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47th CONFERENCE FOR STUDENTS OF AGRICULTURE AND VETERINARY MEDICINE WITH INTERNATIONAL PARTICIPATION

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Council of the University of Novi Sad, Faculty of Agriculture, at its fourth meeting on 18th of February 1981 made a decision that the Conference for students of agriculture and veterinary medicine with international participation, held each year at the Faculty of Agriculture will be held in the memory of academician ***Dr Petar Drezgić***, professor, and the prizes awarded at the Conference will bear his name.

PREFACE

The first Conference for students of agriculture and veterinary medicine with international participation was held in Novi Sad 47 years ago. To the present date, many papers from national and international authors were presented (Hungary – Gödölö and Debrecen, Germany – Kassel, Slovakia – Nitra, Poland – Wroclaw and Warsaw, Romania – Timisoara, Cluj and Bucharest, Bulgaria – Plovdiv, Russia – Moscow and Kemerovo, Macedonia – Skopje and Bitola, Bosnia and Herzegovina – Sarajevo, Banja Luka, Croatia – Zagreb). Since 2009, papers presented at the Conference are published in a special publication named "PROCEEDINGS OF THE 47th CONFERENCE FOR STUDENTS OF AGRICULTURE AND VETERINARY MEDICINE WITH INTERNATIONAL PARTICIPATION" where all published papers have all elements of the original scientific paper. Starting this year, we enabled students and mentors who are interested in benefitting more from this Conference to publish their papers in the journal Contemporary Agriculture published by the University of Novi Sad, Faculty of Agriculture.

This Conference is strongly supported by the Student Parliament of the Faculty of Agriculture, Novi Sad.

As the host and the principal organizer of the Conference University of Novi Sad, Faculty of Agriculture has a special honor and a pleasure to wish a warm welcome to all participants, successful work and amusing socializing in Novi Sad.

Vice-dean for science and international cooperation
Dr Branko Ćupina, full professor

President of the organizing committee
Dr Dragana Budakov, associate professor

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ANTIOXIDANT ENZYME ACTIVITIES IN LEAVES OF WILD PANSY (V*iola tricolor* L.) UNDER WATER STRESS

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SUMMARY

Water stress adversely affects plant metabolism. One of the unavoidable consequences of drought is oxidative stress caused by an imbalance between production of oxygen reactive species and the plants' ability to eliminate their negative impacts. This study was undertaken to evaluate the antioxidant enzyme activities in leaves of wild pansy under drought. The experiment was conducted in a greenhouse at the agricultural experimental station of the Faculty of Agriculture and Food Science in Sarajevo and was designed in such a way that half of the wild pansy plants (six plants) were exposed to drought for ten days, while the other half were not, these plants were regularly watered. The results showed that the guaiacol peroxidase activity in stressed wild pansy plants increased with increasing drought duration. Superoxide dismutase and pyrogallol peroxidase activity were also increased in the first seven and eight days after exposure to drought, respectively, and then started to decrease. Interestingly, in this study, the activity of catalase in wild pansy plants was not affected by water stress.

Key words*: catalase, drought, peroxidases, plant metabolism*

INTRODUCTION

In natural environment, as well as in agricultural production, plants are continually exposed to various unfavorable environmental conditions that negatively affect plant growth and development. As sessile organisms, plants are not able to avoid unfavorable conditions in their habitat; however, they are able to cope with environmental stressors thanks to its behavioral, structural and physiological adaptations (Zhu, 2016).

Drought is undoubtedly one of the most devastating environmental stressors that limit plant growth and productivity. Drought impact is not limited only to the affected area, it has far-reaching negative consequences, particularly on the wider economy and environment. Unfortunately, drought-related risks are expected to increase in the future due to global warming and the climate change (Carrão et al., 2018).

Drought stress adversely affects plant metabolism. Under these conditions, plants actively slow down cell growth primarily due to a decrease in turgor pressure caused by a lack of water in plant cells. Lower turgor pressure also initiates stomatal closure thus preventing

unnecessary water loss. As a result of the closing of the stomata, photosynthetic carbon assimilation decreases, resulting in a lower photosynthetic rate. All these changes negatively affect other plant physiological processes including respiration, carbohydrates metabolism and nutrient uptake and translocation, thereby reducing the plant's chances of survival under drought conditions (Zhang et al., 2020). One of the unavoidable consequences of drought stress in plants is also oxidative stress caused by an imbalance between production of oxygen reactive species (ROS) and the plants' ability to eliminate their negative impacts (Cruz de Carvalho, 2008).

In order to overcome the negative effects of drought, plants have evolved complex morphological and physiological adaptations to adjust to water scarcity. Morphology adaptations are mainly based on reduction of leaf area to minimize water loss and on changes in root architecture to optimize water uptake, while physiological adaptations refer to the plants' ability to maintain cell turgor pressure and detoxify harmful substances via cell osmotic regulation and synthesis of protective molecules. The abovementioned adaptations differ among plant species depending on their genotype, age and growth stage (Seleiman et al., 2021; Bandurska, 2022).

Wild pansy (*Viola tricolor* L.) is one of the most popular plant species for urban gardens and landscapes due to attractive color combination in its flowers. In addition, wild pansy has been widely used in traditional medicine to treat respiratory system diseases (Rimkiene et al., 2003). However, as far as we know, no studies have been performed to assess the impact of water stress on antioxidant enzymes activities in wild pansy plants. Therefore, the aim of this study was to evaluate the response of antioxidant activities of wild pansy to drought stress. The activities of the following enzymes were evaluated: superoxide dismutase, guaiacol peroxidase, pyrogallol peroxidase and catalase. Wild pansy was chosen for this study because its production in Bosnia and Herzegovina has been constantly increasing over the current decade. In this light, the creation and dissemination of new knowledge and information about the wild pansy growth and development, especially under drought conditions, are of great importance for both producers and scientists.

MATERIAL AND METHODS

Experimental design

The experiment was conducted from the mid-September 2023 to end September 2023 in a greenhouse at the agricultural experimental station of the Faculty of Agriculture and Food Science in Sarajevo. Plants used in the experiment were produced in the nursery near the greenhouse and showed no significant difference in size and appearance. Before setting up an experiment, the wild pansy plants were in the early stages of flower development. Over the course of the experiment, air temperature in greenhouse was maintained at 25 ± 5 °C during the day and 18 ± 5 °C during the night.

For the drought treatment experiment, wild pansy plants were grown in pots (10 cm diameter × 9 cm height, one plant per plot) and filled with substrate Florahum-SP. After the acclimation period (3 days), one set of the plants (6 individuals) were exposed to drought for

next 10 days (non-watering), whereas the other set of the plants (also 6 individuals) were not exposed to drought; these plants were regularly watered. Fresh leaves of wild pansy plants were collected daily during the experiment (one leaf per plant) and immediately after cutting were frozen with liquid nitrogen and stored at -20 °C for further analysis. The activities of antioxidant enzymes; superoxide dismutase, guaiacol peroxidase, pyrogallol peroxidase and catalase were carried out in the laboratory of Faculty of Agriculture and Food Sciences, University of Sarajevo.

Chemicals

Potassium dihydrogen phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4), bovine serum albumin, Bradford Reagent, cytochrome C oxidase, xanthine, xanthine oxidase, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), polyvinylpyrrolidone-40 (PVP-40) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals used throughout the analysis, i.e. guaiacol, pyrogallol and hydrogen peroxide, were obtained from Merck (Darmstadt, Germany).

Protein extraction and determination

0.5 g fresh leaves of wild pansy plants leaves were ground into a fine powder in liquid nitrogen using pestle and mortar. In order to extract the soluble protein from the homogenized powder, 1.5 mL of a 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM DTT and 0.05 % (w/v) polyvinylpyrrolidone-40 (PVP-40), was added. The homogenate was centrifuged at 10,000 × g for 10 min at 4 °C and then the supernatant fraction was employed for the subsequent estimation of protein concentration and antioxidant enzyme activities. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Superoxide dismutase (SOD) activity assay

SOD activity was determined spectrophotometrically according to McCord and Fridovich (1969). This method was based on the SOD ability to inhibit reduction of cytochrome C by superoxide anions generated from xanthine-xanthine oxidase system. The reaction assay mixture (860 - 1.000 µl) included 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 50 µM xanthine, 10µM cytochrome C, and xanthine oxidase in an amount sufficient to cause an increase in absorbance at 550 nm of 0.025 min^{-1} in the absence of SOD. The protein extract (10 - 50 µl) were added to the reaction mixture and the rate of reduction of cytochrome c was followed spectrophotometrically at 550 nm. The SOD activity was expressed as unit of SOD activity (U) per mg protein. One unit of SOD activity represents the amount of SOD required to inhibit the reduction rate of cytochrome C by 50%.

Catalase (CAT) activity assay

CAT activity was determined spectrophotometrically according to Aebi (1984). The reaction medium was prepared by mixing 950 µl of the reaction mixture (10 mM H_2O_2 in 50 mM potassium phosphate buffer pH 7.0) and 50 µl of protein extract. CAT activity was assayed by monitoring the decrease in absorbance at 240 nm at an interval of 10 sec up to 120

sec, as a consequence of H_2O_2 consumption. The results were expressed as micromoles decomposed H_2O_2 per min per mg of protein.

Guaiacol peroxidase (GPOD) activity assay

GPOD activity was determined spectrophotometrically according to Chance and Maehly (1955). The reaction medium was prepared by mixing 900 µl of the reaction mixture (50 mM potassium phosphate buffer pH 7.0 amended with 18 mM guaiacol and 5 mM H_2O_2) and 100 µl of protein extract. GPOD activity was assayed by monitoring the increase in absorbance at 470 nm at an interval of 15 sec up to 180 sec, as a result of guaiacol oxidation. The results were expressed as micromoles tetraguaiacol (product of guaiacol oxidation) per min per mg of protein.

Pyrogallol peroxidase (PPOD) activity assay

PPOD activity was determined spectrophotometrically according to Chance and Maehly (1955). The reaction medium was prepared by mixing 950 µl of the reaction mixture (50 mM potassium phosphate buffer pH 7.0 amended with 20 mM pyrogallol and 1 mM H_2O_2) and 50 µl of protein extract. PPOD activity was assayed by monitoring the increase in absorbance at 430 nm, at an interval of 15 sec up to 180 sec, as a result of pyrogallol oxidation. The results were expressed as micromoles purpurogallin (product of pyrogallol oxidation) per min per mg of protein.

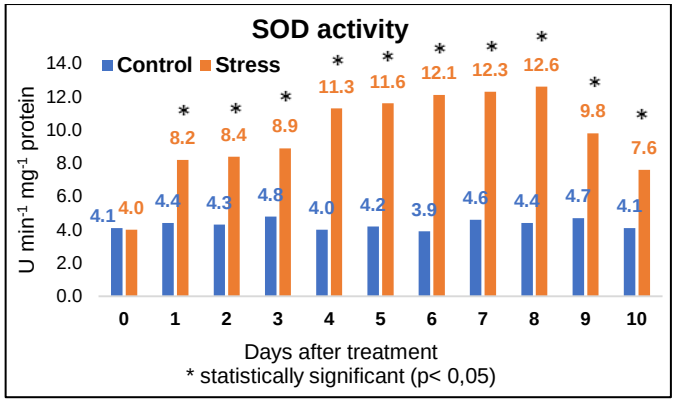
Statistical analysis

All experiments were done in triplicates and the results were expressed as mean ± standard deviation. Microsoft Excel software was used to perform analysis of variance (ANOVA) and differences between treatments were separated by Fisher's least significant difference (LSD) test at a 5% probability level ($P < 0.05$).

RESULTS

Superoxide dismutase activity

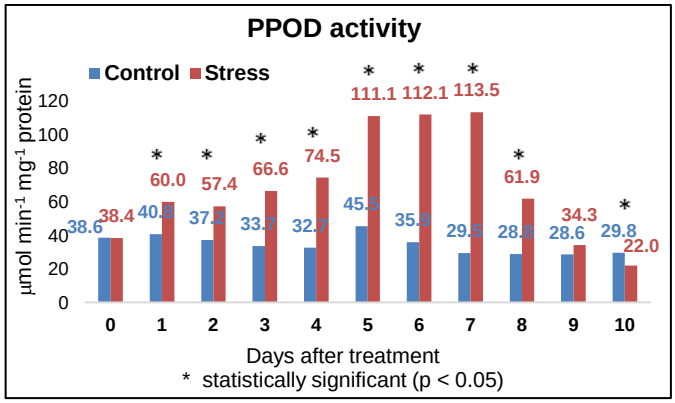
Superoxide dismutase (SOD, EC 1.15.1.1) activity in the leaves of wild pansy increased immediately after drought stress and reached the highest level at day 8. After exposure to drought for more than 8 days, the SOD activity in the leaves of wild pansy began to decrease and continued to decrease until the end of experiment i.e. tenth day of drought stress (graph. 1).



Graph. 1. SOD activity in the leaves of wild pansy

Pyrogallol peroxidase activity

The activity of pyrogallol peroxidase (PPOD, EC: 1.11.1.7) activity in the observed period of 10 days were generally higher in the leaves of wild pansy exposed to drought compared to plants grown under standard growth conditions (without stress), as shown in the graph. 2.

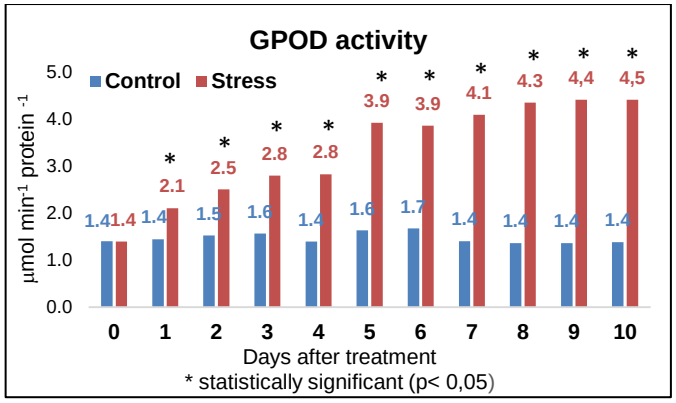


Graph. 2. PPOD activity in the leaves of wild pansy

In this study, an increase in PPOD activity was observed already on the first day of drought stress. Study results also showed that the PPOD activity increased continuously until the seventh day of drought stress, when it reached its maximum, and then started to decrease. Interestingly, PPOD activity in the leaves of wild pansy on the final day of the drought treatment (10 day) was significantly lower than in the control variant where plants were not exposed to drought.

Guaiacol peroxidase activity

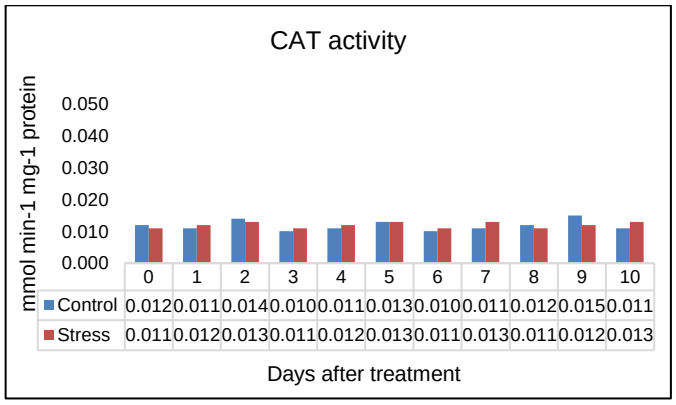
An increase in guaiacol peroxidase (GPOD, EC 1.11.1.7) activity in leaves of stressed wild pansy plants was observed already in the first day of drought stress. With increasing stress duration, GPOD increased continuously until the last day of exposure of wild pansy plants to drought, as shown in graph. 3.



Graph. 3. GPOD activity in the leaves of wild pansy

Catalase activity

In the present study, there were no significant differences between control and stressed wild pansy plants regarding the enzyme activity of catalase (CAT, EC 1.11.1.6) (graph. 4.)



Graph. 4. CAT activity in the leaves of wild pansy

DISCUSSION

Drought stress adversely affects various physiological and biochemical processes in plants, including photosynthesis, respiration, and nutrient uptake and transport in plants. These disorders lead to excessive production of reactive oxygen species (ROS) that can interact with deoxyribonucleic acid, proteins and lipids and cause subsequent cellular damage (Oguz et al., 2022).

In the course of evolution, plants developed different mechanisms that allow them to minimize the harmful effects of ROS and among them enzymatic ROS-scavenging mechanism is undoubtedly one of the most important (Nadarajah, 2020). This mechanism consists of numerous antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), pyrogallol peroxidase (PPOD) and guaiacol peroxidase (GPOD) (Gusti et al., 2021). SOD represents the first line of intracellular defense against ROS, i.e. superoxide radical anions, because it acts as soon as they are generated. This enzyme catalyzes the dismutation of superoxide radical (O_2^-) to molecular oxygen (O_2) and less toxic hydrogen peroxide (H_2O_2) which is subsequently converted to H_2O by CAT or peroxidases (PODs). CAT converts H_2O_2

to water (H_2O) and O_2, whereas PODs reduce H_2O_2 into H_2O using a wide variety of substrates as an electron donor (Suzuki et al., 2020).

In this study, SOD activity in the leaves of wild pansy plants significantly increased already on the first day of drought stress. This result is consistent with previous studies that also found high SOD activity at the initial stage of plant exposure to stress (Li et al., 2006; Lu et al. 2010; Ighodaro and Akinloye, 2018). GPOD and PPOD activities also increased on the first day of stress, however, this increase was less pronounced as compared to SOD. These findings provide further support for the hypothesis that SOD acts as a component of first line defense system against ROS and that GPOD and PPOD play an important role in plant adaptation to drought (Hasanuzzaman et al., 2021).

The results of this study also showed that SOD activity in the leaves of stressed plants increased continuously until the 8th day of drought, and then began to decrease. PPOD activity in leaves of wild pansy plants exposed to drought followed a very similar pattern. The only difference is that the PPOD activity reached its maximum on the 7th day of drought, and then continuously decreased until the end of the experiment. Moreover, the PPOD activity on the 10th day of drought was significantly lower than that of the control. These findings lead to the conclusion that long-term drought causes an imbalance in the plant's defense system which in this particular case is manifested by a decrease in SOD and PPOD activity. These findings are consistent with those of Liu et al. (2011) and Ulusu et al. (2022).

In the current study, the pattern of GPOD activity was slightly different from that of SOD and PPOD. GPOD activity was also observed on the first day of drought and increased with prolonged stress time; however, the rate of increase had slowed down in the last days of the experiment.

Interestingly, in this study, the activity of CAT in leaves of wild pansy plants was not affected by drought stress. Numerous studies have shown that CAT activities can increase, decrease or remain unchanged under drought conditions depending mainly on the experimental conditions and plant species (Song et al., 2022; Mishra et al., 2023). Foyer et al. (1994) reported that the protective action of CAT against ROS is limited, because this enzyme has a relatively poor affinity for H_2O_2. This can be one of the explanations for its low activity in this study. However, it should be noted that CAT has a very high turnover rate since one molecule of this enzyme can convert more than 2 million molecules of H_2O_2 to H_2O and O_2 per second, indicating that CAT represents an essential part of the enzymatic antioxidant defense system in plants (Racchi, 2013).

CONCLUSION

The present investigation revealed that exposure of wild pansy to drought increased SOD, GPOD and PPOD activity in the leaves of plants, indicating that these enzymes play an important role in ROS detoxification and thus in drought tolerance.

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CATTLE BREEDING PREADAPTATION ACCORDING TO THE EUROPEAN GREEN AGREEMENT

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SUMMARY

According to the FAO, livestock contributes 40% of the global value of agricultural output and supports the livelihoods and food and nutrition security of almost 1.3 billion people. Currently, the livestock sector emits an estimated 7.1 GT of CO_2-equivalent per year, representing 14.5% of human-induced greenhouse gas (GHG) emissions. Cattle husbandry is one of the largest GHG emissions from agricultural and livestock sector. Methane and carbon dioxide are both greenhouse gases that contribute to global warming, but as a short-lived flow gas, methane presents an opportunity for positive impact. Enteric methane is the single largest source of direct greenhouse gas (GHG) emissions in the cattle breeding. Methane is emitted on farms through two primary sources: manure degradation and enteric fermentation, the normal digestive process of cattle. According to the present situation in cattle production sector in the world as well as in the R. of N. Macedonia, it is obviously that GHG emissions are increasing and influenced on the climate changing and global warming of our planet. According to the World bank and EU Green Action Plan recommendations, each country has to start own national program for reducing of GHG emissions. In this paper are presented several measures for reducing GHG emissions in cattle production sector.

Key words: *cattle, GHG (green house gas) emission, methane, carbon dioxide, pollution*

INTRODUCTION

In last two decades we are facing with consequences from climate changing, which influences especially in agricultural and animal production too. All of that identifies the steps needed to make the future CAP fully compatible with the Green Deal and its strategies such as "from Farm to Fork" and Biodiversity strategies.

Cattle breeding nowadays are facing with strategic changes according Common Agriculture Policy (CAP) reform proposed by the European Commission in June 2018, and Green Deal from 2020, as a contribution of to the European environmental, climate, and biodiversity protection commitments set in the European Green Deal.

