca(OH)_2$ and $Mg(OH)_2$, respectively. The water solubilities of these hydroxides are 1.20 g L^{-1} and 0.009 g L^{-1}, respectively, and it is much higher compared to $CaCO_3$ (0.014 g L^{-1}) and $MgCO_3$ (0.013 g L^{-1}) (NLA 1990; Beck & Daniels 2008). However, soluble salts are not a problem in rainy areas and well-drained soils when the slag is applied in optimum doses to neutralize the soil pH. Beck & Daniels (2008) report that the soluble salt contents of fine and coarse steel slag are 3.68 and 2.55 dS m^{-1}, respectively. Considering that plants can tolerate soil salinity up to 2dS m^{-1}, serious problems with soil soluble salts should not be expected with the application of steel slag. However, in areas where high doses of steel slag are applied, it is recommended to monitor soluble salts and soil salinity.

It is absolutely required to monitor the amount of heavy metals added to the soil along with the steel slag. Studies show that the amount of heavy metal added to the soil by steel slag is generally well below the permissible limits. If steel slag is used as a liming material, there is no risk for conditions where soil pH is neutral as metals such as Al, Cr, Pb, Cd, Ni, Co, Be, Ba, and Sr have low soil solubility and bioavailability levels (Dimitrova & Mehanjiev 2000; He et al. 2017).

Since steel slag contains a high amount of fertilizer components such as CaO, SiO_2, and MgO, it is used as calcium silicate fertilizer in many regions of the world. Steel slag also includes essential plant nutrient elements such as Fe_2O_3, MnO and P_2O_5 (Shi 2004; Yildirim & Prezzi 2011; Das et al. 2019). Therefore, it has the potential to be used effectively as a fertilizer in cultivated and pasture areas. Studies have shown that steel slag, which is an important environmental problem, can be used as an inorganic fertilizer (Delil et al. 2017). Due to its alkalinity, steel slag is successfully used in agricultural soils instead of limestone to neutralize the acidity of the soil and create more suitable chemical and biological conditions agronomically (Yi et al. 2012; Chand et al. 2015). It has been reported that plant nutrient balance can be achieved rapidly, especially among Si, Fe, Ca, Mg, P, Mn and B in soils where steel slag is applied as calcium silicate fertilizer (Wang et al. 2018b; Das et al. 2020). Although Si is not an essential plant nutrient, it has great importance in promoting photosynthesis, root activity and stem resistance, and preventing yellowing of lower leaves and burn formation in many cultivated plants. For this reason, there are significant increases in yield in terms of quality and quantity, and especially dry matter production and grain yield increase depending on the calcium silicate application. In addition, it is reported that the product yield increases due to Fe, Mn and P supplementation, and the negative effects that lead to product loss, especially root rot and leaf burn, are eliminated (Kostura et al. 2005; Das et al. 2020).

Steel slags are categorized as silicate fertilizer, lime fertilizer, phosphate fertilizer, or Fe-added special fertilizer based on their Ca, Si, Mg, P, Mn, and Fe contents (Ito 2015). In studies on the determination of the efficiency of using steel slag as Fe fertilizer, it has been revealed that very significant increases in yield and plant Fe intake are achieved depending on the application dose in calcareous soils with Fe deficiency. Xian & Qingsheng (2006) applied steel slag directly and in the acidified form to calcareous soil in two different doses (10 and 20 g kg^{-1}) and determined that there were statistically significant increases in the dry matter ratio and plant Fe content of the corn plant at the end of the application.

The Indian Ministry of Agriculture has approved the product, which is obtained by enriching steel slag with nutrients such as Ca, P and Fe and produced by the Indian Fertilizer Association, to be used as a soil amendment. It was determined that the product gave very successful results in acidic soils, and the yield increased by at least 25% (Chand et al. 2015). In their study in Italy and Germany, where they shared long-term field trial results on the use of slag as a mineral fertilizer in agriculture, Branca

et al. (2014) compared BOFS with reference liming material and commercial mineral fertilizers and reported that long-term slag application significantly increased the product amount and feed quality and that no negative effects on soil fertility occurred.

Steel slag can be used directly as a fertilizer, or it can be used more effectively by mixing and preparing compost with vegetable and animal wastes. Composted animal manure is used as a source of N and P. Very successful results can be obtained by mixing steel slag with animal manure and applying it to the soil as a compost enriched with N and P as well as Ca, Si, Mg, Mn, Fe, and other elements (Ito 2015). In the study, Ito (2015) underlined that the temperature of the compost prepared by mixing 15% slag with cattle manure based on weight increased up to 70 ºC at a depth of 20 cm from the surface; in the control group, this temperature could only rise to 58 ºC; even it was mixed every 10 days, the temperature was stabilized at 65-70 ºC after a short decrease; and therefore, the use of slag in composting accelerates the process and compost can be obtained in a shorter time. In the same study, it was determined that compost mixed with slag provided more than 80% germination and increased yield significantly. Therefore, the slag could be successfully used to correct soil acidity, increase soil physical and chemical properties and soil fertility.

5. Considerations in the Use of Steel Slag as a Soil Amendment and Mineral Fertilizer

The most important physical property of steel slag is its particle size distribution. The finer the slag to be used as a liming material in agriculture, the more reactive it will be, and thus, the more its effectiveness in neutralizing soil acidity will increase. In terms of particle size distribution, it is recommended that 90% of the slag passes through a 20 mesh (900 microns) sieve. The bulk density and specific gravity of steel slag are relatively higher than that of agricultural lime material. This situation is directly related to the amount of metal contained in the steel slag. The bulk density of steel slag ranges from 1.6 to 1.9 cm^{-3} while that of agricultural lime material ranges from 1.4 to 1.5 g cm^{-3}. Similarly, the specific gravity of steel slag varies between 3.2 - 3.6 g cm^{-3} while that of agricultural lime material varies between 2.7-2.9 g cm^{-3} (NSA 2021).

The main purpose of applying steel slag to the soil as mineral fertilizer is to provide nutrients to the plant. However, these wastes should not have negative effects on the environment, human, animal, and plant health. Therefore, in order to ensure more effective and sustainable use of steel slag in agriculture, it is necessary to carefully monitor the soil health and groundwater quality due to heavy metal and salt content while examining the effects on yield. Instead of limestone, steel slag has been successfully used for many years to neutralize soil acidity in agricultural soils with high efficiency and low cost. To ensure more effective and sustainable use of steel slag in agriculture, Chand et al. (2015) stated in their study on the behavior and immobilization of heavy metals in steel slag in soil that heavy metal accumulation in the soil was below the permissible limit values depending on the slag application. Wang et al. (2015) stated that steel slag could be used as an effective soil amendment to increase the paddy yield and to decrease the CH_4 and N_2O emission occurring in paddy fields without leading to heavy metal accumulation at concentrations which may produce adverse effects in soils and plants.

It is important in terms of fertilization that the fertilizer produced from steel slag contains nutrients such as Ca and Mg, which are consumed excessively by plants, as well as micro plant nutrients such as Fe and Mn. However, it should not be expected that the fertilizer produced from slag will completely eliminate the deficiency of N, P, and K, which are considered essential nutrients for plants and needed at the macro level. Because the N and K content of the slag is very low, and the P content is not in the amounts that can meet the plant's demands at the optimum level. Therefore, it is clear that it would be much more beneficial to use the fertilizer to be produced together with some N-P-K fertilizer (Ito 2015; Das et al. 2020).

The amount of lime to be added to the soil may vary significantly depending on the characteristics of the lime material used, the thickness of the soil layer to be improved, the soil characteristics, especially the texture and the initial pH of the soil, and the requirements of the product to be grown. The CaO content and particle size of the lime material are very important. As the CaO content of the lime material increases, the amount of lime material to be applied to the unit area decreases. The small particle size will lead to higher chemical efficiency due to the increase in the specific surface area. The thickness of the soil layer to which the lime material will be applied is generally considered as 15-20 cm. This depth is the soil depth where 80% of the root density of many cultivated plants is located, where water and plant nutrients are used intensively, and microbial activity is the highest (USDA 1999; Anderson et al. 2013).

6. Evaluation and Conclusions

Many industrialized countries consider steel slag not as a waste but as a by-product with economic value and use as a liming material in the reclamation of acidic soils and as plant nutrients. The use of slag, which is a by-product of steelmaking facilities, as a soil amendment and mineral fertilizer is of great importance in terms of agriculture, environment, and economy.

Studies on the use of steel slag as a soil amendment and mineral fertilizer indicate that steel slag;

- can be used as an effective and economical soil amendment material in the reclamation of acid soils as an alternative to agricultural lime,

- promotes nutrient uptake, increases plant growth and yield by creating more suitable chemical and biological conditions in acid soils,
- can be used as direct fertilizer since it has a significant amount and variety of essential plant nutrients (P, S, Mn, Fe and Mo), or it can be applied more effectively by mixing and preparing compost with vegetable and animal wastes,
- can be used directly as a calcium and magnesium silicate due to its high amount of CaO, SiO_2, and MgO contents,
- has the potential to be widely used as a source of Si, which has significant effect on quality and quantity of some plants by stimulating photosynthesis in many cultivars, increasing root activity, preventing yellowing of lower leaves, increasing stem strength, and preventing burn formation,
- can increase the crop yield when applied as a mineral fertilizer,
- has the potential to be used as lime fertilizer, phosphate fertilizer, and Fe-containing special fertilizer,
- although it contains certain amounts of heavy metals, the amount of these metals that can be loaded into the soil or taken from the soil by plants is below the maximum allowable amounts.

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References

Anderson N P, Hart J M, Sullivan D M, Christensen N W, Horneck D A & Pirelli G J (2013). Applying lime to raise soil pH for crop production (Western Oregon). Oregon State University, Extension Service, EM9057

Andreas L, Herrmann I, Lidström M & Lagerkvist A (2005). Physical properties of steel slag to be reused in a landfill cover. Tenth International Waste Management and Landfill Symposium. Environmental Sanitary Engineering Centre, Italy

Beck M & Daniels W L (2008). Tube City IMS, LLC steel slag characterization study. Department of Crop and Soil Environmental Sciences. Virginia Tech University, Blacksburg, VA.

Branca T A, Pistocchi C, Colla V, Ragaglini G, Amato A, Tozzini C, Mudersbach D, Morillon A, Rex M & Romaniello L (2014). Investigation of (BOF) converter slag use for agriculture in Europe. *Metallurgical Research and Technology* 111(3): 155-167. doi.org/10.1051/metal/2014022

Carvalho S Z, Vernili F, Almedia B, Demarco M, Silva S N (2017). The recycling effect of BOF slag in the portland cement properties. *Resources, Conservation and Recycling* 127: 216-220. doi.org/10.1016/j.resconrec.2017.08.021

Cebeci M & Sonverdi E (2012). Environmental impacts of integrated iron and steel production processes. International Iron & Steel Symposium, Karabuk, Turkey, 2-4 April

Chand S, Paul B & Kumar M (2015). An overview of use of Linz-Donawitz (LD) steel slag in agriculture. *Current World Environment* 10(3): 975-984. doi.org/10.12944/cwe.10.3.29

Das B, Prakash S, Reddy P S R & Mishra V N (2007). An overview of utilization of slag and sludge from steel industries. *Resources, Conservation and Recycling* 50(1): 40-57. doi.org/10.1016/j.resconrec.2006.05.008

Das S, Kim G W, Hwang H Y, Verma P P & Kim P J (2019). Cropping with slag to address soil, environment, and food security. *Frontiers in Microbiology* 10: 1-7. doi.org/10.3389/fmicb.2019.01320

Das S, Gwon H S, Khan M I, Jeong S T, Kim P J (2020). Steel slag amendment impacts on soil microbial communities and activities of rice (Oryza sativa L.). *Scientific Reports* 10(1): 1–11. doi.org/10.1038/s41598-020-63783-1

Delil A D, Yildirim D & Koleli N (2017). Recovery of plant nutrition elements from steel slag and influence of these elements on the plant growth. *APJES* 5(1): 1–7. doi: 10.21541/apjes.283725

Deus A C F, de Almeida Bertani R M, Meirelles G, Soares A, Moreira L, Bull L & Fernandes D (2018). The comprehensive utilization of steel slag in agricultural soils. In: Y Zhang (Ed.), Recovery and Utilization of Metallurgical Solid Waste, Published by IntechOpen, pp. 1-11

Dimitrova S & Mehanjiev D (2000). Interaction of blast-furnace slag with heavy metal ions in water solutions. *Water Research* 34(6):1957-1961. doi.org/10.1016/s0043-1354(99)00328-0

Euroslag (2021). Statistics 2012. [WWW Document]. URL (http://www.euroslag.com/products/ statistics/2012). (Accessed 17 August 2021).

FHWA (U.S. Department of Transportation Federal Highway Administration) (2017). User guidelines for waste and byproduct materials in pavement construction. Publication Number: FHWA–RD–97–148

Fisher L V & Barron A R (2019). The recycling and reuse of steelmaking slags-A review. *Resources, Conservation and Recycling* 146: 244-255. doi.org/10.1016/j.resconrec.2019.03.010

GDH (The General Directorate of Highways, Turkey) (2017). Use, performance and legislation proposal of steel slag in highway construction. Project Number: 2012–7

Guo J, Bao Y & Wang M (2018). Steel slag in China: treatment, recycling, and management. *Waste Management* 78: 318–330. doi.org/10.1016/j.wasman.2018.04.045

He F, Fang Y, Xie J & Xie J (2012). Fabrication and characterization of glass −ceramics materials developed from steel slag waste. *Materials and Design* 42: 198–203. doi.org/10.1016/j.matdes.2012.05.033

He H, Tam N F, Yao A, Qiu R, Li W C & Ye Z (2017). Growth and Cd uptake by rice (Oryza sativa) in acidic and Cd-contaminated paddy soils amended with steel slag. *Chemosphere* 189: 247-254. doi.org/10.1016/j.chemosphere.2017.09.069

Hemalatha B (2013). Utilization of industrial slag as a soil amendment and source of nutrients in acid soil. University of Agricultural Sciences GKVK, Bangalore.

Ito K (2015). Steelmaking slag for fertilizer usage. Nippon Steel & Sumitomo Metal Technical Report No. 109, 130-136

Jafer H M, Atherton W, Sadique M, Ruddock F & Loffill E (2018). Development of a new ternary blended cementitious binder produced from waste materials for use in soft soil stabilisation. *Journal of Cleaner Production* 172: 516-528. doi.org/10.1016/j.jclepro.2017.10.233

Kim D H, Shin M C, Choi H D, Seo C I & Baek K (2008). Removal mechanisms of copper using steel-making slag: adsorption and precipitation. *Desalination* 223(1-3): 283-289. doi.org/10.1016/j.desal.2007.01.226

Kostura B, Kulveitova H & Lesko J (2005). Blast furnace slags as sorbents of phosphate from water solutions. *Water Research* 39(9): 1795–1802. doi.org/10.1016/j.watres.2005.03.010

Lopez F A, Balcazar N, Formoso A, Pinto M & Rodriguez M (1995). The recycling of Linz-Donawitz (LD) converter slag by use as a liming agent on pasture land. *Waste Management and Research* 13(6): 555-568. doi.org/10.1006/wmre.1995.0052

Makela M, Watkins G, Poykio R, Nurmesniemi H & Dahl O (2012). Utilization of steel, pulp and paper industry solid residues in forest soil amendment: relevant physicochemical properties and heavy metal availability. *Journal of Hazardous Materials* 207-208: 21-27. doi.org/10.1016/j.jhazmat.2011.02.015

Mamatha D, Gowda R C & Shivakumara M N (2018). Effect of basic slag on yield, nutrient status and uptake by paddy in acid soils of Karnataka, India. *International Journal of Current Microbiology and Applied Sciences* 7(2): 2286–2292. doi.org/10.20546/ijcmas.2018.702.276

Manso J M, Ortega-L´opez V, Polanco J A & Seti´en J (2013). The use of ladle furnace slag in soil stabilization. *Construction and Building Materials* 40: 126–134. doi.org/10.1016/j.conbuildmat.2012.09.079

Mengxiao S, Qiang W & Zhikai Z (2015). Comparison of the properties between highvolume fly ash concrete and high-volume steel slag concrete under temperature matching curing condition. *Construction and Building Materials* 98: 649–655. doi.org/10.1016/j.conbuildmat.2015.08.134

Mihalache M, Ilie L, Doru I M, Mihalache D & Ildiko A (2016). Research on heavy metals translocation from soil amended with LF slag in wheat grains. 16th International Multidisciplinary Scientific Geoconference (SGEM 2016), Water Resources, Forest, Marine and Ocean Ecosystems Conference Proceedings, Vol.II; 281–286, Albena, Bulgaria, June 30–July 06.

Munn D A (2005). Steel industry slags compared with calcium carbonate in neutralizing acid mine soil. *The Ohio Journal of Science* 105(4): 79-87

NLA (National Lime Association) (1990). Lime: handling, application and storage. Arlington, VA 22201.

NSA (National Slag Association) (2003). Iron and steel making slag-environmentally responsible construction aggregates. The National Slag Association-Environmental Committee. Environmental Technical Bulletin May 26.

NSA (National Slag Association) (2021). A guide for the use of steel slag in agriculture and for reclamation of acidic lands. [WWW Document]. URL (http://www.nationalslag.org/sites/nationalslag/files/ag_guide909.pdf). (Accessed 01 August 2021)

O'Connor J, Nguyen T B T, Honeyands T, Monaghan B, O'Dea D, Rinklebe J, Vinu A, Hoang S A, Singh G, Kirkham M B & Bolan N (2021). Production, characterisation, utilisation, and beneficial soil application of steel slag: A review. *Journal of Hazardous Materials* 419: 126478. doi.org/10.1016/j.jhazmat.2021.126478

Okochi N C & Mcmartin D W (2011). Laboratory investigations of stormwater remediation via slag: Effects of metals on phosphorus removal. *Journal of Hazardous Materials* 187(1-3): 250-257. doi.org/10.1016/j.jhazmat.2011.01.015

Park J H, Wang J J, Kim S H, Siu C J & Seo D C (2017). Phosphate removal in constructed wetland with rapid cooled oxygen furnace slag. *Chemical Engineering Journal* 327: 713-724. doi.org/10.1016/j.cej.2017.06.155

Pinto M, Rodrigvez M, Besga G, Balcazar N & Lopez F A (1995). Effects of Linz-Donawitz (LD) slag as soil properties and pasture production in the Basque country (Northern Spain). *New Zealand Journal of Agricultural Research* 38(1): 143-155. doi.org/10.1080/00288233.1995.9513113

Pistocchi C, Ragaglini G, Colla V, Branca T A, Tozzini C & Romaniello L (2017). Exchangeable sodium percentage decrease in saline sodic soil after basic oxygen furnace flag application in a lysimeter trial. *Journal of Environmental Management* 203: 896-906. doi.org/10.1016/j.jenvman.2017.05.007

Poulikakos L D, Papadaskalopoulou C, Hofko B, Gschösser F, Cannone Falchetto A, Bueno M, Arraigada M, Sousa J, Ruiz R, Petit C, Loizidou M & Partl M N (2017). Harvesting the unexplored potential of European waste materials for road construction. *Resources, Conservation and Recycling* 116: 32-44. doi.org/10.1016/j.resconrec.2016.09.008

Proctor D, Fehling K, Shay E, Wittenborn J, Green J, Avent C, Bigham R, Connolly M, Lee B, Shepker T & Zak M (2000). Physical and chemical characteristics of blast furnace, basic oxygen furnace and electric arc furnace steel industry slags. *Environmental Science and Technology* 34(8): 1576-1582. doi.org/10.1021/es9906002

Reuter M, Xiao Y & Boin U (2004). Recycling and environmental issues of metallurgical slags and salt fluxes. VII. International Conference on Molten Slags, Fluxes and Salts, pp. 349–356

Shi C (2004). Steel slag-its production, processing, characteristics, and cementitious properties. *Journal of Materials in Civil Engineering* 16(3): 230–236. doi.org/10.1061/(asce)0899-1561(2004)16:3(230)

Song Q, Guo M Z, Wang L & Ling T C (2021). Use of steel slag as sustainable construction materials: A review of accelerated carbonation treatment. *Resources, Conservation and Recycling* 173: 105740. doi.org/10.1016/j.resconrec.2021.105740

Tozsin G, Yonar F, Yucel O & Dikbas A. (2023) Utilization possibilities of steel slag as backfill material in coastal structures. *Scientific Reports* 13(1): 1–11. doi.org/10.1038/s41598-023-31156-z

TSPA (Turkish Steel Producers Association) (2015). Technical Report, Ankara, Turkey

TSPA (Turkish Steel Producers Association) (2018). Technical Report, Ankara, Turkey

Uibu M, Kuusik R, Andreas L & Kirsimae K (2011). The CO_2-binding by Ca-Mg-silicates in direct aqueous carbonation of oil shale ash and steel slag. *Energy Procedia* 4: 925-932. doi.org/10.1016/j.egypro.2011.01.138

Unal S, Yucel O, Kurt M & Gul S (2014). Iron-steel slag from waste to product. Advanced Technologies Workshop, pp. 255–267

USDA (United States Department of Agriculture) (1999). Liming to improve soil quality in acid soils. Technical Note, No:8. Soil Quality Institute, Auburn, AL

Uysal F F & Bahar S (2018). Cüruf çeşitleri ve kullanım alanları. *Trakya University Journal of Engineering Sciences* 19(1): 37-52

Wang W, Sardans J, Lai D Y F, Wang C, Zeng C, Tong C & Liand Y (2015). Effects of steel slag application on greenhouse gas emissions and crop yield over multiple growing seasons in a subtropical paddy field in China. *Field Crops Research* 171: 146-156. doi.org/10.1016/j.fcr.2014.10.014

Wang W, Lai D Y F, Abid A A, Neogi S, Xu X & Wang C (2018a). Effects of steel slag and biochar incorporation on active soil organic carbon pools in a subtropical paddy field. *Agronomy* 8(8): 1-17. doi.org/10.3390/agronomy8080135

Wang W, Sardans J, Wang C, Zeng C, Tong C, Bartrons M & Peñuelas J (2018b). Steel slag amendment increases nutrient availability and rice yield in a subtropical paddy field in China. *Experimental Agriculture* 54(6): 842-856. doi.org/10.1017/s0014479717000412

Wang X, Ni W, Li J, Zhang S & Li K (2021). Study on mineral compositions of direct carbonated steel slag by QXRD, TG, FTIR, and XPS. *Energies* 14(15): 4489. doi.org/10.3390/en14154489

Wen T, Yang L, Dang C, Miki T, Bai H & Nagasaka T (2020). Effect of basic oxygen furnace slag on succession of the bacterial community and immobilization of various metal ions in acidic contaminated mine soil. *Journal of Hazardous Materials* 388:121784. doi.org/10.1016/j.jhazmat.2019.121784

Winkler H (2011). Closed-loop production systems-a sustainable supply chain approach. *Journal of Manufacturing Science and Technology* 4(3): 243–246. doi.org/10.1016/j.cirpj.2011.05.001

Xian W & QingSheng C (2006). Steel slag as an iron fertilizer for corn growth and soil improvement in a pot experiment. *Pedosphere* 16(4): 519-524. doi.org/10.1016/s1002-0160(06)60083-0

Xue P, Xu A, He D, Yang Q, Liu G, Engström F & Björkman B (2016). Research on the sintering process and characteristics of belite sulphoaluminate cement produced by BOF slag. *Construction and Building Materials* 122: 567-576. doi.org/10.1016/j.conbuildmat.2016.06.098

Yang Y, Reijonen I, Yu H, Dharmarajan R, Seshadri B & Bolan N S (2018). Back to basic slags as a phosphorous source and liming material. In: Soil amendments for sustainability: Challenges and Perspectives, CRC Press, Taylor&Francis Group, USA, pp. 237-250

Yi H, Xu G, Cheng H, Wang J, Wan Y & Chen H (2012). An overview of utilization of steel slag. *Procedia Environmental Sciences* 16: 791-801. doi.org/10.1016/j.proenv.2012.10.108

Yildirim I & Prezzi M (2011). Chemical, mineralogical, and morphological properties of steel slag. *Advances in Civil Engineering* 2011: 1-13. doi.org/10.1155/2011/463638

Yonar F (2017). Investigation of electric arc furnace slag utilization in flexible highway layers and mixture performance. Doctoral thesis. Istanbul Technical University, Institute of Science, Turkey, 264 pp

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Determination of Rheological and Chemical Properties of Hemp, Rosehip Seed and Safflower Flours

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ABSTRACT

The use of alternative flour in the production of cereal products such as bread can improve the functional and nutritional properties of bread. However, the addition of substitute flour to wheat flour may have some technological, sensory, and rheological disadvantages. To eliminate these problems, alternative flours should be used in different proportions. The aim of this study is to evaluate the remaining proportions of hemp, safflower and rosehip seeds after oil extraction and to determine their chemical properties (protein, fat, ash) and to investigate the effect on rheological dough properties with Mixolab when used as a substitute for wheat flour. Accordingly, safflower, hemp and rosehip seed flour were added to wheat flour in five different amounts (5, 7.5, 10, 15 and 20%), focusing on displacement. The addition of flour increased the protein, fat, ash, phenolic content, and antioxidant capacity. It was found that the addition of 10% safflower and hemp seed flour and 7.5% rosehip seed flour had a positive effect on rheological properties. For all three flour additions, the dough development time, stability, and percent protein softening values increased, while water holding capacity decreased. Flours added at 15% or more began to negatively affect the rheological properties. This study shows that 3 different flours, which can be added up to 10%, improve the rheological properties, chemically enrich them and improve their functional properties.

Keywords: Alternative flour, Cannabis, Mixolab, Protein weakening

1. Introduction

Wheat is one of the cereals used for bread making. However, breads made from wheat flour dough are considered nutritionally inadequate (Sabanis & Tzia 2009). The partial replacement of wheat flour with non-wheat flour increases the nutritional quality and flavour of bakery products. In this context, blended flours based on wheat and other grains and non-cereal seeds (e.g., sunflower, amaranth, quinoa, lupin, chickpea, flaxseed, chia, hemp, teff) have become popular in baking technology and play several roles, such as improving the rheological properties of the dough and increasing the overall bread quality and nutritional value (Švec & Hrušková 2015). Alternative products are added to wheat flour to improve the properties of various cereal products. The use of small millet flour to improve starch digestibility in bread making (Sharma & Gujral 2019), the use of tomato seed powder to improve dough rheology (Mironeasa & Codină 2019), the production of cookies with barley flour (Jukić et al. 2022), making cakes with chickpea and chestnut flour (Gallego et al. 2022), a flour blend enriched with lentil flour (Bouhlal et al. 2019), making cakes with pigeon pea and sweet potato (Olatunde et al. 2019). However, the rapidly growing global population, the uncertainties of war, changing consumer preferences and the resulting increase in demand for food, and the growing food problem due to drought have accelerated the search for alternatives. Moreover, many projects are being carried out around the world about innovative ways to recycle waste, and the importance of zero waste is a growing concern. In line with this, this study evaluates the waste components of safflower, hemp and rosehip seeds, especially after oil extraction, in the production of cereal-based products.

Safflower (*Carthamus tinctorius* L.) is a member of the family Compositae or Asteraceae. It is an annual herbaceous plant, cultivated mainly for its seed which is used for the production of edible oil and bird seed (Malakian et al. 2011). Safflower contains pigments (cardamom), lignans, polysaccharides, essential oils and fatty acids (arachidonic acid, linoleic acid, linolenic acid, palmitic acid and stearic acid). The nutritional value is associated with a high content of proteins, essential and balanced amino acid contents (Polushkin 2007). The use of its seeds is an intriguing and promising area for the processing branches of the food industry. Although studies on safflower have mainly focused on safflower oil, there are studies on the production of healthy drinks with high antioxidant activity (Kim et al. 2002), breads enriched with functional components (Kutsenkova et al. 2019), and cookies with increased protein content (Kutsenkova et al. 2020) using safflower seeds.

Cannabis sativa L., commonly known as hemp, is an annual herbaceous plant in the Cannabaceae family that is cultivated in China and Canada (Jian et al. 2018). Industrial hemp seeds are a novel variety of cannabis which contain low levels of δ-9-tetrahydrocannabinol, with a concentration of less than 0.3%; industrial hemp seeds are now legally in dozens of countries worldwide (Wei et al. 2021). In recent years, hemp cultivation is permitted in Turkey with the condition of being controlled by the government.

Hemp has been an important source of raw materials for the processing industry, including the use of hemp seeds, fiber, clothing, chemicals, bioenergy, flour, and oil (Grof 2018). Hemp seeds contain 32% protein, 43.7% fat, and 10.3% carbohydrates, as well as a significant amount of dietary fiber, vitamins, and minerals (Bartkiene et al. 2016). The oil from hemp seeds is rich in polyunsaturated fatty acids, proteins, and essential amino acids (Malomo et al. 2014). Moreover, in the last decade, studies on hemp proteins (Mikulec et al. 2019), whose biological value is comparable to that of chicken egg white, have shown that hemp proteins have benefits such as the regulation of cholesterol and serum glucose levels with antioxidant, and anticarcinogen activity (Wei et al. 2021). Due to their high protein, dietary fiber, and oil content, hemp seeds (*Cannabis sativa* L.) have emerged as an alternative product to fortify cereal products by adding hemp flour (Bartkiene et al. 2016; Mikulec et al. 2019) to sourdough (Nionelli et al. 2018), pasta (Schettino et al. 2019), crackers (Radočaj et al. 2014), gluten-free cookies, gluten-free bread (Korus et al. 2017), and energy bars (Norajit et al. 2011).

Rose hips (Rosa spp.) are members of the genus Rosa up to 200 species are cultivated worldwide, of which 25% are found in Turkey (Murathan et al. 2016a). Rosehip fruits are rich sources of bioactive compounds, phytonutrients, and minerals (Murathan et al. 2016b). Due to these compounds, their fruits and seeds have been used for their prophylactic and therapeutic effects against infectious and inflammatory diseases, diabetes, gastrointestinal diseases, colds, diarrhea, and urinary tract diseases (Ilyasoğlu 2014). A daily consumption of 45 g of rosehip powder lowers serum levels of C-reactive protein and ceratin in patients with osteoarthritis (Szentmihályi et al. 2002). Rosehip fruits are generally consumed in dried form as nectar and fruit tea, or after processing into jam, marmalade or gel (Gül & Şen 2017). The inside of the fruit is hairy and has 20-44% seeds (Yıldız & Nergiz 1996). These seeds, by-products of the rosehip industry are generally used as animal feed, containing even greater amounts of specific nutritionally valuable and biologically active components. The seed contains 8.72% crude protein, 31.56-44.05% crude fiber, 7.97% crude oil, and 1.87% ash (Esenbuga et al. 2011). Studies on rose hips generally refer to the fruit itself and the oil extracted from the seed. Bread (Gül & Şen 2017), Turkish noodles (Koca et al. 2018) and probiotic yogurt (Gürbüz 2021) are produced from rosehip seed flour, and there remain few studies in this area.

The aim of this study is to evaluate the changes in rheological properties due to the addition of 5 different amounts of alternative flours to wheat flour using the Mixolab® instrument (gluten, kneading index, flour water removal, amylase activity and retrogradation). No study was found in which the rheological properties of hemp seed flour, rosehip seed meal, and safflower seed meal were determined using the Mixolab® instrument. In this study, the chemical properties of the alternative flours were determined and the changes in rheological properties when 3 different flours were added to wheat flour were investigated on the basis of displacement.

2. Material and Methods

2. 1. Materials

Wheat flour (0.7< % ash ≤0.8) was obtained from Birsan Birlik Gıda San. A.Ş. in Tokat, Turkey, and stored at 4 °C. Hemp seed, rosehip seed and safflower flours were purchased from Hempium Gıda in Amasya, Turkey and stored at 4 °C. All samples were degreased by the cold-pressing method. The parts remaining after oil extraction were used by grinding. All flours were stored in a cool and dry environment until use. Folin-Ciocalteu reagent was obtained from Merck (Germany), 3,4,5-trihydroxybenzoic acid (gallic acid) were obtained from Alfa Aesar (Germany), and the Ankom XT4 cartridges were purchased from Ankom Technology (USA). The other chemicals used were standard analytical standard.

2.1. Analytical methods

The dry matter and ash content of the flours were determined by the gravimetric method (AOAC 2000). The total carbohydrate content of the samples was determined by the phenol-sulfuric acid method (Geater & Fehr 2000). The micro-kjeldahl (Gerhardt KB8, Königswinter, Germany) method was used to analyse the nitrogen content of the samples (AOAC 2000) the crude protein contents of the samples were estimated using a conversion factor of 5.75. The total fat content was determined using an Ankom Fat Analyser (Ankom Technology Corp., Macedon, NY, USA), following the Ankom Technology Method.

2.3. Total phenolic content and antioxidant capacity analysis

The phenolic content of the sample was extracted by mixing 20 mL of acidified methanol (HCl/methanol/water, 1:80:10, v/v) and 1 g of sample, and the mixture was kept in a water bath (Memmert, Germany) at 25 °C for 2 hours. It was then centrifuged at 3000 rpm for 10 minutes. The clear fraction was removed and stored at -18 °C until analysis (Beta et al. 2005).

Determination of total phenolic compounds: Analysis with 2 N Folin-Ciocalteu phenol reagent was performed according to the method described by Singleton and Rossi (1965). The results were calculated as "gallic acid equivalent".

Determination of antioxidant capacity by the method FRAP: It was performed according to the method described by Benzie and Strain (1996), and the results were calculated as "trolox equivalents".

DPPH radical scavenging activity: The antioxidant capacity was determined by the DPPH (2,2 diphenyl-1-picrylhydrazil) method described by Brand-Williams et al. (1995).

2.4. Determination of rheological behaviour using mixolab®

The dough rheological investigations were performed using Mixolab® (Chopin, Tripette et Renaud, Paris, France), which simultaneously determinates dough characteristics during the process of mixing at a constant temperature, as well as during the period of constant heating and cooling. All the measurements were performed using the modified Mixolab® 'Chopin' protocol (ICC No. 173) the parameters of which are presented in Table 1. The alternative flours were added to wheat flour at 5 different rates (5, 7.5, 10, 15, and 20%) on a displacement basis. The amount of flour required for the analysis was calculated by the Mixolab® software based on the values entered for flour mixture moisture and water absorption. Values C1 at 8 min, C2, C3, C4, and C5 were used to calculate Mixolab® parameters protein weakening, breakdown, and retrogradation (Moza & Gujral 2018).

$$\text{Protein weakening (\%)} = (\text{C1 at 8 min} - \text{C2}) / \text{C2} \times 100 \quad (1)$$

$$\text{Breakdown (\%)} = (\text{C3} - \text{C4}) / \text{C3} \times 100 \quad (2)$$

$$\text{Retrogradation (\%)} = (\text{C5} - \text{C4}) / \text{C5} \times 100 \quad (3)$$

where *C1* is the torque at 8 min (Nm), *C2, C3, C4 and C5* is the torque (Nm).

Table 1- Mixolab parameters used in modified Chopin + protocol

Settings	***Values***
Mixing speed	80 rpm
Target torque (for C1)	1100 Nm
Dough weight	75.0 g
Tank temperature	30 °C
Temperature 1st step	30 °C
Duration 1st step	8 min
1st temperature gradient	15 min 4 °C / min
Temperature 2nd step	90 °C
Duration 2nd step	7 min
2nd temperature gradient	10 min -4 °C / min
Temperature 3rd step	50 °C
Duration 3rd step	5 min
Total analysis time	45 min

2.5. Statistical analysis

SPSS statistical program (SPSS, Inc., Chicago, IL, USA) was used, a variance analysis of the results (ANOVA) was performed and the differences between the groups were assessed statistically at a 95% confidence interval using the Duncan multiple comparison test.

3. Results and Discussion

Research on wheat flour substitutes continues in the food industry, which is becoming increasingly important due to global warming and drought. In this study, safflower seeds used in edible oil production and hemp and rosehip seeds used in the cosmetic industry were used. The chemical (moisture, protein, fat, total carbohydrate and ash) and (total phenolic content and total antioxidant activity) functional properties of the mixtures processed into flour were determined and are shown in Table 2.

In the analyses conducted as part of the evaluation of these valuable ingredients, which are commonly used as feed, waste and human food, hemp and safflower seed powders stand out due to their high protein content. While hemp seed powder had a protein content of 36.38% and safflower seed powder had a protein content of 35.27%, the value for rosehip seed flour was 12.93%. The pre-drying process of the solids remaining after oil extraction before milling reduced their moisture content and ranged from 4.53 to 6.96%. As the oils were removed from the products by cold pressing, about 6% oil was detected. The ash content of the flours ranged from 1.50-13.12%. Safflower seed, which stands out with an ash content of 13.12%, has high mineral and fiber properties. Kutsenkova et al. (2020) found that the protein content of the safflower seed was 17.6% and the oil content was 38.3%, while Gül and Şen (2017) in their study found the protein content of rosehip seed flour at 7.4%, the oil content at 4.6%, and the ash content at 2.3%, while Xu et al. (2021) reported the protein content of hemp seed as 24.8%, oil content as 35.5%, and ash content as 5.6%. In another study, rosehip seeds were reported to contain 31.56-44.05% crude fiber, 7.97% crude oil, and 1.87% ash (Esenbuga et al. 2011). The results of the chemical analysis obtained in our study were higher than those reported in the literature. The reason for this is the use of defatted waste products instead of direct seed flours in the study.

The results of the total phenolic content and total antioxidant capacity of the samples are shown in Table 2. The total phenolic content of all three flours was determined in gallic acid equivalents and varied between 2405-5160 µg GAE/g. Rosehip seed flour contains the highest total phenolic content of 5160 µg GAE/g. The total antioxidant capacity was determined by two different methods (DPPH and FRAP). As for total phenolic content, the highest total antioxidant capacity was found in the rosehip seed flour. The DPPH values of the hemp, safflower, and rosehip samples were 61.44, 68.12, and 71.56 µM TE/100g, respectively, and the values of FRAP were 48.32, 65.72, and 65.92 µM TE/100g, respectively. Yu et al. (2013) reported in their study that safflower seeds had a total phenolic content of 55.52 mg GAE/g.

Table 2- Chemical composition of flours

Properties	***Control flour***	***Hemp seed flour***	***Rosehip seed flour***	***Safflower flour***
Moisture (%)	13.48±0.23^{a}	4.53±0.14^{d}	6.96±0.23^{b}	5.14±0.16^{c}
Protein (g/100 g of dry weight)	13.20±0.45^{b}	36.38±1.02^{a}	12.93±0.64^{b}	35.27±0.96^{a}
Fat (g/100 g of dry weight)	0.80±0.10^{d}	5.64±0.61^{c}	6.45±0.30^{a}	6.12±0.46^{b}
T. Carbohydrate (g/100 g of dry weight)	72.23±1.30^{b}	52.13±1.06^{c}	79.12±1.23^{a}	45.49±1.28^{d}
Ash (g/100 g of dry weight)	0.75±0.14^{d}	4.85±0.08^{b}	1.50±0.06^{c}	13.12±0.71^{a}
Total phenolic content (µg GAE/g)	616.47±14.12^{d}	2405±45.78^{c}	5160±51.05^{a}	4762±38.46^{b}
Total antioxidant activity (µM TE/100g)	14.16±0.87^{d}	61.44±0.80^{c}	71.56±1.06^{a}	68.12±0.88^{b}
Total antioxidant activity (µM TE/100g)	29.60±1.32^{c}	48.32±0.48^{b}	65.92±1.08^{a}	65.72±0.75^{a}

^{a,b,c}: Values indicated by different letters in the same line are statistically significantly different ($p<0.05$)

Mixolab® (Chopin Technologies, Villeneuve la Garenne, France) is an instrument that is used to determine the rheological quality of the flour during bread making. The Mixolab technique allows the complete characterization of the flours in terms of the quality of proteins by determining their water absorption, stability, elasticity, and weakening properties; starch behavior during gelatinization and

retrogradation; consistency modification when adding additives and enzymatic activity of the proteases, amylases, etc. (Stoenescu et al. 2011). This device provides, with one single test, a complex analysis of the rheological properties of wheat flour dough, considering dough behavior during mixing, protein coagulation, heating-up behavior at enzyme activity intensification, and starch gelatinization and retrogradation during the final cooling (Blandino et al. 2015).

In this study, hemp seed flour, safflower seed flour, and rosehip seed flour were added to wheat flour in five different ratios (5, 7.5, 10, 15, and 20) and the rheological properties of the obtained flour mixtures were determined using the Mixolab instrument. The rheological results obtained are shown in Table 3. Mixolab torque curves of flours from hemp seed flour, safflower powder and rosehip seed flour are shown Figure 1. As seen in Table 3 the addition of three flours increases the dough development time. The dough development time, which was 1.28 minutes for the control flour, increased to 3.33 minutes for safflower flour, 4.67 minutes for hemp seed flour, and 1.59 minutes for rosehip seed flour. The dough development time increased until the addition of 15% hemp seed flour and began to decrease at 20%. The addition of rosehip seed flour resulted in a smaller increase in dough development time compared to the other two flour additions. This decrease is thought to be caused by the rich phenolic content of rosehip seed flour (Table 2). This can be explained by the ability of the phenolic compounds to react with the sulfhydryl groups of the gluten protein or to increase the rate of protein sulfhydryl-disulfide interchanges (Welc et al. 2022). In a previous study, the addition of phenolic acids to dough was reported to decrease kneading time, tolerance, elasticity, and bread volume (Han & Koh 2011).

Table 3- Results of Mixolab analysis of hemp seed flour, safflower flour and rosehip seed flour added in different amounts

Sample	Ratio	Development time (min)	Stability time (min)	C2 Torque (Nm)	C3 Torque (Nm)	C4 Torque (Nm)	C5 Torque (Nm)	α	β	γ	Water absorption (%)
Control	0.0	1.28±0.01^{h}	3.73±0.05^{n}	0.33±0.01^{d}	1.64±0.01^{cd}	1.41±0.02^{c}	2.11±0.06^{a}	-0.090	0.590	-0.016	57.50±0.06^{a}
Safflower powder ratio (%)	5.0	1.23±0.02^{hı}	6.20±0.11^{jı}	0.27±0.01^{f}	1.54±0.02^{e}	1.30±0.01^{d}	1.75±0.02^{c}	-0.082	0.078	-0.018	56.20±0.05^{b}
	7.5	1.37±0.01^{g}	6.36±0.06^{ı}	0.28±0.03^{f}	1.51±0.03^{f}	1.31±0.01^{d}	1.73±0.03^{cd}	-0.073	0.237	-0.044	56.10±0.04^{b}
	10.0	1.56±0.05^{f}	5.53±0.07^{k}	0.25±0.02^{g}	1.48±0.02^{g}	1.30±0.01^{d}	1.72±0.02^{d}	-0.074	0.106	-0.014	55.60±0.04^{b}
	15.0	3.12±0.08^{e}	5.27±0.02^{l}	0.23±0.03^{gh}	1.41±0.02^{h}	1.27±0.02^{e}	1.73±0.03^{cd}	-0.060	0.060	-0.028	56.30±0.03^{b}
	20.0	3.33±0.06^{d}	4.55±0.08^{m}	0.23±0.02^{gh}	1.34±0.03^{ı}	1.22±0.03^{ef}	1.65±0.04^{e}	-0.062	0.144	-0.060	56.00±0.05^{b}
Hemp seeds flour ratio (%)	5.0	1.53±0.02^{f}	6.88±0.14^{h}	0.34±0.05^{d}	1.60±0.02^{d}	1.40±0.01^{c}	1.74±0.01^{c}	-0.086	0.394	-0.052	57.70±0.06^{a}
	7.5	3.47±0.11^{d}	7.38±0.11^{g}	0.35±0.04^{d}	1.65±0.01^{c}	1.44±0.02^{b}	1.79±0.02^{bc}	-0.100	0.550	-0.004	56.60±0.03^{b}
	10.0	4.53±0.12^{b}	7.46±0.06^{f}	0.32±0.02^{de}	1.57±0.01^{e}	1.47±0.01^{a}	1.81±0.02^{b}	-0.090	0.297	0.014	55.75±0.04^{c}
	15.0	4.67±0.10^{a}	7.88±0.11^{d}	0.30±0.01^{e}	1.53±0.03^{ef}	1.38±0.02^{c}	1.82±0.01^{b}	-0.112	0.282	-0.004	55.20±0.11^{c}
	20.0	3.95±0.09^{c}	6.75±0.09^{h}	0.28±0.01^{f}	1.47±0.02^{g}	1.35±0.01^{d}	1.79±0.02^{bc}	-0.076	0.444	-0.024	54.50±0.13^{c}
Rosehip seed flour ratio (%)	5.0	1.53±0.02^{f}	7.53±0.03^{ef}	0.37±0.01^{c}	1.55±0.01^{e}	1.24±0.01^{e}	1.59±0.01^{f}	-0.088	0.356	-0.036	56.90±0.09^{b}
	7.5	1.44±0.01^{g}	8.28±0.10^{b}	0.38±0.02^{ab}	1.63±0.01^{cd}	1.25±0.02^{e}	1.54±0.01^{g}	-0.089	0.395	-0.067	56.55±0.10^{b}
	10.0	1.07±0.01^{j}	7.60±0.11^{e}	0.38±0.02^{ab}	1.68±0.01^{b}	1.20±0.01^{f}	1.53±0.01^{g}	-0.102	0.424	-0.063	56.10±0.08^{b}
	15.0	1.59±0.03^{f}	8.96±0.15^{a}	0.39±0.02^{ab}	1.70±0.02^{a}	1.10±0.03^{g}	1.46±0.01^{h}	-0.101	0.475	-0.054	55.80±0.08^{c}
	20.0	1.17±0.06^{hı}	8.18±0.11^{c}	0.40±0.01^{a}	1.70±0.02^{a}	0.95±0.04^{h}	1.29±0.05^{ı}	-0.106	0.492	-0.041	55.70±0.10^{c}

$^{a,b,c,\ldots ı}$: Values indicated by different letters in the same column are statistically significantly different ($p<0.05$)

As shown in Table 3, the addition of alternative flours decreases the water holding capacity. The water holding capacity, which was 57.5% for the control flour, decreased to 55.6% for safflower flour, 54.5% for hemp seed flour, and 55.7% for rosehip seed flour. The development time refers to the time elapsed until the dough first begins to form (Rosell et al. 2007). The reduction of it is technologically desirable (Pala 2012). The dough stability (min) is lower than 11% of the maximum consistency reached during the mixing. The dough stability time of the samples are shown in Table 3 and Figure 1. The stability value, which was 3.73 minutes for the control, increased to

6.37 minutes for safflower flour, 7.46 minutes for hemp seed flour, and 8.96 minutes for rosehip seed flour. Rosehip seed flour provided the highest value for dough stability. The high protein content of the added alternative flours increased the stability values.

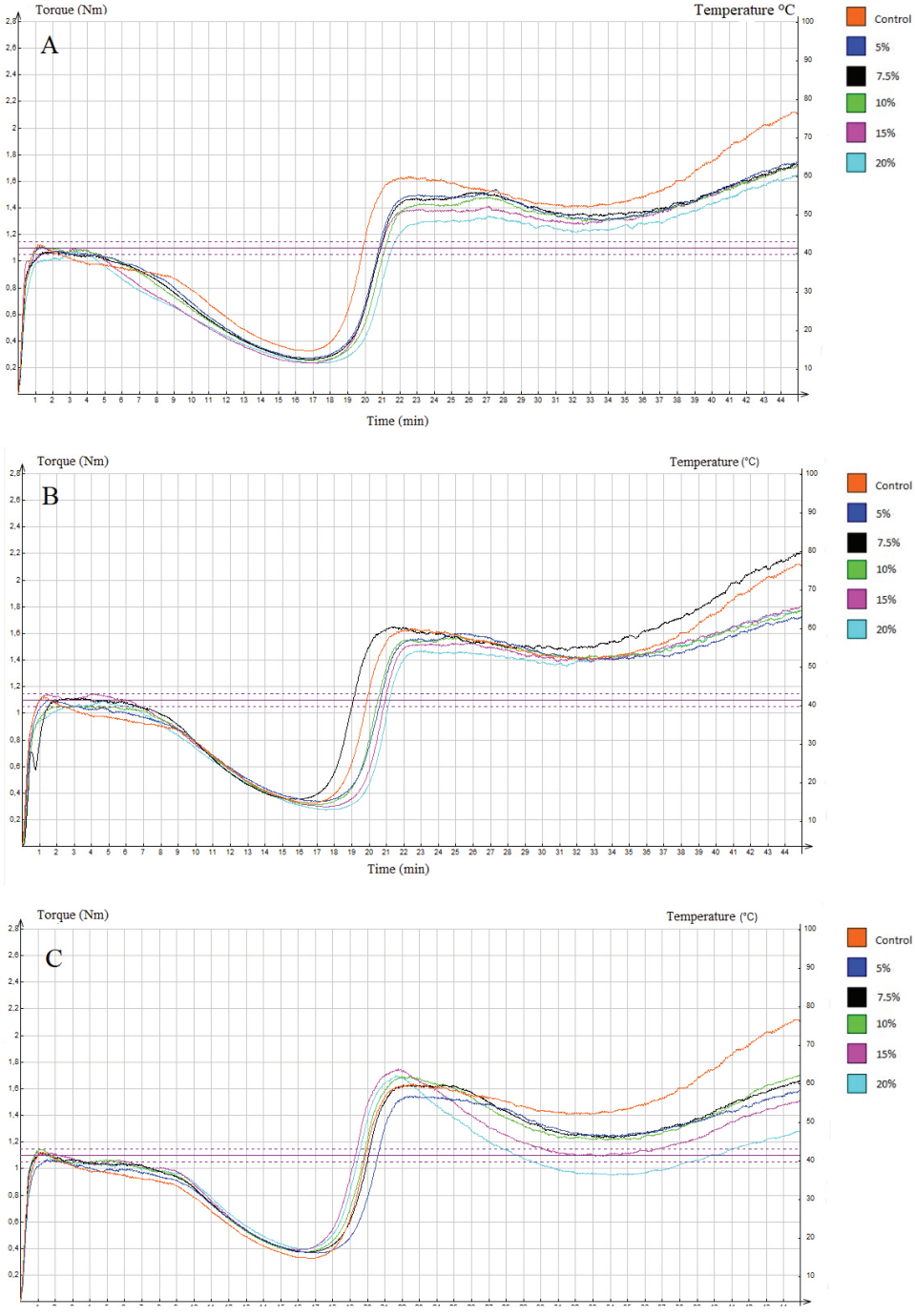

Figure 1- Mixolab torque curves of alternative flours (A) Mixolab torque curves (Nm) of safflower powder flours; (B) Mixolab torque curves (Nm) of cannabis seeds flours; (C) Mixolab torque curves (Nm) of rosehip seed flour

While the C2 torque value of 0.49 Nm in the control flour decreased with the addition of safflower and hemp seed flour, it increased with the addition of rosehip seed flour (Table 3). The rosehip seed flour addition increased the C2 value above 0.40. It is desirable that the proteins in the dough do not weakening during kneading and maintain the network structure (Cappelli et al. 2020). It is predicted that the structural properties of the proteins in rosehip flour are more robust. The C3 torque value, which gives information about the

gelatinization value of the starch, decreased with the addition of safflower and hemp flour, while it increased with the addition of rosehip flour (Table 3). It is thought that the high amount of protein (~35%) in safflower and hemp flours causes this.

It was found that C4 value of 1.41 Nm in the control flour decreased for all three flour additives, with the greatest decrease for rosehip seed flour. It was found that the addition of hemp seed flour lowered the C4 torque value less than other flours and was close to the control flour for some values (Table 3). It is found that the addition of rosehip seeds and safflower flour to the dough causes the starch gel formed during cooking to break down more quickly. It is thought that the addition of hemp seed flour causes the structure of the starch gel to remain stable for a longer period of time (Figure 1).

The viscosity of the dough increased due to the retrogradation of starch as the temperature decreased from 90 °C to 50 °C. C5 value, which was 2.11 Nm for the control flour, decreased to 1.65, 1.74 and 1.29 Nm, respectively, with the addition of safflower, hemp and rosehip seed flour. The addition of safflower, hemp, and rosehip seed flours to wheat flour reduced the water-holding capacity, the mesh structure of the dough was damaged due to the proportional reduction in the amount of gluten, and staling accelerated due to the breaking of the gel structure of the starch.

It has been found that the addition of safflower flour to wheat flour increases the α angle, while the addition of hemp and rosehip seed flour decreases it. β, which was 0.590 for the control flour, decreased to 0.060 with the addition of safflower flour, to 0.282 with the addition of hemp flour, and to 0.356 with the addition of rosehip seed flour (Table 3). The addition of rosehip seed flour is noticeable as the flour that has the least effect on dough viscosity. The gamma angle increased for all three flour additives, with the increase being greatest for rosehip seed flour. It was considered that the increase in the amount of phenolic content, that could act as amylase inhibitor caused this increase. An increase in γ angle may be an indication of slowing amylase activity or a decreasing rate of enzymatic degradation. It should be taken into account that reduced amylase activity may have negative effects on the rising of the dough and the internal pore structure.

Table 4- Mixolab characteristics of hemp seed flour, safflower flour and rosehip seed flour added in different amounts

Sample	*Ratio*	*C1 (Nm)*	*Protein weakening (%)*	*Breakdown (%)*	*Retrogradation (%)*
Control	0.0	0.93±0.01^{c}	64.65	14.17	33.41
Safflower powder (%)	5.0	0.89±0.01^{d}	69.38	15.33	25.71
	7.5	0.88±0.02^{d}	68.89	13.74	24.33
	10.0	0.82±0.03^{e}	69.15	12.17	24.61
	15.0	0.74±0.02^{f}	68.03	9.57	26.11
	20.0	0.71±0.01^{g}	67.32	9.26	26.23
Hemp seeds flour (%)	5.0	0.94±0.03^{c}	63.96	12.62	19.60
	7.5	0.99±0.05^{a}	64.38	10.92	33.50
	10.0	0.96±0.02^{b}	66.42	8.46	20.35
	15.0	0.96±0.02^{b}	69.10	9.66	23.75
	20.0	0.92±0.03^{c}	70.04	8.03	24.26
Rosehip seed flour (%)	5.0	0.94±0.02^{c}	61.25	19.55	22.02
	7.5	0.99±0.01^{a}	61.13	21.40	21.16
	10.0	0.97±0.02^{ab}	61.05	29.36	23.59
	15.0	1.00±0.01^{a}	60.01	33.21	21.51
	20.0	1.01±0.01^{a}	59.76	43.74	25.53

$^{a,b,c,...g}$: Values indicated by different letters in the same column are statistically significantly different ($p<0.05$). C1 is dough consistency after mixing; percent protein weakening is (C1at 8 min-C2)/C1%; C3 is peak consistency; percent breakdown is (C3-C4)/C3%; percent retrogradation is (C5-C4)/C5%

The Mixolab behavior of different flours is shown in Table 4. The value of C1 varied significantly within the varieties and ranged from 0.71 to 1.01 Nm for the alternative flour blends. After 8 minutes of mixing the temperature begins to increase (the viscosity of the dough decreases), excessive kneading leads to a weakening of the protein and a decrease in dough strength, and a minimum torque

C2 is reached. This decrease in dough strength is caused by physical protein breakdown and protein denaturation during heating. Mixolab measures protein weakening as a function of mechanical work (Rosell et al. 2007). The percent protein weakening (%) was calculated for the samples and ranged from 59.76 to 70.04% for alternative flour blends and 64.95% for control flours. The percent protein weakening of rosehip seed flour, which has a lower protein content than the other two flours, was found to be lower. Rosell et al. (2010) reported that the addition of dietary fiber to wheat flour may hinder protein folding and delay protein weakening. The percent breakdown (%) of flours was reported (Table 4) and ranged from 8.03 to 43.74% for alternative flour blends and 14.17% for control flour, respectively. The amylose chains recrystallize upon cooling, resulting in starch gelation (Rosell et al. 2010). The percent retrogradation of the flours was estimated and ranged from 19.60 to 33.50% for the alternative flour blends and 33.41% for the control flour, respectively. Retrogradation is predicted to decrease as a result of the decrease in water-holding capacity and gluten content.

5. Conclusions

This study investigated the changes in chemical, functional, and rheological properties of doughs caused by the addition of alternative flours of hemp, safflower, and rosehip seed waste to wheat flour. The addition of safflower, hemp and rosehip seed flours increased the content of proteins and phenolic compounds. The study showed that the addition of 3 different flours in 5 different amounts affected the rheological properties of the dough. It was found that the added flours increased the percent protein wake value and decreased the percent retrogradation value. It was also found that although the water holding capacity of the dough decreased to some extent, the dough development time and dough stability increased. The results showed that safflower, hemp and rosehip seed flours used as substitutes for wheat flour reduced the starch gelatinization rate. Safflower and hemp flour were found to have a greater effect on rheological properties, while rosehip flour had similar rheological properties to the control flour. Analyzing all the data obtained, it was found that the addition of 7.5% hemp seed flour, 5% safflower powder flour and 10% rosehip seed flour to wheat flour did not negatively affect the rheological properties of the dough. Furthermore, it was found that all added flours improved the protein content and functional properties of the control flour. This study aims to fill the gap in the literature for determining the rheological properties required for bread making by substituting safflower, hemp seed, and rosehip flours for wheat flour. In addition, the results of this study provide data on the potential of using three different flours, which are waste materials, in the food industry.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: A.C., Design: A.C., N.Ş., Data Collection or Processing: A.C., N.Ş., Analysis or Interpretation: A.C., N.Ş., Literature Search: A.C., Writing: A.C., N.Ş.

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References

AOAC (2000). AOAC Official Method. Association of Official Agricultural Chemists. Department of Agriculture

Bartkiene E, Schleining G, Krungleviciute V, Zadeike D, Zavistanaviciute P, Dimaite I, Kuzmaite I, Riskeviciene V & Juodeikiene G (2016). Development and quality evaluation of lacto-fermented product based on hulled and not hulled hempseed (Cannabis sativa L.). *LWT-Food Science and Technology* 72: 544-551. doi.org/10.1016/j.lwt.2016.05.027

Benzie I F F & Strain J J (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry* 239(1): 70-76. doi.org/10.1006/abio.1996.0292

Beta T, Nam S, Dexter J E & Sapirstein H D (2005). Phenolic content and antioxidant activity of pearled heat and roller-milled fractions. *Cereal Chemistry* 82(4): 390-393. doi.org/10.1094/cc-82-0390

Blandino M, Locatelli M, Gazzola A, Coïsson J D, Giacosa S, Travaglia F, Bordiga M, Reyneri A, Rolle L & Arlorio M (2015). Hull-less barley pearling fractions: Nutritional properties and their effect on the functional and technological quality in bread-making. *Journal of Cereal Science* 65: 48-56. doi.org/10.1016/j.jcs.2015.06.004

Bouhlal O, Taghouti M, Benbrahim N, Benali A, Visioni A & Benba J (2019). Wheat-lentil fortified flours: health benefits, physicochemical, nutritional and technological properties. *Journal Materials Environmental Science* 10(11): 1098-1106.

Brand-Williams W, Cuvelier M & Berset C (1995). Use of free radical method to evaluate antioxidant activity. *Food Science and Technology* 28(1): 25-30. doi.org/10.1016/s0023-6438(95)80008-5

Cappelli A, Bettaccini L & Cini E (2020). The kneading process: A systematic review of the effects on dough rheology and resulting bread characteristics, including improvement strategies. *Trends in Food Science & Technology* 104: 91-101. doi.org/10.1016/j.tifs.2020.08.008

Esenbuga N, Macit M, Karaoglu, M, Aksakal V, Yoruk M A, Gül M, Aksu M İ & Bilgin Ö C (2011). A study on possibility of Rosa canina seed use as feed ıngredient in diets of Morkaraman male lambs. *Tropical animal health and production* 43(7): 1379-1384. doi.org/10.1007/s11250-011-9857-6

Gallego C, Belorio M, Guerra-Oliveira P & Gómez M (2022). Effects of adding chickpea and chestnut flours to layer cakes. *International Journal of Food Science & Technology* 57(8): 4840-4846. doi.org/10.1111/ijfs.15719

Geater C W & Fehr W R (2000). Association of total sugar content with other seed traits of diverse soybean cultivars. *Crop Sci* 40(6): 1555-1558. doi.org/10.2135/cropsci2000.4061552x

Grof C P L (2018). Cannabis, from plant to pill. *British Journal of Clinical Pharmacology* 84(11): 2463-2467. doi.org/10.1111/bcp.13618

Gül H & Şen H (2017). Effects of rosehip seed flour on the rheological properties of bread dough. *Scientific Bulletin. Series F. Biotechnologies* 21: 330-335.

Gürbüz B (2021). Production of probiotic yogurt enriched with rosehip seed powder. Master's Degree Thesis. Tekirdağ Namık Kemal University.

Han H M & Koh B K (2011). Effect of phenolic acids on the rheological properties and proteins of hard wheat flour dough and bread. *Jorunal of the Sci. Food Agric* 91(13): 2495-2499. doi.org/10.1002/jsfa.4499

Ilyasoğlu H (2014). Characterization of Rosehip (Rosa canina L.) Seed and Seed Oil. *International Journal of Food Properties* 17(7): 1591-1598. doi.org/10.1080/10942912.2013.777075

Jian F, Divagar D, Mhaiki J, Jayas D S, Fields P G & White N D G (2018). Static and dynamic methods to determine adsorption isotherms of hemp seed (Cannabis sativa L.) with different percentages of dockage. *Food Science & Nutrition* 6(6): 1629-1640. doi.org/10.1002/fsn3.744

Jukić M, Nakov G, Komlenić D K, Vasileva N, Šumanovac F & Lukinac J (2022). Quality Assessment of Cookies Made from Composite Flours Containing Malted Barley Flour and Wheat Flour. *Plants* 11(6): 761. doi.org/10.3390/plants11060761

Kim J H, Park J H, Park S D, Choi S Y, Seong J H & Moon K D (2002). Preparation and antioxidant activity of health drink with extract powders from safflower (Carhamus tinctorius L.) seed. *Korean J. Food Sci. Technol* 34(4): 617-624.

Koca I, Tekguler B, Yilmaz V A, Hasbay I & Koca A F (2018). The use of grape, pomegranate and rosehip seed flours in Turkish noodle (erişte) production. *Journal of Food Processing and Preservation* 42(1): e13343 doi.org/10.1111/jfpp.13343

Korus J, Witczak M, Ziobro R & Juszczak L (2017). Hemp (Cannabis sativa subsp. sativa) flour and protein preparation as natural nutrients and structure forming agents in starch based gluten-free bread. *LWT* 84: 143-150. doi.org/10.1016/j.lwt.2017.05.046

Kutsenkova K S, Nepovinnykh N V, Andreeva L V & Guo Q (2019). Textural Properties of Dough and Sensory Characteristics of Bread Enriched with Crushed Safflower Seeds. *Russian Agricultural Sciences* 45(5): 492-497. doi.org/10.3103/s1068367419050094

Kutsenkova V S, Nepovinnykh N V & Guo Q (2020). Using of safflower seeds as a protein fortifier for shortbread. *Food Hydrocolloids* 105: 105808. doi.org/10.1016/j.foodhyd.2020.105808

Malakian M, Hassanabadi A & Heidariniya A (2011). Effects of safflower seed on performance, carcass traits and blood parameters of broilers. *Res. J. Poult. Sci* 4(2): 18-21. doi.org/10.3923/rjpscience.2011.18.21

Malomo S A, He R & Aluko R E (2014). Structural and functional properties of hemp seed protein products. *Journal of Food Science* 79(8): 1512-1521. doi.org/10.1111/1750-3841.12537

Mikulec A, Kowalski S, Sabat R, Skoczylas Ł, Tabaszewska M & Wywrocka-Gurgul A (2019). Hemp flour as a valuable component for enriching physicochemical and antioxidant properties of wheat bread. *LWT* 102: 164-172. doi.org/10.1016/j.lwt.2018.12.028

Mironeasa S & Codină G G (2019). Dough rheological behavior and microstructure characterization of composite dough with wheat and tomato seed flours. *Foods* 8(12): 626. doi.org/10.3390/foods8120626

Moza J & Gujral H S (2018). Mixolab, retrogradation and digestibility behavior of chapatti made from hulless barley flours. *Journal of Cereal Science* 79: 383-389 doi.org/10.1016/j.jcs.2017.11.003

Murathan Z T, Zarifikhosroshahi M & Kafkas N E (2016a). Determination of fatty acids and volatile compounds in fruits of rosehip (*Rosa* L.) species by HS-SPME/GC-MS and Im-SPME/GC-MS techniques. *Turkish Journal of Agriculture and Forestry* 40(2): 269-279. doi.org/10.3906/tar-1506-50

Murathan Z T, Zarifikhosroshahi M, Kafkas N E & Sevindik E (2016b). Characterization of bioactive compounds in rosehip species from East Anatolia region of Turkey. *Italian Journal of Food Science* 28(2): 314-325

Nionelli L, Montemurro M, Pontonio E, Verni M, Gobbetti M & Rizzello C G (2018). Protechnological and functional characterization of lactic acid bacteria to be used as starters for hemp (Cannabis sativa L.) sourdough fermentation and wheat bread fortification. *International journal of food microbiology* 279: 14-25. doi.org/10.1016/j.ijfoodmicro.2018.04.036

Norajit K, Gu B J & Ryu G H (2011). Effects of the addition of hemp powder on the physicochemical properties and energy bar qualities of extruded rice. *Food chemistry* 129(4): 1919-1925. doi.org/10.1016/j.foodchem.2011.06.002

Olatunde S J, Ajayi O M, Ogunlakin G O & Ajala A S (2019). Nutritional and sensory properties of cake made from blends of pigeon pea, sweet potato and wheat flours. *Food Research* 3(5): 456-462. doi.org/10.26656/fr.2017.3(5).255

Pala A (2012). The effect of the substitution of dried boza powder on dough rheology and bread quality. Master's Degree Thesis. Pamukkale University.

Polushkin P V (2007). The influence of the water regime and density of the state on the productivity of dyeing safflower on light chestnut soils of the Saratov Trans-Volga region. *Extended Abstract of Cand. Sci. (Agric.) Dissertation, Saratov.*

Radočaj O, Dimić E & Tsao R (2014). Effects of hemp (Cannabis sativa L.) seed oil press-cake and decaffeinated green tea leaves (Camellia sinensis) on functional characteristics of gluten-free crackers. *Journal of food science* 79(3): 318-325. doi.org/10.1111/1750-3841.12370

Rosell C M, Collar C & Haros M (2007). Assessment of hydrocolloid effects on the thermo-mechanical properties of wheat using the Mixolab. *Food Hydrocoll* 21(3): 452-462. doi.org/10.1016/j.foodhyd.2006.05.004

Rosell C M, Santos E & Collar C (2010). Physical characterization of fiber-enriched bread doughs by dual mixing and temperature constraint using the Mixolab®. *Eur. Food Res. Technol* 231: 535-544. doi.org/10.1007/s00217-010-1310-y

Sabanis D & Tzia C (2009). Effect of rice, corn and soy flour addition on characteristics of bread produced from different wheat cultivars. Food and Bioprocess Technology 2(1): 68-79 doi.org/10.1007/s11947-007-0037-7

Schettino R, Pontonio E & Rizzello C G (2019). Evaluation of the quality, nutritional value and antioxidant activity of gluten-free biscuits made from corn-acorn flour or corn-hemp flour composites. *European Food Research and Technology* 243(8): 1429-1438. doi.org/10.1007/s00217-017-2853-y

Sharma B & Gujral H S (2019). Modulation in quality attributes of dough and starch digestibility of unleavened flat bread on replacing wheat flour with different minor millet flours. *International journal of biological macromolecules* 141(1): 117-124. doi.org/10.1016/j.ijbiomac.2019.08.252

Singleton V L & Rossi J A (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticuture* 16: 144-158.

Stoenescu G, Ionescu V S & Banu I (2011). Rheological properties of the wheat flour supplemented with different additives. The Annals of the University Dunarea de Jos of Galati. *Fascicle VI-Food Technology* 35(1): 54-62. doi.org/10.35219/foodtechnology.2022.1.03

Švec I & Hrušková M (2015). The Mixolab parameters of composite wheat/hemp flour and their relation to quality features. *LWT-Food Science and Technology* 60(1): 623-629. doi.org/10.1016/j.lwt.2014.07.034

Szentmihályi K, Vinkler P, Lakatos B, Illés V & Then M (2002). Rose hip (Rosa canina L.) oil obtained from waste hip seeds by different extraction methods. *Bioresource technology* 82(2): 195-201. doi.org/10.1016/s0960-8524(01)00161-4

Wei L H, Dong Y, Sun Y F, Mei X S, Ma X S, Shi J, Yan Q, Ji YR, Zhang ZM, Sun HN, Sun XR, Bean S, Tilley M & Song S M (2021). Anticancer property of Hemp Bioactive Peptides in Hep3B liver cancer cells through Akt/GSK3β/β-catenin signaling pathway. *Food Science & Nutrition* 9(4): 1833-1841. doi.org/10.1002/fsn3.1976

Welc R, Kłosok K, Szymańska-Chargot M & Nawrocka A (2022). Effect of chemical structure of selected phenolic acids on the structure of gluten proteins. *Food Chemistry* 389: 133109. doi.org/10.1016/j.foodchem.2022.133109

Xu Y, Li J, Zhao J, Wang W, Griffin J, Li Y, ... & Wang D (2021). Hempseed as a nutritious and healthy human food or animal feed source: A review. *International Journal of Food Science & Technology* 56(2): 530-543. doi.org/10.1111/ijfs.14755

Yıldız H & Nergiz C (1996). Bir gıda maddesi olarak kuşburnu. *Kuşburnu Sempozyumu*. 309-318.

Yu S Y, Lee Y J, Kang S N, Lee S K, Jang J Y, Lee H K & Lee O H (2013). Analysis of food components of Carthamus Tinctorius L. seed and its antimicrobial activity. *Korean Journal of Food Preservation* 20(2): 227-233. doi.org/10.11002/kjfp.2013.20.2.227

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Toxigenic Genes of Coagulase-negative Staphylococci and *Staphylococcus aureus* from Milk and Dairy

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ABSTRACT

The study investigates the prevalence of *Staphylococcus aureus* and coagulase-negative staphylococci in raw milk and dairy products and assesses their toxin-related pathogenic potential and methicillin resistance. A total of 1015, raw milk (260) and dairy samples (325 cheeses, 180 yogurts, 140 ice creams, 110 butter samples) were collected and analyzed. The prevalence of *Staphylococcus aureus* and coagulase-negative staphylococci were 3.2% and 5.3% with mean counts of 3.46 and 3.16 log CFU/mL-g, respectively. Three (*sea, seb, see*) of five (*sea, seb, sec, sed, see*) staphylococcal enterotoxin (SE) genes, two (*tss, etb*) of four (*tss, pvl, eta, etb*) virulence-associated genes, and the absence of methicillin resistance (*mec*A) gene were defined by polymerase chain reaction. SE *sea* (6.9%), *seb* (2.3%) and *see* (1.1%) genes were detected in *Staphylococcus aureus* from one milk and seven different cheese samples. The presence of multiple enterotoxin genes (*sea* and *see*) was detected in a *Staphylococcus aureus* isolate from one cheese. However, the milk-sourced one coagulase-negative staphylococci possessed both the *tss* and *etb* virulence genes. The finding in this study indicates that the frequency of coagulase-negative staphylococci was higher than *Staphylococcus aureus* and moreover, toxin genes associated with human infections were assigned in coagulase-negative staphylococci while enterotoxin genes were determined among *Staphylococcus aureus*. In terms of food safety perspective, coagulase-negative staphylococci are ignored and they are not considered in standard food surveillance analysis. But the presence of virulent coagulase-negative staphylococci in foods is a public health concern. The results obtained from this study are significant as it demonstrates that pathogenic coagulase-negative staphylococci are found in foods, and provides data from Turkey. Additional research is required concerning coagulase-negative staphylococci in the food matrix and clinical isolates.

Keywords: Coagulase-negative staphylococci, Cheese, Milk, Enterotoxin, Virulence

1. Introduction

Staphylococcus aureus is one of the major human pathogens of worldwide bacteriological diseases. Amongst the different types of diseases, staphylococcal enterotoxins (SEs) primarily produced by certain *S. aureus* strains cause a foodborne intoxication referred to as staphylococcal food poisoning (SFP) through consumption of contaminated food (Chieffi et al. 2020). Enterotoxin-producing *S. aureus* strains are associated with food poisoning by producing heat-stable, protease- (such as pepsin and trypsin) and environmental-resistant toxins in foodstuffs (Ahmed et al. 2019; Mahanti et al. 2020). Although there are ten SEs identified, they are primarily classified into five classical types including SEA, SEB, SEC, SED and SEE. These five enterotoxins are responsible for 95% of staphylococcal intoxication cases. The remaining newly described SEG-SEI, SEIJ, SEIQ, SER-SET, and SEIU-SEIV enterotoxins are known to cause only 5% of food poisoning cases (Ahmed et al. 2019; Kou et al. 2021).

The pathogenicity of *S. aureus* strains is not only enterotoxin dependent. This pathogen also secretes strain-specific toxic shock syndrome toxin (TSST-1) which generates an immune hyper-response in the host, a leucocytolytic toxin Panton-Valentine leukocidin

(PVL), exfoliative toxins (*eta* and *etb*) implicated in human skin damage and also the toxins synthesized by most of the strains as α-toxin, γ-toxin, some leukotoxins, and phenol-soluble modulins (PSMs) (Elal Mus et al. 2019).

Antimicrobial resistance is another concern for *S. aureus* strains. In particular, methicillin resistance encoded by the *mec*A gene is widely considered to be a global health problem. The World Health Organization listed methicillin-resistant *S. aureus* as one of the three most difficult infectious agents due to its role in nosocomial infections (Kou et al. 2021).

The pathogen *S. aureus* exists in the mammary glands of dairy animals. Several risk factors associated with *S. aureus* include unhygienic milking procedures, inappropriate preventive techniques, and the lack of antiseptic use before and after milking (Aqib et al. 2018). The pathogenic *S. aureus* strains are found in raw milk and unpasteurized dairy products as a result of primer/seconder contamination (Mahanti et al. 2020). Moreover, several foods are contaminated with enterotoxigenic *S. aureus* via the hands of food handlers (Dorotíkova et al. 2022). European regulations (European Council Regulations 2005) and the Turkish Food Codex (TFC 2011) require the enumeration of coagulase-positive staphylococci and/or detection of SEs in dairy products and some specific foods. However, *S. aureus* toxins still remain one of the most important agents responsible for food poisoning. The European Union One Health Zoonoses Report revealed 43 *S. aureus* toxins outbreaks with 402 human cases in 2020. Four of these outbreaks were strong-evidenced and the number of people hospitalized totaled 32 in European Union Countries (EFSA & ECDC 2021). On the other hand, coagulase-negative staphylococci (CoNS) emerge in foods, especially ready-to-eat products. The presence of toxin genes and production ability among CoNS strains isolated from foods has been observed in recent years (Pyzik et al. 2019; Chajecka-Wierzchowska et al. 2020; França et al. 2021; Banszkiewicz et al. 2022). CoNS are a notorious opportunistic pathogen and can cause infections in immunosuppressed individuals and foreign material implantated patients (Becker et al. 2020). The presence of CoNS in food has become a growing concern in recent years.

The purpose of this study was to determine the distribution and pathogenic potential of CoNS and *S. aureus* in milk and dairy products. To this aim, some staphylococcal toxin genes associated with human infections/food intoxications and methicillin resistance gene were investigated.

2. Material and Methods

2.1. Bacterial isolation

A total of 1015 milk and dairy samples were collected from May 2017 to July 2021 in the Bursa province of Turkey. Samples included raw milk (25 goats, 80 sheep, 155 cows), cheese (325), butter (110), yogurt (180), and ice cream (140). All samples were collected from local bazaars, supermarkets, and local delicatessens. The collected samples were transferred to the laboratory in a cooler with ice packs and prepared for analyses within the same day. The prevalence of *S. aureus* and CoNS was determined using the qualitative detection method according to ISO 6888-1. Briefly, 10 g was obtained randomly from each sample and placed into a sterile stomacher bag containing 90 mL of peptone water. Following homogenization, 0.1 mL of each serial dilution solution was spread in duplicate on Baird-Parker Agar (1.05406, Merck, Germany) supplemented with egg yolk tellurite emulsion and incubated at 37 °C for 48 h. After incubation, five black colonies surrounded by a clear zone from petri dishes were separated for identification. All isolates were subjected to the Gram staining and catalase test. Gram-positive, cocci-shaped, grape-like clusters and catalase-positive isolates were evaluated as *S. aureus*. For the test of coagulase tube test, colonies were taken by inoculation loop and transferred in Brain-Heart Infusion Broth (110493 Merck, Germany) and incubated at 37 °C for 20-24 h. 0.1 mL Brain-Heart Infusion Broth culture mixed with EDTA rabbit plasma (1.13306, Merck, Germany). The *S. aureus* reference strain ATCC 25923 was used for positive control of the coagulase-positive reaction. Tube contents check for coagulation at 37 °C for 4-6 h. The isolates were preserved in Brain-Heart Broth (1.10493, Merck, Germany) supplemented with 20% glycerol at -20 °C for further experiments. The isolates were further confirmed by using polymerase chain reaction (PCR).

2.2. PCR identification and detection of pathogenicity genes

Total DNA from the strains was extracted by using Chelex 100 resin (Sigma Aldrich, USA). PCR was performed in a ThermoCycler (T100, Bio-Rad, USA). Each 25 µL reaction mixture consisted of 1 µL of template DNA, 5 µL of 1.25 U Hot Start Taq DNA polymerase (Bioron, Germany), 1 µL of 1.8 mM $MgCl_2$ (Fermentas, USA), 3µL of 10 mM Tris-HCl (pH 8.9), 4 µL of 200 µ*M* dNTPs (Biolabs, UK), 22 mM KCl, 1 µL of 0.5 mM of each primer (Oligomer, Turkey) and 7 µL of PCR grade water (EURx, Poland). The PCR procedure was carried out with firstly SA gene for confirmation of *S. aureus* isolates, and then the *sea, seb, sec, sed*, *see* genes for detection of enterotoxins and *pvl, tss, eta, etb, mec*A genes for determination of virulence traits. Multiplex-PCR was used to amplify *sea, seb, sec,*

sed, *see* genes and *tss*, *eta*, *etb* genes. The *S. aureus* reference strains ATCC 25923 for *sea*, ATCC 14458 for *seb*, ATCC 19095 for *sec*, ATCC 23235 for *sed*, ATCC 27664 for *see*, ATCC BAA-1747 for *pvl*, MN8 for *tss*, *eta, etb*, ATCC 43300 for *mec*A gene were used as a positive control. The PCR procedures for each primer pair were performed according to the related reference given in Table 1. PCR products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining. 100 bp DNA ladder (Genesta, Germany) was used as a marker.

Table 1- Primers used in the confirmation of *S. aureus* and the determination of enterotoxin and virulence-associated genes

Primers	*Product size (bp)*	*Oligonucleotide sequence (5´-3´)*	*Thermal-cycle protocol*	*Reference*
SA	108	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA	94 *ºC 4 min - (94 ºC 45 s, 50 ºC 45 s, 72 ºC 1 min) x 30 cycles* - 72 *ºC 5 min*	Martineau et al. 1998
sea	102	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	98 *ºC 30 s - (98 ºC 20 s, 60 ºC 20 s, 72 ºC 10 s) x 30 cycles –* *72 ºC 5 min*	Mehrotra et al. 2000
seb	164	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG		
sec	451	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG		
sed	278	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC		
see	209	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC		
tss	326	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	98 *ºC 30 s - (98 ºC 5 s, 57 ºC 10 s, 72 ºC 10 s) x 30 cycles –* *72 ºC 5 min*	Mehrotra et al. 2000
eta	93	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG		
etb	226	ACAAGCAAAAGAATACAGCG GTTTTTGGCTGCTTCTCTTG		
pvl	433	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	98 *ºC 30 s - (98 ºC 5 s, 58 ºC 5 s, 72 ºC 10 s) x 30 cycles –* *72 ºC 5 min*	Lina et al. 1999
mecA	519	TGTCCGTAACCTGAATCAGC GACAAACTCCCACCTATCGC	98 *ºC 30 s - (98 ºC 5 s, 57 ºC 10 s, 72 ºC 10 s) x 30 cycles –* *72 ºC 5 min*	Tsuchizaki et al. 2000

3. Results and Discussion

S. aureus is a worldwide zoonotic pathogen, which can be responsible for approximately 40% of bovine mastitis cases. However, *S. aureus* may exist in the milk of dairy animals after infection and so it contaminates other dairy products (Kou et al. 2021). In the present work, 33 *S. aureus* and 54 CoNS were recovered from 260 raw milk and 755 dairy samples (8.6%) by using the standard culture method, and all *S. aureus* isolates were confirmed by PCR. The mean count of *S. aureus* was 3.30 log CFU/mL-g for all isolated samples (data not shown). A summary of the mean counts by sample kind and coagulase activity results is presented in Table 2. A similar observation has been made in a study by Mercanoglu Taban et al. (2021) who isolated *S. aureus* in 9.4% of milk and dairy collected from Central Anatolia and the Mediterranean Regions of Turkey. Some studies, however, revealed high isolation rates from different countries. Tohoyesseu et al. (2020) found that 36.4% of fermented artisanal dairy products contained *S. aureus* in samples collected from Benin. Research conducted in Egypt showed that the isolation rate of *S. aureus* was 40.5% in milk and dairy products with a 4.12 log CFU/mL-g mean count (Ahmed et al. 2019). Another fairly high result with a 70% rate of *S. aureus* contamination in milk and dairy was published in Saudi Arabia (Alghizzi & Shami 2021).

Table 2- Distribution and counts of staphylococci in the samples

Sample	*Staphylococci counts (log CFU/mL-g)*							
	S. aureus				*Coagulase (-) staphylococci*			
	No of samples	*Min.*	*Mean ± SEM*	*Max.*	*No of samples*	*Min.*	*Mean ± SEM*	*Max.*
Milk (n=260)	8	2	3.35±3.13	4.05	21	2	2.57±2.11	3.43
Cheese (n=325)	16	2	3.54±3.27	4.38	17	2	3.54±3.47	4.70
Butter (n=110)	1	-	2.30±	-	2	3.16	3.25±2.60	3.34
Ice cream (n=140)	8	2	3.46±3.25	3	14	2	2.45±1.82	3
Yoghurt (n=180)	-	-	-	-	-	-	-	-
All (n=1015)	33	2	3.46±3.01	4.38	54	2	3.16±2.97	4.70

Min: Minimum, Max: Maximum, SEM: Standard error of the mean

The prevalence of staphylococci was the highest with an incidence of 15.7% in ice cream, followed by milk (11.2%), cheese (10.1%), and butter (2.7%). None of the yogurt samples contained staphylococci (data not shown). Table 2 presents the distribution of CoNS and *S. aureus* by sample kind. *S. aureus* was observed in 22 out of 140 (15.7%) ice cream samples in this study. Lower incidence rates were reported in China with an incidence of 4.2% (Zhang et al. 2022) and a higher result (74%) was obtained in Egypt (Ahmed et al. 2019). In the present study, 19 of 22 ice cream samples contaminated with *S. aureus* were fruit, chocolate pieces, or cocoa ice cream. The use of improperly washed fruits in ice cream production may be responsible high contamination rate. In our research, 29 (8 were *S. aureus*, 21 were CoNS) out of 260 raw milk samples were found to be contaminated. In Turkey Pehlivanlar Onen et al. (2017) found that 22 out of 40 raw milk samples contained CoNS. Studies on raw milk performed in Turkey showed that 64% of samples contained *S. aureus* (Yildirim et al. 2019), and 95 out of 725 raw milk samples contained *S. aureus* (Tavsanli & Cibik 2022). However, some other researchers reported the presence of *S. aureus* in heat-treated milk. *S. aureus* was determined in pasteurized milk samples by Dai et al. (2019), in 21 marked milk by Ahmed et al. (2019), and also isolated in composite milk by Mahanti et al. (2020). In the current research 17 CoNS and 16 *S. aureus* were identified from 325 cheese samples. The presence of *S. aureus* in various types of cheese was previously reported in Turkey (Pehlivanlar Onen and Aygun 2017; Elal Mus et al. 2019), Egypt (Ahmed et al. 2019; Zayda et al. 2020), Romania (Morar et al. 2021), and China (Cai et al. 2021). In this study, *S. aureus* was detected in 3 of 110 butter samples. Our results were lower than Ranjana et al. (2019), who isolated and identified 11 strains from plain and table butter. A previous study in China revealed that 12.4% of retailed yak butter samples collected from retail stores were contaminated with *S. aureus* (Zhang et al. 2021). We mostly analyzed fresh and homemade yogurt samples to determine the distribution of CoNS and *S. aureus* in this study. Research performed by Pehlivanlar Onen and Aygun (2017) in salted yogurt samples from Turkey showed the absence of staphylococci. This result is in agreement with our findings. Contrary to our yogurt analysis results, Ahmed et al. (2019), Tohoyessou et al. (2020) and Abdulrahman & Sanmi (2021) detected *S. aureus* among yogurt samples. The occurrence of *S. aureus* in dairy products may be caused by various factors, but it is commonly associated with the use of contaminated raw milk and endogenous starter cultures in the production process of dairy products, apart from asymptomatic worker carriers of *S. aureus* (Castro et al. 2020). In addition, the difference in *S. aureus* and CoNS prevalence in milk and dairy may be caused by geographical differences, hygienic conditions of preparation, and storage.

Staphylococci secret the coagulase enzyme and this enzyme is regarded as an indicator of the pathogenicity of *S. aureus* (Ahmed et al. 2019). In the current work, 33 (38%) of 87 *S. aureus* isolates tested positive for coagulase activity. In terms of coagulase activity, the other 54 isolates consisting of 10% of ice cream, 8.1% of milk, 5.2% of cheese, and 1.8% of butter isolates were classified as coagulase-negative. The prevalence of *S. aureus* and CoNS in samples is summarized in Table 2. Besides *S. aureus*, CoNS has been associated with rare cases of food poisoning. However, CoNS is regarded as a major reservoir for toxin production and antimicrobial resistance genes of *S. aureus* as a pathogen (Nasaj et al. 2020; França et al. 2021). Some CoNS isolates from different foods encode toxin-producing genes and these genes can be transmitted to other bacteria (França et al. 2021) Moreover, CoNS causes significant morbidity and socioeconomic losses worldwide (Becker et al. 2020; França et al. 2021).