From September 2021 a new Green Action Plan for Western Balkan countries was published. All European countries have to take seriously implementation of many activities in

the adoption of agriculture as well as cattle breeding according to those new proposed reforms.

Methane and carbon dioxide are both greenhouse gases that contribute to global warming, but as a short-lived flow gas, methane presents an opportunity for positive impact. Enteric methane is the single largest source of direct greenhouse gas (GHG) emissions in the cattle breeding. Methane is emitted on farms through two primary sources: manure degradation and enteric fermentation, the normal digestive process which enables cattle to eat grass and fiber. Next objective is to improve animal productivity and move the dairy and beef sectors towards net zero emissions.

On the planet level, each year 1,6 billion cows releases about 100 kg of methane per head. Methane gas effects on the atmosphere 23 times more than Carbon dioxide. Methane traps in the heat of the Sun and makes the whole planet warmer. Each cow emissions is same as burning of 1000 liters of petrol annually. Several trails considered that cows are responsible for 18% of total greenhouse gasses worldwide. Animal agriculture uses 45% of total Earth's land area (Krinsman et all, 1995).

Shrinking livestock's carbon hoof print worldwide is a big challenge. India, for example, has the world's largest cattle population, but the lowest beef consumption of any country. For example, in India which has the largest cattle population per state in the world, there is the lowest consumption of cattle products per capita. As a result, cows live longer and emit more methane over their lifetime. In addition, cows in tropical regions produce less milk and meat, so it takes them longer to get to market.

Main objectives of the European Green Deal

The common agricultural policy (CAP) reform, proposed by the European Commission in 2018, introduces a more flexible, performance and results-based approach that take into account local conditions and needs, while increasing EU level ambitions in terms of sustainability. Based on this approach and complemented by new CAP tools, the European Commission considers that the CAP reform proposal is compatible with the Green Deal's ambitions**.** The main EU's goals are:

- to ensure food security in the face of climate change and biodiversity loss
- reduce the environmental and climate footprint of the EU food system
- strengthen the EU food system's resilience
- lead a global transition towards competitive sustainability from farm to fork.

The European Commission adopted a set of proposals to make the EU's climate, energy, transport and taxation policies fit for reducing net greenhouse gas emissions by at least 55% by 2030, compared to 1990 levels. Reducing greenhouse gas emissions by at least 55% by 2030 requires higher shares of renewable energy and greater energy efficiency.

The aims of EU member states about renewable energy sources

The aim of EU countries is to increase the binding target of renewable sources in the EU's energy mix to 40%, as well as to promote the uptake of renewable fuels, such as hydrogen in industry and transport, with additional targets. In addition, reducing energy consumption is essential to bring down both emissions and energy costs for consumers and industry. Also the other aim is to increase energy efficiency targets at EU level and make them binding, to

achieve by 2030 an overall reduction of 36-39% for final and primary energy consumption: a) 40% new renewable energy target for 2030, and b) 36-39% new 2030 energy efficiency targets for final and primary energy consumption.

Where livestock methane emissions come from?

The amount of methane emitted by livestock is primarily driven by the number of animals, the type of digestive system they have and the type and amount of feed consumed. Ruminants are the principal source of livestock methane emissions because they produce the most methane per unit of feed consumed. Ruminant livestock (cattle, sheep, buffalo, goats, deer and camels) have a fore-stomach (or rumen) containing microbes called methanogens, which are capable of digesting coarse plant material and which produce methane as a by-product of digestion (enteric fermentation), which is later released by the animal through belching. In Australia emissions, for example, livestock are the dominant source of methane (CH_4) and nitrous oxide (N_2O), accounting for 56% and 73%, respectively.

Cow belching or cow flatulence? Which is a bigger methane source?

Contrary to common belief, according NASA (2021) it's cow belching due to enteric fermentation ("enteric fermentation" is the digestive process of converting sugars into simple molecules for absorption into the bloodstream, which produces methane as a by-product). However, a small percentage of methane is also produced in the cow's large intestine and then expelled. Settling ponds and lagoons for processing manure also produce copious amounts of this greenhouse gas.

MAIN OBJECTIVES

The main objective of this paper is to analyze several different cattle production systems of rearing (intensive, semi-intensive and extensive, as well as dairy, beef or dual-purpose, as well as special systems as cow-calf system of crossbreds and autochthonous breeds as Busha cattle and water buffalo rearing) and production and GHG emissions in the different conditions in 8 agricultural regions of R. of N. Macedonia.

RESULTS AND DISCUSSION

Present situation and cattle population in the R. of N. Macedonia

The present situation and cattle population in the R. of Macedonia is presented in the following tables.

Table. 1 Total number of livestock by categories in the R. of N. Macedonia (AFV, 2020)

Year	**Cattle**	**Sheep**	**Goats**	**Pigs**	**Bee families**
2014	213 578	689 938	74 128	168 581	184 189
2016	228 812	744 396	88 964	109 845	239 216
2017	215 296	733 291	86 479	109 976	233 835
2018	190 455	708 509	83 611	110 886	243 492
2019	164 840	656 459	73 323	125 230	253 489
2020	152 814	653 411	67 911	133 397	309 264

Table. 2 Comparative analyzes of the average capacity of cattle farms between 2014 and 2020 (AFV, 2020)

Year/capacity (heads/farm)	1-5	6-20	21-50	51-100	101-300	101-300	Total no. of cattle
2014	24.11	35.56	22.17	10.99	4.57	2.62	213578
2020	15.63	30.97	25.72	16.23	8.73	2.71	164704

In the R. of N. Macedonia, from the total value of livestock production (180 till 195 million Euros per year), cattle breeding takes 65-70% from total livestock production (115-130 million Euros per year) (Facts and figures, 2019). From total cattle breeding value, more than 91% belongs to dairy production, and the rest is on beef production.

Table. 3 Breed structure of cattle in the R. of Macedonia (AFV, 2021)

Cattle breed	2020	Bo %
Braunvieh	280	0.17
Water buffaloes	55	0.03
Busha	4399	2.67
Crossbreds	84386	51.23
Limousine	43	0.03
Montafon (Brown)	3213	1.95
Grey Tyrol cattle	68	0.04
Simmental	6958	4.22
Beef breeds of cattle	12	0.01
Holstein Friesian	62847	38.16
Hereford	407	0.25
Charolaise	16	0.01
Total no. of cattle	164704	100.00

Table. 4 Nitrous oxide (N2O) emission from fresh waste of dairy cattle (Quinton, 2019)

Parameter	No.	N2O-N(mgN/day)	N2O-N/waste-N(%)
Dry dairy cows	3	2.8 (0-8.3)	0.003
Lactating dairy cows	4	13.1 (6.1-31.9)	0.007

According to Quinton (2019), Nitrous oxide emission from cattle themselves is calculated to be the difference between N_2O emission from the chamber and that from the fresh waste beneath the chamber. From the results of Experiment 1, the daily N_2O emission from the chambers ranged between 4.8 and 12.7 mg N_2O -nitrogen for dry dairy cattle. From the results of Experiment 2, the daily N_2O emission from fresh waste was estimated to be 2.8 mg N_2O -nitrogen for dry cattle. As a result, the daily and yearly N_2O emission from dry cattle were calculated to be 5.2 ± 4.2 (range: 2.0 ~ 9.9) mg N_2O -nitrogen and 2.64 ± 1.65 (0.74 ~ 3.60) g N_2O -nitrogen, respectively.

According to the World bank (2022), The livestock sector is a pillar of the global food system and a contributor to poverty reduction, food security and agricultural development. According to the FAO, livestock contributes 40% of the global value of agricultural output and supports the livelihoods and food and nutrition security of almost 1.3 billion people. Currently, the livestock sector emits an estimated 7.1 GT of CO_2-equivalent per year, representing 14.5% of human-induced greenhouse gas (GHG) emissions. Increasing the efficiency of livestock supply chains is key to limiting the growth of GHG emissions in the future.

EU Action Plan from June 2020

EU Action Plan aims for zero pollution in air, water and soil by 2050!

To steer the EU towards the 2050 goal of a healthy planet for healthy people, the Action Plan sets key 2030 targets to reduce pollution at source, in comparison to the current situation, by:

1. Improving air quality to reduce the number of premature deaths caused by air pollution by 55%;
2. Improving water quality by reducing waste, plastic litter at sea (by 50%) and microplastics released into the environment (by 30%);
3. Improving soil quality by reducing nutrient losses and chemical pesticides' use by 50%;
4. Reducing by 25% the EU ecosystems where air pollution threatens biodiversity;
5. Reducing the share of people chronically disturbed by transport noise by 30%, and
6. Significantly reducing waste generation and by 50% residual municipal waste.

Key initiatives and actions for that purposes are:

- Aligning the air quality standards more closely to the latest recommendations of the World Health Organization,
- Reviewing the standards for the quality of water, including in EU rivers and seas,
- Reducing soil pollution and enhancing restoration,

- Reviewing the majority of EU waste laws to adapt them to the clean and circular economy principles,
- Fostering zero pollution from production and consumption,
- Presenting a Scoreboard of EU regions' green performance to promote zero pollution across regions,
- Reduce health inequalities caused by the disproportionate share of harmful health impacts now borne by the most vulnerable,
- Reducing the EU's external pollution footprint by restricting the export of products and wastes that have harmful, toxic impacts in third countries,
- Launching Living Labs for green digital solutions and smart zero pollution,
- Consolidating the EU's Knowledge Centers for Zero Pollution and bringing stakeholders together in the Zero Pollution Stakeholder Platform,
- Stronger enforcement of zero pollution together with environmental and other authorities.

So, the main two objectives of EU for gasses emissions are: 1) till 2030 EU to deliver a reduction of emissions of at least 55% compared to 1990 levels, and 2) till 2050 EU to become climate neutral.

Measures plan to carry out in agriculture by Climate Action Plan

The each Government's Climate Action Plan outlined a number of measures for agriculture to reduce its Greenhouse Gas emissions by 22-30pc by 2030, set out in 40 actions for the sector over the coming years. Measures include:

- increased uptake of GHG-efficient farming practices; reducing fertilizer use and increasing the use of clover and multi-species swards, improving animal breeding and reducing levels of crude protein in the diet, as well as earlier finishing of animals and increase in organics.
- the average age of slaughter of prime animals is to be reduced from 27 to 24 months by 2030.
- farmers will be expected to reduce their use of chemical nitrogen to <350,000t by 2025 and <325,000t by 2030.
- 65pc of straight Calcium Ammonium Nitrate should be replaced by protected urea (or other protected nitrogen products) while a 90pc uptake of Low Emission Slurry Spreading (LESS) has been outlined.
- increase the number of dairy herds carrying out milk recording from 50pc to 90pc, and increase sucker beef herd weight recording from 30pc to 70pc.
- reduce crude protein content of livestock feeding stuffs to minimize nitrous oxide and ammonia loss, while utilizing feed additives during housing period.
- increase the area farmed organically in Ireland from 74,000 ha to 350,000 ha by 2030.
- contribute agricultural feedstock to the production of 1.6 TWh per annum of indigenous sustainably produced biomethane for injection into the gas grid by 2030.

Further potential measures include:

- A review of diversification opportunities for income and land use for farmers, including in areas such as biomethane and energy production, agroforestry and afforestation.
- Explore the development of a carbon farming model.

Reducing greenhouse gas emissions in livestock production

According to CNBS (2021) methane from cows is a big problem for climate change:

- About 25% of all methane is produced directly from fermentation by cows.
- Seaweed feed is reducing the amount of methane cows produce, according to research being conducted at the University of New Hampshire.
- Methane is a far more destructive greenhouse gas than carbon.

Methane is a major contributor to global heating, and cows produce a lot of it. There may, however, be a way to reduce all that gas: seaweed. On a research farm at the University of New Hampshire, scientists are feeding cows seaweed in an attempt to reduce the amount of methane they produce. Methane is more than 80 times as potent as carbon dioxide in warming the atmosphere, according to the United Nations Economic Commission for Europe, although it breaks down more quickly. About 25% of all methane is produced directly from fermentation by cows. The burps are actually worse than the farts. Seaweed can be part of that solution.

During the activities for reducing methane emissions, we have to be very careful that the seaweed doesn't affect milk production, because farmers are paid based on milk yield, fat and milk protein as well as SCC and bacteria account. Our goal is to reduce methane but at the same time not reduce milk performance. The seaweed may actually improve that performance, according to researchers, because reducing all the burping and farting allows the cows to use that energy instead for milk and beef production. That would be a boon not only for the environment but for the local dairy industry.

How we can reduce livestock greenhouse gas emissions?

There are 4 main approaches to reducing livestock greenhouse gas emissions:

- husbandry (animal breeding, feed supplements, improved pastures)
- management systems (stocking rates, biological control)
- numbers of livestock
- manure management.

Also, measures to change enteric fermentation to reduce emissions may can also increase animal productivity by increasing digestive efficiency.

Reducing the number of livestock to reduce greenhouse gas emissions would be counter to the objectives of the livestock industry. But is it a right solution?

There are some methods for reducing livestock emissions which may lead to increased dry matter intake per animal or provide the farmer with an opportunity to increase stocking rates, resulting in either no net change or even a net increase in methane production.

Also, with improving pasture quality and livestock efficiency, we can also improve productivity and lower emission intensity per unit of product, but the farm's total greenhouse gas emissions may increase due to increased stocking rates.

A) Animal breeding

Many investigations and trials suggest that animal breeding could achieve a 10–20% reduction in methane emissions. There are many variations among animals in methane

emissions per unit of feed intake and these variations suggest that there may be heritable differences in methanogenesis (methane production).
While selection and cattle breeding for reduced methanogenesis may not be compatible with other breeding objectives, breeding for improved feed conversion efficiency (lower net feed intake) should be compatible and is likely to reduce methane emissions and the greenhouse gas intensity of animal products.

B) Diet supplements and feed alternatives

According many trails, supplements like oils, fats, tannins, probiotics, nitrates, enzymes, sea algae and native vegetation, can reduce methane emissions from livestock.
Methaneabatements of 10–25% are possible by feeding ruminants dietary oils, with 37–52% abatement achieved in individual studies. Plant secondary compounds, such as condensed tannins, have been shown to reduce methane production by 13–16%, mainly through a direct toxic effect on methanogens. However, high concentrations of condensed tannins can reduce voluntary feed intake and digestibility.
Plant saponins (natural steroids occurring in several plant families) also potentially reduce methane, and some sources are more effective than others, with methane suppression attributed to combating protozoal infections.
There are approved methodologies for using dietary supplements to reduce greenhouse gas emissions from dairy cows and cattle.
According to Amy Quinton (2019), in USA trail there is up to a 60 percent reduction in methane emissions by using 1 percent of seaweed in the diet. This type of red seaweed, called *Asparagopsis taxiformis*, has one big drawback: a wild harvest is unlikely to provide enough of a supply for broad adoption.

C) Improved pastures

Improved forage quality with lower fiber and higher soluble carbohydrates can reduce methane production in livestock. Being structural fibers, cellulose and hemi-celluloses ferment more slowly than non-structural carbohydrates and yield more methane per unit of feed digested.
Methane emissions are commonly lower with more forage legumes in the diet, partly because of the lower fiber content (faster rate of digestion) and in some cases, the presence of condensed tannins. As improved diet increases animal growth and reduces methane production, it has the effect of reducing the greenhouse gas intensity of the animal products.
Pasture quality can be improved in several ways including by plant breeding, changing from tropical (C4) to temperate (C3) grasses that use different pathways to capture carbon dioxide, or grazing on less mature pastures. Several alternative plant forages, such as broccoli leaves and some native plants (such as *Rhagodia preissii***,** *Eremophila glabra***,** *Acacia saligna* etc.)**,** have been shown to reduce methane emissions in laboratory experiments. Saltbush diets on the other hand, increase methane emissions per unit of organic matter intake.
Also, on cattle rangelands, to help mitigate climate change, the solution is to conserve rangeland ecosystems and keep the carbon that's already stored in rangeland soils safely stored there.

D) Stocking rates

West Balkan countries are faced recently by reducing of total cattle population in last 20 years. Reducing the number of unproductive animals on a farm can potentially improve profitability and reduce greenhouse gas emissions. If productivity increases through nutritional and breeding strategies, the number of livestock can be reduced without losing the quantity of meat that is currently produced.

In dairy cattle, with extended lactation in dairying, where cows calve every 18 months rather than annually, it can reduce herd energy demand by 10%, and so potentially reduce methane emissions by a similar amount.

In beef cattle, with earlier finishing of beef cattle in feedlots, slaughter weights are reached at a younger age, with reduced lifetime emissions per animal and proportionately fewer animals producing methane.

E) Biological control

There are three biological control methods for reducing of methane production from livestock, using:

- viruses to attack the microbes which produce methane
- specialized proteins to target methane-producing microbes
- other microbes (methanotrophs) to break down the methane produced in the rumen into other substances.

A fourth possible option – bovine somatotropin and hormonal growth implants – does not specifically suppress methane formation, but rather improves the animal's performance and reduces the greenhouse gas intensity of the products.

In Canada farmers are feeding cows with seaweed, 1) to reduce costs on farms, and 2) cows produce 20% less methane. Asparagopsis taxiformis reduces the methane to nearly zero (0,01). Farmers start feeding cows with mix of seaweed – like taking 100s of thousands of cars off the roads.

Also, for climate changing in each country is a shortage of WATER (less water - increase of irrigation), less rains, higher temperatures – for more water reduction!

Climate-Smart Agriculture and the World Bank Group

According to the World bank (2022), The livestock sector is a pillar of the global food system and a contributor to poverty reduction, food security and agricultural development. According to the FAO, livestock contributes 40% of the global value of agricultural output and supports the livelihoods and food and nutrition security of almost 1.3 billion people. At the same time, there is wide scope to improve livestock sector practices so that they are more sustainable, more equitable, and pose less risk to animal and human health.

Livestock play a major role in sustainable food systems-for example, manure is a critical source of natural fertilizer, while livestock used as draft animals can help boost productivity in regions where there is low mechanization. Globally, around 500 million pastoralists rely on livestock herding for food, income, and as a store of wealth, collateral or safety net in times of need. Locally, livestock production systems have the potential to contribute to the preservation of biodiversity and to carbon sequestration in soils and biomass. In harsh

environments, such as mountains and drylands, livestock is often the only way to sustainably convert natural resources into food, fiber, and work power for local communities.
Increasing incomes, changing diets, and population growth have led to increased demand and made the livestock sector one of the fastest growing agricultural sub-sectors in middle- and low-income countries. This represents a major opportunity for smallholders, agribusiness, and job creators throughout the livestock supply chain. However, if not properly managed, this growth risks accentuating sustainability issues that span equity, environmental impacts, and public health.
The World Bank Group (WBG) is currently scaling up climate-smart agriculture. In its first Climate Change Action Plan (2016-2020), as well as the forthcoming update covering 2021-2025, the World Bank committed to working with countries to deliver climate-smart agriculture that achieves the triple win of increased productivity, enhanced resilience, and reduced emissions. In 2020, 52 percent of World Bank financing in agriculture also targeted climate adaption and mitigation.

Moving towards environmental sustainability in the livestock sector
The World Bank is committed to improving the livestock sector's contribution to sustainable development. The Bank supports countries to manage and respond to growing demand for animal protein in ways that are significantly less harmful for the environment and contribute significantly less to climate change.
As part of its commitment to helping countries build sustainable, nutritious food systems, the World Bank is moving its livestock investments towards greater sustainability and climate-smart outcomes. All investments are designed with mitigation and adaptation in mind, and an average of 61% of livestock financing over the last three years is directly tied to climate co-benefits (up from 55% in the previous period).
Bank-supported projects seek to improve various dimensions of livestock systems and value chains, using levers such as efficiency gains, balancing of animal rations and sustainable sourcing of feeds, carbon sequestration in agricultural landscapes, energy-efficient technologies and renewable energy sources, animal health and welfare, and better manure management. There are several projects financed by the World bank for this purpose:
Good news - are that Methane lasts only 100 years in the atmosphere (according one group of experts), unlike CO_2 which can stick around for hundreds of years! Carbon remains in the atmosphere for upwards of 1,000 years. Methane is more powerful but remains in the atmosphere for roughly just 12 years (according to the other group of experts).

CONCLUSIONS

Cattle husbandry is one of the largest GHG emissions from agricultural and livestock sector. According to the World bank and EU Green Action Plan recommendations, each country has to start own national program for reducing of GHG emissions. According to the present situation in cattle production sector in the world as well as in the R. of N. Macedonia, it is obviously that GHG emissions are increasing and influenced on the climate changing and global warming of our planet. There are a lot of mentioned measures for reducing GHG

emissions in cattle production sector by we can recommend the most important like: 1) improving the soil structure and fungi's activity for using CH_4 and CO_2 in the soil, 2) improving the pasture structure and cattle grazing systems, 3) improving the breed genetics for better feedstuff utilization, 4) improving the nutrition systems for reducing of CH_4 and CO_2 elimination from cattle, 5) increasing of use of green sea alga's in cattle feeding, 6) Feed additives and supplements that inhibit enteric methane emissions, 7) Feed ingredients that alter metabolic pathways to reduce enteric methane emissions, 8) Increased understanding of microbiome composition and activity in cattle, 9) using technologies such as sensors, robots and precise machines to *monitor enteric methane emissions or related physiological indicator, 10) Socioeconomic analysis of enteric methane mitigation practices and technologies, 11) Genetic selection of cattle that emit less methane, 12) Energy-efficient technologies and renewable energy sources, 13) Better animal health and welfare, and 14) Better manure and zero-west management.

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THE INFLUENCE OF THE STAGE OF LACTATION ON THE NUMBER OF SOMATIC CELLS IN COWS MILK

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SUMMARY

Somatic cell count (SCC) in milk is an indicator of udder health and it is also often used to determine quality payments to dairy producers. SCC is influenced by a large number of factors such as genetic factors, udder infection status, age of the cow, stage and sequence of lactation. The aim of this study was to determine the influence of lactation stage on the SCC in cows' milk. The present research included 25,460 individual milk samples. The SCC was lowest in the first 100 days of lactation (255,450/ml) and after it increased, reaching the highest value at the end of lactation (308,840/ml).

***Key words**: milk, somatic cell count, stage of lactation*

INTRODUCTION

In Serbia, cattle breeding participates in agricultural production with about 33%. Cows are the biggest producers of milk whose proteins have a high biological value. Milk is a very important food item in peoples diets, so much attention is paid to its quality. SCC in milk is an indicator of udder health and frequency of clinical and subclinical mastitis incidence in dairy herds, and it is also often used to determine quality payments to dairy producers (Dakić et al. 2006). Milk from a healthy udder contains less than 200,000 SC/ml, while the total number of microorganisms in raw milk, originating from a healthy udder, immediately after milking is around 5,000/ml (Kelly, 2002). Hortet and Seegers (1998) determined that when the SCC is increased to 400,000/ml, the daily loss per cow is about 1.4 kg of milk, and with 800,000/ml the daily loss is about 2 kg of milk. According to research, the average milk production of cows with less than 400,000 somatic cells/ml was 11% higher than cows with a somatic cell count greater than 400,000/ml (Sharif and Muhammad, 2008). Annual damages caused by mastitis in the USA amount to over 1.3 billion dollars, or about 11% of the value of annual production, and 70% of this amount refers to subclinical mastitis, 11% to discarded milk, and 1.7% to treatment (Majić, 1995). Reduction by SCC selection is slow and difficult to achieve. Selection for smaller udder depth, especially the last quarters, smaller teat spacing, and selection for greater length and smaller teat width, can help reduce the occurrence of mastitis (Monardes et al. 1990). Mastitis can occur as clinical or subclinical

mastitis. These latter clinical mastitis can be classified into mild, moderate, and severe mastitis (Table 1).

Table 1. Definition of the mastitis types with regard to the severity of the symptoms (Le Maréchal, 2011.)

Mastitis type	Definition
Subclinical mastitis	Inflammation of the mammary gland that is not visible and requires a diagnostic test for detection. The most used diagnostic test is the milk somatic cell count. Subclinical mastitis is the most prevalent form of the disease.
Mild clinical mastitis	Observable abnormalities in milk, generally clots or flakes with little or no signs of swelling of the mammary glands or systemic illness. Preferred terminology when describing severity of clinical cases.
Moderate clinical mastitis	Visibly abnormal milk accompanied by swelling in the infected mammary quarter with an absence of systemic signs of illness. The terminology is preferred when describing the severity of clinical symptoms.
Severe clinical mastitis	Udder inflammation characterized by sudden onset with grave systemic and local symptoms. This terminology is preferred for peracute clinical mastitis

The consequences of each type of mastitis on milk quality will differ. For example, clots appear in milk from animals with clinical mastitis whereas none are present in milk from animals with subclinical mastitis. Variations in the SCC can be observed during mastitis with a specific profile for each pathogen. The host immune response to the different pathogens appears to vary among species (Bannerman et al. 2004b) and each pathogen induces specific changes in the milk (Leitner et al. 2006). For example, *C. bovis* does not alter milk composition whereas milk modifications are more marked in the case of *E.coli* mastitis than in the case of mastitis induced by another pathogen (Coulon et al. 2002). In coliform mastitis, the SCC is low before and afterthe clinical mastitis. On the contrary, in the case of clinical *S. aureus* mastitis, the SCC increases before and remains high after cure. In the same way, streptococciinduce a continuous rise in SCC until clinical mastitis is settled and the SCCremains at a high level after mastitis (de Haas et al. 2002). Free fatty acids appear in milk with an increased number of somatic cells and there are changes in the sensory properties of milk This may be explained by the alteration of the milk fat globule membrane by leucocyte lipases or by plasmin through the hydrolysis of lipoproteins, both of which may enhance lipolysis. Nevertheless, results regarding lipoprotein lipase activity in mastitic milk are also contradictory: some authors found that its activity increased (Tallamy and Randolph 1970; Randolph and Erwin 1974; Erwin and Randolph 1975; Azzara and Dimick 1985) during mastitis while others found that it decreased (Fitz-Gerald et al.1981) or found no significant differences (Salih and Anderson 1979). Studies by a large number of authors indicate that the presence of higher SCC in milk is associated with physical and chemical

changes in milk, but there are different data on the intensity of decomposition of individual milk components. Udder infection changes the protein composition of milk, the content of the casein fraction is lower in an infected udder compared to a healthy udder, but the total protein content does not change because the proportion of whey protein increases. It was also determined that increased SCC in milk affects the reduction of fat and lactose content, while the biggest changes are observed in the increase in the amount of mineral content, which leads to a change in the pH value of milk (Mitić et al., 1983; Niketić et al., 2006). In order to preserve the health of the udder and the quality of raw milk, it is necessary to carry out regular analyses. Before taking a sample for analysis, a visual inspection of the milk is performed and sensory properties are determined. If the number of somatic cells is not within the reference values, the producer, purchasers and veterinary inspection are notified (Regulation on the quality of raw milk 2017).

SCC in milk can be influenced by genetic and external factors, the status of udder infection, the age of the cow, the stage and order of lactation, breed, husbandry, season, herd size, stress and other diseases, milking and the time and method of milk sampling. Regarding the influence of the way of holding, Čačić (2003) states that the SCC in a free holding system averages 197,000/ml, and in a tied holding system it is 231,000/ml. And, by analyzing milk from organic and conventional farms, Čubon et al. (2008) found a lower average number of somatic cells in organic milk, 219,000/ml, compared to the average number in conventional milk of 242,000/ml. They conclude that conditions on organic farms have a positive effect on udder health. Seasons have an effect so that cows are more resistant in the summer period, so the SCC in milk is the lowest. In autumn, SCC increases, and in spring it decreases again. Research by Ferreira and DeVries (2015) shows the opposite, that during hot summers with increased humidity, there is a decrease in the amount of milk and an increase in the number of somatic cells in the collected milk. Pavel and Gavan (2011) also determined the highest SCC in July and the lowest in April. It is believed that, also, with the increase in the number of cows in the herd, the SCC in the collected milk also increases. This is explained by the introduction of machine milking and greater infection of the cow's udder. Various forms of stress cause an increase in SCC. The method of sampling also affects the SCC, and it is the highest when milking the last streams and 1-3 hours after milking. Milk samples from evening milking have almost twice the number of somatic cells compared to milk samples from morning milking, which can be attributed to unequal milking intervals. Cows with a high genetic potential for milk production due to the high physiological load on the udder show a greater tendency to get sick with mastitis. By monitoring the number of somatic cells during lactation, two critical periods were determined when the milk physiologically contains increased SCC, namely the beginning and end of lactation. Colostral milk contains an increased number of somatic cells. After the colostral period, SCC decreases, being lowest in the middle and highest at the end of lactation. As a rule, the SCC curve is in the opposite direction of the lactation curve. Given the normally elevated SCC at the beginning of lactation, determination is not recommended in the first six days after calving. After calving, SCC remains elevated for 2 weeks and the average number is 242,000/ml. Schultz et al. (1990) state that first-calf heifers have higher SCC at the beginning of lactation, and multi-calf heifers before weaning. Milk loss related to an increase in SCC was, consequently,

highest toward the end of lactation, both in cows with and cows without a history of CM. (Hagnestam- Nielsen et al., 2009). They suggest that large milk loss in late lactation is related to the fact that the udder is in a catabolic state. The degenerative process taking place might influence both the udder's ability to repair itself after infection and the compensatory ability of uninfected quarters.

The aim of this study was to determine the influence of lactation stage on the number of somatic cells in *cows'* milk, using field data.

MATERIALS AND METHODS

The study included 11 dairy farms located in Vojvodina, with a total of 4,057 Holstein cows. A total of 25,460 individual milk samples were collected at monthly DHI milk tests. Analyses of raw milk samples were carried out on FOSS instruments – CombiFossTMFT+. This device is a combination instrument consisting of the MilcoScanTMFT+ and the FossomaticTMFC. The principle of analyzing of raw milk samples is based on the methodology by mid – infrared spectrometry method (ISO 9622 /2013) and flow cytometry (ISO 13366-2 /2006).For the statistical analysis of SCC data the absolute values were transformed into somatic cell linear scores (Log 2 SCC) by applying the following equation (Sant' Anna and Paranhos da Costa, 2011): Log 2 SCC = log2 (SCC/100.000) + 3. Logarithmic transformations are the most appropriate for the SCC data because they yield normality and homogeneity of the variances, enabling the execution of statistical analysis taking into account the above assumptions (Ali and Shook, 1980). The dataset included: farm code, date of test (season), days in milk (DIM - interval between date of calving and milk test day), daily milk yield, milk fat, protein, lactose, and SNF content, somatic cell count (cells/ml). Lactation was divided into 4 DIM intervals (I - 30 to 100 days, II - 101 to 200 days, III - 201 to 300 days and IV - greater than 300 days). The average values and variability of examined traits (daily milk yield - DMY, milk fat - MF, protein - P, lactose - L, solid non fat - SNF, and somatic cell count - SCC) as well as the effect of factors on mentioned traits were studied by means of the PROC UNIVARIATE and PROC GLM procedures within the Statistic software package (ver. 14 Stat Soft Company 2016). Post–hoc analysis (Duncan test) was used to determine the statistically significant differences between the mean values of different classes, with a significance level at $P<0.05$ and $P<0.01$.

RESULTS AND DISCUSSION

The average results for milk fat, protein, lactose and solids non fat (SNF) percentages, daily milk yield (DMY), and somatic cells count (SCC) are presented in Table 1.

Table 2. Means, Minimum, Maximum, standard deviation (SD) and coefficient of variation (CV) of analyzed variables

Trait	N	Mean	Minimum	Maximum	SD	CV
Fat (%)	25460	3.76	2.00	6.00	0.85	22.61
Protein (%)	25460	3.31	2.00	5.43	0.41	12.39
DMY (kg)	25460	26.75	2.00	67.20	9.88	36.93
SNF (%)	25460	8.74	5.59	10.98	0.47	5.38
Lactose (%)	25460	4.62	2.35	5.44	0.23	4.98
SCC (*1000/ml)	25460	274.84	50.00	1000.00	/	/
Log 2 SCC	25460	3.99	2.00	9.62	1.17	29.32

DMY- Daily milk yield, SNF- Solid non fat, SCC- Somatic cells count

Mean values, determined in this study, for milk fat (3.76%) was little lower and protein contents (3.31%) was little higher than average values for total Holstein population in Vojvodina in 2021 year (milk fat 3.83%, protein 3,26%) given by Main breeding organization (2022). The average SCC in these data was 274,840 cells / ml was higher than reported by Konjačić et al. (2010), but lower than reported by Johnson and Young (2003), Rajala-Schultz and Sville (2003) and Yoon et al. (2004).
Stage of lactation had a significant effect on SCC, and other examined traits in Holstein cows (the values of F-test in all cases are highly significant) (Table 3).

Table 3. Effect of stage of lactation on SCC, milk urea concentration, daily milk yield and milk components

Stage of lactation	N	Fat (%)	Protein (%)	DMY (kg)	SNF (%)	Lactose (%)	SCC (*1000/ml)	Log 2 SCC
1	5845	3.62a	2.99a	32.75a	8.47a	4.68a	255.45a	3.86a
2	8257	3.63a	3.22b	29.51b	8.67b	4.65b	265.82a	3.93b
3	7058	3.84b	3.45c	23.52c	8.85c	4.58c	280.73b	4.04c
4	4300	4.06c	3.68d	18.56d	9.05d	4.51d	308.84c	4.22d
F		328.1**	4267**	2915.2**	1747**	672**	52.50**	91.2**

- a.b.c.d Means within the same column with different superscripts differ significantly (P<0.01)
- significant differences: *P<0.05;**P<0.01;

As it is presented in Table 3., the peak of lactation was in the first 100 days after calving. Some authors found that the peak of lactation was between 4 and 8 weeks after calving

(Čobić and Antov, 1996; Park and Lindberg, 2004), but Piccardi et al. (2014) reported the peak of lactation around 122 days after calving. The SCC was lowest in the first 100 days of lactation (255,450/ml) and after it increased, reaching the highest value at the end of lactation (308,840/ml). The reports by Campos et al. (2006) showed that lactation curves of the content of somatic cells and milk yields usually show opposite patterns. Syridion et al. (2012) and Sitkowska (2008) also concluded that SCC increased with lactation progressing.

CONCLUSION

Based on the present research results, the following conclusions can be drawn, the stage of lactation had significant effects ($P<0.01$) on SCC, milk fat and protein content and daily milk yield.

The number of somatic cells was the lowest (255,450/ml) in the first 100 days of lactation, while the highest (308,840/ml)number was at the very end of lactation.

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BETA-CASEIN GENE POLYMORPHISM IN VOJVODINA HOLSTEIN-FRIESIAN COWS

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SUMMARY

One of the most important milk proteins β-Casein has its several genetic variations of which the two are the most important A1 and A2 type. After the concern about A1 variant on human health, a selection favoring the A2 allele was carried out in different countries. The goal of the research is to determine the frequency of A1 and A2 alleles, and the different types of genotypes inside the population of HF breed on the territory of Vojvodina. Out of 30 samples, A2A2 genotype had frequency 0,97, and A1A2 genotype had frequency 0,03. Alleles' frequency of the A1 and A2 was 0.016 and 0,984.

***Key words**: β-Casein, Holstein-Friesian breed, Polymorphism, selection*

INTRODUCTION

HF breed is the most represented breeds in Vojvodina. According to the annual report of the breeding center, in 2022, in Vojvodina (Main breeding organization), there are 85.858 cattle, which is the 68,48% of the whole population of the cattle under control there.

Milk is the most important nutrient in the people and animals nutrition, especially when it comes to the baby animals which nutrition sometimes consists only of milk. It consists of 3.5% proteins, 80% of which are alpha S1, beta-(β), alpha S2 and kappa-(κ) casein (CSN1S1, CSN2, CSN1S2 and CSN3), while the remaining 20% are whey proteins (α-lactoalbumin and β-lactoglobulin) (Farrell et al. 2004.). When it comes to β-Casein as the milk protein we can say that it is one of the proteins with the most polymorphism. There are 12 variants present in bovine milk including A1, A2, A3, B, C, D, E, F, H1, H2, I and G, of which A1 and A2 are the most common variants (Farrell et al. 2004., Oleński et al. 2012., Singh et al. 2015.). For the A4 allele, found in Korean native cattle, nucleotide substitution is not yet recognized (Kamiñski et al. 2007). It is considered that A2 beta-casein is the original beta-casein protein because it was present before the mutation caused the emergence of A1 beta-casein in European cattle (Bos taurus), several thousand years ago (Malarmathi et al. 2014.). The difference between A1 and A2 beta-casein lies in their amino acid chain composition in position 67: in the former there is histidine and in the latter there is proline. This difference results from the single nucleotide polymorphism (SNP) at codon 67, which is CCT (A2, proline) or CAT (A1, histidine). Therefore, cows with A2A2 genotype produce milk containing A2 beta-casein, unlike cows with A1A1 and A1A2 genotypes, which synthesis A1

beta-casein. The polymorphism that caused the replacement of proline with histidine leads to critical changes in the secondary conformation of the expressed beta-casein gene (Elliott et al. 1999., McLachlan 2001.). The amino acid substitution at a position 67 of β-Cn that defines A1 and A2 variants has a remarkable importance on the potential release of BCM7 (Summer et al 2020.). The presence of histidine causes the release of a bioactive peptide called beta-casomorphin-7 (BCM-7) in the process of gastrointestinal proteolysis of A1 beta-casein in the small intestine of humans, while the presence of proline in A2 protein in position 67 prevents the polypeptide sequence from breaking at this critical site (Thorsdottir et al. 2000.). The amino acid sequence of BCM7 corresponds to the β-Cn sequence 60–66 (Tyr60-Pro61-Phe62-Pro63-Gly64-Pro65-Ile66), and it was originally identified by Henschen et al. (1979.). Given the possible link between BCMs and CNS-associated disorders, much attention has been given to autism and to a lesser extent to the issue of ventilation disorders and sudden infant death syndrome (SIDS) (EFSA, 2009.). If peptides derived from food digestion, specifically BCM7, pass through the intestinal barrier and the BBB (blood brain barrier), they can potentially lead to neurological disorders and altered neuronal development. However, to date, no direct evidence is available on BCM7 ability to cross the BBB, as the effects of BCM7 on the nervous system have been only investigated in vitro (Summer et al. 2020.). Therefore, it is necessary to continue research into the role of BCM-7 (originating from both raw and processed milk, e.g. cheese) for human health. In vivo experiments are necessary to verify the presence of BCM-7 in the blood of animal models fed a diet containing milk with the alternative beta-casein genotype (Kamiñski et al. 2007). After the concern about β-casein A1 variant on human health, a selection favoring the A2 allele was carried out in different countries. To produce the so called A2 milk, cows homozygous for the A2 allele, together with the homozygous and the heterozygous for the A2-type alleles could be used, since no BCM7 will be released (Chessaa et al. 2020.). In general, selection decisions are made on polygenic traits using estimated breeding values (EBVs) that are computed using statistical methods that sum allele effects of genome-wide markers. The EBV for individual traits can also be combined in an economically weighted index to enable a balanced approach to select for multiple traits simultaneously. In some cases, there can be independent intense selection for a single allele or SNP that has a very large effect on a key trait, such as polled phenotype for animal welfare and "A2" milk for perceived human health benefits (Scott et al. 2023.). An implication of this is that intense selection for homozygosity at a given locus (A2) may result in increased inbreeding. Inbreeding can result in a loss of genetic diversity, decreased response to selection, reduced animal performance and ultimately, decreased farm profitability (Scott et al. 2022.).

Results of allelic and genotype frequencies could be useful as a starting point for selecting cows with the desirable A2 allele, in order to encourage small and large dairy farmers to form herds that would produce A2 milk exclusively (Ristanić et al. 2022.). The results of the polymorphism research show higher frequency of A2 (0,604) alleles in regards to A1 (0,396) alleles with the frequencies of the genotypes such as A2A2 (33,02%), A1A2 (54,72%), and the lower frequency of the genotype such as A1A1 (12,26%), (Sistani, 2020). Somewhat newer research shows similar results of the frequency of alleles A2 (0,56) and A1 (0,44), with the frequency of the genotypes A2A2 (31,40%), and A1A2 (48,60%), and the lower

frequency of the A1A1 (20,00%) genotype (Sistani, 2020). Both researches have been performed on the territory of Republic of Serbia for HF breed. The research that was done in Croatia showed the frequency of the alleles A1 (0,350) and A2 (0,650), together with the frequency of the genotypes A2A2 (43,33%), A1A2 (43,33%) and the lower frequency of the genotypes A1A1 (13,33%) (Ivanković et al., 2021).

This research aims to determine the polymorphism of the genes of β-casein present at the Holstein-Friesian breeds in Vojvodina. Type A1 of this gene can have a great impact on people health. Based on this, the selection of the cattle should be aimed at the reduction of the frequency of A1 type in β-casein.

MATERIAL AND METHODS

The samples of this research have been collected on five farms in Vojvodina, all 30 of them. The materials for DNA extraction were the materials collected from the tail hair follicles from the HF breed.

DNeasy Blood & Tissue Kits is used for DNA extraction, and this was done by the protocol issued by a producer. The verification of the quantity and the quality of the extracted DNA samples was done by NanoPhotometer® N60/N50. All the tested samples were used in later analysis. PCR of the amplification was also done by NanoPhotometer® N60/N50. The results of measuring isolated DNA on a spectrophotometer are shown in the Table 1. DNA was detected in each of the samples.

Table 1. The results of the quantity and the quality of the extracted DNA samples.

SAMPLE	Conc. (mg/µl)	A260/A230	SAMPLE	Conc. (mg/µl)	A260/A230
1	142.45	2.374	16	293.00	2.309
2	240.65	2.173	17	184.40	2.330
3	112.80	2.385	18	290.35	2.408
4	426.10	2.195	19	169.40	2.413
5	153.15	2.324	20	278.00	2.404
6	151.95	1.377	21	59.150	3.033
7	91.550	2.753	22	373.30	2.335
8	332.65	2.374	23	159.95	2.232
9	286.85	1.876	24	293.10	2.267
10	216.15	2.375	25	225.75	2.390
11	88.950	2.420	26	221.00	2.393
12	248.95	2.330	27	332.15	2.318
13	102.30	2.514	28	558.30	2.189
14	355.60	2.080	29	308.35	2.348
15	203.10	2.243	30	413.75	2.297

Table 1 shows the spectrophotometry results of all samples. 30 samples are marked in the first and fourth columns. The second and fifth columns shows the concentration (mg/µl) of DNA isolated from each sample, while the third and sixth columns (A260/A230) shows contamination with residues left after the molecule isolation process.