Table 3 - Distribution of toxin-related genes

Genes		No of samples									
		S. aureus					*Coagulase (-) staphylococci*				
		Milk	*Cheese*	*Butter*	*Ice cream*	*Yoghurt*	*Milk*	*Cheese*	*Butter*	*Ice cream*	*Yoghurt*
Enterotoxin genes	sea	1[a]	5[b]	-	-	-	-	-	-	-	-
	seb	-	2[c]	-	-	-	-	-	-	-	-
	sec	-	-	-	-	-	-	-	-	-	-
	sed	-	-	-	-	-	-	-	-	-	-
	see	-	1[d]	-	-	-	-	-	-	-	-
Other toxin genes	tss	-	-	-	-	-	1[e]	-	-	-	-
	eta	-	-	-	-	-	-	-	-	-	-
	etb	-	-	-	-	-	1[e]	-	-	-	-
	pvl	-	-	-	-	-	-	-	-	-	-

[a]S37 isolate number, [b]S7, S14, S25, S44, S56 isolate numbers, [c]S38, S41 isolate numbers, [d]S25 isolate numbers, [e]S3 isolate number

The presence of enterotoxin genes in the *S. aureus* and CoNS isolates is shown in Table 3. SE *sea* gene was detected in one milk (S37) and five (S7, S14, S25, S44, S56) (three soft, one hard, and one semi-hard kind) cheese isolates of *S. aureus*. The SE *seb* gene was assigned in two soft kinds of cheese (S38, S41) and the SE *see* gene in one kind of hard cheese (S25) isolates of *S. aureus*. The *S. aureus* isolated from the hard cheese (S25) possessed both SE *sea* and *see* genes (Figure 1). The coagulase test was positive for all strains carrying the enterotoxin gene. The SE *sec* and *sed* genes were not detected in any isolate from milk and dairy in our research. Two different findings from Turkey showed that *S. aureus* isolates from traditional Turkish dairy-based milk desserts harbored *sea* (5 isolates), *seb* (5 isolates), *see* (1 isolate) and *sea*+*see* (2 isolates) genes (Gucukoglu et al. 2020) and CoNS isolate from goat's milk and cheese had *sed* (1 isolate), *see* (6 isolates); *S. aureus* had *sec* (6 isolates), *sed* (4 isolates) genes (Pehlivanlar Onen et al. 2018). An earlier report from China revealed that 12.9 % of *S. aureus* strains isolated from raw cow (*sea*, *sea*+*sec*, *sec* and *see* genes), horse (*see* gene) and camel (*sea*+*sec* gene) milk had several SE's genes (Kou et al. 2021). The results of research conducted in India demonstrated that *sei* and *seg* genes were found but *sea*, *seb*, *sec*, *sed,* and *see* genes were absent in raw bovine milk samples (Mahanti et al. 2020). A survey from Italy showed that *S. aureus* from raw milk samples harbored enterotoxin genes and these strains' ability to produce sufficient amounts of enterotoxin (Chieffi et al. 2020). Similar to our finding, Ahmed et al. (2019) described SE *sea* and *sed* genes in 13 cheese isolates, also both *sea* and *sed* genes in two different artisanal cheese isolates Zayda et al. (2020) suggested that *S. aureus* in Egyptian raw milk cheeses has *sea, seb, sec, sed, see* and *seg, seh, sei, sej, sep, ser* enterotoxin genes and the presence of multiple enterotoxin genes in isolates was observed in 40%. Again, the detection of various enterotoxin genes in artisan cheese was reported in Brazil (*sea* gene) (Castro et al. 2020) and China (*sea, seb, sec, sed, see* genes) (Cai et al. 2021). Kayili & Sanlibaba (2020) isolated 85 *S. aureus*

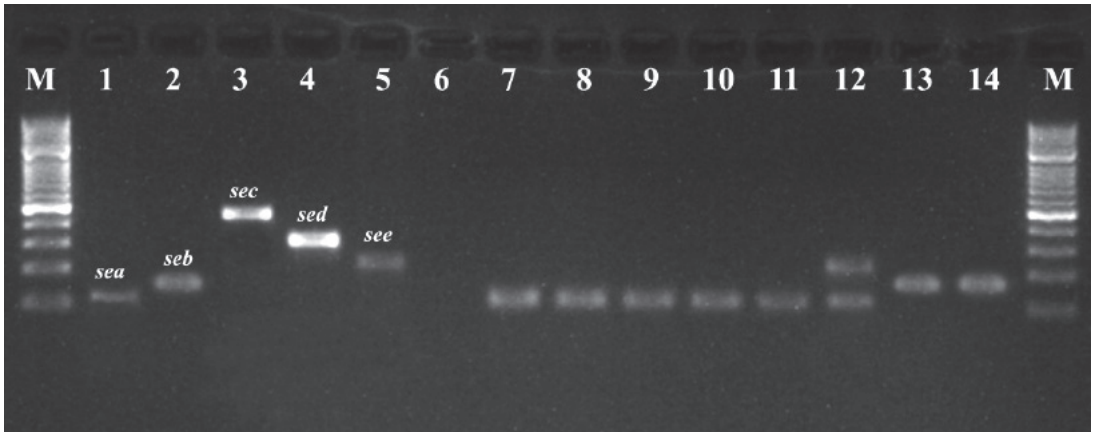

Figure 1- Enterototoxin genes of *S aureus*. M: 100 bp DNA ladder; lane 1: *S aureus* ATCC 25923 for *sea*; lane 2: *S aureus* ATCC 14458 for *seb*; lane 3: *S aureus* ATCC 19095 for *sec*; lane 4: *S aureus* ATCC 23235 for *sed*; lane 5: *S aureus* ATCC 27664 for *see*; lane 6: negative control; lane 7-11: S7, S14, S37, S44, S56 - *sea* positive isolates; lane 12: S25 - *sea*+ *see* positive isolate; lane 13-14: S38, S41 - *seb* positive isolates

from 387 cheese samples and none of the isolates had enterotoxin genes. In the same way, the absence of enterotoxin genes in *S. aureus* from milk and cheese was observed by Yildirim et al. (2019). On the other hand, Muneeb et al. (2021) described the presence of sea, seb, seg, and sei among CoNS isolate from retail market fish. SEs are a tremendously important issue for food safety because they can be active after 30 min boiling and may remain present at 121 ºC for 28 min. This means that SEs were not eliminated through normal pasteurization and sterilization methods. Thus, foods can be contaminated with SEs at production steps before packaging (Necidova et al. 2019). The presence of enterotoxigenic *S. aureus* in cheese which is a ready-to-eat food is a remarkable finding of our research.

The PCR results showed that the frequency of virulence-associated genes was relatively low among isolates. Only one CoNS strain (S3) from raw milk had both *tss* and *etb* virulence genes (Figure 2). The remaining isolates did not represent any investigated virulence genes. The distribution of toxigenic genes in the isolates is shown in Table 3. A study performed in Turkey demonstrated the presence of the *tst* gene (6 goat milk, 1 goat cheese sample) in CoNS and 7 *S. aureus* isolates. The absence of *eta*, *etb* genes (goat milk and cheese) in CoNS and *S. aureus* was also reported (Pehlivanlar Onen et al. 2018). A recent study indicated that one *S. aureus* in cow milk carried the *tst* gene and *pvl*, *eta*, *etb* toxin genes (Gharsa et al. 2019). In Brazil, Castro et al. (2020) declared that 14 (18.4%) artisanal cheese isolates of *S. aureus* harbored the *tsst*-1 gene. In Benin, Tohoyesseu et al. (2020) recorded *eta* and *etb* toxin production in coagulase-positive/negative staphylococci and, also *pvl* toxin secretion in 8.3% of *S. aureus* strains. Similar to our results, the *pvl* gene positive *S. aureus* was not detected in milk by Alghizzi and Shami (2021) but Sadat et al. (2022) reported *pvl* positive *S. aureus* in raw cow milk. The mass food surveillance work including milk and dairy products from China revealed that *S. aureus* isolates carried *tss* (14.3%), *eta* (21.9%), *etb* (12.3%), and *pvl* (16.7%) genes (Liao et al. 2018). Staphylococcal superantigens (SAgs) involve pyrogenic toxins such as *tsst*-1, and SE's, and toxic shock syndrome is caused by *S. aureus* strains that produce *tss*-1 toxin. This syndrome is characterized by rash, fever, hypertension, multiple organ malfunction, and may cause death (Abril et al. 2020). The occurrence of CoNS carrying *tss* and *etb* genes in milk is another considerable finding obtained in our study.

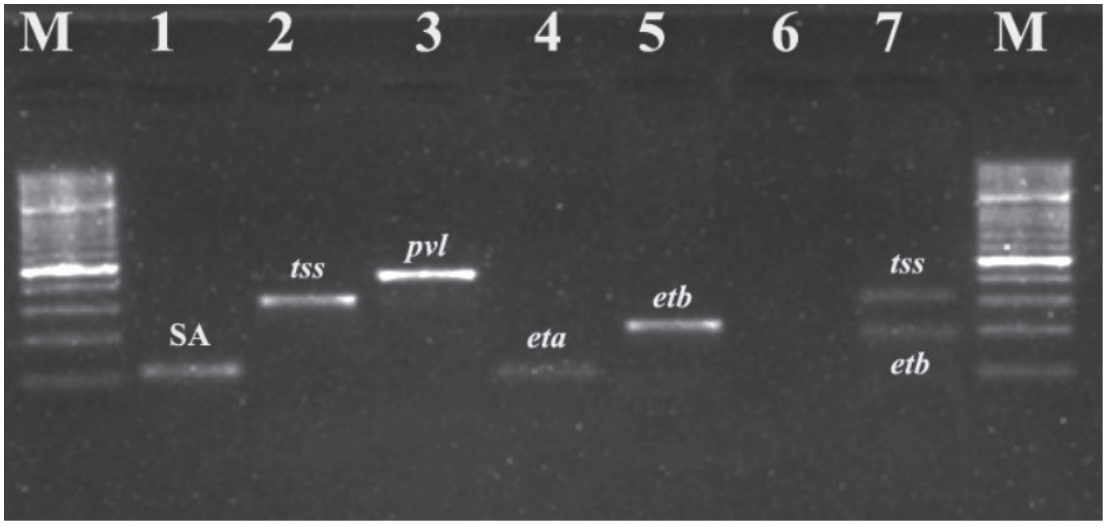

Figure 2- PCR-based screening of virulence genes. Lane M: 100 bp ladder, Lane 1-5: positive controls of SA, *tss, pvl, eta*, and *etb* genes (*S. aureus* ATCC 19095 for SA gene, *S. aureus* ATCC BAA-1747 for *pvl* gene and *S. aureus* MN8 for *tss, eta*, and *etb* genes); lane 6: negative control; lane 7: S3 - *tss* and *etb* positive CoNS isolate

The use of antibiotics still remains to treat or control mastitis, but the impact of antibiotic therapy on *S. aureus* decreases with the accelerating antimicrobial agent resistance across the world (Kou et al. 2021). In particular, methicillin-resistant *S. aureus* is another concern for food safety and public health (Elal Mus et al. 2019). In our research, all *S. aureus* and CoNS isolates were negative for the *mec*A gene responsible for methicillin resistance. Our findings were similar to those obtained by Guncuoglu et al. (2020) reporting the absence of the *mec*A gene carrying *S. aureus* in traditional Turkish dairy-based desserts. As in the current work, Castro et al. (2020) did not observe the *mec*A gene in artisanal cheese isolates of *S. aureus*. In contrast, some current reports indicate the presence of *mec*A gene carrying *S. aureus* isolated from milk and/or dairy products in Turkey (Elal Mus et al. 2019; Tavsanli & Cibik 2022), in Romania (Morar et al. 2021), in Egypt (Ahmed et al. 2019; Zayda et al. 2020; Sadat et al. 2022), in China (Cai et al. 2021; Kou et al. 2021; Zhang et al. 2021), in India (Mahanti et al. 2020), in Benin (Tohoyesseu et al. 2020) and in Algeria (Chenouf et al. 2021). Additionally, Chenouf et al. (2021) reported three *mec*A-positive CoNS isolates from milk in Algeria, and Pyzik et al. (2019) demonstrated that 27.5% of poultry-derived CoNS in Poland carried the *mec*A gene.

4. Conclusions

In conclusion, the present research has revealed the presence of several staphylococcal toxin genes in *S. aureus* and CoNS. Our main findings are the presence of *S. aureus* containing enterotoxin genes from ready-to-eat food (cheese) (S7, S14, S25, S38, S41, S44, S56) and CoNS isolate (S3) carrying TSST-1 and exfoliative toxin genes from milk. From a food safety perspective, the observation of virulent CoNS in foods is noteworthy. Through this work, the surveillance of toxigenic genes of CoNS and *S. aureus* contributed from Turkey. CoNS isolates are also an emerging concern for the transmission of toxins and other pathogenicity-related genes. In terms of one health approach commonly observed but ignored CoNS in animal-derived foods can pose risk to contaminated food consumers, hospitalized patients, juvenile and elderly people, etc. In short, this work acknowledges the pathogenicity of CoNS. The accurate assessment of this suspected bacteria would need further comparative research involving hospital/animal origin, and more data from foods.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: T.E.M., F.C., Design: T.E.M., F.C., Data Collection or Processing: T.E.M., G.E.S., B.E., Analysis or Interpretation: T.E.M., F.C., G.E.S., B.E., Literature Search: T.E.M., G.E.S., B.E., Writing: T.E.M., F.C., G.E.S., B.E.

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References

Abdulrahman F A & Sanmi E (2021). Physicochemical properties, proximate composition and total viable counts of Staphylococcus aureus in nono and yoghurt samples in Kaduna, Nigeria. *Turkish Journal of Agriculture* 9(1): 15-20. doi.org/10.24925/turjaf.v9i1.15-20.3391

Abril A G, Villa T G, Barros-Velázquez J, Cañas B, Sánchez-Pérez A, Calo-Mata P & Carrera M (2020). *Staphylococcus aureus* exotoxins and their detection in the dairy industry and mastitis. *Toxins* 12(9): 537. doi.org/10.3390/toxins12090537

Ahmed A A H, Maharik N M, Valero A & Kamal S M (2019). Incidence of enterotoxigenic Staphylococcus aureus in milk and Egyptian artisanal dairy products. *Food Control* 104: 20-27. doi.org/10.1016/j.foodcont.2019.04.017

Alghizzi M & Shami A (2021). The prevalence of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* in milk and dairy products in Riyadh, Saudi Arabia. *Saudi Journal of Biological Sciences* 28(12): 7098-7104.

Aqib A I, Ijaz M, Farooqi S H & Raza A (2018). Dairy *Staphylococcus aureus*: Epidemiology, Drug Susceptibilities, Drug Modulation, and Preventive Measures. Intechhopen. doi.org/10.5772/intechopen.74552

Banszkiewicz S, Walecka-Zacharska E, Schubert J, Tabis A, Krol J, Stefaniak T, Wesierska E & Bania J (2022). Staphylococcal enterotoxin genes in coagulase-negative staphylococci-stability, expression, and genomic context. *International Journal of Molecular Sciences* 23(5): 2560.

Becker K, Both A, Weibelberg S, Heilmann C & Rohde H (2020). Emergence of coagulase-negative staphylococci. *Expert Review of Anti-Infective Therapy* 18(4): 349-366. doi.org/10.1080/14787210.2020.1730813

Cai H, Kou X, Ji H, Wang X, Wang H, Zhang Y, Lu S, Li B, Dong J, Wang Q, Zhou J & Hu D (2021). Prevalence and characteristics of *Staphylococcus aureus* isolated from Kazak cheese in Xinjiang, China. *Food Control* 123: 107759.

Castro R D, Pedroso S H S P, Sandes S H C, Silva G O, Luiz K C M, Dias R S, Filho R A T, Figueiredo H C P, Santos S G, Nunes A C & Souza M R (2020). Virulence factors and antimicrobial resistance of *Staphylococcus aureus* isolated from the production process of Minas artisanal cheese from the region of Campo das Vertentes, Brazil. *Journal of Dairy Science* 103(3): 2098-2110. doi.org/10.3168/jds.2019-17138

Chajecka-Wierzchowska W, Gajevska J, Wisniewski P & Zadernowska A (2020). Enterotoxigenic potential of coagulase-negative staphylococci from ready-to-eat food. *Pathogens* 9(9): 734. doi.org/10.3390/pathogens9090734

Chenouf N S, Mama O M, Messaï C R, Ruiz-Ripa L, Fernández-Fernández R, Carvalho I, Zitouni A, Hakem A & Torres C (2021). Detection of methicillin-resistant coagulase-negative staphylococci and PVL/mecA genes in cefoxitin-susceptible *Staphylococcus aureus* (t044/ST80) from unpasteurized milk sold in stores in Djelfa, Algeria. *Journal of Dairy Science* 104(3): 2684-2692. doi.org/10.3168/jds.2020-19270

Chieffi D, Fanelli F, Cho G S, Schubert J, Blaiotta G, Franz C M A P, Bania J & Fusco V (2020). Novel insights into the enterotoxigenic potential and genomic background of Staphylococcus aureus isolated from raw milk. *Food Microbiology* 90: 103482. doi.org/10.1016/j.fm.2020.103482

Dai J, Wu S, Huang J, Wu Q, Zhang F, Zhang J, Wang J, Ding Y, Zhang S, Yang X, Lei T, Xue L & Wu H (2019). Prevalence and characterization of Staphylococcus aureus isolated from pasteurized milk in China. *Frontiers in Microbiology* 10: 641. doi.org/10.3389/fmicb.2019.00641

Dorotíkova K, Kamenik J, Bogdanovičová K, Křepelová S, Strejček J & Haruštiaková D (2022). Microbial contamination and occurrence of *Bacillus cereus* sensu lato, Staphylococcus aureus, and Escherichia coli on food handlers' hands in mass catering: Comparison of the glove juice and swab methods. *Food Control* 133: 108567.

Elal Mus T, Cetinkaya F, Karahan H, Gurbuz I B & Degirmenci G (2019). Investigation of mecA gene, virulence traits and antibiotic resistance profiles in methicillin-resistant Staphylococcus aureus isolates from dairy products. *Journal of Food Safety* 39(3): 12620. doi.org/10.1111/jfs.12620

European Council Regulations (2005). No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.

European Food Safety Authority & European Centre for Disease Prevention and Control (EFSA & ECDC) (2021). The European Union One Health 2020 Zoonoses Report. *EFSA Journal* 19(12): 6971. doi.org/10.2903/j.efsa.2021.6971

França A, Gaio V, Lopes N & Melo L D R (2021). Virulence Factors in Coagulase-Negative Staphylococci. *Pathogens* 10(2): 170. doi.org/10.3390/pathogens10020170

Gharsa H, Chairat S, Chaouachi M, Yahia H B, Boudabous A & Slama K B (2019). High diversity of genetic lineages and virulence genes of *Staphylococcus aureus* isolated from dairy products in Tunisia. *Annals of Microbiology* 69: 73-78. doi.org/10.1007/s13213-018-1417-0

Gucukoglu A, Cadirci O, Gulel G T, Uyanik T & Abdullahi A (2020). Enterotoxin gene content and antibiotic resistance profiles of *Staphylococcus aureus* isolated from traditional Turkish dairy-based desserts. *Fresenius Environmental Bulletin* 29(4): 2073-2080.

Kayili E & Sanlibaba P (2020). Prevalence, characterization and antibiotic resistance of *Staphylococcus aureus* isolated from traditional cheeses in Turkey. *International Journal of Food Properties* 23(1):1441-1451. doi.org/10.1080/10942912.2020.1814323

Kou X, Cai H, Huang S, Ni Y, Luo B, Qian B L H, Ji H & Wang X (2021). Prevalence and characteristics of *Staphylococcus aureus* isolated from retail raw milk in Northern Xinjiang, China. *Frontiers in Microbiology* 12: 705947.

Liao F, Gu W, Yang Z, Mo Z, Fan L, Fu X, Xu W, Li C & Dai J (2018). Molecular characteristics of Staphylococcus aureus isolates from food surveillance in Southwest China. *BMC Microbiology* 18: 91. doi.org/10.1186/s12866-018-1239-z

Lina G, Piemont Y, Godail-Gamot F, Bes M, Peter M O, Gauduchon V, Vandenesch F & Etienne J (1999). Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. *Clinical Infectious Diseases* 29: 1128-1132.

Mahanti A, Joardar S N, Bandyopadhyay S, Banerjee J, Ghosh S, Batabyal K, Sar T K, Dutta T K & Samanta I (2020). Characterization of methicillin-resistant and enterotoxins producing Staphylococcus aureus in bovine milk in India. *Journal of Agriculture and Food Research* 2: 100017.

Martineau F, Pickard F J, Roy P H, Ouelette M & Bergeron M G (1998). Species-specific and ubiquitous DNA based assays for rapid identification of *Staphylococcus aureus*. *Journal of Clinical Microbiology* 36(3): 618-623. doi.org/10.1128/JCM.36.3.618-623.1998

Mehrotra M, Wang G & Johnson W M (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *Journal of Clinical Microbiology* 38(3): 1032-1035. doi.org/10.1128/JCM.38.3.1032-1035.2000

Mercanoglu Taban B, Hassankhani A & Aytac S A (2021). Investigation of *mec*A- and *mec*C-positive *Staphylococcus aureus* from raw milk and traditional artisanal dairy foods. *International Journal of Food Properties* 19(1): 954-964. doi.org/10.1080/10942912.2021.1950182

Morar A, Ban-Cucerzan A, Herman V, Tîrziu E, Sallam K I, Abd-Elghany S M & Imre K (2021) Multidrug resistant coagulase-positive *Staphylococcus aureus* and their enterotoxins detection in traditional cheeses marketed in Banat Region, Romania. *Antibiotics* 10(12): 1458. doi.org/10.3390/antibiotics10121458

Muneeb K H, Sudha S, Sivaraman G K, Shome B, Cole J & Holmes M (2021). Virulence and intermediate resistant to high-end antibiotic (teicoplanin) among coagulase-negative staphylococci sourced from retail market fish. *Archives of Microbiology* 203: 5695-5702. doi.org/10.1007/s00203-021-02558-2

Nasaj M, Saeidi Z, Tahmasebi H, Dehbashi S & Arabestani M R (2020). Prevalence and distribution of resistance and enterotoxins/enterotoxin-like genes in different clinical isolates of coagulase-negative Staphylococcus. *European Journal of Medical Research* 25: 48. doi.org/10.1186/s40001-020-00447-w

Necidova L, Bursova S, Harustiakova D, Bogdanovicova K & Lacanin I (2019). Effect of heat treatment on activity of staphylococcal enterotoxins of type A, B, and C in milk. *Journal of Dairy Science* 102(5): 3924-3932.

Pehlivanlar Onen S & Aygun O (2017). Enterotoxin producing ability antimicrobial susceptibility of coagulase-negative staphylococci isolated from goat milk, cheese, and salted yoghurt in Turkey. *International Journal of Scientific and Technology Research* 6(10): 200-206.

Pehlivanlar Onen S, Cantekin Z, Mebkhout F, Aygun O & Ergun Y (2018). Investigation into toxin and slime genes in staphylococci isolated from goat milk and goat cheese in Southern Turkey. *The Thai Journal of Veterinary Medicine* 48(3): 339-346.

Pyzik E, Marek A, Stępień-Pyśniak D, Urban-Chmiel R, Jarosz Ł S & Jagiełło-Podębska I (2019). Detection of antibiotic resistance and classical enterotoxin genes in coagulase-negative staphylococci isolated from poultry in Poland. *Journal of Veterinary Research* 63(2): 183-190. doi.org/10.2478/jvetres-2019-0023

Ranjana K C, Timilsina G, Singh S & Sharma S (2019). Detection of methicillin resistant *Staphylococcus aureus* in dairy products and anterior nares of dairy workers. *Tribhuvan University of Journal of Microbiology* 6:59-62. doi.org/10.3126/tujm.v6i0.26585

Sadat A, Shata R R, Farag A M M, Ramadan H, Alkhedaide A, Soliman M M, Elbadawy M, Abugomaa A & Awad A (2022). Prevalence and characterization of PVL-positive *Staphylococcus aureus* isolated from raw cow's milk. *Toxins* 14(2): 97. doi.org/10.3390/toxins14020097

Tavsanli H & Cibik R (2022). The prevalence, genetic diversity and antibiotic resistance of *Staphylococcus aureus* associated with subclinical bovine mastitis in Balikesir, Turkey. *Veterinarski Arhiv* 92(1):17-25.

Tohoyesseu M G, Mousse W, Sina H, Kona F, Azanghadji T, Guessennd N, Baba-Moussa F, Dadie T, Adjanohoun A & Baba-Moussa L (2020). Toxin production and resistance of *Staphylococcus* species isolated from fermented artisanal dairy products in Benin. *Journal of Pathogens* 2020: 7938149.

Tsuchizaki N, Ishikawa J & Hotta K (2000). Colony for PCR for rapid detection of antibiotic resistance genes in MRSA and enterococci. *The Japanese Journal of Antibiotics* 53(6): 422-429.

Turkish Food Codex (TFC) (2011). Regulation on Microbiological Criteria. Law of Authorization: 5996. Official Gazette of Publication.

Yildirim T, Sadati F, Kocaman B & Siriken B (2019). *Staphylococcus aureus* and staphylococcal enterotoxin detection in raw milk and cheese origin coagulase positive isolates. *International Journal of Science Letters* 1: 30-41. doi.org/10.38058/ijsl.596007

Zayda M G, Masuda Y, Hammad A M, Honjoh K, Elbagory A M & Miyamoto T (2020). Molecular characterization of methicillin-resistant (MRSA) and methicillin-susceptible (MSSA*)* Staphylococcus aureus isolated from bovine subclinical mastitis and Egyptian raw milk cheese. *International Dairy Journal* 104: 104646.

Zhang P, Liu X, Zhang J, Fu X, Wan Y, Pan H, Wu & Wang X (2021). Prevalence and characterization of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* isolated from retail yak butter in Tibet, China. *Journal of Dairy Science* 104(9): 9596-9606.

Zhang P, Liu X, Zhang M, Kou M, Chang G, Wan Y, Xu X, Ruan F, Wang Y & Wang X (2022). Molecular characteristics of *Staphylococcus aureus* from retail ice cream in Shaanxi province, China. *Foodborne Pathogens and Disease* 19(3):217-225. doi.org/10.1089/fpd.2021.0069

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The Least Limiting Water Range to Estimate Soil Water Content Using Random Forest Integrated with GIS and Geostatistical Approaches

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ABSTRACT

Algorithms that exist in every area today have become the center of our lives with technological developments. The uses of machine learning algorithms are being researched with the new developments in the agricultural field. The present study determined the least limiting water range (LLWR) contents of alluvial lands with different soils distributed in the Bafra Plain, where intensive agricultural activities are carried out, and revealed the compression and aeration problems in the area with distribution maps. Also, the predictability of LLWR was evaluated with the random forest (RF) algorithm, one of the machine learning algorithms, and the usability of the prediction values distribution maps was revealed. The LLWR contents of the soils varied in the range of 0.049-0.273 cm^3 cm^{-3} for surface soils. There were aeration problems in 6.72%, compaction problems in 20.16%, and aeration and compaction problems in 0.8% of the surface soils examined in the study area. Furthermore, 72.32% of the soil was under optimal conditions. For the 20-40 cm depth, an aeration problem in 5.88%, a compaction problem in 28.57%, and both an aeration and a compaction problem in 2.52% of the points were detected. In estimating LLWR with the RF algorithm, the root mean square error (RMSE) value obtained for 0-20 cm depth was determined to be 0.0218 cm^3 cm^{-3}, and for 20-40 cm depth, it was 0.0247 cm^3 cm^{-3}. In the distribution maps of the observed and predicted values obtained, the lowest RMSE value was determined by the SK interpolation methods for 0-20 cm depth and the OK interpolation methods for 20-40 cm. The distribution of obtained and predicted values in surface soils was similar. However, variations were found in the distribution of areas with low LLWR below the surface. As a result of the study, it was determined that LLWR can be obtained with a low error rate with the RF algorithm, and distribution maps can be created with lower error in surface soils.

Keywords: Physical properties, Moisture constants, Machine learning, Bafra delta plain

1. Introduction

Today, with the developing technology, evaluating soil and product-yield status has become essential for managing and using soils without losing their functions in the terrestrial ecosystem and the ecosystem services they provide. The aim of applications such as global positioning system, geographic information systems (GIS), remote sensing, yield monitoring, and estimation, and applications such as smart agriculture and precision agriculture for agricultural production is to make crop production within the scope of maximum efficiency and ecological-economic sustainability with minimum input amount. In this context, the physical properties of the soil are critical quality indicators that directly or indirectly affect plant production and yield elements (Şenol et al. 2020). Among the physical parameters of soils, properties such as soil water content, air-filled porosity, temperature, and penetration resistance directly affect plant root growth. In contrast, other properties such as bulk density, texture, aggregate stability, and pore size distribution have an indirect effect (Letey 1958). However, some of these parameters are negatively affected due to compression and compaction due to the pressure applied under heavy field traffic, and the bulk density of the soil increases. From an agricultural point of view, soil or soil layers are considered compacted when the porosity of soils, especially air-filled porosities, is low enough to limit aeration. In such a case, each or both fluids in the soil, air, and water are partially removed from the compacted soil mass, decreasing soil porosity. As a result of the increase in the proportion of small pores in the pore size distribution, aeration, root penetration, water flow, and drainage are prevented.

Aksakal (2004) states that, due to soil compaction, the penetration resistance to root growth increases while the water holding and aeration capacity decreases.

Due to compaction, the soil's changing penetration resistance or bulk density value cannot clearly explain the plant development status. The LLWR feature, a combination of various soil physical properties, is considered one of the soil structural quality indicators. Letey (1958) defined the non-LWR as the water ranges affected by the water content that the plant and the aeration and penetration resistance can take up. Da Silva et al. (1994) developed the LLWR approach by evaluating the soil bulk density in the model. LLWR is the soil water content at which limitations on plant growth in relation to water potential, aeration, and penetration resistance are minimal. The upper limit of the effect of LLWR on root growth was determined as air-filled pore volume (10%) or field capacity, the lower limit was determined as the wilting point or soil water content at which root growth was limited, and a 2 MPa soil penetration resistance occurred. (Da Silva et al. 1994). LLWR decreases with the increasing penetration resistance and bulk density with soil compaction (Haghighi Fashi et al. 2017). Some research has also reported that LLWR's wide variation range enables plants to utilize soil water more effectively and positively affects crop yield (Da Silva & Kay 1997; Chan et al. 2006). Negiş et al. (2020) stated that soil compaction decreased, whereas LLWR increased with the organic material application. However, Alaboz et al. (2021) evaluated that LLWR showed a positive correlation with clay, organic matter, and $CaCO_3$ and a negative correlation with bulk density.

LLWR determination is a quality indicator that is a combination of laborious and time-consuming features. Therefore, studies on the predictability of the LLWR feature by pedotransfer functions have been carried out (Da Silva & Kay 1997; Leão et al. 2005; Tavanti et al. 2019). Alaboz et al. (2021) determined LLWR with deep learning with higher prediction accuracy than ANN. Akar & Güngör (2013) have stated that the random forest (RF) algorithm, one of the machine learning algorithms, is generally preferred since it shows higher accuracy than other approaches. Also, Watts & Lawrence (2008) have stated that the RF algorithm provides high accuracy in determining agricultural regions when applied to an object-oriented approach. RF algorithm, a learning-based approach, is generally used in digital soil mapping (Stum et al. 2010; Machado et al. 2019), and the studies on soil physical properties remain limited.

It is challenging to represent the area of the point samples of soil properties that vary depending on many factors in the field. Also, revealing spatial evaluations instead of point values in determining dynamic features contributes to sustainable management. Geostatistics, which estimates variables by interpolating between variables that do not have observations in a particular observation area and variables with observations, is frequently used to close this gap (Mihalikova et al. 2016; Tunçay et al. 2018). Developing computer, sensor technologies, and programs can easily reveal the variability of soil properties with spatial distribution maps. Alaboz et al. (2020) determined the interpolation methods showing the highest accuracy in the field capacity and wilting point distributions of soils as OK's Gaussian [root mean square error (RMSE): 4.289%] and Cokriging (RMSE: 3.187%), respectively. On the other hand, According to Tunçay et al. (2018) determined the lowest mean absolute error (MAE) and mean squarer error (MSE) values with the regression kriging method in the creation of field capacity distribution maps, while the wilting point was obtained with the Cokriging method. Furthermore, the distribution of the observed values and the values estimated from the algorithms with different methods showed a similar pattern (Alaboz et al. 2020; Şenol et al. 2020).

The present study aimed to; i) determine the least limiting water range (LLWR) contents in the alluvial lands distributed in the Bafra Plain formed on the sediments carried by the Kızılırmak river and reveal the compression and aeration problems in the area with distribution maps, ii) evaluate the predictability of LLWR with the RF algorithm and iii) determine the usability of the distribution maps of the estimated values obtained.

2. Material and Methods

2.1. General characteristics of the study area

The present study was conducted in the Samsun-Bafra delta plains of the Kızılırmak River in the Central Black Sea Region of Turkey (Figure 1).

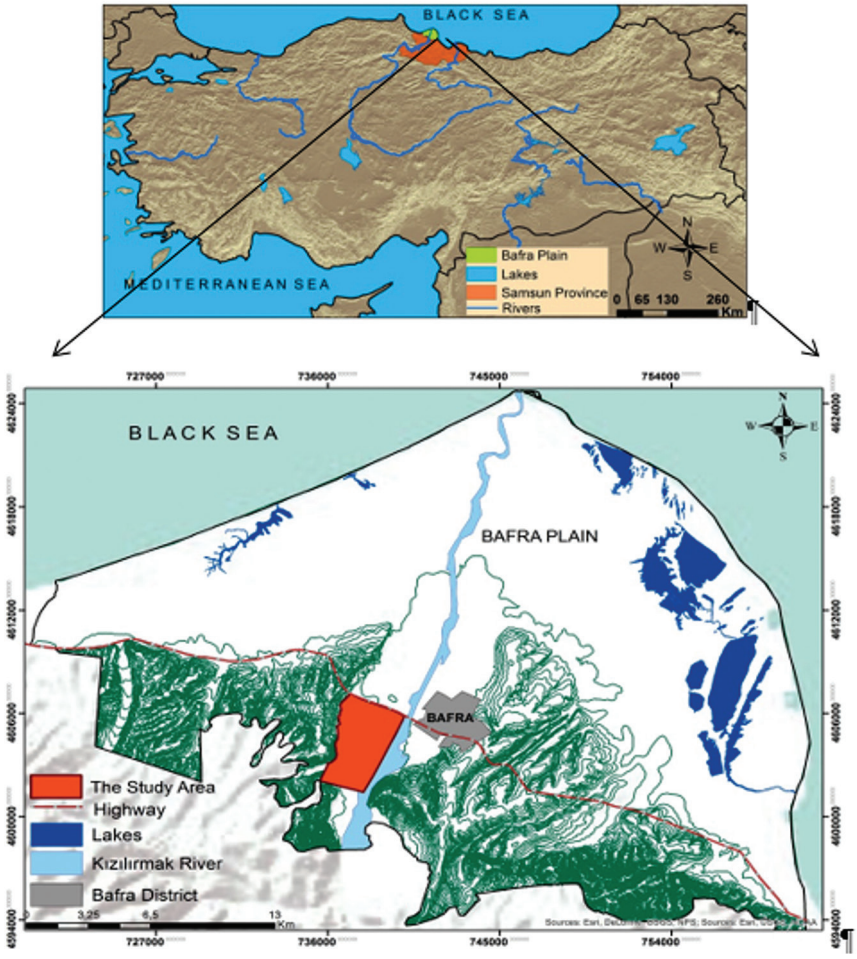

Figure 1- Location maps of the study area

The study area is 30 km from the Samsun city center and covers an area of approximately 1365.4 ha. The climate in the region is semi-humid. The average temperature is 22.2 °C in July and 6.9 °C in January. The annual average temperature is 13.6 °C. Precipitation and evaporation are 764.3 mm and 726.7 mm, respectively (TSMS, 2021). According to the Soil Survey Staff (2014), soil temperature and moisture regimes are mesic and ustic, respectively. The study area is slightly sloping (0.0-2.0%) and is mainly located on the river alluvium carried by the Kızılırmak River. Also, some of the soils of the study area are distributed on colluvial clay deposits from the slopes located in the northwest parts of the area. The soils in the study area were classified as Vertisol, Inceptisol, and Entisol (Soil Survey Staff 2014), and Regosol, Fluvisol, Leptosol, Cambisol, and Vertisol, according to WRB (Figure 2). Intensive agriculture, including vegetables, fruits, and grains, is carried out on the flatlands in the study area.

2.2. Soil sampling and analysis

A total of 214 soil samples were taken from the study area, including surface (0-20 cm) and subsurface (20-40 cm) samples (Figure 2). Soil texture, gravimetric water content, saturated water content-field capacity, and wilting point values were determined by methods described by Gee & Bauder (1986), Blake & Hartge (1986) & Klute (1986), respectively. Penetration resistance measurements were determined by penetrologger (Eijkelkamp 1990). Bulk density was determined using undisturbed soil sampling cylinders.

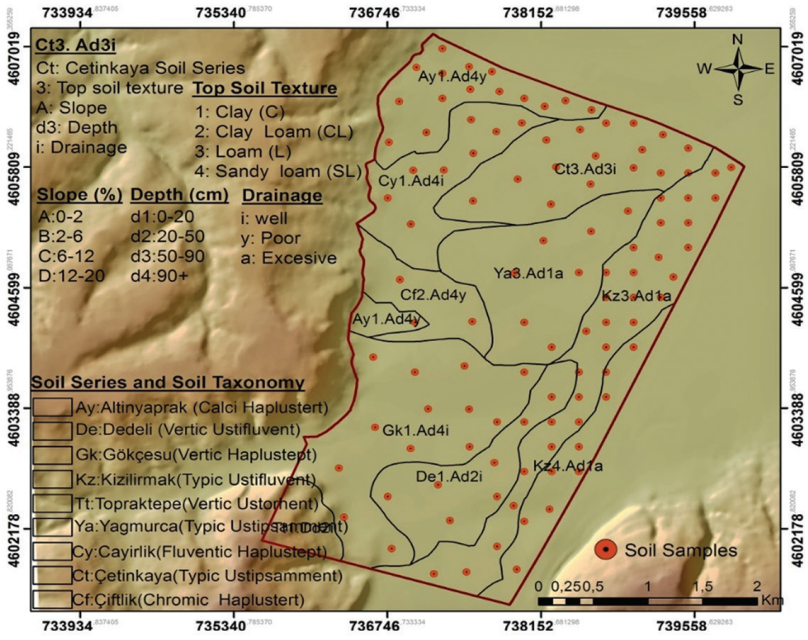

Figure 2- Soil map and soil samples pattern of the study area

The LLWR contents of the soils were determined according to Wu et al. (2003). Using the moisture content at 10% air-filled pore volume (θ_{Ap}), field capacity (θ_{FC}), wilting point (θ_{WP}), and moisture content at 2MPa penetration resistance (θ_{PR}), four possibilities were evaluated as follows, and LLWR was calculated.

1- If $\theta_{Ap} \geq \theta_{FC}$ and $\theta_{PR} \leq \theta_{WP,}$ then LLWR = $\theta_{FC} - \theta_{WP}$ (Available water content of the soil)

2- If $\theta_{Ap} \geq \theta_{FC}$ and $\theta_{PR} \geq \theta_{WP,}$ then LLWR = $\theta_{FC} - \theta_{PR}$ (Penetration resistance limits root development)

3- If $\theta_{Ap} \leq \theta_{FC}$ and $\theta_{PR} \leq \theta_{WP,}$ then LLWR= $\theta_{Ap} - \theta_{WP}$ (Aeration is poor)

4- If $\theta_{Ap} \leq \theta_{FC}$ and $\theta_{PR} \geq \theta_{WP,}$ then LLWR = $\theta_{Ap} - \theta_{PR}$ (Plant growth is limited as both aerations are poor and penetration resistance is high).

To determine the moisture content (θ_{PR}) at 2 MPa, the moisture obtained depending on different depths was calculated according to Busscher's (1990) Equation 1, taking into account penetration resistance measurement and bulk density values. The coefficients of the equation were found in a similar study by Alaboz et al. (2021) evaluated as stated.

$PR = a\theta^{c}D_b^{d}$ Equation (1)

The water content in which the aeration porosity is 10% was calculated by the equation (2):

$\theta_{Ap} = \theta_S - 0.10$ Equation (2)

Here, PR is the penetration resistance (MPa), θis the volumetric water content, D_b is the bulk density (g cm^{-3}), and θ_S is the saturated soil water content (cm^3 cm^{-3}).

2.3. Prediction approach using RF

RF is one of the tree-type learning algorithms. In the RF method, [h(x, θ_k) k=1,…] tree-type classifiers are used. Here, x represents the input data, and θ_k represents the random vector (Breiman 2001). The RF classifier parameters are the number of variables used at each node (mtry) and the number of trees to be developed (ntree) to determine the best split (Pal 2005). The user randomly selects the initial mtry value, increased or decreased, according to the next generalized errors, or the most appropriate mtry is determined by performing the tuning process. Thus, classification precision increases, whereas error decreases. According to Breiman (2001), when choosing the mtry variable value, the total number of variables equal to the square root usually gives optimum results. The RF uses the classification and regression tree (CART) algorithm to develop the largest tree without pruning (Breiman 2001). The CART algorithm divides a node by applying a certain criterion. The RF method adopts the Gini index. The cleavage position with the smallest Gini index is determined

with Gini measurements. When the Gini index reaches zero, the tree-branching process ends when one class remains at each leaf node (Watts & Lawrence 2008). The best branch is determined for each node, and many trees are produced depending on how many trees are desired to be produced (Liaw & Wiener 2002). According to the division criteria determined by using the training data, the nodes are divided into branches, and tree structures are formed (Figure 3).

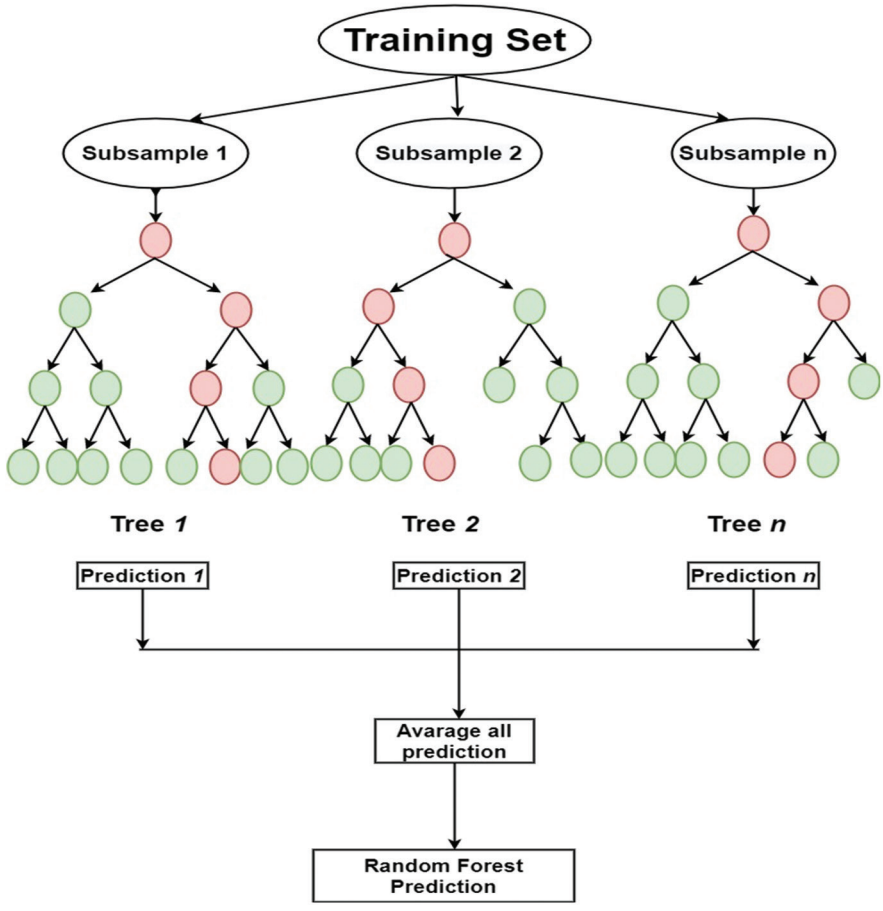

Figure 3- Structure of random forest

The tree with the best performance among the determined trees is assigned to a class (Liaw & Wiener 2002). The RF method has no fixed model, constraint, or pattern. It functions with as many trees as the user wants and is rapid. A RF algorithm is determined using the R package software. In the R Core software, "randomforest (Liaw & Wiener, 2002)", "caret (Kuhn et al. 2020)", and "mice (Van Buuren & Groothuis Oudshoorn 2011)" packages are used. To make the best estimation while creating the model, the tuning process was performed, and mtry was determined as 2, ntree =50 for 0-20 cm, and ntree =60 for 20-40 cm. Furthermore, 70% of the data set was evaluated as training and 30% as the test set. Also, the distribution of soil properties was checked using the Kolmogorov-Smirnov test. The "soil texture" package is used in the texture triangle created in the R software.

2.4. Interpolation models

In the present study, various interpolation [kriging, inverse distance weighting (IDW), and radial base function (RBF)] methods were applied to determine the most suitable model for the creation of spatial distribution maps of LLWR. Kriging, one of the scholastic approaches, uses a linear combination of weights at known points to estimate the value at an unknown point (Oliver & Webster 2015). A semivariogram, a measure of the spatial correlation between two points, is generated. There are several kriging interpolation methods, including simple kriging (SK), ordinary kriging (OK), and universal kriging (UK). Prior to the geostatistical estimation, a variogram was calculated for the distance classes between sample pairs.

SK is based on the logic of trying to estimate the value of a variable at any unknown point, using the values of the known points, similar to other estimation models in general (Li & Heap 2008).

OK (OK makes a calculation very similar to SK, but only OK changes the μ parameter in the μ (x_0) general equation $[1 - \sum_{i=1}^{n} \lambda_i] = 0$ n to $\sum_{i=1}^{n} \lambda_i$=1 instead of the value in the general equation while using the local average (Li & Heap 2008).

In the UK, the OK method cannot be used in case the variable values increase continuously depending on the increasing distance in a certain direction in the study area or space. In case the variable values do not increase continuously at a certain distance, the trends are removed using residual semivariograms, and estimates are made as a result of kriging (Christensen 1990; Brus & Heuvelink 2007).

IDW and RBF models, which are deterministic methods, were also applied in the study.

IDW is one of the most widely used multivariate interpolation methods. IDW is based on estimating the weighted average values of the value of the unknown point from the known point while using the inverse distance functions of the distances. There is a logic that as the distance from the point known to the target point increases, the similarities decrease (Li & Heap 2008).

RBF is currently a method used in the interpolation of multidimensional data. It is generally used for estimating limited data or hard-to-guess area points. The biggest advantage of this method is that it can be easily used in any size due to the low general restrictions (Wright 2003).

In the present study, completely regularized spline, thin plate spline, and spline with tension methods in RBF were evaluated.

ArcGIS 10.5v program was utilized in the creation and evaluation of scattering maps.

2.4. Assessment of the selected models

In the present study, MAE, root means square error (RMSE), and mean absolute percentage error (MAPE) parameters were used to examine the relationships between the predicted and observed values with different interpolation techniques and RF algorithm. Estimates were determined using the following formulas (Equations 3, 4, 5).

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|Zi - Z| \qquad \text{(Equation 3)}$$

$$\text{RMSE} = \sqrt{\frac{\sum (\text{Zi-Z})^2}{\text{n}}} \qquad \text{(Equation 4)}$$

$$\text{MAPE} = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{Zi-Z}{Z}\right| * 100 \qquad \text{(Equation 5)}$$

In the models, *Zi* is the estimation value, *Z* is the observed value, and n is the number of observations.

Also, soil characteristics were calculated in the descriptive statistics of the analysis. The present study used the IBM SPSS 23 software to calculate the values such as minimum, maximum, mean, standard deviation, coefficient of variation, skewness, and kurtosis of the parameters as descriptive statistics. The flow chart of the study is given in Figure 4.

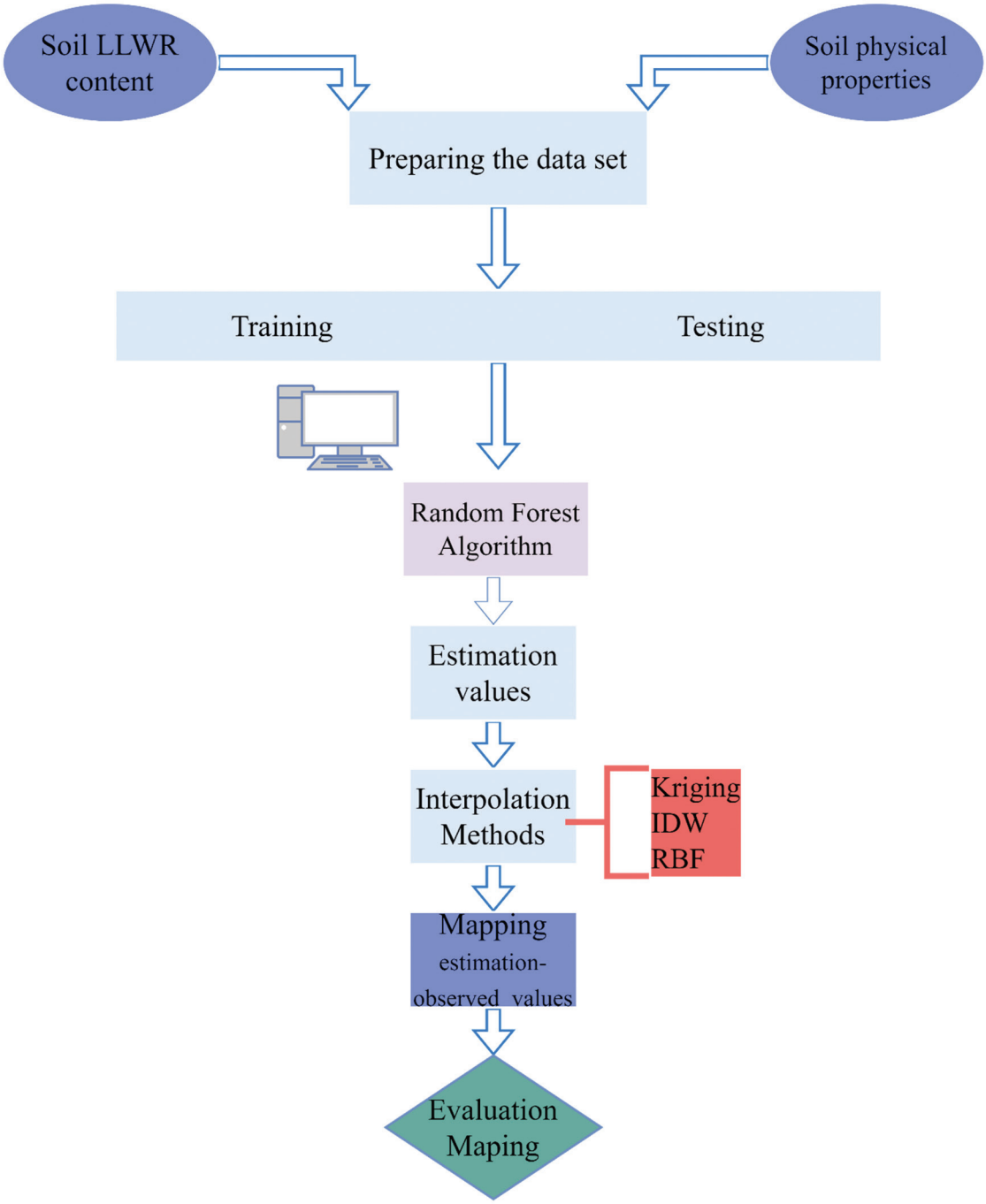

Figure 4- Flow chart

3. Results and Discussion

3.1. Soil physical properties and LLWR

Descriptive statistics for soils of different depths (0-20 cm and 20-40 cm) are given in Table 1. Since the study area is an alluvial land, it shows a significant change, especially in sand and clay distribution rates. Dengiz (2010) has stated that there are significant changes in particle size distribution over short distances in soils formed on sediments transported by rivers. The soils' sand, silt, and clay contents are determined in the ranges of 8.86-78.46%, 11.90-55.79%, and 6.93-63.90%, respectively. On the other hand, the texture class ranged from sandy loam to clay (Figure 5). Medium (loam 31.0%), medium-fine (clay loam 38%), and fine texture (23.52%) are dominant in the study area soils.

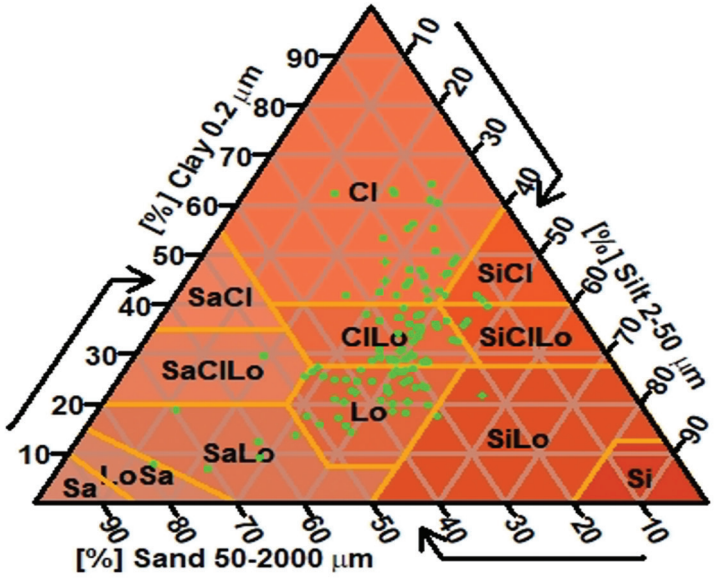

Figure 5- Selected soils in soil texture triangle
Sa: Sand, Lo: Loam, Si: Silt, CI: Clay

The moisture content where the air-filled pore volume is 10% (θ_{Ap}) was 0.330-0.444 cm^3 cm^{-3} for surface soils, and it was determined in the range of 0.265-0.435 cm^3 cm^{-3} at 20-40 cm depth. Depending on the depth, the increase in compaction leads to a decrease in the air-filled pore volume. The bulk density values were determined in the range of 1.20-1.56 g cm^{-3}, and the penetration resistance was determined in the range of 0.26-4.64 MPa in the surface soil. These properties were 1.24-1.68 g cm^{-3} and 0.51-4.40 MPa at 20-40 cm, respectively. As seen from the soil's bulk density and penetration resistance values, depth-dependent compaction was determined. This is thought to be caused by the heavy field traffic applied, especially in the area. Munsuz (1985) has reported that the compression on the surface causes an increase in penetration resistance and bulk density and states that the volume of air-filled pores decreased from 17.3% to 7.2% with the increase in bulk density. The soil's field capacity and wilting point contents were found to be 0.313 and 0.182 cm^3 cm^{-3} on average. It is well known that moisture constants vary depending on texture, organic matter, and structure. Also, the moisture content in the field capacity is significantly affected by the change in the pore volume due to compression (Karahan et al. 2014).

Table 1- Descriptive statistic of soil properties

Properties	*Min*	*Max*	*Mean*	*SD*	*CV*	*Skewness*	*Kurtosis*
0-20 cm							
Sand %	8.86	78.46	30.60	12.83	41.98	1.09	1.93
Clay %	6.93	63.98	32.60	12.57	38.55	0.52	-0.02
Silt %	11.90	55.79	37.50	7.61	20.61	-0.80	1.27
θ_{Ap} cm^3 cm^{-3}	0.330	0.444	0.372	2.90	6.24	-0.07	1.79
θ_{FC} cm^3 cm^{-3}	0.133	0.455	0.332	6.64	18.82	-0.53	0.42
θ_{WP} cm^3 cm^{-3}	0.058	0.347	0.198	5.82	30.25	0.36	-0.13
θ_{PR} cm^3 cm^{-3}	0.037	0.361	0.124	7.01	53.96	0.98	0.46
LLWR cm^3 cm^{-3}	0.049	0.263	0.134	3.87	25.73	-0.35	5.12
BD g cm^{-3}	1.20	1.56	1.40	0.07	4.82	-0.69	0.25
PR MPa	0.26	4.64	1.27	0.88	40.35	1.51	2.46
20-40 cm							
Sand %	9.51	74.08	30.70	13.87	45.24	0.91	0.86
Clay %	6.87	72.26	31.30	13.19	42.14	0.52	-0.06
Silt %	8.34	63.18	38.20	9.68	25.47	-0.58	0.80
θ_{Ap} cm^3 cm^{-3}	0.265	0.435	0.344	3.77	8.14	-1.72	7.59
θ_{FC} cm^3 cm^{-3}	0.137	0.452	0.313	7.03	20.11	-0.52	0.10

Table 1. Continued

θ_{WP} cm^3 cm^{-3}	0.053	0.348	0.182	6.53	34.66	0.20	-0.57
θ_{PR} cm^3 cm^{-3}	0.024	0.380	0160	6.77	41.12	0.62	0.72
LLWR cm^3 cm^{-3}	0.048	0.279	0.129	4.01	28.58	-0.18	2.51
BD g cm^{-3}	1.24	1.68	1.51	0.07	4.89	-0.62	-0.44
PR MPa	0.51	4.40	1.76	0.88	42.34	1.04	0.49

Min: Minimum, Max: Maximum, SD: Standard deviation, CV: Coefficient of variance, θ_{Ap}: Moisture content at 10% air-filled pore volume, θ_{FC}: Field capacity moisture content, θ_{WP}: Wilting point moisture content, θ_{PR}: Moisture content at 2MPa penetration resistance; LLWR: Least limiting water range, BD: Bulk density, PR: Penetration resistance

In the present study, the LLWR contents of the soils varied in the range of 0.048-0.279 cm^3 cm^{-3}. There were aeration problems in 6.72%, compaction problems in 20.16%, and aeration and compaction problems in 0.8% of the surface soils in the study area, whereas 72.32% were determined under optimum conditions. In 20-40 cm depth, aeration problems were detected in 5.88%, compaction problems in 28.57%, and aeration and compaction problems in 2.52%. According to Kay & Anger (2002), LLWR values are classified in the range of "less than" and "good". The LLWR contents are classified as "less than" if <0.1 cm^3 cm^{-3}, "poor" if 0.1-0.15 cm^3 cm^{-3}, "moderate" if 0.15-0.2 cm^3 cm^{-3} ", "good" if >0.2 cm^3 cm^{-3}. Approximately 9.2% of the LLWR contents of the surface soils were <0.1 cm^3 cm^{-3}, and 61% of the soils were determined in the range of 0.1-0.15 cm^3 cm^{-3}. Of the subsurface soils, 16.8% were determined to be "less than" and 58% as "poor". The LLWR contents of >0.02 cm^3 cm^{-3} in surface soils constituted 6.72% while this value decreased to 3.3% at 20-40 cm subsurface depth. In light of the obtained data, one of the significant results was that the amount of water the plant could use under the surface decreased. It is also known that the range of LLWR narrows with the change in pore volume with soil compaction (Kahlon & Chawla 2017). The LLWR contents of soils can exhibit high variability due to textural fraction ratios, compaction, and aeration problems (Alaboz et al. 2021). Negiş et al. (2020), on the other hand, determined significant increases in the lower and upper limits of LLWR with the increase in the doses of organic materials. Also, Silva and Kay (1997) have stated that LLWR exhibited negative correlations with the increase in bulk density and clay content, whereas positive with the organic matter content.

The coefficient of variance (CV) is an important factor in determining the variability of soil properties in the data set. Wilding (1985) classified the CV value as ≤15%, 15-30%, and ≥30% as low, medium, and high variability, respectively. Among the properties examined in the study, θ_{Ap} and BD properties were low, sand, clay, θ_{WP}, and PR were high, and other properties showed moderate variability. The skewness and kurtosis coefficients being close to 0 indicates a normal distribution. The feature closest to the normal distribution was LLWR, whereas the features farthest from the normal distribution were determined to be θ_{Ap} and PR. A negative skewness coefficient indicates skewness to the left and a positive skewness to the right. In contrast, a negative kurtosis coefficient suggests that the curve is flatter than normal and the positivity is steeper.

3.2. Estimation of LLWR with RF

LLWR estimates were carried out using the RF algorithm with the soils' sand, silt, clay, and bulk density values. To make the most appropriate estimation in the model estimation, mtry 2 was selected due to the tuning process. According to Breiman (2001), it was determined that optimum results were obtained when the mtry variable value was chosen as the square root of the total number of variables. Figure 6 shows the ntree (number of trees used) and error rates applied depending on the depths. The error must be stable and at the lowest level to make the most accurate estimation. The lowest error rate was determined with ntree 50 for surface soils and ntree 60 for 20-40 cm depth. In RF, the number of trees (ntree) and mtry are parameters that are often modified to regulate the complexity of the models. mtry indicates the number of randomly sampled indicators as candidates in each compartment. What is sampled for the split at each node is the number of mtry estimators.

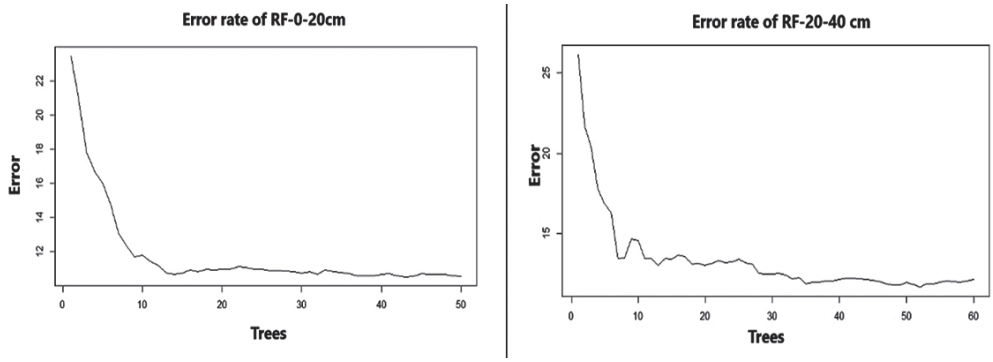

Figure 6- Error rate of trees

The RF model estimates the importance of covariates depending on how good, or bad the prediction will be when one or more variables are removed. It also reveals the errors that may occur by eliminating the good ones (Prasad et al. 2006). The variable importance of the RF algorithm is indicated in Figure 7. Sand and clay were determined as the best predictors for both depths. For 0-20 cm, the order of importance of the features is sand > clay > silt > BD. The feature with the highest error when it was removed from the model was determined as sand. If this feature is not included in the model, approximately an error of 4.5% occurs. The narrow range of variation of the bulk density feature compared to other features is also evident from the CV values. The effect of this narrow range of variation was lower among traits with high variability.

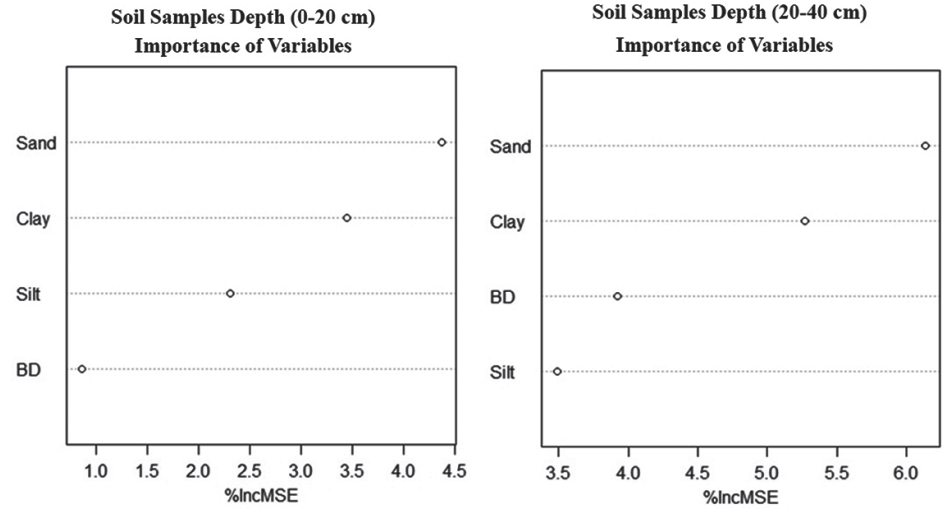

Figure 7- Importance of variables

The order of importance in estimating the LLWR for a soil depth of 20-40 cm is sand > clay > BD > silt. The property with the highest error when removed from the model was determined as sand. If this feature is not included in the model, an error of approximately 6% occurs. It was determined that the increase in compaction in subsurface soils has a defect in the estimation of LLWR. At 20-40 cm soil depth, an error of about 4% is expected as the BD moves away from the model. This value was about 1% in the surface soil, whereas the error showed a four-fold increase at 20-40 cm depth. It has been reported that there were significant negative correlations between bulk density and LLWR. Also, it has been reported that LLWR narrows with increasing bulk density (Haghighi Fashi et al. 2017; Alaboz et al. 2021). Increasing soil compaction depending on the bulk density can increase soil water retention both at field capacity and at wilting point, indicating that it provides higher water retention in the soil with the increase in medium and micro-sized pores due to the reduction of macro-sized pores (Safadoust et al. 2014). However, it is assumed that root development will be inhibited due to the existing compaction of the plant before the soil water content reaches the wilting point. Sand and clay were found to be important in the estimation of LLWR for both depths. Alaboz et al. (2021) determined a statistically significant positive relationship between the clay content of soils and LLWR (r=0.30). The high variability in the sand content of the soils can be understood from the CV value. The highest CV value was determined in the sand among the textural fractions. Therefore, the contribution rate of this feature to the model was found to be high.

3.3 Assessment of models' performance for LLWR estimation

The model estimation performance values obtained in the testing and training phase of the LLWR estimation with the RF algorithm using the sand, silt, clay, and bulk density values of the soils are given in Table 2.

Table 2- Performance assessment of RF model

	Training			***Testing***		
Depth	***RMSE*** ***(cm^3 cm^{-3})***	***MAPE*** ***(%)***	***MAE*** ***(cm^3 cm^{-3})***	***RMSE*** ***(cm^3 cm^{-3})***	***MAPE*** ***(%)***	***MAE*** ***(cm^3 cm^{-3})***
0-20 cm	0.0223	13.645	0.0158	0.0218	13.28	0.0190
20-40 cm	0.0203	12.634	0.0154	0.0247	8.45	0.0167

RMSE: Root mean sequare error, MAPE: Mean absolute percentage error, MAE: Mean absolute error

Similar error rates were determined during the training and testing phases. This shows that the learning performance of the model is high. The RMSE values obtained in the evaluation of the predictive accuracy of the model were determined in the range of 0.0203-0.0247 cm^3 cm^{-3}, and the MAPE values were found to be 8.45-12.645%. Lewis (1982) classified models with a MAPE value of less than 10% are "very good", models between 10-20% are "good", models between 20-50% are "acceptable", and models above 50% are "wrong and faulty". According to the classifications, it was determined that the model's performance was good. In the test phase, the MAE was found to be 0.0190 cm^3 cm^{-3} at 0-20 cm depth and 0.0167 cm^3 cm^{-3} at 20-40 cm depth. Low RMSE, MAPE, and MAE values are desirable for the model's validity in model studies. The more data trained in machine learning algorithms, the higher the probability the model predicts. Alaboz et al. (2021) achieved the best performance with the deep learning algorithm in their studies investigating the predictability of LLWR with artificial neural networks and deep learning algorithms. In another study (Tavanti et al. 2019), LLWR was evaluated with pedotransfer functions, models obtained from the literature, and artificial neural networks were examined. ANN determined the lowest RMSE value to be 0.0142 m^3 m. Akar and Güngör (2013) stated that the RF algorithm exhibits higher accuracy than other approaches. In obtaining successful results, a high tree depth is considered as running the model with many trees in the background.

3.4. Spatial variation of LLWR

Spatial distribution maps of LLWR's observed and predicted values were created according to the most appropriate model using different interpolation models. The data conformity to the normal distribution was checked with the Kolmogorov-Smirnov test, and logarithmic transformation was applied to both data. The RMSE values of the model parameters created for the observed and predicted LLWR values are given in Table 3. In the surface soil (0-20 cm), SK's spherical model was determined as the most suitable model for the distribution of observed values. In contrast, SK's Gaussian model was determined for the distribution of predicted values. Also, it has been determined that OK's Gaussian model is the most suitable model for the spatial distributions of observed and predicted values in subsurface (20-40 cm) soils.

Table 3- Cross-validation and their RMSE values according to different interpolation models

Criteria		***Inverse distance weighing***					***Radial basis function***			
	1		***2***	***3***		***TPS***	***CRS***		***ST***	
LLWR (0-20 cm)	Observe	0.0252	0.0250		0.0249		0.0277	0.0250		0.0250
	Estimate	0.0180	0.0181		0.0183		0.0224	0.0186		0.0184
LLWR (20-40 cm)	Observe	0.0275	0.0280		0.0286		0.0341	0.0283		0.0281
	Estimate	0.0244	0.0250		0.0257		0.0315	0.0255		0.0252
Criteria		***Kriging***								
	Ordinary			***Simple***			***Universal***			
	Gau.	Exp.	Sph.	Gau.	Exp.	Sph.	Gau.	Exp.	Sph.	
LLWR (0-20 cm)	Observe	0.0254	0.0253	0.0253	0.0237	0.0238	0.0236	0.0254	0.0253	0.0253
	Estimate	0.0180	0.0181	0.0181	0.0178	0.0179	0.0179	0.0180	0.0181	0.0180
LLWR (20-40 cm)	Observe	0.0271	0.0277	0.0276	0.0273	0.0272	0.0272	0.0276	0.0277	0.0276
	Estimate	0.0237	0.0240	0.0239	0.0239	0.0238	0.0238	0.0238	0.0240	0.0239

RMSE: Root mean sequare error, Gau.: Gaussian, Exp.: Exponential, Sph.: Spherical, TPS: Thin plate spline, CRS: Completely regularized spline, ST: Spline with tension, LLWR: Least limiting water range

The spatial distribution patterns of the values of both observed (LLWR-O) and estimated (LLWR-E) in surface soils and subsurface soils in the study area, located on alluvial land with different soil properties, showed closeness to each other (Figure 8). In general, low LLWR values in surface (0-20 cm) soils were determined in Ay1, Ad4y, Ct3, Ad3i, and Kz4, which are in the Gold leaf (Calci Haplustert), Çetinkaya (Typic Ustipssament), and Kızılırmak (Typic Ustifluvent) soil series. Low LLWR values were determined in the Ad1a mapping units, while low LLWR values were determined in similar series in the distribution map of the values estimated by RF. Furthermore, examining the LLWR distribution of subsurface soils, which is very important for plant root development, it was seen that low LLWR values are concentrated in the Ct3, Ad3i, and Kz4. Ad1a mapping units located in the Çetinkaya and Kızılırmak soil series and the northeast of the study area. Although a similar case for subsurface (20-40 cm) soils was also seen in the LLWR estimated by RF, the areas with low estimated LLWR values were mostly distributed in the Kızılırmak soil series, which is classified as Typic Ustipssament.

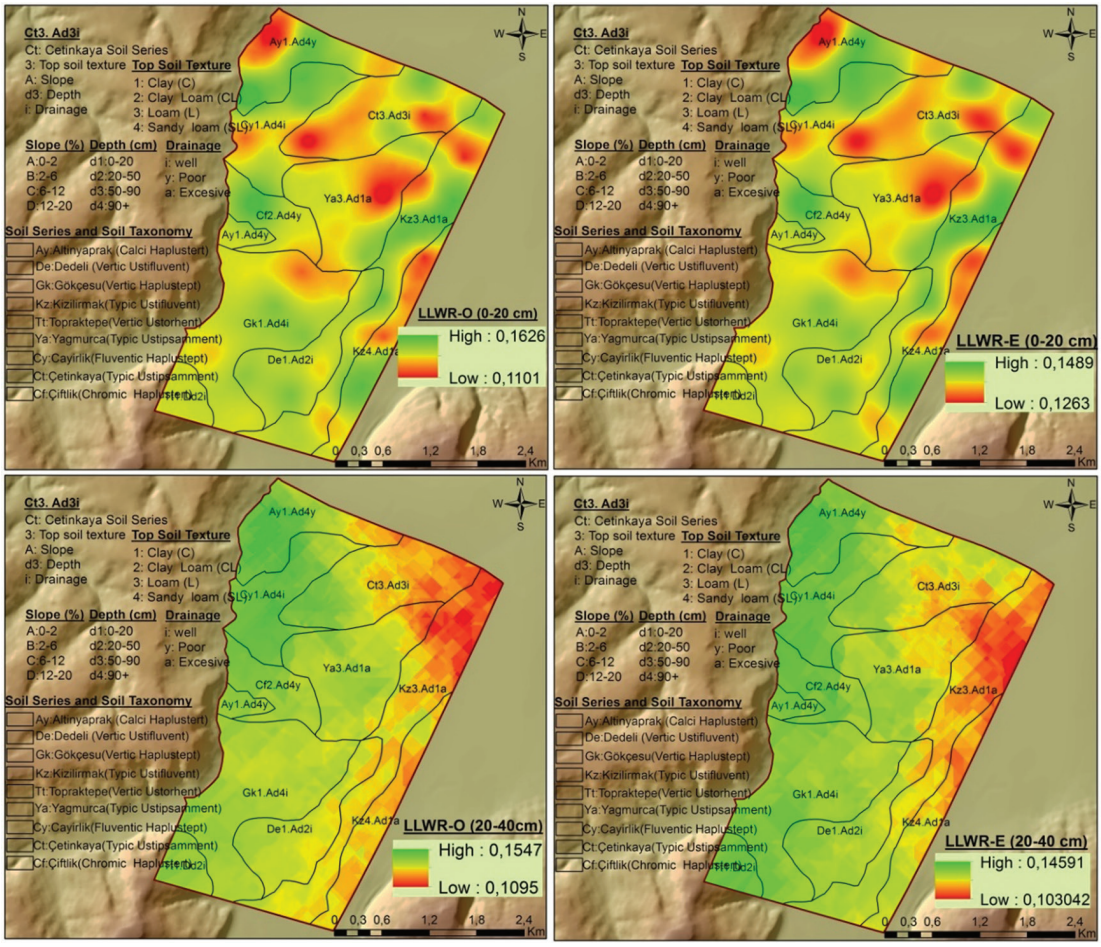

Figure 8- Spatial distribution maps of the LLWR (observed: LLWR-O and estimated: LLWR E for surface and sub-surface soil depths)

4. Conclusions

In the present study, the LLWR contents of the soils distributed on the alluvial lands in the Bafra Delta Plain were evaluated for two different soil depths, and the predictability of LLWR with the RF algorithm, one of the machine learning methods, was investigated. Also, the observed and predicted values of the studied feature were evaluated with different interpolation methods.

As a result, the LLWR contents of the soils were determined in the range of 0.049-0.279 cm^3 cm^{-3}. Aeration problems were determined in 6.72% of the surface soils, compaction in 20.16%, and both aeration and compaction problems in 0.8%. In 20-40 cm depth, aeration problems were detected in 5.88%, compaction problems in 28.57%, and aeration and compaction problems in 2.52%. Soil properties that are effective in estimating LLWR with RF were determined as sand and clay. The importance of BD in the model has increased with the increase of depth-dependent PR. It was shown that the estimation of LLWR from sand, silt, clay, and BD with the RF algorithm could be carried out with high accuracy by training the dataset.

Alluvial soils have unstable properties due to different geological processes. The use of the RF model, which effectively solves complex structures in estimating these properties, was successfully demonstrated. Also, spatial distribution maps were successfully created in alluvial soils using the LLWR estimated values obtained by RF. As a result, it was revealed that there are aeration and compression problems on the surface and subsurface soils in the study area. Also, it was found that spatial distribution maps can be created for the region by successfully estimating LLWR utilizing the RF algorithm. For future studies, it is recommended that spatial distribution maps be updated at regular intervals for sustainable land management, focusing on the possibilities of determining based on LLWR value using the RF algorithm, especially in areas where land traffic will be intense.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: P.A., Design: P.A., O.D., Data Collection or Processing: O.D., Analysis or Interpretation: P.A., O.D., Literature Search: P.A., O.D., Writing: P.A., O.D.

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References

Akar Ö & Güngör Ö (2013). Classification of multispectral images using Random Forest algorithm. *Journal of Geodesy and Geoinformation* 1(2): 105-112. doi.org/10.9733/jgg.241212.1

Aksakal EL (2004). Soil compaction and its importance for agriculture. *Atatürk University Journal of Agricultural Faculty* 35(3-4): 247-252.

Alaboz P, Başkan O & Dengiz O (2021). Computational intelligence applied to the least limiting water range to estimate soil water content using GIS and geostatistical approaches in alluvial lands. *Irrigation and Drainage* 70(5): 1129-1144. doi.org/10.1002/ird.2628

Alaboz P, Demir S & Dengiz O (2020). Determination of Spatial Distribution of Soil Moisture Constant Using Different Interpolation Model Case study, Isparta Atabey Plain. *Journal of Tekirdag Agricultural Faculty* 17(3): 432-444. doi.org/10.33462/jotaf.710411

Blake G R & Hartge K H (1986). Bulk density. In: Klute A, Ed., Methods of Soil Analysis, Part 1—Physical and Mineralogical Methods, 2nd Edition, Agronomy Monograph 9, American Society of Agronomy—Soil Science Society of America, Madison, 363-382.

Baillie I C (2001). Soil survey staff 1999, soil taxonomy: a basic system of soil classification for making and interpreting soil surveys, agricultural handbook 436, Natural Resources Conservation Service, USDA, Washington DC, USA, pp. 869.

Breiman L (2001). Random forests. *Machine Learning* 45(1): 5-32.

Brus D J & Heuvelink G B (2007). Optimization of sample patterns for universal kriging of environmental variables. *Geoderma* 138(1-2): 86-95. doi.org/10.1016/j.geoderma.2006.10.016

Busscher W J (1990). Adjustment of flat- tipped penetrometer resistance data to a common water content. *American Society of Agricultural and Biological Engineers* 33(2): 519-524.

Chan K, Oates A, Swan A, Hayes R, Dear B & Peoples M (2006). Agronomic consequences of tractor wheel compaction on a clay soil. *Soil and Tillage Research* 89(1): 13-21. doi.org/10.1016/j.still.2005.06.007

Christensen R (1990). The equivalence of predictions from universal kriging and intrinsic random-function kriging. *Mathematical Geology* 22(6): 655-664. doi.org/10.1007/bf00890514

Da Silva A, Kay B & Perfect E (1994). Characterization of the least limiting water range of soils. *Soil Science Society of America Journal* 58(6): 1775-1781. doi.org/10.2136/sssaj1994.03615995005800060028x

Da Silva A P & Kay B (1997). Estimating the least limiting water range of soils from properties and management. *Soil Science Society of America Journal* 61(3): 877-883. doi.org/10.2136/sssaj1997.03615995006100030023x

Dengiz O (2010). Morphology, Physico-Chemical Properties and Classification of Soils on Terraces of the Tigris River in the South-East Anatolia Region of Turkey. *Journal of Agricultural Sciences* 16(3): 205-212. doi.org/10.1501/tarimbil_0000001139

Eijkelkamp (1990). Equipment for soil research. Giesbeek (The Netherlands): Eijkelkamp Corporation.

Gee G W & Bauder J W (1986). Methods of soil analysis: physical and minerological analysis. Madison (WI): *American Society of Agronomy Particle-size analysis* 388-409.

Haghighi Fashi F, Gorji M & Sharifi F (2017). Least limiting water range for different soil management practices in dryland farming in Iran. *Archives of Agronomy and Soil Science* 63(13): 1814-1822. doi.org/10.1080/03650340.2017.1308688

Kahlon M S & Chawla K (2017). Effect of tillage practices on least limiting water range in Northwest India. *International Agrophysics* 31(2): 183-194. doi.org/10.1515/intag-2016-0051

Karahan G, Erşahin S & Öztürk H S (2014). Field Capacity Dynamics Affected by Soil Properties. *Journal of Agricultural Faculty of Gaziosmanpasa University* 30(1): 1-9. doi.org/10.13002/jafag177

Kay B D & Anger D A (2002). Soil structure in soil physics companion 249-296.

Klute A (1986). Water Retention: Laboratory Methods. Methods of soil analysis: part 1 physical and minerological methods. 5: 635-662. doi.org/10.2136/sssabookser5.1.2ed.c26

Kuhn M,Wing J, Weston S, Williams A, Keefer C, Engelhardt A, Cooper T, Mayer Z, Kenkel B, & Team R C (2020). Package 'caret'. *The R Journal* 223(7).

Leão T P, Da Silva A P, Perfect E & Tormena C A (2005). An algorithm for calculating the least limiting water range of soils. *Agronomy Journal* 97(4): 1210-1215. doi.org/10.2134/agronj2004.0229

Letey J (1958). Relationship between soil physical properties and crop production. *Advances in soil science* 1: 277-294.

Lewis C D (1982). Industrial and Business Forecasting Methods. Butterworths Publishing.

Li J & Heap A D (2008). A review of spatial interpolation methods for environmental scientists. *Geoscience Australia* 137-145.

Liaw A & Wiener M (2002). Classification and Regression by Random Forest, *R News* 2-3: 18-22.

Machado G, Vilalta C, Recamonde-Mendoza M, Corzo C, Torremorell M, Perez A & VanderWaal K (2019). Identifying outbreaks of Porcine Epidemic Diarrhea virus through animal movements and spatial neighborhoods. *Scientific Reports* 9: 1-12. doi.org/10.1038/s41598-018-36934-8

Mihalikova M, Özyazıcı M A & Dengiz O (2016). Mapping soil water retention on agricultural lands in central and eastern parts of the Black Sea Region in Turkey. *Journal of Irrigation and Drainage Engineering* 142(12): 05016008-1. doi.org/10.1061/(asce)ir.1943-4774.0001094

Munsuz N (1985). Soil Mechanics and Technology. *Ankara University. Faculty of Agriculture Publications*

Negiş H, Şeker C & Çetin A. (2020). Effects of different organic materials on soil compaction and least limiting water range. *Journal of Soil Science and Plant Nutrition* 8(2):118-127. doi.org/10.33409/tbbbd.778834

Oliver M A & Webster R (2015). Basic steps in geostatistics: the variogram and kriging. Springer International Publishing.

Pal M (2005). Random Forest Classifier for Remote Sensing Classification. *International Journal of Remote Sensing* 26(1): 217-222. doi.org/10.1080/01431160412331269698

Prasad A M, Iverson L R & Liaw A (2006). Newer classification and regression tree techniques: Bagging and random forests for ecological prediction. *Ecosystems* 9: 181-199. doi.org/10.1007/s10021-005-0054-1

Safadoust A, Feizee P, Mahboubi A, Gharabaghi B, Mosaddeghi M & Ahrens B (2014). Least limiting water range as affected by soil texture and cropping system. *Agricultural Water Management* 136: 34-41. doi.org/10.1016/j.agwat.2014.01.007

Soil Survey Staff (2014). Keys to Soil Taxonomy. *USDA National Resources Conservation Services*.

Stum A K, Boettinger J L, White MA & Ramsey R D (2010). Random forests applied as a soil spatial predictive model in arid Utah. *Digital Soil Mapping* 179-190.

Şenol H, Alaboz P, Demir S & Dengiz O (2020). Computational intelligence applied to soil quality index using GIS and geostatistical approaches in semiarid ecosystem. *Arabian Journal of Geosciences* 13(23): 1-20. doi.org/10.1007/s12517-020-06214-9

Tavanti R F, Freddi O D S, Tavanti T R, Rigotti A & Magalhães W D A (2019). Pedofunctions applied to the least limiting water range to estimate soil water content at specific potentials. *Engenharia Agrícola* 39(4): 444-456. doi.org/10.1590/1809-4430-Eng.Agric.v39n4p444-456/2019

TSMS (2021). Turkish State Meteorological Service. Ankara, Turkey.

Tunçay T, Başkan O, Bayramin İ, Dengiz O & Kılıç Ş (2018). Geostatistical approach as a tool for estimation of field capacity and permanent wilting point in semiarid terrestrial ecosystem. *Archives of Agronomy and Soil Science* 64(9): 1240-1253. doi.org/10.1080/03650340.2017.1422081

Watts J D & Lawrence R L (2008). Merging random forest classification with an object-oriented approach for analysis of agricultural lands, *The International Archives of the Photogrammetry Remote Sensing and Spatial Information Sciences* 37(B7): 579-582.

Wilding L P (1985). Spatial variability: Its documentation, accommodation and implication to soil surveys. In: Soil spatial Variability, Las Vegas NV, 30 November-1 December 1984. 1985. p. 166-194.

WRB (2014). World reference base for soil resources. International soil classification system for naming soils and creating legends for soil maps. Food *and Agriculture Organization of United Nations* 6: 203.

Wright G B (2003). Radial basis function interpolation: numerical and analytical developments. University of Colorado at Boulder.

Wu L, Feng G, Letey J, Ferguson L, Mitchell J, Mc Cullough-Sanden B & Markegard G (2003). Soil management effects on the nonlimiting water range. *Geoderma* 114(3-4): 401-414. doi.org/10.1016/S0016-7061(03)00052-1

Van Buuren S & Groothuis-Oudshoorn K (2011). mice: Multivariate imputation by chained equations in R. *Journal of Statistical Software* 45(3): 1-67.

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Drone Larvae Homogenate (Apilarnil) as Natural Remedy: Scientific Review

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ABSTRACT
For centuries, honey bee products such as honey, bee pollen, royal jelly, bee bread (Perga), and bee venom have been utilized in natural medicine due to their beneficial properties. A great deal of scientific research has been dedicated to exploring their physico-chemical properties and therapeutic effects. Despite this, drone larvae have not received as much attention from the scientific community. Within a honey bee colony, drones are responsible solely for fertilizing queen bee eggs and consuming food reserves collected by worker honey bees. As a result, beekeepers commonly remove excess drone brood from the hive, which is crucial for preventing and treating varroasis. Lyophilization is the most effective method for preserving drone larvae, and the physicochemical properties of fresh and lyophilized drone larvae were compared. The therapeutic effects of drone larvae, such as androgenic, hepatoprotective, immunostimulatory, and hypolipidemic effects in humans and experimental animals, were summarized. This study aims to summarize current scientific knowledge on drone larvae (apilarnil). The author utilized well-known publication databases like SCOPUS, Google Scholar, and Pub Med to gather research on drone larvae. Furthermore, this review collected information on the chemical composition preservation and bioactive action of drone larvae. Thanks to their high levels of amino acids, fatty acids, vitamins, minerals, and hormones, drone larvae can be considered a potential potency-raising agent.

Keywords: Drone larvae, Apilarnil, Drone brood, Physicochemical properties, Lyophilize, Natural remedy, Varroa control, Hormone

1. Introduction

People prefer to take preventive measures instead of struggling with health problems in order to increase the quality and duration of life. Functional foods attract consumers because they would rather prevent a disease than cure it. Side effects of drugs and increased medical costs are among other important reasons. In addition, in industrialized countries, functional foods are preferred in order to be protected from the pollution in water, air and food, the use of chemicals and hormones or the health risks caused by environmental factors. Similarly, increasing health awareness is important in today's world where access to information becomes easier with developing technologies. As a matter of fact, the increase in demand for these reasons has revitalized the sector and paved the way for the introduction and marketing of novel and different products every day. As a result, the desire of consumers to seek a healthier and higher quality life cannot be ignored.