The mixture used for the PCR is the One*Taq*® 2X Master Mix with Standard Buffer and it is used by the protocol given by the producer for the amount of the PCR substrate up to 25µl. In order to perform the multiplication of the gene fragments, primers were used, the same ones used in the work "An Idea to Explore: Genotyping Bull Sperm to Introduce Basic Molecular Biology Techniques in an Animal Science Course" (De Groef and Grommen 2019.);

CASB122: 5' - GAGTCGACTGCAGATTTTCAACATCAGTGAGAGTCAGGCCCTG - 3 ' Forward

CASB67: 5' - CCTGCAGAATTCTAGTCTATCCCTTCCCTGGGCCCATCG - 3 ' Reverse

The PCR reaction conditions are shown in Table 2.

Table 2. Polymerase chain reaction program for amplification of the exon VII fragment of the β-Casein gene.

Phase	**Temperature (ºC)**	**Time**	**Number of cycles**
Initial denaturation	94	3 minutes	1
Denaturation	94	45 seconds	40
The adoption of primers	62	60 seconds	
The extension of the chain	72	75 seconds	
The final extension	72	10 minutes	1

PCR products (5µl) are mixed with bromophenol blue dye and the analyzed gel electrophoresis by the Gel (Vilber Smart Imaging) with the 2% agarose gel. As the indicator of the length of isolated fragments CSL-MDNA-50BR DNA Ladder RTU (Cleaver Scientific) have been used in this process. For the purpose of splitting the fragments, PCR products have been under the digestion done by TaqI-v2 restriction enzyme following the Time-Saver™ protocol, done on the temperature of 65°C - 15 minutes. The principle of this restriction enzyme is 5e-saver-other/time-saver-qualified-restriction-enzymes/time-saver-qualified-restriction-enzymes"0 minutes. After the incubation done on 3% of the agarose gel, the electrophoresis has been performed together with photographing of the results.

The research was carried out in the Laboratory for Animal breeding, reproduction and physiology which is a part of Department of Animal Science in Faculty of Agriculture, University of Novi Sad.

Descriptive statistics for the quantity and the quality of the extracted DNA samples and allele and genotype frequencies were calculated in the Excel software package.

Figure 1. Laboratory for Animal breeding, reproduction and physiology, Department of Animal Science in Faculty of Agriculture, University of Novi Sad.

RESULTS AND DISCUSSION

The results of measuring isolated DNA on a spectrophotometer are shown in the table 3. Average concentration of DNA in subjects isolates was 243.77 ng/µl, and ranged from 59.15 to 558.30. The average value of the ratio A 260/A 280 was 2.32 and ranged from 1.38 to 3.03. After agarose gel electrophoresis, it was determined that the DNA was visible and complete.

Table 3. Descriptive statistics of measured DNA concentration and of the results of spectrophotometric measurement

Parameters	N	Mean	SE	SD	Min	Max	CV
Conc. (mg/µl)	*30*	243,77	21,04	115,22	59,15	558,30	47,26
A260/A230		2,32	0,05	0,26	1,38	3,03	11,36

Conc. – concentration of DNA; A260/A230 - contamination with residues
N – number of samples; Mean – average mean; SE – standard error; SD – standard deviation; Min – minimum; Max – maximum; CV – coefficient of variation

Analyzing gel electrophoresis of the 30 examined samples, two genotypes have been found: A1A2 and A2A2, whilst the third genotype A1A1 has never been found in this research. The length of the fragments in genotypes is different, 251bp determines the genotype A2A2, 213bp determines the genotype A1A2, whilst 38bp determines the genotype A1A1. As it is shown on the Figure 2. we can conclude that only 1 sample out of 30 samples has genotype A1A2, whilst the others samples (29) were the genotype A2A2.

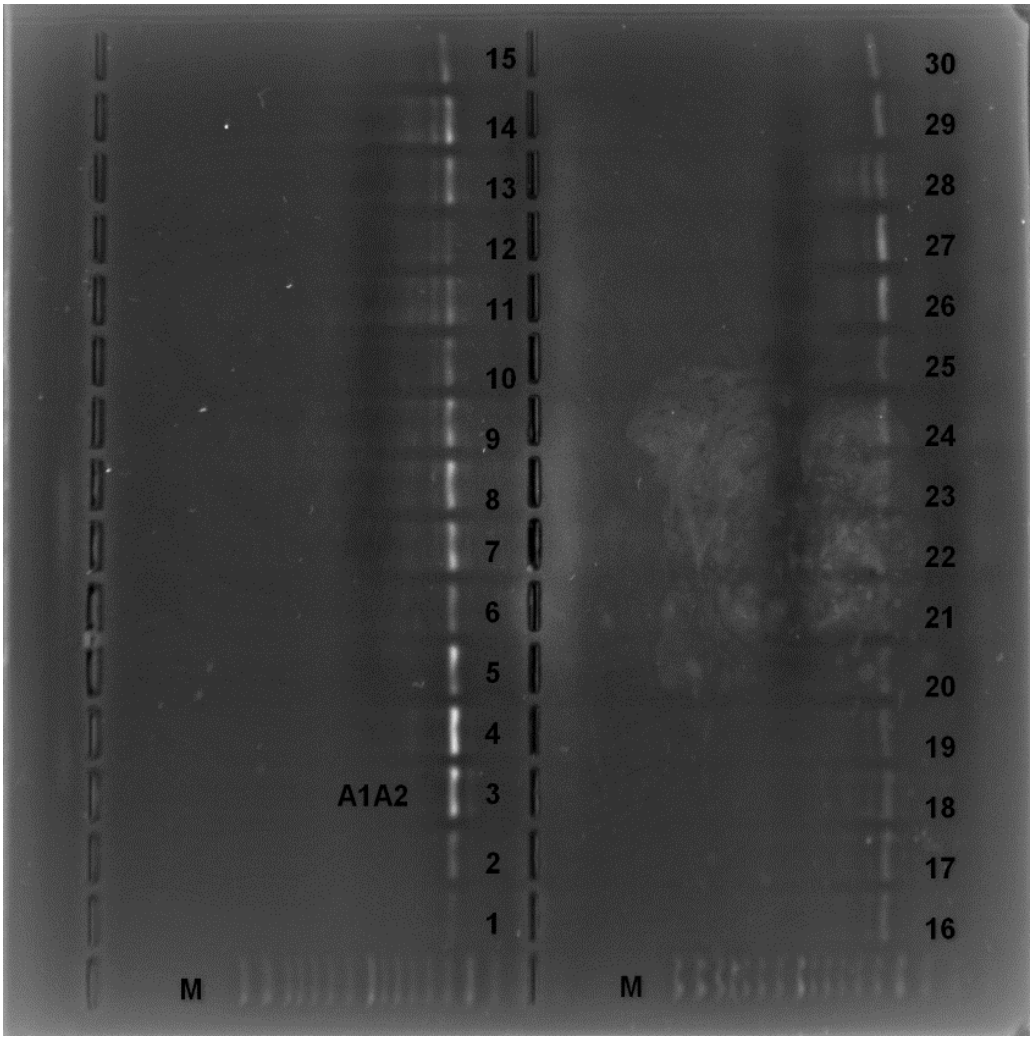

Figure 2. Gel electrophoresis after the digestion using the restrictive enzymes. **M** - CSL-MDNA-50BR DNA Ladder RTU. The samples were marked with numbers from 1-30. Genotype A1A2 is found in the third sample.

Based on this, the frequency of the alleles and genotypes shown in Table 3, was 0,016 and 0,984 (A1 and A2), whilst the frequency of the genotypes of A1A2 and A2A2 was 0,033 and 0,966. The results of the research were analyzed by the Hardy-Weinberg equilibrium, and the population is in balance.

Table 3. Allele frequencies and genotype frequencies.

Genotype	**N**	**Genotype frequencies**	**Allele frequencies**	
			A1=p	**A2=q**
A1A1	0	0	0,016	0,984
A1A2	1	0,033		
A2A2	29	0,966		
SUMA	**30**	**1**	**1**	

N-number of samples

Research of this type has not been done as often as they should be. Just few researches are dedicated to the problem of the frequency of the A1 alleles within the cattle breeds in Serbia and the Balkans. The most important research was published by Ristanić et al. (2020., 2022.), in Serbia. From this research done by Ristanić we can conclude that the A1 allele is present to a lesser extent within the milk cattle compared to A2 alleles. This is the result of this research. The research done in Croatia also shows the lower frequency of A1 alleles (Ivanković et al., 2021). This research, as many others, is done in the Balkans, Asia and Europe, and they show lower frequency of A1 alleles within the milk cows of different breeds.

CONCLUSION

The research of the polymorphism of the β-Casein is not done in such extent in Vojvodina, so we cannot provide much information about the population of the milk cows there. The research on this topic is used in order to raise awareness of the producers, so they pay attention to the β-Casein and its consequence on humans nutrition. So far, the research show that the frequency of the A1 type is low in Vojvodina and the whole region of Serbia. The main purpose of this research is for the producers to pay attention to the selection, especially the selection of those cattle that carry the gene type A2 ofβ-Casein. In many countries the polymorphism of the β-Casein is the most important topic in the milk industry, so the market is classified by the β-Casein type (A2 milk). This procedure should be done in Vojvodina, too, when it comes to milk industry.

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ENVIRONMENTAL ENRICHMENT FOR LABORATORY MICE AND RATS AT UNIVERSITY OF NOVI SAD

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SUMMARY

Laboratory mice and rats are the most commonly used animals in biomedical research. Environmental enrichment is a list of mechanisms for improving the welfare of animals by allowing them to perform their natural, behavioural needs. This study provides information about environmental enrichment for laboratory rats and mice in three scientific – educational institutions in Novi Sad. It has been shown that at least one of these mechanisms is used in these institutions, with a few differences within each one. Environmental enrichment is very important for their well – being, and this study should be an initial point for further research on this topic.

Key words: *laboratory mice and rats, environmental enrichment, welfare, Novi Sad.*

INTRODUCTION

Laboratory mice are the most commonly used animals in biomedical research. They are usually kept in groups, and in one cage there should not be more than 30 individuals. In the cages, it is necessary to provide about 180 cm^2 of living space for one adult, and about 200 cm^2 of living space is needed for one female with young. The bottom of the cages is usually covered with sterilised sawdust, and sometimes they can be given different materials such as paper strips, straw etc., which they use to make a nest. Females usually give birth away from place where they defecate and urinate. Laboratory mice are fed with pellets, *ad libitum,* that are placed in special feeders at the top of the cage. The water is provided in special glass water bottles, also at the top of the cage (Kanački and Samojlik, 2020).

Laboratory rats are also commonly used in research and experiments. Group housing is recommended whenever it is possible because rats are highly social animals. One adult animal needs around 250cm^2 of living space, and female with the youngsters needs about 800cm^2 of living space. They are also fed with pellets, *ad libitum*, that are placed in special feeders, at the top of the cage, and water is also given in glass water bottles, the same way (Kanački and Samojlik, 2020). Rats like to stand on the hind legs, and they need a taller cage to satisfy this behavioural need. They also like to climb, so whenever it is possible, cages with different levels and partitions should be used (Vučinić et al., 2010).

Environmental enrichment for laboratory rats and mice means providing key stimuli that trigger the expression of innate, in order of priority, important forms of animal behaviour,

which animals are always highly motivated to perform. Enriching the living space has several goals, namely increasing the possibility of displaying physiological forms of behaviour, giving animals more opportunities to display high-priority forms of behaviour that is characteristic for the species, preventing the emergence and manifestation of pathological forms of behaviour in the type of compulsive disorders. Enrichment of living space and conditions, can be divided in few categories: physical enrichment, sensory enrichment, manipulative enrichment, nutritional (feeding) enrichment, social enrichment and operant conditioning (Vučinić, 2009),

MATERIAL AND METHODS

The aim of this study was to investigate whether and to what extent certain mechanisms of environmental enrichment are used in three scientific and educational institutions in Novi Sad.

Employees in these three institutions were interviewed about different types of environmental enrichment mechanisms, as well as their personal point of views on this topic, such as:

- their opinion about environmental enrichment,
- if they are using any type of it in their everyday work and to what extent,
- who is deciding about using a certain type of enrichment and how they choose it,
- what are the most problematic aspects of environmental enrichment and how it affects research and experiments,
- why the certain types of enrichment aren't represented enough or at all, and what are the reasons for such situation.

People who were interviewed have different professional qualifications: medical doctors, laboratory technicians, veterinary technicians, biologists and biochemists who all are working with laboratory animals on a daily basis or are doing research on them.

RESULTS AND DISCUSSION

The surveyed institutions of University of Novi Sad were: Department of Pharmacology and Toxicology at Faculty of Medicine, Department of Biology and Ecology at Faculty of Sciences, and National Reference Laboratory for Rabies - Pasteur Institute Novi Sad**.**

After interviewing ten individuals who are working in these institutions, it was found that at least one of the six mechanisms of environmental enrichment is applied in each of these institutions (Table 1.), with few differences within. Most of the people working in these institutions showed interest in learning more about welfare of laboratory animals and how to improve their living conditions in order to make their work easier.

Type	Physical	Sensory	Manipulative	Feeding	Social	Operant conditioning
Faculty of Medicine	Yes	No	No	No	Yes	Yes
Faculty of Sciences	No	No	No	No	Yes	No
Pasteur Institute	No	No	No	No	Yes	Yes

Table 1. Representation of different types of enrichment in surveyed institutions.

At the Department of Pharmacology and Toxicology at the Faculty of Medicine, laboratory mice and rats are kept in the vivarium. Scientists and technicians who work with laboratory animals use physical enrichment tools, such as plastic houses for hiding, for mice. They have used physical enrichment tools for rats in the past also, but at the current moment of the survey, they had none in the cages, due to technical reasons. However, they are planning to get these tools back in the rat cages in the near future, as they've noticed that it is positive for animals. In other research (Vučinić, 2009), physical enrichment as a mechanism of providing any physical materials and items is used in order to increase animal's physical activity, or provide shelters and safe spaces. Partitions can be formed during cage design using various physical enrichment items such as different shelters, nesting boxes, nesting material, tunnels and platforms that provide retreat and viewing areas, for both rats and mice (Baumans, 2005). Beds, climbers, ladders and running wheels can also be used as physical enrichment (Kanački and Samojlik, 2020).

In the same institution, they keep both rats and mice in groups which increases the social enrichment and stimulates natural social behaviour. They keep bigger groups of mice together in one cage (<30 individuals), all of the same sex, and smaller groups of rats, usually consisting of same sex trio, as they don't breed animals. Vučinić (2009) states that social enrichment is a form of enrichment that provides direct contact of animals with animals of the same, or compatible species. Group housing is recommended for laboratory rats because they are highly social mammals. Cloutier et al. (2012) have found that the tickling of rats by the laboratory staff that works with them could mimic the contacts that rats make during play and mutual communication. Tickling is recommended as a good mechanism of social enrichment for laboratory rats that are not in close contact with individuals of the same species or do not live in groups.

Further, researchers train animals to become easier to handle at the beginning of their research, and by that positive reinforcement, the animals become calmer and easier to work with. This is known as operant conditioning form of enrichment.

They also used manipulative tools for enrichment in the past, such as cardboard, but they've had problems with the validity of the experiment and overall health of the animals.

Manipulative enrichment is a form of enrichment that involves inserting objects into the living space that stimulate the animals to display exploratory forms of behaviour and to master new skills (Vučinić, 2009). Olsson and Dahlborn (2002), analysing various experiments related to nest building in mice, came to the conclusion that although nesting material is often given only to pregnant females, nests are built by both males and non-pregnant females. Mice also make nests if there are different hiding places, houses and tunnels, which proves to us that this is a very important form of behaviour for this species. Chmiel Jr. and Noonan (1996) demonstrated that gnawing is a behaviour that is natural to rats and that when given the opportunity, they will perform it. In the experiment, the rats showed an affinity for wooden cubes with holes and wooden balls, which they gnawed on.

Picture 1. Plastic hideouts for laboratory mice, original photo by author.

National Reference Laboratory for Rabies keeps only laboratory mice, as they do a special diagnostic procedure called mouse inoculation test (MIT) for rabies. In this procedure, three-to-ten mice, 3- to 4-weeks old, are anesthetised and inoculated intracerebrally. This is a confirmatory test for rabies, and if mice are inoculated with infected material, they usually show signs of illness on fifth day after inoculation, when they are euthanized. Because of this type of procedure, physical enrichment in this situation is impossible to apply, as technicians and veterinarians have to keep a close eye on inoculated animals, which wouldn't be possible if the animals were given any form of shelter or hideout.

In the past, when animals were given material such as cat litter, to eliminate the odour in order to improve their living conditions, they actually eliminated the natural scents of the animals. As a consequence animals didn't make nests out of sawdust bedding and they didn't

raise young properly. Sensory enrichment implies the introduction of various olfactory, auditory, tactile, visual stimuli and tastes into the living space. (Vučinić, 2009)

As an example of good practice, in this institutions, employees pick up animals by hand and play with them, as a part of handling and their daily work. This is positive reinforcement which enriches the lives of these animals.

Mice are kept in trios, consisting of one male and two females – they are also breeding in these groups. Sometimes females give birth at the same time and they are both feeding and raising all the cubs. The best form of social enrichment for mice involves keeping one male and several females, or several females together (Deacon, 2006) So, it can be said that this is the most natural way of keeping mice, as they tend to have small harems of one male and few females in nature as well.

Picture 2. Trio of mice and their young, original photo by author.

At the Department of Biology and Ecology at the Faculty of Sciences, one type of enrichment is represented, and that is social enrichment. They keep both mice and rats, either in groups or pairs of the same sex individuals. They found the handling training of laboratory rats and mice to be dangerous, because the animal could potentially bite them.

Regarding the feeding enrichment, they think it won't make any difference on animal welfare and their daily work. However, feeding enrichment implies the application of such feeding regimes that maximally stimulate the animals to exhibit all three phases of this form of behavior, namely the appetitive phase /exploratory phase, the consummatory phase /executive phase and the calming phase /final phase (Vučinić, 2009).

On other institutions, scientists and technicians both showed interest in implying the foraging feeding method for both mice and rats. Feeding animals with different types and textures of food can be enriching, as well as stimulating natural foraging feeding behaviour.

CONCLUSION

Based on the results of the research, it can be concluded that the environmental enrichment mechanisms are represented in scientific – educational institutions in Novi Sad. It is shown why some enrichment tools can't be applied at certain situations, and by that is meant that the nature of procedure or experiment, doesn't allow applying these mechanisms, such as MIT test procedure that is done in Pasteur Institute. Some problems have been noted with certain types of enrichment, such as manipulative and sensory, which should be definitely studied in the future. As the science of laboratory animal welfare is still young, further research on this topic is needed. Environmental enrichment is very important for their well – being, and this study could be an initial point for further research. Educations on this topic, and welfare of laboratory animals could be useful.

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TREATMENT OF TAPEWORM (*HYMENOLEPIS ERINACEI*) INFECTION IN A NORTHERN WHITE-BREASTED HEDGEHOG (*ERINACEUS ROUMANICUS*): A CASE REPORT

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SUMMARY

A wild hedgehog was rescued from a dog attack in a backyard of private house in Srbobran, Serbia. In shelter, a cestodosis was suspected after finding segments in animal's faeces. Parasitological examination confirmed the presence of Hymenolepis spp. and nematode eggs and larvae. The hedgehog was successfully treated with praziquantel, with no adverse reactions. After morphological examination of collected tapeworms, the cestode Hymenolepis erinacei was identified. This paper presents the first published record of wild hedgehog tapeworm treatment in our country.

Key words: *Erinaceus roumanicus, hedgehog, tapeworm, Hymenolepis erinacei, praziquantel*

INTRODUCTION

Hedgehogs are small, nocturnal, spiny-coated insectivores classified in family *Erinaceidae* (Hoefer 1994). *Erinaceus roumanicus* has a global distribution extending from central and Eastern Europe, the Baltic and the Balkan Peninsula eastwards through Belarus, Ukraine, and Russia, reaching as far as western Siberia. In the south, its range extends as far as the northern Caucasus and the island of Crete. It is recorded from sea level to at least 1,400 m. Within the Mediterranean region, it ranges from Italy and Slovenia, through the Balkan Peninsula (Croatia, Bosnia and Herzegovina, Serbia, Montenegro, Albania, Macedonia, Bulgaria, Greece, European Turkey) and extending south into the Near East Anatolian Turkey. The northern white-breasted hedgehog inhabits farmland, parks and gardens in rural and urban areas, scrubby habitats at the edge of forests, and shrubby vegetation. (Amori et al. 2021).

Its diet consists of invertebrates, including beetles, caterpillars, and earthworms (Yalden 1976). Because of their feeding habits and environment they are prone to various parasitic infections. Many helminth endoparasites like *Crenosoma striatum, Capillaria aerophila, Capillaria erinacei, Hymenolepis erinacei* (Beck, 2007) and other species of ectoparasites like ticks and fleas (Gaglio et al., 2010). *Hymenolepis* is a cestode belonging to the family *Hymenolepididae*. The genus *Hymenolepis* contains a large number of species occurring mainly in domestic and wild animals (Binkiene et al., 2018). The development

cycle of the hedgehog tapeworm (*Hymenolepis erinacei*) is always with an intermediate host (especially fleas and other insects) which carry cysticercoid forms. In the intestines of the intermediate host, the oncospheres become free and penetrate into the body cavity and develop to infective cysticercoid. Oral ingestion of such insects leads to infection. The sexually mature tapeworms develop in the host and parasitize in the small intestine. Through faeces, proglottids or eggs are released in batches, which are ingested by coprophagous insects (e.g. dung beetles) (Beck, 2007; Prokopic, 1971).

Parasite infections are mainly treated in pet hedgehogs (Beck, 2007). As there are not many published reports of treatment of wild animals, veterinarians have limited experience in treatment of those parasitoses, especially in Serbia. The aim of this paper is to present the case of wild hedgehog infected with *Hymenolepis* spp. caught in a backyard in Srbobran, Vojvodina Province, Serbia, and treatment protocol used for elimination of tapeworm.

MATERIAL AND METHODS

Case description

In late October 2022 in a backyard of a private house in Srbobran (45°32′N 19°47′E), South Bačka District, Vojvodina Province, Serbia, a wild hedgehog was attacked by family's dogs. The hedgehog was kept overnight in a safe enclosure, fed with commercial cat diet and given fresh water. In the next morning, during cleaning of the enclosure, milky-white segments were found in the faeces. Faecal samples were collected and sent to the Laboratory for Parasitology, Department of Veterinary Medicine, Faculty of Agriculture Novi Sad.

Parasitological examination

For initial complete parasitological examination, combined sedimentation-flotation technique (CSFT) (Deplazes, et al. 2016), using three flotation solutions with different specific gravity (SPG): zinc sulphate (SPG 1,18 and 1.35) and Sheather's sugar solution (SPG 1,27). During subsequent controls, the Sheather's sugar solution was used for flotation of cestode eggs. Briefly, 5g of faeces was mixed with water, strained through tea strainer into the plastic test tube, and centrifuged (10 minutes on 800 x g). The supernatant was discarded and the sediment was mixed with flotation solution and again centrifuged. After that, additional flotation solution was added until the upper meniscus appeared. Coverslip 18x18mm was placed on the tube and allowed it to sit for an additional 10 minutes, before removing the coverslip and placing it on a slide. For detection of lungworm larvae, a Baermann test was used (Zajac, et al. 2021). Quantification of nematode eggs was performed using Mini FLOTAC technique using 2 g of faeces and 18 ml of flotation solution (Sheather's sugar and saturated NaCl) (Cringoli, et al. 2017). Cestode eggs were not counted, qualitative labelling of data was performed instead, with "+" for presence, or "-"for absence of eggs in the specific sample. For monitoring of egg shedding, Mini FLOTAC and CSFT technique were performed every other day.

For identification of *Hymenolepis* tapeworm, standard sample staining with carmine was performed (Lalošević, 2008). The tapeworm species was identified according to available keys (Иржавский and Кетенчиев, 2011).

Clinical examination and treatment of the hedgehog

The hedgehog was examined at the Department's Clinic for Small Animals. General examination was performed, and body weight was determined for correct medication dosage. Clinical examination was performed at monthly intervals. The most important was to treat hedgehog's lungworm infection, so antinematode drugs (levamisole and ivermectin) were applied followed with supportive therapy (data not shown).

For the therapy of hymenolepiasis, anticestode drug praziquantel in the form of a tablet intended for dogs and cats (Tenivet®, Evrolek-Pharmacija Doo Šabac, Serbia) was applied off label at a dose of 50 mg for animal heavier than 500 g (Beck, 2007). The drug was applied perorally, after short hibernation period, at the end of November 2022, using a 2 ml syringe.

RESULTS

During clinical examination, it was determined that the hedgehog belongs to the species *Erinaceus roumanicus*, and that it was juvenile male according to his body weight of 575 g. Except of the tapeworm segment found, the animal appeared to be healthy. However, besides the tapeworm from the genus *Hymenolepis*, the results of coprological examination revealed the presence of *Capillaria spp*. eggs and *Crenosoma striatum* first stage larvae (details not shown) (Figure 1).

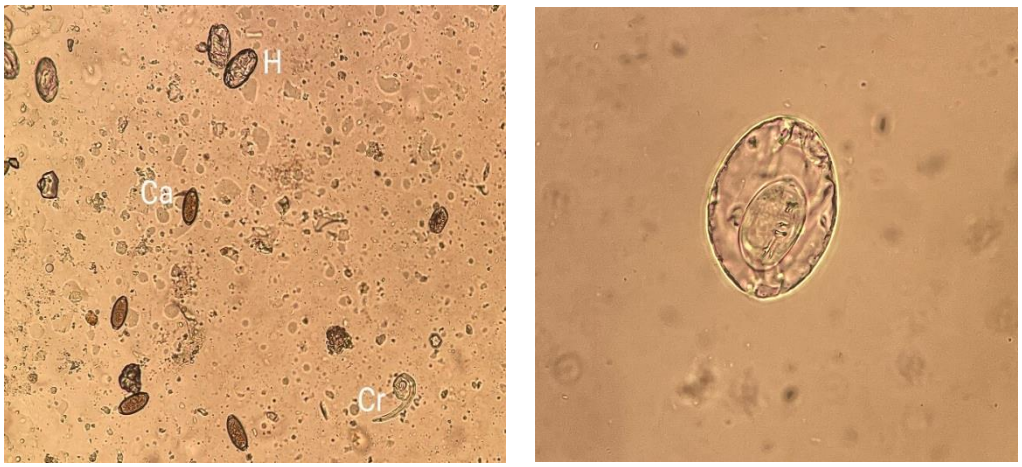

Figure 1. Helminth eggs found in a hedgehog. H- *Hymenolepis erinacei*, Ca- *Capillaria spp*. Cr- *Crenosoma striatum*, magnification 100x (left) and *Hymenolepis erinacei* egg, magnification 400x (right)

Regarding tapeworm shedding, the segments were found daily in the faeces of the hedgehog. The first coprological examination showed the large number of eggs (8690 EPG).

During captivity period, the presence of cestode eggs was noted regularly until the treatment with praziquantel, as shown in Table 1.

Table 1. Presence of *Hymenolepis* eggs and body mass of the hedgehog relative to treatment

	Week 1	Week 2	Week 4	Week 5	Week 7
Presence of eggs	+	+	+*	-	-
Body mass	575g	n.m.	620g	n.m.	711g

***Applied dose of praziquantel**

The treatment with praziquantel had no adverse reactions in the hedgehog. Moreover, a couple of hours after treatment, the animal had expelled a large batch of tapeworms (Figure 2) which were later identified, following morphological analysis, as *Hymenolepis erinacei* (Figure 3).

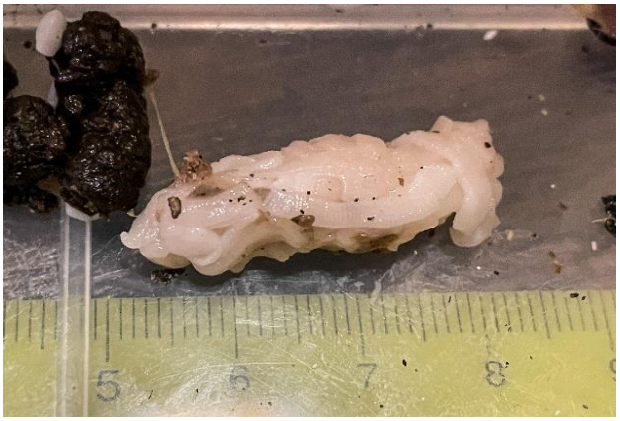

Figure 2. Batch of tapeworms released after applied therapy

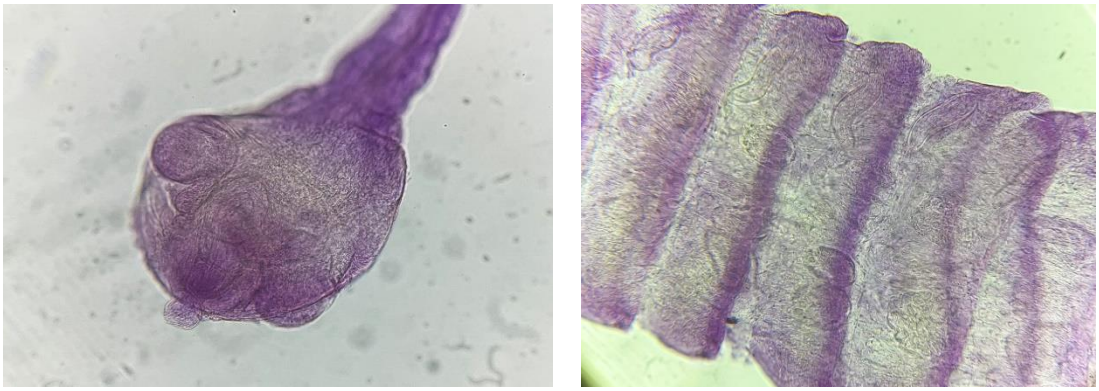

Figure 3. Scolex (left) and proglottids (right) stained by carmine

DISCUSSION

In previous studies, it was determined that the prevalence of the *Hymenolepis erinacei* was around 33,33% on the territory of Serbia (Pavlovic and Savic 2017). This species is usually found at hedgehogs at numerous European countries (Britain, Germany, Italy) and in the Middle East (Turkey, Iran) (Carlson 1980; Boag and Fowler 1988; Cirak, et al. 2010; Youssefi, et al. 2013). The first case of *Hymenolepis erinacei* (there shown as *Vampirolepis erinacei*) in Great Britain (Boag and Fowler 1988) showed the pathological significance on the hedgehog's health which motivated us to try the treatment suggested by Beck 2007. Our study, which was also the first case of successful treatment in Serbia, showed the effectiveness of the praziquantel against this cestode. Knowing the tapeworm life cycle and the diet of hedgehog (e.g. dung beetles) (Prokopic 1971) it is highly suspected that the reinfection might happen. There are also cases of infection in other hedgehog species belonging to the genus *Erinaceus* which don't inhabit Balkan Peninsula (Amori, 2016; Amori, et al. 2021).

This hedgehog species is a protected species in Serbia (Anonymous, 2010). Hedgehogs have a very important role in ecosystems. Fast urbanisation leads to depletion of their natural habitat and much frequent contact with humans so there is a much bigger need for their care and treatment. In Serbia the only places where wild animals are treated are in the clinics for wild animals and by the veterinarians in the zoos. Consequently it is very important to publish the methods of therapy for helminthoses of wild hedgehogs, until shelters and hospitals for wild animals are formed at the national level, as they exist in developed countries.

CONCLUSION

The treatment of wild hedgehog with praziquantel was successful according to expulsion of tapeworms, the absence of their eggs in control examinations and increase in animal weight. Due to the fact that this is the first published case of anticestode therapy for hedgehogs in Serbia, it could be of great benefit to veterinarians, biologists and other people

interested in the health and welfare of these animals. Future tapeworm prevalence studies are needed, including more detailed information on the pathogenic effects of the tapeworms on hedgehog health, which can prove useful in creation of treatment protocols in case of formation of national wildlife rehabilitation centres, similar to ones across Europe.

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QUALITY ASSESSMENT OF IRRIGATION WATER IN CATCHMENT OF WEST MORAVA

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SUMMARY

The groundwater used for irrigation must meet the basic requirement of providing the necessary quantity of water for irrigation as well as its appropriate quality. This research presents the analysis of mineralization of groundwater and it's usability in catchment of West Morava. Two water classifications were used, the FAO classification and Serbian Water Quality Index. The findings from this research emphasize the importance of continuously monitoring water quality and its suitability, even when the results appear positive and suggest a high groundwater quality.

Key words: *Groundwater quality, West Morava, irrigation, FAO classification, Serbian Water Quality Index*

INTRODUCTION

The task of this research is to perform an analysis of the usability of groundwater for irrigation in the West Morava river basin, using available data from reports on the quality of surface and groundwater found on the website of the Environmental Protection Agency. The measurement stations from which the water quality needs to be determined are Kruševac, Sirča, and Stančić selo. The assessment of water quality is conducted using the FAO and SWQI classification for the time period from 2011 to 2020.

The goal of this study is to classify the water intended for irrigation into classes and determine its usability for irrigation, in order to more effectively carry out the irrigation of agricultural crops. Additionally, determining the quality will impact the choice of plant production and the method of irrigation. Evaluating the suitability of irrigation water is gaining increasing importance as a crucial step to prevent potential negative impacts on natural resources, particularly soil, as well as on cultivated crops and agricultural equipment (Fipps, 2003; Joshi et al., 2009; Bauder et al., 2011; Bortolini et al., 2018; Vranesevic et al., 2023).

MATERIAL AND METHODS

In this research, the focus is on the West Morava basin, suitability of groundwater for irrigation. Analyses conducted at measuring stations show the presence of certain parameters

and their concentrations. Water quality parameters, both physical and chemical, were sourced from the Hydrological Yearbook, which is published by the Agency for Environmental Protection, to provide data for analysis (SEPA, 2011-2020). The water classifications that are used in this research are FAO classification and Serbian Water Quality Index (SWQI).

Three hydrological measuring stations are selected, Sirča (viseći most), Stančić selo, and Kruševac. The period for which the research was carried out is for 10 years, from 2011. to 2020. and assessment of groundwater quality were done. The stations to be presented in this research have the following characteristics:

Sirča (viseći most): Hydrological station code 2NP208; Name of groundwater body West Morava- alluvium; Porosity type- Intergranular porosity; Coordinates: 43° 44' 13", 20° 43' 32".

Stančić selo: Hydrological station code 2NP218; Name of groundwater body: West Morava-alluvium; Porosity type- Intergranular porosity; Coordinates 43° 52' 46", 20° 26' 44".

Kruševac: Hydrological station 2NPK-1; Name of groundwater body: West Morava-alluvium; Porosity type- Intergranular porosity; Coordinates 43° 36' 20", 21° 18' 03".

The FAO classification is based on assessing the risk of salinization. The rate of water infiltration into the soil depends on the total salt content in the water and is given special significance. It is expressed by the electrical conductivity of water and the values of SAR (Sodium Adsorption Ratio), which represent the relative ratio of sodium to magnesium and calcium. In this classification, special attention is given to considering the toxicity of individual elements.

In the FAO classification, there are three categories of water quality for irrigation:

I) The first category are waters that can be used for irrigation of all crops without restrictions and do not cause adverse consequences.

II) The second category includes waters that can be used with weak to moderate restrictions. They are mainly applied to areas with a deep groundwater level and good natural drainage. In arid regions and areas with low natural rainfall, periodic salt flushing from the active root zone is necessary. Special attention should be paid to the choice of plant species because sensitive and moderately sensitive crops to salt cannot be irrigated with such water.

III) The third category comprises waters with sharp usage restrictions and can mostly be used on lighter and moderately light soils with a low absorption capacity. In such soils, salt flushing from the root zone is required. Constant monitoring of salt content is also necessary depending on the cultivated species. Under these conditions, tolerant crops to salt are most commonly grown (Bosnjak, 1999).

Using the Serbian Water Quality Index (SWQI) method, ten selected parameters (oxygen saturation, BOD5, ammonium ion, pH value, total nitrogen, orthophosphates, suspended matter, temperature, electrical conductivity and coliform bacteria) represent the properties of surface waters by their quality (q and) , reducing them to one index number. The share of each of the ten parameters on the total water quality does not have the same relative importance, that's why each of them received its own weight (w i) and number of points according to the share in endangering the quality. By summing the product (q and xw) an index of 100 is obtained as an ideal sum of the quality share of all parameters. In the case when quality information is missing for a parameter, the value of the arithmetically measured

SWQI is corrected by multiplying the index with the value 1/x, where x is the sum of the arithmetically measured weights of the available parameters (SEPA, 2011-2020). The formula used to calculate water quality is:

WQI= $\sum_{i=1}^{n}(qi * wi)$

WQI - Water Quality Index, on a scale from 0 to 100

n - Number of parameters

qi - Water quality of the corresponding parameter

wi - Weight assigned to the corresponding parameter

Serbian Water Quality Index categorize water in to a four classes (Table 1).

Table 1: Classification of surface waters using the Serbian Water Quality Index method

WQI - MDK I class		WQI - MDK II class	WQI - MDK III class	WQI - MDK IV class
85-84		74-69	56-44	51 - 35
100-90	89-84	83-72	71-39	38-0
Excellent	Very good	Good	Bad	Very bad
Serbian Water Quality Index(SWQI)				

I) Excellent - water that in its natural state, with filtration and disinfection, can be used to supply water to settlements and in the food industry, while surface water and for breeding noble species of fish;

II) Very good and Good - waters that in their natural state can be used for bathing and recreation of citizens, for water sports, for breeding other types of fish, or which, with modern purification methods, can be used to supply drinking water to settlements and in the food industry;

III) Bad - water that can be used for irrigation, and after modern purification methods, also in industry, except food;

IV) Very bad - waters whose quality adversely affects the environment, and can only be used after applying special purification methods.

RESULTS AND DISCUSSION

An analysis of the essential parameters for water quality, encompassing total dissolved salts, electrical conductivity, cations, and anions, was conducted using statistical methods on the sampled water. To determine their quality, eleven different water quality parameters were taken into consideration. A ten-year analysis of water was considered, specifically from 2011 to 2020, to ensure more accurate and detailed analyses. The minimum, maximum, means, and standard deviation are given in Table 2. A detailed analysis of the parameters required for the application of the two water classification classes for irrigation

has been carried out. A detailed analysis of the parameters required for the application of the two water classification classes for irrigation has been carried out.

Table 2: Analyzed parameters on the measuring points

	Kruševac				Sirča				Stančić selo			
Parameter	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
Ca^{2+} (mg/l)	68,0	97,6	78,9	9,9	60,1	116,0	88,3	20,4	94,4	14401,0	1537,2	4519,9
Mg^{2+} (mg/l)	32,0	63,0	43,9	9,4	22,0	87,0	52,3	21,9	42,0	83,0	66,8	14,1
Na^{+} (mg/l)	11,7	35,8	21,1	7,0	7,1	20,9	12,3	4,6	10,4	28,4	19,0	5,3
K^{+} (mg/l)	1,8	4,2	2,5	0,7	2,1	4,9	3,4	0,9	2,8	3,9	3,2	0,4
HCO_3^- (mg/l)	334,0	472,0	395,4	41,3	320,0	636,0	456,4	105,6	478,0	594,0	547,2	36,2
Cl^- (mg/l)	18,9	41,6	24,8	7,2	10,4	35,9	20,0	8,1	23,8	41,6	35,0	6,1
SO_4^{2-} (mg/l)	40,0	110,0	65,6	19,5	30,0	73,0	54,3	15,8	71,0	97,0	82,2	8,9
NO_3-N (mg/l)	0,4	2,6	0,7	0,7	0,2	0,6	0,4	0,2	0,2	3,6	1,4	1,3
EC (µS/cm)	641	905	750	72	580	981	755	142	1023	1169	1063	52
TDS (mg/l)	375	546	445	51	336	695	457	118	570	756	632	58
pH value	7,2	7,8	7,6	0,2	7,2	7,8	7,4	0,2	6,7	7,6	7,2	0,3

Based on FAO classification, it can be concluded that concerning the risk of water salinity, infiltration, and the toxicity of Na and Cl, the water in Kruševac mostly falls into class I. This water can be used for irrigation with some precautions. At the well in Sirča, the water belongs to both class I and class II, although the infiltration properties are not good. To use this water for irrigation, moderate water restriction is required. In terms of the toxicity of Na and Cl, the water belongs to class I, which means it is not toxic and is suitable for irrigation. At the Stančić selo station, the salinity is very high, classifying the water into class II. In terms of infiltration and the toxicity of Na and Cl, the water is very good, classifying it as class I in this aspect (Figure 1).

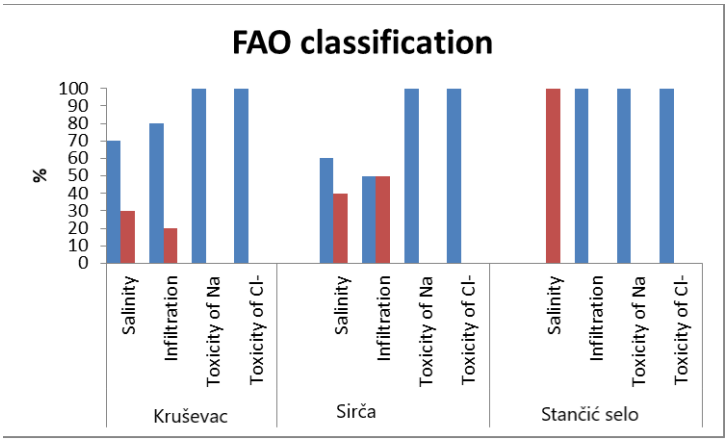

Figure 1: FAO diagram for classification of analysed irrigation waters

Based on the SWQI classification, the water in Kruševac is of good quality and falls into Class II water. In the location of Sirča, the water is also of good quality and belongs to Class II. From these cities, the water can be used for irrigation, fish farming, and recreational purposes for the public. With the help of modern water treatment methods, it can also be used

for supplying residential areas and in industry. In the Stančić Selo the water is of poor quality and falls into Class III water. Despite its poor quality, it can be used for irrigation, and with certain treatment methods, it can be used in industry as well. The results of this classification are shown in Table 3.