Apitherapy, which has existed since the beginning of humanity and whose importance has increased with scientific studies in recent years, draws attention in terms of preventing many diseases and supporting treatment. Natural bee products such as honey, pollen, Perga, propolis, royal jelly and apilarnil have high antioxidant capacity as well as rich nutritional content (Kumazawa et al. 2004; Leja et al. 2007; Eraslan et al. 2017; Özkök & Silici 2017). These valuable products, which honey bees process by collecting nectar and pollen from plants in nature, consist of many bioactive compounds such as protein, carbohydrates, vitamins, enzymes, phenolic compounds, aroma compounds, phytosterols, terpene and terpenoids, fatty acids and aliphatic compounds. Many different biologically active components of honey bee products have been held responsible for different effects such as antioxidant, antimicrobial, antifungal, immunostimulant and anticarcinogenic activities (Eraslan et al. 2008; Koc et al. 2009; Nassar et al. 2012; Miyata & Sakai 2018). The number of scientific studies on Apilarnil a recently discovered bee among the bee products, is very limited. Thus, this review summarizes the current knowledge on the structure, physicochemical content and biological functions of apilarnil, and points out and promotes further research directions.

1.1. Biology of the drone

The order Hymenoptera is known for its haplodiploid system. In the case of honey bees (*Apis mellifera* L.), a colony typically includes a queen bee, numerous sterile female workers, and several hundred drones during the breeding season (Palmer & Oldroyd 2000; Collison 2004). In all castes, developmental stages consist of egg, larva, pupa and adult. A queen bee that has

completed mating lays two types of eggs; fertilized and unfertilized. Queen and worker bees hatch from fertilized eggs, while drones hatch from unfertilized eggs. All hatchlings are fed with royal jelly for the first three days. While queen bee larvae were fed with royal jelly throughout the entire developmental period, the nutrition of worker and drone larvae consisted of a mixture of honey, bee bread and royal jelly (Isidorov 2021). Drones are reared in honeycomb cells that are larger than worker bee cells. In a typical colony's annual cycle, drone production begins 3-4 weeks before the production of new queens at the start of the breeding season. Unlike drones, which mate only once, queen bees mate with an average of 12-14 drones (Rhodes 2002; Tarpy & Page 2000). The development of drones from egg to adult is greater than that of queen (16 days) and workers (21 days), lasting approximately 24 days (De Grandi-Hoffman et al. 1998). Like the males in other Hymenoptera species, spermatogenesis in honey bee drones begins at the larval stage and ends at the pupal stage. The volume of sperm ejaculate in the drone ranges from approximately 0.91 to 1.7 µL per drone, and 3.6-12 million sperm cells are produced. Sperm counts are strongly affected by drone size, larval diet and season (Collins & Pettis 2001; Rhodes et al. 2011). In the first week after hatching, sexual maturation in drones is completed with the development of a pair of mucous glands that protect and nourish sperm along with the migration of sperm to the seminal vesicles (Johnson et al. 2013). Sperm cells provide protection against pathogens due to the proteins found in the seminal fluid. Environmental factors such as climate and diet affect the timing of sexual maturation (Peng et al. 2016). Successful mating ends with the death of the drones, because the drones die soon after mating due to the ejaculation of sperm with great force, and the endophallus remains in the genital tract of the queen. Drones do not have a long proboscis, mouth structure, corbicula, and stingers, which are found in worker bees to collect and transport nutrients. Much of the research examining drone biology and health has been on reproductive quality and ability. Since the only role of drones in the hive is related to reproduction and although they are in the hive temporarily, they have an important function in mating with their queen. The only function of drones in a honey bee colony is to gather hundreds of drones from many different colonies in the air (in drone gathering areas) and mate with the queen bee in flight. It is clear that drones with large, high flight capacity and good maneuverability have a competitive advantage. Thus, a honeybee colony can actually increase reproductive success by producing higher quality and competitive males for reproduction (Winston 1987).

When the colony is without a queen, the worker bees lay unfertilized eggs in the cells of the comb, as the pheromone pressure that prevents the development of the ovaries of the worker bees is removed. These eggs are not drone cells but worker bee cells. The drones grown in these honeycomb cells are smaller. Goins & Schneider (2013) investigated the effects of drones reared in drone cells (DC) and drones reared in worker cells (WC) on potential reproductive quality and caste interactions. Although worker bees initially observed different size and quality potential within the two drone types, they stated that this distinction was not related to acceptance decisions. Utaipanon et al. (2019) showed that drones reared in worker honeybee cells also contribute to mating.

Drone brood production depends on pollen supply to meet protein needs. Colonies regulate drone production according to season and food availability (Hrassnigg & Crailsheim 2005). Optimal drone production depends on climate, colony conditions (size and queen age) and food availability (Boes 2010). Regular removal of drones from a colony helps to regulate drone production. In the Northern Hemisphere, drone production is typically concentrated between May and August. However, the production of drones is ultimately determined by the number of drone honeycomb cells in the colony (Boes 2010). During the drone breeding season, beekeepers can place empty frames in the colony to stimulate the production of drone broods. Removing drone larvae from these combs where Varroa spp. mites laying their eggs can help control their population in the hive. This method has been found to be effective in managing *Varroa* spp. infestations, which can be detrimental to the colony's health (Calderone 2005).

1.2. Functional components of drone larvae

A drone larva (Apilarnil) is obtained by collecting drone larvae 3 to 11 days after hatching. This valuable bee product was discovered by the Romanian scientist Nicolae V. Iliesiu. This name consists of “api” for bee, “lar” for larva and “nil” which is a shortened form of explorer name. However, while drone larvae are collected from the honeycomb cell, they can be collected together with the larval food (drone milk) or it can be obtained by eliminating the larval food. Drone jelly (DM) is a highly nutritious food produced by the hypopharyngeal and mandibular glands of worker bees. It serves as the primary food source for developing drone larvae, and its composition is believed to be linked to the reproductive abilities of drones. While not as well researched as other honeybee products such as honey and royal jelly, drone royal jelly has gained attention in recent years for its potential health benefits. In addition to its nutritional value, drone milk has been shown to have antimicrobial and immunomodulatory properties that may be beneficial in the treatment of various human diseases. Understanding the chemical composition and biological properties of male royal jelly is an important area of research and could lead to the development of new therapeutic agents (Mutsaers et al. 2005).

Larva homogenate has the consistency of creamy milk. Its colour can vary from white to yellowish and it has a distinctive slightly acidic taste (Isidorov 2021). Storage and shelf life information is required for food products. Since apilarnil contains important nutrients and is in a natural state, it is a suitable environment for the development of microorganisms and fungi. Therefore, it can deteriorate under inappropriate storage conditions. The most convenient way to stabilize unstable moist media is lyophilization. Larvae can be stored for 6 days at -2 ºC and up to 10 months at -18 ºC without losing their biological activity (Barnutiu et al. 2013). It can be preserved for 6 months by adding 1-2% volume of honey, which is another method. Krylow et

al. (2007) reported that the addition of homogenate to honey at a concentration of 3-5% preserves its biological properties for up to 6 months at 6-12 ºC. Other than that, the larvae can be mixed with 40% ethyl alcohol at a ratio of 1:1 (Bak & Wilde 2002) or dried in air-circulating dryers and stored for 7 months. Using adsorbent is another method. In this method, it has been reported that the final product obtained with the combination of 1:1 glucose and lactose, larvae and adsorbent (1:6) can be stored for 3 years at room temperature and 3 months in the refrigerator (Lebiediew & Legowicz 2003). The content of main components and physicochemical properties of drone larvae are presented in Table 1 (Finke 2005; Barnutiu et al. 2013; Balkanska et al. 2014; Bogdanov 2016; Isidorov et al. 2016; Margaoan et al. 2017; Sawczuk et al. 2019; Silici 2019; Prikhodko et al. 2020; Koşum et al. 2022). The greatest differences in the physicochemical composition were reported between fresh and lyophilized homogenate in terms of water content. While the water content of fresh apilarnil is between 70.30-76.8%, it decreases to 3.0-5.0% when apilarnil is lyophilized.

Table 1- Chemical composition of drone larvae (fresh and lyophilized) (Balkanska et al. 2014; Silici 2019; Koşum et al. 2022; Margaoan et al. 2017; Barnutiu et al. 2013; Prikhodko et al. 2020; Isidorov et al. 2016; Finke 2005; Bogdanov 2016; Sawczuk et al. 2019)

Characteristics		*Drone larvae*	
		Fresh *Range (Min-Max)*	*Lyophilized* *Range (Min-Max)*
Water, %		65.0-78.5	3.0-5.0
Protein, %		4.6-13.2	32.0-52.4
Lipid, %		1.2-8.38	4.8-24.2
Carbohydrates, %		6.22-12.2	9.30-38.9
	Fructose	0-0.38	-
	Glucose	3.55-7.88	-
	Sucrose	0-0.18	-
Ash, %		0.7-4.1	2.7-4.1
pH		5.8-6.63	7.0
Acidity, mL 0.1 NaOH g^{-1}		0.74-2.61	-
Energy value, $kJ100g^{-1}$		111.9-503.3	501.4-2097.9

Apilarnil is a rich source of protein and amino acids as they are the most abundant nutrients in its composition (Table 2). The protein content of drone larvae is very important for the evaluation of their nutritive properties. According to the analyzes made to date, the protein content is approximately 3.5 times higher in lyophilized apilarnil than the fresh one (Table 1). Studies of chemical analysis of apilarnil showed that relatively low molecular mass proteins consisting of globulin and albumin predominate (Lazaryan et al. 2003; Xu & Gao 2013). It was demonstrated that protein profiles depend on the bee's stages and during larval development its content increases (Ghosh et al. 2016). Comprehensive analysis proved that the total content of amino acids in brood larvae is 37.57-40.57% (Lazaryan et al. 2002). The amino acid composition was characterized by high levels of glutamic acid, valine, aspartic acid, lysine and leucine (Lazaryan et al. 2002; Isidorov 2016). Lazaryan et al. (2002) demonstrated that the content of essential amino acid to be 15.45-16.28. Among all of the amino acids reported in lyophilized apilarnil, the greatest amounts are glutamic acids, leucine, proline, arginine and aspartic acids. Also, valine and lysine are the most abundant (Ghosh et al. 2016). The amino acid lysine contributes to the absorption of calcium in the body and plays an important role in the formation of collagen in bones and connective tissues (Civitelli et al. 1992). Valine, leucine, and isoleucine are essential amino acids that play a crucial role in muscle protein synthesis and repair (Bifari & Nisoli 2017). Animal proteins are reported to have higher nutritional quality than plant-based proteins. This superiority is due to their amino acid composition, digestibility, and ability to carry nutrients such as calcium and iron (Kim et al. 2020). Food proteins are broken down into smaller peptides and individual amino acids by digestive enzymes during the process of digestion. These amino acids are absorbed and utilized for various metabolic processes, including the synthesis of tissue proteins for growth and repair. Amino acids can also be used to synthesize other nitrogen-containing compounds such as neurotransmitters and nucleotides or catabolized for energy production when needed (Atherton et al. 2010). The human body cannot synthesize essential amino acids (EAA), which include isoleucine, leucine, histidine, lysine, methionine, threonine, tryptophan, phenylalanine, and valine. Other amino acids like glycine, proline, arginine, glutamine, and serine are considered "conditionally essential" since they are more important in specific stages of life or conditions such as illness, early development, or stress. The remaining amino acids are classified as "non-essential" because the human body can synthesize them. Therefore, the only way to obtain essential amino acids is through the diet. Animal-based protein sources like meat, eggs, and milk are known as "complete sources of protein" since they contain all nine essential amino acids in sufficient amounts for the body's needs (Wu 2009). Apilarnil is an acceptable product in this group because it contains both high protein content and all nine essential amino acids.

Table 2- Amino acid composition of drone larvae (Lazaryan, 2002; Silici 2019; Ghosh et al. 2016; Fınke 2005; Isıdorov et al. 2016)

Amino acids (g/100g)	*Fresh Range (Min-Max)*	*Lyophilised Range (Min-Max)*	*Amino acids (g/100g)*	*Fresh Range (Min-Max)*	*Lyophilised Range (Min-Max)*
Valine* (Val)	0.49-1.7	0.02-2.27	Asparagine	2.6	3.5
Alanine (Ala)	0.45-1.6	0.08-1.83	Lysine* (Lys)	0.1-1.9	7.2
Glycine (Gly)	0.1-1.4	0.1-1.66	Methionine* (Met)	0.15-2.0	0.5
Leucine* (Leu)	0.43-2.5	0.06-4.0	Tyrosine (Tyr)	0.32-1.5	0.2-2.02
Isoleucine* (Ileu)	0.25-1.6	0.2-2.02	Histidine* (His)	0.18-0.7	0.2-0.99
Proline (Pro)	0.3-2.15	2.0-8.8	Tryptophane*	0.09	0.2
Threonine*(Thr)	0.31-1.6	1.30	Arginine (Arg)	0.4-1.6	3.0
Serine (Ser)	0.21-1.4	0.04-1.61	Cysteine	0.1-0.3	-
Aspartic acid (Asp)	0.76	3.57	Asparaginic acid	0.04	-
Phenylanalanine* (Phe)	0.17-0.33	0.1-1.84	Taurine	0.08	-
Glutamic acid	0.25-1.29	0.1-5.6	Phosphoserine	0.12	-

*: Indicates essential amino acid for human

Proteins are followed by carbohydrates. Among analyzed carbohydrates in apilarnil, fructose and sucrose are at very low levels compared to glucose. Fructose, glucose, sucrose, turanose, maltose, trehalose and isolmaltose contents of fresh apilarnil were determined as 0.6, 3.61,0.14, 0.05, 0.33, 0.44, and 0.11, respectively (Barnitiu et al. 2013). Apilarnil is a rich source of carbohydrates (Balkanska et al. 2014) and is composed of glucose trehalose and glycogen (Lipinski et al. 2008). The carbohydrate level is related to the developmental stage of the larvae. For example, the glycogen level is highest in the youngest larvae (92.2 mg/kg) and decrease by 50% by the fourth day (Lipinksi et al. 2008). The glucose level is low throughout the entire developmental period of the drone. According to Barnutiu et al. (2013) the concentration of glucose in fresh homogenate is 3.61 g/100g. Furthermore, other mono and disaccharides such as α and β-glucopyranose, β-fructofuranose, turanose, glucitol, and maltose have been identified in drone larvae (Barnutiu et al. 2013; Isidorov et al. 2016).

According to the analysis results obtained from the research; the lipid level of apilarnil is approximately 3 times higher in lyophilized form than in fresh one (Table 1). It is an important group of nutrients consisting of lipids, free fatty acids, tri-di- and monoacylglycerols, sterols, phospholipids, and vitamins. Especially the composition and content of fatty acids have an important effect on the functional properties of foods. Alpha linoleic acid (omega 3) and linoleic acid (omega 6) are known as essential fatty acids. The analysis results provided show that drone larvae are a rich source of lipids and fatty acids. (Calder 2015) and lipid contents include free fatty acids, sterols, triacylglycerols, and phospholipids (Isidorov et al. 2016). Fatty acids are energy sources and cell membrane components. It can affect numerous cell characteristics such as metabolism, gene expression, hormone sensitivity and production of biologically active substances. Therefore, they can affect people's health, physiological function, well-being, and disease risk. They are also a source of energy (Calder 2015). The main lipids found in apilarnil include free fatty acids, triglycerides, fatty acid esters and decanoic acids. Apilarnil is rich in fatty acids. Palmitic and stearic acids, which make up almost 50% of the fatty acid content, are unsaturated fatty acids. Apilarnil is also a source of polyunsaturated fatty acids of which linoleic acid (Table 3). In an analysis with GCMS-RI, the oleic acid content of fresh apilarnil was 64.75% and the palmitic acid content was 26.08%, while the saturated fatty acid content was 34.35% and the unsaturated fatty acid content was 65.26% (Koşum et al. 2022). In studies on the fatty acid content of drone larvae, oleic acid and palimitic acid content were determined as 47.5% and 37.3% (Ghosh et al. 2016), and Prikhodko et al. (2020) determined 28.2% and 27.5%. They determined that the basic fatty acids were oleic, palmitic and stearic acids, while the saturated fatty acids were 51.75% and the unsaturated fatty acid level is 46.25%. Otherwise, Ghosh et al. (2016) in oven-dried larvae, lauric acid, myristic acid, palmitic acid, and stearic acid contents were found as 15.5, 116.6, 1844 (37.3%), 584.9 mg/100g, respectively, while hexadecanoic acid and oleic acid contents were 35.1 and 2346.1 (47.5%) detected. Palmitic acid, stearic acid and myristic acid are the most common fatty acids in the diet. Saturated fatty acids are synthesized de novo in humans. The precursor to these fatty acids is acetyl CoA, which is produced in carbohydrate or amino acid metabolism. Saturated fatty acids (lauric, myristic and palmitic acid) in the diet increase LDL concentration, coagulation, inflammation and insulin resistance. Oleic acid is the most common monounsaturated fatty acid (MUFA). Its consumption causes a decrease in low-density lipoprotein (LDL) cholesterol and an increase in high-density lipoprotein (HDL) cholesterol (Morlok 2010). Linoleic acid is an essential fatty acid that lowers blood cholesterol, has an important place in brain development function and skin barrier (Calder 2015). In addition, chemical analyses showed the presence of plant sterols in apilarnil. These sterols are campesterol (5.5 mg/100 g), beta-sitosterol (1.3 mg/100 g), 5-hydroxysitosterol (1.3 mg/100 g), and stigmasterol (0.2 mg/100 g) (Kedzia & Kedzia 2017).

Table 3- Fatty acid content of Apilarnil (Finke 2005; Ghosh et al. 2016)

Fatty acids (mg/100g)	*Range (Min.-Max.)*	*Fatty acids (mg/100g)*	*Range (Min.-Max.)*
Lauric acid (C12:0)	0.2-28.2	Hexadecanoic acid (C16:1)	0.2-72.3
Myrsitic acid (C14:0)	1.2-379.3	Oleic acid (C18:1)	18.2-4701.8
Palmitic acid (C16:0)	14.7-4847.7	Eicosenoic acid (C20:1)	0.1-7.3
Stearic acid (C18:0)	4.3-1277.9	Myristoleic acid (C14:1	2.4-3.1
Arachidic acid (C20:0)	0.2-46.8	Linoleic acid (C18:2)	0.3-46.6
Behenic acid (C22:0)	0.1-16.9	Lİnolenic acid (C18:3)	0.4-153.0
Capric acid (C10:0)	2.0	Eicosadienoic acid (20:2)	0.1
Heptadecanoic acid (C17:0)	4.2-4.3		

Ash analysis provides information on the total mineral content in a food sample. While the ash content was 0.7-4.1 in fresh apilarnil, it was 2.1-4.1 mg/100g in lyophilized samples. Apilarnil is also rich in mineral and vitamin content. When the analyses made to date are evaluated, it is seen that apilarnil contains the highest amount of potassium (140-656 mg/100g) according to the maximum detection limits. The second element's sulfur content is (392.37 mg/100 g). It is followed by magnesium, phosphorus, zinc and calcium. These elements were detected in the range of 20-424, 179-330, 1.5-225.2, and 13.8-139.5 mg/100g, respectively. Na, Fe, Cu and Se contents of other elements analyzed in fresh apilarnil were found in the range of 6.45-106, 1.17-3.2, 0.29-2.4, and 0.01-0.06 mg/100 g, respectively. Minerals are needed for maintaining health and normal functioning of the body. Macrominerals such as Na, Cl, K, Ca, P, Mg, S and trace minerals needed in much smaller amounts are required for normal functions such as cell division, cell metabolism and growth, intracellular K concentration, DNA synthesis, optimal enzyme function and acid-base balance. necessary for cell functions. Magnesium is a cofactor of many enzymes. It is also necessary for synthesis (protein, RNA and DNA), energy metabolism, and maintenance of the electrical potential of cell membranes and nerve tissues in the human body. Selenium is a mineral that protects against the harmful effects of free radicals during stress, infections and tissue injuries. Zinc, which is the basic component of many enzymes, has a function in the synthesis and breakdown of lipids, carbohydrates, proteins and nucleic acids. It is involved in the molecular structure of cellular components and membranes and has an important role in the immune system. Phosphate is an essential component of cell membranes, bones and nucleic acids. It is required in intracellular signalling, cellular energy metabolism, and oxygen release from hemoglobin (Food & Nutrition Board 1997; 2002) Another important mineral is calcium, an element that plays a vital role in blood coagulation, neuromuscular function, and provides hardness to the skeleton together with phosphate salts (Food & Nutrition Board 1997).

Vitamins are needed for a normal and healthy metabolism, their deficiency can lead to serious diseases and even death. Apilarnil is rich in fat and water-soluble vitamins. Data on the vitamin and mineral content of apilarnil are summarized in Table 4. The analyzes have reported that the vitamin A content of drone larvae is 0.01-0.05 mg/100 g. Vitamin A (retinol) is a vitamin that is needed in small amounts. Beta carotene is the main source of provitamin A in the diet. It is necessary for the maintenance of vision and immune function, growth and development, epithelial cell integrity and reproduction. The vitamin D content of apilarnil has been reported to be between 0.03-0.9 mg/kg. Vitamin D is needed in all cells of the body for muscle contraction, mineralization of bone, nerve conduction, and maintenance of blood calcium and phosphate levels (Nordin 1976). Fat-soluble vitamin E, which we can only get through diet, is the main antioxidant in the cell (Food & Nutrition Board 2000). Vitamin C is a water-soluble vitamin that plays an important role in the antioxidant system (Sies 1993). Vitamin E content of apilarnil has been reported as 0.4-1.6 mg/kg. Among the B complex vitamins, the highest amount of B vitamin detected is choline (44.3-68.1 mg/100 g). It was followed by nicotinic acid (Vit B3), riboflavin (Vit B2), pantothenic acid (Vit B5), thiamin (Vit B1) and pyridoxine (Vit B6) and biotin. The need for vitamins and minerals in human nutrition has been determined for water-soluble (vitamin C; 45 mg/day, thiamine; 1.1-1.2 mg/day, riboflavin; 1.1-1.3 mg/day, niacin; 14-16 /NE/day, vitamin B6; 1.3 mg/day, pantothenate; 5mg/day, Vit B12; 2.4 µg/day, folate; 400 µgDFE/day) and fat-soluble vitamins (Vitamin A 500-600 µgRE/day, Vitamin D 5-10 µg/day, Vitamin E is 7.5 mg alpha-TE/day, Vitamin K is 55 µg/day) (WHO 2004). Considering these values, it can be said that apilarnil is an important source of vitamins and minerals.

Table 4- Vitamin and mineral contents in drone larvae (Hu & Li 2001; Bogdanov 2016; Hyrniewicka et al. 2016; Sidor et al. 2021; Prikhodko et al. 2020)

	fresh	*lyophilised*		*fresh*	*lyophilised*
Minerals mg/100g	***Range (Min-Max)***	***Range (Min-Max)***	***Vitamins mg/100g***	***Range (Min-Max)***	***Range (Min-Max)***
Calcium (Ca)	13.8-139.5	556	Retinol (Vitamin A)	0.01-0.05	0.31-14.70
Phosphorus (P)	179-330	-	Beta-carotene (provit A)	0.02-0.9	0.940
Magnesium (Mg)	20-424	126.4	Calciferol (Vitamin D)	0.39-0.6	-
Sodium (Na)	6.45-106	424	α-tocopherol (Vitamin E)	0.4-8	0.53-24.10
Potassium (K)	140-656	-	Thiamin (Vit B1)	0.58-4.1	2.320
Iron (Fe)	1.17-3.2	-	Riboflavin (Vit B2)	0.95-9.1	3.824
Zinc (Zn)	1.5-225.2	900.4	Pantothenic acid (Vit B5)	2.6-13.4	13.396
Manganese (Mn)	0.06-4.4	2.4	Pyridoxine (vit B6)	0.05-1.2	0.220
Copper (Cu)	0.29-2.4	15.2	Choline (Vit B4)	44.3-68.1	-
Selenium (Se)	0.01-0.06	-	Nicotinic acid (Vit B3)	0.06-15.8	0.256
S	392.37	-	Vitamin C	4.02	-
Cr	0.01	-	Coenzyme Q10	2.0	-
Al	0.39	-	Niacin	36.7	-
Cd	0.003	-	Biotine	0.23	-
Pb	0.003	-	Choline	1.68	-
			Ascorbic acid (mg/g)		0.65-3.36
			Coenzyme Q10	20	0.03-114

Scientific research shows that reactive free radicals play a role in many diseases such as heart disease, diabetes and cancer. The cell contains potentially oxidizable substrates such as proteins, fatty acids and DNA (Sies 1993). Therefore, the antioxidant defense system protects it from the harmful effects of free radicals, which are normally produced endogenously in the cell, as well as pollutants and exogenous species such as cigarette smoke. If exposure to free radicals, called oxidative stress, exceeds the protective capacity of the antioxidant defense system, damage to biological molecules may occur. Consuming foods with antioxidant activity that can potentially eliminate or neutralize free radicals may play an important role in disease prevention. Lipophilic molecules such as alpha-tocopherol, retinol and coenzyme Q10 are antioxidants with basic regulatory and metabolic functions in living organism cells. Vitamin E, an endogenous antioxidant, protects lipids in the cell membrane against peroxidation. Along with vitamin E, vitamin A and beta-carotene also protect against oxidation. Another cellular antioxidant is Coenzyme Q10 which acts as an electron carrier in the mitochondrial respiratory chain (Sies 1993). Hryniewicka et al. (2016) determined the content of alpha-tocopherol and coenzyme Q10 in honey bee-derived animal products such as royal jelly, bee bread and drone larvae homogenates by liquid chromatography-tandem mass spectrometry (LC/MS/MS). They found that apilarnil is a rich source of coenzyme Q10. The drone homogenate contained only 8±1 μg/g α-tocopherol and 20±2 μg/g coenzyme Q10.

In addition, there are studies on the bioactivity of Apilarnil. In one of these studies, total phenolic content was 14.35 mgGAE/100 g, DPPH value was 4.93 SC50 mg/mL, ABTS 35.89% and FRAP 0.59 mmol/100 g (Kurtdede & Sevim 2022). In this study, in which the antioxidant capacity of honey, bee pollen, bee bread and apilarnil was evaluated, honey showed the highest antioxidant capacity among the products tested. Bioactivity of a total of 139 apilarnils, 3 times a season, was analyzed from 7 different apilaries in Poland for 3 years. Among the antioxidant tests, DPPH analysis was determined in the range of 2.5-80.40%. In another study, the DPPH value was found to be between 9.2-16.36% in two different antioxidant activity tests, and the FRAP test was 0.80-1.16 (μmolTE/100g), while the total phenolic content was found to be 23462-268.84 mgGAE/100g (Sidor et al. 2021). Haber et al. (2019) reported that the powder of honey bee larvae has high antioxidant activity and polyphenol content.

Apilarnil is a natural substance produced by honeybee larvae, which contains a range of hormones. These hormones include testosterone, which is a male sex hormone, as well as female sex hormones such as estradiol, progesterone, and prolactin. These hormones are important for the development and maintenance of reproductive functions in both males and females, and they have been used in traditional medicine for their potential health benefits (Burmistrova 1999; Budnikowa 2009; Bolatovna et al. 2015). It is known that sex hormones such as testosterone, progesterone and estrogen play important roles in various physiological processes besides reproductive functions and the formation of secondary sex characteristics (Rider & Abdou 2001; Roof & Hall 2000). Testosterone is produced in the ovaries and testicles. Androgens have important roles in muscle development, bone density, production of red blood cells, maturation during puberty, libido and sexual function in both men and women. It also has roles such as regulating menstruation and preventing osteoporosis in women. The other sex hormone, progesterone, receptors have been identified in the brain, cortical and subcortical regions (Woolley & McEwen 1993). This hormone has neuroprotective effects and is effective in promoting nerve regeneration and myelination (Schumacher et al. 2014). Estradiol is an effective hormone in the modulation of neurotransmitter synthesis, release and metabolism, while prolactin is a hormone responsible for breast tissue development and milk production (Glasier et al. 1984; Barth et al. 2015). The content of the hormone of drone larvae is presented in Table 5. Fresh drone homogenate was found to contain 0.31 nmol/100g testosterone, 51.3 nmol/100g progesterone, 410 nmol/100g prolactin and 677.6 nmol/100g estradiol Budnikowa (2009) revealed the dynamics of

sex hormones from larva to pupa, while five-day-old larvae contained 8.2 nmol/l testosterone and 2745 nmol/l, 15-17-day-old pupae contained 15.6 nmol/l testosterone and 343.5 nmol/l estradiol. Drone larvae were found to have more pronounced gonadotropic activity than royal jelly. It has the highest amounts of estradiol and prolactin while the lowest levels of testosterone. The testosterone content of apilarnil is about 0.03 nmol/mL has been reported.

Table 5- Hormone content of apilarnil (Sidor et al. 2021; Bogdanov 2016)

	Fresh	Lyophilised
Hormones (nmol/100g)	**Range (Min-Max)**	**Range (Min-Max)**
Testosterone (nmol/100 g)	0.31-1.10	0.04
Estradiol (nmol/100g)	653.70-680.26	100.3
Progesterone	51.32	8.1
Prolactine	410	79.8

Table 6 of a study shows the enzyme composition of drone larvae. The amylase enzyme, which breaks down starch into glucose, is responsible for amylolytic activity. High amylase activity was found in drone brood based on the study's results (Sidor et al. 2021).

Table 6- Enzyme content of drone larvae* (Sidor et al. 2021)

Enzyme(U/100g w/w)	*Range (Min-Max)*	*Enzyme*	*Range (Min-Max)*
α-D-Glucosidase	47.0-47.8	β- D-Manosidase	0.6-1.0
β- D-Glucosidase	2.2-3.9	N-acetyl-D-hexomaminidase	59.0-68.3
α-D-Galactosidase	0.4-1.4	Alkaline phosphatase	0.1-1.2
β- D- Galactosidase	1.6-2.4	Acid phosphatase	1.6-3.4
α-D-Mannosidase	21.0-22.8	alpha-amylase (U7g)	14.0-17.60

*; 7 days old larvae (n=3)

Semiochemicals (chemical signaling molecules) are naturally produced substances that enable interaction between organisms. Zhang et al. (2019) reported that the only volatile chemical (E)-β-Okimene released by *A. mellifera* larvae. Furthermore, 2 and 3 methyl diacetyl, nonanal, dimethyl sulfide and okimen were determined as active odor compounds. In honey bees (*Apis mellifera*), methylpalmitate (MP), methyloleate (MO), methylololol (ML) and methyl linoleate (MLN) are important pheromone components that trigger the behavior of bees (Haber et al. 2019) Qin et al. (2019) compared these four pheromone components in larvae of worker and drone bees before and after glazing of the comb cells. It was determined that MP, MO and MLN levels were preserved in the closing phase of the eyes and ML was at the highest level in worker bee larvae in glazed cells. They reported that the sum of the four pheromone components increased with aging in worker bee larvae as well as in drone larvae.

1.3. Pharmaceutical activity of drone larvae

Studies conducted to date on the therapeutic activities of apilarnil; estrogenic and androgenic effects, antioxidant capacity, protecting testicular damage, reducing sexual dysfunction, protecting testicular toxicity and liver injury, neurprotective effect, stimulating immune system, antiatherosclreotic activity, etc. (Figure 1).

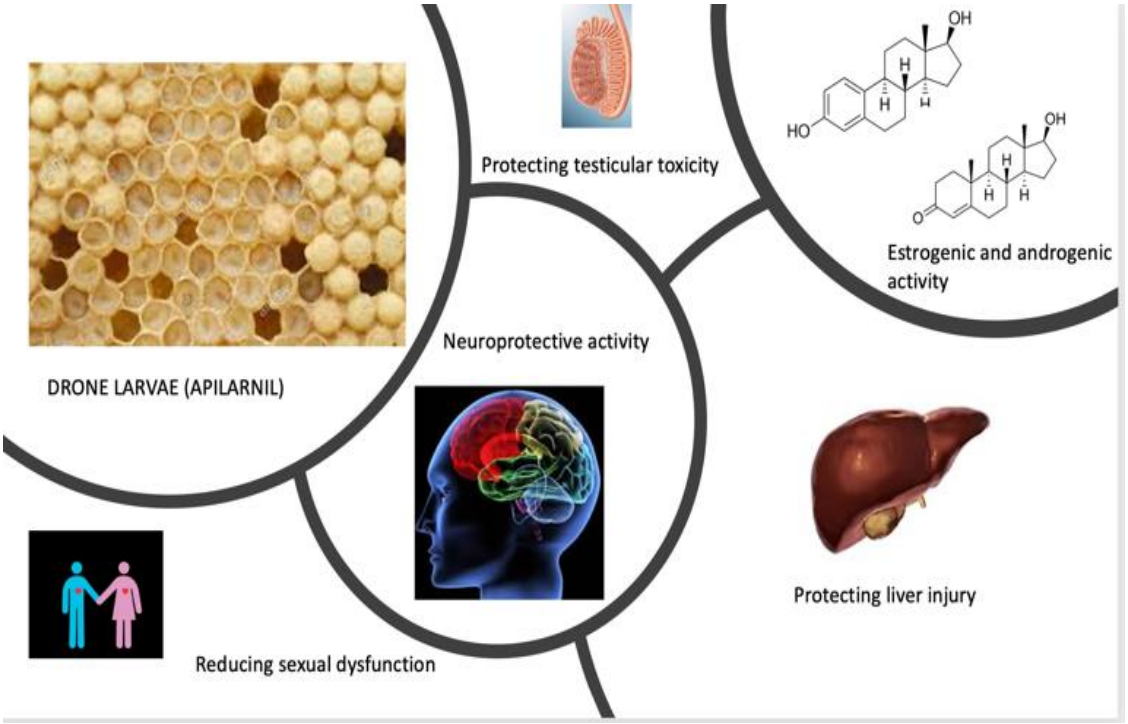

Figure 1- Biological activities of drone larvae (apilarnil)

Androgens have an anabolic effect on skeletal muscle and bone. They increase calcium binding to bones and accelerate protein production. Androgens, together with growth hormone (GH), are the most important endocrine factors affecting bone anabolism. Estrogens are steroid hormones. They are synthesized largely from cholesterol, but also from acetyl CoA. Progesterone and testosterone are synthesized first. All of the testosterone and most of the progesterone are then converted to estrogens in the granulosa cells. Estrogen in most mammalian tissues; has important roles in the growth of cells, embryological development and continuity of life (Fadini et al. 2009). Recent studies have shown that drone larvae have both estrogenic and androgenic effects. Seres et al. (2014) found that DM exhibits significant sexual hormonal effects in rats. Drone milk displayed marked androgenic activity in castrated male rats. They identified the compounds methyl oleate and methyl palmitate which are responsible for its androgenic effect. In a study of the estrogenic effect of apilarnil, this effect was attributed to E-dec-2-enedioic acid (Seres et al. 2013). Another study on drone milk by Seres et al. (2014b) showed that the combination of drone milk and spironolactone has a potent gestagenic effect.

Anabolic substances change the metabolism in the direction of increasing the formation of muscle mass and bone tissue, and in the direction of consumption of fat stores. It has been determined that the administration of drone brood homogenate to pigs affects the hormonal status and increases the growth rate (Zdorovyeva et al. 2018). The addition of drone brood homogenate (25 mg/kg feed) to the pig diet showed an anabolic effect and significantly stimulated the growth rate of the animals (Boryayev et al. 2017). In another study, drone homogenate improved the characteristics of the ejaculate, and had a stimulating effect on the reproductive function of rams (Shoinbayeva 2017). Kosum et al. (2022) showed that drone larvae provide sexual function restoration for Saanen male goat kids. In addition, it was reported that testicular growth and an increase in the production of androgen hormone were obtained in the study. In a study on pigs, it was shown that the supplementation with drone brood homogenate stimulates the early stages of folliculogenesis in gilts, but provokes atresia of follicular development (Kistanova et al. 2020). In male and female broilers, apilarnil did not have a positive effect on growth performance, but it reduced blood glucose and cholesterol levels (Altan et al. 2013). An increase in testicular weight, and testosterone level has been shown to stimulate sexual maturation in the early stages. It was found that dietary apilarnil did not have a positive effect on growth performance in male and female chickens, and apilarnil did not show an anabolic effect.

It is reported that eighty million people worldwide are affected by the inability to have children. It has been reported that the high amount of free oxygen products (ROS), such as hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite, in spermatozoa, is associated with male infertility. In scientific studies, it has been determined that many antioxidants improve sperm quality and prevent sperm damage; such as vitamins E and C, coenzyme Q10, glutathione, folic acid, zinc and selenium, (Agarwal & Sekhon 2010). Coenzyme Q10 has been shown to protect the cell membrane against oxidative stress (Bentinger et al. 2010), folic acid plays a role in DNA synthesis and scavenges free radicals (Joshi et al. 2001). It has been reported that lycopene, vitamins A, C and E are antioxidants used to improve sperm quality, and vitamin E, for example, significantly reduces DNA fragmentation rates and improves sperm quality (Ebisch et al. 2007; Rolf et al. 2009). In recent studies, it has been determined that n-3 PUFA deficiency in the diet affects spermatids (Roqueta-Rivera et al. 2011). Nutrition affects sperm quantity, semen quality, and fertility status (Stevermer et al. 1961). Protein quality is highly dependent on amino acid content and amino

acid bioavailability (Kim et al. 2009). Different amino acid ratios have significant effects on reproductive performance (Ren et al. 2015). In studies on different experimental and farm animals; dietary lysine (Lys) has been shown to improve semen quality from 0.86% to 1.03% (Rupanova 2007). The amino acid composition of seminal plasma significantly affects sperm motility. Li et al. (2003) determined that the addition of amino acids proline, glutamine and glycine) improved sperm membrane and acrosome integrity as well as sperm motility in monkey semen. Research findings suggest that apilarnil has the potential to function as a natural stimulant in the animal endocrine system, which could be beneficial for restoring and improving male sexual desire (Vakina et al. 2020). A preparation containing drone larvae homogenate has been utilized to regulate androgenic activity in females (Elistratov et al. 2017). Androgen deficiency syndrome causes a decrease in the development of the penis and testicles at an early age and prevents puberty. In young people, gynecomastia causes weakness in facial, body or pubic hair and voice development, while in adults, it causes problems such as mood changes, decreased muscle strength, increase in body fat, decreased libido, difficulty in erection, low sperm volume and gynecomastia. Doganyigit et al. (2019) in their study, which tested the protective effect of apilarnil on endotoxic shock, reported that apilarnil reduced testicular damage caused by LPS and this effect was due to the antioxidant capacity of apilarnil.

The biological activity of apilarnil is not limited to its effects on the reproductive system. Doganyigit et al. (2019) reported that apilarnil administered in rats prevented lipopolysaccharide (LPS)-induced liver damage by inhibiting the TLR4/ HMGB-1/ NF-kB signaling pathway. In addition, apilarnil showed protective effect against DNA damage and oxidative stress caused by LPS (Doganyigit et al. 2020). In another study, apilarnil promoted potential renoprotective effects, by the modulation of important markers of the local immune response in the model of LPS-induced sepsis (Inandiklioglu et al. 2021). Similar experimental model, tumor necrosis factor-alpha (TNF-alpha) and brain natriuretic peptide (BNP) expressions were significantly increased in the LPS group, and co-administration of LPS and apilarnil suppressed increased expression levels (Okan et al. 2022). Furthermore, apilarnil increased the activity of the autophagy pathway and showed potential positive effects by providing a significant decrease in protein expression increased by LPS (Doganyigit et al. 2020b).

Vasilenko et al. (2002) demonstrated that the administration of lyophilized apilarnil decreased cholesterol and triglyceride levels. They also demonstrated its hepatoprotective activity and stimulated the immune system. The administration of drone homogenate (Apilarnil) to mutant mice with hereditary hemolytic anemia resulted in a significant increase in the survival rates of the experimental animals. (Andritou et al. 2012). Hamamci et al. (2020) demonstrated the neuroprotective potential of apilarnil.

In another animal experiment, apilarnil proved to have a significant effect on energy production. Animals fed apilarnil have been shown to have greater resistance to fatigue and improve vital parameters. It caused an increase in glycogen depletion and synthesis of glucocorticoid hormones. In another study, it was shown that apilarnil is a powerful energizer, has a strong catabolic effect and stimulates the oxidative process (Kogalniceanu et al. 2010). Apilarnil is used in beverages and in the prophylaxis and treatment of fatigue (Trifonov et al. 2014).

2. Conclusions

Drone larvae (Apilarnil), is a little-known honey bee product rich in nutrients and exhibits many healing and therapeutic properties. It has been used as a cheap, safe and effective natural food against different diseases. Its high protein and essential amino acid content, fatty acid composition, vitamin and mineral richness, and hormonal content differentiate it from among other bee products with apilarnil. The biological and therapeutic activities of drone larvae have been confirmed by performing laboratory and animal/human in vivo experiments. In addition to the many biological activities of this product, it is thought that it may lead to important developments in the future, especially in the field of infertility.

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References

Agarwal A & Sekhon L H (2010) The role of antioxidant therapy in the treatment of male infertility. Human Fertility 13(4): 217-225. https://doi.org/10.3109/14647273.2010.532279

Altan Ö, Yücel B, Açıkgöz Z, Şeremet C, Kösoğlu M & Turgan N (2013). Apilarnil reduces fear and advances sexual development in male broilers but has no effect on growth. Nutiriton and Metabolism 54(3): 355-361. https://doi.org/10.1080/00071668.2013.791382

Andritou C V, Andritou V, Zonda G I, Foia L, Carlan M & Costuleanu M (2012). Experimental treatment involving apitherapy in hereditary hemolyticanemia. *Romanian Journal of Medical and Dental Education* 1: 34-37

Atherton P J, Smith K, Etheridge T, Rankin D & Rennie M J (2010). Distinct anabolic signaling responses to amino acids in C2C12 skeletal muscle cells. Amino acids 38(5): 1533-1539. https://doi.org/10.1007/s00726-009-0377-x

Bak B & Wilde J (2002). Bee brood-An unconventional source of animal protein. Biuletyn Naukowy Uniwersytet Warminsko Mazurski w Olsztynie, Poland 18: 229–237

Balkanska R, Karadjova I & Ignatova M (2014). Comparative analyses of chemical composition of royal jelly and drone brood. Bulgarian Chemical Communications, 46(2): 412-416. https://www.researchgate.net/publication/264577503

Barnutiu L I, Marghitas L A, Dezmirean D, Bobis O, Mihai C & Pavel C (2013). Physico-chemical composition of apilarnil (bee drone larvae). Lucrari Ştiintifice-Seria Zootehnie 59: 199-202

Barth C, Villringer A & Sacher J (2015). Sex hormones affect neurotransmitters and shape the adult female brain during hormonal transition periods. Frontiers in Neuroscience 9:1-20. https://doi.org/10.3389/fnins.2015.00037

Bentinger M, Tekle M & Dallner G (2010). Coenzyme Q- biosynthesis and functions. Communications 396(1): 74-79. https://doi.org/10.1016/j.bbrc.2010.02.147

Bifari F & Nisoli E (2017) Branched-chain amino acids differently modulate catabolic and anabolic states in mammals: a pharmacological point of view. British Journal of Pharmacology 174(11): 1366-1377 https://doi.org/10.1111/bph.13624

Boes K E (2010). Honeybee colony drone production and maintenance in accordance with environmental factors: an interplay of queen and worker decisions. Insectes Sociaux 57:1-9. https://doi.org/10.1007/s00040-009-0046-9

Bogdanov S (2016). Royal Jelly and Bee Brood: Harvest, Composition, Quality. In The Royal Jelly Book; Bee Product Science: Muethlethurnen, Switzerland https://www.researchgate.net/publication/304012318

Bolatovna K S, Rustenov A, Eleuqalieva N, Omirzak T & Akhanov U K (2015). Improving reproductive qualities of pigs using drone brood homogenate. Biology and Medicine 7: 2

Boryayev G, Kıstanova Y, Sdorovyeva Y, Nosov A & Katayev O (2017). Influence of homogenate of drone brood on biochemical parameters of blood and productivity of growing pigs.The 3rd International Symposium on EuroAsian Biodiversity, Minsk, Belarus.

Budnikowa N W (2009). Biologically active compounds in drones. Beekeeping 6:52-53. (In Ukrainian).

Burmistrova L A (1999). Composition and biological activity of drone brood. In Problems of Ecology and Beekeeping Development in Russia; Institute of Beekeeping: Rybnoe, Russia, pp.156-159. (In Russian)

Calder P C (2015). Functional roles of fatty acids and their effects on human health. Journal of Parenteral and Enteral Nutrition 39(15): 18S-32S. https://doi.org/10.1177/0148607115595980

Calderone N W (2005). Evaluation of drone brood removal for management of Varroa destructor (Acari: Varroidae) in colonies of Apis mellifera (Hymenoptera: Apidae) in the northeastern United States. Journal of Economic Entomology 98: 645–650. https://doi.org/10.1603/0022-0493-98.3.645

Civitelli R, Villareal D T, Agnusdei D, Nardi P, Avioli L V & Gennari C (1992). Dietary L-lysine and calcium metabolism in humans. Nutrition 8(6): 400-405.PMID: 148246.

Collison C H (2004). Basics of Beekeeping. The University of Pennsylvania, University Park.

Collins A M & Pettis J S (2001) Effect of Varroa infestation on semen quality. American Bee Journal 141:590-593. ISSN/ISBN: 0002-7626.

De Grandi-Hoffman G, Watkins J C, Collins A M, Loper G M, Martin J H, Arias M C & Sheppard W S (1998). Queen developmental time as a factor in the Africanization of European honeybee (Hymenoptera:Apidae) populations. Annals of the Entomological Society of America 91 (1):52-58. https://doi.org/10.1093/aesa/91.1.52

Doganyigit Z, Silici S, Kaymak E, Okan A, Akın A T & Pandır D (2019). Determination of the protective role of apilarnil against testicular toxicity dur to lipopolysacchardie (LPS) in male rats. Bozok Tıp Dergisi 9:146-154. https://dergipark.org.tr/en/pub/bozoktip/issue/46461/583995

Doganyigit Z, Okan A, Kaymak E, Pandır D & Silici S (2020). Investigation of protective effects of apilarnil against lipopolysaccharide induced liver injury in rats via TLR 4/ HMGB-1/ NF-κB pathway. Biomedicine & Pharmacotherapy 125:109967 https://doi.org/10.1016/j.biopha.2020.109967

Doganyigit Z, Köklü B, Uner A, Kaymak E & Silici S (2020b). The effect of apilarnil on the autophagia against lipopolysaccaride-based sepsis in liver. New Trends in Medicine Sciences 1(2):84-89. ISSN: 2717-8161.

Ebisch IMW, Thomas GMG, Peters WHM, Braat DDM & Steegers-Theunnisen RPM (2007). The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility, Human Reproduction Update 13(2):163-174. https://doi.org/10.1093/humupd/dml054

Elistratov D G, Strukov V I, Trifonov V N, Elistratova Y A, Elistratov K G, Kurus' N V, Fyodorov A V, Krutyakov E N, Andreyeva E S, Elistratova T V, Khomyakova I V, Tolbina G A, Dolgushkina G V, Astafieva A N, Kuptsova T A, Shcherbakova Y G & Smirnova N M (2017). Method of treating androgen deficiency in women. United States Patent Application Publication, US 20170065646 A1.

Eraslan G, Kanbur M, Silici S, Altınordulu S & Karabacak M (2008). Effects of cypermethrin on some biochemical changes in rats:The protective role of propolis. Experimental Animals 57(5):453-460.https://doi.org/10.1538/expanim.57.453

Eraslan G, Kanbur M, Silici S & Karabacak M (2017). Beneficial effect of pine honey on trichlorfon induced some biochemical alterations in mice. Ecotoxicology and Environmental Safety 73(5):1084-1091. https://doi.org/10.1016/j.ecoenv.2010.02.017

Fadini GP, Albiero M, Cignarella A, Bolego C, Pinna C, Boscaro E, Pagnin E, De Toni R, de Kreutzenberg S, Agostini C & Avogaro A (2009). Effects of androgens on endothelial progenitor cells in vitro and in vivo. Clinical Science (Lond).117: 355-364. https://doi.org/10.1042/cs20090077

Finke M D (2005). Nutrient composition of bee brood and its potential as human food. Ecology of Food and Nutrition 44: 257-270. https://doi.org/10.1080/03670240500187278

Food & Nutrition Board (1997). Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D, and fluoride. Washington, DC. National Academy Press. https://doi.org/10.17226/5776

Food & Nutrition Board (2002). Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington, DC, National Academy Press. https://doi.org/10.17226/10026

Food & Nutrition Board (2000). Dietary reference intakes for vitamin C, vitamin E, selenium and carotenoids. A report of the Panel on Dietary Antioxidants and Related Compounds. Washington, DC, National Academy Press. https://doi.org/10.17226/9810

WHO (2004). Vitamin and mineral requirements in human nutrition, 2nd ed. 341 p,. World Health Organization. https://apps.who.int/iris/handle/10665/42716 ISBN:9241546123

Goins A & Schneider S S (2013). Drone "quality" and caste interactions in the honeybee, Apis mellifera L. Insectes Sociaux 60:453-461. https://doi.org/10.1007/s00040-013-0310-x

Ghosh S, Jung C & Meyer-Rochow V B (2016). Nutritional value and chemical composition of larvae, pupae and adults of worker honey bee, Apis mellifera ligustica as a sustainable food source. Journal of Asia-Pacific Entomology 19: 487-495. https://doi.org/10.1016/j.aspen.2016.03.008

Glasier A, McNeilly AS & Howie P W (1984). The prolactin response to suckling. Clinical Endocrinology 21:109–116. https://doi.org.10.1111/j.1365-2265.1984.tb03449.x

Haber M, Mishyna M, Martinez J J & Benjamin O (2019). Edible larvae and pupae of honeybee (Apis mellifera): Odor and nutritional characterization as a function of diet. Food Chemistry 292:197-203 https://doi.org/10.1016/j.foodchem.2019.04.041

Hamamcı M, Doğanyiğit Z, Silici S, Okan A, Kaymak E, Yılmaz S, Tokpınar A, Inan L E (2020). Apilarnil: A novel neuroprotective candidate. Acta Neurol Taiwan 29: 33-45.

Hrassnigg N & Crailsheim K (2005). Differences in drone and worker physiology in honeybees (Apis mellifera). Apidologie 36:255-277. https://doi.org/10.1051/apido:2005015

Hryniewicka M, Karpinska A, Kijewska M, Turkowicz M J & Karpinska J (2016) LC/MS/MS analysis of alpha tocopherol and coenzyme Q10 content in lyophilized royal jelly, beebread and drone brood. Journal of Mass Spectrometry 51(11):1023-1029.
https://doi.org/10.1002/jms.3821

Hu F & Li Y (2001). Nutritive value and pharmacological actions of Italian worker bee larvae and pupae. In Proceedings of the 37th International Apicultural Congress p.28

Inandiklioglu N, Doganyigit Z, Okan A, Kaymak E & Silici S (2021). Nephroprotective effect of apilarnil in lipopolysaccharide-induced sepsis through TLR4/NF-κB signaling pathway Life Sciences 284: 119875. https://doi.org/10.1016/j.lfs.2021.119875

Isidorov VA, Bakier S & Stocki M (2016). GC-MS investigation of the chemical composition of honeybee drone and queen larva homogenate. Journal of Apicultural Science 60:111–120. https://doi.org/10.1515/jas-2016-0011

Isidorov V A (2021) Honeybee alchemy. IBRA and Northern Bee Books. 274 p. ISBN: 9781913811020.

Joshi R, Adhikari S, Patro BS, Chattopadhyay S & Mukherjee T (2001) Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. Free Radical Biology and Medicine 30(12): 1390-1399. https://doi.org/10.1016/S0891-5849(01)00543-3

Johnson RM, Dahlgren L, Siegfried B D & Ellis M (2013). Effect of in-hive miticides on drone honey bee survival and sperm viability. Journal of Apicultural Research 52(2):88-95. https://doi.org/10.3896/ibra.1.52.2.18

Kedzia B & Hołderna-Kedzia E (2017). Less Known Bee Products; Sadecki Bartnik: Stróze, Poland (In Polish)

Kim W, Wang Y & Selomulya C (2020). Dairy and plant proteins as natural food emulsifiers. Trends in Food Science and Technology 105:261-272. https://doi.org/10.1016/j.tifs.2020.09.012

Kim M Y, Park K S, Yeo W S, Choo H & Chong Y (2009). In vitro solubility, stability and permeability of novel quercetin-amino acid conjugates. Bioorganic and Medicinal Chemistry 17(3): 1164-1171. https://doi.org/10.1016/j.bmc.2008.12.043

Kistanova E, Zdoroveva E, Nevitov M, Nosov A, Vysokikh M, Sukhanova I, Vishnyakova P, Abadjieva D, Ankova D, Rashev P & Boryaev G (2020). Drone brood fed supplement impacts on the folliculogenesis in growing gilts. Veterinarski Arhiv 90(6): 583-592. https://doi.org/10.24099/vet.arhiv.0886.

Kogalniceanu S, Lancrajan I & Ardelean G (2010). Changes of the glucidic metabolism determined by the physical effort of the treatment with aslavital and apilarnil. Arad Medical Journal 13: 33-41.

Koc A N, Silici S, Ercal B D, Kasap F, Hormet-Oz H T & Mavus-Buldu H (2009). Antifungal activity of Turkish honey against Candida spp. and Trichosoron spp: an in vivo evaluation. Medical Mycology 47(7): 707-712. https://doi.org/10.3109/13693780802572554

Koşum N, Yucel B, Kandemir C, Taskın T, Duru M E, Kucukaydin S, Margaoan R & Cornea-Cipcigan M (2022). Chemical composition and androgenic effect of bee drone larvae (Apilarnil) for Goat male kids. Chemistry and Biodiversity 19(8):e202200548. https://doi.org/10.1002/cbdv.202200548

Krylow W N, Agafonow A W & Kriwcow N I (2007). Theory and Methods of Apitherapy; GNU: Moscow, Russia pp. 168–180. (In Russian)

Kumazawa S, Hamasaka T & Nakayama T (2004). Antioxidant activity of propolis of various geographica origins. Food Chemistry 84(3):329-339. https://doi.org/10.1016/S0308-8146(03)00216-4.

Kurtdede E & Sevin S (2022). Evaluation of antioxidant properties and total phenolic and flavonoid contents of honeybee hive products collected from the Ankara region. Kocatepe Veterinary Journal 15(3):342-347. https://doi.org/10.30607/kvj.1138870

Lazaryan D S, Sotnikova E M & Ertushenko N S (2003). Standardization of bee bread homogenate composition. Pharmaceutical Chemistry Journal 37: 614-616.
https://doi.org/10.1023/B:PHAC.0000016077.99039.4b

Lazaryan D S (2002). Comparative amino acids analysis of bee bread. Pharmaceutical Chemistry Journal 36: 680–682. https://doi.org/10.1023/A:1023469931357.

Lebiediew W I & Legowicz M A (2003). Harvesting a drone license is profitable. Beekeeping 6: 52-53. (In Russian).

Leja M, Mareczek A, Wyzgolik G, Klepacz-Banjak J & Czekonska K (2007). Antioxidative properties of bee pollen in selected plant species. Food Chemistry 100(1):237-240. https://doi.org/10.1016/j.foodchem.2005.09.047

Li Y, Zhang X, Dinneys A & Ji W (2003). Effect of amino acids on cryopreservation of cynomolgus monkey (Macaca fascicularis) sperm. American Journal of Primatology 59(4): 159-165. https://doi.org/10.1002/ajp.10073.

Lipinski Z, Zoltowska K, Wawrowska J & Zaleska M (2008). The concentration of carbohydrates in the developmental stages of the Apis mellifera carnica Drone brood. Journal of Apicultural Science 52(1):5-11. https://www.researchgate.net/publication/287511615

Margaoan R, Marghitas L A, Dezmirean D S, Bobis O, Bonta V, Catana C, Urcan A, Muresan C I & Margin M G (2017). Comparative study on quality parameters of royal jelly, apilarnil and queen bee larvae triturate. Bulletin of University of Agricultural Sciences and Veterinary Medicine- Cluj-Napoca Animal Science and Biotechnologies 74 (1): 51–58. https://doi.org/10.15835/buasvmcn-asb:12622

Miyata Y & Sakai H (2018). Anti-cancer and protective effects of royal jelly for therapy-induced toxicities in malignancies. International Journal of Molecular Sciences 19(10):3270. https://doi.org/10.3390/ijms19103270

Morlok K M (2010). Food Scientist's Guide to Fats and Oils for Margarine and Spreads Development. Kansas State University Food Science, Manhattan, Kansas, USA, 82 p.

Mutsaers M, Blitterswijk H, Leven L, Kerkvliet J & Waerdt J (2005). Bee products-properties, processing and marketing. Agromisa Foundation, Wageningen, ISSN: 90-8573-028-7

Nassar S A, Mohammed A H, Soufy H, Nasr S & Mahran K M (2012). Immunostimulant effect of Egyptian propolis in rabbits. The Scientific World Journal 901516, 9 pages. https://doi.org/10.1100/2012/901516

Nordin BEC (1976) Nutritional considerations. In: Nordin BEC, ed. Calcium, phosphate and magnesium metabolism. Edinburgh, Churchill Livingstone pp. 1-35

Okan A, Kaymak E, Uner AK, Silici S & Doğanyiğit Z (2022). Apilarnil prtotects the LPS induced endotoxemic heart. Turk Hijyen ve Deneysel Biyoloji Dergisi 79(4): 720-729. https//doi.org/10.5505/TurkHijyen.2022.78200

Özkök D & Silici S (2017). Antioxidant activities of honeybee products and their mixtures. Food Science and Biotechnology 26(1):201-206. https://doi.org/10.1007/s10068-017-0027-0

Palmer K A & Oldroyd B P (2000). Evolution of multiple mating in the genus Apis. Apidologie 31:235-248. https://doi.org/10.1051/apido:2000119

Peng Y, Grassl J, Millar A H & Baer B (2016). Seminal fluid of honeybees contains multiple mechanisms to combat infections of the sexually transmitted pathogen Nosema apis. Proceedings Royal Society B 283(1823):20151785.

Prikhodko A, Yankina O, Kim N, Koltun G & Skolov A (2020). Chemical composition of the far eastern homogenate of drone brood. E3S Web of Conferences 203, 04015. https://doi.org/10.1051/e3sconf/202020304015

Qin Q H, He X J, Barron A B, Guo L, Jiang W J & Zeng Z J (2019). The capping pheromones and putative biosynthetic pathways in worker and drone larvae of honey bees Apis mellifera. Apidologie 50: 793–803. https://doi.org/10.1007/s13592-019-00686-9

Ren B, Cheng X, Wu D, Xu SY, Che LQ, Fang Z F, Lv G, Dong H J & Lin Y (2015). Effect of different amino acid patterns on semen quality of boars fed with low-protein diets. Animal Reproduction Science 161: 96-103. https://doi.org/10.1016/j.anireprosci.2015.08.010

Rhodes J W (2002). Drone honey bees rearing and maintenance. Agnote NSW Agriculture Livestock Officer, Apiary Products ISSN. 1034-6848

Rhodes J W, Harden S, Spooner-Hart R, Anderson D L, Wheen G (2011). Effects of age, season and genetics on semen and sperm production in Apis mellifera drones. Apidologie 42: 29-38. https://doi.org/10.1051/apido/2010026

Rider V & Abdou N I (2001). Gender differences in autoimmunity: molecular basis for estrogen effects in systemic lupus erythematosus. International Immunopharmacology 1(6):1009-24. https:// doi.org/10.1016/s1567-5769(01)00046-7. PMID: 11407298

Rolf C, Cooper T G, Yeung C H & Nieschlag E (1999). Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study, Human Reproduction 14(4): 1028-1033. https://doi.org/10.1093/humrep/14.4.1028

Roof R L & Hall E D (2000). Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. Journal of Neurotrauma 17(5):367-88. https://doi.org/ 10.1089/neu.2000.17.367. PMID: 10833057

Roqueta-Rivera M, Stroud C, Haschek A M, Akare SJ, Segre M, Brush R S, Agbaga M P, Anderson R E, Hess R A & Nakamura M T. (2010) Docosahexaenoic acid supplementation fully restores fertility and spermatogenesis in male delta-desaturase null mice. Journal of Lipid Research 51: 360-367 https://doi.org/10.1194/jlr.M001180

Rupanova M (2007). Influence of different lysine's levels in the compound feeds for boars on quantity and quality of the semen. Journal of Animal Science 43(4):45-50

Sawczuk R, Karpinska J & Miltyk W (2019). What do we need to know about drone brood homogenate and what is known. Journal of Ethnopharmacology 245: 111581. https://doi.org/10.1016/j.jep2018.10.042

Schumacher M, Mattern C, Ghoumari A, Oudinet J P, Liere P, Labombarda F, Sitruk-Ware R, De Nicola A F & Guennoun R (2014). Revisiting the roles of progesterone and allopregnanolone in the nervous system: resurgence of the progesterone receptors. Progress in Neurobiology 113:6-39. https://doi.org/10.1016/j.pneurobio.2013.09.004

Seres A B, Ducza E, Bathori M, Hunyadi A, Beni Z, Dekany M & Gaspar R (2013). Raw drone milk of honeybees elicits uterotrophic effect in rats: Evidence for estrogenic activity. Journal of Medicinal Food 16(5):404-409 https://doi.org/10.1089/jmf.2012.0232

Seres A, Ducza E & Gaspar R (2014b) Investigation of gestagenic effect of raw drone milk in rats. Acta Pharnaceutica Hungarica 84(2):77-81. PMID: 25167703

Seres AB, Ducza E, Bathori M, Hunyadi A, Beni Z, Dekany M, Hajagos-Toth J, Verli J & Gaspar R. (2014) Androgenic effect of honeybee drone milk in castrated rats: Roles of methyl palmitate and methyl oleate. Journal of Ethnopharmacology 153(2): 446-453. https://doi.org/10.1016/j.jep.2014.02.050

Shoinbayeva K B (2017). Biologically active preparation and reproductive function of stud rams. Asian Journal of Pharmaceutics 11(3): 184-191. https://doi.org/10.22377/ajp. v11i03.1402

Sidor E, Milek M, Zagula G, Bocian A & Dzugan M (2021). Searching for differences in chemical composition and biological activity of crude drone brood and royal jelly useful for their authentication. Foods 10: 2233. https://doi.org/10.3390/foods10092233

Sies H (1993). Oxidative stress: an introduction. In: Sies H, ed. Oxidative stress: oxidants and antioxidants. London, Academic Press, pp: 15–22. https://doi.org/10.1113/expphysiol.1997.sp004024

Silici S (2019). Chemical content and bioactive properties of drone larvae (apilarnil). Mellifera 19: 14-22

Tarpy D R & Jr Page R E (2000). No behavioral control over mating frequency in queen honeybees (Apis mellifera L.) implications for the evaluation of extreme polyandry. American Naturalist 155:820-827. https://doi.org/10.1086/303358

Trifonov V N, Elistratova J A, Elistratov K G, Kurus N V, Homykova I V & Elistratova, T V (2014). Dietary Beverage. United States Patent Application Publication, US 2014/0186458 A1

Utaipanon P, Holmes M J & Oldroyd B P (2019). Queenless colonies contribute to the male breeding population at honeybee drone congregation areas. Insectes Sociaux 66(4): 593-599. https://doi.org/10.1007/s00040-019-00720-0

Vakina T N, Petrova E V, Trifonov V N, Krutiakov E N, Fedorov A V, Andreeva E S, Elistratova T V, Khomykova I V & Tolbina G A (2020). Restoring male sex drive (libido) using a food supplement for restoring male sex drive (libido) (European Union Patent No. EP2886114B1).https://doi.org/10.17504/protocols.io.b4muqu6w

Vasilenko Yu K, Klimova O V & Lazaryan DS (2002). Biological effect of drone brood under chronic hyperlipidemia conditions. Pharmaceutical Chemistry Journal 36(8): 434–436. https://doi.org/10.1023/A:1021214728006.

Winston M L (1987). The biology of the Honeybee. Harvard University Press, Cambridge, 294 pages ISBN: 9780674074095

Woolley C S & McEwen B S (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. Journal of Comparative Neurology 336(2):293-306. https://doi.org/10.1002/cne.903360210

Wu G (2009). Amino acids: metabolism, functions, and nutrition. Amino acids 37:1-17. https://doi.org/10.1007/s00726-009-0269-0

Xu X & Gao Y (2013). Isolation and characterization of proteins and lipids from honeybee (Apis mellifera L.) queen larvae and royal jelly. Food Research International 54: 330–337. https://doi.org/10.1016/j.foodres.2013.07.030

Zdorovyeva E V, Boryaev G I, Nosov A V, Kataev O G, Meloyan G M, Zemlyanova J V & Kistanova EK (2018). Hormonal status and productive qualities of young pigs at inclusion in a diet feding homogenate drone brood. Agrarnyy Nauchnyy Zhurnal 2:3-7. ISSN: 2313-8432 (Russian). https://doi.org/10.28983/asj.v0i2.366

Zhang H, Hou C, Dai P, Liu Y, Wu Y, Pang Y & Diao Q (2019). Volatiles from different ınstars of honeybee worker larvae and their food. Insects 10: 118. https://doi.org/10.3390/insects10040118

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The Recent Advances to Increase Nutrient Utilization of Dietary Plant Proteins by Enzyme Supplementation and Fermentation in Rainbow Trout (*Oncorhynchus mykiss*): A Review

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ABSTRACT
Aquaculture is the fastest growing animal production sector globally. However, its sustainability heavily relies on the development of nutritionally balanced cost-effective and environmentally friendly aqua feeds for fish and crustacean species that are already being farmed or future candidate species for intensive farming around the world. Therefore, feeds produced for farmed aquatic species should be highly digestible in terms of nitrogen and phosphorous contents in order to avoid excessive release of these nutrients into the water column through solid and soluble discharge. Excessive nitrogen and phosphorous in the water are the main reason for eutrophication occurring and causing severe depletion of oxygen and creating hypoxia for many aquatic organisms living inside a water column. Strategies like formulating aqua feeds on required DP/DE (Digestible Protein/Digestible non-protein Energy) basis for farmed species and using synthetic enzymes as feed additives in order to make plant phosphorous bioavailable for fish are being utilized by the commercial aqua feed producers around the world. Fermenting plant protein ingredients with microorganisms and using prebiotics and probiotics as feed additives are also considered a viable option to reduce the nutrient load of aquafarms since these have been shown to increase the digestibility of feed ingredients via increased gut health maintaining the optimal composition and environmental conditions for gut microbiome. In this regard, this review is intended to emphasize the importance of the sustainability efforts of aquaculture production from the perspectives of environmentally friendly aqua feed formulations and improvements based on recent knowledge gathered for the effects of dietary external enzyme supplementation and fermentation of plant ingredients on the growth and wellbeing of rainbow trout (*Oncorhynchus mykiss*) throughout the world.

Keywords: Rainbow trout, Plant proteins, Biotechnology, Nutrient utilization, Sustainability

1. Introduction

Fisheries and aquaculture production reached a new record of 214 million tons in 2020. More than 157 million tones (89%) of total aquatic animal production was used for human consumption and aquaculture is the fastest growing food production sector in the world (FAO 2022). Demand for aquatic animal protein has been increasing at a much faster pace than the supply of capture fisheries throughout the world and the gap has been filled by aquaculture for the last two decades (FAO 2022). However, aquaculture activities specifically done in open sea (mariculture) and lakes could have a huge impact on the ecosystems in these areas due to many interactions of aquaculture with the environment. Among those interactions (Figure 1), nutrient pollution stemming from uneaten food, faeces and ammonia excretion is the most complex in nature because the phosphorous, ammonium, nitrate and nitrite severely disrupt the trophic balances, create eutrophic conditions and become toxic to the organisms (Soto & Norambuena 2003; Pandey & Satoh 2008; Wang et al. 2012; Braña et al. 2021). Intensive fish and crustacean farming totally rely on the nutritionally balanced good quality aqua feeds. Feed cost is the biggest expenditure in aqua farms averaging almost 70% of all the farm expenditures. For this reason, the wastage through uneaten pellets and diets that are imbalanced and prepared using unsustainable ingredients like fish meal and oil and poorly digestible ingredients is seen a major obstacle for achieving the cost effectiveness and environmentally friendly status of aqua farms globally (Braña et al. 2021; Kurniawan et al. 2021).

Main considerations in reducing the impact of aquafarms into the aquatic ecosystems have historically been to improve feed production and feeding technology and to optimize the feed composition (Wang et al. 2012). In this respect, feed production based on the knowledge concerning the optimal nutrient requirements of farmed species supplied by highly digestible feed ingredients has gained momentum in parallel to the global developments in methodology of fish nutritional studies over the past twenty years. Therefore, diet formulations based on the optimum DP/DE (Digestible Protein/Digestible non-protein Energy ratio) determined for each farmed species using *in vivo* apparent ingredient nutrient digestibility values have become the standards of

the commercial aqua feed formulations (Engin & Carter 2001; 2006; Glencross et al. 2008). Rainbow trout production in Türkiye reaches approximately 166.000 tones constituting the 35% of the total inland and marine aquaculture production (Yıldırım & Çantaş 2022). In this respect, this review will primarily focus on the strategies available for optimizing dietary nutrient bioavailability in rainbow trout (*Oncorhynchus mykiss*), the most widely cultured freshwater species not only in Türkiye but around the world.

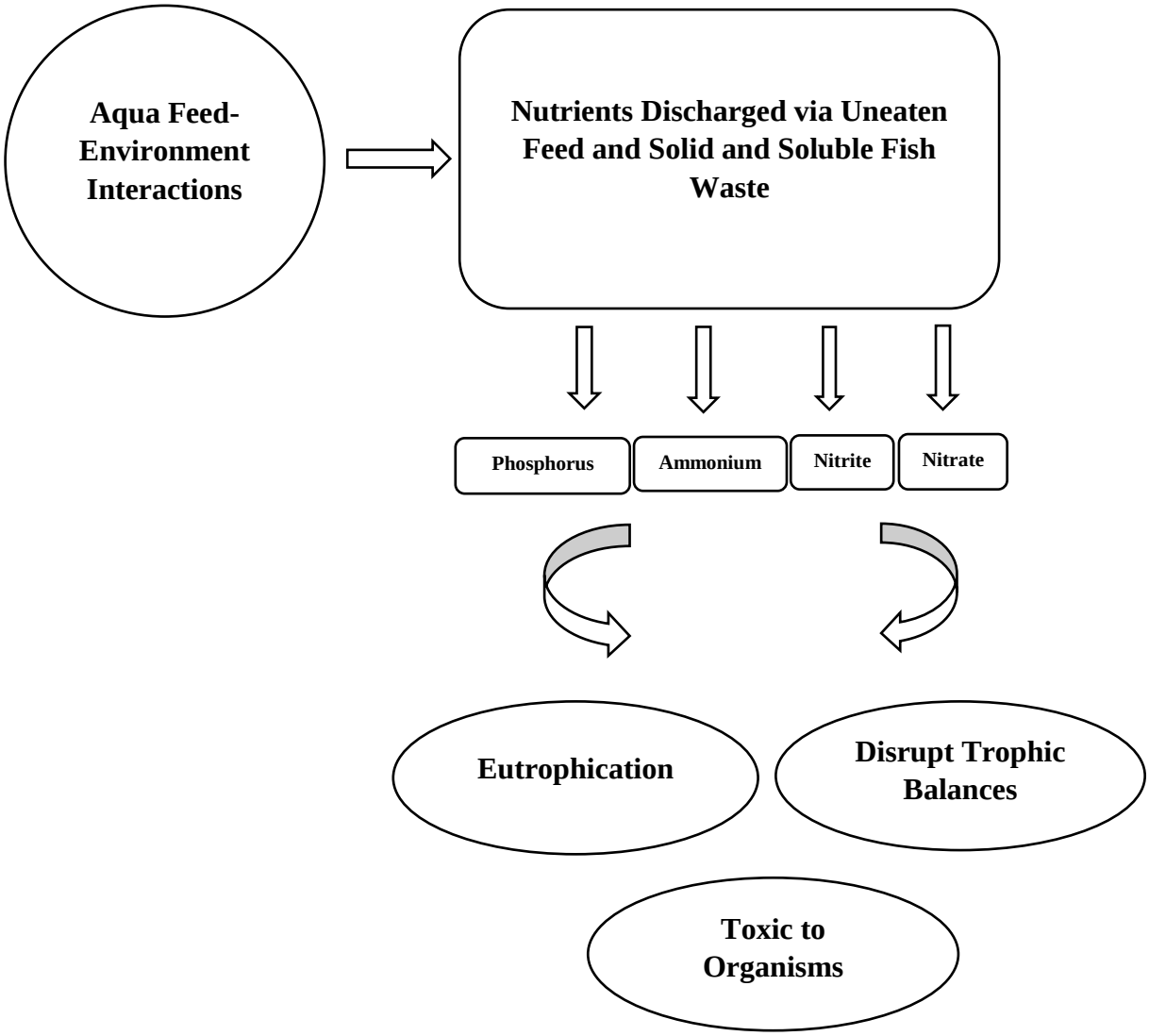

Figure 1- The flow chart of nutrient-environment interaction in freshwater lakes and offshore sea fish farming cages (modified after Braña et al. 2021).

2. Alternative Feed Ingredients to Fish Meal in Rainbow Trout Diets: Efforts to increase plant protein use by increasing the nutrient retention

2.1. Dietary exogenous enzyme use in plant protein based diets

Sustainable and eco-friendly fish farming necessitates the finding of alternative protein and oil feed ingredients to fishmeal and oil that are highly digestible in farmed species (Morales et al. 2018). Fish meal use in commercial salmonid diets has plummeted from almost 65% to as low as 15% over the last three decades thanks to the extensive scientific research conducted in Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*) (Davies et al. 2021). The biggest fishmeal replacer in commercial salmonid diets has been the full fat or defatted soybean meal and its by-products and, to a lesser, extent rape and sunflower seed meals and wheat and corn gluten meals have been utilized (Gatlin et al. 2007; Kumar et al. 2020; Davies et al. 2021). Although terrestrial plant proteins are increasingly being used in commercial aqua feeds for salmonids, this was mainly achieved by industrial and technical measures applied to these ingredients to alleviate the Anti Nutritional Factors (ANFs) that they contain. Many plant proteins including soy products contain ANFs such as lectins, saponins, oligosaccharides and trypsin inhibitors that could severely disrupt the absorption process of dietary nutrients in fish (Stone et al. 2005; Gatlin et al. 2007; Yiğit & Ölmez 2011; Glencross et al. 2012; Barrows & Frost 2014). In addition, phosphorus, an indispensable micronutrient that

is required for energy generating cellular metabolic pathways in all living organisms, is found as phytates in plants and classified as non-bioavailable inositol phosphate salts (Morales et al. 2018). Therefore, effective use of alternative terrestrial plant protein sources to fishmeal in aqua feeds necessitates the total removal or reduction of above mentioned ANFs in plant ingredients using numerous methods varying from mechanical to microbiological ones. In this respect, the majority of the latest investigations in rainbow trout appears to be related to the improvement in the retention efficiency of nutrients of dietary plant protein ingredients through fermentation and other processing techniques or dietary addition of external enzymes (Tanemura et al. 2016; Morales et al. 2018; Greiling et al. 2019; Lee S A et al. 2020). Decades earlier scientists were able to demonstrate that the high level of fish meal phosphate (tri-calcium phosphate in the form of bone P source) significantly lowered N and P retention efficiencies in rainbow trout compared to that of fish fed low-P plant ingredients (defatted soybean meal and corn gluten meal) indicating dietary total P surpassing the optimal requirement could actually prevent its bioavailability to the trout (Satoh et al. 2003). Recent study by Morales et al. (2018) demonstrated that dietary 4 $g.kg^{-1}$ inclusion of external P sources as monosodium phosphate, monocalcium phosphate and monoammonium phosphate to the plant protein based diets making the total dietary P content up to 10 $g.kg^{-1}$ significantly increased the growth rate and feed conversion in rainbow trout. The authors also concluded that the bioavailability of P, measured as P digestibility was significantly higher in fish fed monosodium and monoammonium phosphates compared to that of fish fed monocalcium phosphate indicating the higher solubility of P salt and absorption and lower particulate P discharge to water through faeces for monosodium and monoammonium phosphate sources in trout when used as dietary additives (Sugiura et al. 2001; Kals et al. 2012; Morales et al.2018).

Besides finding the best possible dietary P source, research objectives have also focused on the increase of bioavailability of myo-inositol-hexakis dihydrogen phosphate to farmed fish, classified as phytic acid, a commonly found P source in all dietary plant protein ingredients used in aqua feeds as the amount used increased greatly over the years (Hua & Bureau 2010; Greiling et al. 2019). The forefront of the methodological approach to make phytic acid more bioavailable to farmed fish has been specifically through the dietary enzyme addition to diets or the fermentation of the plant proteins with several strains of microorganisms. Phytic acid salts are the primary form of Phosphorus ($InsP_6$) in plant seeds and raw feed ingredients originating from plant seeds (Cheng & Hardy 2003; Okungoya et al. 2006; Singh 2008; Rodehutscord et al. 2016; Greiling et al. 2019; Lee S A et al. 2020). The hydrolysis of $InsP_6$ is needed for the $InsP_6$ bound P to be released and absorbed in digestive tract of fish (Greiling et al. 2019). Enzymes such as phytases and phosphatases catalyse the hydrolytic cleavage of $InsP_6$ and its salts to myo-inositol via several phosphorylated intermediary products enabling P to be released from the ring and become available for absorption (Greiner & Konietzny 2010; Greiling et al. 2019). Because fish in general lack these enzymes in their digestive track, the bioavailability of myo-inositol bound P as P source is limited in fish. However, it was reported that tilapia was able to digest the $InsP_6$-P on a certain extent compared to species that belong to *cyprinidae* and *Salmonidae* families (Hua & Bureau 2010). Rodehutscord & Pfeffer (1995) demonstrated that trout fed diets containing 60% of dietary P as phytate P supplied by plant protein ingredients mostly from soybeans considerably increased P digestibility and utilization from 25 to 57% and 19 to 49% respectively at 15 °C water temperature when microbial phytase included in diets compared to those fish fed the control diet not supplemented with phytase. The authors also found that the P utilization at 10 °C increased from 6 to 25% but feed intake and growth rate of trout were very low regardless of phytase supplementation to the experimental diets. Similarly, Cheng & Hardy (2003) reported that total dietary P and phytate P digestibility significantly increased in trout fed diets containing extruded full fat and expelled soybeans supplemented with microbial phytase (0 to 1000 FTU/kg diet increased by 200 FTU/kg diet). The authors also concluded that the optimal dosage to include diets for trout appeared to be around 400 FTU/kg diet in order to release the P and other minerals effectively when extruded full fat soybean is used in diets (Cheng & Hardy 2003). However, the apparent digestibility coefficients for total phosphorus and phytate phosphorus appeared to be significantly lower in trout fed the diet containing expelled soybean meal and supplemented with the phytase at 200 FTU/kg level compared to that of fish fed diets containing extruded full fat soybean meals supplemented with increasing amount of microbial phytase (Cheng & Hardy 2003). Diets containing two types of pre-dephytinized soy proteins with very low amount of fishmeal have also been demonstrated to significantly improve the growth, feed utilization, P and Zn retention in the juvenile rainbow trout indicating the effective reduction of phytic acid contents in microbial phytase pre-treated soy products compared to non-treated counterparts (Vielma et al. 2004). A recent study by Greiling et al. (2019) hypothesized that dietary supplementation with mineral phosphorus as monoammonium phosphate (MAP, 1 g/kg DM Diet) and/or *Aspergillus oryzae* 6-phytase (2800 FTU kg/ DM Diet) might increase the clearance of dietary phytic acid thereby retaining the P in Atlantic Salmon and trout. The results showed that trout were better able to hydrolyse phytic acid ($InsP_6$) and its salts (phytate-P) therefore absorbing P in the presence of dietary phytase and/or MAP than Atlantic salmon were owing to having a much lower stomach pH, living in freshwater and higher optimal ambient temperature (Greiling et al. 2019). A study by Lee S A et al. (2020) also found that trout fed completely plant protein based diets not supplemented with inorganic P source but containing advanced *Escherichia coli* phytase at 500 and 2500 FTU/kg at two different ambient temperatures (11 vs 15 °C) retained P around 19 to 29% and 23 to 25% higher compared to that of fish fed diets supplemented with inorganic P source (positive control) and negative control without phytase supplementation respectively. Authors also concluded that significantly higher N (nitrogen) and P retention efficiencies with phytase supplementation in trout at both temperatures indicated the efficacy of the microbial phytase, which may differ widely according to the source and biotechnological methods used in the production (Griener & Konietzny 2010; Morales et al. 2011), in this experiment, even at 11 °C (Lee S A et al. 2020) contradicting the findings of similar investigations earlier (Yiğit et al. 2018). Furthermore, a study by Demir & Yılayaz (2020) demonstrated that dietary phytase supplementation at 1000 FTU/kg diet effectively reduced the particulate P discharge whilst increasing the dissolved P and N discharge in trout fed diets containing hazel nut and soybean meal as main plant protein sources in diets. Besides temperature and animal related factors such as species,

age and gastric retention time, it is understood that the efficacy of the exogenous phytases may also appear to be closely linked to the dietary source of protein that high fishmeal diets actually provide reasonable amount of protein and available P to meet the requirements limiting the response to phytase by the fish (Morales et al. 2013; Dersjant-Li et al. 2015; Yiğit et al. 2018; Lee S A et al. 2020).

Although its use in fish diets is new, dietary exogenous supplementation of proteases has been widely documented in poultry (Mahagna et al. 1995; Simbaya et al. 1996; Kaczmarek et al. 2014; Mahmood et al. 2017; Walk et al. 2018). Proteases are expected to have an effect on ANF's specifically the protease inhibitors that exist in most of the raw and mechanically processed plant protein sources used in aqua feeds by breaking down macromolecular proteins. They may also compensate the endogenous enzyme deficiency in young animals. A recent study by Lee S. et al. (2020) investigated the effects of dietary supplementation of 175 mg protease complex/kg diet on ingredient apparent nutrient digestibility coefficients (ADC) in rainbow trout. The ingredients used in the study consisted of two different fish, animal, animal by-products and an insect meal (feather meals, two poultry by-product meals, two meat and bone meals, sardine meal, menhaden meal, black soldier fly larvae meal), single cell protein meal (*Methanococcus maripaludis*), plant protein meals (soybean meal, canola meal, distiller's dried grains with soluble (DDGS), cotton seed meal, peanut meal, sunflower meal) and an algae meal (Lee S et al. 2020). The results indicated that dietary supplementation of protease complex significantly increased ingredient-specific ADC's for dry matter, energy, most of the essential amino acids and aspartic and glutamic acids in rainbow trout with most ingredients having improved digestibility of at least one amino acid (Lee S et al. 2020). Ingredient wise, soybean meal was found to have the most profound improvement for nutrient ADC's in rainbow trout by dietary supplementation of protease complex (Lee S et al. 2020). Several commercial carbohydrate enzyme complexes specifically targeting the fibrous components of plant protein meals used in aqua feeds were also investigated for nutrient absorption and retention efficiencies in rainbow trout (Ogunkoya et al. 2006; Denstadli et al. 2011; Collins et al. 2018; Kumar et al. 2020). Carbohydrases are also reported to increase gut and overall health of animals promoting the growth of beneficial bacteria among the microbiome community of the intestine (Adeola & Cowieson 2011; Zhou et al. 2013; Castillo & Gatlin 2015). A commercial enzyme cocktail reported to have xylanase, amylase, cellulase, protease and β-glucanase activity was tested at two different doses in a study designed to improve the nutrient retention efficiencies in trout fed 0, 100 and 200 g/kg diet soybean meal (Ogunkoya et al. 2006). The authors found that enzyme cocktail coated diets (at 1 and 2.5 g/kg diet) containing soybean meal did not improve trout growth rate at 15 °C water temperature but solid P and N wastes were significantly lower in fish fed diets specifically coated with 2.5 g/kg enzyme cocktail (Ogunkoya et al. 2006). They also postulated that the very little effect of enzyme supplementation on growth and nutrient digestibility in trout might be related to the pellet making through extrusion or ingredients used are being highly digestible for trout. The effectiveness of exogenous supplementation of enzymes on the hydrolysis of the bonds between biomolecules in dietary plant ingredients is thought to be closely related to the degree of previous mechanical treatment that the ingredients are exposed to probably during protein and oil extraction procedures or expansion and gelatinization as a result of dietary production (Stone et al. 2003; 2005; Castillo & Gatlin 2015). The effects of soybean meal, rapeseed meal, pea meal and sun flower cake treated with or without a commercial enzyme cocktail (β-glucanase, hemicellulase and pectinase) in semi-moist (45% water) and wet conditions (85% water) at different ambient temperature and durations on the hydrolysis of NSP (Non-Starch Polysaccharides) were also investigated *in vitro* and *in vivo* growth experiments in rainbow trout (Denstadli et al. 2011). The *in vitro* experiment indicated that the degradation of NSP's in these plant ingredients appeared not to be limited by time, temperature and the amount of water in pretreatments but rather the complex nature of NSPs showing the most prominent changes occurred with the uronic acid, xylose and arabinose in all pretreatment conditions tested in the investigation (Denstadli et al. 2011). The trout growth was unaffected by the ingredient pretreatment and the enzyme supplementation probably due to released substrates of small carbohydrate polymers during enzyme pretreatment of diets either being excreted or bound to other nutrients thus lowering the absorption specifically at water temperature of 12 °C (Glencross et al. 2003; Denstadli et al. 2011).

Recent application of enzyme bioprocessing technology developed by Ohio Soybean Council was applied to soybean meal and demonstrated to be effective on substantially lowering many ANFs present in unprocessed soybean meal such as TI, lectins, oligosaccharides and phytic acid (Hulefeld et al. 2018; Kumar et al. 2019; 2020). This EnzoMeal was also investigated for its effect on trout growth performance and growth related gene expressions by Kumar et al. (2020). Authors specifically tested the low and high dietary inclusion levels of EnzoMeal (8 and 16%) or the equal combination of SBM and EnzoMeal replacing up to 18% of fishmeal in the highest inclusion level. Control diet included 25% of fishmeal and contained neither the unprocessed soybean meal nor the EnzoMeal. EnzoMeal significantly improved trout growth performance and nutrient retention compared to fish fed diets containing same amount of traditional SBM (Soybean meal) but the effect was even greater at high dietary inclusion levels indicating improved nutrient quality index as a result of reduction of major ANFs in EnzoMeal. *Camelina sativa*, an oil seed, is a rich source of linolenic (18:3n3) acid and its pressed cakes after oil extraction and the oil itself are increasingly being considered as fishmeal and oil replacer in aqua feeds for many farmed fish species including rainbow trout (Hixson et al. 2014; 2015; Ofori-Mensah et al. 2020). A study by Collins et al. (2018) investigated the effects of the water, carbohydrases and *Rhizopus oligosporus* treated high oil residue camelina meal (HORM) on the growth performance and gut health of the juvenile rainbow trout. They were able to demonstrate that 80 g/kg inclusion of all the treated HORM products resulted in similar growth and nutrient retention rates and villus structure was not compromised compared to that of fish fed the fishmeal control diet but rainbow trout fed untreated HORM and *Rhizopus oligosporus* treated HORM diet attained lower growth and SGR respectively (Collins et al. 2018). Regardless of mechanical, enzymatic or microbiological treatments, previous studies pointed out that rainbow trout were able to utilize both the solvent extracted and the high oil residue camelina meal better than Atlantic salmon

depending on primarily the size of the fish and the water salinity levels (Hixson et al. 2014; 2015; Brown et al. 2016; Ye et al. 2016).