Table 3: Classification of water using the SWQI method

Serbian Water Quality Index					
Descriptive indicator	Excellent	Very good	Good	Bad	Very bad
Numerical indicator	(100-90)	(84-89)	(72-83)	(39-71)	(0-38)
Kruševac			73		
Sirča (viseći most)			73		
Stančić selo				67	

CONCLUSION

In order to make irrigation safer and more efficient, it is necessary to determine the water quality. Assessing water quality is a process that begins with water sampling. Based on the values of various parameters, the sampled water is classified into different classes. There are various methods for water classification, and in this study, FAO and SWQI classifications were used. Based on the calculations and water classification, it can be concluded that groundwater in the West Morava basin is of satisfactory quality for irrigation. Unfortunately, Serbia has not fully utilized its irrigation potential. The reasons for this include significant investments required for irrigation systems and limited government support. It should be noted that water quality for irrigation depends not only on the natural properties of water but also on all of us.

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ANTIOXIDANT CAPACITY OF DIFFERENT TYPES OF MICROGREENS

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SUMMARY

Microgreens represent young plants (seedlings) used in human nutrition as a food supplement, enriching the visual aspect, improving the texture and increasing the taste. In addition, they are popular in nutrition due to the high content of bioactive components with a high antioxidant potential. Seeds of different plant species are used to grow microgreens. Therefore, the aim of this work was to determine the influence of the plant species on the content of some bioactive components and the antioxidant potential of microgreens. The following plant species are represented in the research: oats, wheat, flax, peas, basil and buckwheat. Microgreens are grown in controlled laboratory conditions of the Faculty of Agriculture and Food Sciences in Sarajevo. The research determined the total content of phenols, flavonoids and antioxidant activity. The research results show that the content of bioactive components and antioxidant value significantly depended on the type of microgreens. The content of total phenols ranged from 42.32 (oats) to 140.12 mg GAE g-1 (basil). The lowest content of flavonoids was recorded in wheat (28.68 mg CAE g-1), while the highest was recorded in purple basil (80.42 mg CAE g-1). The researched species also differed significantly in activoxidative activity, ranging from 132.99 (oats) to 399.60 µM Fe^{2+} g^{-1}.

***Key words**: microgreens, phenols, flavonoids, antioxidant activity, health*

INTRODUCTION

Nowadays, an increasing number of people are becoming increasingly aware of the importance of a healthy diet and its impact on general health. In this context, there is a growing interest in different aspects of nutrition, especially in microgreens. Microgreens are young vegetables that consist of roots, stems and cotyledon leaves. The stems and leaves of microgreens are concentrated in nutrients, making them a powerful superfood. Microgreens are the first real leaves produced from the seeds of the plant that are about 5-8 cm tall (Kumar et al., 2022). These tiny sprouts are rich in nutrients and, at the same time, bring a special aroma and colour to the plate.

Due to all the complications in the agricultural and health sector, microgreens are attributed to functional food that is rich in chemical compounds, which can be expected to provide a certain solution for various types of diseases, etc. (Bhaswant et al., 2023)

Microgreens are easy to grow and mature quickly. Growing these does not require a lot of space, so they can also be grown in urban areas. This is particularly significant in the context of self-sustaining food and vertical farming. Microgreens bring colour, texture and freshness to every dish. Their presence on the plate makes the meal more attractive and tempting. In addition, different types of microgreens offer a variety of tastes, from spicy to sweet notes, which allows creativity in the kitchen. (Renna et al., 2023)

Growing microgreens requires fewer resources compared to the traditional growing of mature plants. A smaller amount of water, light and space is needed, which makes this type of cultivation more environmentally friendly. In addition, the need for long transport is reduced, and the amount of waste in the food supply chain is reduced.

Microgreens are a true "superfood" source of nutrients. Even though they are small, they are appreciated because they are rich in various nutrients and minerals. (Di Gioia et al., 2023). These small plants have concentrations of nutrients that are normally much higher than mature plants (Kumar at al, 2022). For example, microgreens like broccoli sprouts contain high levels of vitamins C, K, folic acid and carotenoids. Microgreens are known for their increased content of phenols and flavonoids, which makes them an even more valuable food in the human diet.

These compounds are known for their antioxidant properties and potential benefits for human health. Phenols and flavonoids are compounds in many plants, and their concentration depends on many factors. Therefore, the aim of this work was to determine the influence of the type of microgreens on the content of total phenols, flavonoids and antioxidant Dcapacity.

MATERIAL AND METHODS

The experiment was conducted in the agricultural laboratory of the Faculty of Agriculture and Food Sciences, University of Sarajevo. Young shoots (microgreens) are grown under room conditions. The research was conducted with 6 different plant species: oats/oats (*Avena sativa* L.), wheat (*Triticum aestivum* L.), flax (*Linum usitatissimum* L.), peas (*Pisum sativum* L.), basil (*Ocimum basilicum* L. cv *red rubin* and *genovese*) and Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn). Sowing was done in plastic containers (0.4 L) intended to produce seedlings. The containers were previously filled with sowing substrate (Klasman potgrond H). The research was carried out in three repetitions. Harvesting and sampling for analyses of young shoots were conducted at technological maturity (developed cotyledons). After harvesting, the samples were dried and ground. The following quality indicators were analyzed: content of total phenols, flavonoids and antioxidant activity.

Extraction of bioactive compounds. Ethanol extracts were prepared as follows: 1 g of sample was added to a volumetric flask. Then, 50 mL of 60% ethanol was added, and

everything was mixed. After incubation at room temperature for 24 hours, the extracts were filtered and used for chemical analyses.

Determination of the content of total phenols. The content of total phenols in the extracts was determined by the modified Folin-Ciocalteu method (Gavrić et al. 2021), and the results were expressed as mg of gallic acid equivalents (GAE) per gram of sample.

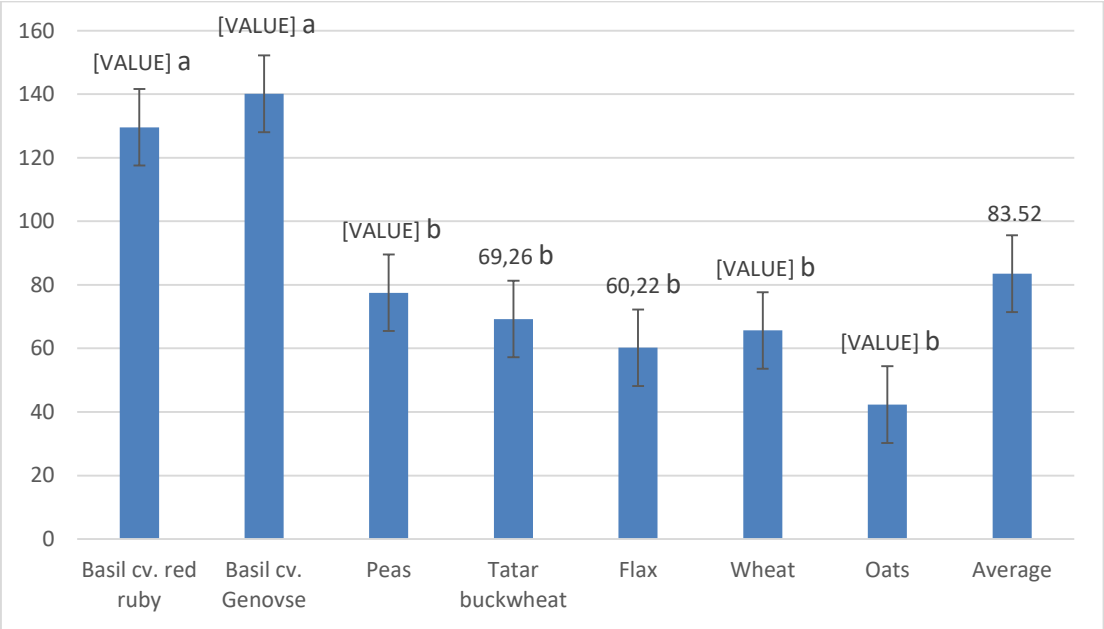
Determination of flavonoid content. The flavonoid content was determined by a modified spectrophotometric method based on the coloured reaction of flavonoids with $AlCl_3$ (Zhishen, Mengcheng and Jianming 1999), and the results were expressed in mg of catechin acid equivalents (CAE) per gram of sample.

Determination of antioxidant capacity. The antioxidant capacity of the extract was measured using a modified FRAP method (Benzie and Strain 1996). The results are expressed as μM Fe^{2+} g^{-1}.

Statistical processing of the data was performed by analysis of variance (ANOVA test) with a significance level of 5% ($P<0.05$). The SPSS 22.0 program (IBM, USA) was used for statistical data processing.

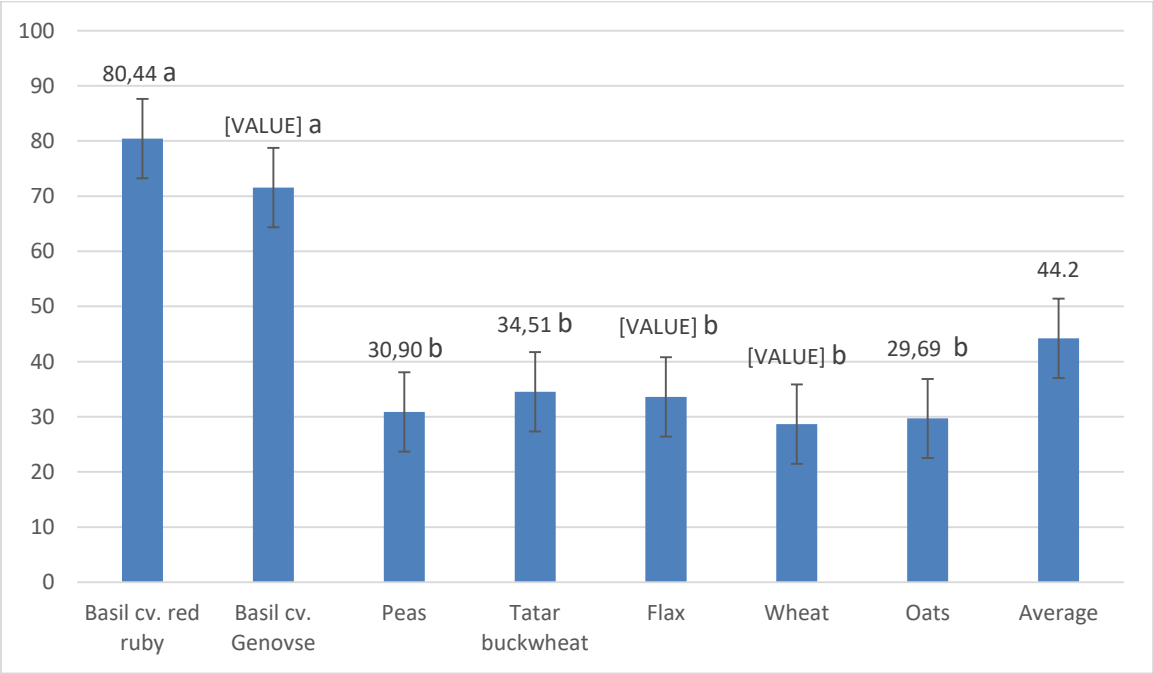
RESULTS AND DISCUSSION

The research results shown in the graphs show that the content of total phenols and flavonoids in microgreens significantly depended on the researched species. The lowest content of total phenols was recorded in oat microgreens (42.32 mg GAE g^{-1}) and the highest in genovese basil (140.12 mg GAE g^{-1}). A high content of total phenols was also recorded in the second investigated basil variety (red rubin) (129.57 mg GAE g^{-1}). Statistical analysis of the data found that both researched varieties of basil had a significantly higher phenol content than other types of microgreens. The obtained results are following Ghoora et al. (2020). They investigated microgreens of ten different types and concluded that the content of total phenols significantly depends on the researched species.



Graph 1. Content of total phenols, mg GAE g^{-1} (Different letters a-b indicate a statistically significant difference, $p<0.05$)

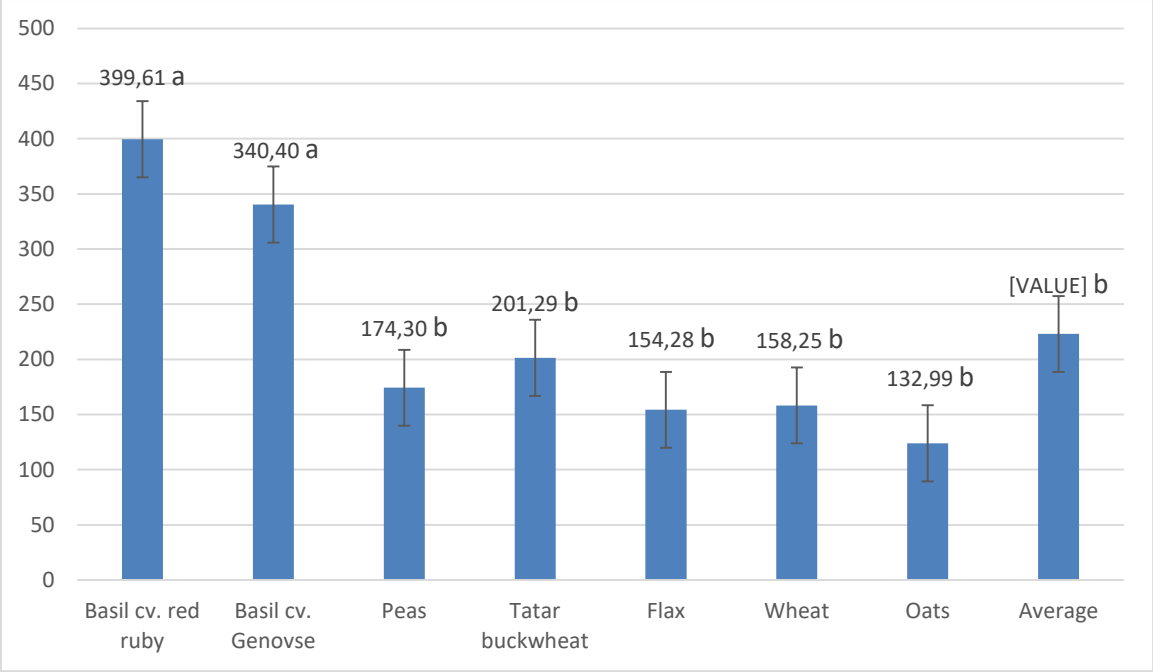
The content of flavonoids in the plant depends significantly on the species and the environmental conditions in which the plant thrives (Gavrić et al. 2018). Given that in this study, the environmental conditions, such as temperature, humidity and light, were constant for all investigated species, it can be said that the content of flavonoids significantly depended on the studied species. In our research, the highest flavonoid content was recorded in basil microgreens samples (71.56 and 80.44 mg CAE g^{-1}) and the lowest in samples of wheat microgreens (28.68 mg CAE g^{-1}).



Graph 2. Content of total flavonoids, mg CAE g^{-1} (Different letters a-b indicate a statistically significant difference, $p<0.05$)

Based on the data on antioxidant capacity, this investigated property also significantly depended on the type of microgreens. The antioxidant capacity ranged from 132.99 (oats) to 399.61 µM Fe2+ g (basil). The highest values of antioxidant capacity were recorded in the investigated basil cultivars. Kwee and Niemeyer (2011) studied 15 different basil cultivars and concluded that all investigated cultivars have high antioxidant activity.

Based on the obtained data, it can be said that the content of total phenols, flavonoids, and antioxidant capacity is relatively high in the investigated microgreens. This fact is particularly interesting because the microgreens of basil, peas, buckwheat, flax, wheat and oats can be a valuable source of phenolic compounds with potential medicinal effects. It is believed that the consumption of food rich in phenolic compounds alleviates and cures diseases such as heart disease (Javanmardi et al., 2003), high blood pressure (Miranda et al., 2016), neurodegenerative diseases (Tamuly et al., 2013) and diabetes (Tebogo et al., 2016).



Graph 3. Antioxidant capacity, µM Fe^{2+} g^{-1} (Different letters a-b indicate a statistically significant difference, $p<0.05$)

CONCLUSION

The content of total phenols, flavonoids and antioxidant capacity of microgreens significantly depended on the type of plant. The highest values of bioactive components were found in basil microgreens, followed by buckwheat, peas, wheat, flax and oats. All researched types of microgreens can be a valuable potential source of antioxidants in human nutrition.

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EFFECT OF BEDDING MATERIAL ON FOOTPAD DERMATITIS IN BROILER CHICKEN

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SUMMARY

This research was conducted to determine whether different types of bedding material affect the incidence and severity of FPD. A total of 255 one-day old Ross 308 broilers were allocated to 3 tretmants with 5 replicates, with 17 chickens per pen. The investigated treatments were un-chopped straw, peat, and peat with wood shavings. The incidence and severity of FPD were measured at 28, 35 and 42 days of age. The results showed that straw had the highest scores of FPD, and peat had the lowest. It can be concluded that the type of bedding significantly affects the occurrence and severity of FPD.

***Key words**: bedding material; footpad dermatitis; peat; wheat straw*

INTRODUCTION

One of the most important factors in broiler production is the quality of the bedding. Bedding material has a significant role in broiler production due to its influence on growth, carcass quality, health and welfare (Garcês i sar., 2013). In addition, Boussaada et al. (2022) state that litter affects the concentration of ammonia and dust in the air. A number of authors state that factors such as the amount and type of bedding material, mixture composition, housing system, health status, stocking density and age at slaughter can significantly affect the quality of the litter (Grimes et al., 2006; Bilgili et al., 2009; Škrbić et al., 2010; Škrbić et al., 2012).

De jong et al. (2014) argue that type of bedding material and moisture content have been identified as the most significant factors in the development of footpad dermatitis (FPD). On the other hand Shepherd et al. (2010) state that particle size is one of the most important factors in the occurrence of FPD. Very large and coarse bedding materials may, however, downgrade carcass quality due to their abrasive effects to foot pads (Diarra et al., 2021).

Footpad dermatitis is a type of contact dermatitis that causes necrotic lesions on the footpads of broilers (Greene et al., 1985). In the earlier stages, there is a change in the color of the foopad, while in the later stages, necrosis of the epidermis occurs, and in more severe

cases, inflammation of the subcutaneous tissue may occur (Ekstrand et al., 1997). Shepherd and Fairchild (2010) state that FPD is a condition that has multifactorial causes, however, dry bedding material is considered the most significant factor in reducing its occurrence. More severe lesions can lead to lameness, which significantly affects welfare (Bassler et al., 2013), as well as reduced growth as a consequence of reduced food consumption due to pain (Martland, 1985).

Contact dermatitis is caused primarily by moisture and irritating chemical substances in the litter (Bessei, 2006; Taira et al., 2014). The activity of microorganisms is intensified in wet litter, which results in temperature increase and ammonia production (Matkovic et al., 2015). The level of litter moisture depends on a number of factors, including litter type and amount, type of drinking and ventilation system, microclimate conditions, season, chicken age, nutrition, health, and stocking density (Bessei, 2006; Shepherd & Fairchild, 2010; Dunlop et al., 2016).

Wheat straw is the most common choice of bedding material due to its wide distribution, low price and relatively good characteristics. It is preferable to chop the straw, and Žikić et al. (2017) found that a length of less than 2 cm had positive effects on the occurrence of footpad dermatitis, which was the result of a better ability to absorb moisture. Kyvsgaard et al. (2013) found a higher incidence of FPD in broilers reared on straw compared to chickens reared on peat and sawdust. Kaukonen et al. (2017) found that broilers on peat had a significantly lower incidence of footpad dermatitis compared to sawdust and straw.

Birds spend most of their life in close association with the bedding or litter material. Hence, the most obvious contributor to FPD may be the type, quantity, or substandard quality of bedding material. Bedding materials with sharp edges may contribute to FPD through their abrasive action. A wide range of materials could be used as broiler litter: rice hulls, ground corncobs, stump chips, pine sawdust, wood shavings, pine bark, sand, coconut husk, newspaper, corn cob, wheat straw, ground rapeseed straw (Grimes et al., 2002; Sirri et al., 2007; Meluzzi et al., 2008; Garcês et al., 2013). Of these, litter may be the most important because broilers spend most of their time on the litter and their foot pads, hock and breast are in constant contact with the litter. Therefore, if the type, quantity and quality of litter material are not optimal there is a considerable risk that birds will develop contact dermatitis and breast blisters (Meluzzi and Sirri, 2009).

Various types of litter materials are used in different countries. In Slovenia, wood shavings and sawdust are the most common materials used as litter in commercial broiler production. However, these preferred litter materials are becoming expensive and limited in supply. Therefore, appropriate substitutions need to be considered. Various forms of recycled paper and chopped straw have proven to be good litter materials. Various litter amendments such as chemicals - aluminum sulfate (Madrid et al., 2012), sodium bisulfate (Nagaraj et al., 2007; Li et al., 2012), zeolite (Li etal., 2008), microbiological preparation (Iwaczuk-Czernik et al., 2007), or a commercial ammonia binding agent (Lazarevic et al., 2014) are used to reduce litter moisture, pH and NH3 emission in broiler houses. Nagaraj et al., 2007 state that such litter treatments might have positive effects on the occurrence of FPD.

The aim of this research was to determine the influence of different types of bedding materials on the occurrence and severity of footpad dermatitis in broilers.

MATERIAL AND METHODS

The experiment was conducted at the experimental farm of Faculty of Agriculture in Novi Sad. A total of 255 one-day old Ross 308 broilers were allocated to 3 tretmants with 5 replicates. Broilers were arranged in floor pens with 17 chickens per pen. The first treatment was un-chopped wheat straw, which was added in the amount of 2.5 kg/m^2. The second treatment was peat, which was added in an amount of 8.5 kg/m^2, and the third treatment was peat with wood shavings in an amount of 8.5kg/m^2.

The incidence and severity of FPD were measured at 28, 35 and 42 days of age using the scoring method described in Welfare Quality® Assessment protocol for poultry (2009): no evidence of footpad dermatitis (score 0), minimal evidence of lesions (score 1 and 2), and evidence of lesions (score 3 and 4).

All analyses were performed using StatSoft computer package (STATISTICA 14, 2020). Differences with $P<0.05$ were considered statistically significant. Differences between groups were analyzed using Mann-Whitney U test.

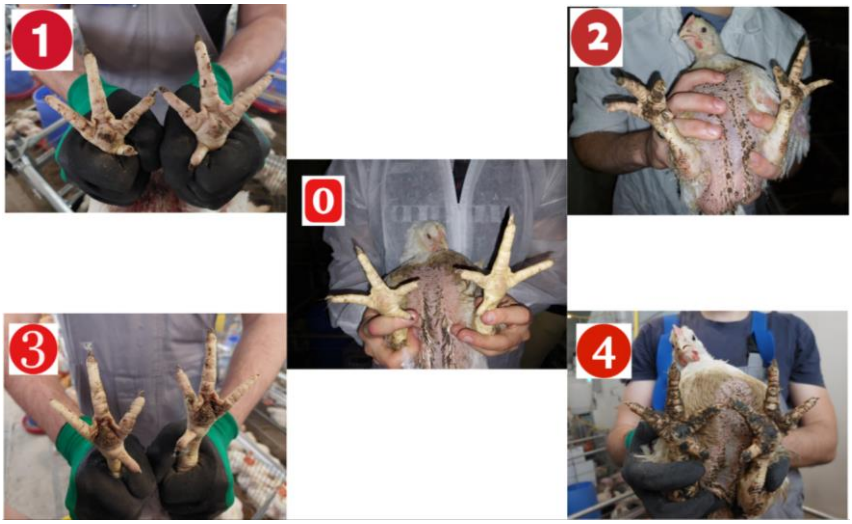

Figure 1 – Macroscopic evaluation of footpad dermatitis

RESULTS AND DISCUSSION

From table 1. it can be seen that broilers reared on straw had significanty higher incidence of footpad dermatitis (1.12) compared to peat (0.05) and peat with wood shavings (0.07) ($P<0.05$). In addition, the severity of lesions was higher in broilers reared on straw. These results are in line with Kaukonen et al. (2017) who found a significantly lower incidence of FPD in broilers reared on peat compared to straw. Kyvsgaard et al. (2013) also found a significantly higher incidence of footpad lesions in broilers reared on straw compared to peat and wood shavings. The reason for the higher occurrence of lesions in chickens raised on straw can be found in the high content of lignin in the straw, which reduces the ability to bind

water (Diarra et al., 2021). No differences where found between peat and peat with wood shavings (P>0.05).

Table 1. Effect of different bedding material on incidence and severity of FPD at 4 weeks of age

Score	Treatments					
	Straw		**Peat**		**Peat with wood shavings**	
	No.	%	No.	%	No.	%
0	11	13.25	77	95.06	79	92.94
1	49	59.04	4	4.94	6	7.06
2	22	26.51	0	0.00	0	0.00
3	1	1.20	0	0.00	0	0.00
4	0	0.00	0	0.00	0	0.00
Total	83	100.00	81	100.00	85	100.00
Average	1.12[a]		0.05[b]		0.07[b]	

[a,b,c] Means within the same row with different superscript differs significantly (p<0.05)

Straw length can significantly affect the occurrence and severity of FPD, and reducing straw length to 2 cm or less significantly reduces the occurrence of FPD (Žikić et al., 2017). Đukić Stojčić et al. (2016) state that chopping straw results in a smaller number of animals with lesions and the severity of lesions is lower.

Table 2. Effect of different bedding material on incidence and severity of FPD at 5 weeks of age

Score	Treatments					
	Straw		**Peat**		**Peat with wood shavings**	
	No.	%	No.	%	No.	%
0	5	6.10	62	80.52	57	70.37
1	24	29.27	14	18.18	24	29.63
2	37	45.12	1	1.30	0	0.00
3	15	18.29	0	0.00	0	0.00
4	1	1.22	0	0.00	0	0.00
Total	82	100.00	77	100.00	81	100.00
Average	1.84[a]		0.20[b]		0.29[b]	

[a,b,c] Means within the same row with different superscript differs significantly (p<0.05)

In the fifth week of age broilers reared on straw had the highest occurrence of FPD, while broilers reared on peat had the lowest incidence (P<0.05). There was a slight increase in

broilers reared on straw with FPD compared to the fourth week of age. In addition, the number of individuals with scores 2 and 3 has also increased. However, the largest number of broilers on peat and peat with wood shavings had a score of 0, while scores of 3 and 4 were not recorded. Taira et al. (2013) state that a significant increase in the occurrence of FPD occurs after 21 days of age.

Table 3. Effect of different bedding material on incidence and severity of FPD at 6 weeks of age

Score	Treatments					
	Straw		**Peat**		**Peat with wood shavings**	
	No.	%	No.	%	No.	%
0	0	0.00	56	72.73	46	59.74
1	20	24.39	19	24.67	25	32.47
2	35	42.68	1	1.30	4	5.19
3	23	28.05	1	1.30	2	2.60
4	4	4.88	0	0.00	0	0.00
Total	82	100.00	77	100.00	77	100.00
Average	2.05^{a}		0.26^{b}		0.44^{b}	

[a,b,c] Means within the same row with different superscript differs significantly ($p<0.05$)

With an average score of 2.05 straw had the highest occurence of FPD, followed by peat with wood shavings with an average of 0.44 and peat with an average of 0. Score 4 was established only in broilers reared on straw, while it was not established in broilers reared on peat and peat with wood shavings which is in line with Kaukonen (2017) who found a lower occurrence of FPD on peat compared to wood shavings and straw, however, a small percentage of lesions with a score of 4 were found in both litter types.

CONCLUSION

The highest incidence and severity of FPD was found in broilers reared on wheat straw, while the lowest was recorded in broilers reared on peat. Based on the results obtained in this study, it can be concluded that the type of bedding material significantly affects the occurrence and severity of FPD. Therefore, choosing an adequate bedding material is extremely important because broilers spend their whole life in direct contact with the litter. Further testing of different types of bedding materials is needed to find bedding that have positive effects on broiler welfare and reduces the incidence and severity of FPD.

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ELICITATION OF HEMP UNDER LED AND ITS EFFECT ON CANNABINOID CONTENT

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SUMMARY

Cannabis sativa plants with a THC content of up to 1%, according to Czech law, were grown in a closed environment under LED lights with a controlled climate, such as temperature humidity, for three consecutive growing cycles. Each cycle had the same conditions, including light intensity and a nutritional plan that was divided into a growth phase and a flowering phase. The growth phase lasted four weeks, and the flowering phase lasted eight weeks. Twenty-four hours before the harvest, plants were divided into two groups, one of which was treated with methyl jasmonate at a concentration of 44.9mg.L-1 and a second group that was not treated at all. The plants were then dried for seven days, after which samples were taken and sent to the commercial laboratory in Austria to determine the cannabinoid content. The analyses revealed exciting results between the groups when the first treatment with MeJa decreased CBD by 35% and THC by 30% but increased the content of CBG by 66%. 2nd treatment with MeJa increased CBD by 21% and THC by 20% but decreased the content of CBG by 22%, and the third treatment with MeJa increased CBD by 48%, THC by 58%, and CBG by 318%. However, these results were not statistically proven.

***Key words**: methyl jasmonate, cannabidiol, cannabigerol, tetrahydrocannabinol*

INTRODUCTION

Higher plants contain carbohydrates, lipids, and proteins in the form of amino acids, minerals, and, most importantly, low molecular weight compounds called secondary metabolites. Secondary metabolites are not necessary for plant growth and development. However, the plant will synthesize them due to the environment affecting the plant. The environment can stress the plant and trigger a reaction in the increased synthesis of the bioactive compounds, which are valuable in the pharmaceutical industry.

Stress in the plant induces an enhanced production of secondary metabolites, called elicitation. There are many forms of stress, both biotic and abiotic. Biotic stress is when a plant is exposed to living organisms, including fungi, bacteria, insects, etc.; abiotic stress is when a plant is exposed to drought, salinity, low or high temperature, or another environmental extreme (Siboza et al. 2014).

Using chemicals, for example, acetylsalicylic acid (Khan et al. 2012a, b, c), methyl jasmonate (Wasternack 2014; Danaee et al. 2015), jasmonic acid (Pirbalouti et al. 2014) or ethylene (Yoo et al. 2009) to increase the content of secondary metabolites has been shown as a more precise way than using drought, high or low temperatures, or another form of both biotic and abiotic stressors.

Trying to increase the content of secondary metabolites in the cannabis plant is meaningful mainly when cultivating hemp, which is generally characterized by low THC content, typically from 0.2% to 1%. Such hemp can be extracted, and many cosmetics products can be made, provided they do not contain THC.

As cannabinoids are prohibited in food according to Regulation (EU) 2015/2283 on novel foods, and THC is prohibited in cosmetics according to Regulation (EC) 1223/2009, this gives cannabis manufacturers few options for working with cannabis extracts.

However, even these limitations allow manufacturers to market cosmetic products to support the treatment of certain diseases. This is where elicitation can be applied, as it is possible to obtain extracts with an exciting composition of cannabinoids from which THC is removed. It is much better to use a mix of cannabinoids due to the so-called entourage effect, which is a synergistic phenomenon in which multiple cannabis compounds interact with each other to modulate therapeutic action (Simei et al. 2023).

It was also shown that cannabinoids in combination, for example, CBD and CBG, have a higher anti-inflammatory effect than CBD or CBG used alone (Cabrera et al. 2021).

The most crucial factor for plant cultivation is light. In closed systems, using artificial light growers has many possibilities, for example, metal halide light (MH), high-pressure sodium lights (HPS), plasma lights, or light-emitting diodes (LED). LEDs are becoming increasingly popular when growing all kinds of plants due to their high efficacy and low electrical cost compared to HPS lights. Plants need light for their photosynthesis, and because photosynthetic spectra are known today (Hoover, 1937; McCree, 1972; Inada, 1976), using LEDs can adjust the light spectrum as desired (Bugbee, 2016).

Effects of light spectrum on plant morphology or yields are today well known (Dougher and Bugbee, 2001; Barnes and Bugbee, 1992).

Many growers, however, cannot change the light's spectrum, so another essential factor - PPFD (photosynthetic photon flux density) is used. It was proven by Chandra et al. (2008) that the highest photosynthetic efficiency in cannabis is observed in less than 1 500 PPFD, which is usually expressed in $\mu mol/m^2/s$. However, the effects of PPFD are not linear, meaning the effect of light intensity becomes less significant when a PPFD of 1 500 $\mu mol/m^2/s$ of photons is reached (Chandra et al. 2008).

Another essential factor for cannabis cultivation is nutrition. Cannabis nutrition is often misunderstood by commercial growers who overfertilize the plants, mainly in the flowering stage, with massive amounts of phosphorus, believing it will increase yield (Westmoreland and Bugbee, 2022).

The research was done on phosphorus uptake (Shiponi and Berntein 2021; Bernstein et al. 2019; Cockson et al. 2020; Veazie et al. 2021), but so far, it was not proven that high concentrations of phosphorus (over 30mg) have any effect on cannabis yield.

Last but not least, one of the cardinal factors is the environment, temperature, and humidity. Temperature can be a limiting factor for net photosynthesis (Pn). Low temperatures slow Pn, while high temperature stops Pn (Chandra et al. 2011). It is needless to say that temperature and net photosynthesis are cultivar/strain-specific (Chandra et al. 2011).

Humidity is another factor many commercial growers need to learn more about. The term VPD (vapor pressure deficit) is becoming increasingly popular. VPD combines the relative humidity and air temperature and describes the difference between the actual maximum amounts of water the air can hold for a given temperature (Jin et al. 2019).

MATERIAL AND METHODS

Cannabis cuttings were made in a Czech commercial cannabis facility, where they were grown. For this research, several dozen cannabis cuttings from the cultivar Peach Goliath were made. It took them seven days to root when the plants lacked nutrition. After rooting, 48 plants were sown into 11L pots with coco coir and perlite in ratio 60:40. Photoperiod was set for 18/6h for four weeks. For light supply, Sunpro Sundocan LED 900w was used. 48 plants were divided into four groups of 12, while 12 plants were under one Sundocan, which meant 12 plants per $1m^2$.

The power of each Sundocan light was set to 360 watts. The height of the Sundocan was adjusted to get a PPFD of 500 µmol/s/m2. After setting up the high of the light, each week, as plants grew, measurements were made to keep the same intensity. The average light intensity (PPFD) during the growth phase was 489 µmol/s/m2. The Apogee ePAR sensor MQ-610: 400 – 750nm ePAR Meter was used to measure the light intensity. To measure the light intensity, Sundocan was measured in each corner and the middle, and an average was made from those measurements.

Plants were watered daily during the first week when the amount of nutrient solution was 50mL per plant. In the second week, plants were watered daily when the amount of nutrient solution was 100mL per plant. Plants were also topped, removing the top of the plant on the main stem. In the third week, plants were watered every second day with a nutrient solution of 200mL per plant. In the fourth week, the last week in the growth phase, the plant was watered three times a week when the amount of nutrient solution was 300mL per plant.

The electric conductivity of the nutrient solution during the growth phase did not change and was 1.6 mS/cm when pH was between 6.1 and 6.3.

The average temperature during the growth phase was 23.4 °C when the lights were on and 20.6 °C when the lights were off.

The average humidity during the growth phase was 78.7% when the lights were on and 54.3% when the lights were off.

Table 1. Composition of macronutrients in nutrient solution (in 50mL) for the first week. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
12.25mg	0.4mg	1.84mg	13.53mg	6.74mg	3.56mg	1.26mg

Table 2. Composition of macronutrients in nutrient solution (in 100mL) for the second week. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
24.50mg	0.8mg	3.70mg	27.05mg	13.50mg	7.13mg	2.43mg

Table 3. Composition of macronutrients in nutrient solution (in 200mL) for the third week. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
49mg	1.57mg	7.36mg	54.19mg	26.96mg	21.38mg	7.3mg

Table 4. Composition of macronutrients in nutrient solution (in 300mL) for the fourth week. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
73.50mg	2.40mg	11.04mg	81.12mg	40.44mg	21.38mg	7.30mg

On the first day of the fifth week, the photoperiod was changed to 12/12h. The power of each Sundocan light was set to 900 watts. The height of the Sundocan was adjusted to get a PPFD of 1200 µmol/s/m2. After setting up the high of the light, for the first three weeks, as plants were still growing, measurements were made to keep the same intensity. Plants were then put into nets to ensure proper growth without bending. Light intensity was then measured once in two weeks. The average light intensity (PPFD) during the flowering phase was 1229 µmol/s/m2.

Plants were watered three times a week when the nutrient solution was changed. For the first three weeks of the flowering phase, the amount of nutrient solution was 500mL. During the fourth and fifth weeks of the flowering phase, the amount of nutrient solutions was 700mL. In the fifth week, plants were pruned from the bottom to 1/3 of their height, where all branches and leaves were removed. In the sixth and seventh weeks of flowering, the amount of nutrient solution was 900mL; in the last week, the eighth week of flowering, only water was used.

The electric conductivity of the nutrient solution during the flowering phase did not change and was 2.4 mS/cm when pH was between 6.0 and 6.2.

The average temperature during the flowering phase was 26.1 °C when the lights were on and 22.3 °C when the lights were off.

The average humidity during the flowering phase was 58.5% when the lights were on and 48.4% when the lights were off.

Table 5. Composition of macronutrients in nutrient solution (in 500mL) for week 1 to 3. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
73mg	4.36mg	85.96mg	152.55mg	74.63mg	35.52mg	51.2mg

Table 6. Composition of macronutrients in nutrient solution (in 700mL) for weeks 4 to 5. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
102.86mg	6.11mg	120.39mg	213.58mg	104.49mg	53.92mg	71.68mg

Table 7. Composition of macronutrients in nutrient solution (in 900mL) for week 6 to 7. Nutrients were calculated in their pure form, not in the form of oxides.

$N - NO_3^-$	$N - NH_4^+$	$P - PO_4^{-2}$	K^+	Ca^{2+}	Mg^{2+}	$S - SO_4^{-2}$
132.5mg	7.86mg	154.79mg	274.6mg	134.34mg	69.33mg	92.16mg

Twenty-four hours before harvest, a methyl jasmonate (Sigma-Aldrich) solution was prepared in concentration 44,9 mg.L-1, and half of the plant was treated.

When harvesting, plants were divided into treated and non-treated groups. Only the flowers from plants were removed and put into the Sunflower Trimmer, where the flowers were cleaned from leaves. After this process, plants were transferred into a drying room, where they were dried for seven days. Temperature was set at 23.5 °C for the whole drying process, and humidity was set at 55%. Temperature and humidity while drying was set the same for cycle two and three. After seven days of drying, samples were taken from treated and non-treated groups and were sent for laboratory testing.

After harvest, the growing process was repeated two more times. Changes in temperature, humidity, and light intensity are shown in Table 8. Sage genetic material was used, meaning cuttings from the same mother plant.

Table 8. Changes in environmental conditions between cycles

	Growth phase				
	Temperature		Humidity		Light intensity
	Day	Night	Day	Night	
Cycle 1	23.4°C	20.6°C	78.7%	54.3%	489 µmol/s/m2
Cycle 2	23.8°C	20.9°C	76.3%	52.1%	524 µmol/s/m2
Cycle 3	23.1°C	20.3°C	77.1%	52.9%	513 µmol/s/m2
	Flowering phase				
	Temperature		**Humidity**		**Light intensity**
	Day	Night	Day	Night	
Cycle 1	26.1°C	22.3°C	58.5%	48.4%	1 229 µmol/s/m2
Cycle 2	26.8°C	22.6°C	61.2%	50.3%	1 189 µmol/s/m2
Cycle 3	26.4°C	22.7°C	57.8%	51.9%	1 218 µmol/s/m2

Treatment of methyl jasmonate was used to ideally increase the content of either CBD or CBG or both.

The STATISTICA program (version 13.2, StatSoft, Inc., California, USA) was used to analyze measured data. Tukey's honest significant difference (HSD) was used to identify significantly different mean values, $P < 0.05$, on the probability level. The results were not significant, which is why we did not present them in the study. A box plot tool identifying mean and standard deviation was used, too.