The results of studies regarding to dietary exogenous enzyme inclusions on the growth performance of rainbow trout summarized in the text are short-listed in Table 1.

Table 1- The effects of dietary exogenous enzyme supplementation on growth parameters of rainbow trout (*Oncorhynchus mykiss*) fed plant protein based diets

Enzymes and Treatment Conditions	*Dietary Plant Protein*	*Effects*	*Reference*
Aspergillus niger Phytase at 10 and 15 °C water temperatures	Soybean Meal	Considerably higher P digestibility and retention at 15 °C	Rodehutscord & Pfeffer (1995)
Microbial phytase (Natuphos 5000 l) Included at increasing dosage to evaluate nutrient digestibility Activity 5.537 FTU/g	Raw, Extruded and Expelled Full Fat Soybean Meals	Significantly higher ADCs of Total P Phytate-P, Mg, Mn, Zn for Extruded SBM, Optimal dosage 400 FTU/kg	Cheng & Hardy (2003)
Pre-treatment of dietary plant-proteins with microbial phytase (Natuphos)	Two different commercial soy protein	60 and 40 % increase in weight gain and P retention for both Soy proteins	Vielma et al. (2004)
Top coating the diets with a commercial enzyme mix showing xylanase, amilase, cellulose, protease and β-glucanase activity at 1 and 2.5 g.kg-1 levels	0, 10 and 20 % dietary inclusion of Soybean Meal	Marginal effects of soybean meal and external enzyme on growth and nutrient retention but reduced faecal cohesiveness and sinking speed	Ogunkoya et al. (2006)
Semi-moist (45% water at 45 °C) pre-treatment of dietary plant proteins with enzyme RONOZYME®	Soybean Meal Rape Seed Meal Sun Flower Cake	No effects on pellet quality and nutrient digestibility but decreased growth and feed utilization were observed	Denstadli et al. (2011)
Interactive effect of dietary *Aspergillus oryzae*-6-Phytase (2800 FTU/kg DM diet) and MAP (Monoammonium Phosphate 1 g P/kg DM diet) on disappearance of Phytic acid in plant protein only diets	Soy Protein Concentrate Wheat Gluten Whole Fava Beans Corn Gluten	Increased InsP6-P hydrolysis with Phytase supplementation and additive effect of MAP and Phytase on digested P	Greiling et al. (2019)
Dietary commercial enzyme treated soybean meal and (EnzoMeal™) and normal soybean meal replacing fishmeal at 8 and 16% levels	Soybean Meal Enzyme treated SBM Soy Protein Concentrate	Increased growth and protein retention with EnzoMeal™ at both replacement level compared to SBM	Kumar et al. (2020)
Measurement of nutrient ADCs using commercial Protease complex at 175 g.kg-1 dietary inclusion level	Soybean Meal Peanut Meal Cottonseed Meal Canola Meal Sunflower Meal DDGS	Ingredient specific improvements on nutrient digestion with SBM showing the most profound increments specifically on dry matter and most of the EAA ADCs with protease supplementation	Lee S. et al. (2020)
Post pellet liquid application of *Escherichia coli* phytase at 0, 500 and 2500 FTU/kg to plant protein only based diets with low P levels at 11 and 15 °C	Soy Protein Concentrate Pea Protein Concentrate	Increased growth and enhanced N and P utilization even at lower temperatures by enzyme supplementation	Lee S.A. et al. (2020)

2.2. Fermentation of dietary plant meals with microorganisms

Fermentation of fibrous plant feed ingredients with one type or the mixture of different types of microorganisms and their effects on growth and intestinal health is an active research area in ruminants and poultry (Villas-Bôas et al. 2002; Nigam & Pandey 2009; Hooge et al. 2010; Picoli et al. 2022). However, research in farmed finfish gathered pace only after the partial replacement of dietary fishmeal with oil extracted or full fat oil seeds and legumes was demonstrated as a possibility without compromising the growth and intestinal health of fish (Yamamoto et al. 2010; Barnes et al. 2015; Tanemura et al. 2016; Wang et al. 2016; 2019). Fermentation is reported to increase the nutritional value of unconventional plant feed ingredients by increasing protein and lipid contents mainly because of reduction in the fibre and ANF content (Dawood & Koshio 2020). It is generally defined as useful bioconversion or metabolic decomposition through which complex substrates are transformed into simple compounds by the microorganisms (Balakrishnan & Pandey 1996). During the fermentation process, there may also be increase in the population of probiotic microorganisms and secondary metabolites with prebiotic activity that could result in the increment of overall absorption and therefore the ADCs of dietary nutrients in fish (Picoli et al. 2022). Furthermore, fermentation using mixture of different types of microorganisms composed of bacteria and yeast are reported to further increase the useful metabolic

decomposition of dietary plant ingredients because better use of substrates is possible as a result of the production of different enzymes by these organisms capable of attacking wide variety of compounds (Fossi et al. 2014; Dawood & Koshio 2020; Picoli et al. 2022). It appears that research targeting nutritional improvement of dietary plant ingredients using several fermentation techniques in salmonid species including rainbow trout is lagging behind compared to those conducted for other farmed carnivorous and omnivorous fish and crustacean species in the literature (Wang et al. 2016; Dawood & Koshio 2019).

Recent investigations on the use of fermented plant dietary ingredients in rainbow trout targeted the possibility of the inclusion of traditional plant ingredients used in aqua feeds such as soybean and lupin meals in higher amounts by way of using special fermentation conditions and methods like Solid State Fermentation (Sealey et al. 2009; Yamamoto et al. 2010; Barnes et al. 2015; Tanemure et al. 2016; Davies et al. 2021). A study by Sealey et al. (2009) hypothesized that juvenile rainbow trout fed probiotic supplemented soybean meal containing starter diets (up to 20% dietary inclusion on DM basis) could develop immune mediated soybean tolerance making it possible to include higher levels of dietary soybean meal in their feeds during the grow-out period. The authors were able to demonstrate that trout exposed to soybean in starter diets supplemented with probiotics (combination of naturally occurring *Saccharomyces cerevisiae*, *Enterococcus faecium* and *Lactobacillus* sp.) showed less severe pathological changes and increased protein digestion and absorption when encountering much higher (43% DM basis) dietary soybean meal during the grow-out period probably indicating intestinal colonization by putative probiotics that could principally help the host in adjusting to dietary changes (Heikkinen et al. 2006; Sealey et al. 2009). Although it is much debated, the probiotic mechanism appears to occur in several multifaceted processes including in the production of anti-bacterial inhibitory compounds and competition for chemicals and adhesion sites leading to an improved microbial balance as well as host immune modulation and the modification of dietary components to increase utilization by the host organisms (Verschuere et al. 2000; Heikkinen et al. 2006; Sealey 2009). This hypothesis was put to the test in a recent investigation conducted by Özil et al. (2023) in rainbow trout. The study demonstrated that a dietary inclusion of 1.1% probiotic mixture (Table 2) in combination with 1% either sage (*Salvia officinalis*) or myrtle berry (*Myrtus communis*) powder as medicinal plants showed a positive effect on the growth performance, intestinal microflora and histology, antioxidant enzyme activities and disease resistance in juvenile rainbow trout fed 30% soybean meal as the main dietary plant protein source (Özil et al. 2023). Previously different medicinal plants from different geographical locations not only in Turkey but around the world have been studied for their effects on digestive enzyme activities, intestinal health, immunomodulatory response and antioxidant capacities in trout extensively and it remains an active research topic in aqua feed additives (Awad et al. 2012; Sönmez et al. 2022; Filogh et al. 2023)

The effects of two different fermentation conditions of defatted and heat-treated soybean meal on growth and nutrient digestibility and physiological conditions of juvenile rainbow trout were investigated by Yamamoto et al. (2010). In the study, the soybean meal was fermented using 30 and 45% ($w.w^{-1}$) water for about 7 and 10 h respectively until the material temperature reach 80 °C in both fermentation conditions and almost 48% (on wet weight basis) non-fermented and fermented soybean meals constituted the experimental diets except the fishmeal control diet. The results showed that increased water and duration in fermentation process appeared to influence the growth performance and dietary carbohydrate and lipid digestibility values positively as well as making the morphological abnormalities in distal intestine and liver tissues associated with soybean consumption less visible in trout (Yamamoto et al. 2010). The beneficial effects of the fermentation conditions (temperature and duration) of soybean products using *Lactobacillus plantarum* and a mixture of several yeast and bacterial species on growth performance, intestinal histopathological changes and other related physiological mechanisms including antioxidant capacities of Turbot, Nile tilapia and orange spotted grouper were also reported by several authors (Shiu et al. 2015; Wang et al. 2016; Picoli et al. 2022). The most recent investigation by Davies et al. (2021) tested the effectiveness of biotechnologically developed dietary additive (Synergen™) manufactured based on Solid State Fermentation with *Aspergillus niger* technology on the growth performance, nutrient digestibility and intestinal morphology using nutritionally balanced diets containing yellow lupin and soybean meals at 300 and 181 $g.kg^{-1}$ of the diets respectively in the juvenile rainbow trout. The product was added into nutritionally balanced diets at two different concentrations (1 vs 5 $g.kg^{-1}$ of diets DM basis). It appeared that 0.5% dietary inclusion of Synergen™ significantly improved growth, crude protein and fibre digestibility and serum glucose levels in trout compared to that of fish fed the same diets with 0.1% supplementation or without supplementation indicating the effective hydrolysis of cellulosic and hemicellulosic oligosaccharides in plant proteins thereby absorbing the glucose more freely into the blood (Davies et al. 2021). This was also supported with the significantly lower number of goblet cells found in fish fed the diet supplemented with 0.5% synergen™ in this study because the goblet cell numbers and their proliferation rate are closely associated with high dietary fibre content in mono gastric animals. However, this reduction of goblet cells could also be influenced by the microbiome characteristics indicating a reduced investment in mitigating the effect of stressors within the lumen (Davies et al. 2021).

Apart from investigating the effects of fermentation process itself on the nutritional value of fermented plant ingredients on fish performance, research specifically in trout recently concentrated on the identifying the comparative response to same fermented soybean meal by different strains (Barnes et al. 2015). In the investigation, dietary fishmeal was replaced by 35 and 50% of commercially produced fermented soybean meal and fed by hand to satiation once per day to rainbow trout from Shasta and McConaughy strains at 23.4 and 15.9 g initial average weight respectively for a 94-d grow-out period. The results showed that both strains of rainbow trout gained significantly lower weight when fed 50% dietary fermented soybean compared to fish fed fishmeal control and 35% fermented soybean meal diets. However, the much lower weight gain and higher HSI (Hepatosomatic index) values achieved by the McConaughy strain than that of those achieved by the Shasta strain at each dietary

fermented soybean meal replacement levels probably indicated that increased fat deposition due to the slow growth and decreased feed efficiency in the McConaughy strain of rainbow trout (Barnes et al. 2015). They also reported that distal intestine morphology scores of fish fed diet containing 35% fermented soybean meal in both strains of rainbow trout were similar to that of fish fed fishmeal control diet but at 50% replacement level the Shasta strain had significantly higher lengths of lamina propria of simple folds and connective tissue at base of folds indicating this strain, compared with the McConaughy strain, might be more susceptible to saponins that is a compound associated with soybean meal induced enteritis (Krogdahl et al. 2003; Knudsen et al. 2008) in fish (Barnes et al. 2015). Recently dietary 30 and 50% of fish meal replaced by several treated or not treated plant protein concentrates before fermentation process and supplemented with shrimp soluble extract were also investigated for their effects on rainbow trout growth and nutrient retention, intestinal histology, non-specific immune response and haematological analysis by Moniruzzaman et al. (2018). An equal mixture of soybean and corn gluten meal fermented with *Bacillus subtilis* according to the solid-state fermentation technique and the same ingredients pre-treated through acid hydrolysis to increase the solubility of substrate and their substitution with 2% shrimp soluble extract constituted the isonitrogenous and isolipidic main four dietary treatments in this investigation (Moniruzzaman et al. 2018). The total number of experimental diets increased to ten with four replicates at each replacement level and the LT-FM and Vietnam FM diets as positive controls (Monurizzaman et al. 2018). Trout whole body nutrient composition and blood biochemistry parameters did not significantly change with regards to pre-treatment of plant protein concentrates replacing dietary fish meal at 30 and 50% replacement levels but final body weight and percentage weight gain of trout fed fermented soybean and corn gluten meal diets with supplemented shrimp soluble extract at both replacement levels were significantly higher than that of fish fed not-supplemented diets indicating the positive effect of some of the essential amino acids that are missing in plant proteins but sufficiently existed in shrimp protein (Tulli et al. 2010; Kader et al. 2012; Jo et al. 2017; Moniruzzaman et al. 2018). It appears that higher than 35% dietary inclusion of fermented plant protein sources in trout diets lowers the growth rates simply by changing the morphology of villus preventing the effective nutrient absorption giving way to decreased non-specific immune response measured as SOD (superoxide dismutase), lysozyme and MPO (myeloperoxidase) enzyme activities as similarly reported in other salmonids and farmed bream species (Knudsen et al. 2008; Krogdahl et al. 2010; Kader et al. 2012; Kokou et al. 2012; Khosravi et al. 2015; Moniruzzaman et al. 2018).

In vivo and *in vitro* nutrient digestibility measurements are an inseparable part of the nutritional evaluation of novel dietary ingredients before these ingredients are widely included in commercial feed formulations manufactured for many farmed animals including fish. The fermentation of plant protein ingredients specifically with *Lactobacillus* spp. is reported to increase nutrient digestibility most likely through the alteration of the composition of microbiota in the gastrointestinal tract or the digestive enzyme activities or the chelation of minerals and the increased solubility of nutrients due to increased acidification caused by lactic acid production in the process (Ringø 1991; Yanbo & Zirong 2006; Pandey & Satoh 2008; Lim et al. 2015; Latorre et al. 2016; Zhou et al. 2016; Lin & Chen 2022). In addition, *Bacillus* spp. has previously been associated with the detoxification of potentially harmful dietary components and producing vitamin B complexes specifically vitamin B12 and biotin which are in return known to improve overall feed utilization and digestibility of feed ingredients (El-Haroun et al. 2006; Han et al. 2015; Dawood & Koshio 2020). The mechanism behind the beneficial effects of naturally occurring organic acids during fermentation with microorganisms on nutrient absorption and utilization was hypothesized in rainbow trout fed low fishmeal diets by Pandey & Satoh (2008) using dietary supplementation of Lactic acid (LA) and Citric acid (CA), Methionine Hydroxy Analog (MHA) and Liquid Trace Elements (LTE) at 1% inclusion levels. The authors also included the diet supplemented with calcium phosphate at 0.5% and non-supplemented with any P-source as positive and negative controls in the investigation. The better growth performance achieved by trout fed diets supplemented with CA and LTE compared to that of fish fed the other dietary treatments was implicated as the positive effects of increased P and N retention efficiencies found in fish fed these diets (Pandey & Satoh 2008). It was also postulated that CA and LTE in acid solutions effectively improved the de-phosphorylation of phytate P in plant ingredients increasing the bioavailability of P to trout compared to inorganic P thereby reducing the amount excreted into the environment (Pandey & Satoh 2008). In a recent study the effects of fermentation of several conventional (*i.e*. soybean meal and rapeseed meal) and non-conventional (algal and macrophyte meals) dietary plant ingredients with three species of white-rot fungi on *in vitro* and *in vivo* nutrient digestibility was investigated in rainbow trout (Tanemura et al. 2016). The species of white-rot fungi used in the study were *Tramates coccinea*, *Lentinula edodes* and *Pleurotus sajor-caju* and the meals were fermented semi-anaerobically at 38 °C (for *T. coccinea*) and 28 °C (for *L. edodes* and *P. sajor-caju*) in an incubator maintained under 90-100% humidity. *In vitro* degradability rates of NDFs (Neutral Detergent Fiber) in all the fermented dietary ingredients decreased significantly except the macrophyte meal probably indicating the fungi utilized non-NDF carbohydrates such as starch for their growth (Tanemura et al. 2016). It is also speculated that certain ingredients like macrophyte meal rich in minerals, specifically Mn and Fe, might prevent fungi breaking down the cellulose during fermentation (Dillon et al. 1988; Manubens et al. 2007; Tanemura et al. 2016). Authors were also able to demonstrate that CP (crude protein) contents of all the fermented ingredients increased compared to corresponding non-fermented control groups because of decreased yield rates increasing the relative amount of CP in fermented ingredients. However, decreased CP digestibility measurements in juvenile rainbow trout fed diets containing fermented ingredients indicated that CP contributed by the fungal mycelium growth during fermentation was not utilized as efficiently as intact proteins in the ingredients itself (Tanemura et al. 2016). It is documented that mycelium may contain N-containing chitin for up to 5% of its dry weight and is not digested well by all mono-gastric animals including fish (Lindsay et al. 1984; Yoshida et al. 1987). Furthermore, a study by Tanemura et al. (2016) re-iterated that the P bioavailability in trout fed diets containing fermented rapeseed and soybean meals significantly increased compared to that of fish fed diets containing non-fermented counterparts indicating the ability of fungi breaking down the Phytate-P and making it bioavailable to the fish regardless of the species of fungi.

The results of the investigations concerning the dietary inclusion of fermented plant proteins on the growth performance and several physiological mechanisms in rainbow trout summarized in the text are short-listed in Table 2.

Table 2- The results of dietary inclusion of fermented plant proteins on the growth performance and several physiological mechanisms in rainbow trout (*Oncorhynchus mykiss*)

Fermentation method and conditions	*Plant Protein*	*Effects*	*Reference*
Induction of soy tolerant juveniles by dietary supplementation of commercial probiotics containing live *Saccharomyces cerevisiae* and several *Lactobacillus* spp. in the starter diets followed by exposition to 43% of the same plant ingredient in grow-out period	Soybean Meal	Improved soybean meal utilization and less severe pathological changes in fish exposed to SBM with probiotic supplementation in the starter period	Sealey et al. (2009)
Pre-fermentation of the plant ingredient with *Bacillus* spp. at 80 °C using two different water levels and time duration	Defatted and heat treated Soybean Meal	Increased water and time during fermentation improved growth with no visible distal intestine abnormalities and regardless of fermentation method both carbohydrate and lipid digestibility increased	Yamamoto et al. (2010)
Dietary fishmeal replacement by commercially available fermented, soybean meal at 0, 35 and 50 % for Shasta and McConoughy strains of rainbow trout	Fermented Soybean Meal	Up to 35 % inclusion appeared to be feasible considering similar growth feed utilization and distal intestine morphology indices to fishmeal control in both strains	Barnes et al. (2015)
Fermentation of several plant meals by three species of white-rot fungi at 38 or 28 °C for 6 weeks to evaluate *in vitro* and *in vivo* nutrient ADCs	Soybean Meal Rapeseed Meal Algal Meal (*Gelidiaceae* spp.)	Trametes coccinea and Lentinula edodes were more effective in lowering NDFs but increased ash as a result of reduction in yield rates after fermentation decreased organic and dry matter and crude protein but significantly increased P digestibility	Tanemura et al. (2016)
Replacement of dietary fish meal by fermented plant protein source (soybean and corn gluten mixing ratio 1:1) using *Bacillus subtilis* with solid state fermentation technique at 30 and 50 % levels	Soybean Meal Corn gluten	Supplementation of diets with shrimp soluble extract significantly improved growth at both replacement levels but villus morphological structure compromised at 50% replacement level	Moniruzzaman et al.(2018)
Commercial Solid State Fermentation product used as feed additive at 1 and 5 g.kg-1 levels in diets containing 53 % plant protein sources	Yellow Lupin Meal Soybean Meal	Improved growth, crude protein and fibre digestibility and decrease in goblet cell numbers were observed at 5 g.kg-1 dietary inclusion level	Davies et al. (2021)
1.1 % dietary supplementation of Probiotic mixture in combination with 1 % of either sage or myrtle powder; Probiotic mixture include: *Lactobacillus* spp, *Lactococcus* spp *Lactobacillus acidophilus*, *Bifidobacterium* spp. *Kluyveromyces marxianus* (isolated from kefir) and *Lactobacillus sakei* (isolated from trout intestines)	Soybean Meal Wheat Gluten	Improved growth and significantly increased villi length and width and goblet cell numbers and total bacteria count	Özil et al. (2023)

3. Conclusions

The need to reduce the impact of aqua feeds into the aquatic environment whilst maintaining the general health and wellbeing of farmed fish and crustacean species is a priority research area in fish nutrition studies. The widespread use of plant protein sources as an alternative to fishmeal is only made possible with increased nutrient digestibility and decreased ANFs in these dietary ingredients for fish. Dietary external supplementation of enzymes that are specific to certain nutrient compounds in plant protein sources and known to be not inherited to exist in digestive tract of fish is an active research topic in nutritional studies for fish since the replacement of certain amounts of dietary fishmeal by these plant protein sources has been demonstrated previously (Romarheim et al. 2006; Gatlin et al. 2007). The fermentation of plant protein sources with numerous microorganisms

such as *Lactobacillus* spp., *Aspergillus niger* and *Saccharomyces cerevisiae* and *Rhizopus oligosporus* has also been used as a strategy to increase the amounts to be used in fish diets without compromising growth and overall health and other related physiological mechanisms. Although not studied as extensively as in terrestrial farm animals, research targeting the possibility of dietary inclusion of biotechnologically produced enzymes and fermented plant ingredients in farmed fish has been growing over the past twenty years. Research has so far demonstrated that up to 50% of dietary fishmeal could be replaced by plant proteins in rainbow trout following dietary external supplementation of proteases, carbohydrases and phytases and fermentation of these protein sources with several species of yeast and bacteria including *Saccharomyces* spp. and *Lactobacillus* spp. without compromising growth and the gastrointestinal integrity of the fish (Sealey et al. 2009; Barnes et al. 2015; Collins et al. 2018; Moniruzzaman et al.2018; Kumar et al. 2020). Increased P digestibility and retention was also observed in these investigations indicating specifically the ability of external supplementation of phytases on the de-phosphorylation of phytate-P (Greiling et al. 2019; Demir & Yılayaz 2010). However, the standardization of these biotechnological innovations for aqua feeds is far from being realized since the effectiveness of enzymes and fermentation methods immensely depends on the species differences and their farming conditions specifically the environmental temperature and pH as well as the technology and the fermentation conditions and the type of microorganisms to produce these novel ingredients. In order to understand the true potential of these biotechnologically developed ingredients for fish feed, research addressing the effects of these differences on the degradation of NDFs and some of the ANFs (Non-Starch Polysaccharides, phytic acid and trypsin inhibitors) in plant proteins in a wide variety of farmed fish species is required. Future research specifically targeting the bio compounds that generates following the fermentation processes by different microorganisms and their interaction with other nutrients therefore the nutrient absorption and other physiological mechanisms in fish is also much needed.

References

Adeola O & Cowieson A J (2011). Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *Journal of Animal Science* 89: 3189-3218. https://doi.org/10.2527/jas.2010-3715

Awad E, Austin B & Lyndon A (2012). Effect of dietary supplements on digestive enzymes and growth performance of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Journal of American Science* 8(12): 858-864.

Balakrishnan K & Pandey A (1996). Production of biologically active secondary metabolites in solid-state fermentation. *Journal of Scientific and Industrial Research* 55: 365–372.

Barnes M E, Brown M L & Neiger R (2015). Comparative performance of two rainbow trout strains fed fermented soybean meal. *Aquaculture International* 23: 1227-1238. https://doi.org/10.1007/s10499-015-9879-6

Barrows F T & Frost J B (2014). Evaluation of the nutritional quality of co-products from the nut industry, algae and an invertebrate meal for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 434: 315-324. https://doi.org/10.1016/j.aquaculture.2014.08.037

Braña C B C, Cerbule K, Senff P & Stolz I K (2021). Towards Environmental Sustainability in Marine Finfish Aquaculture. *A review article Front. Mar. Sci.* doi:10.3389/fmars.2021.666662

Brown T D, Hori T S, Xue X, Ye C L, Anderson D M & Rise M L (2016). Functional genomic analysis of the impact of camelina (Camelina sativa) meal on Atlantic salmon (*Salmo salar*) distal intestine gene expression and physiology. *Marine Biotechnology*. 18: 418–435. https://doi.org/10.1007/s10126-016-9704-x

Castillo S & Gatlin III D M (2015). Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: A review. *Aquaculture* 435: 286-292. https://doi.org/10.1016/j.aquaculture.2014.10.011

Cheng Z J & Hardy R W (2003). Effects of extrusion and expelling processing, and microbial phytase supplementation on apparent digestibility coefficients of nutrients in full-fat soybeans for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 218: 501-514. https://doi.org/10.1016/s0044-8486(02)00458-1

Collins S A, Xie S, Hall J R, White M B, Rise M L & Anderson D M (2018). Evaluation of enzyme- and *Rhizopus oligosporus*-treated high oil residue camelina meal on rainbow trout growth performance and distal intestine histology and inflammatory biomarker gene expression. *Aquaculture* 483: 27-37 https://doi.org/10.1016/j.aquaculture.2017.09.017

Davies S J, El-Haroun E R, Hassaan M S & Bowyer P H (2021). A Solid-State Fermentation (SSF) supplement improved performance, digestive function and gut ultrastructure of rainbow trout (*Oncorhynchus mykiss*) fed plant protein diets containing yellow lupin meal. *Aquaculture* 545, 737177. https://doi.org/10.1016/j.aquaculture.2021.737177

Dawood M A O & Koshio S (2020). Application of fermentation strategy in aqua feed for sustainable aquaculture. *Reviews in Aquaculture* 12: 987-1002 https://doi.org/10.1111/raq.12368

Demir O & Yılayaz A (2020). Effects of the use of feeds containing phytase enzyme from different protein sources on nitrogen and phosphorus discharge of rainbow trout (*On corhynchus mykiss*) juveniles. *Iranian Journal of Fisheries Sciences* 19(4): 2089-2105.

Denstadli V, Hillestad M, Verlhac V, Klausen M & Øverland M (2011). Enzyme pretreatment of fibrous ingredients for carnivorous fish: Effects on nutrient utilization and technical feed quality in rainbow trout (*Oncurhynchus mykiss*). *Aquaculture* 319: 391-397 https://doi.org/10.1016/j.aquaculture.2011.07.012

Dersjant-Li Y, Awati A F, Schulze H F & Partridge G (2015). Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *Journal of Science Food Agriculture* 95: 878–896 https://doi.org/10.1002/jsfa.6998

Dillon C R, Maurice D V & Jones J E (1988). Composition of *Egeria densa*. *Journal of Aquatic Plant Management* 26: 44–45

El-Haroun E, Goda A S & Chowdhury K (2006). Effects food dietary probiotic Biogensupplementation as a growth promoter on growth performance and feed utilization in Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Research* 37: 1473-1480 https://doi.org/10.1111/j.1365-2109.2006.01584.x

Engin K & Carter C G (2006). Growth and food utilization of the Australian short-finned eel, *Anguilla australis australis* (Richardson) given paired iso-energetic diets with increasing crude protein content. *Animal Science* 82: 169-174 https://doi.org/10.1079/asc200528

Engin K & Carter C G (2001). Ammonia and urea excretion rates of juvenile Australian short-finned eel (*Anguilla australis australis*) as influenced by dietary protein level. *Aquaculture* 194: 123-136. https://doi.org/10.1016/s0044-8486 (00)00506-8

FAO (2022). Fishery and Aquaculture Statistics. Global aquaculture production 1950-2020 (FishStatJ). In: FAO Fisheries and Aquaculture Division [online]. Rome. Updated 2022. www.fao.org/fishery/statistics/software/fishstatj/en

Filogh A, Bilen S, Sönmez A Y & Elp M (2023). Growth, Blood Parameters, Immune Response and Antioxidant Enzyme Activities in Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1792) Fed Diets Supplemented with Fumitory (*Fumaria officinalis*). *Journal of Agricultural Science*s (Tarim Bilimleri Dergisi), 29(1):47-59. DOI: 10.15832/ankutbd.982032

Fossi B T, Tavea F, Fontem L A, Ndjouenkeu R & Wanji S (2014). Microbial interactions for enhancement of α-amylase production by Bacillus amyloliquefaciens 04BBA15 and *Lactobacillus fermentum* 04BBA19. *Biotechnology Reports* 4: 99–106 https://doi.org/10.1016/j.btre.2014.09.004

Gatlin D M, Barrows F T, Brown P, Dabrowski K, Gaylord T G, Hardy R W, Herman E, Hu G, Krogdahl Å, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza E J, Stone D, Wilson R & Wurtele E (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research* 38: 551–579 https://doi.org/10.1111/j.1365-2109.2007.01704.x

Glencross B D, Boujard T B & Kaushik S J (2003). Influence of oligosaccharides on the nutritional value of lupin meals when fed to rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 219: 703–713. https://doi.org/10.1016/s0044-8486(02)00664-6

Glencross B, Rutherford N & Bourne N (2012). The influence of various starch and nonstarch polysaccharides on the digestibility of diets to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 356–357: 141–146 https://doi.org/10.1016/j.aquaculture.2012.05.023

Greiling A M, Tschesche C, Baardsen G, Kröckel S, Koppe W & Rodehutscord M (2019). Effects of phosphate and phytase supplementation on phytate degradation in rainbow trout (*Oncorhynchus mykiss* W.) and Atlantic salmon (*Salmo salar* L.). *Aquaculture* 503: 467-474 https://doi.org/10.1016/j.aquaculture.2019.01.035

Glencross B, Hawkins W, Evans D, Rutherford N, McCafferty P, Dods K, Karopoulos M, Veitch C, Sipsas S & Buirchell B (2008). Variability in the composition of lupin (*Lupinus angustifolius*) meals influences their digestible nutrient and energy value when fed to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 277: 220-230 https://doi.org/10.1016/j.aquaculture.2008.02.038

Greiner R & Konietzny U (2010). Phytases: biochemistry, enzymology and characteristics relevant to animal feed use. In: Bedford, M.R., Partridge, G.G. (Eds.), Enzymes in Farm Animal Nutrition. CABI, Wallingford, pp. 96–128 https://doi.org/10.1079/9781845936747.0096

Han B, Long W-q, He J-y, Liu Y-j, Si Y-q & Tian L-x (2015). Effects of dietary Bacillus licheniformis on growth performance, immunological parameters, intestinal morphology and resistance of juvenile Nile tilapia (*Oreochromis niloticus*) to challenge infections. *Fish and Shellfish. Immunology* 46: 225-231 https://doi.org/10.1016/j.fsi.2015.06.018

Heikkinen J, Vielma J, Kemiläinen O, Tiirola M, Eskelinen P, Kiuru T, Navia- Paldanius D & von Wright A (2006). Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 261: 259–268 https://doi.org/10.1016/j.aquaculture.2006.07.012

Hixson S M, Parrish C C & Anderson D M (2014). Full substitution of fish oil with camelina (*Camelina sativa*) oil, with partial substitution of fish meal with camelina meal, in diets for farmed Atlantic salmon (*Salmo salar*) and its effect on tissue lipids and sensory quality. *Food Chemistry* 157: 51–61 https://doi.org/10.1016/j.foodchem.2014.02.026

Hixson S M, Parrish C C, Wells J S, Winkowski E M, Anderson D M & Bullerwell C N (2015). Inclusion of camelina meal as a protein source in diets for farmed salmonids. *Aquaculture Nutrition* 22: 615–630 https://doi.org/10.1111/anu.12276

Hooge D M, Pierce JI & McBride K W (2010). Meta-analysis of laying hen trials using diets with or without Allzyme® SSF enzyme complex. *International Journal of Poultry Science* (9): 824–827. https://doi.org/10.3923/ijps.2010.824.827

Hua K & Bureau D P (2010). Quantification of differences in digestibility of phosphorus among cyprinids, cichlids, and salmonids through a mathematical modelling approach. *Aquaculture* 308: 152–158 https://doi.org/10.1016/j.aquaculture.2010.07.040

Hulefeld R, Habte-Tsion H M, Lalgudi R, Cain R, McGraw B, Tidwell J H & Kumar V (2018). Nutritional evaluation of an improved soybean meal as a fishmeal replacer in the diet of Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture Research* 49: 1414–1422 https://doi.org/10.1111/are.13593

Jo H, Lee S, Yun H, Hong J, Moniruzzaman M, Bai S C & Jeon T (2017). Evaluation of dietary fishmeal analogue with addition of shrimp soluble extract on growth and nonspecific immune response of rainbow trout, *Oncorhynchus mykiss*. *Journal of the World Aquaculture Society* 48: 583–591 https://doi.org/10.1111/jwas.12355

Kaczmarek S A, Rogiewicz A, Mogielnicka M, Rutkowski A, Jones R O & Slominski BA (2014). The effect of protease, amylase, and non-starch polysaccharide degrading enzyme supplementation on nutrient utilization and growth performance of broiler chickens fed corn-soybean meal-based diets. *Poultry Science* 93: 1745–1753 https://doi.org/10.3382/ps.2013-03739

Kader A, Koshio S, Ishikawa M, Yokoyama S, Bulbul M, Nguyen B T & Laining A (2012). Can fermented soybean meal and squid by-product blend be used as fishmeal replacements for Japanese flounder (*Paralichthys olivaceus*). *Aquaculture Research* 43: 1427–1438 https://doi.org/10.1111/j.1365-2109.2011.02945.x

Kals J, Heinsbroek L T N & Zwart S (2012). Phosphorus digestibility and retention of three inorganic phosphorus sources in rainbow trout (*Oncorhynchus mykiss*); Does MAP outperform DCP and MCP. In: ISFNF (Ed.), Poster Presentations, Book of Abstracts XV International Symposium on Fish Nutrition and Feeding, Molde, Norway, pp. 114.

Khosravi A, Safari M, Khodaiyan F & Gharibzahedi S M T (2015). Bioconversion enhancement of conjugated linoleic acid by *Lactobacillus plantarum* using the culture media manipulation and numerical optimization. *Journal of Food Science Technology* 52: 5781–5789 https://doi.org/10.1007/s13197-014-1699-6

Knudsen D, Jutfelt F, Sundh H, Sundell K, Koppe W & Frøkiaer H (2008). Dietary soya saponins increase gut permeability and play a key role in the onset of soybean-induced enteritis in Atlantic salmon (*Salmo salar* L.). *British Journal of Nutrition* 100:120–129 https://doi.org/10.1017/s0007114507886338

Kokou F, Rigos G, Henry M, Kentouri M & Alexis M (2012). Growth performance, feed utilization and non-specific immune response of gilthead seabream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. *Aquaculture* 364: 74–81 https://doi.org/10.1016/j.aquaculture.2012.08.009

Krogdahl Å, Bakke-McKellep AM & Baeverfjord G (2003). Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition* 9: 361–371 https://doi.org/10.1046/j.1365-2095.2003.00264.x

Krogdahl Å, Penn M, Thorsen J, Refstie S & Bakke A M (2010). Important anti-nutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. *Aquaculture Research* 41: 333–344 https://doi.org/10.1111/j.1365-2109.2009.02426.x

Kumar S, Sahu N, Pal A, Choudhury D & Mukherjee S (2006). Non-gelatinized corn supplemented with α-amylase at sub-optimum protein level enhances the growth of *Labeo rohita* (Hamilton) fingerlings. *Aquaculture Research* 37: 284–292 https://doi.org/10.1111/j.1365-2109.2005.01434.x

Kumar V, Wang H-P, Lalgudi R, Cain R, McGraw B & Rosentrater KA (2019). Processed soybean meal as an alternative protein source for yellow perch (*Perca flavescens*) feed. *Aquaculture Nutrition* https://doi.org/10.1111/anu.12911.

Kumar V, Lee S, Cleveland B M, Romano N, Lalgudi R S, Benito M R, McGraw B & Hardy R W (2020). Comparative evaluation of processed soybean meal (EnzoMealTM) vs. regular soybean meal as a fishmeal replacement in diets of rainbow trout (*Oncorhynchus mykiss*): Effects on growth performance and growth-related genes. *Aquaculture* 516: 734652 https://doi.org/10.1016/j.aquaculture.2019.734652

Kurniawan S B, Ahmad A, Rahim N F M, Said N S M, Alnawajha M M, Imron M F, Abdullah S R S, Othman A R, Ismail N I & Hasan H A (2021). Aquaculture in Malaysia: Water-related environmental challenges and opportunities for cleaner production. *Environmental Technology and Innovation* 24: 101913 https://doi.org/10.1016/j.eti.2021.101913

Latorre J D, Hernandez-Velasco X, Wolfenden R E, Vicente J L, Wolfenden A D, Menconi A, Bielke L R, Hargis B M & Tellez G (2016). Evaluation and selection of Bacillus species based on enzyme production, antimicrobial activity and biofilm synthesis as direct-fed microbial candidates for poultry. *Frontiers in Veterinary Science* 3:95 doi:10.3389/fvets.2016.00095

Lee S, Kabir Chowdhury M A, Hardy R W & Small B.C (2020). Apparent digestibility of protein, amino acids and gross energy in rainbow trout fed various feed ingredients with or without protease. *Aquaculture* 524: 735270 https://doi.org/10.1016/j.aquaculture.2020.735270

Lee S A, Lupatsch I, Gomes G A & Bedford M R (2020). An advanced *Escherichia coli* phytase improves performance and retention of phosphorus and nitrogen in rainbow trout (*Oncorhynchus mykiss*) fed low phosphorus plant-based diets, at 11 °C and 15 °C. *Aquaculture* 516: 734549 https://doi.org/10.1016/j.aquaculture.2019.734549

Lin Y-H & Chen Y-T (2022). *Lactobacillus* spp. fermented soybean meal partially substitution to fishmeal enhances innate immune responses and nutrient digestibility of white shrimp (*Litopenaeus vannamei*) fed diet with low fishmeal. *Aquaculture* 548: 737634. https://doi.org/10.1016/j.aquaculture.2021.737634

Lindsay G J, Walton M J, Adron, J W, Fletcher T C, Cho C Y & Cowey C B (1984). The growth of rainbow trout (*Salmo gairdneri*) given diets containing chitin and its relationship to chitinolytic enzymes and chitin digestibility. *Aquaculture* 37: 315–334https://doi.org/10.1016/0044-8486(84)90297-7

Lim C, Lückstädt C, Webster C D & Klesius P (2015). Organic Acids and Their Salts. In: Lee, Cheng-Sheng, Lim, C., Gatlin III, D. M., Webster, C. D. (Eds.) Dietary Nutrients, Additives, and Fish Health, Wiley-Blackwell, pp. 305-319. https://doi.org/10.1002/9781119005568.ch15

Mahagna M, Nir I, Larbier M & Nitsan Z (1995). Effect of age and exogenous amylase and protease on development of the digestive tract, pancreatic enzyme activities and digestibility of nutrients in young meat-type chicks. *Reproduction Nutrition Development* 35:201–212 https://doi.org/10.1051/rnd:19950208

Mahmood T, Mirza M A, Nawaz H, Shahid M, Athar M & Hussain M (2017). Effect of supplementing exogenous protease in low protein poultry by-product meal based diets on growth performance and nutrient digestibility in broilers. *Animal Feed Science and Technology* 228: 23–31 https://doi.org/10.1016/j.anifeedsci.2017.01.012

Manubens A, Canessa P, Folch C, Avila M, Salas L & Vicuña R (2007). Manganese affects the production of laccase in the basidiomycete *Ceriporiopsis subvermispora*. *FEMS Microbiology Letters* 275: 139–145 https://doi.org/10.1111/j.1574-6968.2007.00874.x

Moniruzzaman M, Bae J H, Won S H, Cho S J, Chang K H & Bai S J (2018). Evaluation of solid-state fermented protein concentrates as a fishmeal replacer in the diets of juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquaculture Nutrition* 24: 1198-1212 https://doi.org/10.1111/j.1574-6968.2007.00874.x

Morales G A, Moyano F J & Marquez L (2011). In vitro assessment of the effects of phytate and phytase on nitrogen and phosphorus bio accessibility within fish digestive tract. *Animal Feed Science and Technology* 170: 209–221 https://doi.org/10.1016/j.anifeedsci.2011.08.011

Morales G A, Rodrigañez M S D, Márquez L, Díaz M & Moyano F J (2013). Solubilization of protein fractions induced by *Escherichia coli* phytase and its effects on in vitro fish digestion of plant proteins. *Animal Feed Science and Technology* 181: 54–64 https://doi.org/10.1016/j.anifeedsci.2013.02.004

Morales G A, Azcuy R L, Casaretto M E, Márquez L, Hernández A J, Gómez F, Koppe W & Mereu A (2018). Effect of different inorganic phosphorus sources on growth performance, digestibility, retention efficiency and discharge of nutrients in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 495: 568-574 https://doi.org/10.1016/j.aquaculture.2018.06.036

Nigam P S N & Pandey A (2009). Biotechnology for Agro-Industrial Residues Utilization. Springer Science and Business Media B.V, Netherlands.

Ofori-Mensah S, Yıldız M, Arslan M & Eldem V (2020). Fish oil replacement with different vegetable oils in gilthead seabream, *Sparus aurata* diets: Effects on fatty acid metabolism based on whole-body fatty acid balance method and genes expression. *Aquaculture* 529: 735609. https://doi.org/10.1016/j.aquaculture.2020.735609

Ogunkoya A, Page G I, Adewolu M A & Bureau D P (2006). Dietary incorporation of soybean meal and exogenous enzyme cocktail can affect physical characteristics of fecal material egested by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 254: 466-475 https://doi.org/10.1016/j.aquaculture.2005.10.032

Özil Ö, Diler Ö, Kayhan M H, Kök Taş T, Seydim Z B & Didinen B I (2023). Effects of Dietary Sage, Myrtle and/or Probiotic Mixture on Growth, Intestinal Health, Antioxidant Capacity, and Diseases Resistance of *Oncorhynchus mykiss*. *Journal of Agricultural Sciences* (Tarim Bilimleri Dergisi), 29(2):721-733. DOI: 10.15832/ankutbd.1120481

Pandey A & Satoh S (2008). Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. *Fish Science* 74: 867-874 https://doi.org/10.1111/j.1444-2906.2008.01601.x

Picoli F, Marques S O, Oliveira A D, Nunes C G, Serafini S, Klein B, Oliveira N S, Santos N N O, Zampar A, Lopes D L A & Fabregat T H P (2022). Mixed culture microorganisms fermented soybean meal improves productive performance and intestinal health of Nile tilapia (*Oreochromis niloticus*) juveniles fed plant-based diets in a bio floc system. *Aquaculture Research* 00: 1–13. https://doi.org/10.1111/are.15859.

Ringø E (1991). Effects of dietary lactate and propionate on growth, and digesta in Arctic charr, *Salvelinus alpinus* (L.). *Aquaculture* 96: 321–333 https://doi.org/10.1016/0044-8486 (91)90161-y

Rodehutscord M & Pfeffer E (1995). Effects of supplemental microbial phytase on phosphorus digestibility and utilization in rainbow trout (*Oncorhynchus mykiss*). *Water Science* and *Technology* 31: 143–147 https://doi.org/10.2166/wst.1995.0371

Rodehutscord M, Rückert C, Maurer H P, Schenkel H, Schipprack W, Bach Knudsen B E, Schollenberger M, Laux M, Eklund M, Siegert W & Mosenthin R (2016). Variation in chemical composition and physical characteristics of cereal grains from different genotypes. *Archives in Animal Nutrition* 70: 87–107 https://doi.org/10.1080/1745039x.2015.1133111

Romerheim O H, Skrede A, Gao Y, Krogdahl Å, Denstadli V, Lilleeng E & Storebakken T (2006). Comparison of white flakes and toasted soybean meal partly replacing fish meal as protein source in extruded feed for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 256: 354-364 https://doi.org/10.1016/j.aquaculture.2006.02.006

Satoh S, Hernández A, Tokoro T, Morishita Y, Kiron V & Watanabe T (2003). Comparison of phosphorus retention efficiency between rainbow trout (*Oncorhynchus mykiss*) fed a commercial diet and a low fishmeal based diet. *Aquaculture* 224: 271–282 https://doi.org/10.1016/s0044-8486(03)00217-5

Sealey W M, Barrows F T, Smith C E, Overturf K & LaPatra S E (2009). Soybean meal level and probiotics in first feeding fry diets alter the ability of rainbow trout *Oncorhynchus mykiss* to utilize high levels of soybean meal during grow-out. *Aquaculture* 293: 195-203 https://doi.org/10.1016/j.aquaculture.2009.04.013

Shiu Y-L, Hsieh S-L, Guei W-C, Tsai Y-T, Chiu C-H & Liu C-H (2015). Using Bacillus subtilis E20-fermented soybean meal as replacement for fishmeal in the diet of orange spotted grouper (*Epinephelus coioides*, Hamilton). *Aquaculture Research* 46: 1403–1416 https://doi.org/10.1111/are.12294

Simbaya J, Slominski B, Guenter W, Morgan A & Campbell L (1996). The effects of protease and carbohydrase supplementation on the nutritive value of canola meal for poultry: in vitro and in vivo studies. *Animal Feed Science and Technology* 61: 219–234 https://doi.org/10.1016/0377-8401 (95)00939-6

Singh P K (2008). Significance of phytic acid and supplemental phytase in chicken nutrition: a review. *Worlds of Poultry Science Journal* 64: 553–580 https://doi.org/10.1017/s0043933908000202

Soto D & Norambuena F (2003). Evaluation of salmon farming effects on marine systems in the inner seas of southern Chile: a large-scale measurative experiment. *Journal of Applied Ichthyology* 20: 493-501 https://doi.org/10.1111/j.1439-0426.2004.00602.x

Sönmez A Y, Bilen S, Özdemir K Y, Alagöz K & Özçelik H (2022). Effect of Aqueous Methanolic Extract of Pomegranate Peel (*Punica granatum*) and Veratrum (*Veratrum album*) on Oxidative Status, Immunity and Digestive Enzyme Activity in Rainbow Trout (*Oncorhynchus mykiss*). *Journal of Agricultural Sciences* (Tarım Bilimleri Dergisi) 28 (2): 159-170. DOI: 10.15832/ankutbd.870923

Stone D A J, Allan G I & Anderson A J (2003). Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). IV. Can dietary enzymes increase digestible energy from wheat starch, wheat and dehulled lupin. *Aquaculture Research* 34: 135–147 https://doi.org/10.1046/j.1365-2109.2003.00777.x

Stone D A, Hardy R W, Barrows F T & Cheng Z J (2005). Effects of extrusion on nutritional value of diets containing corn gluten meal and corn distiller's dried grain for rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Aquaculture* 17 (3): 1–20 https://doi.org/10.1300/j028v17n03_01

Sugiura S H, Gabaudan J, Dong F M & Hardy R W (2001). Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout (*Oncorhynchus mykiss* (Walbaum)) fed soybean meal-based diets. *Aquaculture Research* 32: 583–592 https://doi.org/10.1046/j.1365-2109.2001.00581.x

Tanemura N, Akiyoshi Y, Okano K & Sugiura, S (2016). Effects of culturing rapeseed meal, soybean meal, macrophyte meal, and algal meal with three species of white-rot fungi on their in vitro and in vivo digestibility evaluated using rainbow trout. *Aquaculture* 453: 130-134 https://doi.org/10.1016/j.aquaculture.2015.12.001

Tulli F, Messina M, Calligaris M & Tibaldi E (2010). Response of European sea bass (*Dicentrarchus labrax*) to graded levels of methionine (total sulfur amino acids) in soya protein-based semi-purified diets. *British Journal of Nutrition* 104: 664–673 https://doi.org/10.1017/s0007114510001029

Verschuere L, Rombaut G, Sorgeloos P & Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology Molecular Biology* 64(4): 655–671 https://doi.org/10.1128/mmbr.64.4.655-671.2000

Vielma J, Ruohonen K & Peisker M (2004). Dephytinization of two soy proteins increases phosphorus and protein utilization by rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 204: 145-156 https://doi.org/10.1016/s0044-8486 (01)00653-6

Villas-Bôas S G, Esposito E & Mitchell D A (2002). Microbial conversion of lignocellulosic residues for production of animal feeds. *Animal Feed Science and Technology* 98: 1–12 https://doi.org/10.1016/s0377-8401(02)00017-2

Walk C L, Pirgozliev V, Juntunen K, Paloheimo M & Ledoux D R (2018). Evaluation of novel protease enzymes on growth performance and apparent ileal digestibility of amino acids in poultry: enzyme screening. *Poultry Science* 97: 2123-2138 https://doi.org/10.3382/ps/pey080

Wang X, Olsen L M, Reitan K I & Olsen Y (2012). Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. A review article. *Aquaculture and Environment Interaction* 2: 267-283 https://doi.org/10.3354/aei00044

Wang L, Zhou H, He R, Xu W, Mai K & He G (2016). Effects of soybean meal fermentation by Lactobacillus plantarum P8 on growth, immune responses, and intestinal morphology in juvenile turbot (*Scophthalmus maximus* L.). *Aquaculture* 464: 87-94 https://doi.org/10.1016/j.aquaculture.2016.06.026

Wang P, Zhou Q, Feng J, He J, Lou Y & Zhu J (2019). Effect of dietary fermented soybean meal on growth, intestinal morphology and microbiota in juvenile large yellow croaker, *Larimichthys crocea*. *Aquaculture Research* 50: 748–757 https://doi.org/10.1111/are.13929

Yanbo W& Zirong X (2006). Effects of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Animal Feed Science and Technology* 127: 283-292 https://doi.org/10.1016/j.anifeedsci.2005.09.003

Yamamoto T, Iwashita Y, Matsunari H, Sugita T, Furuita H, Akimoto A, Okamatsu A & Suzuki N (2010). Influence of fermentation conditions for soybean meal in a non-fish meal diet on the growth performance and physiological condition of rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 309: 173-180 https://doi.org/10.1016/j.aquaculture.2010.09.021

Ye C, Anderson D M & Lall S P (2016). The effects of camelina oil and solvent extracted camelina meal on the growth, carcass composition and hindgut histology of Atlantic salmon (*Salmo salar*) parr in freshwater. *Aquaculture* 450: 397–404 https://doi.org/10.1016/j.aquaculture.2015.08.019

Yıldırım Ö & Çantaş İ B (2022). Türkiye'de Gökkuşağı Alabalığı yetiştiriciliğinin üretim ve ekonomik göstergelerinin incelenmesi. Acta Aquatica Turcica, 18(4): 461-474. https://doi.org/10.22392/actaquatr.1101098

Yiğit N O & Ölmez M (2011). Effects of cellulase addition to canola meal in tilapia (*Oreochromis niloticus* L.) diets. *Aquaculture Nutrition* 17: e494-e500 https://doi.org/10.1111/j.1365-2095.2010.00789.x

Yiğit N O, Koca S B, Didinen B I & Diler İ (2018). Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed soybean meal-based diets. *Journal of Applied Animal Research* 46 (1): 29-32 https://doi.org/10.1080/09712119.2016.1256292

Yoshida H, Sugahara T & Hayashi J (1987). Changes in carbohydrates and organic acids during development of mycelia and fruit-bodies of shiitake mushroom (*Lentinus edodes* (Berk.) Sing.). *Journal of Japanese Society of Food Science and Technology* 34: 274–281 https://doi.org/10.3136/nskkk1962.34.5_274

Zhou Y, Yuan X, Liang X, Fang L, Li J, Guo X, Bai X & He S (2013). Enhancement of growth and intestinal flora in grass carp: the effect of exogenous cellulase. *Aquaculture* 416–417: 1–7

Zhuo Li-C, Liu K& Lin Yu-H (2016). Apparent digestibility of soybean meal and *Lactobacillus* spp. fermented soybean meal in diets of grouper, *Epinephelus coioides*. *Aquaculture Research* 47: 1009-1012 https://doi.org/10.1111/are.12543

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Determination of Physicochemical, Rheological, Microbiological and Sensory Properties of Low Protein Yoghurt Substitutes Produced for PKU (Phenylketonuria) Patients

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ABSTRACT
Treatment of PKU (Phenylketonuria) is a lifelong special diet program starting from the newborn period. The aim of this study was to produce yoghurt substitute for PKU patients. A commercial low protein milk substitute, xanthan gum (1%), commercial yoghurt gelling agent (1.5%), starch (4%) and pectin (1.6%) were used to produce yoghurt substitute. Control yoghurt was produced from cow's milk. The fermentation of all samples was completed at the end of the 5th hour. The pH of the samples decreased during storage. The total solid matter of the corn starch and pectin added samples were higher than the those of others. Syneresis values of the samples with xanthan gum, pectin and commercial gelling agent were negligible. The shear stress values of xanthan gum, commercial gelling agent and starch added samples were found close to each other. The shear stress of the pectin added sample was the highest among the samples. L* values of the control and starch added samples, a* and b* values of pectin added sample were higher than those of other samples. The amount of protein and phenylalanine was higher in the pectin added sample than the other samples containing gelling. However, their values in all yoghurt substitute samples were found to be well below the upper limit value that can be consumed. Although the amount of phenylalanine tolerated in the body varies according to age, gender, weight and the degree of phenylketonuria, it is stated that up to 1000 mg per day. While numbers of *Lactobacillus delbrueckii* ssp. *bulgaricus* increased during storage, numbers of *Streptococcus thermophilus* increased by pectin addition and decreased in other samples. In terms of general acceptability in sensory analyses, the most preferred sample was the sample added containing commercial gelling agent. This sample was followed by the samples with corn starch and pectin. It was concluded that these yoghurt substitutes could support the missing alternative product range for the patients.

Keywords: Phenylketonuria, PKU patients, Hydrocolloid, Stabilizer, Milk substitute

1. Introduction

Phenylketonuria (PKU) is one of the most common congenital metabolic disorders which is a disease caused by mutations in the gene encoding the enzyme phenylalanine hydroxylase (Scriver and Kaufman, 2001). As a result of deficiency of this enzyme, the amino acid phenylalanine, which does not turn into tyrosine, and its metabolites (phenylpyruvic, phenyl lactic acid, phenyl acetic acid) formed as a result of its transamination, accumulate in the blood, urine and other body fluids of the patient, causing mental-motor retardation (Müslümanoğlu et al. 2014; Parlak 2018). When PKU is not treated, irreversible mental retardation, microcephaly, motor impairment, eczematous rash, autism, seizure development, developmental problems, abnormal behavior and psychiatric symptoms (Van Wegber et al. 2017; Erdal & Caferoğlu 2018), light hair, skin and eye color, widely spaced teeth, mold-like urine due to defect in melanin formation and sweat odor (Gaw et al. 1999; Davis et al. 2005) occurs.

The general principle in the treatment of PKU is special diet programs that should be started with the diagnosis in the neonatal period and their lifelong continuity. This diet aims to keep the blood Phe level within normal limits by minimizing the amount of Phe ingested with food (Lee & Newman 2003; Seçkin 2007; Özer et al. 2008). The diet program is different for each patient. A diet list is prepared according to the patient's height, age, body weight, type of phenylketonuria and blood Phe level (Anonymous 2017a). Products with high Phe content are removed from the diet lists of individuals with PKU. Milk and dairy products are among these products (Scriver & Kaufman 2001; Waisbren et al. 2007). This amino acid, which is found in 4% to 5% in almost all proteins, is 5.4 g / 100 g in cow's milk protein and forms the building block of many proteins, especially together with tyrosine (Kavas & Kınık 2005). The protein requirement necessary for the growth and development of the individual is met with special amino acid mixtures without Phe (Üstüner Top & Küçük Alemdar 2015).

The daily Phe amounts that patients with classical, moderate and mild phenylketonuria can tolerate are 20 mg/kg, 20-25 mg/kg and 25-50 mg/kg, respectively. (Thöny & Blau 2006; Akış 2012). According to recommendations for UK, Germany, the USA, France, the Netherlands, and the 2016 ESPKU (European Society for Phenylketonuria and Allied Disorders Treated as Phenylketonuria) guidelines, the allowed amount of Phe is 130-400 mg/day for 0-2 years old, 200-400 mg / day for 3-9 years old, 350-800 mg/day for 10-15 years old, 450-1000 mg/day for adolescent/adult and 120-400 mg/day for pregnancy. Tolerance for pregnancy will usually increase in later stages of pregnancy. Since Phe is an essential amino acid, excessive restriction is also harmful and, particularly in infancy, will result in impaired growth and cognitive development (Burgard et al. 2016).

There are home-made yoghurt substitutes recipes for PKU patients that are not industrially produced. Milk powder (20 g), yoghurt maker gel (3 g), lactose (1 g), and water (200 mL) are boiled. When the yogurt reaches the fermentation temperature, normal yoghurt (30 g) is added and a fermentation is carried out for 5 to 6 hours. In another way, 500 g of milk substitute (Taranis dalia), 40 g of starch and salt are boiled. Fifty grams of yogurt as a starter culture is added and left to ferment (Anonymous, 2017b). In another recipe, 400 mL of milk substitute and 40 g of corn starch are boiled for 5 minutes. Twenty g of yoghurt, 5 mL of lemon juice, 3 g of salt and 5 g of granulated sugar mixture is added to the starchy jelly that has come to the temperature of yoghurt fermentation. Fermentation takes 3 hours. In all recipes, yoghurts are stored at +4 ^{o}C after fermentation. In the last recipe, the protein, Phe and energy content of 1 serving of yoghurt is 0.22 g, 107.34 Kcal and 10-15 mg, respectively (Anonymous 2020).

It has been reported that the prevalence of PKU varies according to ethnic groups, being higher in white and native Americans, and lower in blacks, Asians and Spain (Walter et al. 2006). According to data released by Ministry of Health in 2006 revealed that prevalence decreased and rate was 1/4500. However, Turkey is still in the list of countries with the highest prevalence (TCSB 2006).

Lifelong diet is very important in PKU disease. Many foods are on the banned food list of PKU patients. There is a great need for alternative low-protein products for the limited diet lists of PKU patients. However, today there are not enough studies on the products that PKU patients can consume. Studies for low-protein milk and dairy products are very few. There is no industrially produced yoghurt substitute for PKU patients. These patients consume yoghurt substitutes produced in different formulations at home. Under these conditions, a standard product cannot be obtained. This situation can be dangerous for the health of very sensitive patients.

Scortegagna et al. (2021) observed in their study named “Evaluation and acceptability of alternative food recipes for patients with phenylketonuria” that there are few studies aiming to detail food products for the PKU population. They noted that as diet has a significant impact on the daily life of these patients, the number of studies to develop food products for patients with PKU needs to be increased.

In this study, it was aimed to produce yogurt substitutes that PKU patients can consume by using different stabilizers and to determine the properties of those yogurt substitutes.

2. Material and Methods

2.1. Materials

The research material consists of low protein milk substitute, yoghurt culture, low methoxylated pectin, xanthan gum, commercial gelling agent and corn starch. Since commercial gelling agent is an expensive product, in this study it was compared with other gelling agents that may be alternative in terms of their contribution to yoghurt substitution Because cow's milk contains high amounts of protein, low-protein milk substitute (The Taranis Dalia Low Protein Milk Substitute, Lactalis Nutrition Santé LNS, France) was used in yoghurt production. This milk substitute is made up of water, cream, lactose, curdled milk powder, maltodextrin, mono and diglycerides of fatty acids (palm and / or rapeseed oil). One hundred mL of low protein milk substitute has 200 mg of protein, 6.4 mg of phenylalanine, 6.7 mg of threonine, 6400 mg of carbohydrate, 2600 mg of fat and 17.5 mg of calcium. UHT milk (cow milk) supplied by Pınar Süt Mamülleri San. A.Ş. was used for the production of control sample. Low-protein milk substitute was not used in the control sample production. In the production of other samples, only low protein milk substitute was used as milk. As starter culture, a yoghurt culture (Doğadan Bizim Gıda ve Süt Ürünleri San. ve Tic. Ltd. Şti., Istanbul, Turkey) consisting of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* was used. Low methoxylated pectin (Arosel Gıda Katkı Maddeleri ve Mak. San. Dış Tic. Ltd. Şti., Istanbul, Turkey), xanthan gum (Çağdaş Kimya ve Gıda San Tic Ltd Şti., Istanbul, Turkey), commercial gelling agent and corn starch (Pak Gıda Üretim Pazarlama A. Ş., Istanbul, Turkey) were used as gelling agents. Low methoxylated pectin was chosen because it produces a stable gelation at low sugar concentrations, using very little calcium within wide pH limits (pH 2.5-6.5). Xanthan gum is preferred due to its high viscosity in small concentrations, its immediate dissolution in hot and cold water, its resistance to deterioration that may occur due to heat and physical applications, and its usability between pH 1-11. Commercial gelling agent yoghurt maker gel was obtained from Nestle Nutrition GmbH (Frankfurt, Germany). It is a vegetable based gelling agent which contains carob powder and calcium lactate. One hundred g of it contains 1.5 g carbohydrate and 5.3 g protein. This gel is preferred because it is a low

protein yoghurt ingredient. Corn starch was preferred due to its ability to make a gel in a wide pH range with a more fluid structure that is resistant to breakage.

2.2. Methods

2.2.1. Preparation and fermentation of milk substitute

For the low protein yoghurt substitute, before starting the study, yoghurt pre-tests were made using various proportions of gelling agents and it was decided to use 1% xanthan gum, 1.5% commercial gelling agent, 4% starch and 1.6% pectin. The lyophilized yoghurt culture was inoculated into some low protein milk heated to 42 ºC and activated for 1.2 hours in the incubator at 42 ºC. On the other hand, some low protein milk substitute was poured in equal amounts (1000 mL each) into four different containers. The consistency of the milks was increased by adding 1% xanthan gum, 1.5% commercial gelling agent, 4% starch and 1.6% pectin into each and heating milks. The low protein milk substitutes added xanthan gum, commercial gelling agent and starch turned into a gel after being heated at 82 ºC for 5 minutes, 88 ºC for 5 minutes and 94 ºC for 5 minutes respectively. Heat treatment was continued until gelation was achieved. The low protein milk substitute to which pectin was added did not turn into a gel when kept at 99 ºC for 2 minutes. It turned into a gel when cooled after inoculation. Then, the activated yoghurt culture was inoculated (2%) to the viscous milks brought to the inoculation temperature (42 ºC). After inoculation, the milks were separated into plastic containers (PP material) and left to incubate at 42 ºC. The same procedures were applied to the UHT milk for the control sample. pH values of the samples were followed during fermentation. After 5 hours of fermentation, yogurt sample produced from UHT milk and other yogurt substitute samples were stored at +4 ºC. The analyses of yoghurt and yoghurt substitutes were carried out on the 1st, 7^{th}, 14^{th} and 21^{st} days of storage.

The pH, syneresis, total solid matter, stable phase rheological properties and microbiological analyses were performed on the 1^{st}, 7^{th}, 14^{th} and 21^{st} days of storage. Sensory and color analyses of the samples were carried out on the 1^{st} day of storage, crude protein and Phe analyses were performed on the 7^{th} day of storage.

2.2.2. Physicochemical analyses

pH measurements were carried out by means of a digital pH meter named 300/310 branded WaterproofHand heldpH / mV / TemperatureMeter (Kosikowski 1982). Total solid amount of samples were carried out with using AOAC method (1990). Crude protein analysis was performed using the Kjeldahl method (IDF 1986). Phe content was calculated using HPLC system (Waters, USA) (Goldar et al. 2016). For the determination of syneresis values, 5 g of sample was weighed and centrifuged at 4500 rpm and 10 °C for 30 minutes and then serum amount calculated as% by modifying the method stated by Verbeken et al. (2006). Konica-Minolta Chroma MeterCR-5 device was used for color determination. The samples were homogenized and placed in the measuring cup. The measurement was recorded 5 times. L* (brightness), a* (+ red, - green) and b* (+ yellow, - blue) values of yoghurt samples were determined (Cueva & Aryana 2008).

2.2.3. Rheological analyses

Steady phase analysis was performed using a temperature controlled (peltier system) rheometer (TA Instruments DHR2, USA) in a specified parallel plate configuration (cone diameter 40 mm), 1-100 s^{-1} shear rate and 5 °C degrees (Barnes et al. 1993).

2.2.4. Microbiological analyses

Microbiological analyses were carried out by applying the method of spreading on the surface (TGK 2003). The anaerobic environment required for microbiological analyses was provided by Microbiology Anaerocult A kits obtained from Merck Germany. 35 mL of sterile water was transferred to the kits and the kits were put into anaerobic jars. De Man-Rogosa Agar (MRS, Oxoid CM 361) was used for *Lactobacillus delbrueckii* ssp. *bulgaricus* growth. The pH of the medium was lowered to 5.2 by using HCl. Petri plates were left to incubate for 72 hours at 37 ºC under anaerobic conditions. Growing colonies (30-300) were counted (Dave & Shah 1996). M17-Agar (Merck, Germany) was used for the growth of *S. thermophilus*. Petri plates were left to incubate at 37 ºC for 3 days under aerobic conditions. Round colonies (30-300) formed after incubation were counted (TGK 2003).

2.2.5. Sensory analyses

The samples were evaluated for color, appearance, taste, odor, texture, mouth consistency, spoon consistency and general acceptability. In sensory evaluation, the ratings of very unsatisfactory, unsatisfactory, average, good and very good were given 1, 2, 3, 4 and 5 points, respectively. This sensory evaluation was carried out by 6 panelists (Meilgaard et al. 2006).

2.2.6. Statistical analyses

In the statistical evaluation of sensory and color analyses results, the difference between samples was determined using one-way ANOVA analysis. During the evaluation of phsicochemical and microbiological analyses results, the difference between the groups was determined using the univariate general linear model procedure of the SPSS statistical software programme (SPSS Statistics 21.0 Inc., Chicago, IL, USA). Duncan's multiple comparison test was used to determine significant differences among the means at P<0.05 (Düzgüneş et al. 1978). All trials and measurements were repeated in triplicates.

3. Results and Discussion

3.1 Physicochemical properties

The fermentation was carried out in a controlled manner by observing the pH values of the yoghurt samples during the fermentation. Table 1 shows the pH values observed during fermentation.

Table 1- pH values of yoghurt and low protein yoghurt substitute samples during fermentation

Fermentation time (h)	*K*	*A1*	*A2*	*A3*	*A4*
0	6.42±0.03 ^{Aa}	6.31±0.03 ^{Ab}	6.04±0.02 ^{Bc}	6.34±0.01 ^{Ab}	5.83±0.02 ^{Ad}
1	6.21±0.02 ^{Ba}	6.00±0.00 ^{Bc}	6.11±0.01 ^{Ab}	6.03±0.05 ^{Bc}	5.76±0.04 ^{Bd}
2	5.95±0.03 ^{Ca}	5.66±0.02 ^{Cc}	5.66±0.03 ^{Cc}	5.87±0.01 ^{Cb}	5.59±0.05 ^{Cd}
3	5.16±0.03 ^{Dc}	5.21±0.01 ^{Dc}	5.37±0.02 ^{Da}	5.31±0.03 ^{Db}	5.16±0.04 ^{Dc}
4	4.84±0.02 ^{Ec}	4.63±0.02 ^{Ee}	4.78±0.03 ^{Ed}	4.89±0.01 ^{Eb}	5.05±0.02 ^{Ea}
5	4.50±0.04 ^{Fc}	4.51±0.02 ^{Fc}	4.52±0.03 ^{Fc}	4.60±0.03 ^{Fb}	4.80±0.04 ^{Fa}

K (the control group, the difference from other samples is that stabilizer was not used); A1 (1% xantham gum); A2 (1.5% commercial gelling agent); A3 (4% corn starch), A4 (1.6% pectin).

Lower-case letters present the differences between the different samples in the same fermentation time and upper-case letters show differences between the fermentation times of each samples (P<0.01).

At the end of the 5th hour, the fermentation was terminated. At the end of fermentation, the control sample had the lowest pH value. The sample with the closest pH value was the one xanthan gum. At the end of the 5th hour, the pH of the sample to which pectin was added was at the highest level.

pH values of yoghurt samples decreased during storage (Table 2). Generally, the highest pH was detected in the pectin added sample and the lowest pH in the control sample during storage. The difference between yoghurt types and difference between storage days in terms of pH value was found to be significant at the P<0.05 level. Commercial gelling agent, starch and pectin added yoghurts were found to be statistically similar. While it was determined that the control yoghurt was different from these, xanthan gum added yoghurt was found to be similar to both groups.

The commercial gelling agent contains Ca-lactate. Pathomrungsiyounggul et al. (2010) reported that Ca-lactate has a pH-lowering effect. The protective activity of lactates has been explained by various researchers as the addition of lactate reduces the water activity (aw) of the product to prevent microbial growth and provides microbial inhibition by lowering the intracellular pH (de Wit & Rombouts 1990). Lactates are both Gram (+) and Gram (-) a bacteriostatic agent that can inhibit bacteria is an agent. In the studies, it was determined that the effect of lactates on the product increased with the increase of its concentration, depending on the properties of the product (Bingöl & Bostan 2012). Although the commercial gelling agent contains calcium lactate, these effects were not observed in the sample containing the commercial gelling agent. The pH values and microorganism counts of that sample were not significantly lower from the other samples (Table 2). This may be due to the low calcium lactate ratio.

Goldar et al. (2016) produced a special yoghurt for PKU patients. Each of the yoghurt milks contained equal amounts of milk (5%), starch (2%), inulin (2%), butter (3.5%) and transglutaminase (0.1%). While two of the samples contained 4% permeate (composed of water, sugar, some minerals and non-protein nitrogen compounds), 1.5% and 2% non-dairy creamer, the other two samples contained 5% permeate 1.5% and 2% non-dairy creamer. There was water in the ratio of 80.4-81.9% in yoghurt milk. *L. delbrueckii ssp. bulgaricus* and *S. thermophilus* were used as starter. After incubated for 4 h at 42 °C, they were stored at 4 ºC for 2 weeks. The highest pH reduction (0.15 unit) was seen in the sample containing 4% permeate and 2% non-dairy creamer during storage. The reduction in pH can be attributed to activity of bacteria in yoghurt and production of lactic acid from lactose in permeate. The pH values of the samples in that study were considerably lower than those in this study. Cogan (1996) stated that yoghurt bacteria are also active at refrigerator temperature and can cause a significant decrease in pH.

Dry matter values of the samples are shown in Table 2. Among the samples to which gelatin was added on the 1st day of storage, the dry matter of the starch added sample was the highest. This was followed by the pectin added sample. The xanthan gummy sample had the lowest dry matter. It is an expected result that the solid matter values of the samples differ due to the differences in the water binding capacity of the substances used as thickener. When hydrocolloids are used as thickeners in foods, water is physically bound and the structure of the food material changes (Çakmakçı 2012). In a study conducted by Goldar et al. (2016) solid matter ratios of the samples varied between 14.80-17.00%. Total solid matter (%) of samples containing corn starch and pectin in this study were similar to those of that study. The solid matter of other samples in this study was significantly lower than those in that study. Difference between solid matter ratios in both studies may be due to the different materials used.

Table 2- Some physicochemical and microbiological properties of yoghurt substitute samples during the storage

Treatments	*Storage Periods (days)*	*pH*	*Total Solid Matter (%)*	*Syneresis (%)*	*L. delbrueckii ssp. bulgaricus (log cfu/g)*	*S. thermophilus (log cfu/g)*
Control	1	4.40 ± 0.20^{Ab}	10.80 ± 0.26^{Ca}	2.07 ± 0.02^{Eb}	4.98 ± 0.05^{Ca}	6.18 ± 0.05^{Bb}
	7	4.29 ± 0.02^{Aab}	12.00 ± 0.20^{Cb}	2.81 ± 0.03^{Ea}	5.52 ± 0.03^{Cc}	6.30 ± 0.10^{Bb}
	14	4.17 ± 0.01^{Aab}	11.30 ± 0.10^{Cb}	2.43 ± 0.02^{Ea}	5.30 ± 0.02^{Cb}	6.48 ± 0.20^{Bb}
	21	4.18 ± 0.73^{Aa}	12.21 ± 0.01^{Cb}	2.44 ± 0.02^{Ea}	5.00 ± 0.02^{Ca}	5.30 ± 0.02^{Bb}
Xantan gum	1	4.35 ± 0.01^{ABb}	11.10 ± 0.10^{Aa}	0.00 ± 0.00^{Db}	4.74 ± 0.05^{Aa}	6.84 ± 0.02^{Aa}
	7	4.33 ± 0.02^{ABab}	10.90 ± 0.21^{Ab}	0.00 ± 0.00^{Da}	5.04 ± 0.01^{Ac}	5.60 ± 0.05^{Aa}
	14	4.18 ± 0.01^{ABab}	10.80 ± 0.00^{Ab}	0.01 ± 0.00^{Da}	4.65 ± 0.02^{Ab}	5.60 ± 0.01^{Aa}
	21	4.24 ± 0.02^{ABa}	10.53 ± 0.09^{Ab}	0.02 ± 0.00^{Da}	4.40 ± 0.05^{Aa}	4.90 ± 0.03^{Aa}
Commercial gelling agent	1	4.45 ± 0.02^{Bb}	11.38 ± 0.01^{Ba}	0.03 ± 0.02^{Cb}	4.87 ± 0.06^{Ba}	7.30 ± 0.30^{Da}
	7	4.38 ± 0.02^{Bab}	11.40 ± 0.00^{Bb}	0.04 ± 0.01^{Ca}	5.11 ± 0.01^{Bc}	5.70 ± 0.01^{Da}
	14	4.41 ± 0.02^{Bab}	11.30 ± 0.03^{Bb}	0.12 ± 0.02^{Ca}	4.93 ± 0.05^{Bb}	5.41 ± 0.01^{Da}
	21	4.36 ± 0.00^{Ba}	11.18 ± 0.00^{Bb}	0.01 ± 0.00^{Ca}	4.82 ± 0.02^{Ba}	5.26 ± 0.03^{Da}
Starch	1	4.44 ± 0.02^{Bb}	16.70 ± 0.10^{Ea}	1.43 ± 0.01^{Bb}	4.60 ± 0.02^{Aa}	7.00 ± 0.03^{Ca}
	7	4.36 ± 0.01^{Bab}	16.58 ± 0.02^{Eb}	2.19 ± 0.01^{Ba}	4.70 ± 0.04^{Ac}	5.48 ± 0.02^{Ca}
	14	4.34 ± 0.01^{Bab}	16.52 ± 0.08^{Eb}	1.53 ± 0.01^{Ba}	4.74 ± 0.01^{Ab}	5.30 ± 0.05^{Ca}
	21	4.30 ± 0.01^{Ba}	16.98 ± 0.19^{Eb}	1.66 ± 0.03^{a}	4.62 ± 0.03^{Aa}	4.48 ± 0.02^{Ca}
Pectin	1	4.58 ± 0.03^{Bb}	14.20 ± 0.20^{Da}	0.03 ± 0.01^{Ab}	4.70 ± 0.02^{Ba}	4.30 ± 0.06^{Aa}
	7	4.52 ± 0.02^{Bab}	14.30 ± 0.30^{Db}	0.00 ± 0.00^{Aa}	5.48 ± 0.02^{Bc}	5.70 ± 0.03^{Aa}
	14	4.38 ± 0.02^{Bab}	14.19 ± 0.09^{Db}	0.01 ± 0.00^{Aa}	4.38 ± 0.02^{Bb}	5.48 ± 0.06^{Aa}
	21	4.54 ± 0.03^{Ba}	13.87 ± 0.10^{Db}	0.01 ± 0.00^{Aa}	4.20 ± 0.05^{Ba}	5.11 ± 0.03^{Aa}

Upper-case letters present the differences between the different samples in the same storage time and lower-case letters show differences between the storage times of each samples ($P<0.01$).

The syneresis is a structural defect and can be defined as the separation of the liquid phase held in the protein network spontaneously from the gel structure without any external effects (Lucey 2002). The syneresis is an undesirable feature that affects consumer choice of the product (Nunes et al. 2006).

The syneresis values of yoghurt samples during storage are shown in Table 2.

The syneresis values were higher in control yoghurt and starchy yoghurt than in the others. The effect of days and yoghurt varieties on the syneresis was found to be significant at the $P<0.01$ level. Syneresis of the samples at 7, 14 and 21 days were the same, while the 1st day was different from the others.

The product obtained in this study is a yoghurt substitute product and hydrocolloids were used to obtain a yoghurt-like structure. Hydrocolloids are compounds that can improve the texture of yoghurt. These compounds include long and branched molecules, which are able to establish links with each other or with other molecules present in the environment in the form of an emulsion. Additions of hydrocolloids to yoghurt are effective in absorbing water, increasing viscosity and strengthening and improving the texture of yoghurt (Mortazavian & Sohrabvandi 2006; Bahrami et al. 2013).

In the study conducted by Goldar et al. (2016) it has been observed that the rate of syneresis in yoghurt samples produced for patients with PKU decreased during the 14-day storage period. The reduction in serum separation may be due to the corn starch and inulin used in the formulation. In this study, irregularities were observed in serum separation between the storage days of the samples. In some samples no serum separation was observed on some days of storage. The reason for this is that the use of milk substitutes, not milk, in the production of yoghurt substitutes, differences in product composition or the level of stabilizers may have been high.

Crude protein, nitrogen and phenylalanine analyses were performed on yoghurt samples on the 7th day of storage. The results obtained are shown in Table 3.

Table 3- Crude protein, nitrogen and phenylalanine values of yoghurt substitute samples

Samples	*Nitrogen (%)*	*Crude Protein (%)*	*Phenylalanine (mg/L)*
K	0.50±0.10 [b]	3.19±0.02 [c]	60.03±2.47 [d]
A1	0.07±0.03 [a]	0.44±0.04 [a]	21.90±1.03 [b]
A2	0.07±0.02 [a]	0.44±0.01 [a]	15.59±0.92 [a]
A3	0.08±0.01 [a]	0.51±0.03 [a]	23.00±1.81 [b]
A4	0.12±0.04 [a]	0.76±0.02 [b]	28.78±1.01 [c]

K (the control group, without stabilizer); A1 (1% xantham gum); A2 (1.5% commercial gelling agent), A3 (4% corn starch); A4 (1.6% Pectin). ($P<0.01$)

In this study, the Phe amounts of yoghurt substitute samples are well below the recommended amounts. Considering that 1 serving (a bowl) of yoghurt is 180 grams, it has been determined that PKU patients can consume all yoghurt substitutes with stabilizer produced in this study when the values of Phe are considered.

Abdel-Salam & Effat (2010) prepared milk-based drink for PKU patients. Ultrafiltration was applied to a mixture of buffalo and cow milk (1:1) at 50-55 ºC. Glycomacropeptides (a protein source) (2.5%) and corn germ oil (3%) were added to ultra-filtered (UF) milk permeate (consisting of water, sugar, some minerals and non-protein nitrogen compounds) and emulsification was applied. The final product was then heated to 85 °C for 15 minutes, then rapidly cooled and stored at 4 °C. The protein value of the final product was 2.5%, and the amount of Phe 12 mg/100 mL. The protein and Phe values detected in that study are well above those in this study. This may be due to the use of buffalo and cow's milk in that study.

In the study conducted by Pimentel et al. (2014), low-protein foods were prepared according to recipe books specifically designed for phenilketonuria patients. Later, these foods were analyzed. Low protein homemade yoghurt (100 g) (daily basic food) contains corn starch (5.4 g), egg substitute (1.2 g), low protein milk substitute (84.4 g), natural yoghurt (9.0 g). Pimentel et al. (2014) reported that low-protein homemade yoghurt contained 0.9% protein and 21.3 Phe mg/g protein. In study conducted by Goldar et al. (2016), the protein amounts of the samples varied between 2.87-2.99%, while the Phe amounts varied between 24.09-27.64 mg/100g. Protein and Phe amounts in this study were also considerably lower than in that study.

Low-protein milk substitute differs from cow's milk in color. Therefore, the color of the low-protein yogurt substitute will be different from the color of yogurt produced from cow's milk. In addition, color analyses were carried out to determine whether the gelling agents used had an effect on the color. Color analyses were performed on yoghurt samples on the first day of storage. The highest L * values were seen in control yoghurt, the highest a * and b * values were seen in low protein yoghurt with pectin. The lowest L * value was observed in pectin added low protein yoghurt, the lowest a * value in control yoghurt and the lowest b * value in starch added low protein yoghurt. Results of color analyses are shown in the Table 4.

Table 4- Color properties of yoghurt and yoghurt substitute samples

Samples	*L**	*a**	*b**
K	91.11±0.00[e]	-1.93±0.01[a]	10.14±0.01[a]
A1	83.95±0.01[c]	0.09±0.01[c]	15.89±0.00[d]
A2	82.31±0.02[b]	0.09±0.01[c]	15.21±0.01[c]
A3	85.85±0.04[d]	-0.02±0.00[b]	13.75±0.02[b]
A4	81.98±0.01[a]	1.22±0.00[d]	17.04±0.01[e]

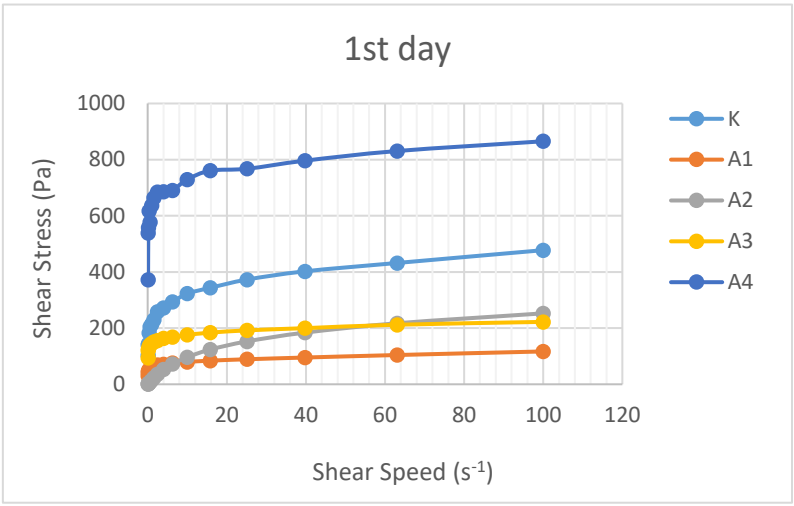
K (the control group, without stabilizer); A1 (1% xantham gum); A2 (1.5% commercial gelling agent); A3 (4% corn starch); A4 (1.6% pectin).

The difference between yoghurt types in terms of color was found statistically significant at $P\leq0.01$ level. Different stabilizers used may cause color change. In terms of a* values, only low protein yoghurt samples containing xanthan gum and commercial gelling agent were similar. In study conducted by Macit & Bakirci (2017), the L* value of the yoghurt sample containing xanthan gum was lower than that of the yoghurt sample containing starch. In that study, the b* value of the yoghurt sample containing xanthan gum was higher than that of the yoghurt sample containing starch. The results of their study were similar to the results of this study. It can be said that these two studies are similar in terms of the effect of xanthan gum and starch on values.

3.2. Rheology steady shear properties

The steady phase analysis in yoghurts was examined at 5 °C, between 1-100 s^{-1} shear rate. The shear stress graphs of yoghurt substitutes on the 1st and 21st days of storage at 1-100 (1/s) are shown in Figure 1.

a



b

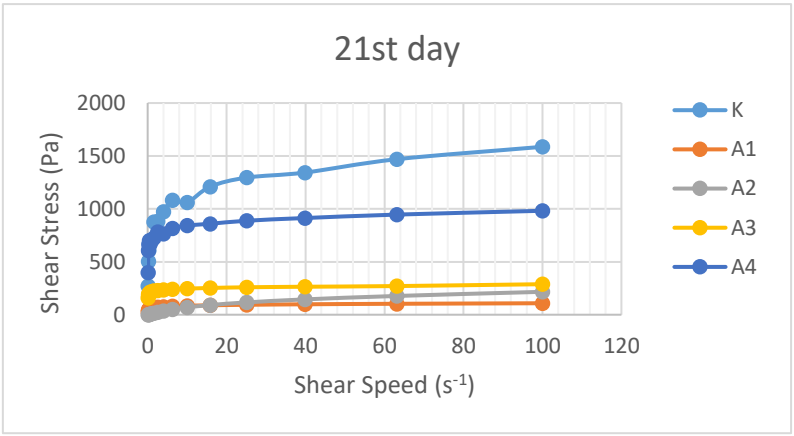


Figure 1- Shear rate-shear stress graph of yoghurts with different stabilizer added during the storage a: 1st day, b: 21st day K (the control group, without stabilizer), A1 (1% xantham gum), A2 (1.5% commercial gelling agent), A3 (4% corn starch), A4 (1.6% pectin).

When the shear stresses at shear rates on the 1st and 7th days of storage were examined, it was observed that the highest shear stress was in the pectin added low protein yoghurt sample. It was determined that the lowest shear stress was in the starch added low protein yoghurt sample. When the shear stresses at all shear rates were examined on the 14th and 21st days of storage, it was seen that the highest shear stress was in the control yoghurt. It was followed by a sample of pectin-added yoghurt. The fact that the shear stress of pectin added low protein yoghurt was high on the 1st and 7th days of storage, because the consistency was very dense. The decrease in its shear stress on the 14th and 21st days of storage can be explained by the deterioration of its structure during storage.

3.2. Microbiological properties

L. delbrueckii ssp. *bulgaricus* numbers in yoghurt samples generally increased until the 7th day of storage and then decreased. *L. delbrueckii* ssp. *bulgaricus* count was found in the control sample on all days of storage. This was followed by the yoghurt sample with commercial gelling agent. The difference between samples and days was found to be significant at the $P<0.01$ level. Statistically, while xanthan gum and starch added low protein yoghurt samples were similar, commercial gelling agent and pectin added low protein yoghurt samples were similar to each other. Control yoghurt was different from all of these samples.

On the first day of storage, the sample with commercial gelling agent had the highest number of *S. thermophilus*, while the sample with the lowest number of *S. thermophilus* had pectin added sample. In the control sample and the pectin supplemented sample, an increase, then a decrease was observed during storage. The *S. thermophilus* number of other samples decreased during storage. While the number of *S. thermophilus* is expected to increase, the reason for the decrease may be that the free water available by the bacteria in the environment is bound by thickeners.

In this study, the *S. thermophilus* counts changed more than the *L. delbrueckii* ssp. *bulgaricus* numbers. Similar results were obtained in the study of Macit & Bakirci (2017). They produced set type yoghurt using seven different stabilizers (sodium caseinate, gelatin, carrageenan, xanthan gum, guar gum, locust bean gum (LBG), native corn starch). Reducing water activity of stabilizers affected negatively the development of *S. thermophilus*. *S. thermophilus* needs higher water activity than *L. delbrueckii* ssp. *bulgaricus* (Macit & Bakirci 2017). The difference between the cultivars and between days was found to be significant at the P<0.01 level. Similarity was observed in xanthan gum and pectin added low protein yoghurt samples.

3.7. Sensory properties

Sensory properties of yoghurt and low protein yoghurt substitute samples were determined on the first day of storage. The results obtained by applying the hedonic test in terms of color, appearance, taste, odor, texture, consistency in mouth, consistency on spoon and general acceptability are shown in Figure 2.

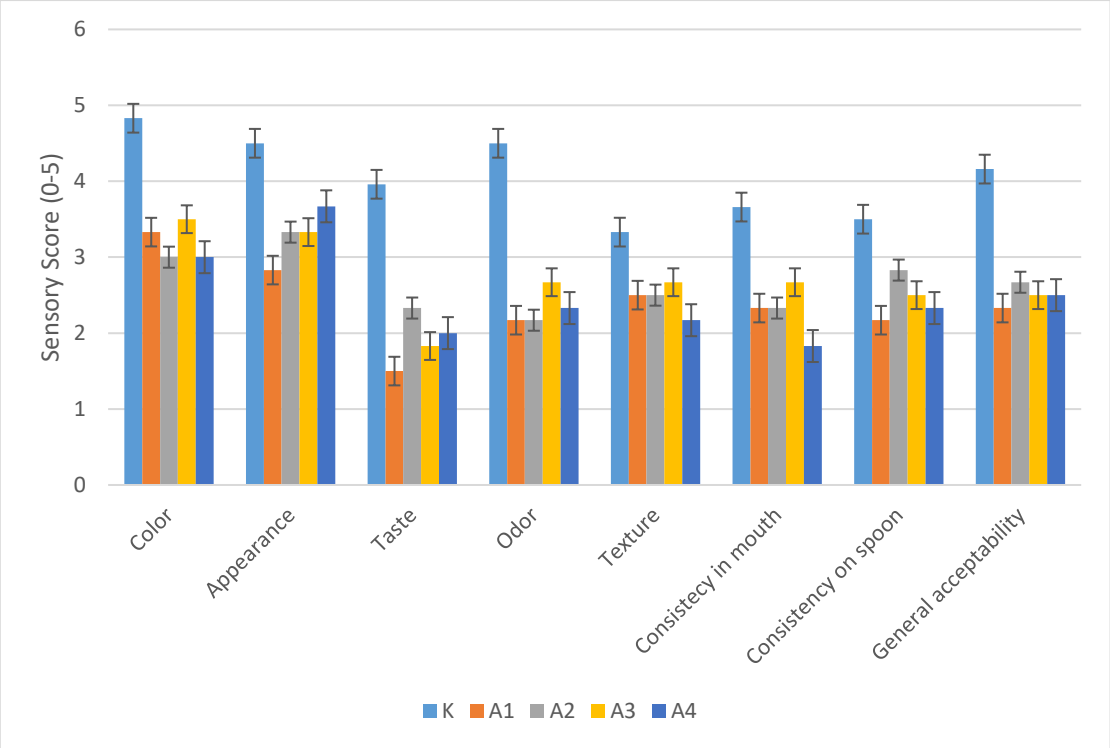


Figure 2- Sensory properties of yoghurt and low protein yoghurt substitute samples on the first day of storage
K (the control group, without stabilizer); A1 (1% xantham gum); A2 (1.5% commercial gelling agent); A3 (4% corn starch), A4 (1.6% pectin).

Control yoghurt became the most admired yoghurt in terms of all properties. It was thought that yoghurts produced using milk substitute are not as appreciated as the control yoghurt, since the sensory analyses of yoghurt substitute samples were performed by people who could consume yoghurt and did not accustomed to consuming milk substitute. In terms of taste, the commercial gelling agent supplemented sample followed the control sample with the highest score. It was followed by the example with pectin added. In terms of odor, texture and consistency in mouth, the starch-added sample was the most popular example of stabilizer-added yoghurt substitutes.

As a result of the sensory analyses, it was concluded that while xanthan gum, commercial gelling agent and starch were used at appropriate rates in the production of yoghurt substitutes, it was concluded that pectin was used at a high rate. The consistency of the pectin added low protein yoghurt sample was found to be harder and more brittle than desired, and it was proposed to reduce the amount used for possible subsequent applications.

As a result of the variance analysis performed, it was determined that the difference between samples was statistically significant at the P≤0.01 level in terms of color, taste and odor, while the difference between samples was statistically significant at the P≤0.05 level in terms of appearance and general acceptability. It was determined that the difference between the samples in terms of other criteria was not significant.

Macit & Bakirci (2017) examined the odor, appearance, consistency, taste and general acceptability properties of the set type yoghurts with stabilizer added. As in this study, the control example in that study was the most liked in terms of all features. In terms of all properties, the scores of the samples with starch added were higher than the scores of the samples with xsantan gum.

Those results are similar to those determined in this study. Xanthan gum particles are larger than casein fractions. When they enter the network of casein micelles, they cause a heterogeneous structure. Therefore, microstructural features are negatively affected. This may negatively affect the sensory properties of yoghurt with xanthan gum added (Macit & Bakirci 2017).

According to the sensory evaluations, in terms of all criteria, the samples with the average score from the highest to the lowest were the control samples, starch, commercial gelling agent, pectin and xanthan gum added low protein yoghurt substitute samples, respectively. Panelists have suggested that sweeteners can be added to low-protein yoghurt substitute samples in order to improve the taste-aroma. Evans et al. (2018) determined the food habits of children with PKU and their parents. In that study, it was found that children liked sweet foods more. Sweetened low-protein yogurt substitutes can be a particularly viable option for children. It was thought that it would be appropriate to use any sweetener that does not contain Phe or lactose as sweetener.

4. Conclusions

PKU patients consume yogurt substitutes prepared under home conditions. Industrial production must be done to obtain a standard product. The protein and phenylalanine amounts of the low-protein yogurt substitute samples produced in this study are at a level that PKU patients can consume. It was concluded that if sweetener is added, yogurt substitutes would be more appreciated in terms of taste. In future studies, the production of yogurt substitutes using probiotic bacteria can be tried. Since there are very few studies on this subject, it is necessary to increase the studies on the subject in order to standard production.

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References

Abdel-Salam A M & Effat L K (2010). Preparation and evaluation of a novel therapeutic dairy-based drink for phenylketonuria. North American Journal of Medical Science 2(2): 66-70. doi:10.4297/najms.2010.266

Akış M (2012). Sağlıklı ve Fenilketonürili Çocuklarda Fonksiyonel B12 Vitamin Eksikliğinin Plazma Metilmalonik Asit ve Homosistein Düzeyleri İle Araştırılması, Yüksek Lisans Tezi, Dokuz Eylül Üniversitesi Sağlık Bilimleri Enstitüsi, İzmir (in Turkish)

Anonymous (2017a). Low Proteinli Diet Principles. PKU Family Association. Retrieved in July, 14, 2017 from https://www.pkuaile.com/dusuk-proteinli-diyet-prensipleri

Anonymous (2017b). Yoghurt recipe for PKU patients. Retrieved in April, 27, 2017 from https://youtu.be/tGzR953W3N8

Anonymous (2020). Yoghurt Recipe. Vitafrends PKU. Retrieved in December, 25, 2020 from https://www.vitafriendspku.com/tr/tarifler/yogurt/

AOAC (1990). Official methods of analysis of the association of official analytical chemists. Helrich K (Ed.) Published by the Association of Official Analytical Chemists Inc. Arlington, p. 980

Bahrami M, Ahmadi D, Alizadeh M & Hosseini F (2013.) Physicochemical and sensorial properties of probiotic yogurt as affected by additions of different types of hydrocolloid. Korean Journal for Food Science 33(3): 363-368. doi:10.5851/kosfa.2013.33.3.363

Barnes H A, Hutton J F & Walters K F R S (1993). An Introduction to Rheology. Elsevıer Science Publichers B.V./ Physical Sciences & Engineering Division, Sara Burgerhartstraat 25, Third impression, AM Amsterdam, The Netherlands.

Bingöl E B & Bostan K (2012). Bir gıda katkı maddesi olarak laktatların et ve et ürünlerinde kullanımı. Journal of Faculty of Veterinary Medicine, Istanbul University 38(1): 79-88

Burgard P, Lachmann R H & Walter J (2016). Hyperphenylalaninaemia. In: Saudubray J M, Baumgartner M R & Walter J (Eds.) Inborn Metabolic Diseases, Springer, Berlin, Heidelberg, pp. 251–263

Cogan TM (1996). History and taxonomy of starter cultures. In: Congan T M & Accolas J (Eds.) Dairy Starter Culture. VCH Publishers Inc., New York, pp. 1-20

Cueva O & Aryana K J (2008). Quality Attributes of a Heart Healthy Yogurt. LWT-Food Science and Technology 41(3): 537-544. doi:10.1016/j.lwt.2007.04.002

Çakmakçı S (2012). Gıda Katkı Maddeleri 7. Baskı. Atatürk Üniversitesi Ziraat Fakültesi Ders Yayınları. Atatürk Üniversitesi Ziraat Fakültesi Ofset Tesisi, No: 164, Erzurum, Turkey (in Turkish)

Dave R I & Shah N P (1996). Evaluation of media for selective enumeration of Streptococcus thermophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus and bifidobacteria. Journal of Dairy Science 79(9): 1529-1536. doi:10.3168/jds.S0022-0302(96)76513-X

Davis L E, King M K & Schuttz J L (2005). Fundamentals of Neurologic Disease Demos Medical Publshing, New York

de Wit J C & Rombouts F M (1990). Antimicrobial effect of sodium lactate. Food Microbiology 7: 113-120. doi:10.1016/0740-0020(90)90017-C

Düzgüneş O, Kesici T, Kavuncu O & Gürbüz F (1987). Araştırma ve Deneme Metodları (İstatistik Metodları II) Atatürk Üniversitesi Ziraat Fakültesi Yayınları No: 1021, Ders Kitabı No: 295, Ankara (in Turkish)

Erdal B & Caferoğlu Z (2018). Fenilketonüride yeni bir tedavi: Pegvaliase. Erciyes Üniversitesi Sağlık Bilimleri Fakültesi Dergisi 5(1): 42-53 (in Turkish)

Evans S, Daly A, Chahal S, Ashmore C, MacDonald J & Macdonald A (2018). The influence of parental food preference and neophobia on children with phenylketonuria (PKU). Molecular Genetics and Metabolism Reports 14(2018): 10-14. doi:10.1016/j.ymgmr.2017.10.007

Goldar P, Givianard M H & Shams A (2016). Effect of ultrafiltered milk permeate and non-dairy creamer powder concentration on low phenylalanine yoghurt's physicochemical properties during storage. Journal Food Science Technology 53(7): 3053–3059. doi:10.1007/s13197-016-2278-9

IDF (1986). Determination of nitrogen content (Kjeldahl method) and calculation of crude protein content Standard 20A, International Dairy Federation. Brussels, Belgium.

Kavas G & Kınık Ö (2005). İnek sütü ve peynir suyu proteinlerindeki esansiyel amino asitlerin beyin fonksiyonları, psikiyatrik hastalıklar ve süte uygulanan teknolojik parametrelerle ilişkileri. Gıda 30(3): 173-179 (in Turkish)

Kosikowski F V (1982). Cheese and Fermented Milk Foods. 2ed published by F.V. Kosikowski and Associates, New York, USA

Lee A & Newman J M (2003). Celiac diet: Its impact on quality of life. Journal of the American Dietetic Association 103(11): 1533-1535. doi:10.1016/j.jada.2003.08.027

Lucey J A (2002). Formation and physical properties of milk protein gels. Journal of Dairy Science 85: 281-294. doi:10.3168/jds.S0022-0302(02)74078-2

Macit E & Bakirci İ (2017). Effect of different stabilizers on quality characteristics of the set-type yogurt. African Journal of Biotechnology 16(46): 2142-2151. doi:10.5897/AJB2017.16197

Meilgaard M C, Civille G V & Carr B T (2006). Sensory Evaluation Techniques, 4rd edn. CRC Press LLC Publishing, Boca Raton, London.

Mortazavian A M & Sohrabvandi S (2006) Probiotics and food probiotic products: based on dairy probiotic products, Eta Press, Tehran, pp. 31-69 (in Persian)

Müslümanoğlu M H, Çine N, Özdemir M, Çilingir O, Başaran N, Durak B, Solak M & Artan S (2014). Fenilketonüri hastalığında prenatal-postnatal tanıda VNTR bağlantısı ve direkt mutasyon analizleri birlikteliğinin avantajları. The Medical Journal of Kocatepe 5(2): 19-23 (in Turkish)

Nunes M C, Raymundo A & Sousa I (2006). Gelled vegetable desserts containing pea protein, kapa carrageenan and starch. European Food Research and Technology 222: 622–628. doi:10.1007/s00217-005-0170-3

Özer E A, İbanoğlu Ş & İbanoğlu E (2008). Fenilketonüri hastalığı ve fenilalanin kısıtlı diyet. In:Türkiye 10. Gıda Kongresi, 21-23 Mayıs, Erzurum pp. 1139-1140 (in Turkish)

Parlak Ö (2018). Fenilketonüri hastaları (PKU) ve çölyak hastaları için bisküvi üretimi. Yüksek Lisans Tezi, Bursa Teknik Üniversitesi, Fen Bilimleri Enstitüsü, Bursa (in Turkish)

Pathomrungsiyounggul P, Grandison A S & Lewis M J (2010). Effect of calcium carbonate, calcium citrate, tricalcium phosphate, calcium gluconate and calcium lactate on some physicochemical properties of soymilk. International Journal of Food Science & Technology 45(11): 2234-2240. doi:10.1111/j.1365-2621.2010.02399.x

Pimentel F B, Alves R C, Costa A S G, Fernandes T J R, Torres D, Almeida M F & Oliveira M B P P (2014). Nutritional composition of low protein and phenylalanine-restricted dishes prepared for phenylketonuria patients. LWT Food Science Technology 57: 283-289. doi:10.1016/j.lwt.2013.12.031

Scortegagna M L, de Oliveria V R, Lobato C M D & Doneda D (2021). Evaluation and acceptability of alternative food recipes for patients with phenylketonuria. Journal of Food and Nutrition Research 60(2):131-137

Scriver C R & Kaufman S (2001). Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver C R, Beaudet A L, Sly S W & Valle D (Eds.) The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill, 1667-724

Seçkin Y (2007). Fenilketonürili çocukların psiko-pedegojik sorunları ve çözümleri. In: IX. Uluslararası Katılımlı Beslenme ve Metabolizma Kongresi, 22-25 Ekim, İstanbul, pp. 39-43

TCSB (2006). Neonatal Tarama Programı Genelgesi 2006/130, Türkiye Cumhuriyeti Sağlık Bakanlığı, Ankara, Turkey. Retrieved in Fabruary, 25, 2021 from https://www.saglik.gov.tr/TR,11079/neonatal-tarama-programi-genelgesi-2006--130.html

TGK (2003). Fermente sütler tebliği S: 24512, Türk Gıda Kodeksi. Tebliğ No: 2001/21 Ankara, Turkey.

Thöny B & Blau N (2006). Mutations in the BH4–metabolizing genes GTP cyclohydrolase I, 6-pyruvoyl-tetrahydropterin synthase, sepiapterin reductase, carbinolamine-4a-dehydratase, and dihydropteridine reductase. Human Mutation 27: 870-878. doi:10.1002/humu.20366

Üstüner Top F & Küçük Alemdar D (2015). Fenilketonürili çocuğu olan ailelerinin yaşadıkları güçlükler: Niteliksel bir çalışma. Hemşirelikte Eğitim ve Araştırma Dergisi 12(1): 62-68. doi:10.5222/HEAD.2015.062 (in Turkish)

Van Wegber A M J, MacDonald A, Ahring K. Bélanger-Quintana A, Blau N, Bosch A M, Burlina A, Campistol J, Feillet F, Gizewska M, Huijbregts S C, Kearney S, Leuzzi V, Maillot F, Muntau A C, van Rijn M, Trefz F, Walter J H & van Spronsen F J (2017). The complete European guidelines on phenylketonuria: Diagnosis and treatment. Orphanet Journal of Rare Diseases 12: 162. doi:10.1186/s13023-017-0685-2

Verbeken D, Bael K, Thas O & Dewettinck K (2006). Interactions between κ-carregeenan, milk proteins and modified starch in sterilized dairy desserts. International Dairy Journal 16(2006): 482-488. doi:10.1016/j.idairyj.2005.06.006

Waisbren S E, Noel K, Fahrbach K, Cella C, Frame D, Dorenbaum A & Levy H (2007). Phenylalanine blood levels and clinical outcomes in phenylketonuria: a systematic literature review and meta-analysis. Moleculer Genetics Metabolism 92(1): 63-70. doi:10.1016/j.ymgme.2007.05.006

Walter J H, Lee P J & Burgard P (2006). Hyperphenylalaninaemia. In J. Fernandes, J. M. Saudubray, G. van den Berghe, and J. H. Walter (Eds.), Inborn Metabolic Diseases; Diagnosis and Treatment (4th ed., pp. 222-232). Heidelberg, Germany: Springer

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Compositional Changes of the Jujube Fruit During Solar and Tray Drying

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ABSTRACT
This study aims to determine the changes in some components of jujube fruit after drying in the solar and tray dryer, and to choose the appropriate drying method in terms of component loss. In this study, jujube fruit *(Zizyphus jujuba Mill.)* was used as material and were obtained from producers in the Çivril-Denizli regions of Turkey. Firstly, we analysed the total soluble solids, dry matter, titratable acidity, pH, total phenolic content, organic acids (malic, citric, succinic and tartaric acid), sugars (glucose, fructose and sucrose) and water-soluble vitamins (ascorbic acid, riboflavin, niacin, pyridoxine and thiamine) in fresh jujube fruit. Secondly, the fresh jujube fruits were dried in the solar and tray dryer (50, 60 and 70 °C). Following the drying processes, changes of the above-mentioned parameters were evaluated. The total phenolic content was determined using the spectrophotometric method. Sugars, organic acids and water-soluble vitamins content were determined using a high-performance liquid chromatography (HPLC) instrument. The solar drying of the jujube fruit resulted in glucose and sucrose content decrement and in fructose content increment, while tray drying resulted in decrement in glucose, fructose and sucrose content. A high decrement in organic acids and water-soluble vitamins content of the jujube fruit both in the solar and tray drying process was determined. In addition, solar drying resulted in more vitamin content decrement compared to the tray drying.

Keywords**:** HPLC, *Zizyphus jujuba Mill.*, Organic acids, Sugars, Water-soluble vitamin

1. Introduction

Jujube (*Zizyphus jujuba Mill.*), a Chinese-origin plant with about 400 different culture varieties, is known to have been grown in China for around 4000 years. Jujube also grows in Russia, India, North Africa, Southern Europe, the Middle East, and Anatolia, which is a natural spreading area. Although it is not native to Turkey, it can be grown in the Marmara, and West and South Anatolia regions (Genç 2005; Yücel 2005). Jujube is known in different cultures with various names such as jujube, hong zao, chinese date, tara, liane crocs chien, nan tsao, azufaifo, pomme malcadi, petite pomme, ta tsao, annap, ünnap (Hernandez et al. 2016).

Jujube is a fruit with high nutritional value for the fruit is important in many places for human nutrition because of its rich source of ascorbic acid, total phenolic compounds, antioxidant capacity, carotenoids, and minerals. Jujube fruits contain essential trace elements such as magnesium, zinc, copper, iron, phosphorus, and substances such as niacin and riboflavin necessary for the body's enzyme and hormone system. In addition, they contain mucilage and pectin (Promyou et al. 2012; Gao et al. 2013).

Jujube fruits, which are rich in water-soluble vitamins, minerals, and many more organic and inorganic substances, can be used in the treatment of many diseases such as liver and heart, vascular disorders, and cholesterol disorders in the blood. It is used among people as a breast softener, sputum and diuretic, constipating, aphrodisiac, and a good toxin suppressant cough (Omid 1997).

Jujube is used as an additive in the production of many medicinal drugs and has an anti-inflammatory, pain-relieving effect (Abd-Alrahman et al. 2013). Several studies report that the fruit can be effective in the treatment of diseases such as diabetes (Miri 2018; Shahrajabian et al. 2019; Proestos 2020), jaundice (Rahman et al. 2018) diarrhea, wounds, and ulcers (Kaur et al. 2019; Soni & Malik 2021).

Drying is the oldest preservation method and has been used since ancient times. Through the drying process, the shelf life extends, water activity reduces, microbiological safety increases and transportation costs are reduced. The choice of drying method is crucial for the preservation of quality parameters. Solar drying and hot air drying are traditional drying methods (Elmas et al. 2019). Drying with hot air provides some advantages compared to solar drying, such as being free from climatic effects

and hygienic conditions. However, hot air drying also brings disadvantages such a longer drying time, loss of nutritional and bioactive value, and changes in sensory properties (Wang et al. 2016).

Although fresh jujube fruit contains a high amount of vitamin C, this fruit is typically consumed as nuts. Therefore, it is important to know the changes that occur in its composition during the drying process. To the best of our knowledge, there is no published research study about the water-soluble vitamin determination of jujube fruits during solar and tray drying. The objectives of this study are (a) to determine some physical and chemical properties of jujube fruits during solar and tray drying, and (b) to determine the changes of organic acids, sugars, and water-soluble vitamins during solar and tray (50, 60, and 70 °C) drying.

2. Material and Methods

2.1. Material

Approximately 200 kg of Jujube fruit was obtained from jujube producers in the Çivril district of Denizli and shipped to the laboratory in crates. The samples were harvested in 2013. Jujube fruits collected under controlled conditions from the garden in the Çivril region were used for solar and tray drying. Mature stage jujube fruits were collected from 10 different predetermined trees. The collection process was carried out homogeneously from all sides of the tree (north, south, east, and west). Then, the jujube fruits were used in both the initial analysis and drying process following the selection, sorting, and washing processes.

2.2. Drying procedure

Two different methods, namely solar and tray drying, were used to dry the fruits. In the solar drying process, the fruits were laid on wooden exhibitions and dried in the shade. The drying time of jujube fruit for solar and tray drying is 7-11 days and 60-72 hours, respectively.

The working temperature range, relative humidity range, and air-speed range of the oven used for the drying were 40 °C - 120 °C, 20-95%, and 0-2 m/s, respectively. The drying conditions were carried out according to the method suggested by Fang (2009). Tray drying experiments were carried out in a hot air dryer (Yücebaş Makine Ltd. İzmir, Turkey) at temperatures of 50, 60, and 70 °C. The hot air dryer consists of a centrifugal fan, electric heater, and electronic proportional controller to ensure airflow. A constant airflow of 0.2 m/s and a constant 20% relative humidity were used during the drying process. The weight of the jujube fruits were regularly recorded during hot air drying. The drying process was applied until the sample weight reached a moisture content of 15 g/100 g. Three replicates for each of the experiments were performed.

Care was taken to ensure that the jujube fruits, which will be dried, had the same degree of maturity. The fruits were stored in a refrigerator at 4 °C until they were to be dried in the solar and tray dryer. In the hot air-drying processes, approximately 4500 grams of jujube samples were used for each temperature and 4000 grams in the solar drying processes. Samples were dried to an approximately 20% moisture content. The moisture content of the final product was determined by the rapid moisture determination method in order not to be affected by ambient humidity.

2.3. Extraction for organic acids and sugars

Organic acid and sugar analyses were carried out according to the method suggested by Soyer et al. (2003) and Sturm et al. (2003). Fresh and dried jujube fruits were weighed 20 g and ultrapure water was added at a ratio of 1/1 (w/w). Then, it was homogenized with a blender (Waring-USA). After 10 g of the obtained mixture was taken, 50 mL of ultrapure water was added and centrifuged at 13440 G-force for 15 minutes (core NF800R-Turkey). Following the centrifugation, 5mL of the supernatant was filtered in amber vials through a 0.45µm PTFE (Sartorius, SM16555Q, Germany) syringe-type filter. The obtained extracts were stored at -20 °C until analysis. The extracts were carried out in triplicate.

2.4. Determination of organic acids

In order to determine the organic acid content of the jujube fruit, calibration solutions were prepared at five different concentrations with tartaric, malic, citric, and succinic acid standards. The prepared solutions were injected into the HPLC device, a linear regression analysis was applied to the obtained data and the equation definition curve was created. The reliability of the method was confirmed by the recovery tests. The recoveries for malic, citric, succinic, and tartaric acid were determined as 99.5%, 98.32%, 98.96%, and 98.18%, respectively. The detection limits for each organic acid, based on a signal-to-noise ratio (S/N) of 3, were 0.035 g/L for malic acid, 0.040 g/L for citric acid, 0.040 g/L for succinic acid, and 0.050 g/L for tartaric acid. The HPLC conditions of organic acid analyses are given in Table 1. The linearity of the standard curve, limit of detection, recovery, coefficient of variation, and relative error in the determination of organic acids are given in Table 2.

Table 1- HPLC conditions for the analysis of sugars, organic acids, and water-soluble vitamins

Apparatus/Condition and Pump	*Sugar*	*Organic acid*	*Water-Soluble vitamin*
Liquid Chromatography	Shimadzu, LC20AD/Japan	Shimadzu, LC20AD /Japan	Shimadzu, LC20AD /Japan
Column	Bio Rad Aminex HPX-87 ion exclusion column (300x7.8 mm)	Bio Rad Aminex HPX-87 ion exclusion column (300x7.8 mm)	Nucleosil, C-18 (250 x 4.6 mm, ID) Macherey-Nagel
Degasser	Shimadzu, DGU-20A3	Shimadzu, DGU-20A3	Shimadzu, DGU-20A3
System controller	Shimadzu, CBM, 20Alite	Shimadzu, CBM, 20Alite	Shimadzu, CBM, 20Alite
Detector	Shimadzu, RID-10A Detector	Shimadzu, Photo Diode Array (PDA) Detector, SPD-M20A, 214 nm	Shimadzu, Photo Diode Array (PDA) Detector, SPD-M20A, 214 nm
Column oven-temperature	Shimadzu, CTO-20A, 85 °C	Shimadzu, CTO-20A, 25 °C	Shimadzu, CTO-20A, 25°C
Flow rate	1 mL/min	1 mL/min	0.6 mL/min
Mobile Phase	Isocratic, Acetonitrile: Ultra pure water (80:20 v/v)	Isocratic, 0.01 N H_2SO_4	Isocratic: KH_2PO_4-Asetonitril (99:1, v/v)
Injection volume	20 µL	20 µL	20 µL
Signal to Noise Ratio	3	3	3

2.5. Determination of sugars

The external standard method was used to determine the sugar concentration in the jujube fruits. To draw the standard calibration curves, solutions of 5 different concentrations were prepared from glucose, fructose, and sucrose standards. The prepared standard solutions were injected into the HPLC device and the equation defining curve was calculated by applying linear regression analysis. Recovery experiments have used the confirmation the reliability of the method. Recoveries for glucose, fructose, and sucrose were determined as 98.48%, 99.96%, and 98.18%, respectively. The detection limits for each sugar based on a signal-to-noise ratio (S/N) of 3, were 0.2 g/L for glucose, 0.3 g/L fructose, and 0.15 g/L sucrose. The HPLC conditions of the sugar analysis are given in Table 1. The linearity of the standard curve, the limit of detection, the recovery, the coefficient of variation, and the relative error in determining the sugars are given in Table 2.

Table 2- Linearity of the standard curve, detection limit, recovery, coefficient of variation, and relative error of determination of organic acid, sugars, and water-soluble vitamin in jujube fruits

Parameter	*Linear range[xy]*	*R*	*r^2*	*Detection limit [xy]*	*Recovery (%) Mean SD[b]*	*Relative error (%)*	*CV (%)*
Malic acid	0.0-100.0	0.9993	99.40	0.035	99.5±1.88	0.50	1.89
Citric acid	0.0-100.0	0.9935	99.54	0.040	98.32±1.68	1.68	1.71
Succinic acid	0.0-50.0	0.9987	99.80	0.040	98.96±0.11	0.11	0.11
Tartaric acid	0.0-100.0	0.9987	99.86	0.050	98.18±1.80	1.80	1.83
Ascorbic acid	0.0-200.0	0.9992	99.90	0.1	96.68±1.61	3.32	1.67
Riboflavin	0.0-40.0	0.9998	99.94	0.2	96.36±0.91	5.60	0.94
Niacin	0.0-200.0	0.9996	99.93	0.1	96.24±1.93	3.79	2.00
Pyridoxine	0.0-30.0	0.9990	99.82	0.2	95.94±0.67	3.96	1.04
Thiamine	0.0-50.0	0.9985	99.69	0.5	97.44±1.19	2.40	1.22
Glucose	0.0-250.0	0.9982	99.69	0.2	98.48±3.04	0.11	3.09
Fructose	0.0-250.0	0.9992	99.67	0.3	99.96±3.22	0.40	3.22
Sucrose	0.0-25.0	0.9989	99.83	0.15	98.18±1.87	1.80	1.90

[x]: Malic, citric, succinic, tartaric, glucose, fructose, and sucrose concentration (g L^{-1}); [y]: Ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine concentration (mg L^{-1}); [b]: Mean ± standard deviation; [CV]: Coefficient of variation

2.6. Extraction of water-soluble vitamins

Seven different concentrations of ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine standards, were obtained from Sigma-Aldrich Chemie GmbH (Deisenhafen, Germany) and prepared in pure water. A water-soluble vitamin analysis was performed according to the method of Kadakal et al. (2004). Ultra-pure water was added to fresh, solar, and tray-dried jujube fruits in a ratio of 1:9 and homogenized by shredding with a laboratory blender (Waring-USA) for 4 min. Following filtering through the coarse filter, the filtrate was centrifuged (core NF800R- Turkey) for 15 min at 13440 G-force. A 5 mL of supernatant was passed through a 0.45 µm PTFE (FP 30/45 CA-S, Schleicher & Schuell, Darmstadt, Germany) syringe-type filter and transferred to 5 mL vials. The extracts were stored at -20 °C until analysis. The extracts were carried out in triplicate and the HPLC conditions of the water-soluble vitamins analysis are given in Table 1. The linearity of the standard curve, the limit of detection, the recovery, the coefficient of variation, and the relative error in determining the water-soluble vitamins are given in Table 2.

2.7. Recovery of organic acids, sugars, and water-soluble vitamins

The standard addition model was used to determine the recovery rates of organic acid, sugar, and vitamin analyzes. For this purpose, standard solutions were added to the jujube samples in which the organic acid, sugar, and vitamin content were known. Then, the prepared solutions were injected into the HPLC device under the same conditions. Linear regression analysis was applied to the obtained data and the equation definition curve was created. The reliability of the method was confirmed by the recovery tests.

2.8. Total phenolic substance analysis

The Folin-Ciocalteu spectrophotometric method was used to determine the total amount of phenolic substances (Singleton & Rossi 1965). 5 g of dried jujube samples were weighed and a 1:9 (w/v) methanol solution was added. After disintegration in the homogenizer, it was centrifuged at 10 °C at 9000 rpm. Following centrifugation, the samples were filtered through coarse filter paper. 300 µL of the samples prepared by methanol extraction was taken and 1500 µL of Folin-Ciocalteu solution (1:10, Folin-Ciocalteu reagent: ultrapure water) was added and waited for 5 minutes. Then, 1200 µL of 7.5% sodium bicarbonate solution was added and incubated for 2 hours in the dark at room temperature. After incubation, the absorbance values of the samples were determined in a spectrophotometer device (PG Instruments T80 UV/VIS, UK) at a wavelength of 760 nm. In order to calculate the results, the gallic acid standard obtained from the Sigma-Aldrich company was used. Solutions of different concentrations were obtained from the gallic acid standard and the gallic acid standard calibration curve was drawn.

2.8. Physicochemical analysis

The analysis of water-soluble solid (°Bx), total solid (%), pH, and titratable acidity (dry tartaric acid) were realized according to the method of the Association of Official Analytical Chemists (1990).

2.9. Statistical analysis

The experiments were carried out in triplicate and repeated twice. The obtained data were analyzed with variance for the comparison of all data. Following the revealed significant effect ($P<0.05$) of variance, the least significant difference test was used (Statistical Analysis Software 1985).

3. Results and Discussion

The water-soluble dry matter and total dry matter values increased both in solar and tray drying. Similarly, an increase in pH values of jujube fruit dried in solar and tray dryer was observed. In contrast, there was a decrease in titratable acidity of jujube fruits by solar and tray drying process. However, the pH increase in tray drying at lower temperatures was higher than those drying at high temperatures. The increase in pH and decrease in titratable acidity are parallel in the tray drying and the solar drying processes depending on the drying temperatures. Changes in dry matter, °Bx, pH, titratable acidity, and total phenolic content of the jujube fruit through solar and tray drying are given in Table 3. A high amount of total phenolic substance (1968.5 mg GAE / 100 g) was detected in fresh jujube fruit. However, as seen in Table 3, there is a considerable loss of total phenolic substance both in the tray drying at 50, 60, and 70 °C and in the solar drying process. The loss in the solar drying process is far higher than the tray drying. This may be due to the fact that the drying time of jujube fruits dried in the sun is longer. There is no similar study in the literature showing the change in pH, titratable acidity, °Bx, and total phenolic substance depending on drying, but Guclu et al. (2021) studied the phenolic characteristics of fresh and powdered sweet red peppers. In their study, organic and conventional peppers were dried by hot air, intermittent microwave and infrared drying methods. While phenolic compounds were not found in red peppers dried by intermittent microwave method, they were detected in samples dried with hot air and infrared. Their study revealed that the drying method was effective on phenolic compounds. Studies are generally concerned with the content of fresh ripe fruit or its change due to ripening. The pH, titratable acidity, and °Bx value of fresh ripe jujube fruit were given as 6.4, 1.7 (citric acid L^{-1}), and 18.2, respectively (Hernandez et al. 2015). The total phenolic content of fresh jujube fruit was reported as 600.5 GAE/100 g (Gao et al. 2011).

Table 3- Changes in dry matter, °Bx, pH, titratable acidity, and total phenolic content of jujube fruit with solar and tray drying

Drying Method		*Dry matter (%)*	*°Bx*	*pH*	*Titratable acidity (%)*	*Total phenolic (mg GAE/100g)*
Control		19.4 ± 0.2c[y]	9.1 ± 0.1c	2.50 ± 0.06b	3.16 ± 0.07a	1968.5 ± 12.4a
Solar drying		76.4 ± 0.1b	65.1 ± 0.1b	2.92 ± 0.05ab	1.63 ± 0.04b	718.2 ± 8.7d
Tray drying (°C)	50	79.8 ± 0.3a	69.8 ± 0.2a	3.07 ± 0.08a	1.12 ± 0.05c	1482.6 ± 18.5b
	60	80.2 ± 0.2a	70.0 ± 0.2a	2.94 ± 0.06ab	1.26 ± 0.09c	1410.1 ± 25.1b
	70	80.6 ± 0.2a	70.2 ± 0.3a	2.88 ± 0.08ab	1.54 ± 0.06b	1233.7 ± 14.8c

*: Results are given on dry basis; y: Different letters on the same column are statistically different ($P<0.05$)

Changes in sugar values of jujube fruit with solar and tray drying are given in Table 4. A decrease in glucose and sucrose content and an increase in fructose content in dried jujube fruits were determined. The glucose, fructose, and sucrose content of the jujube fruit dried in the tray dryer at 50, 60, and 70 °C were decreased in all three temperatures. The decrease in glucose and fructose content drying at 50 °C is higher than drying at 70 °C. The loss of glucose, fructose, and sucrose decreases as the drying temperature increases. Results are given on g/100 mg DW. However, the sucrose content of jujube fruit, which has an initial sucrose content of 29.8 mg/100 g, decreased to 3.2 mg/100 g with drying at 50 °C in the tray dryer. The sucrose content of jujube fruits dried at 60 and 70 °C was found below the detectable limit. In particular, the decrease in sucrose content can be explained by the inversion of sucrose with the effect of temperature. Non-reducing carbohydrates such as sucrose are broken down into glucose and fructose under the influence of temperature, pH, and water in the environment. For this reason, a large decrease in sucrose content is observed (Artık et al. 2011). Gao et al. (2012) found that the glucose amount of jujube fruit dried with different drying techniques decreased from 274.5 mg/100 g dry weight (DW) to 229.5 mg/100 g DW. Elmas et al. (2019) reported that the amount of fructose and sucrose in jujube fruit dried at 60, 70 and 80 °C decreased due to increasing drying temperature and air velocity. Tepe & Ekinci (2021) similarly found that there was a decrease in the content of sucrose, fructose, and glucose with the hot air drying of jujube fruit. They reported that the highest loss of glucose and fructose was determined at 50 °C, and the highest loss of sucrose was detected in samples dried at 70 °C.

Table 4- Changes in sugar, organic acid, and water-soluble vitamin values of jujube fruit with solar and tray drying

Parameters		*Control*	*Solar drying*	*Tray drying (°C)*		
				50	*60*	*70*
Sugars (mg/100 g)	Glucose	1426.7±3.4a[y]	1370.6±6.0b	1211.4±2.8d	1271.8±4.0cd	1304.8±4.3bc
	Fructose	242.6±4.3ab	256.9±4.0a	201.8±4.9c	209.3±5.2bc	222.9±4.3b
	Sucrose	29.8±1.4a	Nd	3.2±0.3b	Nd	Nd
Organic acids (mg/100g)	Malic acid	186.5±2.4a	118.3±3.9c	171.3±2.8b	169.1±3.5b	164.4±3.0b
	Citric acid	178.7±4.6a	121.3±2.6c	170.5±4.1ab	163.8±5.0b	160.2±3.8b
	Succinic acid	17.5±0.5a	10.7±0.3c	15.0±0.5ab	14.2±0.3b	13.8±0.4b
	Tartaric acid	40.8±0.5a	34.2±0.6b	40.6±0.7a	39.5±0.9a	39.0±0.5a
Water-soluble vitamins (mg/100g)	Ascorbic acid	71.2±0.5a	9.6±0.1d	28.7±0.5b	19.3±0.4c	12.1±0.2d
	Riboflavin	0.036±0.002a	0.025±0.01b	0.024±0.001b	0.024±0.001b	0.022±0.001b
	Niacin	0.82±0.04a	0.33±0.02d	0.70±0.04b	0.59±0.05bc	0.52±0.02c
	Pyridoxine	0.076±0.002a	Nd	Nd	Nd	Nd
	Thiamin	0.018±0.002a	0.013±0.001c	0.018±0.002a	0.018±0.001a	0.015±0.001b

[y]: Different letters on the same line are statistically different (P<0.05); *: Results are given on dry basis; Nd: Not detectable limit

Changes in the organic acid content of jujube fruit with solar and tray drying are given in Table 4. There was a decrease in the amount of tartaric, malic, citric, and succinic acid in jujube fruits that were dried both in the solar and tray drying. The decreases in malic, citric, succinic, and tartaric acid content at 70 °C, which is the highest drying temperature, are more than 50 °C. That is to say that, as the drying temperature increases, the loss of malic, citric, succinic, and tartaric acid is increased. The reason for the decrease in organic acids can be explained by temperature and oxidation reactions (Levent 2017).

Changes in the water-soluble vitamin content of jujube fruit with solar and tray drying are given in Table 4. Solar drying caused a decrease in the ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine content of jujube fruits. Solar drying caused a decrease in all analyzed vitamins. Moreover, solar drying caused a 100% decrease in pyridoxine content. As a result of drying jujube fruits at 50, 60, and 70 °C in the tray drying, there was a decrease in ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine content in all three temperatures. The decreases in ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine content at 70 °C, which is the highest drying temperature, are more than 50 °C. That is to say that, loss of ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine are related to the increase in drying temperature. It was reported that the loss of vitamin C in jujube fruit dried with different drying techniques increased (Fang et al. 2009; Wojdylo et al. 2016). However, the least loss was reported in jujube fruits dried at 70 °C (Fang et al. 2009). Ascorbic acid is accepted as one of the main indicators in determining quality loss due to its thermal sensitivity. In general, the fact that the loss of ascorbic acid is low after the applied process shows that the loss of other nutritional elements is also low. Tepe & Ekinci (2021) reported that drying temperature has a great effect on B complex vitamins (especially on pyridoxine) and they found that the highest losses were observed in thiamine, riboflavin, and niacin contents at 70 °C, while the lowest loss at 50 °C.

4. Conclusions

Solar drying of jujube fruit caused a decrease in glucose and sucrose content of fruits and an increase in fructose content while drying on a tray caused a decrease in glucose, fructose, and sucrose content. Solar drying caused significant losses in tartaric,

malic, citric, and succinic acids compared to tray drying (50, 60, and 70 °C). A high decrement in ascorbic acid, riboflavin, niacin, pyridoxine, and thiamine content of jujube fruit both in the solar and tray drying process was determined. With this in mind, solar drying of jujube fruit can be considered an alternative method in terms of efficient use of energy and energy saving. However, it is recommended to dry the jujube fruit in a tray dryer in terms of nutrient content, product quality, and component loss. In the literature, there are a limited number of studies on the drying of jujube fruit. We believe that our article may be useful to close the gap in the literature. Apart from this, it is industrially important to determine the best drying method in terms of nutritional value and product quality as a result of drying the jujube fruit, which is mostly consumed dry, in the solar and on a tray. In future studies, it is recommended to determine the physical and chemical properties of jujube fruits grown in different regions and to dry them using innovative drying methods.

References

Abd-Alrahman S H, Salem-Bekhit M M & Elhalwagy M E (2013). Chemical composition and antimicrobial activity of Ziziphus jujuba seeds extract. *Journal of Pure and Applied Microbiology* 7: 379-385

Artık N, Bayındırlı L & Mert İ (2011) Carbohydrates, Corn Sugar and Food Industry Use, *Türkiye Gıda ve İçecek Sanayii Dernekleri Federasyonu*, Ankara, 104 pp. (In Turkish)

Association of Official Analytical Chemists—AOAC (1990). Official methods of analysis. *Association of official analytical chemists*, IAC, Arlington

Elmas F, Varhan E & Koç M (2019). Drying characteristics of jujube (*Zizyphus jujuba*) slices in a hot air dryer and physicochemical properties of jujube powder. *Journal of Food Measurement and Characterization* 13(1): 70-86. https://doi.org/10.1007/s11694-018-9920-3

Fang S, Wang Z & Hu X (2009). Hot air drying of whole fruit Chinese jujube (Zizyphus jujuba Miller): Thin-layer mathematical modelling. *International Journal of - Science and Research* 44(9): 1818-1824. https://doi.org/10.1111/j.1365-2621.2009.02005.x

Fang S, Wang Z, Hu X & Datta A K (2009). Hot-air drying of whole fruit chinese jujube (Zizyphus jujuba Miller): Physicochemical properties of dried products. *International journal of food science & technology* 44(7): 1415-1421. https://doi.org/10.1111/j.1365-2621.2009.01972.x

Genç M (2005). Ornamental Plant Cultivation. Basic Crafting Techniques. *Süleyman Demirel University Publication*, 1. Vol, Isparta, pp. 369 (In Turkish)

Gao Q H, Wu P T, Liu J R, Wu C S, Parry J W & Wang M (2011). Physico-chemical properties and antioxidant capacity of different jujube (Ziziphus jujuba Mill.) cultivars grown in loess plateau of China. *Scientia Horticulturae* 130(1): 67-72. https://doi.org/10.1016/j.scienta.2011.06.005

Gao Q H, Wu C S, Wang M, Xu B N & Du LJ (2012). Effect of drying of jujubes (*Ziziphus jujuba Mill*.) on the contents of sugars, organic acids, α-tocopherol, β-carotene, and phenolic compounds. *Journal of Agricultural and Food Chemistry* 60(38): 9642-9648. https://doi.org/10.1021/jf3026524

Gao Q H, Wu C S & Wang M (2013). The jujube (*Ziziphus jujuba* Mill.) fruit: A review of current knowledge of fruit composition and health benefits. *Journal of Agricultural and Food Chemistry* 61(14): 3351-3363. https://doi.org/10.4324/9780429160509-6

Guclu G, Keser D, Kelebek H, Keskin M, Sekerli Y E, Soysal Y & Selli S (2021). Impact of production and drying methods on the volatile and phenolic characteristics of fresh and powdered sweet red peppers. *Food Chemistry* 338, 128129. https://doi.org/10.1016/j.foodchem.2020.128129

Hernández F, Noguera-Artiaga L, Burló F, Wojdyło A, Carbonell-Barrachina Á A & Legua P (2016). Physico-chemical, nutritional, and volatile composition and sensory profile of Spanish jujube (*Ziziphus jujuba Mill*.) fruits. *Journal of the Science of Food and Agriculture* 96(8): 2682-2691. https://doi.org/10.1002/jsfa.7386

Kadakal Ç, Poyrazoğlu E S, Artık N & Nas S (2004). Effect of Activated Charcoal on Water-Soluble Vitamin Content of Apple Juice. *Journal of Food Quality* 27: 171–180. https://doi.org/10.1111/j.1745-4557.2004.tb00647.x

Kaur S, Kumar M, Pandit K, Kumar A & Kaur S (2019). Potential health benefits of nutraceuticals for human health. *Environmental Contaminants and Natural Products*; Bentham Science Publishers: Sharjah, United Arab Emirates pp. 193-208. https://doi.org/10.2174/9789811410963119010012

Levent O (2017). The effect of process conditions on the physical, chemical, bodied, organic and rheological properties of apricot sauces obtained from Malatya apricots. PhD Thesis, *İnönü University*, (Published), Ankara (In Turkish)

Miri M S (2018). Cultivation, chemical compositions and health benefits of jujube (*Ziziphus jujuba Mill*.). In Ist *National Congress and International Fair of Medicinal Plants and Strategies for Persian Medicine that Affect Diabetes*, 9-11 Oct, Mashhad, Iran.

Omid B (1997). Approach the production and processing plants. Tehran, *Tarahan Publisher*, Iran, pp. 109-110

Proestos C (2020). The benefits of plant extracts for human health. *Foods* 9(11): 1653. https://doi.org/10.3390/foods9111653

Promyou S, Supapvanich S, Boodkord B & Thangapiradeekajorn M (2012). Alleviation of Chilling Injury in Jujube Fruit by Dipping in 350 °C Water. *Kasetsart Journal - Natural Science* 46: 107-119

Rahman E, Momin A, Zhao L, Guo X, Xu D, Zhou F & Ji B (2018). Bioactive, nutritional composition, heavy metal and pesticide residue of four Chinese jujube cultivars. *Food Science Biotechnology* 27(2): 323-331. https://doi.org/10.1007/s10068-017-0256-2

SAS® (1985). Institute. SAS user's guide. Statistics. Version 5th Ed. Cary, NC, SAS Institute Inc

Shahrajabian M H, Khoshkharam M, Zandi P, Solar W & Cheng Q (2019). Jujube, a super-fruit in traditional Chinese medicine, heading for modern pharmacological science. *Journal of Medicinal Plants Studies* 7(4):173-178

Singleton V L & Rossi J R (1965). Colorimetry of total phenolics with phosphomolibdic - phosphothungstic acid. *American Journal of Enology and Viticulture* 16: 144-158

Soni H & Malik J K (2021). Phyto-Pharmacological Potential of Zizyphus jujube: A Review. *Scholars International Journal of Biochemistry* 4(2):1-5. https://doi.org/10.36348/sijb.2021.v04i01.001

Soyer Y, Koca N & Karadeniz F (2003). Organic Acid Profile of Turkish White Grapes and Grape Juices. *Journal of Food Composition and Analysis* 16: 629-636. https://doi.org/10.1016/S0889-1575(03)00065-6

Sturm K, Koron D & Stampar F (2003). The composition of fruit of different strawberry varieties depending on maturity stage. *Food Chemistry* 83: 417-422. https://doi.org/10.1016/S0308-8146(03)00124-9

Tepe B & Ekinci R (2021). Drying characteristics and some quality parameters of whole jujube (*Zizyphus jujuba Mill.*) during hot air drying. *Italian Journal of Food Science* 33(1): 1-15. https://doi.org/10.15586/ijfs.v33i1.1947

Wang B, Huang Q, Venkitasamy C, Chai H, Gao H, Cheng N, Cao W, Lv X & Pan Z (2016). Changes in phenolic compounds and their antioxidant capacities in jujube (*Ziziphus jujuba Miller*) during three edible maturity stages. *LWT - Food Science and Technology* 66: 56-62. https://doi.org/10.1016/j.lwt.2015.10.005

Wojdyło A, Figiel A, Legua P, Lech K, Carbonell-Barrachina Á A & Hernández F (2016). Chemical composition, antioxidant capacity, and sensory quality of dried jujube fruits as affected by cultivar and drying method. *Food Chemistry* 207: 170-179. https://doi.org/10.1016/j.foodchem.2016.03.099

Yücel E (2005). Trees and Shrubs. *Türmatsan*, Eskişehir, 301 pp. (In Turkish)

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Response of Different Substrates and Irrigation Water Levels on Yield and Oil Quality of Ginger Grown in Greenhouse

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ABSTRACT
Growing media and irrigation water levels are the most important factors affecting plant yield and quality throughout the world. The present research was conducted in a greenhouse located in the Batı Akdeniz Agricultural Research Institute between the 2019 and 2020 growing season. The study aims to determine the effects of different substrates and irrigation levels on yield and phenolic and essential oil compounds of ginger (*Zingiber officinale*) irrigated by means of a drip irrigation system. In order to investigate the effects of different substrates and irrigation levels on the physiological characteristics and yield of ginger, an experiment was conducted as factorial, in which the main factor was three substrates (S_1: 75% cocopeat + 25% perlite, S_2: 50% peat + 50% perlite, and S_3: 25% zeolite + 75% peat) and the sub factor was four irrigation levels (I_1:100% I_2: 75%, I_3: 50%, and I_4:25%) were applied experimental plots according to the daily solar radiation values reaching the greenhouse, with 3 replications. The evapotranspiration values ranged between 49.7-198.7 L plant^{-1} and 51.7-206.9 L plant^{-1} in the 2019 and 2020 years, respectively. Rhizome fresh weight values for I_1, I_2, I_3 and I_4 were determined as 134.8, 94.7, 71.2 and 31.1 g in 2019 and 164.5, 148.1, 95.1 and 74.9 g in 2020, respectively. Water deficit stress significantly ($P \leq 0.01$) increased the 6-gingerol, 6-shogaol, α-zingiberene, α-farnesene, and geranly-acetate contents while it decreased the β-sesquiphellandrene and β-bisabolene content. It was found that the essential oil yield of ginger decreased depending on the increasing irrigation water stress levels.

Keywords: Gingerol, Growing media, Solar radiation, Water deficit, *Zingiber officinale*

1. Introduction

Ginger (*Zingiber officinale*) is one of the most popular spices worldwide, and is widely used as both a spice and a medicinal herb. The plant, which is widely used in food, medicine, and beverage in the world, grows naturally in India, China, South East Asia, and Mexico (Hayden et al. 2004; Ghosh 2011; Nair 2013). The total cultivated area of ginger amounts to 385,172 ha and a production total of 4,081,374 tons worldwide (Malhotra et al. 2021). Ginger cultivation in Turkey is relatively new, where the spice is frequently used in food and in detox tea, with a total planting area of 200 ha (Uysal Bayar et al. 2021).

Ginger is usually consumed as young ginger or mature ginger. Ginger contains 80.9% moisture, 2.3% protein, 0.9% fat, 1.2% mineral, 2.4% fiber and 12.3% carbohydrates. The minerals found in ginger include iron, calcium, and phosphorus, while it also contains vitamins such as thiamine, riboflavin, niacin and vitamin C. Ginger also possesses several interesting bioactive constituents and health-promoting properties. 6-gingerol is a major pungent ingredient in ginger, also possesses potent anti-oxidant, anti-cancer, analgesic, anti-pyretic, anti-inflammatory, cytotoxic, anti-diabetic, anti-obesity, anti-nausea, anti-gastric and anti-proliferative activities (Puengphian & Sirichote 2008). The composition of ginger may vary depending on the species, variety, growing conditions, drying and storage conditions (Ghosh 2011).

Ginger is made up of 1-3% essential oil which contains several active ingredients. The main active ingredients in ginger oil are sesquiterpenes: bisabolene, zingiberene and zingerol. The phenolic compounds found in ginger are shogaol and gingerol components. The proportions of active ingredients and phenolic components vary according to the irrigation, nutrition, and cultural practices (Kemper 1999).

There are an increasing number of consumers of ginger products in the world. Water management is one of the major factors affecting ginger production in arid and semiarid regions. Deficit irrigation adversely affects many physical and chemical processes related to water use efficiency of ginger cultivation (irrigated via micro sprays), thus leading to a decrease in plant yield and quality (Meneghelli et al. 2020). Islam et al. (2015) studied the effects of two irrigation treatments (I_1: irrigation in a

dry period, 7 days before planting and 60 days after planting and I_2: no irrigation) on ginger (irrigated by hose pipe which has 2.5 cm diameter). They found that the highest weight of rhizome (268 g plant^{-1}) was obtained from I_1 irrigation treatments. Kumar et al. (2018) reported that ginger crops demand a large amount of irrigation water, requiring continuous irrigation throughout the growing periods. Gatabazi et al. (2019) investigated the effects of different irrigation water levels (T_1: 2025% maximum allowable depletion, T_2: 40–45% maximum allowable depletion, T_3: 60–65% maximum allowable depletion, and T_4: 80–85% maximum allowable depletion) on the yield and quality of ginger and found that water use (WU) ranged between 219 and 509 mm in greenhouse conditions. Meneghelli et al. (2020), in their study in Brazil, determined five irrigation depths (50%, 75%, 100%, 125% and 150% of crop evapotranspiration) on ginger and noted that total water use ranged between 919 and 1564 mm in open field conditions. Mohd and Sembok (2020) investigated three irrigation frequencies (WF_2 = two times a day applications, WF_4 = four times a day applications and WF_6 = six times a day applications) and three volumes of irrigation water (A_1 = 300 mL, A_2 = 600 mL and A_3 = 1200 mL) on yield and quality of ginger in soilless culture (100% of coir dusts) conditions. They reported that a combination of 6 times per day of irrigation frequency and 1200 mL irrigation water gave the best ginger plant growth performance and rhizomes weight in the soilless culture system.

On the other hand, the soilless culture system is the most intensive production method in agriculture. Soilless culture system can result in higher yields even under a limited and adverse growing environment. Significant factors persuading plant growth in soilless culture systems are water availability, nutrient content, moisture and soil aeration (Tüzel et al. 2019). Ravindran et al. (2004) argued that ginger growth improved under constant elevated moisture root and water availability to the plant in soilless culture. Yaseer Suhaimi et al. (2012) evaluated five combinations of substrates (100% coir dust; 100% burnt paddy husks; 70% coir dust + 30% burnt paddy husks; 30% coir dust + 70% burnt paddy husks; and 50% coir dust + 50% burnt paddy husks) on ginger and reported that the highest shoot height, shoot fresh weight, and rhizome weight were obtained plants grown in 100% coir dust. Supriya et al. (2020) studied three different substrates (cocopeat – 100%, cocopeat + perlite – 75:25 and cocopeat + sand – 75:25) on ginger and determined that the highest plant height, number of leaves, number of tillers, leaf area, and fresh rhizome weight per plant were recorded in a cocopeat + sand (75:25) combination.

Gatabazi et al. (2019) note there is limited information available on the response of ginger species that are subjected to varying water stress regimes. Information regarding the plant's response to water stress regimes and drought tolerance mechanisms can help to devise appropriate irrigation management strategies and be useful in breeding programs for the selection of genotypes that can withstand extreme conditions. Research concerning the effects of different irrigation water levels and substrates on son ginger have not been found in Turkey. For this reason, the aims of this study were (i) to evaluate different irrigation levels of ginger under greenhouse conditions (ii) to examine the effects of different growing media on ginger yield and (iii) to determine phenolic compounds, essential oil, and oil components of ginger under greenhouse conditions.

2. Material and Methods

2.1. Experimental area and climatic conditions

The study was conducted in a greenhouse located at the Batı Akdeniz Agricultural Research Institute, Antalya, Turkey in 2019-2020. The research area was located at a latitude of 36 56' N and a longitude of 30 53' E, and an altitude of 28 m. The average temperature and relative humidity inside the greenhouse for the study period are presented in Figure 1. The temperature and relative humidity ranged between 13.2-36.8 °C and 24.2-84.7% in 2019, respectively. In 2020, temperature and relative humidity ranged between 13.5-36.2 °C and 24.8-82.5%, respectively.

2.2. Treatments and experimental design

Three different substrates consisted of volumetric mixtures of cocopeat (C) and perlite (P) (S_1: 75% C + 25% P), peat (Pe) and perlite (S_2:50% Pe + 50% P), and zeolite (Z) and peat (S_3:25% Z + 75% Pe) and four irrigation levels (I_1:100%, I_2: 75%, I_3: 50%, and I_4:25%) were used in the study. Some properties of the different substrates used in study are given in Table 1. Different substrates formed by main plots were designed according to the randomized block design whereas the irrigation levels were designed as sub-plots. Thus, 4×3 split plots were applied and each treatment was replicated three times in the experiment (Figure 2).

2.3. Planting and growing conditions

Ginger rhizome was planted on 15 March 2019 and 6 March 2020. Each of the rhizomes was cut into smaller pieces of about 3-4 cm long and 35-45 g in weight before planting. The rhizomes were planted in 2.43 m^3 polypropylene bags (12.0 m long × 0.45 m wide × 0.45 m deep) with one row (0.45 x 0.45 m spacing, 5 plants m^{-2}) and a distance between the adjacent polypropylene bags of 50 cm. The polypropylene bags were filled with three different substrates. Each polypropylene bag contained 27 plants. The polypropylene bags were placed in gutters for the collection of drainage water.

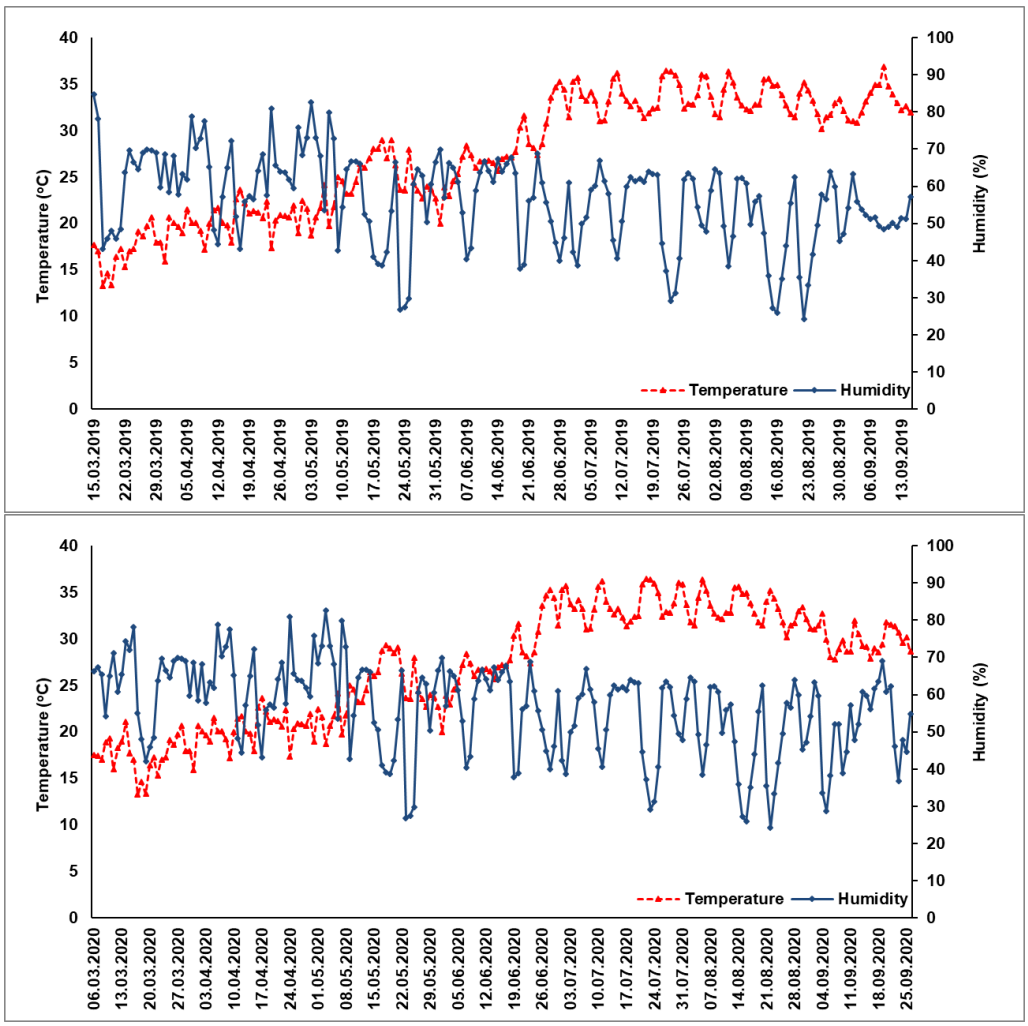


Figure 1- Average temperature and relative humidity measured inside the greenhouse

Table 1- Some properties of different substrates used in the study

Properties	*S_1*	*S_2*	*S_3*
pH	5.50	6.10	6.20
Electrical Conductivity (micromhos cm^{-1})	940.00	445.00	340.00
Humidity (%)	14.60	10.30	18.50
Dry matter (%)	85.40	89.70	81.50
Organic matter (%)	29.20	78.80	66.80
Ash (%)	70.80	21.20	33.20
Total Nitrogen (%)	0.17	0.83	0.52
Carbon (%)	16.90	45.70	38.80
Carbon/Nitrogen	100.30	54.80	74.90
Total Iron (ppm)	929.00	844.00	1593.00
Total Manganese (ppm)	14.00	27.00	28.00
Total Zinc (ppm)	6.00	11.00	7.00
Total Copper (ppm)	0.00	8.00	6.00

S_1: 75% cocopeat + 25% perlite, S_2: 50% peat + 50% perlite, and S_3: 25% zeolite + 75% peat)

Figure 2- Experimental design used in the study S_1: 75% cocopeat + 25% perlite, S_2: 50% peat + 50% perlite, and S_3: 25% zeolite + 75% peat), I_1: Irrigated at 100%, I_2: Irrigated at 75%, I_3: Irrigated at 50%, and I_4: Irrigated at 25%

2.4. Nutrient management and irrigation

The plant nutrient solution recommended for ginger was 119 mg L^{-1} N, 83 mg L^{-1} P, 163 mg L^{-1} K, 193 mg L^{-1} Ca, 48 mg L^{-1} Mg, 6 mg L^{-1} Fe, 0.9 mg L^{-1} Mn, 0.3 mg L^{-1} B, 0.08 mg L^{-1} Zn, 0.06 mg L^{-1} Cu and 0.04 mg L^{-1} Mo (Hayden et al. 2004). The prepared stock nutrient solutions were used in irrigation practices for a balanced nutritional level in each treatment. Each irrigation level was provided with a tank of nutrient solution (1000 L) and a pump. The solution pH in each irrigation tank was arranged between 5.5-6.0 by the addition of nitric acid. All treatments were irrigated at the same time by a drip irrigation system having an in-line dripper discharging 1.6 L h^{-1} at a pressure of 0.1 MPa. The irrigation frequency was based on solar radiation achieved in greenhouse. The amount of water applied was calculated to meet the solar radiation. The irrigation scheduling was automatically implemented by a digital timer. A radiation-based evapotranspiration method was used to determine the applied irrigation water. For this purpose, a solar radiation sensor, placed in the greenhouse was used to apply the four irrigation rates 25% (I_4), 50% (I_3), 75% (I_2), and 100% (I_1) times the standard rate. The applied irrigation water (L) was determined using the following equation (Guyot 1998).

$$I = \frac{R_i}{\lambda} \times A \quad (1)$$

Where; I is the applied irrigation water (L), R_i is the incoming solar radiation inside the greenhouse (MJ m^{-2} day^{-1}), $\boldsymbol{\lambda}$ is the latent heat of water vaporization (MJ kg^{-1}), and A is the area of the polypropylene bags (m^2).
Plant water consumption (L $plant^{-1}$) was calculated using the following equation.

$$PWC = \frac{I-D}{PN} \quad (2)$$

Where; PWC is the plant water consumption (L plant^{-1}), I is the applied water (L), D is the drainage water (L) and PN is the plant number per polypropylene bags.

2.5. Harvesting and measurements

The harvest was performed when the leaves turned yellow and started to dry 50%. Twenty plants were harvested from each plot on September 15, 2019 and September 26, 2020. The fresh rhizome weight was measured in precision digital scale (0.1 g accuracy) for each treatment. Plant height was measured via ruler and the results given in terms of cm. The number of brunch per plant was counted one by one from selected 20 ginger samples.

2.6. Determination phenolic and essential oil compounds

The rhizomes of the ginger plants were dried and grinded before extraction. The rhizomes were dried in an air-circulated (7.272 m^3/hr) drying oven (Venticell-404 Standard, MMM group, Germany) at 40 °C until the humidity level was approximately 10%. After drying procedure, the rhizomes were ground. The grinding was realized at grinder (Retsch Grindomix GM 200) at 10.000 rpm during 1 minute.

The extraction of the phenolic compounds was realized using rhizome powders. The phenolic compounds of the powders were extracted using a methanol-water mixture (80:20). The extraction was made in an orbital shaker (Heidolph Unimax 2010) over a period of 1 hour. After extraction, the extracts were centrifuged at 5000 rpm for a total of 5 minutes. Later, the liquid phase was taken. The methanol-water mixture was added to the residual part and the same procedures were repeated 3 times. After this procedure, the extracts were taken to the 50 mL volumetric flask and it was diluted to the volume of the volumetric flask (Cemeroğlu 2010). In this part of the study, 6-gingerol and 6-shogaol contents of the rhizomes were determined. The compounds were detected in Liquid Chromatography (Agilent 1290)-Mass Sepctrometry (6430 Triple Quadropole) (LC-MS/MS) device with Zorbax RRHD Eclipse Plus C18 column (3 µm 2.1x100 mm) by using the method developed by Fischer et al. (2011). The calibration solutions of the compounds were prepared, firstly. The MS parameters (polarity, fragmentor voltage, product ions, collision energies) were determined. The calibration curve was plotted using calibration solutions and MS parameters. The quantitative contents of the compounds were calculated using calibration curves.

Essential oil extractions of the rhizome powders were realized at Clevenger apparatus (Isotex, 98-IV-B). The rhizome powders (20 g) were placed into the Clevenger apparatus and 200 mL of deionized water was added. The hydrodistillation was made during 2 hours. The essential oil content was calculated as (v/w, %) (Anonymous 2011). The essential oils were diluted with hexane as 1:100. The essential oil components were determined using Gas chromatography (Agilent 7890A)-mass detector (Agilent 5975C)/flame ionization detector (GC-MS/FID) device with capillary column (HP Innowax Capillary; 60.0 m x 0.25 mm x 0.25 µm). Helium was used as carrier gas with 0.8 mL/min flow rate, and the samples were injected into the device as 1 µL with 40:1 split rate. The injection block temperature was 250 °C, column temperature programme was adjusted as 60 °C (10 minute), from 60 °C to 220 °C with 4 °C/minute increasing rate and 220 °C (10 minute). The scanning range was 35-450 atomic mass unit and 70 eV was used as electron bombardment ionization, WILEY7 and OIL ADAMS libraries data were used in identification of the essential oil components. The components percentage ratios were determined using a FID detector and the identification of the components was made using an MS detector (Özek et al. 2010).

2.7. Statistical analyses

The experiment was carried out in a randomized block design with three replications in 36 experimental plots. During the experiment, the rhizome weight (g plant^{-1}), plant height (cm), number of branch (per plant), plant water consumption (L plant^{-1}), oil content (%), phenolic and essential oil compounds were determined. The collected data were subjected to the analysis of variance (ANOVA) using SPSS Statistics Base v23 (SPSS Inc., Chicago, IL, USA), and significant differences between means were compared through an LSD test ($P<0.05$) (Dean et al. 2017).

3. Results and Discussion

3.1. Solar radiation and plant water consumption

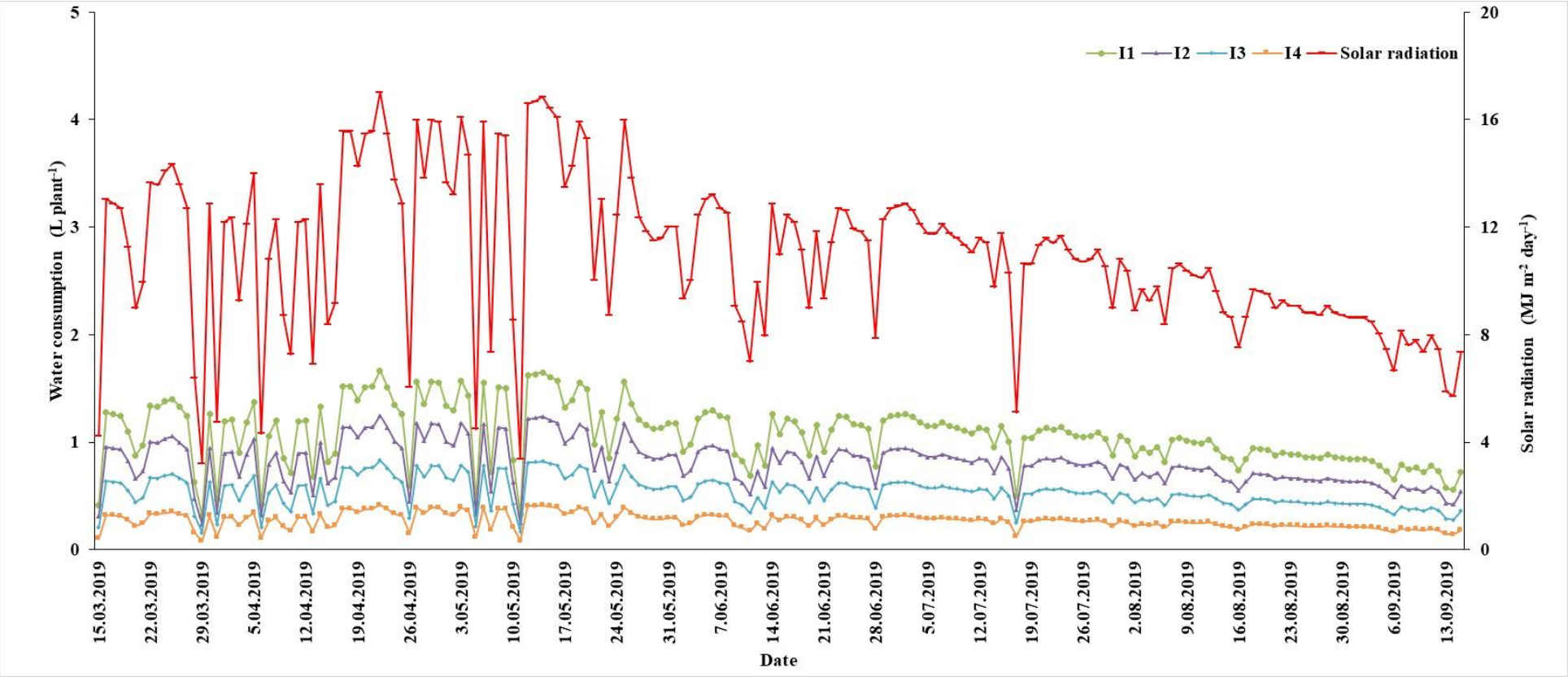
Daily solar radiation and plant water consumption are given in Figure 3. The daily solar radiation ranged from 4.32 to 17.02 MJ m^{-2} day^{-1} and 1.98 to 16.33 MJ m^{-2} day^{-1} in the 2019 and 2020 growing seasons, respectively. The highest solar radiation was measured in 22 April 2019 and 13 April 2020. Plant water consumption varied between 49.7-198.7 L plant^{-1} (248-994 mm) and 51.7-206.9 L plant^{-1} (258-1034 mm) in 2019 and 2020 growing season, respectively. Kandiannan et al. (1996) noted that the water requirement of ginger has been estimated by the Queensland Irrigation and Water Supply Commission to be between 1320-1520 mm during a complete crop cycle. Gatabaziet al. (2019) found that ginger is highly sensitive to water stress and deficit irrigation levels effect on yield and quality (plant height, stems per plant, number of leaves, leaf area index) of ginger. They

determined that the total water consumption of ginger varied between 219 and 549 mm (31.8 and 13.7 L plant^{-1}) in open field conditions. Meneghelli et al. (2020) reported that the total water depth applied in the range of 1100–1200 mm favors the development of ginger plants, providing the highest yields of total and export rhizomes, the greatest average mass of export quality rhizome and lowest production of small rhizomes.

Table 2- Effects of different substrates and irrigation levels on yield and quality parameters

Treatments	*Years*							
	2019				*2020*			
	RW	*PH*	*SN*	*EO*	*RW*	*PH*	*SN*	*EO*
S_1	48.6 c^x	67.7 b	2.9 c	0.8 c	91.2	71.2	5.3	0.9 b
S_2	85.9 b	71.4 a	4.0 b	0.9 b	128.0	71.3	5.7	0.9 b
S_3	114.4 a	72.7 a	4.4 a	1.0 a	142.7	74.5	6.1	1.0 a
Substrates (S)	**	*	**	**	NS	NS	NS	**
LSD (0.05)	4.35	3.44	0.20	0.08				0.09
I_1	134.8 a	79.7 a	5.7 a	1.1 a	164.5 a	77.1	7.1 a	1.2 a
I_2	94.7 b	74.2 b	3.6 b	1.0 ab	148.1 a	75.5	6.4 b	1.0 b
I_3	71.2 c	66.5 c	3.3 c	0.8 b	95.1 b	73.2	5.1 c	0.8 c
I_4	31.1 d	62.1 d	2.4 d	0.7 c	74.9 b	63.5	4.1 c	0.6 d
Irrigations (I)	**	**	**	**	**	NS	**	**
LSD (0.05)	5.02	3.97	0.23	0.09	50.84		1.86	0.12
S_1I_1	79.4 d	69.6 de	3.4 cd	1.0	132.4	64.5	7.0	1.1
S_1I_2	45.6 e	68.6 ef	3.4 cd	0.9	124.6	67.4	5.7	0.8
S_1I_3	40.4 ef	67.4 ef	2.8 ef	0.7	74.3	79.3	5.0	0.8
S_1I_4	29.0 g	65.4 eg	1.8 g	0.6	33.6	73.4	3.7	0.7
S_2I_1	137.9 b	82.4 ab	6.6 a	1.1	166.4	82.8	7.0	1.0
S_2I_2	93.8 c	78.6 bc	3.4 cd	1.0	148.7	78.5	6.7	1.0
S_2I_3	81.0 d	66.1 ef	3.2 de	0.9	104.7	72.8	5.0	1.0
S_2I_4	30.0 g	58.5 g	2.6 f	0.7	92.1	51.0	4.0	0.9
S_3I_1	187.1 a	87.0 a	7.0 a	1.2	194.8	83.9	7.3	1.0
S_3I_2	144.6 b	75.5 cd	4.0 b	1.0	171.0	80.6	7.0	0.9
S_3I_3	92.3 c	66.0 ef	3.8 bc	0.9	106.2	67.3	5.3	0.9
S_3I_4	33.6 fg	62.5 fg	2.8 ef	0.7	98.9	66.0	4.7	0.8
S×I	**	**	**	NS	NS	NS	NS	NS
LSD (0.05)	8.70	6.88	0.40					

RW: Rhizome weight (g plant^{-1}); PH: Plant height (cm); SN: Stem number (number plant^{-1}); EO: Essential oil (%); S_1: 75% cocopeat + 25% perlite, S_2: 50% peat + 50% perlite, and S_3: 25% zeolite + 75% peat); I_1: Irrigated at 100%; I_2: Irrigated at 75%; I_3: Irrigated at 50%, and I_4: Irrigated at 25%; NS: not significant; *: significant at P<0.05; **: significant at P<0.01; ^{x} : Within each column, the levels containing the same letter form a group of means within which there are no statistically significant differences (95% confidence level)


I1
I2
I3
I4
Solar radiation
Water consumption (L plant-1)
Solar radiation (MJ m-2 day-1)
Date
15.03.2019
22.03.2019
29.03.2019
5.04.2019
12.04.2019
19.04.2019
26.04.2019
3.05.2019
10.05.2019
17.05.2019
24.05.2019
31.05.2019
7.06.2019
14.06.2019
21.06.2019
28.06.2019
5.07.2019
12.07.2019
19.07.2019
26.07.2019
2.08.2019
9.08.2019
16.08.2019
23.08.2019
30.08.2019
6.09.2019
13.09.2019

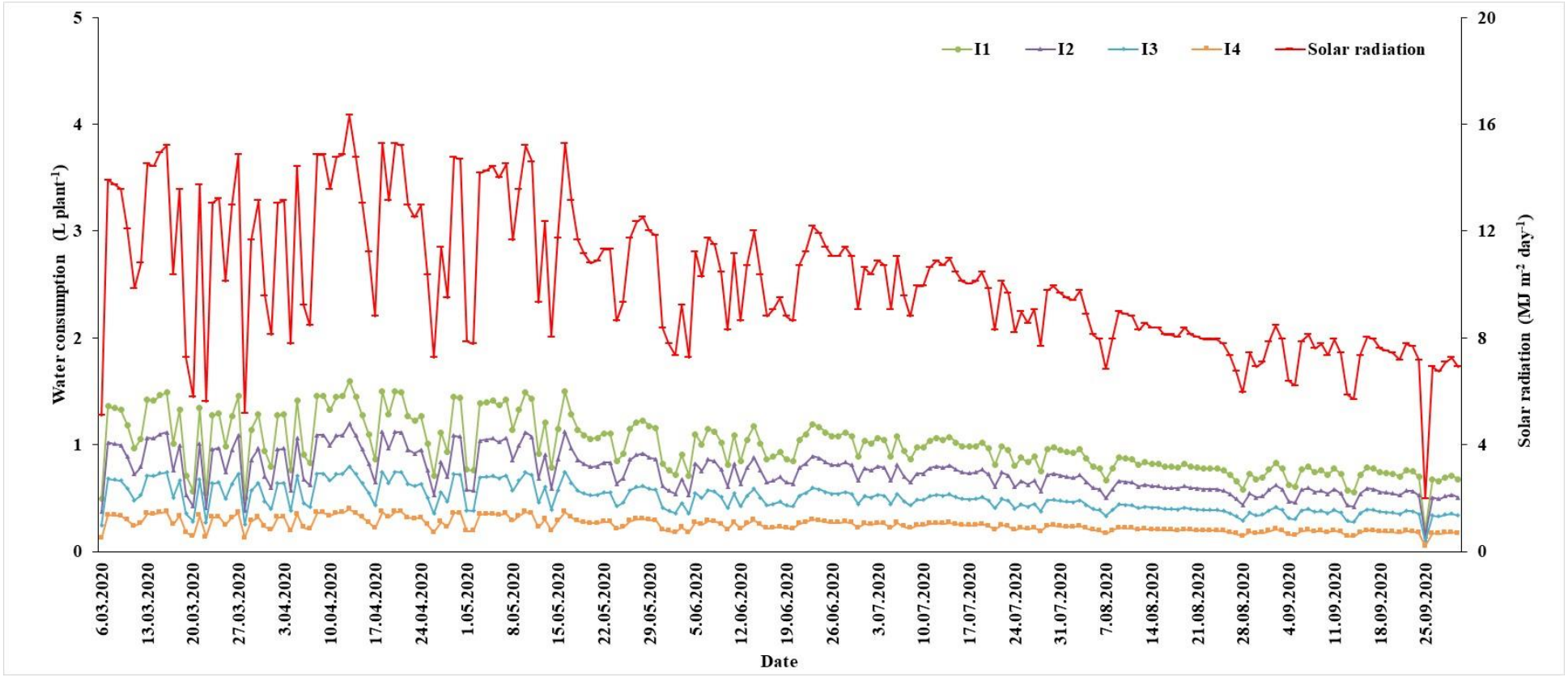


Figure 3- Daily solar radiation and cumulative water consumption (I_1: Irrigated at 100%, I_2: Irrigated at 75%, I_3: Irrigated at 50%, and I_4: Irrigated at 25%)

3.2. Yield, quality parameters, and essential oil

The average rhizome weight obtained from the different substrates and irrigation levels taken in the experiment and the variance analysis results of these yields are given in Table 2.

It was found that interactions were statistically different ($P<0.01$) for rhizome weight, plant height, and stem number in 2019, with the exception of essential oil, although no difference was found in 2020 (Table 2). The rhizome weight changed between 29.0-187.1 g plant^{-1} in the first year and 33.6-194.8 g plant^{-1} in the second year of the study. The highest rhizome weight was obtained as 187.1 and 194.8 g plant^{-1} from S_3I_1 treatment while the lowest rhizome weight was 29.0 and 33.6 g plant^{-1} from S_1I_4 treatment in the study. The reduction in the quantity of irrigation water resulted in a relatively lower rhizome weight. Rhizome weights in the second year are higher than in the first year. Temperature is one of the most important climatic factors for the development of ginger, which is a tropical plant. In the second year of the study, higher temperatures (Figure 1) from the planting date (first 15 days) compared to the first year may be the reason for the increase in rhizome weight. Beardsell et al. (1979) stated that sufficient water in a substrate is crucial for plant growth and development. Prasad et al. (2008) reported that deficit irrigation affects plant growth stages. They also stated that besides physiological reactions, plants underwent morphological changes, vegetative growth was reduced and the development of plant reproductive organs was inhibited under water stress. As can be seen, in both years of the experiment, as the stress of irrigation water increased, the rhizome weight per plant decreased. Baloyi (2004) reported the rhizome weight of wild ginger as 161.5, 121.1, 163.8, 178.0, and 76.5 g under 0.25 L day^{-1}, 1 L day^{-1}, 2 L day^{-1}, 2 L 2nd day^{-1}, and 2 L week^{-1}, respectively. Manjunatha (2010) determined the rhizome weight as 123.3, 165.0 and 230.7 g under 12, 18 and 24 L m^{-2} day^{-1}, respectively. Yaseer Suhaimi et al. (2012) obtained the highest rhizome weight from plants grown in 100% cocopeat medium with 1340 g, while the lowest rhizome weight was obtained from 30% cocopeat + 70% burnt rice hull with 1090 g. Islam et al. (2015) determined the rhizome weight as 268.07 g and 60.80 g, respectively, under irrigated and non-irrigated conditions. Gatabazi et al. (2019) determined the rhizome weight as 250 and 100 g plant^{-1}, under fully irrigated and 80% water constraint conditions, respectively. Meneghelli et al. (2020) determined the rhizome weight as 316 g plant^{-1} under control conditions, and determined the rhizome weight as 263 g plant^{-1} under 50% water constraint conditions. Similar results were found by Mishra & Mishra (1982) and Ghosh (1996).

According to Table 2, deficit irrigation treatments showed significant difference ($P<0.01$) for plant height in the first year of the experiment. While in the first year of the study, the plant height changed between 58.5 and 87.0 cm, in the second year, it ranged from 51.0 to 83.9 cm. Depending on the irrigation levels, the highest plant height was obtained from the I_1 irrigation level in the first and second year of the study. The highest plant was obtained as 87.0 and 83.9 cm from S_3I_1 treatment while the lowest plant was 58.5 and 51.0 cm from S_1I_4 treatment in the study. Plant height is a good indicator for determining the effect of water stress on the plant and is among the most important parameters affecting the weight. It was observed that as the irrigation level decreased, plant height values also decreased. Water stress, which is one of the abiotic stress sources, causes an increase in the osmotic pressure in the plant root zone and in this case makes it difficult for the plant roots to take water and plant nutrients (Sonneveld et al. 1999). The vegetative growth of the plant is limited as a result of the decrease in water intake. Plant height of ginger varied from Baloyi (2004) and Manjunatha (2010) determined plant height varied from 49.9 to 59.8 cm and 51.3 to 58.2 cm, respectively. Islam et al. (2015) found that plant height was significantly affected by irrigation and that the plant height was 60.5 cm in non-irrigated conditions and 71.6 cm in irrigation conditions. Similarly, Gatabazi et al. (2019) determined the plant height as 68 and 42 cm under full irrigation and water stress conditions, respectively. Yaseer Suhaimi et al. (2012) obtained the tallest plants from plants grown in a 100% cocopeat with 123 cm, while the shortest plant length was 105 cm and those grown in a mixture of 30% cocopeat + 70% burnt paddy husks. Other studies reported that deficit irrigation shortened plant height in ginger (Pawar 1990; Ghosh 1996; Chandra et al. 2001).

The stem number obtained from the study is given in Table 2. In the first year of the experiment, the effect of substrates, irrigation level, and substrates–irrigation levels interaction on the stem number was statistically significant. Examining the stem number with respect to irrigation level in the first year, the highest stem number was obtained from I_1 (4.4), while the lowest stem number was from I_1 (2.9). In the second year of the experiment, the effects of substrates and substrates-irrigation levels on stem number were not statistically significant; while the effect of irrigation level on stem number was statistically significant. The highest stem number was found in I_1 (7.1), while the lowest stem number was in I_4 (4.1). In substrates–irrigation levels interaction, the highest stem number was obtained from S_3I_1 treatments. Reducing the amount of irrigation water negatively affects plant growth by reducing the moisture content in the substrates and thus restricting the water uptake through plant roots. At the same time, water stress decreases plant growth by causing a decrease in photosynthesis rate and a decrease in cellular expansion. Mokgehle et al. (2017) stated that irrigation water plays an important role in the physiological processes of plants and that well-watered ginger easily maintains its normal physiological functions. Gatabazi et al. (2019) stated that the first sign of water stress usually causes a decrease in cell swelling, thus causing a decrease in cell growth, especially thinning of the stem and leaf number. The same researchers found that the number of stem was 8.8 and 5.6 under fully irrigated and 80% water constraint conditions, respectively.

The essential oil obtained from the different substrates and irrigation levels taken in the experiment and the variance analysis results of these yields are given in Table 2. It was found that interactions were not statistically different for the essential oil in 2019 and 2020. The essential oil changed between 0.6-1.2% in the first year and 0.7-1.1% in the second year of the study. The

highest essential oil was obtained as 1.2% from the S_3I_1 treatment while the lowest essential oil was 0.6% from the S_1I_4 treatment. Depending on the irrigation levels, the highest essential oil was obtained from the I1 irrigation level in the first and second year of the study. The highest essential oil was obtained from the S3 substrate in the first and second year of the study. A reduction in the quantity of irrigation water resulted in a relatively lower essential oil content in our study. There is a dilemma about the change in essential oil content as some of the studies reporting that essential oil content t increases with an increase in water stress whereas others show essential oil contents decrease as the water stress decreases. Lawrence (1984) argued that decreased irrigation water amount increased the essential oil content whereas Kumar et al. (2018) reported that irrigation water deficiency has reduced the essential oil yield.

3.3. Phenolic and essential oil compounds

The influence of irrigation levels on phenolic and essential oil compounds in ginger is given in Table 3. Two phenolic and twenty-two essential oil compounds were positively identified after analysis via liquid and gas chromatography, respectively. Five essential oil compounds of more than 2% in abundance level are given Table 3. The effect of irrigation treatments was significant ($P<0.01$) for all phenolic and essential oil compounds in 2019 and 2020. In 2019, the highest 6-gingerol (9.03 mg g^{-1}), 6-shogaol (1.48 mg g^{-1}), α-zingiberene (36.98%), α-farnesene (6.82%) and geranly-acetate (7.27%) content was obtained from the I_4 irrigation treatment; β-sesquiphellandrene (24.72%) and β-bisabolene (8.44%) content from I_1 irrigation treatment. In 2020, the highest 6-gingerol (6.16 mg g^{-1}), 6-shogaol (1.39 mg g^{-1}), α-zingiberene (32.24%), α-farnesene (5.06%) and geranly-acetate (8.68%) content was obtained from the I_4 irrigation treatment; β-sesquiphellandrene (22.34%) and β-bisabolene (7.72%) content from the I_1 irrigation treatment. 6-gingerol and 6-shogaol are the most pungent phenolic compounds of ginger and have potent antioxidant activity and health promoting properties. Decreasing the irrigation levels also resulted in a significant increase in 6-gingerol and 6-shogaol content. It was found that the substrates were not statistically different for 6-gingerol and 6-shogaol content in 2020. Ginger cultivated in the S_1 media showed a slightly higher 6-gingerol content. The widely accepted idea there is a widespread increase in phenolic compounds in response to water stress is most often incorrect, since phenolic compounds may experience either a decrease or no changes in concentration when subjected to water stress (Albergaria et al. 2020). Sharizan et al. (2014) and Yaseer Suhaimi et al. (2018) suggested that secondary metabolites, such as 6-gingerol and 6-shogaol content and accumulation, were not affected by the substrates.

Gatabazi et al. (2022) found that water stress may help to improve the phenolic content for ginger species. The total phenolic content was lower in the full irrigation treatments in the study. The decrease in the total phenolic content under full irrigation treatments conditions observed in the current study aligns with previous findings that suggest that increased irrigation can limit specific components to improve secondary metabolites (Battaieb et al. 2010; Gatabazi et al. 2022). On the other hand, Jiang & Huang (2001) and Weidner et al. (2009) determined water stress either decreases or increases the content of phenolic and oil compounds. Additionally, Albergaria et al. (2020) carried out a systematic review on the effect of water stress on the contents of total phenolic and oil compounds in medicinal plants, concluding that that the acceptance that there is a widespread increase in phenolic and oil compounds in response to water stress is most often incorrect.

4. Conclusions

This study analyzed the effects of deficit irrigation levels and different substrates in ginger grown in greenhouse on yield, quality parameters. The effects of different irrigation levels on rhizome weight, plant height, stem number, essential oil, phenolic and essential oil compounds were found to be statistically significant. The maximum rhizome weight was obtained from I_1 treatments. The phenolic and essential oil compounds content increased as the amount of water deficiency increased, whereas β-sesquiphellandrene and β-bisabolene content decreased as the amount of water deficiency increased. Compared with the I_1 irrigation treatment, the mean relative rhizome weight decreases were 19, 45, and 65% and the essential oil content decreases were 13, 20, and 43% for I_2, I_3, and I_4 treatments, respectively. However, water deficit treatments caused an increase in the phenolic content (6-gingerol and 6-shogaol) in ginger. Substrate S_3 (containing 25% zeolite + 75% peat) showed good growth and increased the rhizome yield up to 46% and 17% compared to S_1 (containing 75% cocopeat + 25% perlite) and S_2 (containing 50% peat + 50% perlite). It can be concluded that the best performance in terms of ginger yield was obtained in S_3 substrate with I_1 irrigation treatments in soilless culture system.

Table 3- Effects of different substrates and irrigation levels on phenolic and essential oil compounds

Treatments	*2019*							*2020*						
	Phenolic compounds		*Essential oil compounds*					*Phenolic compounds*		*Essential oil compounds*				
	6-gingerol (mg g^{-1})	*6-shogaol (mg g^{-1})*	*A-zingiberene (%)*	*β-sesquiphellandrene (%)*	*β-bisabolene (%)*	*α-farnesene (%)*	*geranly-acetate (%)*	*6-gingerol (mg g^{-1})*	*6-shogaol (mg g^{-1})*	*α-zingiberene (%)*	*β-sesquiphellandrene (%)*	*β-bisabolene (%)*	*α-farnesene (%)*	*geranly-acetate (%)*
S_1	7.50 a^x	1.15 b	34.77 a	21.92 b	7.72	6.62 b	5.02 b	5.44 a	1.19	31.10	20.74	6.84 b	4.57	7.78 a
S_2	6.82 c	1.13 b	32.78 b	21.54 c	7.60	6.40 c	5.84 a	5.26 b	1.09	30.20	20.72	7.26 a	4.42	7.16 a
S_3	7.13 b	1.31 a	33.59 b	22.76 a	7.62	6.82 a	4.82 c	5.11 c	1.15	30.30	21.44	7.32 a	4.84	6.04 b
Substrates (S)	**	**	**	**	NS	**	**	NS	NS	NS	NS	*	NS	**
I_1	5.65 d	0.99 d	30.49 d	24.72 a	8.44 a	6.58 b	3.72 d	4.35 d	0.87 b	29.22 b	22.34 a	7.72 a	4.16 c	5.20 d
I_2	6.22 c	1.07 c	32.82 c	22.55 b	7.63 b	6.62 b	4.70 c	5.12 c	0.96 b	29.64 b	21.93 a	7.35 a	4.56 b	6.04 c
I_3	7.71 b	1.24 b	34.56 b	21.20 c	7.32 b	6.74 a	5.14 b	5.45 b	1.36 a	31.02 a	20.44 b	7.02 b	4.73 ab	7.28 b
I_4	9.03 a	1.48 a	36.98 a	19.82 d	7.28 b	6.82 a	7.27 a	6.16 a	1.39 a	32.24 a	19.12 c	6.52 c	5.06 a	8.68 a
Irrigations (I)	**	**	**	**	**	**	**	**	**	**	**	**	**	**
S_1I_1	6.59 g	1.00 gh	30.82 fg	23.49 c	8.06	7.16 ab	3.98 h	4.35 i	0.97 e	28.98	22.91	7.30	3.98	5.04
S_1I_2	6.70 fg	1.10 e	34.74 cd	22.35 d	7.71	6.83 cd	4.86 f	4.71 g	1.06 de	29.89	22.54	7.09	4.46	6.43
S_1I_3	7.92 d	1.11 de	35.78 bc	21.43 e	7.19	6.41 e	4.95 f	5.29 e	1.38 ac	32.23	18.58	6.66	4.60	7.51
S_1I_4	8.81 b	1.41 b	37.75 a	20.30 f	7.84	6.19 ef	6.34 c	6.08 b	1.35bc	33.16	18.96	6.20	4.97	9.63
S_2I_1	5.23 h	1.00 gh	29.04 g	24.85 b	8.80	5.99 f	4.15 h	4.31 i	0.90 ef	29.55	21.50	7.72	3.96	5.95
S_2I_2	6.81 ef	1.03 fg	31.56 ef	21.35 e	7.36	6.09 f	5.64 e	5.23 f	0.94 e	29.24	21.20	7.34	4.30	6.09
S_2I_3	6.90 e	1.17 d	33.07 de	20.25 f	7.13	6.73 d	6.03 d	5.42 d	1.23 cd	30.04	21.10	7.15	4.48	7.63
S_2I_4	8.33 c	1.29 c	37.43 ab	19.60 g	7.00	6.92 bd	7.19 b	6.09 b	1.28 bc	31.90	18.88	6.70	4.92	8.67
S_3I_1	5.11 h	0.95 h	31.60 ef	25.80 a	8.47	6.22 ef	2.94 l	4.40 h	0.75 f	29.00	22.43	8.07	4.22	4.51
S_3I_2	5.15 h	1.08 ef	32.15 ef	23.68 c	7.63	6.76 cd	3.65 i	5.41 d	0.88 ef	29.73	21.85	7.35	4.70	5.56
S_3I_3	8.32 c	1.45 b	34.83 cd	21.93 d	7.51	6.98 bc	4.45 h	5.63 c	1.45 ab	30.86	21.64	7.22	5.06	6.40
S_3I_4	9.94 a	1.73 a	35.77 bc	19.48 g	6.75	7.39 a	8.00 a	6.30 a	1.53 a	31.62	19.50	6.54	5.16	7.56
S×I	**	**	*	**	NS	**	**	**	*	NS	NS	NS	NS	**

S_1: 75% cocopeat + 25% perlite, S_2: 50% peat + 50% perlite, and S_3: 25% zeolite + 75% peat); I_1: Irrigated at 100%; I_2: Irrigated at 75%; I_3: Irrigated at 50%, and I_4: Irrigated at 25%; NS: not significant; *: significant at P<0.05; **: significant at P<0.01; ^{x}: Within each column, the levels containing the same letter form a group of means within which there are no statistically significant differences (95% confidence level)

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Albergaria E T, Oliveira A F M & Ulysses Paulino Albuquerque U P (2020). The effect of water deficit stress on the composition of phenolic compounds in medicinal plants. *South African Journal of Botany* 131:12-17. https://doi.org/10.1016/j.sajb.2020.02.002

Anonymous (2011). TSE EN ISO 6571-Spices, condiments and herbs - Determination of volatile oil content (hydro distillation method). Turkish Standards Institute, Ankara, Türkiye

Baloyi T C (2004). Growth, anatomy, quality and yield of wild ginger (*Siphonochilus aethiopicus*), in response to nitrogen nutrition, fertigation frequency, and growing medium. MSc Agric Thesis, University of Pretoria, South Africa

Battaieb I N, Zakhama W A, Wannes M E & Marzouk B (2010). Water deficit effect on *Salvia officinalis* fatty acids and essential oils composition. *Scientia Horticulturea* 120: 271-275. https://doi.org/10.1016/j.scienta.2008.10.016

Beardsell D V, Nicholas D G & Jones D L (1979). Physical properties of nursery potting mixture. *Scientia Horticulturae* 11: 1-8. https://doi.org/10.1016/0304-4238(79)90048-7

Cemeroğlu B (2010). Food Analysis. Food Technologies Association, 657 p., Ankara, Türkiye

Chandra R, Sheo G, Chandra R & Goving S (2001). Effect of mulching on yield of ginger. *Journal of Spices Aromatic Crops* 10(1): 13-16

Dean A, Voss D & Dragulji D (2017). Design and Analysis of Experiments. Springer International Publishing, New York

Fischer U A, Carle R & Kammerer D R (2011). Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MS. *Food Chemistry* 127(2): 807-821. https://doi.org/10.1016/j.foodchem.2010.12.156

Gatabazi A, Marais D, Steyn J M, Araya H T & Mokgehle S N (2019). Growth and yield responses of two ginger species to different levels of nitrogen. *South African Journal of Plant and Soil* 36: 289-298. https://doi.org/10.1080/02571862.2019.1566501

Gatabazi A, Marais D, Steyn M, Araya H, du Plooy C, Ncube B & Mokgehle S (2022). Effect of water regimes and harvest times on yield and phytochemical accumulation of two ginger species. *Scientia Horticulturae* 304(October): 111353. https://doi.org/10.1016/j.scienta.2022.111353

Ghosh K S (1996). Effect of spacing and mulching on the growth and yield of ginger (*Zingiber officinale* Rosc.). M.S. Thesis, Department of Horticulture, BAU, Mymensingh pp. 38

Ghosh A K (2011). *Zingiber officinale*: A natural gold. *International Journal of Pharma and Bio Sciences* 2(1): 283-194

Guyot G (1998). Physics of the environment and climate. John Wiley, Chichester, USA, 632p.

Hayden A L, Brigham L A & Giacomelli G A (2004). Aeroponic cultivation of ginger (*Zingiber officinale*) rhizomes. *Acta Horticulturae* 659: 397-402. https://doi.org/10.17660/actahortic.2004.659.52

Islam M A, Rahim M A & Iqbal T M T (2015). Effect of irrigation and mulching on growth and yield of ginger. *Bangladesh Agronomy Journal* 18(1): 27-36. https://doi.org/10.3329/baj.v18i1.25564

Jiang Y & Huang B (2001). Effects of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. *Journal of Experimental Botany* 52(355): 341-349. https://doi.org/10.1093/jexbot/52.355.341

Kandiannan K, Sivaraman K, Thankamani C K & Peter K V (1996). Agronomy of ginger (*Zingiber officinale* Rose.): A review. *Journal of Spices and Aromatic Crops* 5(1): 1-27

Kemper K J (1999). Ginger (*Zingiber officinale*). *Longwood Herbal Task Force* 3: 1-18

Kumar M, Dubey S, Dwivedi P K, Yadav A K & Kumar M (2018). Influence of different mulch materials on vegetative growth and yield of ginger (*Zingiber officinale* Rosc.) under drip irrigation system. *International Journal of Current Microbiology and Applied Sciences* 7(2): 2546-2551. https://doi.org/10.20546/ijcmas.2018.702.310

Lawrence B M (1984). Major tropical spices-ginger (*Zingiber officinale* Rose.). *Perfumer and Flavorist* 9(5): 1-40

Malhotra S K, Cheriyan H, Meena B, Manojkumar K & Sreekumar S (2021). Spices Statistics at a Glance 2021. Directorate of Arecanut and Spices Development, Calicut, Keral

Manjunatha M (2010). Evaluatıon and standardization of different levels of drip irrigation and fertigation wıth mulching on growth, yield and quality of ginger (*Zingiber officinale* Rosc.). MsC Thesis, University of Agricultural Sciences GKVK, Bangalore 153 p.

Meneghelli C M, Krause M R Schmidt J, Colombo J N & Vieira G H S (2020). Performance of ginger crop under different irrigation depths. *Bioscience Journal* 36(3): 783-791. https://doi.org/10.14393/bj-v36n3a2020-47717

Mishra S & Mishra S S (1982). Effect of mulching and weedicides on growth and fresh rhizome yield of ginger. In: The Abstract of Papers Annual Conference of Indian Society of Weed Science 33 p., Bihar, India

Mohd Y S & Sembok W Z W (2020). Effects of irrigation frequency and nutrient solution volume on the growth and yield of ginger (*Zingiber officinale* Roscoe) cultivated using soilless culture system. *International Journal of Scientific and Research Publications* 10(12): 702-706. https://doi.org/10.29322/ijsrp.10.12.2020.p10883

Mokgehle S N, Tesfay S Z, Araya H & du Plooy C P (2017). Antioxidant activity and soluble sugars of African ginger (*Siphonochilus aethiopicus*) in response to irrigation regimen and nitrogen levels, *Acta Agriculturae Scandinavica Section B-Soil & Plant Science* 67(5): 1-11. https://doi.org/10.1080/09064710.2017.1293723

Nair K P (2013). The Agronomy and Economy of Turmeric and Ginger. The Invaluable Medicinal Spice Crops. Elsevier, Amsterdam, 514 c. https://doi.org/10.1007/978-3-030-29189-1_26

Özek G, Demirci F, Özek T, Tabanca N, Wedge D E, Khan S I, Başer K H C, Duran A & Hamzaoglu E (2010). Gas chromatographic–mass spectrometric analysis of volatiles obtained by four different techniques from *Salvia rosifolia* Sm., and evaluation for biological activity. *Journal of Chromatography A* 1217: 741-748. https://doi.org/10.1016/j.chroma.2009.11.086

Pawar H K (1990). Use of plastic as a mulch in scheduling of irrigation to ginger in semi- arid climate. Proceedings of the 11th International Congress on the Use of Plastics in Agriculture, E.99- E.109, New Delhi, India

Prasad P V V, Staggenborg S A & Ristic Z (2008). Impacts of drought and/or heat stress on physiological, developmental, growth, and yield processes of crop plants. In Response of Crops to Limited Water: Understanding and Modeling Water Stress Effects on Plant Growth Processes; American Society of Agronomy: Madison, WI, USA, 2008; pp. 301-355. https://doi.org/10.2134/advagricsystmodel1.c11

Puengphian C & Sirichote A (2008). 6-gingerol content and bioactive properties of ginger (*Zingiber officinale* Roscoe) extracts from supercritical CO_2 extraction. *Asian Journal of Food and Agro-Industry* 1(1): 29-36. https://doi.org/10.17660/actahortic.2010.875.48

Ravindran P N, Nirmal Babu K & Shiva K (2004). Botany and Crop Improvement of Ginger. In Ginger: The Genus Zingiber, Ravindran, P. N. and Nirmal Babu (Eds.). CRC Press, pp. 15-86. https://doi.org/10.1201/9781420023367

Sharizan A, Aminah A, Nor Fadhilah S & Noor I N H E A (2014). Simple method determine of pungency content in local ginger. *Buletin Teknologi MARDI* 6(2014): 77-82

Sonneveld C, Baas R, Nijssen H M C & De Hoog J (1999). Salinity of flower crops insoilless culture. *Journal of Plant Nutrition* 22: 1033-1048. https://doi.org/10.1080/01904169909365692

Supriya G S, Bhoomika H R, Ganapath, M, Nataraj S K & Ravi C S (2020). Effect of different media and nutrients on growth and yield of ginger (*Zingiber officinale* Rosc.) in soilless culture under protected structure. *Journal of Pharmacognosy and Phytochemistry* 9(6): 2004-2007

Tüzel Y, Gül A, Tüze I H & Öztekin G B (2019). Different soilless culture systems and their management. *Journal of Agricultural, Food and Environmental Sciences* 73(3): 7-12. https://doi.org/10.55302/jafes19733007t

Uysal Bayar F, Kaya A S & Çınar O (2021). The effects of different growing conditions on yield and quality parameters of ginger (*Zingiber officinale*). *Mediterranean Agricultural Sciences* 34: 93-100. https://doi.org/10.29136/mediterranean.737788

Weidner S, Kordala E, Brosowska-Arendt W, Karamac M, Kosinska A & Amarowicz R (2009). Phenolic compounds and properties of antioxidants in grapevine roots (*Vitis vinifera* L.) under low-temperature stress followed by recovery. *Acta Societatis Botanicorum Poloniae* 78: 279-286. https://doi.org/10.5586/asbp.2009.036

Yaseer Suhaimi M, Mohamad A M, Mahamud S & Khadzir D (2012). Effects of substrates on growth and yield of ginger cultivated using soilless culture. *Journal of Tropical Agriculture and Food Scienc*e 40(2): 159-168

Yaseer Suhaimi M, Adzemi M A & Wanzaliha W S (2018). Effect of organic substrates on ginger growth, yield and 6-gingerol content cultivated using soilless culture system. *AGROFOR International Journal* 3(1): 122-131. https://doi.org/10.7251/agreng1801122Y

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Transfer Learning based Image Classification of Diseased Tomato Leaves with Optimal Fine-Tuning combined with Heat Map Visualization

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ABSTRACT
Plant disease detection and disease classification at initial stages for sensitive commodities like tomatoes and potatoes is highly mandated as the harvest losses have a direct impact on the price fixation of the vegetables. The most identified limitation in the study of plant pathology is the availability of datasets with visual symptoms that covers all the possible diseases of one crop or plant species. Computer Vision systems and advancements in deep learning-based modeling methodologies gained significant attention in smart farming. It is presumed that the implementation of deep learning algorithms demands a large amount of data to learn complex features automatically and this can pose a challenge for applications with lesser data to achieve generalization. In such cases, Transfer Learning with optimum regularization techniques and fine-tuning mechanisms is the solution to overcome the limitations of smaller datasets. The objective of the work is to develop Tomato Disease Classification System using a transfer learning approach for ten tomato disease classes of the PlantVillage dataset downloaded from the Kaggle platform. Inception V3, a pre-trained transfer learning model is used to classify this multi-class, imbalanced, tomato plant disease based on the leaf symptoms such as dark brown lesions, concentric rings, etc. Geometrical data augmentation is used as a regularization technique to expand the size of the dataset. Significant improvement in the performance metrics is observed when the finetuning is optimum. The training accuracy and validation accuracy of the model before and after fine-tuning are 97.08%, 83.52%, and 98.19%, 95.93% respectively. The average accuracy with augmentation and optimal fine-tuning is 98%. In addition, prediction scores in terms of precision, recall, and F1-score are obtained to visualize the rate of mispredictions across the disease classes. It is observed that the misprediction rate is high across the classes early blight, late blight, and Septoria spot due to similar visual symptoms. Further, activations are used to generate an attention map in the form of Heat Maps which are included as a post-processing step before the classification of the output. Plant Leaf Disease Classification- A web application is deployed using Streamlit Python library and Ngrok services.

Keywords: Image classification, Transfer learning, InceptionV3, Attention maps

1. Introduction

Tomato (Solanum lycopersicum) is the most profitable commercial crop in India. This is a sensitive commodity as the harvest losses have a direct impact on the price fixation of the vegetables. Early disease symptoms appear on leaves and if it is potentially identified it prevents spreading. It is observed that Tomato Leaf Curl Virus has a dreadful impact (Yang et al. 2019) and leads to yield losses. The most identified limitation in the study of plant pathology is the availability of datasets with visual symptoms that covers all the possible diseases pertaining to one crop or plant species. Investigations prove that effective learning happens from intermediate to higher order layers in terms of statistical strength both qualitatively and quantitatively (Bengio et al. 2011). Transfer learning in computer vision applications is based on the previous insight which re-uses a model pre-trained on large image datasets. The knowledge learned from the pre-trained model (Pan et al. 2009) using Transfer Learning (TL) approach works on a lesser number of images (Hussain et al. 2018) and the training time is also reduced substantially. This TL approach lies under two broad categories namely Homogeneous and Heterogeneous transfer learning (Wang et al. 2019) (Zhuang et al. 2020). A homogeneous method aims at reducing the difference between marginal and conditional probabilities of source and target domains and heterogeneous transfer learning (Sukhija et al. 2006). aims at reducing the gap between the feature spaces of the source and the target. The above categories can further be subdivided into a few more classes of approach and one such is feature – based transfer learning. This approach applies to heterogeneous and homogeneous (Feuz & Cook 2015) (Oruba et al. 2014) transfer learning. The heterogeneous Transfer Learning approach is used in this tomato disease classification system.

Frontiers in plant disease classification include the use of typical ML models (Saleem et al. 2020) such as Support Vector Machines (SVM), Decision Tree (DT), K-Nearest Neighbours (K-NN), etc. These models have prominent results with small-scale datasets and the study signifies the need for qualitative and quantitative analysis of disease classification. Since CNN models automatically extract features with no complex pre-processing steps, most of the investigations use either CNN or CNN-enhanced models for disease prediction. Segmentation-based study of lesions and their geometric properties using a sliding window is used to highlight the affected leaf portions. Histogram Equalization, Colour to grey conversion, K-Means clustering, Discrete Wavelet Transforms, and Contouring are also used along with CNN models and SVMs for image classification (Harakannanavar et al. 2022) (Liu et al. 2021). However, misinterpretation occurs as leaves rapid color change occurs due to environmental conditions. SVM classifier for Tea leaves disease detection through feature reduction technique (Hossain et al. 2018) Regional-CNN to segregate weeds from paddy farms based on spatial information (Saleem et al. 2020) semantic segmentation and encoder – decoder model for weed separation (Guo et al. 2018) are also, some of the DL models and methods used in precision agriculture using image recognition. These models however lack quantitative analysis across each disease class such as misprediction rate. A recent study on the classification and prediction of (Jiang et al. 2019) diseased apple plants based on visual symptoms conclude that CNN based Inception Module provides enhanced classification performance on background clutter, occlusion in leaf images, and poor lighting environment (Astani et al. 2022). Inception V3 is a 48- layer dense CNN-based TL model and the model's capability in learning feature representations through the TL approach is studied. It is observed that recognition of cervical cancer cell structures using InceptionV3 (Dong et al. 2020) based on the TL approach has a performance outcome of 98% with better generalization. TL approach-based food images classification usingInceptionV3 has an enhanced classification performance of 98% in 100 epochs (Goh et al. 2021) Another work on face mask detection (Jignesh Chowdary et al. 2020) using InceptionV3 based on the TL approach has a classification accuracy of 99% on masked face dataset. Pulmonary classification using images based on TL and InceptionV3 (Wang et al. 2019) methodology has attained improved sensitivity and specificity scores. With the above insights, the work on the identification of leaf disease symptoms of the Tomato plant using the InceptionV3 model is performed. This multi-class image dataset downloaded from the Kaggle platform covers frequently occurring disease symptoms on tomato leaves such as Bacterial spots, Early Blight, Late Blight, Spider Mites, Target Spots, Tomato Yellow Leaf Curl Virus, and so on. The diseased leaf image dataset is trained using pre-trained weights of InceptionV3 on the target network (Nguyen et al. 2018) (Zhang et al. 2018), and the performance of leaf disease classification is measured. This image classification problem is also deployed as a web-based application using Streamlit python library and Ngrok cloud services. The main contribution of the proposed investigation involves:

- Developing and validating InceptionV3 model through a transfer learning approach to closely suit the real-time field scenarios with uneven distribution of images across disease classes and variable image resolutions.
- Signifying the role of the transfer learning approach for applications with lesser data since it is presumed that deep learning-based investigations demand larger datasets.
- Evaluating heterogeneous transfer learning method between cross domains with appropriate performance indicators.

2. Proposed Methodology

Fine-tuning of a way to transfer learning is performed based on the weights of the previously trained layer to minimize the loss during the training process. The final feature map layer provides the state of the model being trained and it is later fine-tuned and flattened, and the output is fed to the end fully connected layer of the classifier model. A dropout layer is added to the hidden layers for regularization. This form of regularization achieves optimum performance that minimizes the variance in the validation set by preventing dense co-adaptations on training data. NumPy-based argmax prediction is used on random test images to predict the disease class and other performance metrics such as precision, recall, and F1 score. To visualize the performance of the model in the between-layers activation maps as heat maps are being generated for the layer just before the last layer of the model. Th classifier developed is deployed as a web application using Streamlit, a python library. The required libraries are imported, and the application is developed using %%writefile app.py, and the features required on the webpage being defined. The trained model is loaded, to predict the class of output on random images downloaded from the internet. This web application development depends on Ngrok, which enables cross-platform application development, and it is used as a tunnel to the Streamlit port. The technical elaborations of all the key modules are discussed in the forthcoming sections. The flow of the entire process carried out in this paper is shown as block diagram in Figure 1.

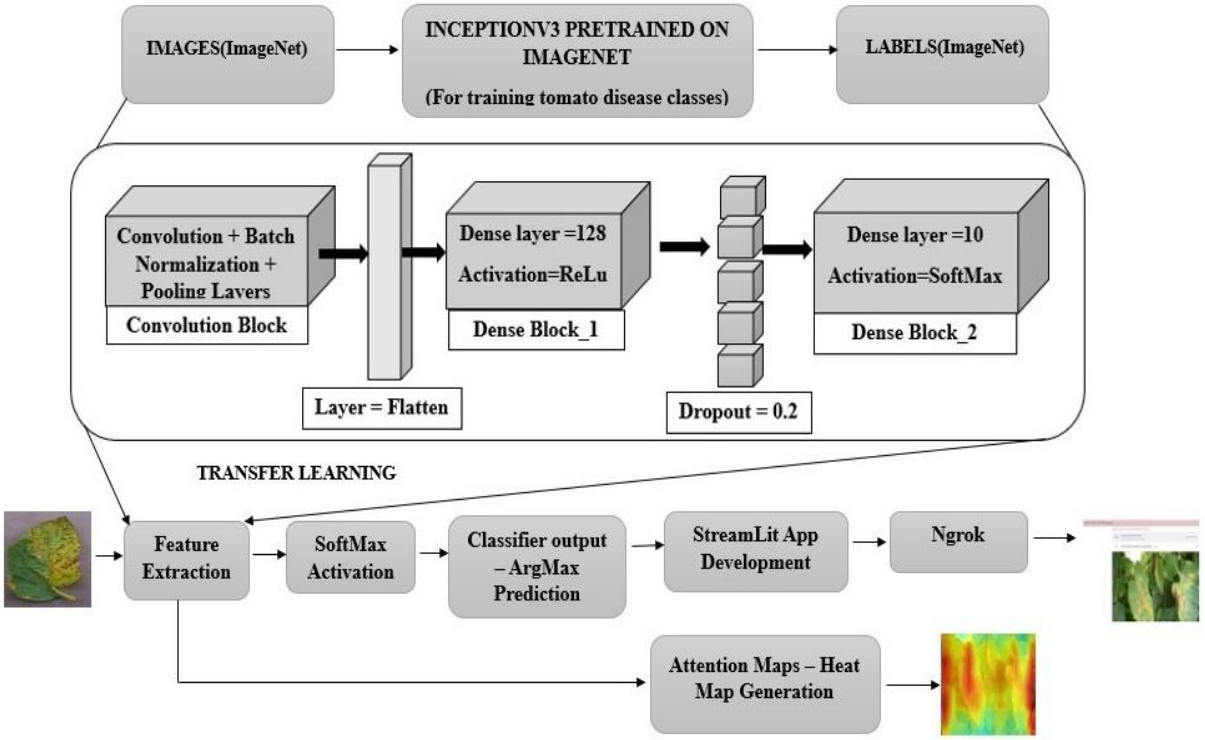


Figure 1- Image Classification of Diseased Tomato Leaves – Methodology

3.1. Convolution Neural Networks (CNN), the base model of Inception V3

3.1. Transfer learning – Notation, definition and approach used

The objective of the transfer learning is to improvise the conditional probability distribution P(Yt|Xt) of the target domain Dt (corresponding learning task is Tt) with the information learned from source domain Ds (corresponding learning task is Ts). Here (Dt≠Ds) and (Tt≠Ts).

In a more generalized way, when two tasks are different the respective label spaces are also different (Yt≠Ys), and its conditional probability distribution (Wiatowski & Bölcskei 2017) is also different. (P(Yt|Xt) ≠P(Ys |Xs)).

In this paper, a feature-based heterogeneous transfer learning approach is performed on the Inception V3 model pre-trained on the ImageNet dataset. The features extracted from the final convolution layer of the InceptionV3 model being utilized for transfer learning. The early convolution layers are frozen as these layers will extract more general or low-level features and as training happens the later layers are focused on specific features.

3.2. InceptionV3 model as image feature extractor

ImageNet is a research resource for the computer vision domain that can label, recognize, and classify around 22,000 object categories. It is one of the largest anthologies of images which is around 10 million with 3.2 million cleanly annotated images as of 2020. This ImageNet fosters the development of many robust and sophisticated models and one such is the Inception V3 model.

The key concept of InceptionV3 is to reduce the computational costs (i.e) the number of deeper networks say filters of size 5x5 or 7x7 into 1x7 and 1x5 smaller filters of asymmetric size as shown in Figure 2 used. A 1x1 filter is used to reduce the channel depth (Szegedy et al. 2016). For example, a 1x1 filter is used to reduce 100 M into 10M by a factor of 10 and thus shrinks the number of channels.

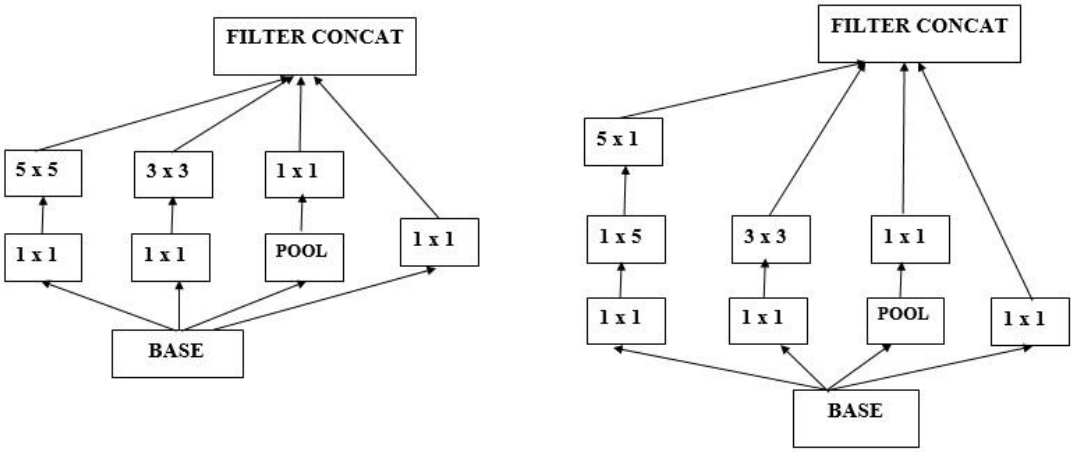


Figure 2- InceptionV3 - Symmetric Factorization and fig(b)InceptionV3 Asymmetric Factorization where 5x5 block is Factorized into 5x1 and 1x5.

The model has attained around 78% accuracy in 170 epochs on the ImageNet dataset and it is a culmination of symmetrical and asymmetrical layers consisting of convolution layers, average and max pooling layers, SoftMax for loss computation, and batch normalization is used throughout for stabilizing the process. In the InceptionV3 factorization model, batch normalization is used in auxiliary classifiers. The role of the auxiliary classifier is it acts as a small CNN inserted during the training and the loss incurred due to the inclusion of this classifier will be added to the main loss. But in Inception V3, this auxiliary classifier acts as a regularizer (Zhang et al. 2019) which aids the loss module to avoid or prevent over-fitting.

The term Accuracy is the ratio of the number of correct predictions to the total number of predictions. This performance parameter assesses the model's ability to function across various classes, the relationship between each parameter, and pattern prediction. The InceptionV3 model has attained 78% Top-1 accuracy on the ImageNet validation dataset. Further, the Top-5 Accuracy is extended to 93.7%. The flip-flop of accuracy is the error parameter and for having the good insight into the model, the accuracy parameter provides a comprehensive perspective. The error rate in terms of accuracy is given as Error Rate= 1-Accuracy. The Top-1 and Top-5 accuracy on ImageNet Validation dataset for various models is given in Table 1.

Table 1- Top-1 and Top-5 accuracy of various models

S.No.	*Model*	*Number of Parameters*	*Top-1 accuracy*	*Top-5 Accuracy*
1	VGG-16	138 357 544	71.8%	90.1%
2	Inception V3	23 851 784	77.9%	93.7%
3	ResNet50	25 636 712	74.9%	92.1%
4	AlexNet	62 378 344	63.3%	84.6%
5	GoogLeNet	23 000 000	74.8%	92.2%
6	InceptionResNetV2	55 873 736	80.3%	95.3%
7	ResNet-152	25 000 000	78.57%	98.2%
8	DenseNet	8 062 504	76.39%	93.34%
9	Xception	22 910 480	79.00%	94.5%

4. Image Dataset, Diseased Leaf Symptoms and Data Augmentation

4.1. Tomato- Diseased image dataset

The Plant Village dataset contains 38 categories of healthy and unhealthy leaf images of apples, potatoes, pepper plants, etc. This paper focuses on 10 categories of Tomato Leaf classes inclusive of the healthy tomato images the train folder consists of 14472 images and the test set consists of 3616 images, both belonging to 10 classes. The distribution of images under 10 categories is shown in Figure 3.

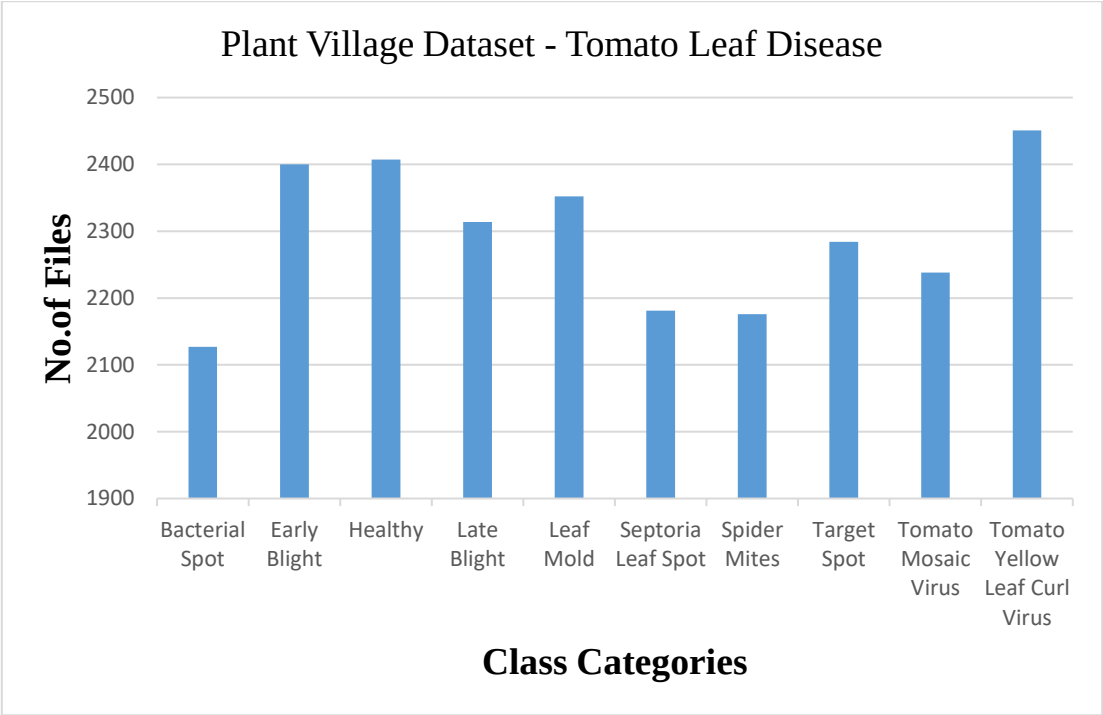


Figure 3- Distribution of Images of PlantVillage Dataset focusing on Tomato Leaf Disease under various classes

4.2. Image data augmentation

Image augmentation is carried out as an initial step to prevent over-fitting and to generalize the model on the output classes (Shorten & Khoshgoftaar 2019). Random and appropriate transformations such as flips, shifts, and zooms are performed on the actual images of the PlantVillage dataset using Keras ImageDataGenerator and it is shown in Figure 4. The data generators act as inputs to the model which also performs normalization operations on the augmented dataset. To create train and test generators flow_from_directory is used.

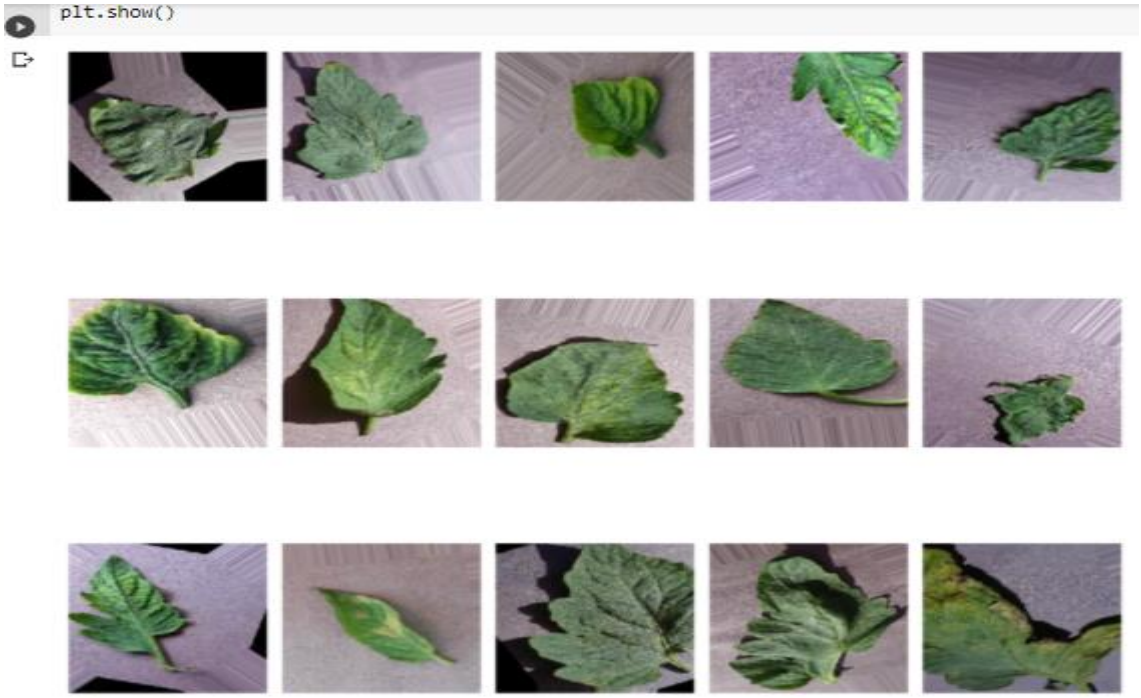
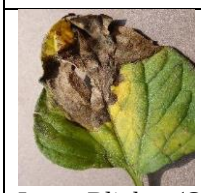
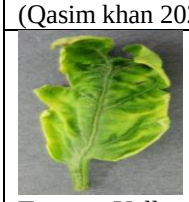


Figure 4- Results of Data Augmentation on a Training Image

4.3. Diseased tomato leaf visual appearance (Symptoms)

Early symptoms appear on the leaves with some changes in foliar structures. Early blight begins with dark brown lesions beneath the leaves and in the case of late blight wet blotches appear it is identified as one of the most destructive diseases since this blight is airborne and the spores spread at a faster rate. Septoria leaf spot and early blight may occur simultaneously. Bacterial spot is another disease with wet large blotches on matured plants and it usually appears on the leaf margins. Thus, each disease has its unique symptoms caused by various pathogens. The 10 broad classes in the image dataset used for the analysis and their visual symptoms are shown in Table 2.

Table 2- Diseased Tomato Leaves and their Visual Symptoms

 Bacterial Spot (Qasim khan 2022)	Scientific Name: *Xanthomonas (X. euvesicatoria, X. gardneri, X. perforans, and X. vesicatoria)* • The spots appear as wet-looking circular areas (Potnis et al. 2015) (Osdaghi et al. 2021) with the scabby wart-like surface. • Initially starts with yellow green discoloration and later turns into brownish red. Since plants with bacterial spots cannot be cured, infected plants should be identified and removed to prevent further spread.
 Early Blight (Qasim khan 2022)	Scientific Name: Alternaria solani • Discoloured spots or rings and a few small brown lesions (Adhikari et al. 2017) appear on the leaves. • Does not affect the fruits initially if it is potentially identified and treated to prevent spreading. • Known as Bull's eye disease as it starts with the appearance of spots and two concentric rings around in matured leaves. These concentric rings can spread to stems and fruits and further the spots can combine and make patches.
 Late Blight (Qasim khan 2022)	Scientific Name: *Phytophthora infestans* (Montagne) Bary • Steady brown spots (Mazumdar et al. 2021) that cover a major part of the fruit • Irregular spots which turn mushy and dark brown or blackish purple lesions • Traces of white fungal growth
 Leaf mold (Qasim khan 2022)	Scientific Name: Passalora fulva • Foliage, pale green, and yellow spots on the upper side of the leaves. The color of the upper side of the leaf is olive green and finally curls. • The leaf will have irregular borders and in severe cases, the spots enlarge, and the fruit is black with rot (Yoshida et al. 2021) in the stem.
 Septoria Leaf Spot (Qasim khan 2022)	Scientific Name: Septoria • A destructive disease where leaf spots with dark brown (Ibrahim 2019) outlines and a greyish centre appears on the lower side of the leaf. • As the disease spreads spots spread and eventually the leaf color turns yellow, later brown, and withers.
 Tomato Yellow Leaf Curl (Qasim khan 2022)	Scientific Name**:** Tomato yellow leaf curl virus (TYLCV) • Stunted growth, leaf size reduces (Yang et al. 2019) (Mariyappan et al. 2013), and leaf curls upwards. • Lead to chlorosis and finally tomato production.

 Spider mites (Qasim khan 2022)	Scientific Name: TETRANYCHUS EVANSI • This polyphagous pest disease lays eggs on the top side of the leaf (Liu et al. 2020). • It lays eggs on the bottom side and the leaf underneath turns yellow or tannin color. • This causes a blotchy color pattern.
 Target spot (Qasim khan 2022)	Scientific name: Corynesporacassiicola • Early symptoms are like early blight and many other fungal spots. • The target spot (Weeraratne et al. 2020) is concentric rings with the innermost brown lesions surrounded by yellow circles. • As the disease spreads the spots enlarge and club with other spots thereby covering a major area of the leaves.
 Tomato Mosaic Virus (Qasim khan 2022)	Scientific Name: Tobamovirus • This pathogenic virus causes irregular ripening of fruits. • The symptoms (Kubota et al. 2003) include leaves with light green and yellow mosaics on the leaves with a prominent reduction of the leaf curvatures. • The slight fern-shaped impaired affect fruit yield by 2 to 23%.

4.4. Performance metrics - Precision, recall, and F1- score

Prediction metrics is significant for evaluating the trained model on multi-class image dataset. RoC Curve is considered efficient for binary classifiers and on balanced datasets. Precision-Recall metrics and F1-Score shown in Table 4, provide better evaluation insights on class predictions irrespective of balanced or imbalanced datasets (Saito & Rehmsmeier 2015) as compared to RoC curves. True Positives (TP), True Negatives (TN), False Positives (FP), and False Negatives (FN) shown in Table 3 are the four basic parameters required for calculating the metrics say Accuracy, Precision, Recall, and F1-Score (Liang et al. 2022) shown in Table 4.

True Positives (TP): The model predicts the positive class when both the Predicted and Actual classes are the same.
True Negatives (TN): The model predicts the negative class when both prediction and Actual are No or Negative.

False Positives (FP): Also known as Type 1 error. Wrong prediction of the Negative class occurs when the actual class is Negative, but prediction outcomes are Positive.

False Negatives (FN): Also known as Type II error. Wrong prediction of the Positive class occurs when actual outcomes are Positive, but prediction results are Negative.

Table 3- Basic Parameters required for Computing Performance Metrics

	Predicted Class			$TPR = \frac{TP}{Actual\ Positive} = \frac{TP}{TP+FN}$
Actual Class		**Class = Positive**	**Class = Negative**	$FNR = \frac{FN}{Actual\ Positive} = \frac{FN}{TP+FN}$
	Class = Positive	**True Positive**	**False Negative**	$TNR = \frac{TN}{Actual\ Negative} = \frac{TN}{TN+FP}$
	Class = Negative	**False Positive**	**True Negative**	$FPR = \frac{FP}{Actual\ Negative} = \frac{FP}{TN+FP}$

Table 4- Performance Metrics used for Model's Evaluation

Performance Metrics	
Accuracy	$\frac{TP+TN}{TP+FP+TN+FN}$
Precision	$\frac{TP}{TP+FP}$
Recall	$\frac{TP}{TP+FN}$
F1 Score	$\frac{2*(Precision*Recall)}{(Precision+Recall)}$

The above values are used to compute True Positive Rate (TPR), False Positive Rate (FPR), True Negative Rate (TNR), and False Negative Rate (FNR). TPR is also known as Sensitivity and TNR is known as Specificity (Altman, Douglas et al. 1994).

- Accuracy: It is the ratio of correctly predicted observations to the total number of observations
- Precision: Ratio of relevant observations to the retrieved observations. It is a measure of quality.
- Recall (Sensitivity): It is the measure of total relevant results correctly classified by the model. It is a measure of quantity.
- F1 Score: It is the weighted average of Precision and Recall. The score is high only when both precision and recall are high.

4.5. Evaluation methodology

While training the data it is quite important to achieve optimum fit over the model. A model initially will learn the correlation between the input samples (x) and the target values(y). This is done by evaluating the model on the test data. In this study, the percentage of data used for training and testing is 70:30 respectively. The evaluation methodology implemented during the model training and validation phase is detailed in the following algorithm.

Tomato Disease Classification Algorithm

Input Data: Tomato Disease Dataset (PlantVillage) images (X, Y); where Y= Predicted class {y/yϵ Tomato Disease Classes}
Pre-processing steps:
Step1: Set the image size s_i and set layer. trainable = False
Step2: Import Keras Sequential Model, Layers, ImageDataGenerators, and optimizer.
Step 3: Perform Geometrical data augmentation on X images and obtain X'
Step4: Gain the information from the source domain D_s and learning task T_s of Inception V3
Step5: Apply the information learned to target Domain D_t with X' and the corresponding target learning task T_t where $D_s \neq D_t$
Step 6: Train the model for N epochs for the augmented dataset X'.
Step 7: Compute the values $y(true)$ and $y(pred)$ for the output classes.
Step 8: Estimate the deviation and apply categorical cross-entropy loss function and RMSProp optimizer on Y predicted classes.
Step 8: Fine-tune the model for M epochs to obtain a balanced fit.

5. Results and Discussion

The entire implementation is performed on Tesla T4 and the features extracted using the InceptionV3 model and Keras API is used for loading the model with pre-trained weights the model compilation requires the parameters optimizer, loss, and performance metrics. The model is optimized on the augmented multi-class dataset using a Categorical cross-entropy loss function, RMSprop optimizer with a learning rate of 0.0001 and decay rate of 1e-6. The values for both the learning rate and decay parameter is fixed based on the over-fitting results obtained during the implementation. Learning rate decay is independent of the optimizer. Model Checkpoint and the callback function is used to save the model and the tensor board information after each epoch. A definite file path is used to save the model in h5 format. The function callbacks list is used in aggregation with model. Fit function. This feature facilitates obtaining performance metrics such as training and validation for both accuracy and loss parameters. The number of epochs is set to 50 and the batch size is 32 for the model training process. The intermediate results during the training process are studied to learn the impact of data augmentation. Optimal fine-tuning is performed for additional 10 epochs based on the accuracy curves. Table 5 shows the training and validation accuracy graphs.

During the training process for the model. Fit function, the steps per epoch during the is fixed based on the length of the training samples divided by batch size and validation steps are fixed based on the length of the validation samples divided by the batch size. The dropout regularization technique of 0.7 was initially used on hidden layers. Since validation accuracy was greater than the training accuracy which depicts the over-fitting scenario, an appropriate and ideal value of 0.5 is used on the hidden layers. NumPy-based argmax and the corresponding prediction scores are initially obtained and the before and after fine-tuning results

with and without image data augmentation are tabulated in Table 6. The model showed improved performance on training and validation accuracy after fine-tuning.

Table 5- Image Classification Intermediate Result – Training and Validation Accuracy

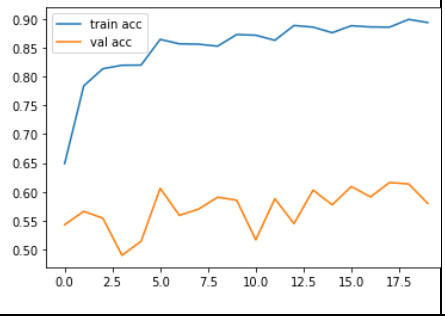
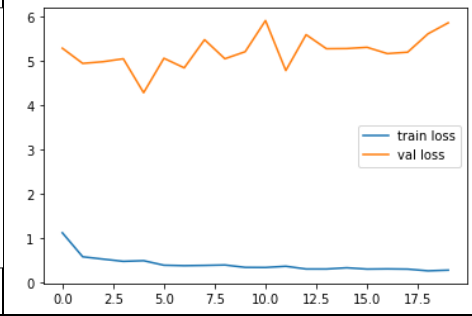
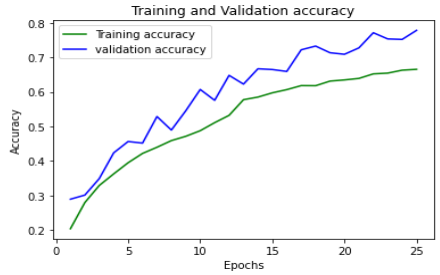
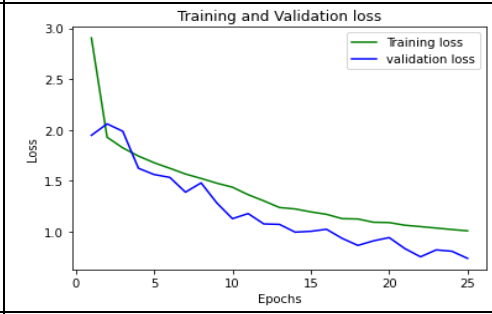
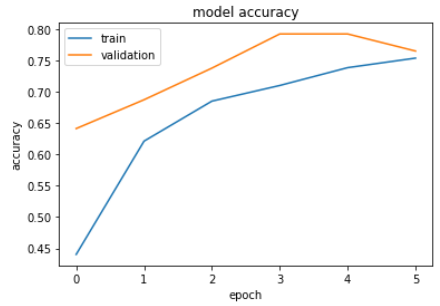
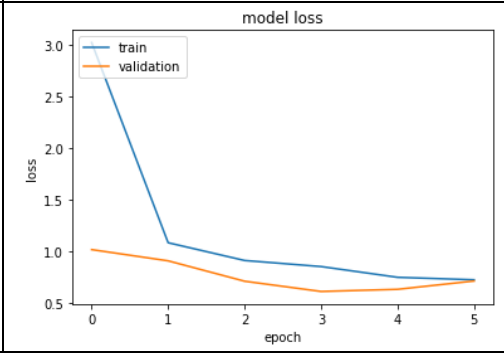
Model	*Accuracy – Training vs Validation*	*Loss – Training vs Validation*	*Observations*
Underfit (Result taken after 17 epochs)			No regularization and data augmentation used. Training accuracy of 80% is obtained. However, validation accuracy is very low.
Balanced fit (Result taken after epoch 25)			Optimal regularization with data augmentation implemented. It is observed that training accuracy is greater than validation accuracy
Over fit (Result taken after epoch 5)			The accuracy declined during the initial epochs due to high regularization and use of all geometrical augmentation techniques on every image.

Table 6- Image Classification Final Results – Before and After Finetuning

Before Finetuning	`[ ]` `# Model evaluation`
	`scores_train = model.evaluate(train_generator,verbose=1)`
	`scores_validation = model.evaluate(validation_generator,verbose=1)`
	`print("Train Accuracy: %.2f%%" % (scores_train[1]*100))`
	`print("Validation Accuracy: %.2f%%" % (scores_validation[1]*100))`
	
	`68/68 [==============================] - 41s 593ms/step - loss: 0.6732 - accuracy: 0.9708`
	`17/17 [==============================] - 10s 605ms/step - loss: 0.9510 - accuracy: 0.8352`
	`Train Accuracy: 97.08%`
	`Validation Accuracy: 83.52%`

After Finetuning	`[ ]` `# Model evaluation`
	`scores_train = model.evaluate(train_generator,verbose=1)`
	`scores_validation = model.evaluate(validation_generator,verbose=1)`
	`print("Train Accuracy: %.2f%%" % (scores_train[1]*100))`
	`print("Validation Accuracy: %.2f%%" % (scores_validation[1]*100))`
	
	`44/44 [==============================] - 365s 8s/step - loss: 0.3768 - accuracy: 0.9819`
	`11/11 [==============================] - 90s 8s/step - loss: 0.4093 - accuracy: 0.9593`
	`Train Accuracy: 98.19%`
	`Validation Accuracy: 95.93%`

In addition, the model's performance on this multi-class dataset is evaluated in terms of TP, TN, FP, and FN. The classification metrics say Accuracy, Precision, Recall and F1 score results are shown in Figure 5. The results show the highest sensitivity, precision, and F1 score is recorded against Tomato Yellow Leaf Curl Virus followed by Tomato Mosaic Virus which leads to high yield loss and require immediate attention to prevent further spreading to other plants.

```
report = metrics.classification_report(true_classes, predicted_classes, target_names=class_labels)
print(report)
```

```
                                                precision    recall  f1-score   support

                       Tomato___Bacterial_spot       0.85      0.89      0.87       340
                         Tomato___Early_blight       0.89      0.51      0.65       384
                          Tomato___Late_blight       0.71      0.88      0.79       370
                            Tomato___Leaf_Mold       0.89      0.88      0.89       325
                    Tomato___Septoria_leaf_spot       0.72      0.89      0.80       349
Tomato___Spider_mites Two-spotted_spider_mite       0.80      0.85      0.83       348
                          Tomato___Target_Spot       0.76      0.79      0.77       365
         Tomato___Tomato_Yellow_Leaf_Curl_Virus       1.00      0.87      0.93       392
                   Tomato___Tomato_mosaic_virus       0.95      0.96      0.96       358
                               Tomato___healthy       0.94      0.92      0.93       385

                                       accuracy                           0.84      3616
                                      macro avg       0.85      0.84      0.84      3616
                                   weighted avg       0.85      0.84      0.84      3616
```

Figure 5- Precision, Recall, and F1 Score

Figure 6. shows a 10x10 confusion matrix generated for 10 disease classes where each row corresponds to the predicted disease class and the column corresponds to the actual class. It is known as a statistical error matrix with results for the 10 classes generated in order (class names as listed in Figure 5) between actual values and predicted values. The matrix element at (2, 2) corresponding to Early blight shows 196 correct predictions, 68 early blight images are misinterpreted as late blight and 47 images are misinterpreted as Septoria leaf spot. Similar mispredictions due to close symptom similarities for all the 10 classes are also shown in Figure 6.

```
cm = confusion_matrix(true_classes, predicted_classes)
print(cm)
```

```
[[301   1   8   0  22   0   7   1   0   0]
 [ 32 196  68  10  47  12  18   0   1   0]
 [  2  12 326   9   5   3   8   0   4   1]
 [  1   2  12 285   9   7   4   0   4   1]
 [  5   6  14   4 310   2   5   0   2   1]
 [  0   0   5   3  10 297  25   0   3   5]
 [  7   3  12   1  11  30 288   0   1  12]
 [  6   1  10   3   9  16   4 340   2   1]
 [  0   0   1   4   3   1   3   0 345   1]
 [  0   0   2   0   3   3  19   0   2 356]]
```

Figure 6 - Precision, Recall, and F1 Score The performance of the proposed method is compared with other models, and it is tabulated in Table 7

Table 7- Comparison with Other Models

Authors	***Proposed Model***	***No. of Images/ Tomato Classes***	***Accuracy***	***Mechanism used***	***Performance Metrics***	***Augmentation***	***Fine-tuning***
Jiang Ding et al. 2020	Resnet-50	3000 images/3 classes	98	Leaky ReLu and 11 x 11 convolution layer	Accuracy	Nil	Nil
Al-gaashani, Mehdhar SAM 2022	MobileNetV2 and NASNetMobile	1152 images / 6 classes	97%	Concatenated features of both classifiers used.	Accuracy	Nil	Nil
Agarwal, Mohit, Abhishek Singh et al. 2020	Modified CNN architecture	10 classes	91.2	Augmentations on Modified architecture	Accuracy	✓	Nil
Basavaiah, Jagadeesh, et al. 2020	Decision tree classifier	9 classes	90	-	Accuracy	Nil	Nil
	Random forest classifier		94	-	Accuracy	Nil	Nil
Rangarajan, Aravind Krishnaswamy et al. 2018	VGG16	13 262	97.29	Accuracy dropped as weight and bias learning rate is increased.	Accuracy	Nil	✓
	AlexNet	13,262	97.49	Reduced execution time but fine-tuning decreased performance	Accuracy	Nil	✓
Our Method Transfer Learning Approach	**InceptionV3**	**18 088/10 classes**	**98**	**Optimal Finetuning and Regularization Method improved the performance**	**Accuracy, Precision, Recall, F1-Score**	✓	✓

Attention maps aid in visualizing the models learning traits on the extracted high- and low-level features of an image. These interpretable attention maps provide us an insight into the local fine-grained details which are significant in enhancing the model's accuracy. Attention heat maps are generated using Activation Mapping. For a given input image, obtain the output activations (Zagoruyko & Komodakis 2016) in the form of (H', W', C) where C represents the number of channels. To obtain the spatial attention map g which focuses on the activations within the same layer, convert the image into (H', W'). This attention map is applied to the last convolution layer. The activation A is given as, $A_i = A[;;i]$. Here I represents the index values of the channel dimension. Then the evaluation (Zhou et al. 2016) is given as

$$g(A) = \sum_{i=1}^{C} |A_i| \quad (1)$$

For IncepionV3 used in plant disease classification, the heat maps are generated for the last convolution layer and a test image of class early blight is used for which the visual leaf disease symptom is very poor. This scenario requires insight into the model's interpretation in the between layers. Compared to the initial heat maps the later heat map images shown in Figure 7 focus more on the weak early blight symptoms and the leaf boundaries as the actual image has shadowing effects.

To examine the generalization of the model's performance, a random image, is downloaded from google images, and heat map visualization is shown in Figure 8. The last rows of the heat maps are centered on the visual symptoms and the leaf boundaries.

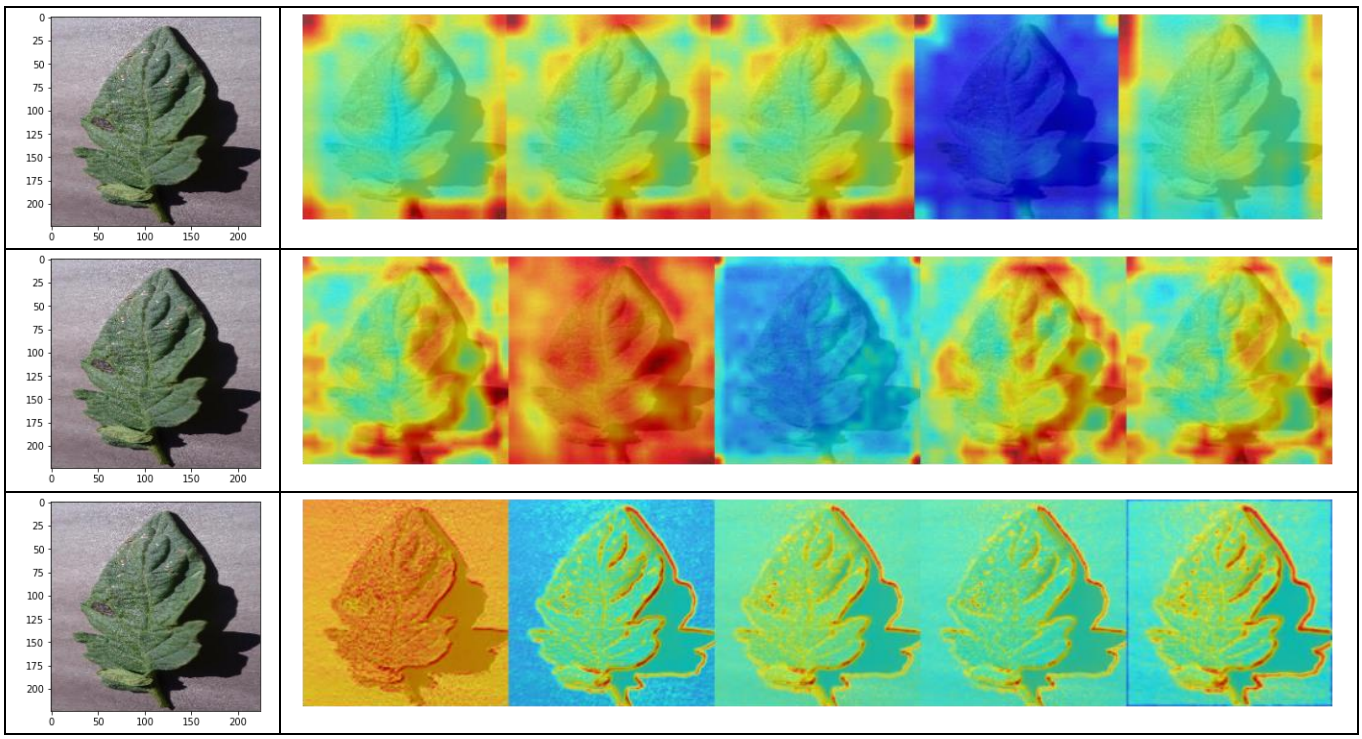


Figure7- Heat Map Generations on a Test Image

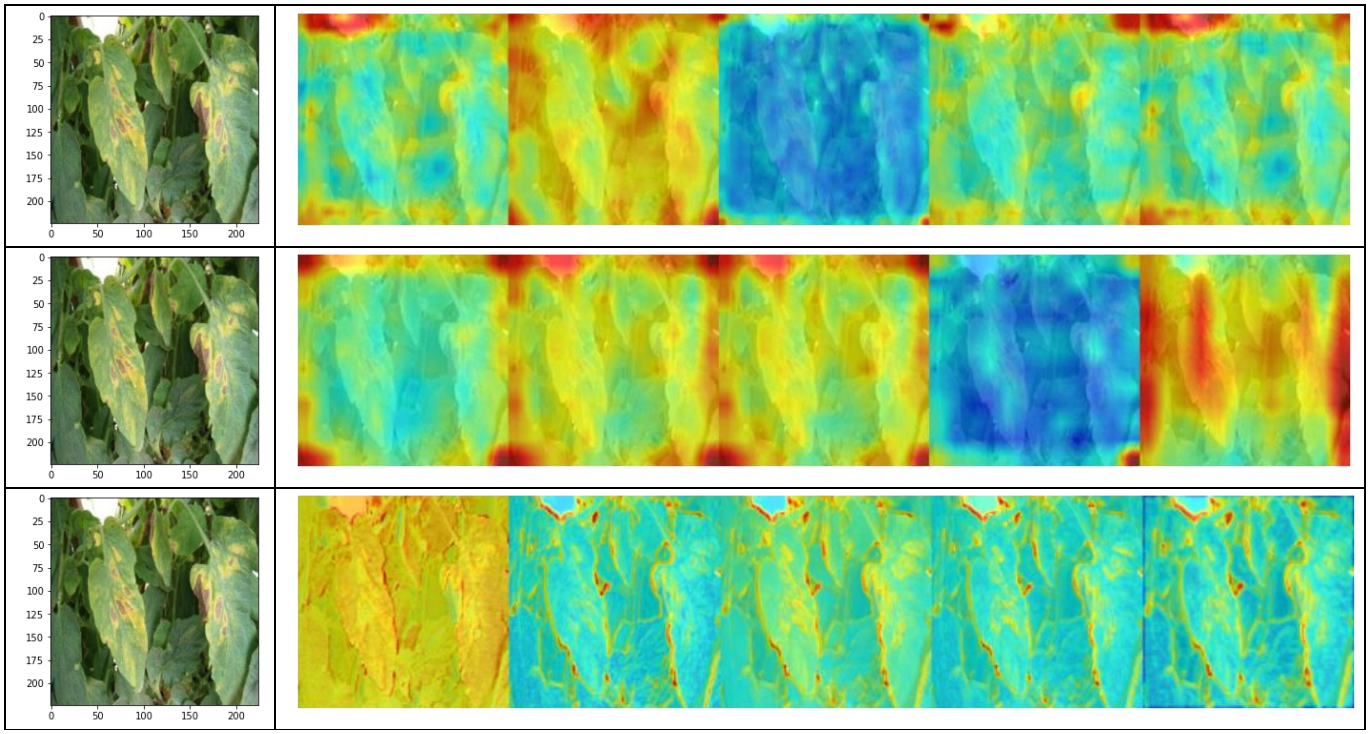


Figure 8- Heap Map Generations on a Random Image

The same random image used in Figure 7 is used as a test image for the web application developed using the Streamlit python library. Ngrok provides a secure tunnel for which an authentication token shown in Figure 7 is obtained and the. yml configuration file is available.

The Ngrok is tunneled to port number 8501. The required web page application features are written using %%writefile app.py and is shown in Figure 9.

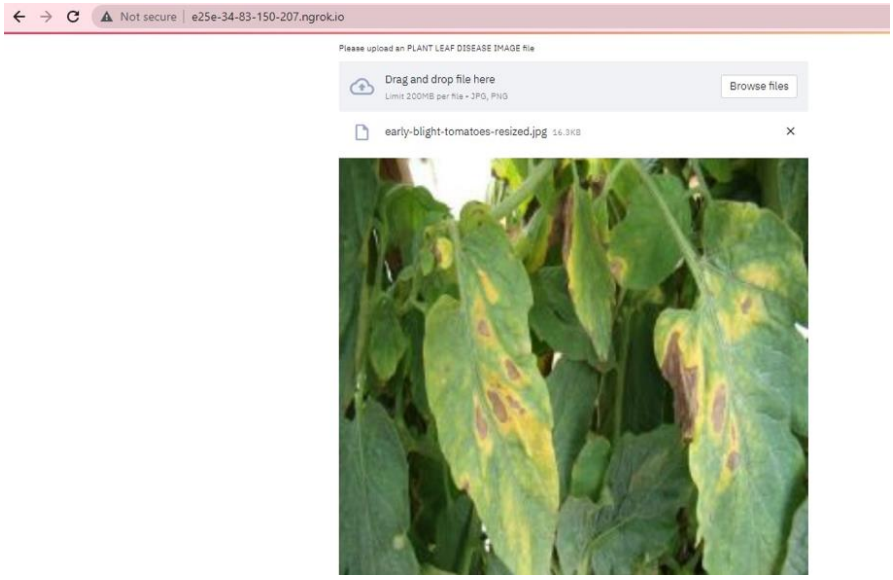


Figure 9- Plant Leaf Disease Classification: Web-based Application Showing Random Images Uploaded from the Internet

6. Conclusions

This paper investigates on the effectiveness of the Transfer Learning approach for classification systems with limited data for cross- domains. The investigation and comparison with related works proves InceptionV3's classification potential is enhanced for multi-class, slightly imbalanced dataset with appropriate regularization and fine-tuning mechanisms. The classification report and confusion matrix show that the highest classification scores are reported for the disease classes Tomato Yellow Curl Virus and Mosaic Virus. Maximum mispredictions due to similar visual symptoms are recorded between Early Blight, Late Blight, and Septoria leaf spot. The overall training and validation accuracy before and after fine-tuning are 97.08%, 83.52% and 98.19%, 95.93% respectively. The results also show that implementing sophisticated methods to improve accuracy during the training process led to over-fitting situations. To evaluate and understand this kind of scenario and the model's insight during the process of training requires visualization attention such as Heat map generation. The classifier model is deployed as a web application. As the next step in this process, the attention mechanisms must be further examined by combining convolution mechanisms and transformer encoders such as Vision Transformer for the areas like image classification and image captioning.

Data availability statement

The 10 tomato disease classes are downloaded from the Kaggle Platform (Link: https://www.kaggle.com/datasets/cookiefinder/tomato-disease-multiple-sources). This is a publicly available dataset with the license CC0: Public Domain.

Disclosure statement

The authors received no specific funding for this study and declare that they have no conflicts of interest to report with the present study.

References

Adhikari P, Oh Y & Panthee D R (2017). Current status of early blight resistance in tomato: an update. International journal of molecular sciences 18(10): 1-22 https://doi.org/10.3390/ijms18102019

Agarwal, Mohit, Abhishek Singh, Siddhartha Arjaria, Amit Sinha & Suneet Gupta (2020). ToLeD: Tomato leaf disease detection using convolution neural network. Procedia Computer Science 167(2020): 293-301. https://doi.org/10.1016/j.procs.2020.03.225

Al-gaashani, Mehdhar S A M, Fengjun Shang, Mohammed S A Muthanna, Mashael Khayyat & Ahmed A. Abd El-Latif (2022). Tomato leaf disease classification by exploiting transfer learning and feature concatenation. IET Image Processing 16(03): 913-925. https://doi.org/10.1049/ipr2.12397

Altman, Douglas G & J Martin Bland (1994). Diagnostic tests. 1: Sensitivity and specificity. BMJ: British Medical Journal 308(6943): 1552. doi: 10.1136/bmj.308.6943.1552.

Astani M, Hasheminejad M & Vaghefi M (2022). A diverse ensemble classifier for tomato disease recognition. Computers and Electronics in Agriculture, 198, 107054. https://doi.org/10.1016/j.compag.2022.107054

Basavaiah, Jagadeesh & Audre Arlene Anthony (2020). Tomato leaf disease classification using multiple feature extraction techniques. Wireless Personal Communications 115(1): 633-651

Bengio Y, Bastien F, Bergeron A, Boulanger–Lewandowski N, Breuel T, Chherawala Y & Sicard G (2011). Deep learners benefit more from out-of-distribution examples. In Proceedings of the Fourteenth International Conference on Artificial Intelligence and Statistics. JMLR Workshop and Conference Proceedings, pp. 164-172

Dong N, Zhao L, Wu C H & Chang J F (2020). Inception v3 based cervical cell classification combined with artificially extracted features. Applied Soft Computing 93: 106311. https://doi.org/10.1016/j.asoc.2020.106311

Feuz K D & Cook D J (2015). Transfer learning across feature-rich heterogeneous feature spaces via feature-space remapping (FSR). ACM Transactions on Intelligent Systems and Technology (TIST) 6(1): 1-27. https://doi.org/10.1145/2629528.

Goh A M & Yann X L (2021). Food-image Classification Using Neural Network Model. Int. J. of Electronics Engineering and Applications 9(3): 12-22

Guo Y, Liu Y, Georgiou T & Lew M S (2018). A review of semantic segmentation using deep neural networks. International journal of multimedia information retrieval 7(1): 87-93. https://doi.org/10.1007/s13735-017-0141-z

Harakannanavar S S, Rudagi J M, Puranikmath V I, Siddiqua A & Pramodhini R (2022). Plant leaf disease detection using computer vision and machine learning algorithms. Global Transitions Proceedings 3(1): 305-310. https://doi.org/10.1016/j.gltp.2022.03.016.

Hossain S, Mou R M, Hasan M M, Chakraborty S & Razzak M A (2018). Recognition and detection of tea leaf's diseases using support vector machine.2018 IEEE 14th International Colloquium on Signal Processing & Its Applications (CSPA): 150-154. doi: 10.1109/CSPA.2018.8368703

Zhang H, Zhang L & Jiang Y (2019). Overfitting and Underfitting Analysis for Deep Learning Based End-to-end Communication Systems. 11th International Conference on Wireless Communications and Signal Processing 1-6. doi: 10.1109/WCSP.2019.8927876

Hussain M, Bird J J & Faria D R (2018). A study on cnn transfer learning for image classification. In UK Workshop on computational Intelligence UK, Springer, Cham pp. 191-202. https://doi.org/10.1007/978-3-319-97982-3_16

Ibrahim H (2019). Susceptibility Studies on Two Varieties of Tomato (Lycopersicon esculentum) to Fungal Leaf Spots. EAS Journal of Nutrition and Food Sciences 1(1): 11-15. doi: 10.36349/easjnfs. 2019.v01i01.003

Jiang P, Chen Y, Liu B, He D & Liang C (2019). Real-time detection of apple leaf diseases using deep learning approach based on improved convolutional neural networks. IEEE Access 7(1): 59069-59080. doi: 10.1109/ACCESS.2019.2914929

Jiang, Ding, Fudong Li, Yuequan Yang & Song Yu (2020). "A tomato leaf diseases classification method based on deep learning." Chinese control and decision conference (CCDC) pp. 1446-1450. IEEE, 2020. DOI: 10.1109/CCDC49329.2020.9164457

Jignesh Chowdary G, Punn N S, Sonbhadra S K & Agarwal S (2020). Face mask detection using transfer learning of inceptionv3. In Big Data Analytics: 8th International Conference, BDA 2020, Sonepat, India, December 15–18, 2020, Proceedings 8: 81-90. Springer International Publishing. https://doi.org/10.1007/978-3-030-66665-1_6

Kubota K, Tsuda S, Tamai A & Meshi T (2003). Tomato mosaic virus replication protein suppresses virus-targeted posttranscriptional gene silencing. Journal of virology, 77(20): 11016-11026. https://doi.org/10.1128/JVI.77.20.11016-11026.2003

Liu J, Chafi R, Legarrea S, Alba J M, Meijer T, Menken S B & Kant M R (2020). Spider mites cause more damage to tomato in the dark when induced defenses are lower. Journal of chemical ecology, 46, pp.631-641. https://doi.org/10.1007/s10886-020-01195-1

Liu J & Wang X (2021). Plant diseases and pest detection based on deep learning: a review. Plant Methods 17(1): 1-18. https://doi.org/10.1186/s13007-021-00722-9

Liang, Jingsai. Confusion Matrix: Machine Learning." POGIL Activity Clearinghouse 3(4) (2022). https://pac.pogil.org/index.php/pac/article/view/304

Mariyappan D, Ganapathy T & Ramalingam R (2013). Survey and incidence of tomato leaf curl virus in Tamil Nadu.Pestology, 37, (7), pp. 10-15

Mazumdar P, Singh P, Kethiravan D, Ramathani I & Ramakrishnan N (2021). Late blight in tomato: insights into the pathogenesis of the aggressive pathogen Phytophthora infestans and future research priorities. Planta, 253(6), pp.1-24. https://doi.org/10.1007/s00425-021-03636-x

Nguyen L D, Lin D, Lin Z & Cao J (2018). Deep CNNs for microscopic image classification by exploiting transfer learning and feature concatenation. In 2018 IEEE International Symposium on Circuits and Systems (ISCAS). pp 1-5. doi: 10.1109/ISCAS.2018.8351550

Osdaghi E, Jones J B, Sharma A, Goss E M, Abrahamian P, Newberry E A, Potnis N, Carvalho R, Choudhary M, Paret M L & Timilsina S (2021). A centenary for bacterial spot of tomato and pepper. Molecular Plant Pathology, 22(12): 1500-1519. doi: 10.1111/mpp.13125

Pan S J & Yang Q (2009). A survey on transfer learning. IEEE Transactions on knowledge and data engineering 22(10): 1345-1359. doi:10.1109/TKDE.2009.191

Potnis N, Timilsina S, Strayer A, Shantharaj D, Barak J D, Paret M L, Vallad G E & Jones J B (2015). Bacterial spot of tomato and pepper: diverse X anthomanias species with a wide variety of virulence factors posing a worldwide challenge. Molecular plant pathology, 16(9): 907-920. https://doi.org/10.1111/mpp.12244

Qasim Khan (2022), Tomato Disease Multiple Sources, CC0: Public Domain https://www.kaggle.com/datasets/cookiefinder/tomato-disease-multiple-sources

Rangarajan, Aravind Krishnaswamy, Raja Purushothaman & Anirudh Ramesh (2018). "Tomato crop disease classification using pre-trained deep learning algorithm." Procedia computer science 133 (2018): 1040-1047. https://doi.org/10.1016/j.procs.2018.07.070

Saito T & Rehmsmeier M (2015). The precision-recall plot is more informative than the ROC plot when evaluating binary classifiers on imbalanced datasets. PloS one, 10(3), p.e0118432. https://doi.org/10.1371/journal.pone.0118432

Saleem M H, Potgieter J & Arif K M (2020). Plant disease classification: A comparative evaluation of convolutional neural networks and deep learning optimizers. Plants 9(10): 1319. https://doi.org/10.3390/plants9101319

Saleem M H, Khanchi S, Potgieter J & Arif K M (2020). Image-based plant disease identification by deep learning meta-architectures. Plants 9(11): 1451. https://doi.org/10.3390/plants9111451

Shorten C & Khoshgoftaar T M (2019). A survey on image data augmentation for deep learning. Journal of Big Data, 6(1):1-48. https://doi.org/10.1186/s40537-019-0197-0

Sukhija S, Krishnan N C & Singh G (2016). Supervised Heterogeneous Domain Adaptation via Random Forests. In Proceedings of the Twenty-Fifth International Joint Conference on Artificial Intelligence, pp. 2039-2045. http://localhost:8080/xmlui/handle/123456789/2879

Szegedy C, Vanhoucke V, Ioffe S, Shlens J & Wojna Z (2016). Rethinking the inception architecture for computer vision. In Proceedings of the IEEE conference on computer vision and pattern recognition, pp. 2818-2826.

Wang C, Chen D, Hao L, Liu X, Zeng Y, Chen J & Zhang G (2019). Pulmonary image classification based on inception-v3 transfer learning model. IEEE Access, 7(1): 146533-146541. DOI: 10.1109/ACCESS.2019.2946000

Wang K, Gao X, Zhao Y, Li X, Dou D & Xu C Z (2019). Pay attention to features, transfer learn faster CNNs. In International Conference on Learning Representations, pp. 1-14

Weeraratne W A P G, Wijerathne W M S D K & Dissanayake D M K K (2020). Occurrence of target spot of tomato caused by Corynespora cassiicola in Sri Lanka. *Ceylon Journal of Science* 49(5): 397-400. http://doi.org/10.4038/cjs.v49i5.7807

Wiatowski T & Bölcskei H (2017). A mathematical theory of deep convolutional neural networks for feature extraction. IEEE Transactions on Information Theory, vol. 64, no. 3. pp. 1845-1866. DOI: 10.1109/ACCESS.2020.3024111

Yang Y, Liu T, Shen D, Wang J, Ling X, Hu Z, Chen T, Hu J, Huang J, Yu W & Dou D (2019). Tomato yellow leaf curl virus intergenic siRNAs target a host long noncoding RNA to modulate disease symptoms. PLoS pathogens, 15(1): 1-22. https://doi.org/10.1371/journal.ppat.1007534

Yoshida K, Asano S, Sushida H & Iida Y (2021). Occurrence of tomato leaf mold caused by novel race 2.4. 9 of Cladosporium fulvum in Japan. *Journal of General Plant Pathology* 87(1): 35-38. https://doi.org/10.1007/s10327-020-00963-x

Zagoruyko S & Komodakis N (2016). Paying more attention to attention: Improving the performance of convolutional neural networks via attention transfer. conference paper at ICLR, arXiv preprint arXiv:1612.03928. https://doi.org/10.48550/arXiv.1612.03928

Zhang H, Zhang L & Jiang Y (2019). Overfitting and underfitting analysis for deep learning based end-to-end communication systems. In 2019 11th International Conference on Wireless Communications and Signal Processing (WCSP): 1-6. DOI: 10.1109/WCSP.2019.8927876

Zhang K, Wu Q, Liu A & Meng X (2018). Can deep learning identify tomato leaf disease? Advances in Multimedia, Special issue pp. 1-10. https://doi.org/10.1155/2018/6710865

Zhou B, Khosla A, Lapedriza A, Oliva A & Torralba A (2016). Learning deep features for discriminative localization. In Proceedings of the IEEE conference on computer vision and pattern recognition, pp. 2921-2929

Zhuang F, Duan K, Xi D, Zhu Y, Zhu H, Xiong H & He Q (2020). A comprehensive survey on transfer learning. Proceedings of the IEEE 109(1): 43-76. DOI: 10.1109/JPROC.2020.3004555

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Validity of the Phillips Curve in the Agricultural Sector and Asymmetric Effects: The Case of Türkiye

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ABSTRACT
Although the employment share of agriculture, which has historically been the main job creator sector, has decreased over time, it still plays an essential role in newly industrializing countries such as Türkiye. However, the depth of the decline in employment is due to the economic impact of agriculture rather than its share of GDP. One way to measure this effect is to adapt the Phillips curve (PC) for the agricultural sector. Therefore, the aim of this study is to investigate the validity of the Phillips curve in the Turkish agricultural sector and the short- and long-term linear and non-linear relationships caused by shocks on unemployment-inflation. The study was conducted via the augmented Dickey-Fuller, Phillips-Perron, and Zivot-Andrews unit root tests as well as the autoregressive distributed lag model (ARDL) and the nonlinear autoregressive distributed lag model (NARDL) via the Turkish Statistical Institute's (TURKSTAT) 2014Q1-2021Q3 dataset. The cointegration coefficient was negative, yet statistically significant. Also, the short-term imbalances were eliminated, and the system converged to the equilibrium values in the long-run with significant fluctuations. The long-run negative cointegrated relationship and PC validity in negative shocks are the most significant results of the study.

Keywords**:** Inflation, Unemployment, ARDL-NARDL, Negative shocks, Negative long-run cointegration

1. Introduction

Inflation and unemployment are two economic problems that are closely associated and thought to have the most mutual interaction among the concepts of growth, unemployment, and inflation that are accepted as the most three essential indicators in macroeconomics literature. In the literature, the approach to estimate the inflation-unemployment relationship via the Phillips curve method has a wide research base. Despite the decades that have passed since the original Phillips curve was created, the relationship that has developed continuously and remained as one of the main arguments of policy makers still maintains its validity today. Therefore, governments are often keen to determine the optimal combination of unemployment and inflation. In this regard, in Türkiye, where high inflation has been experienced for many years, combating inflation and unemployment has been one of the main objectives of government programs. However, this relationship did not receive critical attention until the astronomical increase in agricultural product prices experienced in the recent period in Türkiye, and even scientific studies in this area remained considerably penurious. The main motivation for this study is the curiosity about whether the Philips curve, which has obvious practical importance yet has barely been studied for the agricultural sector in the literature, is valid in the recent era. It is also essential to carry out both linear and non-linear studies in agriculture in terms of revealing the effects of shocks and having a different policy-developer perspective to reduce unemployment in the agricultural sector.

Thus, the purpose of this study is to investigate the unemployment-inflation relationship through the validity of the Phillips curve and asymmetric effects between 2014 and 2021 in Türkiye when food price inflation became more noticeable. In this context, unemployment in the agricultural sector and the rates of price increases agricultural products were compiled from the Turkish Statistical Institute (TURKSTAT) database and the relationship between them was analyzed by employing the nonlinear autoregressive distributed lag (NARDL) and autoregressive distributed lag (ARDL) tests.

The nexus of price changes, unemployment, and the level of production discussions that started with Humphrey (1986) gained a different basis with the empirical evaluation of the subject in the 20th century. In 1926, Irving Fisher found a strong causal relationship between price changes and employment due to the costs are lagged adjusted to changes in prices. However, Tinbergen (1936), in the first researcher-conducted econometric study, determined causality from unemployment to wage inflation due to demand pressure in the labor market. Moreover, the relationship between wage inflation and unemployment rate was diagramed by A. J. Brown (1955), but the stable tradeoff was first plotted by P. Sultan (1957).

A.W. Phillips conducted a study in 1958 to determine the relationship between nominal wages and unemployment rates in the British economy. He calculated via simple regression the existence of a long-run, negative, and non-linear static relationship between money wages and unemployment rate in three different periods (1861-1913, 1913-1948, and 1948-1957) in which there were structural breaks. In other words, he, like his predecessors, examined the relationship between nominal wages and unemployment rate and, like other researchers, detected the inverse and non-linear relationship. However, Phillips differed from their findings not in that he proved or drew the relationship with the data, but that he found the high frequency and inversely correlated relationship was stable (Frisch 1977). Two years after the publication, Samuelson & Solow (1960), slightly differentiating Phillips' work, and examined the relationship between inflation and unemployment variables for the United States. The designed equation was as follows:

$\pi_t = \propto -\gamma(U_t - U^*)$ π_t - current inflation, $\propto$ - position of the curve

Where; γ - coefficient is the ratio of inflation to the deviation of unemployment from the natural rate, Ut - current unemployment rate, and U^* - the natural rate of unemployment. A negatively sloped curve that is referred to as the Phillips curve in the literature that presents the negative relationship between inflation and unemployment was structured (Samuelson & Solow 1960).

Until the late 1960s, this approach was used and considered quite frequently in estimating how wages and prices might be used to offset each other since orthodox Keynesian economists thought that the curve represented a stable relationship valid in both the short and long run (Akkuş 2012). Lipsey (1960) found a negative relationship between monetary wages and the unemployment rate in the analysis based on the relationship between excess demand in the labor market and money wages and the same surplus and unemployment rate in the same market. So, according to this type of Keynesian thought (that was surprisingly in favor of the Samuelson-Solowian framework rather than the original Phillipsian approach), a realistic and permanent decrease in unemployment could only be achieved by putting up with an increase in inflation levels. The reason why the Phillips curve was easily accepted by the orthodox Keynesian view was that this view believed in the strong explanatory power of the investment-saving and liquidity preference-money supply (IS-LM) model including the concepts of inflation and market price (Mankiw 1990).

However, with the reduction of world oil supply in the early 1970s, an unprecedented rise in the price of oil and petroleum products caused the production volume to decline due to scarcity of resources as well as the shrinking demand due to prices. The decline in production volume decreased the demand for labor, and layoffs increased. Hence, the validity of the Phillips curve was weakened by the stagflation and was subjected to many criticisms. The theoretical and practical inadequacy of the Phillips curve has led economists to seek new economic solutions. M. Friedman (1968) and E. S. Phelps (1967) made the initial criticisms as well as new interpretations. Independently of each other, they included adaptive expectations in the Phillips' literature and caused a paradigm shift in the field (Mankiw 1990). The essential criticism of these two scholars is that the Phillips curve does not take expectations into consideration, but monetarists accept that inflation expectations depend on past inflation rates (Dornbusch et al. 2016). In a wage bargain, when the expected and actual inflation rates are initially the same, the Phillips curve will shift upward in the short run since a move to reduce the unemployment rate will push the expected inflation above the actual inflation rate, and this situation will continue until the unemployment rate is equal to the equilibrium unemployment rate. Therefore, the long-run curve will always remain vertical at the natural rate of unemployment, and unemployment remains constant in the long run at its natural rate (Gordon 2018). The confirmation of the monetarists' propositions in the 1970s caused governments to rely less on the curve.

On the other hand, new classical economists reject the adaptive expectation hypothesis, which is based on the assumption that economic decision-makers fail and constantly repeat their mistakes when analyzing the information they have, arguing that the hypothesis is not based on the micro-foundations of economics (Tokatlıoğlu & Özturk 2015). They believe in the optimization of individuals and the clearing of markets since their goal was to rebuild macroeconomics starting from the microeconomic primitives of choice and technology (Mankiw 1990). According to their rational expectations theory, economic agents use all the information available, not just that from previous periods (Muth 1961), because it is uncertain if the previous period information flow will lead to definite future results (Parkin 2011). Therefore, individuals benefit from learning from each mistake and not making the same mistake again. Hence, Lucas (1972) included rational expectations in the analysis because expected inflation is a mix of the information that economic agents had in the previous period and the information they have in the current period ($\dot{P}_t^e = (\dot{P}t\,|\Omega t - 1)$). This study presents this theoretical background in depth in order to clarify which school's view in the literature is closer to this study's findings while testing the validity of the Phillips curve for the agricultural sector.

The Phillips curve comes to the fore during unemployment-inflation trade-off discussions in agriculture, which is a sector that is very sensitive to increases in the general level of prices and changes in the underproduction capacity. In fact, the original Phillips curve developed its analysis from agricultural production. The Phillips curve model suggests that the increase in the general level of prices resulting from the increase in domestic agricultural product prices has no effect on the nominal wage change rates. Because changes in imports and food prices affect the cost of living, this is reflected in wages. When imports and food prices are excluded from the system, the increase in the rate of change in monetary wages in years when the retail price index is high appears to be cost related. In this context, the only exceptions to the monetary wage-unemployment relationship are imports and the price-wage spiral (Phillips 1958).

When agriculture is evaluated in light of this information, many factors from imports to the price-wage spiral affect the sector. To define the agricultural sector as it is most widely accepted in the literature, it is a labor-intensive field that is shaped according to geographical conditions and that has intensive use of inputs and tools diversified depending on technology. Tools such as tractors or harvesters, which replaced the ox-scythe duo used in the past to cultivate-harvest agricultural land, also left the industry dependent on gasoline-derived energy. This state of dependency, which is seen not only in production but also in the transportation of the final products, created fluctuations in energy prices, and this cost increase in the prices of agricultural products caused an upward movement. Moreover, the intermediate goods are affected by exchange rate increases and the high level of indirect taxes, which are two other factors in the production cost increase. The impact of these cost increases on the level of coverage of wages and product prices is disturbing in that they directly affect three goods groups for public consumption: food, rent, and transportation. Within the researched years, food and transportation expenditures in Türkiye are the first two expenditures in the consumer price index (CPI) (TURKSTAT 2022). In reality, the increase started in 2002, and thereafter, the prices of food products in international markets have increased continuously due to extreme climate changes. The fact that the imbalance between supply and demand is not unique to Türkiye has led to discussions in the foreign literature that food prices will compete with or even surpass oil prices in the future (eg. Baumeister & Kilian 2013). Additionally, with "the abolition of import taxes on agriculture and/or the reduction of the import tax rate" (Martin 2011) in Türkiye, many agricultural products cannot be produced profitably to meet domestic consumption. Also the synthetic price increases of the sellers in the Turkish food sector, which has an oligopolistic structure, adversely affect the entire supply process of agricultural products from the field to the table.

Fundamentally, since agricultural activities mostly involve domestic or seasonal work, labor contracts are short and lead to post-harvest unemployment. Due to the structural transformation experienced after 1980 in Türkiye, the sectoral structure of employment has changed, and the agricultural sector has regressed. This regression continued and deepened in the 1990s and 2000s. On the other hand, the declines in the GDP and employment in the agricultural sector become ordinary when the development level of the country increases. However, the search for profit above the cost increases has also caused the people engaged in agriculture to migrate and give up the land and goods they used in the agricultural sector. This process played an essential role in the increase in the general level of prices due to the shortage of supply. Therefore, the expected situation for countries such as Türkiye is that as Phillips (1958) stated, the analysis cannot be valid in an agricultural sector where imported products, synthetic price increases, and wage increases due to cost increases are intense. Yet, in the absence of these obstacles, the curve becomes valid.

As mentioned in the preceding section, Tinbergen is crucial to the subject of the Phillips curve because he was the person who conducted the first econometric study on unemployment and inflation. Another important aspect of Tinbergen's research is that his aim was to bring optimal solutions especially to the public sector. Efforts of employment-price relationship modelling gained importance through Tinbergen's policy implications for an agricultural country such as Holland and for developing countries that were once dominated by the agricultural sector such as Türkiye. Since the causality in the price-employment relationship moves from unemployment to wage inflation due to the demand pressure from the labor market in such countries, the transformation initiated by Tinbergen has accelerated.

According to TURKSTAT (2022), the agricultural sector created employment for nearly a quarter of the workforce from 2014-2021. Theoretically, it is likely that agriculture's share of GDP will decrease relatively as national economies get stronger, yet, in Türkiye, the sectoral share of GDP related to agriculture was considerable and ranged between 6% and 7% from 2014 to 2021 (TMFA 2022). This stable structure can be explained both by the claim that the country is an agricultural country and by the role of the state in agriculture. From 1923 to the end of the 1970s, Türkiye adopted state-supported agricultural policies. However, while the main purpose of agriculture in the state-supported period was centered on the production of strategic products, the nutrition of society, and the storage and protection of goods, the government support provided to agriculture was deliberately reduced and resources were mostly used in the industrial and service sectors after 1980. But, in the 1980s, the agricultural sector was considered in the framework of food security, agricultural revenue, and supply and pricing of agricultural products, rather than providing economic development or contributing to development. For this reason, the evaluation of the Phillips curve—which was structured econometrically by Tinbergen, developed with Phillips and Samuelson-Solow, and shaped by the Monetarist and New Classical movements—in terms of the state-supported agricultural sector in Türkiye is important because of its economic return and employment share. Thus, it is unsurprising that many studies have examined the validity of the Phillips curve in the economies of various countries, but surprisingly few studies have analyzed its validity for the agricultural

sector, especially in the case of Türkiye. For this reason, this study presents chronologically domestic and foreign studies investigating the validity of the Phillips curve econometrically.

The first group presented in Table 1 investigates the relationship between inflation and unemployment.

Table 1- Studies on the Validity of the Phillips Curve

Author	*Year*	*Data Range*	*Country*	*Result*
Uysal & Erdoğan	2003	1980-2002	Türkiye	The Phillips Curve for 1990-2002 is valid.
Kuştepeli	2005	1980-2003	Türkiye	Phillips Curve is not valid
Önder	2006	1987-2004	Türkiye	Phillips Curve is not valid
Hepsağ	2009	2000-2007	Türkiye	In the long run, the Phillips Curve is valid.
Arabacı & Eryiğit	2012	1991-2010	Türkiye	Phillips Curve is valid
Mangır & Erdoğan	2012	1990-2011	Türkiye	Phillips Curve is not valid
Bayrak & Kanca	2013	1970-2010	Türkiye	While the Phillips Curve is invalid in the long run, it is valid in the short run.
Şentürk & Akbaş	2014	2005-2012	Türkiye	There is a bidirectional causality relationship between inflation and unemployment.
Öztürk & Emek	2016	1997-2006	Türkiye	Phillips Curve is valid
Göçer	2016	2005-2015	Türkiye	Phillips Curve is valid
Tabar & Çetin	2016	2003-2016	Türkiye	Phillips Curve is not valid
Alper	2017	1987-2006	Türkiye	Phillips Curve is valid
Saraç & Yıldırım	2017	2005-2016	Türkiye	Phillips Curve is valid
Eygü	2018	1990-2017	Türkiye	Phillips Curve is valid
Özer	2020	2006-2017	Türkiye	Phillips Curve is valid
Polat	2019	2008-2017	Türkiye	Phillips Curve is valid
Salman & Uysal	2019	2006-2018	Türkiye	Phillips Curve is not valid
Kayacan & Birecikli	2020	1998-2016	Türkiye	Phillips Curve is not valid
Şengönül & Tekgün	2021	2005-2011	Türkiye	Phillips Curve is valid
Ozan & Bakırtaş	2021	1995-2019	Türkiye	Phillips Curve is valid
Uğur	2021	1993-2018	Türkiye	Phillips Curve is not valid
Yıldız	2021	2006-2020	Türkiye	Phillips Curve is not valid

In sum, there is no consensus on the validity of the Phillips curve. In fact, the research methods used in these studies are also different from each other. For instance, Uysal & Erdoğan (2003) and Göçer (2016) examined the validity of the Phillips curve for Türkiye by using Granger causality analysis. However, Şengönül and Tekgün (2021), Ozan & Bakırtaş (2021), Hepsağ (2009), and Alper (2017) reached similar results by employing the ARDL boundary test approach. The validity of the curve for Türkiye has also been established by Eygü (2018) and Özer (2020) via the OLS method; by Ozturk & Emek (2016) via unit root ADF and co-integration tests; by Arabacı & Eryiğit (2012) via linear and non-linear regression tests; by Şenturk & Akbaş (2014) via Zivot-Andrews unit root tests with structural breaks and bootstrap causality tests; by Saraç & Yıldırım (2017) via a Markov-switching test; and by Polat (2019) via panel data analysis.

On the other hand, there are several other studies that claim the invalidity of the Phillips curve in the case of Türkiye. In fact, there is no unity in research method and style among this group just as there was no unity among those who defend the validity of the curve. For example, while Kuştepeli (2005) claimed that the curve is not valid for Türkiye based on the results she obtained from linear and non-linear regression analysis, Önder (2006) made the same determination with structural breaks and Markov-switching tests. Similarly, the invalidity of the curve for Türkiye was supported by Mangır & Erdoğan (2012) with causality analysis; by Tabar & Çetin (2016) with structural break tests; by Salman & Uysal (2019) with VAR tests; by Kayacan & Birecikli (2020) with unobserved compositional models; by Uğur (2021) with the panel causality test; and by Yıldız (2021) with Toda-Yamamoto causality analysis. Among these two groups, only Bayrak & Kanca (2013) reported a bilateral finding with short-term validity and long-term invalidity in their analysis using cointegration and OLS tests.

Although unemployment and inflation are a general problem for Türkiye, they are also a substantial agricultural economic problem with the presence of seasonal and permanent workers, the unemployed, and the increase in food prices both locally and

globally. In addition, we determined that the unemployment and inflation studies related to the agricultural sector in Türkiye have been conducted bilaterally or discussed under the framework of unemployment hysteria while we were conducting a literature review for this study. Since there are very few studies on the validity of the Phillips curve in agriculture, we will present all national and international studies in this section.

The earliest studies in this area date back to the early 2000s. In the study by Terzi & Oltulular (2004), they determined by Granger causality analysis a negative relationship between inflation and unemployment between 1923 and 2003 in three sectors, namely agriculture, industry, and services. Similar results were also found in the study of Bilman (2008) conducted on the validity of the Phillips curve between 1990 and 2008 using the Hodrick–Prescott filter and Markov-switching model. In the study, although the labor force participation rate in Türkiye was gradually decreasing, the authors claimed that the reason for this was the inability to create an employment environment that would allow the increasing population to work, especially in non-agricultural sectors. Additionally, the study found that although unemployment increased even in the agricultural sector, the general level of prices increased and the curve was valid.

Özaksoy (2015) investigated the validity of the Phillips curve between 1955 and 2014 with the ARDL method within the framework of the minimum underemployment rate (MURI) approach by taking agriculture as a variable of the Turkish labor market. The basic logic of MURI is the analysis of the backward-curved Phillips curve. The slope and inflection points of this Phillips curve depend on how quickly workers show real-wage resistance. If workers resist real wages, even at low wage levels, the Phillips curve will become steeper, and relatively low inflation will reverse at a high unemployment rate. Although there is evidence for the existence of a negative relationship between inflation and unemployment in Türkiye in the long run, the findings have not been explained in terms of agriculture specifically.

Şengönül & Tekgün (2021), previously mentioned above in the first group of Phillips curve studies, dealt with the agricultural sector in a very limited part of their studies. In their research by regions, they figured that since 16 regions were rural areas engaged in agriculture and animal husbandry, inflation and unemployment in these regions showed trade-offs in accordance with the Phillips curve theory.

Similar to Türkiye, there are very few researchers who have directly investigate the relationship between the agricultural and the Phillips curve throughout the world. And not surprisingly the vast majority of those researchers choose the agricultural sector as a subject in the sectoral sense. The reason of not researching agriculture in terms of the curve comes from its structure. Agriculture mostly dependents on climatic conditions and includes the seasonal worker factor.

In Table 2, there are several selected foreign studies that relate agriculture and the Phillips curve. Only those that are marked with (**) have made evaluations directly and specifically in the field of agriculture. The rest of the studies have made analyses by considering agriculture together with other sectors.

Table 2- Studies dealing with both Agricultural Sector and Phillips Curve

Author	*Year*	*Data Range*	*Country*	*Result*
Geary & Jones**	1975	1953-1972	Ireland	PC is weakly valid
Miroljub	1989	1965-1985	Yugoslavia	PC is not valid
Dua & Gaur	2009	1990-2005	Japan, Hong Kong, Singapore, Philippines, Thailand, China, India	PC is valid
Imbs et al.	2011	1978-2005	France	PC is valid
Durevall & Sjö**	2012	2000-2010	Ethiopia and Kenya	PC is valid
Pogorelyy	2013	2003-2013	U.S.A	PC is valid
Mawejje & Lwanga	2015	2000-2012	Uganda	PC is not valid
Ochuodho & Lantz**	2015	2006-2051	Canada	PC is valid for 2006-2015
Duncan et al.	2019	2006-2016	Kenya	PC is valid
Sasongko & Huruta	2019	1984-2017	Indonesia	PC is not valid
Hirata et al.	2020	2003-2017	Japan	PC is valid both in the short-run and in the long-run
Hyder & Hall	2020	1973-2013	Pakistan	PC is valid

** represents the papers which made analyses solely on agriculture

As can be seen, Durevall & Sjö (2012) for Ethiopia and Kenya, Pogorelyy (2013) for the United States, and Ochuodho & Lantz (2015) for Canada have claimed that the Phillips curve is valid in the agricultural sector. Of the studies that solely worked on the relationship between agriculture and the Phillips curve, Geary & Jones (1975) indicated a rather weak relationship for

Ireland. Also, Miroljub (1989) explained that this relationship was not valid for Yugoslavia in the 1965-1985 period, with prices and the general level of employment being determined by the central planning administration.

The general opinion about the validity of the Phillips curve in the agricultural sector also exists in studies that tested the validity of the Phillips curve in the agricultural sector along with other sectors. Dua & Gaur (2009) for Japan, Hong Kong, Singapore, Philippines, Thailand, China, and India; Imbs et al.(2011) for France; Duncan et al.(2019) for Kenya; and Hyder & Hall (2020) for Pakistan all determined the negative trade-off between unemployment and inflation. Conversely, Mawejje & Lwanga (2015) and Sasongko & Huruta (2019) insisted on the invalidity of the curve in terms of agricultural inflation and unemployment for both Uganda and Indonesia. However, Hirata, Maruyama, and Mineyama (2020) conducted a study on Japan that revealed a different result compared to the rest. These authors declared the validity of the curve in the short term yet invalidity in the long term. The inability to reach a consensus among the studies mentioned above, both in Türkiye and abroad, reinforces the curiosity about the findings of this study.

2. Material and Methods

We constructed inflation and unemployment data sets for the period 2014Q1-2021Q3 in the agricultural sector by employing the data pool of TURKSTAT. Specifically, we collected agricultural inflation data through the "Agricultural Products Producer Price Index According to Twelve-Month Averages (%) (AINF)," and we retrieved agricultural unemployment data via the "Monthly Agricultural Unemployment (AUR)." The agricultural unemployment figures were used to represent unemployment rates in the Phillips curve, while for the inflation rate, the logarithms of the agricultural price index values were taken and shown with the abbreviations of *loginf* and *logun*.

The model used to measure the validity of the Phillips curve in the Turkish agricultural sector is given in Equation 1.

$$loginf = \beta_0 + \beta_1 logun + \mathcal{E}_t \quad (1)$$

The descriptive statistics of the variables are presented in Table 3, and the graphs by level values are shown in Figures 1 and 2.

Table 3- Descriptive Statistics and Tests

Descriptives	***loginf***	***logun***
Mean	4.855797	11.23005
Median	4.781904	11.20934
Maximum	5.412185	11.88581
Minimum	4.453258	10.53269
Std. Dev.	0.290554	0.269958
Skewness	0.448119	0.145515
Kurtosis	1.934917	3.836660
Jarque-Bera	2.502792	1.013569
Probability	0.286105	0.602430
Observation	31	31

As can be seen from Table 3, the data have a normal distribution, as the Jarque Bera statistic indicated. Also, Figure 1 presents the CPI index, showing a continuous graph of increase, and can be considered to contain a unit root. However, as can be seen in Figure 2, while the agricultural inflation rate follows an increasing trend over time, the agricultural unemployment rate follows a stagnant course.

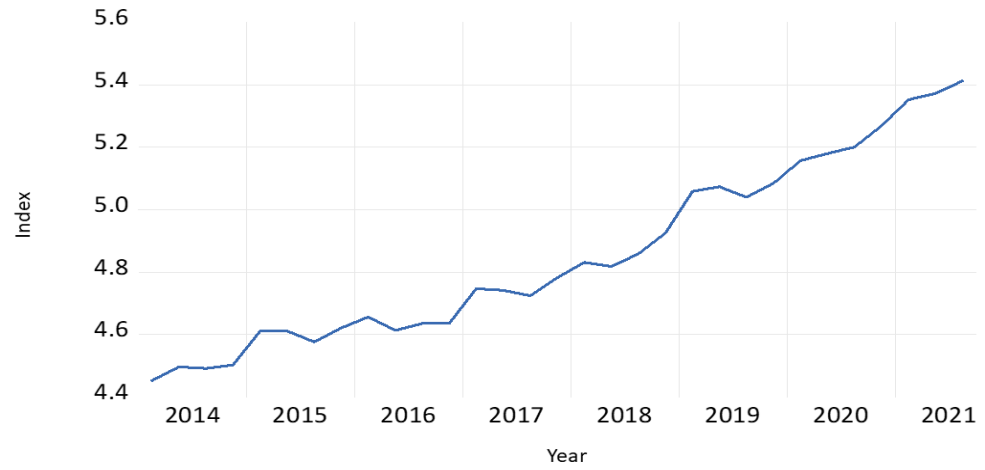

Figure 1- Agricultural Price Index (2014Q1-2021Q3)

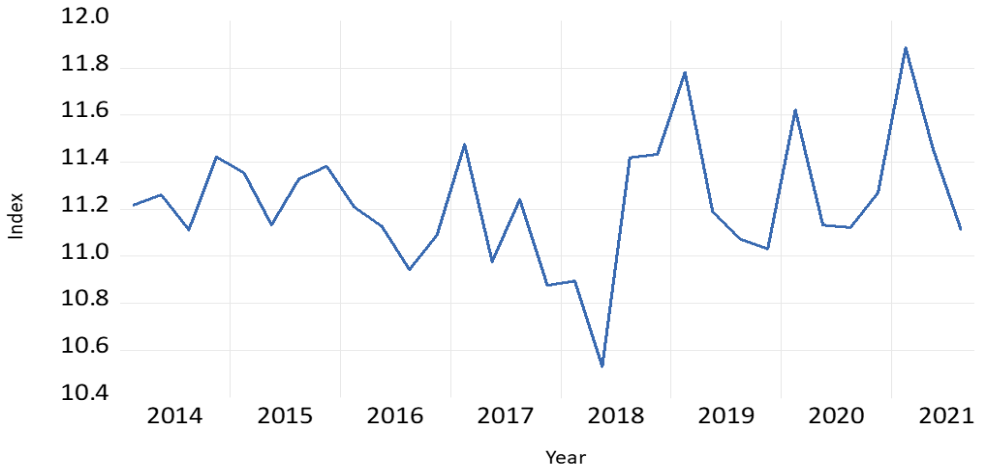

Figure 2- Agricultural Unemployment (2014Q1-2021Q3)

Before analyzing whether the Phillips curve is valid in the agricultural sector, we tested the stationarity of the variables used. Immediately after the test, we estimated the NARDL model.

2.1. Unit root tests and NARDL model

In order to test the stationarity in the study, we applied the augmented Dickey-Fuller (ADF) unit root test developed by Dickey & Fuller (1979, 1981) and the Phillips-Perron (PP) (1988) unit root test. The main difference between the PP and the ADF tests is that the lagged values of the error terms are treated as serially correlated and with heteroscedasticity. Additionally, although lagged values are included in the ADF test to eliminate the autocorrelation, the problem is tried to be solved by modifying the test statistics in the PP test. Finally, we applied the Zivot-Anders unit root test to determine whether there were structural breaks.

The NARDL model is an extended version of the ARDL model developed by Pesaran & Shin (1999) and Pesaran et al. (2001), including asymmetric relationships. The asymmetric cointegration regression developed by Shin et al. (2011) can be represented as follows within the framework of the variables used in this study:

$$loginf_t = \theta_0 + \theta_1 logun_t^+ + \theta_2 logun_t^- + \varepsilon_t \quad (2)$$

In the formulation, θ_i, $logun_t^+$, and $logun_t^-$ represent the long-run coefficient vector and the partial sums of the positive and negative changes in unemployment, respectively. In this model, the decomposition into partial sums is determined as follows:

$$logun_t^+ = \sum_{i=1}^{t} \Delta logun_t^+ = \sum_{i=1}^{t} \max(\Delta logun_i, 0) \quad (3)$$

$$logun_t^- = \sum_{i=1}^{t} \Delta logun_t^- = \sum_{i=1}^{t} \min(\Delta logun_i, 0) \quad (4)$$

When the asymmetric regression shown in Equation 2 is added to the unconstrained error correction model proposed by Pesaran et al. (2001), the NARDL model proposed by Shin et al. (2011) is obtained and shown as follows:

$$\Delta loginf_t = \alpha_0 + \alpha_1 loginf_{t-1} + \alpha_2 logun^+_{t-1} - \alpha_3 logun^-_{t-1} + \sum_{i=1}^{p} \alpha_{4i} \Delta\, loginf_{t-i} + \sum_{i=1}^{q} \alpha_{5i} \Delta\, logun^+_{t-i} + \sum_{i=1}^{m} \alpha_{6i} \Delta\, logun^-_{t-i} + u_t \quad (5)$$

Where; *p*, *q*, and *m* are lag lengths determined according to Akaike information criteria (AIC) or Schwarz information criteria (SIC). The long-term effects (θ_1 and θ_2) of the positive and negative shocks in unemployment, shown in Equation 2, can be calculated as follows with the help of the coefficients obtained from the estimation of Equation 2: $\theta_1 = -\alpha_2/\alpha_1$, $\theta_2 = -\alpha_3/\alpha_1$. The short-term effects of positive changes in unemployment can be represented by $\sum_{i=0}^{q} \alpha_{5i}$, and the short-term effects of negative changes are also obtained through $\sum_{i=0}^{m} \alpha_{6i}$. One of the main advantages of the NARDL model is that it allows estimation of the asymmetrical relationships among variables not only for the long run but also for the short run.

In this study, the following stages were followed chronologically while estimating in the NARDL model. Prelusively, a unit root test was conducted to determine the stationarity of the variables. It is not essential that the variables are stationary at different degrees in the NARDL model as in the ARDL model. However, to estimate the long-term coefficients, the quadratic difference of a variable should not be stationary (Narayan & Narayan, 2004). Secondly, the unconstrained asymmetric error correction model shown in Equation 5 was estimated, and the optimal lag length was determined according to the AIC information criteria. Afterwards, the bound test recommended by Pesaran et al. (2001) and Shin et al. (2011) was applied to determine whether there was a long-term relationship among the variables. While applying the bound test, we placed a zero constraint on the lagged coefficients of the dependent and independent variables ($H_0 = \alpha_1 = \alpha_2 = \alpha_3 = 0$), and the *F* statistic was obtained. If the statistical value obtained from the mentioned result was greater than the critical upper value, then we assumed that there was a long-term relationship among the variables, and the long- and short-term effect coefficients would be calculated accordingly.

3. Results

Two of the traditional unit root tests, ADF and PP, were applied to determine the stationarity of the variables as mentioned. Additionally, the Zivot-Anders structural break unit root test was applied to detect the presence of structural breaks. The outcomes are summarized in Tables 4 and 5.

Table 4- Traditional Unit Root Test Results

	ADF	***PP***
loginf	***2.020***[a]	***2.809***[a]
logun	-4.409[b]	-4.590[b]
Δ*loginf*	-6.099[a]	-5.692[a]
Δ*logun*	-	-

Notes: *Optimal lag lengths are selected automatically according to SIC in ADF test and Newey-West method in the PP test. Both test statistics were compared with the table values of MacKinnon (1996) at the 5% confidence interval. a represents the test format with a constant term, b the model result with a constant term and trend.*

Table 5- Zivot-Anders Unit Root Test Results

Variables	***Level***		***First Difference***		***Result***
	Test Stats.*	***Critical Value*****	***Test Stats.****	***Critical Value*****	
loginf	***-2.24*** (2016Q2)	-4.93	-7.29 (2015Q3)	-4.93	***I(1)***
logun	***-3.35*** (2019Q1)	-4.93	-7.40 (2018Q3)	-4.93	***I(1)***

Notes: * *These are test statistics with fixed terms. Terms in parentheses indicate periods of structural break; **: 5% critical values*

According to the traditional unit root test results, although only the inflation variable was first-order stationary, when structural breaks are taken into account, both variables were found to be stationary in the first order. In terms of level values, the inflation variable showed a structural break in the 2016Q2 period, and the unemployment variable showed a structural break in the 2019Q1 period. Therefore, a dummy variable that took the value of 1 in the mentioned periods and 0 in other periods was added to the model while making the estimation.

After determining the degrees of stationarity, we began the estimation phase of both the ARDL and NARDL models in order to examine the relationships among the variables since there was the probability of finding asymmetrical relationships even if there was no linear relationship among the variables. To that end, first, we determined the optimal lag lengths for both models,

and then the models were estimated. While determining the optimal lag length, we considered the smallest AIC value without autocorrelation. In the analysis where the maximum lag length was taken as 4, we determined that the optimal lag number was 3 for the ARDL model and 2 for the NARDL (see Table 6).

Table 6- Optimal Lag Length for ARDL and NARDL Models

	ARDL		*NARDL*	
p	***AIC***	***LM***	***AIC***	***LM***
1	-3.8198	0.1978	-3.8927	0.1034
2	-3.8198	0.1978	**-4.4257**	**0.8926**
3	**-4.0666**	**0.8395**	-4.6234	0.5827
4	-4.0666	0.8395	-5.0599	0.3615

The results of the bound test performed to determine the long-term relationships for linear and asymmetric models are presented in Table 7. As can be seen, in the ARDL model, there was a long-term relationship only at 5% and 10% confidence intervals while the inflation and unemployment variables were cointegrated in all confidence intervals in the NARDL model.

Table 7- Bounds Test Results for ARDL and NARDL Models

Model	***F Stats.***	***Critical Values***					
		1%		***5%***		***10%***	
		I(0)	***I(1)***	***I(0)***	***I(1)***	***I(0)***	***I(1)***
ARDL(3,1,0)	6.2529	6.34	7.52	4.87	5.85	4.19	5.06
NARDL (2,2,2,1)	7.8044	5.17	6.36	4.01	5.07	3.47	4.45

Tables 8 and 9 summarize the estimated ARDL and NARDL models in order to test the validity of the Phillips relationship in the agricultural sector. Before proceeding to estimating the long-term effects, we applied some additional tests to check the suitability of the models. The R^2 values were reasonably high in both models, which implies that the explanatory power of the independent variables was quite significant. Similarly, other fit tests also indicated that the models work smoothly. Moreover, among the tests, the Breusch-Godfrey LM tests inferred no autocorrelation, the ARCH-LM tests identified no heteroscedasticity problem, and the Jarque-Bera test demonstrated residuals normally distributed. Finally, according to the Ramsey RESET test, the setup of the models was correct, and the coefficients were stable.

Table 8- ARDL (3, 1, 0) Model Estimation Results

Variable	***Coefficient***	***Std. Error***	***t-Stats.***	***Prob.***
loginf(-1)	0.575139	0.177499	3.240242	0.0041
loginf(-2)	-0.202853	0.180949	-1.121049	0.2755
loginf(-3)	0.459151	0.148443	3.093112	0.0057
logun	0.061414	0.022331	2.750164	0.0123
logun(-1)	0.035158	0.026992	1.302510	0.2075
dummy	0.061523	0.034866	1.764558	0.0929
constant	-0.323053	0.500008	-0.646096	0.5256
Diagnostic Tests				
R^2: 0.9923				
Adj. R^2:0.9896				
F-statistic: 368.75				
Prob.(F-stat.): 0.00				
D-W: 2.092098				
Autocorrelation: *Breusch-Godfrey LM*: $\chi_1^2 = 0.196(0.66), \chi_2^2 = 0.176(0.83), \chi_3^2 = 0.265(0.84), \chi_4^2 = 0.202(0.93)$				
Heteroskedasticity – *ARCH LM*: $\chi_1^2 = 0.003(0.95), \chi_2^2 = 0.272(0.76), \chi_3^2 = 0.476(0.70), \chi_4^2 = 0.529(0.71)$				
Normality: *Skewness*: 0.5577, *Kurtosis*: 3.9644, *Jarque-Bera*: 2.5470 (0.2812)				
Stability: *Ramsey-Reset Test*: $\chi_1^2 = 1.6825(0.21)$,				

Table 9- NARDL (2, 2, 2, 1) Model Estimation Results

Variable	*Coefficient*	*Std. Error*	*t-Stats.*	*Prob.*
loginf(-1)	-0.065862	0.217058	-0.303429	0.7655
loginf(-2)	-0.352521	0.173446	-2.032447	0.0591
logunpos	0.119845	0.031408	3.815768	0.0015
logunpos(-1)	0.050586	0.031574	1.602144	0.1287
logunpos(-2)	0.209580	0.054548	3.842153	0.0014
logunneg	0.034656	0.042034	0.824474	0.4218
logunneg(-1)	0.174066	0.054952	3.167619	0.0060
logunneg(-2)	-0.101765	0.032343	-3.146449	0.0062
dummy	-0.095660	0.043864	-2.180823	0.0445
dummy(-1)	-0.054759	0.031314	-1.748726	0.0995
constant	6.373053	1.264862	5.038536	0.0001
Diagnostic Tests				
R^2: 0.9959				
Adj. R^2:0.9931				
F-statistic: 359.04				
Prob.(F-stat.): 0.00				
D-W: 1.957708				
Autocorrelation: *Breusch-Godfrey LM*: $\chi_1^2 = 0.162(0.69)$, $\chi_2^2 = 0.114(0.89)$, $\chi_3^2 = 0.531(0.66)$, $\chi_4^2 = 0.577(0.68)$				
Heteroskedasticity – *ARCH LM*: $\chi_1^2 = 0.114(0.73)$, $\chi_2^2 = 0.965(0.39)$, $\chi_3^2 = 0.659(0.58)$, $\chi_4^2 = 0.422(0.79)$				
Normality: *Skewness*: 0.5824, *Kurtosis*: 3.068, *Jarque-Bera*: 1.588 (0.451)				
Stability: *Ramsey-Reset Test*: $\chi_1^2 = 1.735(0.1032)$,				

When the long-term estimation results of the models employed in the study were examined, we noticed quite remarkable findings.

The linear ARDL model results presented in Table 10 showed a long-term relationship among the variables. Because the calculated cointegration was negative and statistically significant, accordingly, the imbalances that will occur in the system disappear in the long term, and the system converges to its long-term values. Although agricultural unemployment has a positive effect on inflation, this effect is not statistically significant.

Table 10- ARDL (3, 1, 0) Model Long-run Estimation Results

Variable	*Coefficient*	*Std. Error*	*t-Stats.*	*Prob.*
logun	0.572909	0.395764	1.447603	0.1632
dummy	0.364985	0.435301	0.838465	0.4117
CointEq(-1)	-0.168563	0.037108	-4.542555	0.0002
*EC = loginf - (0.5729*logun + 0.3650*dummy)*				

In contrast, the coefficients obtained in the NARDL model, whose estimation results are shown in Table 11, were statistically significant. Accordingly, both increases and decreases in agricultural unemployment positively affected the agricultural price inflation rate. However, the greater effect of positive shocks in unemployment provided strong evidence that the traditional Phillips relationship was invalid in the agricultural sector. Thus, an increase of 10% in agricultural unemployment, for example, was reflected in the price inflation rate at a level of approximately 3%. This situation can be evaluated in the context of increases in food prices, especially because of increases in agricultural unemployment and therefore in production. Since the increase in agricultural unemployment might adversely affect agricultural production, this increase would likely result in an increase in food prices. However, a 10% decrease in the unemployment rate caused price inflation to increase by about 1%. In other words, the traditional Phillips relationship is valid only for negative shocks in unemployment in the agricultural sector. In other respects, the statistical significance of the dummy variable showing structural breaks can be considered to indicate that structural breaks in the agricultural sector will affect inflation negatively.

Table 11- NARDL (2, 2, 2, 1) Model Long-run Estimation Results

Variable	***Coefficient***	***Std. Error***	***t-Stats.***	***Prob.***
logunpos	0.267919	0.021264	12.59991	0.0000
logunneg	0.075408	0.038275	1.970174	0.0664
dummy	-0.106050	0.032394	-3.273745	0.0048
CointEq(-1)	-1.418382	0.232957	-6.088598	0.0000
*EC = loginf - (0.2679*logunpos + 0.0754*logunneg -0.1060*dummy*				

Finally, the cointegration coefficient calculated for the NARDL model was negative and statistically significant. Accordingly, the short-term imbalances were eliminated in the long-term, and the system converged to the long-term equilibrium values. Nonetheless, the fact that the coefficient was greater than one indicates that there were significant fluctuations in the convergence of the system to the long-term equilibrium.

4. Conclusions

There are close similarities between the macroeconomic target sizes of almost all countries throughout the world, regardless of their level of development. For instance, economic growth, external balance, price inflation, and unemployment are used as indicators in the magic diamond approach used by the Organization for Economic Co-operation and Development (OECD) to evaluate the macroeconomic performance of countries. The Phillips curve directly analyzes the relationship between two of these basic quantities. Therefore, the validity of the curve plays a vital role in determining the applicable economic policies. Also, understanding the relationship suggested by the curve has an even more important role for economic politics, especially in countries such as Türkiye, which have shifted from an agriculture base to an industrial base over time and whose population engaged in agriculture has decreased despite the general population increase even though the share of employment in agriculture is still undeniably high. In addition, evaluation of the validity of the curve—which was damaged theoretically by the oil crises, by the Monetarist claim of its short-term validity, and by the New Classicists' rejection of the theory but which re-emerged with the contribution of Lucas to rational expectations—for a sector offers a significant opportunity for researchers in terms of identifying which economic school's approach is most valid today.

In this study carried out in light of these insights, we investigated the validity of the Phillips curve in the agricultural sector in Türkiye and the short- and long-term linear and non-linear relationships caused by positive and negative shocks on unemployment-inflation. Our investigation used the ARDL and NARDL approaches using quarterly data from the first quarter of 2014 through the third quarter of 2021. First, the long-term relationship among the variables was questioned, and we determined that since the null hypothesis was strongly rejected in both models, the variables were related in the long run. Afterwards, we tested for the long-term effects of the explanatory variable in both models. ARDL test findings provided an expected result since, as Phillips (1958:299) claimed, import products in the agricultural sector, synthetic price increases, and wage increases due to cost increases were intense in countries such as Türkiye and showed the validity of the theory. The fact that this result has not been examined in other field studies on this subject is one of the contributions of this study to the literature. Also, although the long-term effects were found to be insignificant in the linear model, we note that the asymmetric shocks that occurred were significant in the long-term.

Furthermore, we determined that price inflation rates had a serious breakdown in the second quarter of 2016, and unemployment displayed similar diffraction in the first quarter of 2019. In fact, when analyzed in terms of price inflation, this was a period in which double-digit figures were reached again in the relevant years. In addition, the unemployment figures in 2019 (after 2009) reached the highest levels since the 1990s. Overall, the findings of the analysis indicated that a 10% rise in agricultural unemployment raised price inflation by about 3% while a 10% decrease in agricultural unemployment raised price inflation by approximately 1% (0.75%). In other words, while the nexus was valid for negative shocks, it was not valid for positive shocks. In terms of these findings, a different result was determined by the studies of Şengönül & Tekgün (2021), Ozan & Bakırtaş (2021), Hepsağ (2009), and Alper (2017), all of which were conducted via the ARDL method. However, similar results were reached in the short term by the NARDL method by Kuştepeli (2005), Önder (2006), Salman & Uysal (2019), and Kayacan & Birecikli (2020). In terms of linear results, our findings are consistent with the results of Geary & Jones (1975),

Miroljub (1989), Mawejje & Lwanga(2015), and Sasongko & Huruta (2019) for Ireland, Yugoslavia, Uganda, and Indonesia, respectively. This study, which is one of the few studies related to the agricultural sector in Türkiye, differs from all other field studies in terms of significance and validity of negative shocks in the long term.

Although the agricultural sector is still an important economic sector in Türkiye, it is gradually losing its power, and citizens seem to prefer to produce in different sectors. Therefore, in order to analyze the employment in this sector, it is necessary to analyze the changes in the general level of prices. Shocks that cause the labor market to fluctuate and the asymmetric relationship between vacant jobs and unemployment include factors that increase labor supply and/or decrease labor demand. Although the changes in the economy cause fluctuations in production with shocks, these changes occur via the effect of negative shocks and cause asymmetric effects. Some of the factors that emerge with the asymmetric effects are labor prices and sectoral-capacity utilization. Labor prices become a policy tool used by governments in inflationary or deflationary regimes. As capacity utilization in the economy increases—that is, moves closer to full employment—the economy becomes stronger. In this study, we detected that there was an asymmetrical trend in unemployment rates due to inflationary-deflationary periods in the economy. Moreover, we noted that inflation increased in the long run regardless of the increase or decrease in agricultural unemployment. These findings point to the existence of other structural problems besides unemployment, which should be carefully investigated in the agricultural sector in Türkiye.

Additionally, although according to TURKSTAT data 30% of Türkiye's population lived in villages or towns, that hosted agricultural workforces, in 2007, this rate dropped dramatically to 7% by 2021. Also, elderly rate which is the dependent population aged 65 and over in the rural area was 9% in 2007 but 19% in 2021. Undoubtedly, one of the main reasons behind the increase in the elderly population and the departure of the working population from the region is unemployment caused by the decrease in profit margins in the agricultural sector.

The proof of the validity of the curve indicates that it would be more appropriate for policies in the agricultural sector to apply new-Keynesian recommendations. The accurate analysis of the relationship between price inflation and unemployment and the determination of policies suitable for the sector will also be instrumental in the development of the agricultural sector and its higher contribution to the national product.

The limitation of this study is that it excludes the dependent population from the evaluation. In this regard, we think that it will be an opportunity to consider the dependent population data for Türkiye, in case TURKSTAT publishes it, in terms of developing the Phillips curve in future studies. In addition, we believe that this study will shed light on the deeper economic insights to be made based on the determination of the validity of the Phillips curve.

References

Akkuş E (2012). Phillips Curve: A Theoretical Analysis of the Inflation-Unemployment Exchange (In Turkish). İstanbul Üniversitesi İktisat Fakültesi Mecmuası 62(2): 99-151

Alper F Ö (2017).The Relationship Between Inflation and Unemployment: ARDL Boundary Test Approach for Türkiye (In Turkish) . *Uluslararası Ekonomi ve Ticaret Araştırmaları Dergisi* 1(2): 71-80

Arabacı Ö & Eryiğit K Y (2012). A Threshold Regression Estimation of Phillips Curve: Türkiye Case. *Eskişehir Osmangazi Üniversitesi İİBF Dergisi* 7: 29-47

Bayrak M & Kanca O C (2013). An Application on the Phillips Curve in Turkey (In Turkish). *Eskişehir Osmangazi Üniversitesi İİBF Dergisi* 8(3): 97-115

Baumeister C & Kilian L (2013) Do Oil Price Increase Cause Higher Food Prices? Bank of Canada Working Paper 52 https://www.bankofcanada.ca/wp-content/uploads/2013/12/wp2013-52.pdf

Bilman A S (2008). Policy Recommendation of the Phillips Curve and Asymmetric Effects: The Case of Türkiye (In Turkish). Dokuz Eylül Üniversitesi Sosyal Bilimler Enstitüsü İktisat Ana Bilim Dalı Yüksek Lisans Tezi

Dickey D A & Fuller A W (1981). Likelihood Ratio Statistics for Autoregressive Time Series With A Unit Root. Econometrica 49(4): 1057-1072. doi.org/10.2307/1912517

Dickey D A & Fuller A W (1979). Distribution of The Estimators For Autoregressive Time Series With A Unit Root. *Journal of The American Statistical Association* 74: 427-431. doi.org/10.2307/2286348

Dornbusch R, Fischer S & Startz R (2016). Macroeconomics. 12 ed. İstanbul: Literatür Yayınları.

Dua P & Gaur U (2009). Determination of Inflation in an Open Economy Phillips Curve Framework: The Case of Developed and Developing Asian Countries. Centre for Development Economics Delhi School of Economics, Working Paper No. 178

Duncan H O, Li F & Sali M W (2019). Trade-off Philips Curve, Inflation and Economic Implication: The Kenyan Case. *International Journal of Economics and Finance* 11(4): 60-73. doi.org/10.5539/ijef.v11n4p60

Durevall D & Sjö B (2012). The Dynamics of Inflation in Ethiopia and Kenya. African Development Bank Group Working Paper Series 151

Eygü H (2018). Examining the Relationship Between Inflation, Unemployment and Foreign Trade: The Case of Turkey (1990-2017).(In Turkish). . Kastamonu Üniversitesi İktisadi İdari Bilimler Fakültesi Dergisi, 20(2): 98-112. doi.org/iibfkastamonu.408823

Fisher I (1926). A Statistical Relation between Unemployment and Price Changes. International Labor Review 13. June (6): 785-792. doi.org/10.1086/260048

Friedman M (1968). The Role of Monetary Policy. American Economic Review 58: 1-17

Frisch H (1977). Inflation Theory 1963-1975: A Second Generation Survey. *Journal of Economic Literature* 4(15): 1289-1317

Geary P T & Jones R M (1975). Appropriate measure of unemployment in an Irish Phillips Curve. Economic and Social Review 7(1): 55-63

Gordon R J Friedman and Phelps on the Phillips Curve Viewed from a Half Century's Perspective. NBER Working Paper Series 24891, pp. 1-15

Göçer İ (2016). Is Phillips Curve Theory Valid in Turkey? An Econometric Analysis with Structural Breaks (In Turkish).. Aydın İktisat Fakültesi Dergisi 1(1): 50-62

Hepsağ A (2009). Analysis of the Relationship Between Inflation and Unemployment in Turkey: Bounds Test Approach (In Turkish). İktisat Fakültesi Mecmuası 59(1)

Hirata W, Maruyama T & Mineyama T (2020). Flattening of the Wage Phillips Curve and Downward Nominal Wage Rigidity: The Japanese Experience in the 2010s. Bank of Japan Working Paper Series 20-E-4

Humphrey T (1986). From Trade-offs to Policy Ineffectiveness: A history of the Phillips Curve. Monograph: Federal Reserve Bank of Richmond: Richmond

Hyder K & Hall S G (2020). Estimates of the New Keynesian Phillips Curve for Pakistan. Empirical Economics 59(1): 871-886. doi.org/10.1007/s00181-019-01659-8

Imbs J, Jondeau E & Pelgrin F (2011). Sectoral Phillips Curve and the Aggregate Phillips Curve. *Journal of Monetary Economics* 58(4): 328-344. doi.org/10.1016/j.jmoneco.2011.05.013

Kuştepeli Y (2005). A Comprehensive Short-Run Analysis of a (Possible): Turkish Phillips Curve. Applied Economics 37: 581-591. doi.org/10.1080/000368404200030749

Lipsey R G (1960). The relation between unemployment and the rate of change of money wage rates in the United Kingdom 1862-1957: A further analysis. Economica 27(105): 1-31 doi.org/10.2307/2551424

Lucas R E (1972). Expectations and the Neutrality of Money. *Journal of Economic Theory* 4(2): 103-124. doi.org/10.1016/0022-0531(72)90142-1

Mangır F & Erdoğan S (2012). The Relationship Between Inflation and Unemployment in Turkey (1990-2011) (In Turkish). Finans Politik & Ekonomik Yorumlar 49(570): 77-86

Martin W (2011) Export Restrictions and Price Insulation during Commodity Price Booms. The World Bank Policy Research Working Paper 5645 https://www.imf.org/external/np/seminars/eng/2011/trade/pdf/session1-martin-paper.pdf

Mankiw N G (1990). A Quick Refresher Course in Macroeconomics. *Journal of Economic Literature* 28(2): 1645-1660 doi.org/10.3386/w3256

Mawejje J & Lwanga M M (2015). Inflation Dynamics and Agricultural Supply Shocks in Uganda. EPRC Research Series No. 120.

Miroljub H (1989). The Wage-Push Inflation in Yugoslavia 1965-85. Institute of Social Studies Working Paper no. 67.

Muth J F (1961). Rational Expectations and the Theory of Price Movements. Econometrica 29(3): 315–335. doi.org/10.2307/1909635

Ochuodho T O & Lantz V A (2015). Economic impacts of climate change on agricultural crops in Canada by 2051: A global multi-regional CGE model analysis. Environmental Economics 6(1): 113-125. doi.org/10.1016/j.forpol.2015.12.005

Ozan E C & Bakırtaş İ (2021). Is New Keynesian Wage Phillips Curve Valid in Turkey? Findings from the Ardl Bounds Test Approach (In Turkish). Hacettepe Üniversitesi İktisadi ve İdari Bilimler Fakültesi Dergisi 39(2): 237-251. doi.org/ 10.17065/huniibf.785756

Önder A Ö (2006). "The Stability of The Turkish Phillips Curve and Alternative Regime Shifting Models" Ege University Working Papers in Economics 2006, Working Paper No: 06/02

Özaksoy F (2015). A New Look at the Labor Market from the New Keynesian Phillips Curve to MURI (In Turkish). Yıldız Teknik Üniversitesi Sosyal Bilimler Enstitüsü İktisat Ana Bilim Dalı Yüksek Lisans Tezi.

Özer M O (2020). Analysis of the Long-Run Relationship Between Inflation and Unemployment Rates in Turkey: Fourier Approach to the Phillips Curve (In Turkish).. *Pamukkale Üniversitesi Sosyal Bilimler Enstitüsü Dergisi* 39: 179-192. doi.org/10.30794/pausbed.560093

Öztürk S & Emek M L (2016). Phillips Curve Analysis for Türkiye with Data for April and October 1997-2006 (In Turkish). *Balkan Sosyal Bilimler Dergisi* 5(10): 64-74

Parkin M (2011). Economics. (Ö. Uzun, S. Demir, S. Güneş, & Ş. Sezgi, Çev.) Ankara: Akademi Yayıncılık

Pesaran M H, Shin Y & Smith R J (2001). Bounds Testing Approaches to The Analysis of Level Relationships. *Journal of Applied Econometrics* 16(3): 289-326. doi.org/10.1002/jae.616

Phelps E S (1967). Philips Curves, Expectations of Inflation, and Optimal Unemployment over Time. Economica 34: 254-81. doi.org/10.2307/2552025

Phillips A W (1958). The Relation between Unemployment and the Rate of Change of Money Wage Rates in The United Kingdom, 1861-1957. Economica, New Series, 25(100): 283-299. doi.org/10.2307/2550759

Pogorelyy M U (2013). About the State of the World Wheat Market at the Current Stage. *Russian Journal of Agricultural and Socio-Economic Sciences* 11(23): 24-30. doi.org/10.18551/rjoas.2013-11.04

Polat E (2019). The Relationship Between Unemployment and Inflation: An Empirical Analysis for NUTS-2 Regions in Türkiye (In Turkish).. *Yönetim ve Ekonomi Dergisi* 26(3): 783799. doi.org/10.18657/yonveek.544446

Salman G & Uysal D (2019). Determining the Relationship Between Inflation and Unemployment in Türkiye: 2006:Q1-2018:Q2 Var Model Analysis (In Turkish). *Uluslararası Sosyal Bilimler Akademik Araştırmalar Dergisi* 3(1): 1-20

Samuelson P A & Solow R M (1960). Analytical Aspects of Anti-Inflation Policy. The American Economic Review 50(2): 176-194. jstor.org/stable/1815021

Saraç T B & Yıldırım A E (2017). The Relationship Between Inflation and Unemployment: The Case of Türkiye (In Turkish). Turan-Sam Uluslararası Bilimsel Hakemli Dergisi 8(32): 15-20

Sasongko G & Huruta A D (2019). The casuality between inflation and unemployment: The Indonesian evidence. Verslas: Teorija ir praktika / Business: Theory and Practice 20(1): 1-10. doi.org/10.3846/btp.2019.01

Şengönül A & Tekgün B (2021). Panel ARDL Analysis of the Phillips Curve: A Cross-Regional Application in Türkiye (In Turkish).. *Uluslararası Ekonomi Siyaset İnsan ve Toplum Bilimleri Dergisi* 4(2): 81-97

Şentürk M & Akbaş Y E (2014). Evaluation of the Reciprocal Relationship Between Unemployment-Inflation and Economic Growth: The Case of Türkiye (In Turkish). *Journal of Yasar University* 9(34): 5820-5832. doi.org/10.19168/jyu.43548

Tabar Ç K & Çetin I (2016). Phillips Curve Analysis in Turkish Economy.(In Turkish). *Journal of Life Economics* 3(4): 79-100

Terzi H & Oltulular S (2004). Economic Growth-Inflation Process in Türkiye: An Econometric Analysis by Sectors (In Turkish). *Bankacılar Dergisi* 50: 1-16

Tinbergen J (1936). Kan hier te lande, al dan niet na overheidsingrijpen, een verbetering van de binnenlandse conjunctuur intreden, ook zonder verbetering van onze exportpositie? Welke lering kan ten aanzien van dit vraagstuk worden getrokken uit de ervaringen van andere landen? In Vereeniging voor de Staathuishoudkunde en de Statistiek, Prae-adviezen (pp. 62–108): -Gravenhage: Martinus Nijhof.

TMFA, Republic of Türkiye Ministry of Agriculture and Forestry (2022). Agricultural GDP and Share of Agriculture at Current Prices https://www.tarimorman.gov.tr/SGB/Belgeler/Veriler/GSYH.pdf

Tokatlıoğlu İ & Ozturk F (2015). Long-Run Unemployment and the Phillips Curve: Performance Comparison of Inflation Forecasts for Türkiye (In Turkish). Ekonomik Yaklaşım 26(94): 1- 26

Turkish Statistical Institute (2022). Employment, Unemployment and Wages Data. Statistical Data Portal. https://data.tuik.gov.tr/Kategori/GetKategori?p=Istihdam,-Issizlik-ve-Ucret-108

Uğur B (2021). BRICS and the Relationship Between Inflation and Unemployment in Türkiye: Panel Causality Analysis (In Turkish). *Erzincan Binali Yıldırım Üniversitesi İktisadi ve İdari Bilimler Fakültesi Dergisi* 3(2): 1-14

Uysal D & Erdoğan S (2003). The Relationship Between Inflation and Unemployment and the Case of Türkiye (1980-2002). (In Turkish). *Sosyal ve Ekonomik Araştırmalar Dergisi* 6: 35-47

Yıldız Ş (2021). The Analysis of the Validity of the Phillips Curve in Türkiye via the Fourier Cointegration and Causality Tests. *İnsan ve Toplum Bilimleri Araştırmaları Dergisi* 10(4): 3173-3190. doi.org/10.15869/itobiad.918514

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Regional Drought Analysis with Standardized Precipitation Evapotranspiration Index (*SPEI*): Gediz Basin, Turkey

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ABSTRACT
In this study, regional drought analysis was performed with the Standard Precipitation Evapotranspiration Index (*SPEI*) and L-moment techniques by using the monthly average temperature and monthly total rainfall amounts collected from five sites in the Gediz basin in Turkey. Using the monthly average temperatures, the Potential Evapotranspiration (*PET*) amounts obtained by the Thornthwaite method and the monthly total rainfall amounts were divided into 5 different reference periods as 1, 3, 6, 9 and 12 months. Expressing the difference between rainfall and potential evapotranspiration amounts, the water balance (D_i) series showed that almost all of the 9 and 12-month periods suffered from water deficiency and the 3-month period was water excessive. After determining the distributions that provide the best adaptation to the water balance series, according to the *SPEI* values obtained, near-normal conditions prevailed in all sites, while moderate and severe arid and humid conditions sometimes occurred, while extremely humid and arid conditions were rarely seen. In the regional drought analysis using L-moment techniques with the *SPEI* values obtained, a region of five sites were accepted and the discordancy and heterogeneity measures showed that the basin was acceptable homogeneous. *SPEI* values are generally the best fit generalized extreme values (GEV) for 1 and 3-month periods, generalized normal (GNO) for 6-month period, generalized logistics (GLO) for 9-month period, Pearson type 3 (PE3) distributions for 12-month period. According to the regional *SPEI* values for reference periods, it has been found near-normal in 1.11, 1.25 and 2 years, moderately humid in 1.04 years, very humid conditions for 1.01 and 1.02 years, moderately dry in 4 and 5 years, severe arid in 10 years, and extremely dry conditions in 20 and longer periods.

Keywords: Potential evapotranspiration, Water balance, *SPEI*, Index-Flood, Regional homogeneity, Drought duration, Drought frequency

1. Introduction

As in the world, the rapid increase in the population in Turkey also causes an increase in the need for water. As a result of incorrectly applied water policies and unconscious water consumption, the amount and quality of water that can be used is decreasing day by day. The effects of global warming, which is the biggest climatic problem of today, are felt every day, and precipitation decreases as the temperature increases, or the irregularity in precipitation is seen in the form of storms, tornadoes and hail that damage agricultural lands. Due to the increase in temperature and the lack of sufficient precipitation, drought is observed in many basins of Turkey or the risk of drought is high. In recent years, there are predictions that the drought will increase gradually and that the 22nd century will be the dry century, and that agricultural production will decrease accordingly. Experts mention that if global warming continues like this, problems will arise in many fields other than agriculture (Redmond 2000). Drought is one of the most important problems for humanity arising from climate change. Although drought does not occur suddenly like other natural disasters (floods, storms, etc.), it is one of the highest cost disasters in the world, threatening more people and nature than other natural disasters, and reaching an annual average of 8-10 million dollars (Wilhite 2000). Drought has been increasing in Turkey in recent years, and it is predicted that there will be a significant lack of precipitation, especially in Southeastern and Central Anatolia regions, and recently Aegean region. Global warming combined with the current lack of precipitation; Agricultural production constitutes an important obstacle to meeting the water required for drinking-use. Located in the Western Anatolia part of Turkey, the Gediz Basin has a semi-arid climate and the decrease in water resources restricts agricultural activities. The dry period, which started in the late 1980s and continued until the mid-1990s, caused many problems in terms of irrigation water. There are approximately 110000 hectares of agricultural land in the basin. Droughts of varying severity and duration in the basin from time to time cause problems in reaching irrigation water and as a result, economic losses (Çetinkaya et al. 2008).

Many indices have been developed to describe drought. The indices, which are made by using some meteorological data, provide short and useful information about drought. Methods such as the Standard Precipitation Index (*SPI*), which are widely

used to indicate drought and only consider precipitation, cannot fully explain drought. In recent years, method such as Standard Precipitation Evapotranspiration Index (*SPEI)* has been used, which consider not only precipitation but also precipitation and other important climatic parameters for agriculture. Many studies have been carried out on *SPEI* in recent years. Vicente-Serrano et al. (2010) suggested *SPEI*, which was based on precipitation and temperature data, unlike other drought indices. The biggest advantage of this index is that it can be combined with other data such as precipitation and evapotranspiration, primarily to include the effects of different temperature values in drought forecasts. Potop & Možn (2011) proposed to use the potential evapotranspiration in the study of drought severity in the Czech Republic using drought index, *SPEI*. In the study, they calculated the potential evaporation (ET_0), water balance, D_i (P-ET_0) series at different time scales, and fitted the water balance to the logarithmic logistic distribution to obtain the *SPEI* index series. *SPEI* and water balance series were calculated according to short (1, 3 and 6 months) and long (12 and 24 months) time scales to evaluate the variation of drought conditions over time. Begueria et al. (2014) mentioned that the use of the *SPEI* in climatology and hydrology studies has become widespread. They presented probability distribution parameters to obtain standardized values, ET_0 calculation methods, and methods for estimating probability parameters used in calculating *SPEI* at different time scales. Meixiu et al. (2014) made a drought calculation based on monthly precipitation and air temperature values at 609 points in China during the 1951–2010 period with *SPEI*. They stated that severe and extreme droughts have become more serious for the whole of China since the late 1990s. Stagge et al. (2014) considered whether *SPEI* differed significantly from *SPI* and used five *PET* methods commonly used at 3950 sites in Europe. They found that *SPEI* differed significantly from *SPI*. Li et al. (2015) used *SPEI* to characterize drought conditions in Southwest China between 1982 and 2012 and carried out the calculations using precipitation and temperature data for various periods. Their results showed that Southwest China is prone to drought, and they found that the average *SPEI* values across all five different time scales decreased significantly. Liu et al. (2015) stated in their study that China is one of the countries with the highest drought propensity, and to investigate this, they analyzed the regionalization and spatiotemporal variations of drought based on *SPEI* covering 810 sites in China between 1961 and 2013. Stagge et al. (2015) used the univariate probability distribution to normalize the index for comparison among climates, using *SPI*, a meteorological drought index recommended by the World Meteorological Organization, and *SPEI*, a newer climatic water balance variant. They said that the selection of the inappropriate probability distribution may cause errors in the index values by increasing or decreasing the drought severity. Yang et al. (2015) analyzed the monthly average precipitation and monthly average temperature of 47 meteorological stations in and around the Haihe Basin in the background of climate change, using *SPEI* to obtain the temporal and spatial distribution of different drought levels for the last 50 years with the support of geographic information systems. Anli (2017) explained that the analysis of the temporal variation of reference evapotranspiration (ET_0) is of great importance in arid and semi-arid regions where water resources are limited. *SPEI* method was used with the temporal variation of reference evapotranspiration (ET_0) and L-moment parameter estimation, with parametric and non-parametric tests, and conducted regional drought analyzes in the semi-arid Konya closed basin. Miah et al. (2017), to overcome the lack of long-term climate data in Bangladesh, they conducted an *SPEI* analysis for the years 1901-2011 and found that the frequency and intensity of drought was higher in the northwestern part of the country. This made the region vulnerable to both extreme and severe droughts. Based on the results obtained, *SPEI*-based drought intensity and frequency analyzes were conducted by considering the northwest region of the country to get an idea about drought assessment in Bangladesh. Bae et al. (2018) analyzed the intensity, variability and trends of drought using *SPEI_TH* and *SPEI_PM* in their study in South Korea and compared the results. *SPEI_PM* showed slightly more intense drought than *SPEI_TH* except Chuncheon and Gwangju. At five stations except Cheoncheon, Gwangju and Jinju, the probable cumulative probability increased significantly from 1981-1995 to 1996-2010 with *SPEI_PM* below 1.5 value.

In recent years, significant progress has been made in drought studies on both at-site and regional scales. The spatiotemporal ones of these studies are as follows; Bacanli et al. (2017) precipitation and drought trend analysis in the Aegean region in standard periods, Dikici (2020) drought analysis for different indices and different durations in the Asi basin, Eris et al. (2020) spatiotemporal analysis of meteorological drought in Küçük Menderes river basin in the Aegean region, Aksoy et al. (2021), a new technique with *SPI* method to develop critical drought severity-duration-frequency curves, Katipoğlu et al. (2021) meteorological studies and spatiotemporal analysis of hydrological droughts in the Euphrates river basin, Aksu et al. (2022) using long-term high-resolution Climate Hazard Group Stationary Infrared Precipitation (CHIRPS) data , spatiotemporal drought analysis in the Küçük Menderes river basin and Zeybekoğlu (2022) spatiotemporal analysis of meteorological drought in the Hirfanlı dam basin were performed.

Statistical methods become the most important tool in climatological frequency analyzes to be carried out. Frequency analysis can be defined as specifying the intervals at which a climatological event will occur. The climatological data to be used should cover a long period of time to qualify the event. In frequency analysis studies, it is of great importance to specify the parameter estimation method along with the selection of the probability distribution function. The most commonly used of these methods are moments, maximum likelihood, probability-weighted moments and L-moments (Anlı & Öztürk 2011). L-moments technique gives the characteristics of climatological data and the distribution parameters of these data in a simple and effective way (Greenwood et al. 1979). In some cases, there is not enough data to strongly describe the frequency of an extreme event that may occur at a station, and in some stations, there is no data at all. In addition, in cases where the observation time at a station is shorter than the expected return period, the quantile amounts are not reliable when at-site frequency analyzes are applied (Hosking 1990, Vogel et al. 1993). In this method, which is called regional frequency analysis, the principle is that it should be applied in cases where data from different measurement stations have similar frequencies. Thus, more accurate results are obtained by using regional characteristics at each measuring station, as well as within an appropriately defined region, even in

basins with no data and no measuring stations on them (Hosking & Wallis 1993). Most of the studies conducted in Turkey examine the drought as at-site analysis, and there are few regional drought frequency studies. In this study, a period of approximately 27-58 years, collected from five climate stations in the Gediz basin; A regional drought analysis was carried out using the Standard Precipitation Evapotranspiration Index (*SPEI*) and L-moments technique for 5 different reference periods (1-month, 3-month, 6-month, 9-month and 12-month) with monthly total precipitation and monthly average temperature data. The aim of the study is to make an accurate regional estimation of drought in order to minimize the drought conditions that may occur in the basin in the medium and long term. As a novelty in the study, the regionalization of the values obtained by the *SPEI* method was carried out using the index-flood method. Thus, according to the appropriate homogeneity, drought estimation will be made regionally, not on at-site frequency.

2. Study area and the dataset

Located in the Western Anatolia part of Turkey, the Gediz Basin has a semi-arid climate and the scarcity of accessible water resources limits agricultural activities. The dry period, which started in the late 1980s and continued until the mid-1990s, caused significant problems in terms of irrigation water. There is approximately 110000 hectares of agricultural land in the basin (Çetinkaya et al. 2008). In the study, monthly total precipitation amounts and monthly average temperature data given in Table 1 with at least 25 years of observation and collected from non-intervened stations in the Gediz Basin were used as material. In Figure 1, the approximate locations of the meteorological stations in the basin and used for the study were given.

Table 1- Characteristics of stations located in the Gediz Basin

Station	*Code*	*Observation period*	*Number of observations*	*Elevation (m)*	*Latitude*	*Longitude*	*Mean annual rainfall (mm)*
Akhisar	17184	1960-2018	59	92	38.9118	27.8233	576.8
Demirci	17746	1992-2018	27	855	39.0349	28.6482	640.4
Gediz	17750	1972-2018	46	736	38.9947	29.4003	565.2
Manisa	17186	1960-2018	59	71	38.6153	27.4049	735.3
Salihli	17792	1960-2018	57	111	38.4831	28.2340	493.1

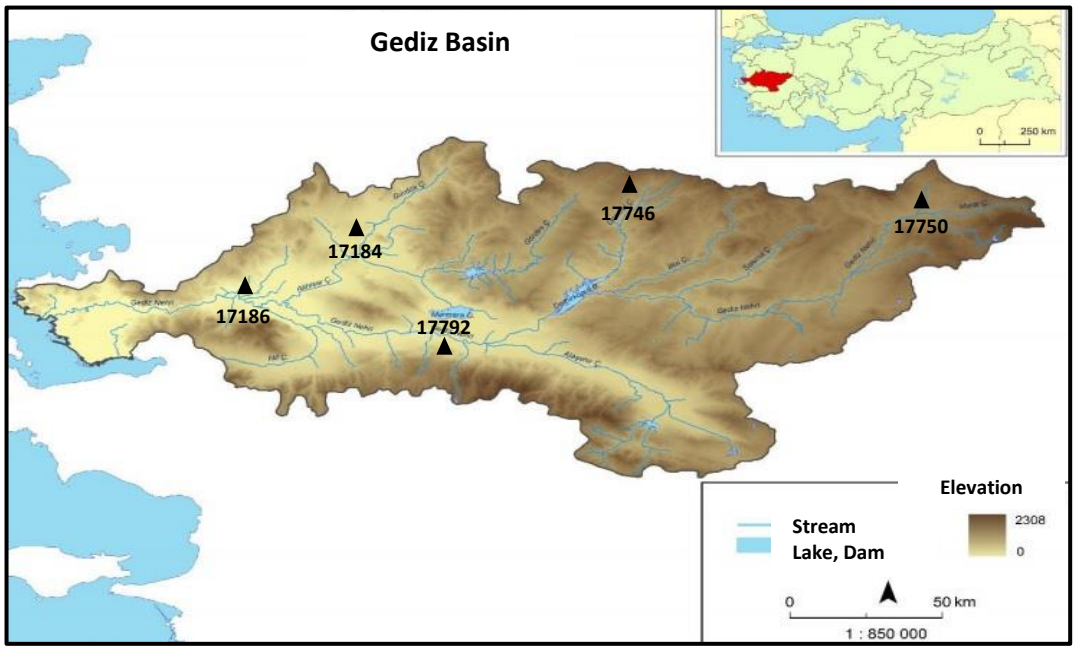

Figure 1- Location of the climate stations in Gediz Basin (SYGM 2016)

3. Methods

3.1. Calculation to SPEI

To calculate *SPEI*, 5 reference periods were determined by taking the 1, 3, 6, 9 and 12-month sums of the monthly total rainfall amounts (P_i). Potential Evapotranspiration (PET_i) amounts were determined by the Equation 1-3 as given in Thornthwaite (1948) and Thornthwaite & Mather (1955, 1957) by using the monthly average temperatures taken from the stations in the Gediz basin.

$$PET_i = 16 \times \left(\frac{10 \times t}{I}\right)^a \times G \quad (1)$$

$$a = 6.7510 \times 10^{-7} \times I^3 - 7.7110 \times 10^{-5} \times I^2 + 1.791210 \times 10^{-2} \times I + 0.49239 \quad (2)$$

$$I = \sum_1^{12} \left(\frac{t}{5}\right)^{1.514} \quad (3)$$

Where; t, Monthly average temperature (°C); I, Annual temperature index; G, Latitude correction coefficient. The latitude correction coefficient (G) was taken as 1.04 for Akhisar station, 0.85 for Demirci and Gediz stations, 1.24 for Manisa station and 1.23 for Salihli station (Thornthwaite 1948, Thornthwaite & Mather 1957). Various reference periods of the potential evapotranspiration (PET_i) amounts calculated with the Thornthwaite method using the relevant reference periods of the monthly total rainfall amounts (P_i) measured at the stations used in the study in the Gediz basin for calculating *SPEI*. The difference between the periods (Water Balance, D_i series) showing the excess or lack of water was calculated with Equation 4 (Vicente-Serrano et al. 2010).

$$D_i = P_i - PET_i \quad (4)$$

The most appropriate probability distribution for these series was estimated for D_i series. Normal (N), 3-parameter Normal (N3), 3-parameter Gamma (G3), Logistic (LO) and 3-parameter Logarithmic Logistic (LLO3) distributions were used. The most appropriate distribution was determined by Anderson-Darling (AD) test statistics. The probability of each D_i value was calculated by the cumulative distribution function $F(x)$ of the probability distribution, with the help of the parameters of the most suitable distribution among the distributions that fit the series. By using the standard values of the cumulative distribution functions $F(x)$ and using the Equation 5 and 6, *SPEI* series of 1, 3, 6, 9 and 12-month were obtained.

$$SPEI = W - \frac{(C_0 + C_1 W + C_2 W^2)}{(1 + d_1 W + d_2 W^2 + d_3 W^3)} \quad (5)$$

$$\text{For } P \leq 0.5, \quad W = (-2lnP)^{0.5}, \quad P = 1 - F(x) \quad (6)$$

If P>0.5, the P value changes to 1-P and the sign of the calculated *SPEI* value is inverted.

Where; C_0, 2.515547; C_1, 0.802853; C_2, 0.010328; d_1, 1.432788; d_2, 0.189269; d_3, 0.001308; P, Probability level.

Drought categories for the calculated *SPEI* values were given in Table 2.

Table 2- *SPEI* drought categories (Li et al. 2015)

Moisture category	***Code***	***SPEI***
Extremely wet	EW	*SPEI* > 2.00
Very wet	VW	1.50 < *SPEI* < 1.99
Moderately wet	MW	1.00 < *SPEI* < 1.49
Near normal	NN	-0.99 < *SPEI* < 0.99
Moderately drought	MD	-1.00 < *SPEI* < -1.49
Severely drought	SD	-1.50 < *SPEI* < -1.99
Extremely drought	ED	*SPEI* < -2.00

3.2. Regional analysis

The index-flood method was applied for the regionalization of the *SPEI* series in the Gediz basin (Dalrymple 1960). The basis of index-flood method is that the stations form an approximately homogeneous region and the frequency distribution of the stations in this region is the same except for a certain scale factor belonging to that station (Equation 7).

$$Q_i(F) = \mu_i q(F) \qquad i=1,\ldots, N \quad (7)$$

It is the indicator value representing the average of the frequency distribution at station μ_i. The q (F) value, which is the same for each station; shows the probability of being exceeded (Wallis & Wood 1985). The regionalization process is generally defined as the determination of climatological homogeneous regions, the selection of the regional frequency distribution and the estimation of the quantiles of indices. In this study, regional drought analysis was carried out with the *SPEI* series obtained by using L-moments. Hosking & Wallis (1993) stated probability-weighted moments in Equation 8, their linear combinations, L-moments, in Equation 9, and L-moment ratios in Equation 10.

$$b_r = n^{-1} \sum_{j=1}^{n} x_{(j)} \frac{(j-1)(j-2)...(j-1)}{(n-1)(n-2)...(n-i)} \tag{8}$$

After finding the probability-weighted moments (b_0, b_1, b_2 and b_3), which are the first four b_r values (r= 0, 1, 2, 3), the first four L-moment statistics, symbolized by ℓ for any distribution, are determined by the equations given below.

$$\begin{aligned} \ell_1 &= b_0 \\ \ell_2 &= 2b_1 - b_0 \\ \ell_3 &= 6b_2 - 6b_1 + b_0 \\ \ell_4 &= 20b_3 - 30b_2 + 12b_1 - b_0 \end{aligned} \tag{9}$$

Dimensionless L-moment ratios (L-coefficient of variation, L-skewness and L-kurtosis) are also estimated as given below.

$$\begin{aligned} t &= \ell_2 / \ell_1 \quad \text{(L-coefficient of variation)} \\ t_3 &= \ell_3 / \ell_2 \quad \text{(L-skewness)} \\ t_4 &= \ell_4 / \ell_2 \quad \text{(L-kurtosis)} \end{aligned} \tag{10}$$

In this study, in the regional analysis of the *SPEI* series; (1) Measures of Discordancy and Heterogeneity to determine climatological homogeneous regions, (2) Measure of Goodness of Fit to select regional frequency distribution, and (3) Regional L-moment algorithm for estimation of quantiles of indices. In this study, five stations located in the Gediz basin were firstly accepted as a single (one) region, and the analyzes were carried out according to this condition.

The discordancy measure (D) is the determination of the discordant stations within a region and it is explained by Hosking & Wallis (1997) as in Equation 11.

$$D = \frac{1}{3} N \left(u_i - \bar{u}\right)^T K^{-1} \left(u_i - \bar{u}\right) \tag{11}$$

u_i is the vector of moment ratios of the station i, K is the covariance matrix of this vector, and the mean of the vector. For a station to be considered discordant, the discordancy measure (D) must be greater than the critical table value, which varies depending on the number of stations in the region. Since five stations were considered in this study, the critical value was taken as 1.333 (Hosking & Wallis 1993). After specifying a suitable region according to the discordancy measure, the heterogeneity measure was applied to evaluate whether the region was homogeneous. Heterogeneity measure; It was calculated for three different L-statistics: L-coefficient of variation and H_1, L-coefficient of variation and combination of L-skewness ratios, H_2, and combination of L-kurtosis and L-skewness ratios, and H_3. From here, the H statistic for all three cases is written as in Equation 12.

$$H = \frac{\left(V_{obs} - \mu_v\right)}{\sigma_v} \tag{12}$$

V_{obs}; is the weighted standard deviation obtained from the regional data according to the L-moment ratios, μ_v and σ_v; It shows the mean and standard deviation of the number of simulations of the V_{obs} statistics. In this study, the four-parameter Kappa probability distribution, which is a strong distribution, was used while performing the simulation, since it represents many distributions in the frequency analysis of climatological events. In order to estimate μ_v and σ_v values reliably, the number of

simulations was considered as 500 for a region. According to this test, the region; if $H < 1$, it is considered to be acceptably homogeneous, if $1 \leq H < 2$, it is probably heterogeneous, and if $H \geq 2$, it is decidedly heterogeneous (Hosking 1994).

The Z^{DIST} statistic, which is determined depending on the L-kurtosis and frequency distribution from the detection of the homogeneous region, was given as in Equation 13.

$$Z^{DIST} = \left(\tau_4^{DIST} - t_4^R + B_4\right)/\sigma_4 \tag{13}$$

t_4^R, regional mean L-kurtosis ratio of sample, B_4, σ_4 regional mean L-kurtosis ratio bias value and standard deviation of sample, respectively, and were expressed in Equation 14 and 15.

$$B_4 = N_{sim}^{-1}\sum_{m=1}^{N_{sim}}\left(t_4^{(m)} - t_4^R\right) \tag{14}$$

$$\sigma_4 = \left[\left(N_{sim}-1\right)^{-1}\left\{\sum_{m=1}^{Nsim}\left(t_4^{(m)} - t_4^R\right)^2 - N_{sim}B_4^2\right\}\right]^{1/2} \tag{15}$$

N_{sim} represents the number of simulations performed with the help of Kappa distribution, and m represents the number of simulated regions. In this study, Generalized Logistic (GLO), Generalized Extreme Values (GEV), Generalized Pareto (GPA), Generalized Normal (GNO) and Pearson type 3 (PE3) distributions with three parameters were applied. If absolute $Z^{DIST} \leq 1.64$ in any frequency distribution, this distribution is considered suitable for the regional distribution, but the distribution that provides the absolute Z^{DIST} value closest to zero among the considered distributions is chosen as the most appropriate (best-fit) distribution.

The quantiles of the *SPEI* values were estimated with the regional L-moment algorithm. In a region with N stations, an i station has n_i data, the sample mean ℓ_1^i, sample L-moment ratios $t^{(i)}, t_3^{(i)}, t_4^{(i)}$ are calculated, and the L-moment regional average ratios t^R, t_3^R, t_4^R are determined as weighted according to the sample size of the stations, and their mathematical explanation has been written in Equation 16-18.

$$t^R = \sum_{i=1}^{N} n_i t^{(i)} / \sum_{i=1}^{N} n_i \tag{16}$$

Regional average ℓ_1^i is 1;

$$t_r^R = \sum_{i=1}^{N} n_i t_r^{(i)} / \sum_{i=1}^{N} n_i \qquad r = 3, 4,... \tag{17}$$

and the regional population (λ_i and τ_i) and sample L-moment ratios (ℓ_i^R, t_i^R) are equalized.

$$\begin{aligned} \lambda_1 &= \ell_1^R \\ \tau &= t^R \\ \tau_3 &= t_3^R \end{aligned} \tag{18}$$

Equation 19, in which regional dimensionless development curves are determined, is written as follows:

$$\hat{Q}_i(F) = \ell_1^i q(F; \ell_1^R, t^R, t_3^R, t_4^R) \tag{19}$$

While performing regional analyzes, routines written by Hosking (2005) with FORTRAN 77 source codes were used. These routines were compiled and run under a main executable program. A basic flow chart of the methodology of the study was given in Figure 2.

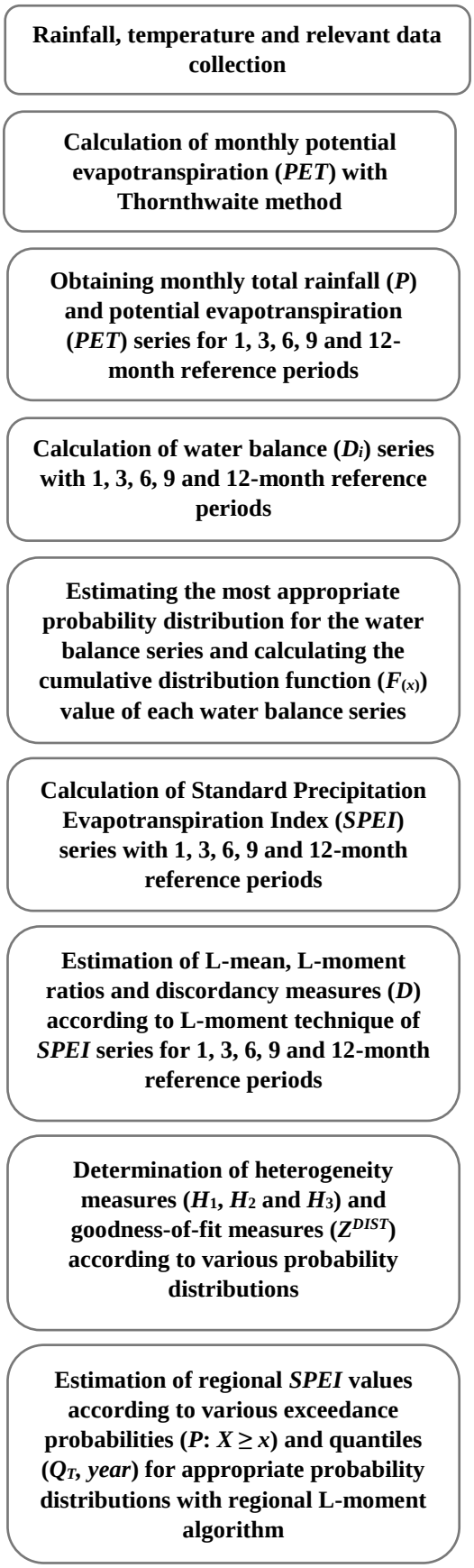

Figure 2- A basic flow chart of the methodology

4. Results and Discussion

As a preliminary information, some descriptive statistics and distribution characteristics of the annual total precipitation and annual average temperature data used in the study, calculated according to L-moment approach, were given in Table 3.

Table 3- Some descriptive statistics and distribution characteristics of annual total rainfall and annual mean temperature data

Site name	*L-mean*	*Standard error of L-mean*	*L-standard deviation*	*L-coefficient of variation*	*L-skewness*	*L-kurtosis*	*Best-fit frequency distribution*
Annual total rainfall (mm)							
Akhisar	576.8	17.4	131.5	22.80	0.42	-0.21	Generalized Pareto
Demirci	640.4	23.2	120.7	18.85	-0.33	-0.88	Log-logistic 3
Gediz	565.2	16.4	111.2	19.67	0.22	-0.36	Log-normal
Manisa	735.3	22.1	169.6	23.06	0.40	-0.06	Generalized gamma
Salihli	493.0	12.2	92.0	18.65	0.29	-0.05	Gamma
Annual mean temperature (ºC)							
Akhisar	16.19	0.09	0.728	4.50	0.31	-0.46	Generalized Pareto
Demirci	13.69	0.17	0.861	6.29	-0.26	-0.16	Generalized extreme values
Gediz	12.72	0.12	0.832	6.54	0.37	0.01	Log-logistic 3
Manisa	16.83	0.08	0.612	3.64	0.30	-0.15	Weibull 3
Salihli	16.26	0.15	1.149	7.07	-1.12	3.11	Log-logistic 3

The water balance series (D_i) obtained by using the monthly total rainfall amounts and the potential evapotranspiration amounts estimated according to the Thornthwaite method were given in Figure 3 for various reference periods.

Water shortages were observed in 1990 and 1992 in the 1-month period, in 1961, 1964, 1966, 1967, 1969, 1972, 1975-1977, 1983, 1985, 1988-1994, 1996, 1997, 2000-2002, 2006-2008, 2011, 2017 and 2018 in the 6-month period. Water deficiency was observed in all years in the 9-month period, and in all years except 1965 and 1981 in the 12-month period at Akhisar station (Figure 3a).

Water shortage has been observed in 1992 and 2001 in the 1-month period, in 2001, 2002, 2007 and 2008 in the 6-month period, in all years except 2016 in the 9-month period, in all years except 1997, 1998, 2005 and 2009 in the 12-month period at Demirci station (Figure 3b). As seen in Figure 3c, water shortage occurred in 2015 in the 1-month period, in 1976, 1977, 1989, 1990, 1992, 2001, 2002, 2007, 2008 and 2014 in the 6-month period. There was water deficiency in all years in the 9-month period, and water deficiency was observed in all years except 1978, 1981, 1983, 2005 and 2009 in the 12-month period at the Gediz station. As seen in Figure 3d, there was a shortage of water in 1989 and 1992 in the 1-month period, and in 1964, 1972, 1977, 1985, 1989-1992, 1994, 1996, 2001, 2002, 2007 and 2008 in the 6-month period. Water deficiency was observed in all years except 1965 and 1978 in the 9-month period, and in all years in the 12-month period at Manisa station. When Figure 3e is examined, water shortage was observed in the year 1992 and 2001 in the 1-month period, and, except for the years 1960, 1962, 1965, 1966, 1968, 1978, 1984, 1993, 2003, 2010, 2012, 2015 in the 6-month period. Water shortage occurred in all years in 9- and 12-months' periods at Salihli station. There was no shortage of water in all stations, and excess water was observed throughout the observation period in the 3-month period. In order to determine the *SPEI* series for various reference periods, the frequency distributions that best fit the water balance (D_i) series were determined.

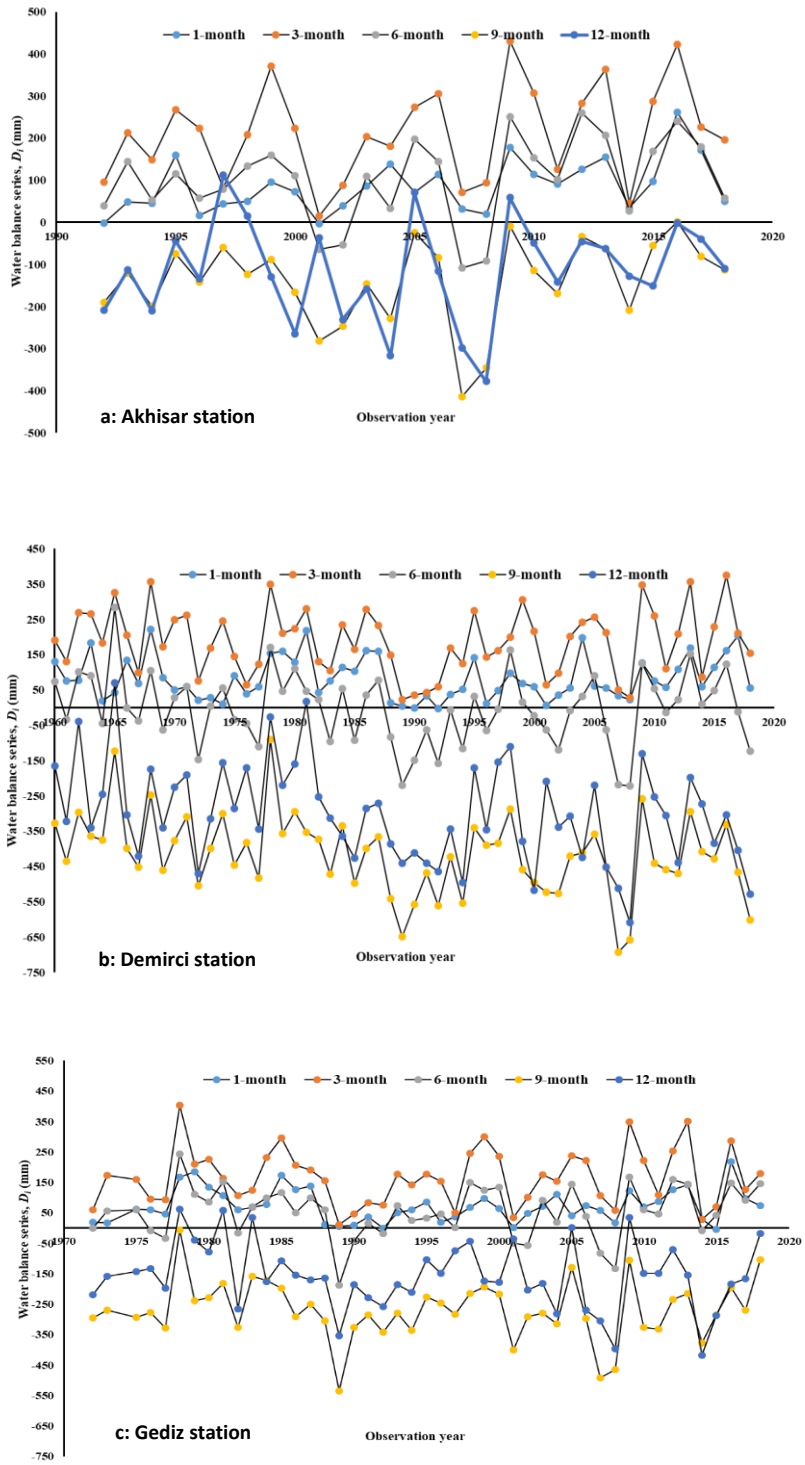

Figure 3- The water balance series (D_i) for stations according to various reference periods

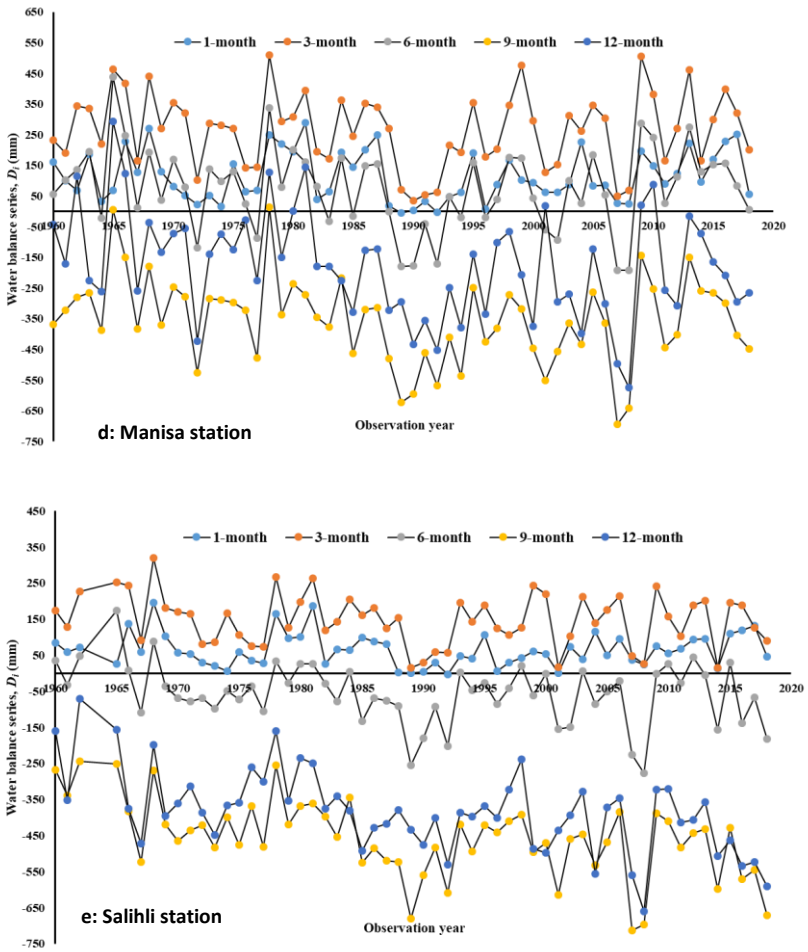

Figure 3 (continued)- The water balance series (D_i) for stations according to various reference periods

Normal (N), 3-parameter Normal (N3), 3-parameter Gamma (G3), Logistic (LO) and 3-parameter Logarithmic (LLO3) distributions were applied and the most appropriate distribution was calculated with Anderson-Darling (AD) test statistic and the results were given in Table 4.

The cumulative distribution functions $F(x)$ of each water balance D_i series were calculated by using the parameters of the frequency distributions selected for the stations according to the reference periods. *SPEI* values were determined for each station and reference periods by using the values corresponding to $F(x)$. The calculated *SPEI* series were given in Figure 4 for Akhisar, Demirci, Gediz, Manisa and Salihli stations, respectively. Moderate drought conditions were observed in 1964, 1972, 1988, 1996, 2008, severe drought in 1974, 1989, 2001, and extreme drought conditions in 1990, 1992 for the 1-month period. Moderate drought was observed in 1972, 1976, 1992, 2001, and severe drought in 1989-1991, 2007-2008 for the 3-month period. When the 6-month period is examined, moderate drought occurred in 1972, 1977, 1990, 1994, 2002, 2018, severe drought in 1992, and extreme drought in 1989, 2007-2008. In the 9-month period, moderate drought occurred in 1988, 1990, 1992, 1994, 2001-2002, severe drought in 2018, and extreme drought in 1989, 2007-2008. In the 12-month period, moderate drought was observed in 1972, 1989, 1991, 1992, 1994, 2016, 2012, severe drought in 2000, 2007, 2018, and extreme drought in 1985 and 1991 (Figure 4a). Moderate drought in 1996, 1998, severe drought in 1992 and extreme drought in 2001 were observed for the 1-month period. Moderate drought occurred in 1992, 1997, 2002, 2007, 2008, 2014, and severe drought in 2001 for the 3-month period. Severe drought was observed in 2001, 2002 and 2008, and extreme drought in 2007 for the 6-month period. Moderate arid conditions were observed in 2002 and 2004, severe arid conditions in 2001 and extremely dry conditions in 2007-2008 for the 9-month period.

Table 4- Frequency distributions applied to water balance (D_i) series for various reference periods and evaluation criteria

Candidate distribution	*Station*	*Evaluation criteria*	*Reference period (month)*				
			1	*3*	*6*	*9*	*12*
Normal (N)	**Akhisar**	**AD**	1.321	**0.243**	0.181	0.351	0.237
		P	<0.005	0.758	0.91	0.459	0.778
	Demirci	**AD**	0.479	**0.260**	0.338	0.485	**0.217**
		P	0.217	0.683	0.477	0.209	0.824
	Gediz	**AD**	0.644	0.3	0.296	0.687	0.517
		P	0.087	0.567	0.58	0.068	0.18
	Manisa	**AD**	1.256	**0.263**	0.357	0.408	0.158
		P	<0.005	0.69	0.443	0.336	0.949
	Salihli	**AD**	0.639	**0.199**	0.543	0.576	0.566
		P	0.091	0.881	0.156	0.128	0.136
3-parameter Normal (N3)	**Akhisar**	**AD**	0.370	0.267	0.195	0.346	**0.147**
		P	*	*	*	*	*
	Demirci	**AD**	**0.204**	0.287	0.357	0.499	0.231
		P	*	*	*	*	*
	Gediz	**AD**	0.296	0.201	0.298	0.678	0.509
		P	*	*	*	*	*
	Manisa	**AD**	0.64	0.278	0.38	0.414	**0.108**
		P	*	*	*	*	*
	Salihli	**AD**	**0.237**	0.215	0.565	0.59	0.487
		P	*	*	*	*	*
3-parameter Gamma (G3)	**Akhisar**	**AD**	**0.338**	0.449	0.658	4.078	0.598
		P	*	*	*	*	*
	Demirci	**AD**	0.221	0.299	2.134	14.098	2.918
		P	*	*	*	*	*
	Gediz	**AD**	**0.268**	**0.179**	18.775	13.522	3.623
		P	*	*	*	*	*
	Manisa	**AD**	**0.555**	0.435	0.561	3.609	1.387
		P	*	*	*	*	*
	Salihli	**AD**	0.269	0.447	9.403	4.107	3.998
		P	*	*	*	*	*
Logistic (LO)	**Akhisar**	**AD**	1.208	0.311	**0.155**	**0.151**	0.247
		P	<0.005	>0.25	>0.25	>0.25	>0.25
	Demirci	**AD**	0.429	0.295	**0.288**	**0.339**	0.218
		P	0.243	>0.25	>0.25	>0.25	>0.25
	Gediz	**AD**	0.529	0.299	**0.246**	**0.369**	0.444
		P	0.135	>0.25	>0.25	>0.25	0.228
	Manisa	**AD**	1.279	0.334	**0.307**	**0.285**	0.176
		P	<0.005	>0.25	>0.25	>0.25	>0.25
	Salihli	**AD**	0.461	0.274	**0.368**	**0.345**	0.369
		P	0.21	>0.25	>0.25	>0.25	>0.25
3-parameter Log-Logistic (LLO3)	**Akhisar**	**AD**	0.417	0.316	0.169	0.159	0.202
		P	*	*	*	*	*
	Demirci	**AD**	0.244	0.308	0.3	0.352	0.224
		P	*	*	*	*	*
	Gediz	**AD**	0.335	0.267	0.254	0.373	**0.432**
		P	*	*	*	*	*
	Manisa	**AD**	0.682	0.336	0.326	0.3	0.146
		P	*	*	*	*	*
	Salihli	**AD**	0.277	0.278	0.39	0.361	**0.342**
		P	*	*	*	*	*

AD: Anderson-Darling test statistic; **P:** probability value; *****: indicates non-computable probability level; Bold numbers show the best-fit frequency distributions for D_i series according to Anderson-Darling test statistic.

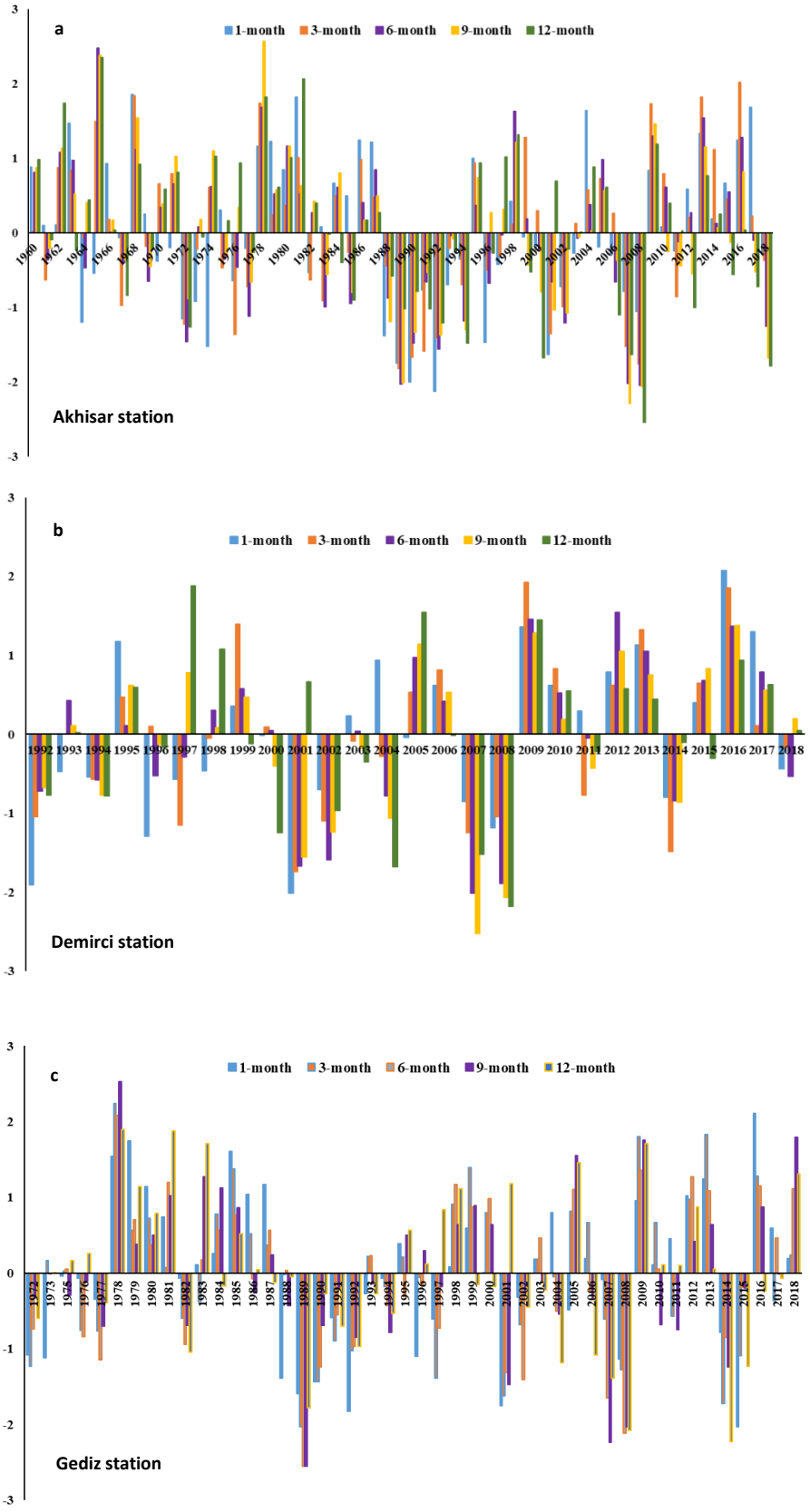

Figure 4- *SPEI* series according to various reference periods

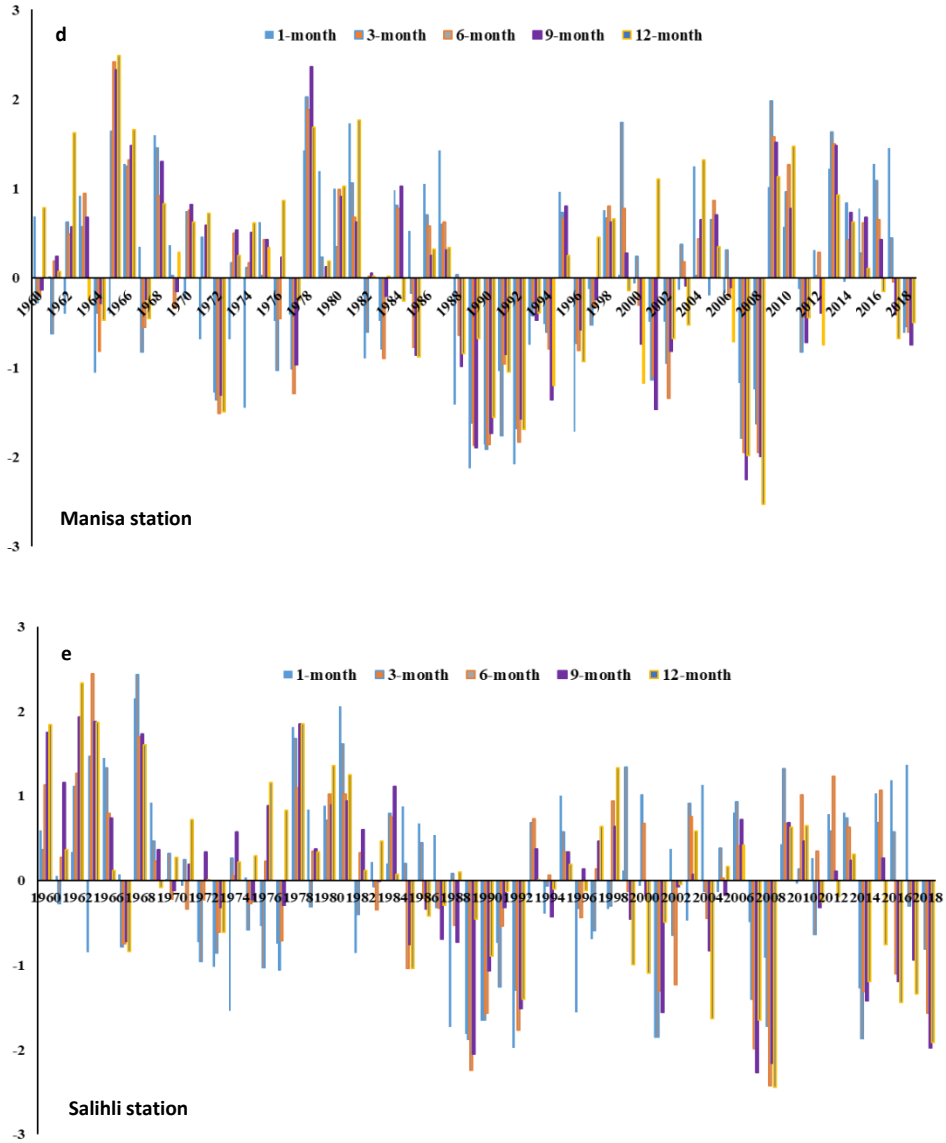

Figure 4 (continued)- *SPEI* series according to various reference periods

There was moderate drought in 2000, severe drought in 2004, 2007, and extreme drought in 2008 for the 12-month period (Figure 4b). Moderate drought was observed in 1972, 1973, 1988, 1990, 1996, 2008 severe drought in 1989, 1992, 2001, and extreme drought in 2015 for the 1-month period. Moderate drought was observed in 1972, 1990, 1992, 1997, 2008, 2015 severe drought in 2001, 2014, and extreme drought in 1989 for the 3-month period. Moderate drought occurred in 1977, 1990, 2001, 2002, severe drought in 2007, and extreme drought in 1989 and 2008 for 6-month period. Moderate drought conditions occurred in 2001, 2014, and extreme drought conditions occurred in 1989, 2007, 2008 for the 9-month period. Moderate drought was experienced in 1982, 2004, 2006, 2007, 2015, severe drought in 1989, and extreme drought in 2008, 2014 for the 12-month period (Figure 4c). Moderate drought occurred in 1964, 1972, 1974, 1988, 1991, 2007, 2008, severe drought in 1990, 1996, and extreme drought in 1989 and 1992 for the 1-month period. Moderate drought was observed in 1972, 1976-1977, 2001, and severe drought in 1989-1992, 2007, 2008 for the 3-month period. Moderate drought conditions were observed in 1977, 2001, 2002, and severe drought conditions in 1972, 1989, 1990, 1992, 2007, 2008 for the 6-month period. Moderate drought was experienced in 1972, 1994, 2001, severe drought in 1989, 1990, 1992, 2008, and extreme drought in 2007 for 9-month period. Moderate drought occurred in 1972, 1991, 1994, 2000, severe drought in 1990, 1992, 2007, and extreme drought in 2008 for the 12-month period (Figure 4d). Moderate drought was observed in 1973, 2014, and severe drought in 1974, 1988-1990, 1992, 1996, 2001 for the 1-month period. In 1976, 1977, 1991, 1992, moderate drought conditions occurred in 2007, severe drought conditions occurred in 1989-1990, 2001, 2008, 2014 for the 3-month period. Moderate drought was observed in 1985, 2001, 2002, 2014-2016, severe drought in 1990, 1992, 2007, 2018, and severe drought in 1989, 2008 for the 6-month period. Moderate drought was observed in 1990, 2014, 2016, severe drought in 1992, 2001, 2018, and extreme drought in 1989, 2007, 2008 for the 9-month period. Moderate drought occurred in 1985, 1992, 2000, 2014, 2016-2017, severe drought in 2004, 2007, 2018, and extreme drought in 2008 for the 12-month period (Figure 4e).

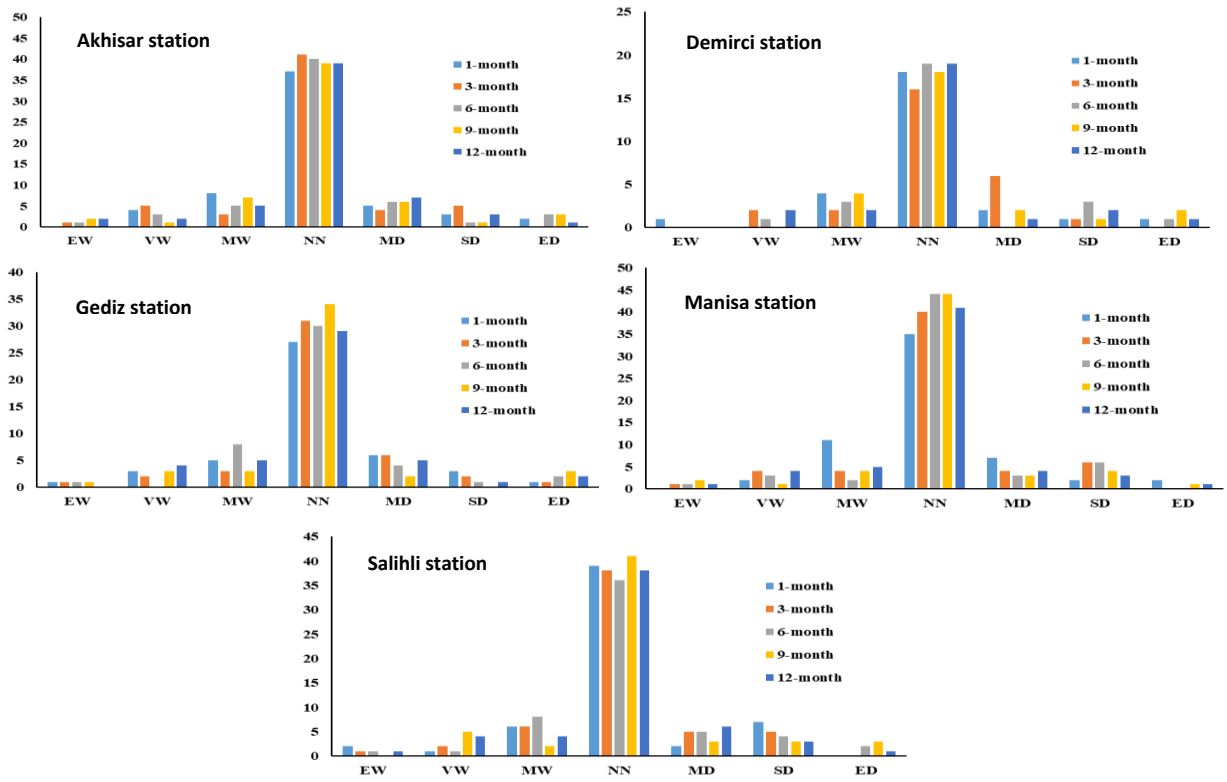

Figure 5- The frequency histograms of dry and wet years according to the *SPEI* series

The frequency histograms of dry and wet years according to the *SPEI* series were also given in Figure 5. It is seen that there are mostly near-normal (NN) conditions in all stations according to the *SPEI* series in Figure 5. For the regional drought analysis of the *SPEI* series, first of all, the five stations used in the study were accepted as a region and the discordancy, heterogeneity and goodness-of-fit measure tests were carried out according to this condition. The mean, L-moment ratios and discordancy measures (*D*) for the stations according to various reference periods were given in Table 5.

Table 5- Mean, L-moment ratios and discordancy measures (*D*) for various reference periods by stations for *SPEI* values

Reference period	*Station*	*L-mean*	*L-coefficient of variation*	*L-skewness*	*L-kurtosis*	*D**
1-month	**Akhisar**	-0.51	0.1288	-0.0006	0.0505	1.09
	Demirci	-0.48	0.1375	0.0279	0.1647	1.33
	Gediz	-0.50	0.1409	-0.0189	0.0640	0.95
	Manisa	-0.54	0.1379	-0.0146	0.0516	0.30
	Salihli	-0.46	0.1202	0.0274	0.1208	1.32
3-month	**Akhisar**	-0.46	0.1168	-0.0098	0.0980	0.56
	Demirci	-0.59	0.1338	-0.0245	-0.0150	1.32
	Gediz	-0.54	0.1291	-0.0424	0.1175	1.27
	Manisa	-0.41	0.1169	-0.0629	0.1094	1.02
	Salihli	-0.49	0.1230	0.0177	0.0766	0.84
6-month	**Akhisar**	-0.54	0.1335	-0.0614	0.1437	0.44
	Demirci	-0.48	0.1230	-0.1221	0.1482	1.13
	Gediz	-0.48	0.1355	-0.0202	0.1289	0.80
	Manisa	-0.49	0.1095	-0.0469	0.1144	1.33
	Salihli	-0.51	0.1362	-0.0512	0.1039	1.31
9-month	**Akhisar**	-0.51	0.1387	-0.0288	0.1516	1.32
	Demirci	-0.56	0.1385	-0.1678	0.1468	1.12
	Gediz	-0.59	0.1275	0.0445	0.2152	1.24
	Manisa	-0.47	0.1170	-0.0078	0.1670	0.68
	Salihli	-0.58	0.1244	-0.1126	0.1406	0.64
12-month	**Akhisar**	-0.47	0.1279	-0.0027	0.1317	1.29
	Demirci	-0.56	0.1256	-0.0478	0.1383	1.21
	Gediz	-0.54	0.1358	-0.0442	0.0858	1.13
	Manisa	-0.42	0.1199	-0.0257	0.1235	1.12
	Salihli	-0.47	0.1260	-0.0457	0.1106	0.24

* *D*, critical value for the discordancy measure: 1.333 (Hosking & Wallis 1997)

The means of *SPEI* values show that the drought status of the basin is Near Normal (NN) from the minus side according to the stations and reference periods. When the L-skewness values are examined, it is seen that there is a left skewness in *SPEI* values in general, but this skewness is not much. When the L-kurtosis values are examined, it is seen that the *SPEI* values are generally flat. When the discordancy measures are examined, although there are stations that are very close to the critical value (1.333) for different reference periods, no discordant stations were found (Table 5). Since no discordant stations were found for the reference periods according to the discordancy measure, the homogeneous region was simulated 500 times using the Kappa distribution, and the regional Kappa probability distribution parameters were determined. By using the calculated Kappa distribution parameters, heterogeneity measures were determined according to the number of 500 simulations for each reference period and are given in the Table 6.

Table 6- Heterogeneity measures for various reference periods

Reference period	*H1*	*H2*	*H3*
1-month	-0.2911	-0.2896	-0.9545
3-month	-0.1912	-0.1881	-0.8758
6-month	-0.2156	-0.2369	-2.1049
9-month	-0.1132	-0.1097	-0.4652
12-month	-0.0882	-0.0869	-2.3861

According to all heterogeneity measures, all reference periods were found to be acceptably homogeneous (Table 6). The fact that the heterogeneity measures in Table 6 have negative values is considered to indicate that the separation between the L-coefficients of variation between stations is less than expected from a homogeneous region and that there is a positive correlation between the data at different stations.

Goodness-of-fit measures were determined after the suggested regions were found to be homogeneous at an acceptable level according to the reference periods. The goodness-of-fit measures (Z^{DIST}) of the frequency distributions applied in the study were given in Table 7.

Table 7- Goodness-of-fit measures (Z^{DIST}) of frequency distributions used in the study for reference periods

Frequency distribution	*Code*	*Reference period*				
		1-month	*3-month*	*6-month*	*9-month*	*12-month*
Generalized logistic	**GLO**	4.84	4.50	2.17	**0.13***	2.61
Generalized extreme values	**GEV**	**1.50***	**1.05***	**-1.08***	-2.73	**-0.63***
Generalized normal	**GNO**	2.37	2.07	**-0.01*#**	-1.82	**0.35***
Pearson type 3	**PE3**	2.35	2.03	**-0.10***	-1.88	**0.31*#**
Generalized Pareto	**GPA**	-4.50	-4.99	-6.60	-7.63	-6.27

*: Suitable distributions; #: The best-fit distribution

Generalized extreme value in 1 and 3-month periods, generalized extreme value in 6 and 12-month periods, generalized normal and Pearson type 3, generalized logistic distributions in 9-month period were determined as appropriate distributions. The general Pareto distribution was not suitable for any reference period. According to the appropriate regional distributions, regional weighted parameters were estimated at 90% acceptance level. The probable regional *SPEI* values obtained for various exceedance probabilities (P: $X \geq x$) and durations (Q_T, year) according to these distributions with the regional L-moment algorithm technique were given in Table 8.

Table 8- Regional *SPEI* values obtained at various return probabilities and periods according to the most appropriate distributions

Exceedance probability	*Return period*	*Frequency distribution / Reference period*				
		GEV		*GNO*	*GLO*	*PE3*
(P: X ≥ x)	*(year)*	*1-month*	*3-month*	*6-month*	*9-month*	*12-month*
0.99	**1.01**	1.84	1.57	1.62	1.87	1.74
0.98	**1.02**	1.62	1.39	1.41	1.54	1.50
0.96	**1.04**	1.34	1.17	1.16	1.20	1.23
0.90	**1.11**	0.88	0.78	0.76	0.70	0.80
0.80	**1.25**	0.41	0.38	0.37	0.27	0.38
0.50	**2**	-0.51	-0.46	-0.45	-0.49	-0.45
0.25	**4**	-1.23	-1.15	-1.16	-1.14	-1.15
0.20	**5**	-1.40	-1.32	-1.34	-1.31	-1.33
0.10	**10**	-1.86	-1.76	-1.84	-1.81	-1.80
0.05	**20**	-2.22	-2.12	-2.27	-2.29	-2.21
0.01	**100**	-2.87	-2.78	-3.13	-3.40	-2.99

Table 8 shows that the *SPEI* values obtained according to the most appropriate frequency distributions for all reference periods can be very wet conditions in 1.01 years, moderately wet in 1.02 and 1.04 years, and near-normal in 1.11, 1.25 and 2 years. In addition, it is possible to experience moderately dry conditions in 4 and 5 years, severe dry in 10 years, and extremely dry in 20 and longer years in the basin.

5. Conclusions

In this study, a regional drought analysis was carried out with *SPEI* by using monthly average temperature and monthly total rainfall amounts obtained from Akhisar, Demirci, Gediz, Manisa and Salihli meteorological stations with long observation periods in the Gediz basin. The results from these analyzes are summarized as follows:

- The water balance series showed that there was little water deficiency in only a few years in the 1-month period. Water deficiency and excess water conditions in some years in the 6-month period. While there was a shortage of water in almost all of the 9 and 12-month periods, there was no shortage of water at any of the stations in the 3-month period, and excess water condition was detected.

- Anderson-Darling test statistics were applied to determine the distribution that best fits the water balance series. According to the AD test statistics results, the logistic distribution provided the best fit with the water balance series obtained for the 6 and 9-month reference periods at all stations. The dominant distribution adapting to the 3-month period was the normal distribution, 3-parameter gamma and 3-parameter normal distributions for the 1-month period, 3-parameter logarithmic logistic and 3-parameter normal distributions for the 12-month period provided the best fit.

- The cumulative probability distribution function of each water balance variant was determined and *SPEI* values were obtained by using these functions and related equations. According to the *SPEI*, near-normal conditions were the most dominant at all stations, while moderate and severe drought conditions and moderate and very wet conditions were occasionally observed, while extremely wet and dry conditions were rarely observed.

- Regional frequency analysis was carried out by using the *SPEI* values obtained from the points according to the stations and using the L-moment parameter estimation method. The five stations used in the study were accepted as a region and the Gediz basin was determined to be acceptable homogeneous at an acceptable level for these stations, according to the Discordancy and Heterogeneity measure tests applied with L-moment ratios.

- In order to determine the distributions that best-fit the *SPEI* values, 5 regional frequency distributions were applied and according to the results of the Goodness-of-fit Measure test; Generalized Extreme Values for 1 and 3-month period, Generalized Normal for 6-month period, Generalized Logistics for 9-month period and Pearson type 3 for 12-month period provided the best-fit.

- Regional *SPEI* values were obtained according to the parameters of the regional frequency distributions that provide the best-fit for the reference periods, and according to the L-moment algorithm technique, various probability of exceedance and recurrence interval.

- According to the regional *SPEI* values, it can be said that for the reference periods 1.11, 1.25 and 2 years Near Normal, 1.04 years Moderate Wet, 1.01 and 1.02 years Very Wet conditions can occur. It is thought that Moderate Dry conditions may occur within 4 and 5 years, Severe Dry conditions within 10 years, and Extreme Dry conditions for more than 20 years.

The results show that dry conditions will occur from short to long duration Similar results were found in Dikici (2020), Eris et al. (2020) Aksoy et al. (2021) Aksu et al. (2022) and Zeybekoğlu (2022). Establishment of a drought crisis center should be considered as a priority in order to minimize the dry conditions that may occur in the basin in the medium- and long-term period. In addition, the irrigation methods applied in the basin should be switched to mostly advanced pressurized methods, irrigation efficiency rate should be increased and irrigation training courses should be given to farmers. Pipeline conveyance systems should be used and land consolidation techniques should be done in order to minimize the losses in the conveyance of water from the water source to the agricultural land. A plant pattern should be created according to the climatic and water resources conditions of the region. The water obtained by macro basin water harvesting techniques should be used for irrigation purposes for dry periods. Water pricing should be planned according to water volume, not irrigation area. By not allowing construction and buildings around water resources, easier collection and protection of water should be ensured, and all the suggestions should be sustainable.

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References

Aksoy H, Cetin M, Eris E, Burgan H I, Cavus Y, Yildirim I & Sivapalan M (2021). Critical drought intensity–duration–frequency curves based on standardized precipitation index. *Hydrological Sciences Journal* 66(8): 1337–1358. https://doi.org/10.1080/02626667.2021.1934473

Aksu H, Cavus Y, Aksoy H, Akgul M A, Turker S & Eris E (2022). Spatiotemporal analysis of drought by CHIRPS precipitation estimates. T*heoretical and Applied Climatology* 148: 517–529. https://doi.org/10.1007/s00704-022-03960-6

Anlı A S & Öztürk F (2011). Regional Frequency Analysis for Annual Maxima Precipitation Data Measured in Ankara Province (In Turkish) *Journal of Agricultural Faculty of Gaziosmanpasa University* 28(1): 61-71

Anli A S (2017). Temporal variation of reference evapotranspiration and regional drought estimation using SPEI method for semi-arid Konya closed basin in Turkey. European Water 59: 231-238

Bacanli Ü G (2017). Trend analysis of precipitation and drought in the Aegean region, Turkey. Meteorological Applications 24: 239–249. https://doi.org/10.1002/met.1622

Bae S, Lee S, Yoo S & Kim T (2018). Analysis of Drought Intensity and Trends Using the Modified SPEI in South Korea from 1981 to 2010. Water, *Open Access Journal* 10(327): 1-17. https://doi.org/10.3390/w10030327

Begueria S, Vicente-Serrano S M, Reig F & Latorre B (2014). Standardized precipitation evapotranspiration index (SPEI) revisited: parameter fitting, evapotranspiration models, tools, datasets and drought monitoring. *Int. J. Climatol* 34: 3001–3023. https://doi.org/10.1002/joc.3887

Çetinkaya C P, Fistikoglu O, Fedra K & Harmancioglu N B (2008). Optimization methods applied for sustainable management of water-scarce basins. *Journal of Hydroinformatics* 10 (1): 69-95. https://doi.org/10.2166/hydro.2007.011

Dalrymple T (1960). Flood frequency analyses. Water Supply Paper 1543-A, U.S. Geological Survey, Reston, Va.

Dikici M (2020). Drought analysis with different indices for the Asi Basin (Turkey). Scientific Reports 10, 20739. https://doi.org/10.1038/s41598-020-77827-z

Eris E, Cavus Y, Aksoy H, Burgan H I, Aksu H & Boyacioglu H (2020). Spatiotemporal analysis of meteorological drought over Kucuk Menderes River Basin in the Aegean Region of Turkey. Theoretical and Applied Climatology 142: 1515–1530. https://doi.org/10.1007/s00704-020-03384-0

Greenwood J A, Landwehr J M, Matalas N C & Wallis J R (1979). Probability weighted moments: Definition and relation to parameters of several distributions expressable in inverse form. Water Resources Research 15: 1049-54. https://doi.org/10.1029/wr015i005p01049

Hosking J R M (1990). L-Moments: Analysis and Estimation of Distributions Using Linear Combinations of Order Statistics. *Journal of the Royal Statistical Society* (Series B) 52: 105-124. https://doi.org/10.1111/j.2517-6161.1990.tb01775.x

Hosking J R M (1994). The four-parameter kappa distribution. *IBM Journal of Research and Development* 38: 251-8. https://doi.org/10.1147/rd.383.0251

Hosking J R M (2005). FORTRAN routines for use with the method of L-moments, Version 3.04. Research Report RC 20525, IBM Research Division, T.C. Watson Research Center, Yorktown Heights, N.Y.

Hosking J R M & Wallis J R (1993). Some statistics useful in regional frequency analysis. Water Resources Research 29: 271-81. https://doi.org/10.1029/92wr01980

Katipoğlu O M, Acar R & Şenocak S (2021). Spatio-temporal analysis of meteorological and hydrological droughts in the Euphrates Basin, Turkey. Water Supply 21(4): 1657–1673. https://doi.org/10.2166/ws.2021.019.

Li X, He B, Quan X, Liao Z & Bai X (2015). Use of the Standardized Precipitation Evapotranspiration Index (SPEI) to Characterize the Drying Trend in Southwest China from 1982–2012. Remote Sens. 7: 10917-1093; https://doi.org/10.3390/rs70810917

Liu X, Wang S, Zhou Y, Wang F, Li W & Liu W (2015). Regionalization and Spatiotemporal Variation of Drought in China Based on Standardized Precipitation Evapotranspiration Index (1961–2013). Hindawi Publishing Corporation, Advances in Meteorology, Volume 2015, Article ID 950262, 18 pages, https://doi.org/10.1155/2015/950262

Meixiu Yu, Qiongfang Li, Hayes M J, Svobodab M D & Heime R R (2014). Are droughts becoming more frequent or severe in China based on the Standardized Precipitation Evapotranspiration Index: 1951–2010? *Int. J. Climatol* 34: 545–558. https://doi.org/10.1002/joc.3701

Miah Md G, Abdullah H M & Jeong C (2017). Exploring Standardized Precipitation Evapotranspiration Index for Drought Assessment in Bangladesh. Springer International Publishing, Environ Monit Assess 189: 547. 1–16. https://doi.org/10.1007/s10661-017-6235-5

Potop V & Možn M (2011). The Application a New Drought Index-Standardized Precipitation Evapotranspiration Index in The Czech Republic. Mikroklima A Mezoklima Krajinných Struktur a Antropogenních Prostředí. Skalní Mlýn, 2. 4.2. 2011, ISBN 978-80-86690-87-2

Redmond K T (2000). Integrated climate monitoring for drought detection, in Drought: A Global Assessment, Hazards Disasters Ser., vol. I, edited by D. A. Wilhite, pp. 145 – 158, Routledge, New York.

Stagge J H, Tallaksen L M, Xu C & Van Lanen H A J (2014). Standardized precipitation-evapotranspiration index (SPEI): Sensitivity to potential evapotranspiration model and parameters. Hydrology in a Changing World: Environmental and Human Dimensions Proceedings of FRIEND-Water 2014, Montpellier, France, October 2014. 367-374.

Stagge J H, Tallaksen L M, Gudmundsson L, Van Loon A F & Stahl K (2015). Candidate Distributions for Climatological Drought Indices (SPI and SPEI). *Int. J. Climatol.* 35: 4027–4040. https://doi.org/10.1002/joc.4267.

SYGM (2016). The Impact of Climate Change on Water Resources Project. Project Final Report, Appendix-7 Gediz Basin (In Turkish). Ministry of Forestry and Water Management, General Directorate of Water Management

Thornthwaite C W (1948). An Approach toward a Rational Classification of Climate. Geographical Review 38:1, 55-94. https://doi.org/10.2307/210739.

Thornthwaite C W & Mather J R (1955). The Water Balance. Laboratory of Climatology Publ. 8. Centerton, NJ.

Thornthwaite C W & Mather J R (1957). Instructions and Tables for Computing Potential Evapotranspiration and the Water Balance. Drexel Institute of Technology, Laboratory of Climatology, Publications in Climatology 10(3): 311 pp.

Vicente-Serrano S M, S Beguería & J I López-Moreno (2010). A Multiscalar Drought İndex Sensitive to Global Warming: The Standardized Precipitation Evapotranspiration İndex. J. Climate, 23: 1696-1718. https://doi.org/10.1175/2009jcli2909.1

Vogel R M, Thomas W O & McMahon T A (1993). Flood-flow frequency model selection in Southwestern United States. *Journal of Water Resources and Management* 119(3): 353-66. https://doi.org/10.1061/(asce)0733-9496(1993)119:3(353)

Wallis J R & Wood E F (1985). Relative accuracy of log Pearson III procedures. *Journal of Hydraulic Engineering* 111: 1043-56. https://doi.org/10.1061/(asce)0733-9429(1985)111:7(1043)

Wilhite D A (2000). Drought as a Natural Hazard: Concepts and Definitions, (Chapter 1). In: D.A. Wilhite (ed.), Drought: A Global Assessment (Volumes 1 and 2), Routledge Publishers, London

Yang M, Yan D, Yu Y & Yang Z (2015). SPEI-Based Spatiotemporal Analysis of Drought in Haihe River Basin from 1961 to 2010. Hindawi Publishing Corporation Advances in Meteorology Volume 2016, Article ID 7658015, 10 pages. https://doi.org/10.1155/2016/7658015
Zeybekoğlu U (2022). Spatiotemporal analysis of droughts in Hirfanli Dam basin, Turkey by the Standardised Precipitation Evapotranspiration Index (SPEI). Acta Geophysica 70: 361–371, https://doi.org/10.1007/s11600-021-00719-x

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Effect of Adding Lactic Acid Bacteria to Maize Silage on Nutritive Guality, Fermentation Properties and in Vitro Digestibility

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ABSTRACT
This study aimed to determine the effects of adding lactic acid bacteria to maize silage on the nutritional quality, fermentation properties, and its *in vitro* organic matter digestion (IVOMD). Pre-fermented juices (PFJ) prepared from different water-soluble carbohydrate (WSC) sources at the rate of 5% and commercial homofermentative and heterofermentative lactic acid bacteria (LAB) were added to silages. Groups were designed as (I) control, (II) Glucose-PFJ, (III) Fructose-PFJ, (IV) Sucrose-PFJ, (V) Molasses-PFJ, (VI) Homofermentative LAB (HoLAB) and (VII) Heterofermentative LAB (HetLAB). Lactic acid bacteria (LAB) count, lactic acid (LA), acetic acid (AA), LA/AA ratio, pH and yeast values of the natural fermented lactic acid bacteria liquids prepared by adding 5% of different easily soluble carbohydrate sources to meadow grass showed significant variations. The differences among the groups in the crude ash (CA), acid detergent fiber (ADF), IVOMD and methane (CH_4) values of the silage groups prepared by adding PFJ were also found to be statistically significant. The differences in the fermentation characteristics of the silages (pH, ammonia-nitrogen (NH_3-N), LA, AA, LA/AA, CO_2, and total yeast mold after aerobic stability) were statistically significant too. When all parameters were examined, it was concluded that the addition of PFJ, which is prepared by adding 5% fructose to the meadow grass plant, to the maize silage has positive effects on IVOMD, ME, CH_4, LA and yeast-mold and can be used instead of commercial inoculants.

Keywords: Epiphytic microorganisms, Inoculant, *In vitro* digestibility, Methane, Mikrobiota

1. Introduction

The maize plant is the predominant grain crop for ensiling worldwide, compared to other forage crops due to its fermentation efficiency in animal nutrition. Since the maize plant has a proportionally higher dry matter content, a low buffer capacity (resistance to acidification) and sufficient water-soluble carbohydrate (WSC) content necessary for lactic acid (LA) fermentation, it can be easily ensiled. The use of bacterial inoculants in ensiling provides a rapid pH decrease by producing lactic acid, preventing the growth of undesirable epiphytic microorganisms, reducing proteolysis and increasing dry matter gain (Muck 2013).

Lactic acid bacteria (LAB) are divided into two main groups according to the types of saccharolytic fermentation (Axelsson 1998). Members of the mandatory homofermentative (HoLAB) or facultative heterofermentative LAB group include *Lactobacillus acidophilus, L. delbrueckii, L. helveticus, L. farciminis, L. lactis and L. bovis*. Only in some special cases (when there is insufficient sucrose in the environment), microorganisms in this group, called facultative heterofermentative LAB (HetLAB), acquire heterofermentative properties. The most important members of this group are *L. plantarum. L. alimentarius, L. casei, L. curvatus, L. sakei, L. paralimentarius and L. pentosus* are also found in this group. Members of the mandatory HetLAB group include *L. brevis, L. buchneri, L. fermentum, L. reuteri, L. fructivorans, L. sanfranciscensis* and *Leuconostoc mesenteroides*. The effects of bacterial inoculants used as silage additives on silage fermentation vary according to the characteristics of the LAB contained in the inoculant. Results are inconsistent regarding silage fermentation of Homofermentative LAB use in maize silage (Kleinschmit & Kung 2006). Although the use of HetLAB increases aerobic stability by preventing the growth of yeasts in the silage, fermentation losses were observed due to CO_2 formed during fermentation (Blajman et al. 2020). Another aspect to consider is the number of bacteria in the product and per gram of silage. A comparison of the products available on the market for silage additives reveals a range of 10^5 to 10^6 cfu/g of silage (DLG 2011; Aragón 2012). Natural fermented lactic acid bacteria liquid (PFJ) is more effective in LA production especially in silages with a high moisture content, since it contains many epiphytic LAB species that act synergistically, can be prepared in a practical and economical way and used as an alternative to commercial LAB inoculants. Its use has become widespread in recent years due to its benefits (Sun et al. 2021). This study was conducted to determine the effects of the addition of LAB to maize silage on the silage quality, its fermentation properties and it's *in vitro* organic matter digestion.

2. Material and Methods

2.1. Study design and silage preparation

In this study, the maize plant was used as the raw silage material. The buffering capacity of the fresh maize plant used in the present study was determined according to the method reported by Playne & McDonald (1966). The total number of LABs in the fresh silage material and in the experimental groups was measured in four replications per group according to the tempo automatic bacterial count test method reported by Güney & Ertürk (2020). The addition-free maize plant constituted the control group in the study, while the experimental groups consisted of maize silages to which fructose PFJ (F-PFJ), molasses PFJ (M-PFJ), sucrose PFJ (S-PFJ), glucose PFJ (G-PFJ), commercial HoLAB, and HetLAB inoculants were added. The PFJs used in this study were prepared according to the method reported by Masuko et al. (2002). After the PFJs were added to the meadow grass plant with pure water at a ratio of 1:1 and passed through the blender, 5% of different easily soluble carbohydrate sources (glucose, fructose, molasses, and sucrose) were added to the resulting liquid and left to incubate at 30°C for five days. The microbiota content of the PFJs were determined according to the Shotgun method (Sun et al. 2021). The PFJs, HoLAB and HetLAB inoculants were applied to the silages at 1 mL/kg. Inoculants were used by inoculating 1 mL of silage with 10cc distilled water to ensure homogeneity. The commercial HoLAB inoculant that was used as an additive contained *Lactobacillus plantarum* DSM 18112, *Lactobacillus plantarum* DSM 18113, *Lactobacillus plantarum* DSM 18114, *Lactobacillus plantarum* ATCC 55943, *Enterococcus faecium* ATCC 55593 and *Enterococcus faecium* ATCC 53519 water. HetLAB inoculant used as additive contained the *Lactobacillus buncheri* ATCC PTA-2494 strain.

Control and experimental groups were compressed into 1.5-liter glass jars in 4 replicates. The silages were fermented in a dark environment for 60 days before opening. During this time, the silages were stored at room temperature.

2.2. Fermentation profile analysis

After the silages were opened, a 3-5 cm section was discarded from the top of the jar. After the silages were poured into a container, approximately 25 g of silage sample was mixed homogeneously with 100 mL of distilled water with the help of a blender. The pH value of the shredded silage liquid was rapidly recorded with pH meter measuring device. In addition to the silage liquid, 0.1 mL of 1M HCl was added to the tubes prepared for ammonia nitrogen analysis. For the analysis of LA and volatile fatty acids (VFA), 0.25 mL of 25% metaphosphoric acid was added to the prepared tubes. The tubes prepared for ammonia nitrogen, LA and VFA assessment were stored in the deep freezer until analyses. NH_3-N/TN determination of the silage samples were performed according to the method reported by Broderick & Kang (1980). VFA such as propionic acid (PA), acetic acid (AA) and butyric acid (BA) and LA were determined as reported by Suzuki & Lund (1980). For this reason, high performance liquid chromatography (HPLC) analyser (Shimadzu LC-20 AD HPLC pump, Icsep Coregel (87H3 colon), Shimadzu SIL-20 ADHT Autosampler, Shimadzu cto-20ac Colum oven, Shimadzu SPD M20A Detector (DAD), Türkiye) was used. The silages obtained in the study were subjected to aerobic stability test in order to determine the CO_2 production values. For this purpose, silages were exposed to oxygen for 5 days according to the method developed by Ashbell et al. (1991).

While the raw nutrient contents of the silages (such as dry matter, crude ash, crude protein) were determined according to the method reported by AOAC (2005), the ADF and NDF analyzes of the silages were performed as reported by Van Soest et al. (1991). Before the raw nutrient analysis, the silages were dried at room temperature and ground in a laboratory mill to pass through a 1 mm sieve and made ready for analysis.

The gas production values of the silages and alfalfa herbage were determined through the method described by Menke & Steingass (1988) using four glass syringes as replicates. The rumen fluid used in the analysis was collected with the help of a rumen pump from 2 rams who were provided with a training diet (60% forage, 40% concentrate) for 2 weeks. The in vitro organic matter digestibility (IVOMD) (g/kg OM) and metabolizable energy (ME) (MJ/kg DM) of silages were calculated using equations reported by Menke et al. (1979) as:

$$ME\ (MJ/kg\ DM) = 2.20 + 0.136 \times Gp + 0.057 \times CP + 0.0029 \times CP2,$$
$$IVOMD\ (\%) = 14.88 + 0.889 \times Gp + 0.45 \times CP + 0.0651 \times XA,$$

Where; CP is CP in g/100 g DM, crude ash in g/100 g DM and gas production is the net gas production (mL) from 200 mg DM after 24 h of incubation. After recording 24-h gas production values, gas inside the syringe was measured by three-way syringe system and total gas was injected into computer-assisted infrared methane gas meter (Sensor Europe GmbH, Erkrath, Germany) and then methane content was determined as a percentage of 24 h the total amount of gas formed (Goel et al. 2008). Yeast and mold contents of silages were determined using the method reported by Filya et al. (2000).

2.3. Statistical analysis

One Way Analysis of Variance (One Way Anova) was used to determine whether the data obtained from the groups were significantly different. Duncan's multiple comparison tests were used to control the significance of the differences among the

groups, and for this purpose, the SPSS (1991) software program was used.

3. Results and Discussion

The amount of LAB, LA, AA, LA/AA ratio, pH, total yeast and mold values of the PFJs prepared by adding different easily soluble carbohydrate sources (glucose, fructose, molasses and sucrose) to the meadow grass plant are presented in Table 1. Microbiota graphics of the LAB liquids prepared by adding different easily soluble carbohydrate sources (fructose, sucrose and molasses) to the meadow grass plant are shown in Figure 1.

When the microbiota contents of the PFLs prepared by adding 5% easily soluble carbohydrate sources (fructose, molasses, sucrose and glucose) to the meadow grass plant were examined, it was observed that they were of different types and ratios. As indicated in Figure 1, in S-PFJ, *Lactiplantibacillus plantarum* 38%, *Lactiplantibacillus pentosus* 19%, *Lactiplantibacillus paraplantarum* 2%, *Lactiplantibacillus plagiomi* 2%, *Lactiplantibacillus argentoratensis* 2%, *Lactiplantibacillus argentoratensibacillus* 2% *fermocicillus* 7%, *Levilactobacillus brevis* 2%, *Levilactobacillus parabrevis* and *Levilactobacillus hammesii* species were identified.

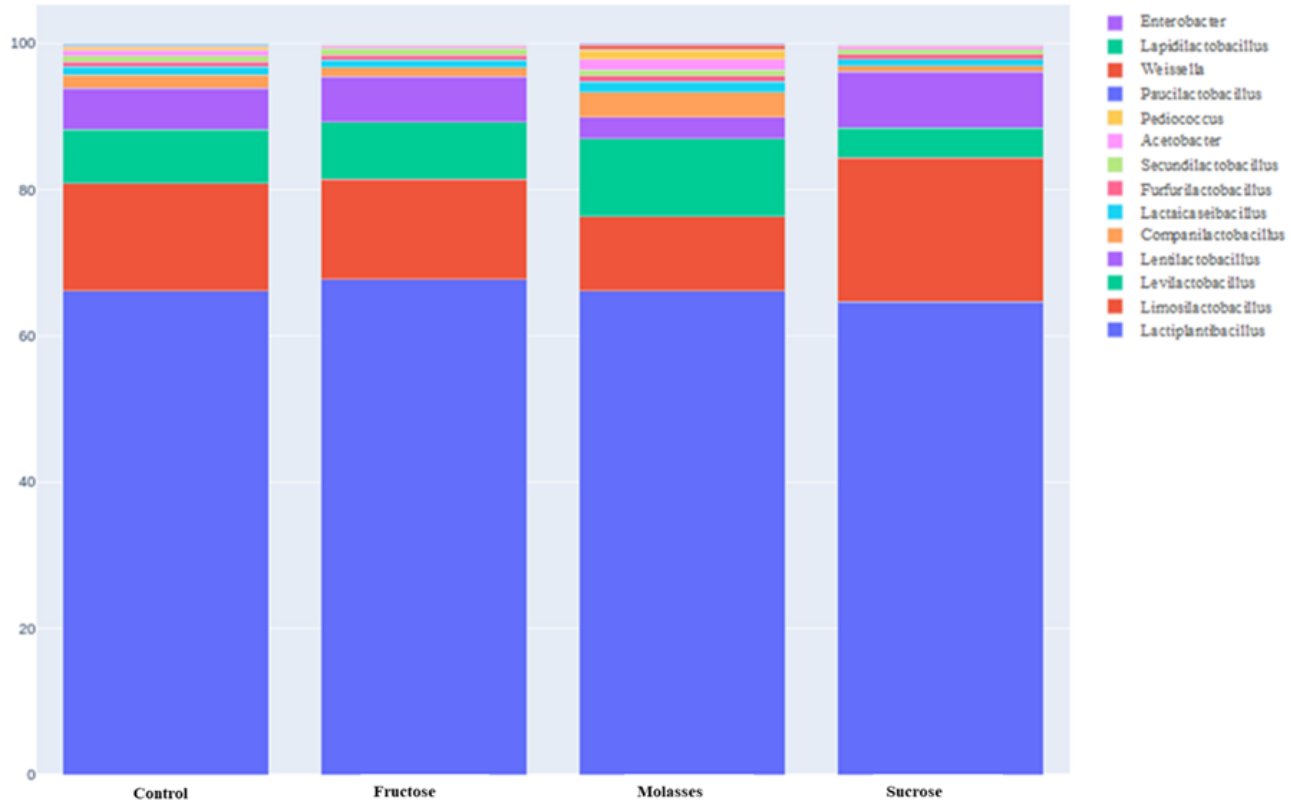

Figure 1- Microbiota graphs of LAB liquids prepared by adding 5% easily soluble carbohydrate sources (fructose, sucrose and molasses) to the meadow grass plant

As shown Figure 1, *Lactiplantibacillus plantarum* 41%, *Lactiplantibacillus pentosus* 19%, *Lactiplantibacillus paraplantarum* 2%, *Lactiplantibacillus plagomomi 2%*, *Lactiplantibacillus argentoratensis* 2%, *Lictiplantibacillus argentoratensibacillus* 2% *fermoscinercillus* 5%, *Levilactobacillus brevis* 2% and *Levilactobacillus zymae* 5% species were identified in the microbiota content of F-PFJ.

When the microbiota content of M-PFJ were examined in Figure 1, *Lactiplantibacillus plantarum* 42%, *Lactiplantibacillus pentosus* 18%, *Lactiplantibacillus paraplantarum* 3%, *Lactiplantibacillus argentoratensis* 2%, *Limosilactobacillus levbuchilactiobacillus* 8%, *brevis* 4%, *Levilactobacillus zymae* 5%, *Companilactobacillus kimchii* 5% *Lactobacillus pontis, Lactobacillus frumenti, Levilactobacillus parabrevis, Levilactobacillus hammesii, Levilactobacillus namurensis* and *Levilactobacillus senmaizukei* species were identified.

Similarly, Parvin & Nishino (2010) detected *Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus brevis* and *Lactococcus lactis* species in Rhodes grass in their study. In a study by Fabiszewska et al. (2019) *Lactiplantibacillus plantarum, Lactobacillus acidophilus, E. faecium, P. Acidilactici, P. pentosaceus Lentilactobacillus buchneri, Limosilactobacillus reuteri, Lacticaseibacillus casei Levilactobacillus zymae, Apilactobacillus kunkeei, Levilactobacillus*

acidifarinae, *Levilactobacillus namurensis*, *Levilactobacillus brevis*, *Levilactobacillus spicheri*, *Fructilactobacillus fructivorans*, *Fructilactobacillus fructivorans* and *Levilactobacillus hammesii* were identified as key species used to effectively and vigorously improve silage quality. Similar species were detected in the PFJs of the present study.

Table 1- Fermentation values of PFJs prepared by adding 5% different easily soluble carbohydrate sources to the meadow grass plant

Groups	*LAB*	*LA*	*AA*	*LA/AA*	*pH*	*Yeast*	*Mold*
G-PFJ	12.52^{a}	754.77^{a}	75.05^{b}	10.05^{a}	3.48^{b}	7.03^{c}	<10
F-PFJ	12.33^{b}	351.16^{d}	45.88^{d}	7.80^{b}	3.29^{c}	7.60^{b}	<10
S-PFJ	12.01^{d}	411.01^{c}	71.79^{c}	5.80^{d}	3.25^{d}	7.82^{a}	<10
M-PFJ	12.18^{c}	700.00^{b}	108.95^{a}	6.40^{c}	3.72^{a}	5.82^{d}	<10
SEM	0.058	52.946	6.75915	0.493	0.055	0.235	…
P-Value	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**…**

$^{a-d:}$ Values with different letters in the same column were found to be different (P<0.05); **LAB:** Lactic acid bacteria log10 cfu/mL, **LA:** Lactic acid g/kg DM, **AA:** Acetic acid g/kg DM, **Yeast:** log10 cfu/mL, **Mold:** log10 cfu/mL.

When the fermentation values such as LAB number, LA, AA, LA/AA ratio, pH and yeast of the PFJs prepared by adding 5% different easily soluble carbohydrate sources to the meadow grass plant were examined, the differences among the groups were statistically significant. In this study, when the LAB values in the natural LAB fluids were examined, the lowest LAB value was obtained from the S-PFJ, and the highest value was obtained from the G-PFJ. The total LAB values in the PFJ obtained in this study were found to be higher than the values obtained in the study of Bureenok et al. (2005a), and similar to the values reported in the study of Aydın & Denek (2022). It is known that LAB species degrade different carbohydrates at different levels. The higher the molecular weight of the carbohydrate type, the lower the level of fermentation, that is, degradation. More complex carbohydrates such as sucrose and polysaccharides are more difficult to break down than monosaccharides, and microbial and plant enzymes play an important role in this breakdown process (Kılıç 1986). Detection of the lowest LAB count in the sucrose PFJ group supports this hypothesis. Bureenok et al. (2005b) found the highest LAB value in the glucose supplemented group and the lowest LAB value in the S-PFJ group, which is consistent with the current study. When the LA values of the natural LAB fluids were examined, the highest LA value was found in the G-PFJ group, and the lowest LA value was found in the F-PFJ group. A possible reason for the difference could be attributed to the fact that LAB can decompose glucose into LA more efficiently than other carbohydrate sources (Müller & Lier 1994). The fact that the highest amount of LA was in the G-PFJ group supports this statement. While the highest AA value in the natural LAB fluids was detected in the M-PFJ group, the lowest AA value was observed in the F-PFJ group. This result can be explained by the fact that molasses contains not only sucrose but also nitrogenous compounds that can be used by microorganisms (Otero et al. 1993). Our results are in accordance with these reported by Bureenok et al. (2005a). When Table 1 is examined, the LA/AA ratio in the PFJ groups was within the range of 5.80-10.05. It is reported that homolactic fermentation occurs when the LA/AA ratio is greater than 3.0 and heterolactic fermentation occurs when the LA/AA ratio is less than 3.0 (Zhang et al. 2010). In this study, the LA/AA ratio being greater than 3 in all groups reveals that homolactic is more intense than heterolactic activity. When the pH values of the PFJs were examined, the lowest value (3.25) was obtained in the S-PFJ, and the highest value (3.72) was obtained in the M-PFJ group. Differences in pH values in PFJs may be due to the type and amount of LAB used, the plant species, the easily soluble carbohydrate source and amount, and the fermented incubation time (Can 2010). According to Denek et al. (2011), the pH values (3.45-3.76) in PFJs prepared by adding glucose were comparable with that of the present study. When Table 1 was examined, the highest yeast value in the PFJs was obtained from the S-PFJ, and the lowest yeast value was obtained from the M-PFJ. In addition, molds were not found in any PFJ group. The determination of the lowest yeast and the highest acetic acid values in the M-PFJ group can be explained by the fact that the amount of acetic acid formed as a result of LAB fermentation has an inhibitory effect on the growth and activity of yeasts (Ali et al. 2020). Yeast and mold values in PFJ prepared by adding 2% glucose to alfalfa plant are similar to the values in this study (Tao et al., 2017). The analyses of the maize plant used as silage raw material in this study are shown in Table 2.

Table 2- Analysis values of the maize plant used as silage material in the study

Silage Material	***BC***	***DM***	***CA***	***CP***	***ADF***	***NDF***	***IVOM***	***ME***	***CH_4***	***LAB***	***Yeast***	***Mold***
Maize	210	28.15	6.70	7.95	26.25	56.14	58.72	8.61	9.94	$7.2*10^{6}$	$2*10^{6}$	$4.2*10^{5}$

BC: Buffer capacity; **DM:** Dry matter, %; **CA:** Crude ash, DM%; **CP:** Crude protein, DM%; **ADF:** Acid detergent insoluble fiber, DM%; **NDF:** Neutral detergent insoluble fiber, DM%; **IVOMD:** *In vitro* organic matter digestion, %; **ME:** Metabolizable energy MJ/kg DM; **CH_4:** *In Vitro* methane gas, %

The nutrient content and IVOMD, ME and *in vitro* CH_4 values of the silages prepared by adding LAB liquid to the maize plants are presented in Table 3.

Table 3- Nutrient content and IVOMD, ME and CH_4 values of the silages prepared by adding LAB liquid to the maize plants

Groups	*DM*	*CA*	*CP*	*ADF*	*NDF*	*IVOMD*	*ME*	*CH_4*
Control	27.84	6.09^{b}	7.25	25.28^{a}	54.56	56.21^{d}	8.59	10.28^{a}
F-PFJ	27.20	6.28^{b}	7.25	24.33^{a}	46.85	66.86^{a}	9.81	7.33^{c}
M-PFJ	27.44	6.13^{b}	7.37	24.88^{a}	46.77	64.27^{abc}	9.73	9.16^{ab}
S-PFJ	28.07	6.23^{b}	7.37	25.26^{a}	49.15	61.22^{bc}	9.09	9.01^{ab}
G-PFJ	27.51	6.80^{a}	7.44	23.78^{ab}	42.84	63.88^{abc}	9.26	8.65^{bc}
HoLAB	27.15	6.40^{ab}	7.28	20.79^{bc}	51.06	65.60^{ab}	9.13	8.07^{bc}
HetLAB	26.51	6.51^{ab}	7.77	19.28^{c}	52.74	60.15^{cd}	9.12	8.23^{bc}
SEM	0.152	0.063	0.045	0.550	1.141	0.809	0.122	0.222
P	0.125	0.021	0.100	0.002	0.077	0.001	0.105	0.004

$^{a-c:}$ Values with different letters in the same column were found to be different (P<0.05); **DM:** Dry matter, %; **CA:** Crude ash, DM%; **CP:** Crude protein, DM%; **ADF:** Acid detergent insoluble fiber, DM%; **NDF:** Neutral detergent insoluble fiber, DM%; **IVOMD:** *In Vitro* organic matter digestion, %; **ME:** Metabolizable energy MJ/kg DM; **CH_4:** *In Vitro* methane gas, %.

When Table 3 was examined, the differences among the groups in the CA, ADF, IVOMD and CH_4 values of the silages were statistically significant (P<0.05), while the differences in DM, CP, NDF and ME values were not significant (P>0.05). When the CA values of the silages were examined, an increase was observed in G-PFJ group due to the addition of additives compared to the controls. ADF values of the silages were found to be statistically significant in all groups. ADF values of HoLAB and HetLAB groups showed a decrease compared to the other groups. The hypothesis that HetLAB strains produce ferulate esterase and that ferulate esterase can reduce the cell wall coverage (Ding et al. 2019) was in agreement with the present study. Jalč et al. (2009a) reported that inoculants reduced ADF and NDF levels in their silage study using three microbial inoculants (*Lactobacillus plantarum* CCM 4000, *L. fermentum* LF2 and *Enterococcus faecium* CCM 4231). When the IVOMD values of the silages were examined, increases were observed in all experimental groups compared to the control group, but the highest IVOMD value was observed in the group with the addition of F-PFJ. It is considered that the main fermentation product in the silages was LA and therefore IVOMD values were increased. While Sucu (2009) reported that LAB inoculants increased the OM digestibility of maize silage, Altınçekiç (2006) reported that LAB inoculants significantly reduced the *in vitro* OM digestibility of maize silage. However, depending on the type of microorganism, strain, and substrate used, *in vitro* responses to the effects of silage inoculants have varied considerably (Ellis et al. 2016). When the CH_4 values of the silages were examined, the lowest value was observed in the F-PFJ group, and the highest value was observed in the control group. Research reveals that LAB has varying impacts on CH_4 production. According to Cao et al. (2011), the application of the *L.* plantarum Chikuso-1 inoculant along with vegetable residual silage resulted in a 46.6% decrease in methane generation when compared to the control group. Significant drops in CH_4 were also observed, according to Jalč et al. (2009b), after inoculating grass silage with *E. faecium* (CCM 4231), *L. fermentum*, or *L. plantarum* (CCM 4000). Nevertheless, Jalč et al. (2009c) discovered no significant increases in CH_4 for the same LAB when used as an inoculant with maize. Huyen et al. (2020) found that the ryegrass silage prepared by adding *Lactiplantibacillus plantarum* (LMG P-20353), *Lactiplantibacillus plantarum* (CECT 4528), *Lactococcus lactis subsp. lactis* and *Lactococcus lactis subsp. lactis* (DSM 33083) strains reduced the CH_4 value. These results are consistent with that reported by Doyle et al. (2019). It has been hypothesized that LAB can reduce CH_4 production in ruminants by altering rumen fermentation, directly inhibiting rumen methanogens, inhibiting specific rumen bacteria that produce H_2 or methyl-containing compounds that are substrates for methanogenesis, and producing bacteriocins that inhibit methanogens or by affecting other rumen microorganisms that produce substrates required for methanogenesis (Doyle et al. 2019).

Within the scope of this study, the fermentation characteristics of the silages prepared by adding PFJ and commercial HoLAB and Het LAB inoculant to the maize plants and the correlation results of the analyses are provided in Tables 4 and 5.

Table 4- The effect of the silages prepared by adding LAB liquid to the maize plants on fermentation properties

Groups	*NH_3-N*	*CO_2*	*PH*	*LA*	*AA*	*LA/AA*	*PA*	*BA*	*Yeast-Mold*
Control	8.57^{a}	2.34^{ab}	3.61^{b}	49.96^{d}	10.38^{d}	4.80^{b}	-	-	9.66^{b}
F-PFJ	8.52^{ab}	2.28^{ab}	3.63^{b}	58.84^{a}	14.83^{b}	3.96^{f}	-	-	3.85^{f}
M-PFJ	7.51^{c}	1.66^{ab}	3.61^{b}	56.90^{b}	12.90^{c}	4.60^{c}	-	-	6.13^{d}
S-PFJ	7.67^{bc}	1.28^{b}	3.62^{b}	26.85^{f}	5.90^{f}	4.41^{e}	-	-	9.10^{c}
G-PFJ	7.92^{abc}	1.05^{b}	3.73^{a}	39.44^{e}	8.76^{e}	4.50^{d}	-	-	8.95^{c}
HoLAB	6.57^{d}	4.26^{a}	3.63^{b}	54.87^{c}	4.78^{g}	11.47^{a}	-	-	10.24^{a}
HetLAB	7.95^{abc}	1.30^{b}	3.81^{a}	24.37^{g}	18.35^{a}	1.32^{g}	-	-	5.70^{e}
SEM	0.151	0.290	0.017	2.560	0.867	0.550	-	-	0.433
P	**0.001**	**0.028**	**0.000**	**0.000**	**0.000**	**0.000**	**-**	**-**	**0.000**

^{a,b,c,d,e,f,g}: Values with different letters in the same column were found to be different (P<0.05); **NH_3-N/TN:** Ammonia nitrogen; **CO_2:** Carbon dioxide, g/kg DM; **LA:** Lactic acid, g/kg DM; **AA**: Acetic acid, g/kg DM; **PA:** Propionic acid, g/kg DM; **BA:** Butyric acid, g/kg DM, **Yeast-Mold:** log10 cfu/g

Table 5- Correlation relationship between the fermentation properties and yeast and mold values of the silages prepared by adding LAB liquid to the maize silages

Silage Parameters		*pH*	*NH_3-N*	*LA*	*AA*	*CO_2*	*Yeast-Mold*	*IVOMD*	*ME*	*CH_4*
pH	**PC**	1	0.173	-0.528**	0.415*	-0.197	-0.151	-0.148	-0.075	-0.149
	P		0.379	0.004	0.028	0.314	0.444	0.454	0.705	0.448
NH_3-N	**PC**		1	-0.036	0.446*	-0.192	-0.368	-0.435*	-0.147	0.216
	P			0.856	0.017	0.327	0.054	0.021	0.456	0.270
LA	**PC**			1	-0.086	0.404*	-0.136	0.371	0.246	-0.071
	P				0.665	0.033	0.491	0.052	0.206	0.721
AA	**PC**				1	-0.278	-0.839**	-0.068	0.202	-0.177
	P					0.153	0.000	0.729	0.303	0.368
CO_2	**PC**					1	0.219	0.127	-0.042	-0.061
	P						0.263	0.518	0.831	0.759
Yeast-Mold	**PC**						1	-0.299	-0.462*	0.420*
	P							0.122	0,013	0.026
IVOMD	**PC**							1	0.818**	-0.475*
	P								0.000	0.011
ME	**PC**								1	-,181
	P									,356
CH_4	**PC**									1

PC: Pearson correlation; ***:** The correlation is significant at the 0.05 level; ****:** The correlation is significant at the 0.01 level; **NH_3-N/TN:** Ammonia nitrogen; **CO_2:** Carbon dioxide, g/kg DM; **LA:** Lactic acid, g/kg DM; **AA:** Acetic acid, g/kg DM; **IVOMD:** *In Vitro* organic matter digestion, %; **ME:** Metabolizable energy, MJ/kg DM; **CH_4:** *In Vitro* methane gas, %, **Yeast-Mold:** log10 cfu/g

When the fermentation properties (pH, NH_3-N, LA, AA, LA/AA, CO_2, and total yeast-mold after aerobic stability) of the silages prepared by adding LAB liquid to the maize plants were examined, the differences among the groups were statistically significant (P<0.05).

The pH values of the silages were within the range of 3.61-3.81. The reason for the highest pH value (3.81) in the silage group with heterofermentative additives was that in the HetLAB silage plant structure, secondary products are produced such as ethyl alcohol, acetic acid, diacetyl and carbon dioxide, as well as lactic acid, which is the main product from WSCs. When the correlation of analyses of the silages was examined in Table 5, the positive correlation (R:0.415) between silage pH and AA supported this finding. In addition, the reason for the low pH value in all silage groups is related to the low buffering capacity of the maize plant and its sufficient WSC content for fermentation. Moreover, it is thought that the bacterial inoculants used ensure silage fermentation with rapid pH decrease by using WSCs in the plant.

When the NH_3-N values of the silages were examined, a decrease was observed in M-PFJ, S-PFJ and HoLAB groups compared to the control group. The lowest NH_3-N value was identified in the group with the addition of the HoLAB additive. As a result of the LA production rate and rapid pH decrease in the silo environment depending on the decrease in the efficiency of proteolytic enzymes, proteolysis decreases and thus the degradation of proteins also decreases (Reich & Kung 2010). Carpintero et al. (1979) reported that the silage NH_3-N/TN% value should be lower than 11% to be classified in the good quality silage class. McDonald et al. (1991) reported that lower pH values inhibited protein degradation in silages. Therefore, in the experiment, all maize silages had low ammonia-N contents.

When the LA and AA values of the silages were examined, the highest LA value (58.84) was found in the group in which F-PFJ was added to the maize silage, and the lowest LA value (24.37) was found in the group with the HetLAB addition to the maize silage. The highest AA value (18.35) of the silages was identified in the group with the HetLAB inoculant addition. The silage with the lowest AA value (4.26) was found in the HoLAB group. Bernardi et al. (2019) reported that the addition of the HoLAB inoculant increases LA and decreases AA and that the addition of the HetLAB inoculant increases AA by reducing LA. These results support the findings of the present study. In Table 4, the LA/AA ratio of the silages was within the range of 1.32-11.47. The highest homofermatative activity was found in the group with the HoLAB addition. The highest heterolactic activity was observed in the group with the HetLAB inoculant addition. Studies that added inoculants to maize silage support the results of the current study (Bernardi et al. 2019; Xu et al. 2021). In the present study, on day 5 of aerobic stability, the CO_2 formation amounts of the silages varied between 1.05 and 4.26 g/kg DM, and the highest value was detected in the group with the HoLAB inoculant supplementation while the lowest value was detected in the group with G-PFJ added to the maize silage and in the group with the HetLAB inoculant addition. Ali et al. (2020) reported that the amount of acetic acid produced by HetLAB fermentation in silages has an inhibitory effect against microorganisms that cause silage deterioration, inhibits the growth and activity of yeast and molds, and reduces CO_2 production, that is, improves aerobic stability values. When the correlation table is examined, the negative correlation of AA between CO_2 and yeast-mold supports this statement. In the present study, the highest CO_2 and total yeast and mold amounts after aerobic stability and the lowest AA value were identified in the group with the added HoLAB inoculant. Adesogan & Arriola (2020) reported that HoLAB inoculants reduce the amount of AA that inhibits yeast production but increase the amount of LA and decrease aerobic stability. The report that increased levels of LA are used as a substrate for yeast growth and that LA is decomposed by yeasts to CO_2 and water is in line with the present study (Adesogan & Arriola 2020). In most of the studies examining the effect of inoculant use on the aerobic stability of silage, aerobic stability was negatively affected by the addition of HoLAB (Filya 2002; Filya & Sucu 2010; Filya et al. 2004).

4. Conclusions

This study aimed to determine the effects of the addition of LAB to maize silage on the silage quality, its fermentation properties and its *in vitro* organic matter digestion.

When all parameters were examined, it was concluded that the addition of F-PFJ, which was prepared by adding 5% fructose from easily soluble carbohydrate sources to the meadow grass plant, to maize silage has the most positive effects on silage fermentation and *in vitro* organic matter digestion.

References

Adesogan A T & Arriola K G (2020). Bacterial inoculants for improving silage quality and animal performance. In: Proceedings of the 82nd Cornell Nutrition Congress 19-22 October, New York, pp. 42-57

Ali N, Wang S, Zhao J, Dong Z, Li J, Naza M & Shao T (2020). Microbial diversity and fermentation profile of red clover silage inoculated with reconstituted indigenous and exogenous epiphytic microbiota. Bioresource Technology 314: 123606. https://doi.org/10.1016/j.biortech.2020.123606

Altınçekiç E (2006). The effect of combination of organic acid-bacterial inoculant on the fermentation, aerobic stability and feed value of maize silage. Master's Thesis, Uludağ University, Bursa, Türkiye. (In Turkish) https://acikerisim.uludag.edu.tr/bitstream/11452/3441/1/202289.pdf

AOAC (2005). Official methods of the association of official analytical chemists, 16th Edn. Arlington, TX: Association of Official Analytical Chemists.

Aragón Y A (2012). The use of probiotic strains as silage inoculants. In: E C Rigobelo (Eds.), Probiotics in Animals, Croatia, pp. 1-32. http://dx.doi.org/10.5772/50431

Ashbell G, Weinberg Z G, Azrieli A, Hen Y & Horev B (1991). A simple system to study the aerobic determination of silages. Canadian Agricultural Engineering 34: 171-175

Axelsson L (1998). Lactic acid bacteria: classification and physiology. In: S. Salminen & A. von Wright, Lactic Acid Bacteria: Microbiology and Functional Aspects, 2nd (Edn), Revised and Expanded, Marcel Dekker, New York pp. 1–72. https://doi.org/10.1201/9780824752033.ch1

Aydin S S & Denek N (2022). Amount of lactic acid bacteria in fermented natural lactic acid bacteria liquids prepared with varying sucrose inclusion at different incubation periods. Medycyna Weterynaryjna 78(9): 456-460. http://dx.doi.org/10.21521/mw.6686

Blajman J E, Vinderola G, Paez R B & Signorini ML (2020). The role of homofermentative and heterofermentative lactic acid bacteria for alfalfa silage: a meta-analysis. *Journal of Agricultural Science* 158(1-2): 107-118. https://doi.org/10.1017/S0021859620000386

Bureenok S, Namihira T, Tamaki M, Mizumachi S, Kawamoto Y & Nakada T (2005a). Fermentative quality of guineagrass silage by using fermented juice of the epiphytic lactic acid bacteria (FJLB) as a silage additive. *Asian-Australasian Journal of Animal Sciences* 18(6): 807-811. https://doi.org/10.5713/ajas.2005.807

Bureenok S, Namihira T, Kawamoto Y & Nakada T (2005b). Additive effects of fermented juice of epiphytic lactic acid bacteria on the fermentative quality of guineagrass (Panicum maximum Jacq.) silage. Grassland Science 51(3): 243-248. https://doi.org/10.1111/j.1744-697X.2005.00032.x

Broderick G A & Kang J H (1980). Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. Journal of Dairy Science 63(1): 64-75. https://doi.org/10.3168/jds.S0022-0302(80)82888-8

Can L (2010). The effects of bacterial inoculants and/or enzymes on the fermentation, aerobic stability and in vitro organic matter digestability characteristics of triticale:hungarian vetch silages, Master's thesis. Namık Kemal University, Tekirdağ. (In Turkish). https://acikerisim.nku.edu.tr/xmlui/bitstream/handle/20.500.11776/652/0031672.pdf?sequence=1&isAllowed=y

Cao Y, Cai Y, Takahashi T, Yoshida N, Tohno M, Uegaki R, Nonaka K & Terada F (2011). Effect of lactic acid bacteria inoculant and beet pulp addition on fermentation characteristics and in vitro ruminal digestion of vegetable residue silage. Journal of Dairy Science, 94(8): 3902-3912. https://doi.org/10.3168/jds.2010-3623

Carpintero C M, Henderson A R & McDonald P (1979). The effect of some pre-treatments on proteolysis during the ensiling of herbage. Grass and Forage Science 34(4): 311-315. https://doi.org/10.1111/j.1365-2494.1979.tb01483.x

Denek N, Can A, Avci M, Aksu T & Durmaz H (2011). The effect of molasses-based pre-fermented juice on the fermentation quality of first-cut lucerne silage. Grass and Forage Science 66(2): 243-250. https://doi.org/10.1111/j.1365-2494.2011.00783.x

Ding Z T, Xu D M, Bai J, Li F H, Adesogan A T, Zhang P, Yuan X J & Guo X S (2019). Characterization and identification of ferulic acid esterase-producing Lactobacillus species isolated from Elymus nutans silage and their application in ensiled alfalfa. *Journal of Applied Microbiology* 127(4): 985-995. https://doi.org/10.1111/jam.14374

DLG (Deustsche LandwiethshaftsGesellschaft) (2011). Praxishandbuch Futter-und Substratkonservierung. DLG-Verlags-GmbH, Frankfurt am Main, Germany, pp. 284-327.

Doyle N, Mbandlwa P, Kelly W J, Attwood G, Li Y, Ross R P, Stanton C & Leahy S (2019). Use of lactic acid bacteria to reduce methane production in ruminants, a critical review. Frontiers in Microbiology, 2207. https://doi.org/10.3389/fmicb.2019.02207

Ellis J L, Bannink A, Hindrichsen I K, Kinley R D, Pellikaan W F, Milora N & Dijkstra J (2016). The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. Animal Feed Science and Technology 211: 61-74. https://doi.org/10.1016/j.anifeedsci.2015.10.016

Fabiszewska A U, Zielińska K J & Wróbel B (2019). Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: a minireview. *World Journal of Microbiology and Biotechnology* 35(5): 1-8. https://doi.org/10.1007/s11274-019-2649-2

Filya İ (2002). The effects of lactic acid bacterial inoculants on the fermentation, aerobic stability and in situ rumen degradability characteristics of maize and sorghum silages. *Turkish Journal of Veterinary and Animal Sciences* 26(4): 815-823.

Filya I, Ashbell G, Hen Y & Weinberg Z G (2000). The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. Animal Feed Science and Technology 88(1-2): 39-46. https://doi.org/10.1016/S0377-8401(00)00214-5

Filya I & Sucu E (2010). The effects of lactic acid bacteria on the fermentation, aerobic stability and nutritive value of maize silage. Grass and Forage Science, 65(4): 446-455. https://doi.org/10.1111/j.1365-2494.2010.00763.x

Filya I, Sucu E & Karabulut A (2004). The effect of Propionibacterium acidipropionici, with or without Lactobacillus plantarum, on the fermentation and aerobic stability of wheat, sorghum and maize silages. *Journal of Applied Microbiology* 97(4): 818-826. https://doi.org/10.1111/j.1365-2672.2004.02367.x

Goel G, Makkar H P S & Becker K (2008). Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin-rich fractions from different plant materials. *Journal of Applied Microbiology* 105(3): 770-777. https://doi.org/10.1111/j.1365-2672.2008.03818.x

Güney F & Ertürk Ö (2020). Determination of the effects of propolis ethanolic extract on some properties of fruit yoghurt during storage. *Mustafa Kemal University Journal of Agricultural Sciences* 25(2): 145-152. https://doi.org/10.37908/mkutbd.694712

Huyen N T, Martinez I & Pellikaan W (2020). Using lactic acid bacteria as silage inoculants or direct-fed microbials to improve in vitro degradability and reduce methane emissions in dairy cows. Agronomy 10(10): 1482. https://doi.org/10.3390/agronomy10101482

Jalč D, Laukova A, Pogány Simonová M, Váradyová Z & Homolka P (2009a). Bacterial inoculant effects on corn silage fermentation and nutrient composition. Asian-Australasian Journal of Animal Sciences 22(7): 977-983. https://doi.org/10.5713/ajas.2009.80282

Jalč D, Laukova A, Varadyova Z, Homolka P & Koukolova V (2009b). Effect of inoculated grass silages on rumen fermentation and lipid metabolism in an artificial rumen (RUSITEC). Animal Feed Science and Technology, 151(1-2): 55-64. https://doi.org/10.1016/j.anifeedsci.2008.11.004

Jalč D, Váradyová Z, Lauková A, Homolka P & Jančík F (2009c). Effect of inoculated corn silage on rumen fermentation and lipid metabolism in an artificial rumen (RUSITEC). Animal Feed Science and Technology 152(3-4): 256-266. https://doi.org/10.1016/j.anifeedsci.2009.04.019

Kılıç A (1986). Silo yemi (Öğretim, öğrenim ve uygulama önerileri). Bilgehan Basımevi, İzmir, pp.327.

Kleinschmit D H & Kung Jr L (2006). The effects of Lactobacillus buchneri 40788 and Pediococcus pentosaceus R1094 on the fermentation of corn silage. *Journal of Dairy Science* 89(10): 3999-4004. https://doi.org/10.3168/jds.S0022-0302(06)72443-2

Masuko T, Hariyama Y, Takahashi Y, Cao L M, Goto M & Ohshima M (2002). Effect of addition of fermented juice of epiphytic lactic acid bacteria prepared from timothy (Phleum pratense) and orchardgrass (Dactylis glomerata) on fermentation quality of silages. Journal of Japanese Society of Grassland Science pp. 120-125

McDonald P, Henderson A R, Heron S J E (1991) Plant Enzymes. In: McDonald P, Henderson AR, Heron SJE (Eds.), The Biochemistry of Silage. Abersytwyth, UK pp. 48-80

Menke K H, Raab L, Salewski A, Steingass H, Fritz D & Schneider W (1979). The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. The Journal of Agricultural Science 93(1): 217-222. https://doi.org/10.1017/S0021859600086305

Menke K H & Steingass H (1988). Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development 28: 7-55. https://doi.org/10.2508/chikusan.79.483

Muck R E (2013). Recent advances in silage microbiology. Agricultural and Food Science 22(1): 3-15. https://doi.org/10.23986/afsci.6718

Müller M & Lier D (1994). Fermentation of fructans by epiphytic lactic acid bacteria. *Journal of Applied Microbiology* 76(4): 406-411. https://doi.org/10.1111/j.1365-2672.1994.tb01647.x

Otero M A, Reyes A, Carrera E & Leon M A (1993). Composition and properties of cane molasses from Northeastern Cuba. *International Sugar Journal* 95(1129E): 4-8

Parvin S & Nishino N (2010). Succession of lactic acid bacteria in wilted rhodesgrass silage assessed by plate culture and denaturing gradient gel electrophoresis. Grassland Science 56(1): 51-55. https://doi.org/10.1111/j.1744-697X.2009.00173.x

Playne M J & McDonald P (1966). The buffering constituents of herbage and of silage. *Journal of the Science of Food and Agriculture* 17(6): 264-268. https://doi.org/10.1002/jsfa.2740170609

Reich L J & Kung Jr L (2010). Effects of combining Lactobacillus buchneri 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. Animal Feed Science and Technology 159(3-4): 105-109. https://doi.org/10.1016/j.anifeedsci.2010.06.002

SPSS (1991). Inc. Statistical package for the social sciences (SPSS/PC+). Chicago, IL.

Sucu E (2009). The effects of lactic acid bacterial ınoculants on fermentation, aerobic stability and rumen ecology of maize silage. PhD Thesis, Bursa Uludag University, Bursa. (In Turkish) https://acikbilim.yok.gov.tr/handle/20.500.12812/690419

Sun L, Jiang Y, Ling Q, Na N, Xu H, Vyas D, Adesogan A T & Xue Y (2021). Effects of adding pre-fermented fluid prepared from red clover or lucerne on fermentation quality and in vitro digestibility of red clover and lucerne silages. Agriculture 11(5): 454. https://doi.org/10.3390/agriculture11050454

Suzuki M & Lund C W (1980). Improved gas-liquid chromatography for simultaneous determination of volatile fatty acids and lactic acid in silage. *Journal of Agricultural and Food Chemistry* 28(5): 1040-1041. https://doi.org/10.1021/jf60231a023

Tao L, Zhou H, Zhang N, Si B, Tu Y, Ma T & Diao Q (2017). Effects of different source additives and wilt conditions on the Ph value, aerobic stability, and carbohydrate and protein fractions of alfalfa silage. *Animal Science Journal* 88(1): 99-106. https://doi.org/10.1111/asj.12599

Van Soest P V, Robertson J B & Lewis B A (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74(10): 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Xu D, Wang N, Rinne M, Ke W, Weinberg Z G, Da M, Bai J, Zhang Y, Li F & Guo X (2021). The bacterial community and metabolome dynamics and their interactions modulate fermentation process of whole crop corn silage prepared with or without inoculants. Microbial Biotechnology 14(2): 561-576. https://doi.org/10.1111/1751-7915.13623

Zhang Y G, Xin H S & Hua J L (2010). Effects of treating whole-plant or chopped rice straw silage with different levels of lactic acid bacteria on silage fermentation and nutritive value for lactating Holsteins. *Asian-Australasian Journal of Animal Sciences* 23(12): 1601-1607. https://doi.org/10.5713/ajas.2010.10082