RESULTS AND DISCUSSION

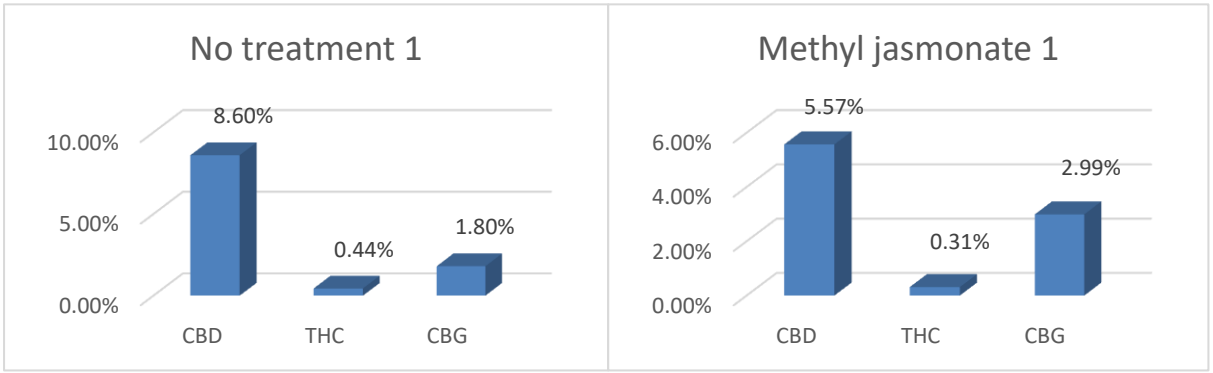

Figure 1. Content of cannabinoids in not treated group vs treated group, cycle 1

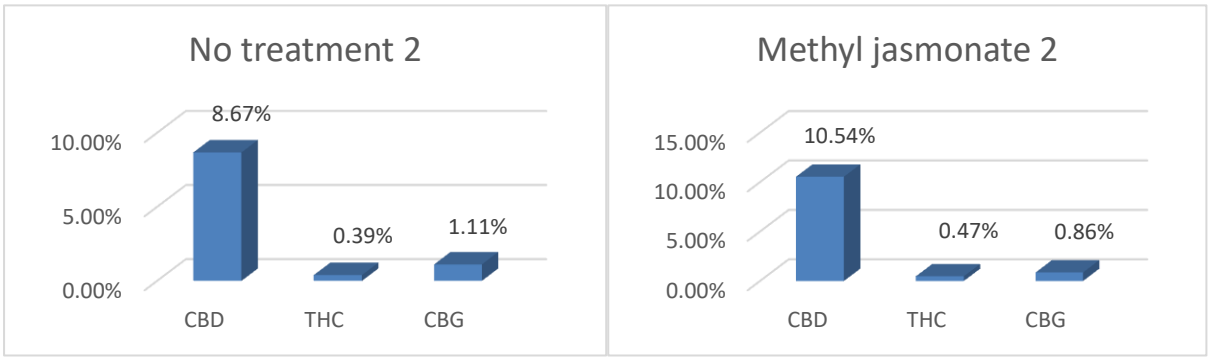

Figure 2. Content of cannabinoids in not treated group vs treated group, cycle 2

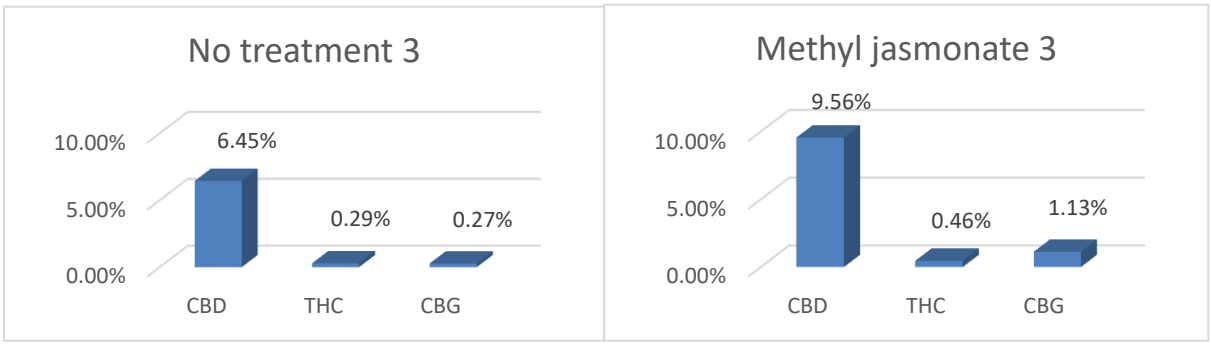

Figure 3. Content of cannabinoids in not treated group vs treated group, cycle 3

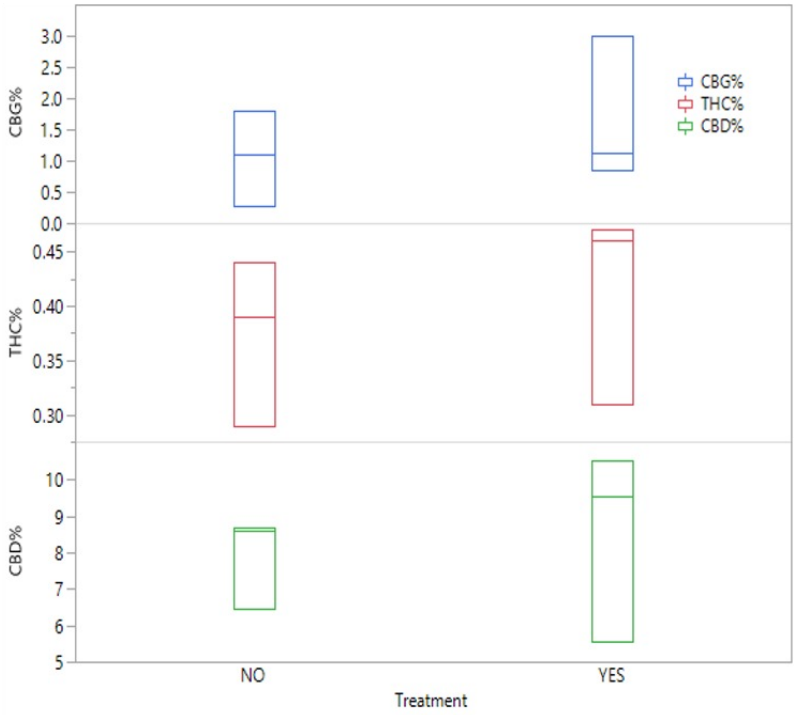

Figure 4. Effect of treatment (mean ± standard errors)

Although Figures 1, 2, 3, and 4 show a change in the cannabinoid content, the standard deviation fluctuates. Based on the Tukey HSD test the results are statistically not significant and do not correspond with the research of Khan et al. (2012a, b, c), Wasternack (2014), Danaee et al. (2015), Pirbalouti et al. (2014) and Bailey (2019), where all those researchers were able to prove the effects of elicitations on secondary substances in various plant, cannabis sativa included.

CONCLUSION

Affecting the composition of cannabinoids using elicitation is complicated, and each cannabis genetic may respond differently. While increasing nonpsychoactive cannabinoids in the plant might be attractive for some hemp growers, elicitation should be used mainly for research purposes to try to understand better how cannabinoids are synthesized in the cannabis plant.

Elicitation might serve in the research of cannabinoids in the future; however, there is a need for much more extensive research on this topic.

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INFLUENCE OF *AZOTOBACTER* SPP. FROM ACIDIC SOIL ON GERMINATION AND INITIAL GROWTH OF DIFFERENT AGRICULTURAL CROPS

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In the world, especially in the European Union, more and more space is being given to agriculture based on the principles of sustainable development. This kind of agriculture implies the absence or minimization of the application of all agrotechnical measures that negatively affect the environment, the reduction or complete absence of the use of chemical fertilizers and pesticides, as well as the increase in the use of effective biopreparations, which include microbiological preparations. The aim of this research was the isolation and physiological characterization of Azotobacter spp. from acidic soil and the examination of the effect of their application on the germination and initial growth of wheat (Triticum sp. L.), corn (Zea mays L.) and oilseed rape (Brassica napus L.). Isolation of Azotobacter isolates from acidic soil, determination of their biochemical properties, evaluation of isolates influences on seed germination and on the parameters of initial growth of wheat, corn and rapeseed seeds, were done. The results of this study reveal that Azotobacter strains isolated from acidic soil have multiple biochemical and plant growth-promoting (PGP) properties. The application of Azb 3, Azb5 and Azb10 isolates had the best effect on the germination of all three seeds. On average, the application of Azb 3 and Azb10 isolates had the best effect on the stem and root length of all three plants.

***Key words**: PGPR, inoculation, wheat, corn, oilseed rape*

INTRODUCTION

Today, agriculture faces a double challenge: to reduce the application of agrotechnical measures that negatively affect ecological systems and the quality of life, and to adapt to the expected consequences of climate change, primarily intense rains and drought. Precisely for these reasons, in the world, especially in the European Union, more and more space is being given to agriculture based on the principles of sustainable development. This kind of agriculture implies the absence or minimization of all agrotechnical measures that negatively affect the environment, the reduction or complete absence of the use of chemical fertilizers and pesticides, as well as the increase in the use of effective biopreparations, which include microbiological preparations (Nivetha et al., 2021). Microbiological preparations contain effective strains of microorganisms, which positively affect the growth and health of the plant, as well as its yield. These microorganisms are known as PGP microorganisms (Plant Growth Promoting) (Kumar et al., 2022).

The mechanisms by which PGPR promote plant growth are not fully understood, but it is believed that the PGPR enhance plant growth and yield either by direct or indirect mechanisms. The direct growth-promoting mechanisms are: the ability of microorganisms to produce phytohormones like indoleacetic acid, gibberellin, cytokinins and ethylene, N2 fixation, antagonism against phytopathogenic microorganisms by the production of siderophores, and also solubilization of mineral phosphates and other nutrients (Basu et al., 2021). The indirect mechanisms are: the extracellular production of antibiotics, synthesis of antifungal metabolites, production of fungal cell wall lysine enzymes, competition for sites on roots and induced systemic resistance (Meena et al., 2020).

Different bacteria that have been reported as PGPR belong to the following genera: *Pseudomonas, Bacillus, Azospirillum, Agrobacterium, Azotobacter, Arthrobacter, Serratia, Rhizobium, Frankia, Beijerinckia, Burkholderia, Klebsiella, Streptomyces, Staphylococus, Xanthomonas, Trichoderma, Fusarium, Penicillium, Aspergillus, Glomus, Gigaspora, Scutellospora, Acaulospora,* etc. The most studied bacteria and exploited as biocontrol agents include the species of *Azotobacter* genus (Glick, 2012).

Azotobacter is found in neutral to weakly alkaline soils, in aquatic environments, in the rhizosphere of plants and in the phyllosphere. According to the research of Stamenov (2014), the number and activity of *Azotobacter* depend primarily on the pH reaction. Bacteria of the genus *Azotobacter* belong to the group of free nitrogen fixers. Esmailpour et al. (2013) determined that *Azotobacter* sp. can fix up to 20 kg N/ha per year and that it can be successfully used in the production of various agricultural crops. Many results have shown that *Azotobacter* spp. produce growth hormones, such as gibberellins, auxins and cytokinins (Remus et al., 2000), as well as siderophores (Hajnal Jafari et al., 2020). According to Prajapati et al. (2008), bacteria of the *Azotobacter* genus have a significant impact on increasing soil fertility, through the biosynthesis of biologically active substances, phytopathogenic inhibitors, hormones, as well as the degradation of pesticides and heavy metals.

The application of *Azotobacter* sp. gave good results in the production of various plants. In the production of agricultural and vegetable plants using multiple inoculants, i.e. a mixture of *Azotobacter* spp. and phosphomineralizers, the supply of nitrogen and phosphorus as well as the yield of plants increased (Hamidi et al., 2022). In the work of Song et al. (2021) inoculation with *A. chroococcum* improved nutrient uptake by the plant and led to an increase in corn (*Zea mays* L.) yield. Similar to this research, Abdiev et al. (2019) found that the co-inoculation of *Rhizobium* sp. and *Azotobacter* sp. led to an increase in the yield of chickpeas (*Cicer arietinum* L.). Inoculation of wheat (*Triticum aestivum* L.) with *Azotobacter* sp. led to an increase in yield even in conditions of high soil salinity (El-Nahrawy and Yassin, 2020).

The aim of this research was to isolate and characterize isolates of *Azotobacter* from acidic soil and monitor the effects of their application on the germination and initial growth of wheat (*Triticum* sp. L.), corn (*Zea mays* L.) and oilseed rape (*Brassica napus* L.).

MATERIAL AND METHODS

Isolation and biochemical characterization of the isolates

The *Azotobacter* bacteria were isolated from the soil having the following characteristics: 1,08% $CaCO_3$; 2,59% humus; 0,13% N; 3,17 mg P_2O_5 in 100g of soil; 14,78 mg K_2O in 100g of soil; pH in H_2O 6,89; pH in KCl 5,47.

N-free medium was used for *Azotobacter* sp. isolation (K2HPO4 0.3 g L-1, CaHPO4 0.2 g L-1, MgSO4 0.3 g L-1, NaCl 0.5 g L-1, FeCl3 0.1 g L-1, CaCO3 2.5 g L-1, solution of microelements 1 ml L-1, agar 16 g L-1). Morphology characterization and identification of the isolates were examined using light microscopic observation (Motic BA210).

Biochemical characterization of the selected isolates, such as growth at different temperatures (4, 10, 28, 37, and 45 °C), pH levels (5.5, 7 and 9), and salt concentrations (3, 7 and 10% NaCl), were examined using standard methods (Benson, 2002).

Evaluation of isolates for their PGP potential

Wheat (*Triticum* sp. L.), corn (*Zea mays* L.) and oilseed rape (*Brassica napus* L.) seeds, were used as plant material. Seeds used in experiments were taken from the collection of the Laboratory of Microbiology, Faculty of Agriculture, Novi Sad. The effect of selected isolates on seed germination and initial growth was examined in controlled conditions. Before inoculation, the seed sterilization was performed with 70% ethanol and 0.1% HgCl2 solution and rinsed with sterile water. Fifty seeds of wheat, twenty of corn and one hundred oilseed rape seeds were placed on sterile filter paper discs previously placed in Petri dishes. After that, 10 ml of inoculum, titer 10^9 CFU/ml, was introduced into each Petri box. The Petri dishes were then placed in a thermostat at 22ºC. After two days, the number of germinated seeds was determined. The stem and root length (mm) of germinated seeds were measured after 3, 5 and 7 days.

Statistical analysis

The data were statistically processed using the Statistics 13.3 software (TIBCO Software Inc.).

RESULTS AND DISCUSSION

Slimy, milky-white colonies, with Gram-negative, coccoid-shaped with a mucous capsule were selected as *Azotobacter* isolates. In this study, 12 isolates of the genus *Azotobacter* were isolated from the soil sample and were denoted by Azb1 to Azb12.

The physiological characterization of the isolates (the influence of temperature, concentration of hydrogen ions and NaCl on the growth of isolates) is presented in Table1.

Table 1. Physiological characterization of isolates

	Temperature (°C)*					NaCl (%)			pH		
	4	10	28	37	45	3	7	10	5.5	7	9
Azb1	-	-	++	++	+	-	-	-	+	++	++
Azb2	+	-	++	++	+	-	-	-	+	++	++
Azb3	-	+	++	++	+	-	-	-	+	++	+++
Azb4	-	+	++	+	+	-	-	-	+	++	+
Azb5	-	-	++	++	+	-	-	-	+	++	++
Azb6	-	-	++	++	+	-	-	-	+	++	+
Azb7	+	+	++	++	+	-	-	-	+	++	+++
Azb8	-	-	++	+	-	-	-	-	++	++	+
Azb9	-	-	++	+	-	-	-	-	+	++	+
Azb10	-	-	++	+++	-	-	-	-	++	++	+++
Azb11	-	-	++	-	-	-	-	-	+	++	+
Azb12	-	-	++	-	-	-	-	-	+	++	+

* - absence of growth; + minimal growth; ++ optimal growth; +++ intense growth;

Azotobacter isolates had optimal growth at 28°C, while at 4 and 45°C minimal growth or complete absence of growth were recorded. Most of the isolates had optimal growth at 37°C, but only Azb10 had intense growth at this temperature. These results are in accordance with the results of Saribay (2003), whose investigation showed that *Azotobacter* species are sensitive to temperature.

The results of this study showed that *Azotobacter* isolates could not tolerate salinity. In contrast to our study, in the work of Aasfar et al. (2021), *Azotobacter salinestris* could grow in 8% NaCl solution, and some species even at higher concentrations (10%).

In the present study, all isolates could grow at different pH. The optimum pH for the vast majority of isolates was 7 and 9. The isolate Azb10 tolerated a wide range of pH, indicating its ability to survive in the extreme conditions of the soil. Minimal growth of most isolates was determined at a pH 5.5, except for isolates Azb8 and Azb10. These isolates had optimal growth. Three isolates (Azb3, Azb7, Azb10) showed intense growth at pH 9. This is in accordance with the research of many authors who claim that *Azotobacter* species are very sensitive to low pH values and that their presence in acidic soil is very weak, and often cannot even be determined (Miličić, 2009). Becking (2006) claims that the optimal pH of soil for the growth of *Azotobacter* species is 7. According to Aquilanti et al. (2004) for a more intensive growth of *Azotobacter* sp., a neutral environment is the most favorable.

Depending on physiological properties, to examine their effect on seed germination, root and stem length of seedlings, 6 representative isolates: Azb1, Azb2, Azb3, Azb5, and Azb10 were chosen.

In this study, the application of the isolates enhanced the number of germinated seeds (Table 2).

Table 2. The influence of isolates on the germination of wheat, corn and oilseed rape seeds

Isolate	Wheat		Corn		Oilseed rape	
	No.	%	No.	%	No.	%
Azb1	49b	98	12b	60	94a	94
Azb2	50a	100	11b	30	92a	92
Azb3	50a	100	16a	80	94a	94
Azb5	50a	100	18a	90	100a	100
Azb7	50a	100	17a	85	98a	98
Azb10	50a	100	18a	90	98a	98
Control	47ab	94	5c	25	69b	69

Note. Values in the same row followed by different letters indicate significant differences ($p < 0.05$) between the means.

After 2 days, all isolates enhanced the number of germinated seeds. The highest germination percentage of wheat seeds (100%) was obtained in the variant with the Azb2, 3, 5, 7 and 10 isolates, while the smallest number was determined in the control variant (94%). This increase was not statistically significant. A statistically significant increase in germination of maize seeds in all variants was observed. The highest germination percentage (90%) was determined in the variants with isolates Azb5 and Azb10, while in the control variant, the percentage was the lowest (25%). The introduction of *Azotobacter* isolates led to a statistically significant increase in germination of oilseed rape seeds. The best result was achieved using the Azb5 isolate (100%), and the weakest using the Azb2 isolate (92%). Similar to these results, the positive effect of bacterial inoculation on the germination of medicinal plants was determined by Lenin and Jayanthi (2012). Also, Khaosaad et al. (2006) found a positive effect of PGP bacteria on germination, stem length and plant biomass of oregano.

The effect of isolates application (Azb1, Azb2, Azb3, Azb5, Azb7, Azb10) on the initial growth of wheat, corn and oilseed rape seed are presented in Tables 3, 4 and 5.

Application of all isolates had a positive effect on the stem and root length of wheat germinated seeds during all three measurements (Table 3). The best effect was achieved by using isolates Azb3, Azb5 and Azb10. Compared with the control variant, in these variants there was a statistically significant increase in the determined parameters after all three measurements. The weakest effect was achieved using the isolate Azb1.

Table 3. The influence of isolates on the stem and root length (mm) of wheat (*Triticum* sp.L.) seed

Isolates	3 days		5 days		7 days	
	Stem	Root	Stem	Root	Stem	Root

	length	length	length	length	length	length
Azb1	2,5c	8,8d	20,7c	33,2bcd	33,7c	34,2c
Azb2	4,7c	16,5c	19,0c	27,3cd	51,2b	30,2c
Azb3	18,7	23,0b	41,3b	46,0ab	43,5b	57,7a
Azb5	16,6ab	19,8bc	45,8ab	50,2a	51,2ab	52,2ab
Azb7	12,2b	19,0a	47,5ab	40,3abc	51,5ab	43,7bc
Azb10	20,5a	28,3a	51,5a	42,8ab	56,5a	52,3ab
Control	2,2c	8,2d	19,0c	24,2d	34,2c	33,5c

Note. Values in the same row followed by different letters indicate significant differences ($p < 0.05$) between the means.

The best effect of the isolates application on the initial growth of corn seeds was achieved with isolates Azb3, Azb5 and Azb10 (Table 4). In these variants, during all three measurements, a statistically significant increase in the stem and root length of the germinated corn seeds was determined.

Table 4. The influence of isolates on the stem and root length (mm) of corn (*Zea mais* L.) seed

Isolates	3 days		5 days		7 days	
	Stem length	Root length	Stem length	Root length	Stem length	Root length
Azb1	2,5c	8,8d	20,7c	33,2bcd	33,7c	34,2c
Azb2	4,7c	16,5c	19,0c	27,3cd	51,2b	30,2c
Azb3	18,6a	23,0b	41,3b	46,0ab	43,5b	57,7a
Azb5	16,7ab	19,8bc	45,8ab	50,2a	51,2ab	52,2ab
Azb7	12,2b	19,0a	47,5ab	40,3abc	51,5ab	43,7bc
Azb10	20,5a	28,3a	51,5a	42,8ab	56,5a	52,3ab
Control	2,2c	8,2d	19,0c	24,2d	34,2c	33,5c

Note. Values in the same row followed by different letters indicate significant differences ($p < 0.05$) between the means.

It was observed that all applied isolates positively affected the initial growth of oilseed rape (Table 5). On average, the best results were achieved with Azb3, Azb5 and Azb10 isolates. Application of Azb1, Azb2 and Azb7 isolates led to an increase in stem and root length, but the measured increases were not always statistically significant.

Table 5. The influence of isolates on the stem and root length (mm) of oilseed rape (*Brassica napus* L.) seed

Isolates	3 days		5 days		7 days	
	Stem length	Root length	Stem length	Root length	Stem length	Root length
Azb1	2,5c	8,8d	20,7c	33,2bcd	33,7c	34,2c
Azb2	4,7c	16,5c	19,0c	27,3cd	51,2b	30,2c
Azb3	18,7a	23,0b	41,3b	46,0ab	43,5b	57,7a
Azb5	16,7ab	19,8bc	45,8ab	50,2a	51,2ab	52,2ab
Azb7	12,2b	19,0a	47,5ab	40,3abc	51,5ab	43,7bc
Azb10	20,5a	28,3a	51,5a	42,8ab	56,5a	52,3ab
Control	2,2c	8,2d	19,0c	24,2d	34,2c	33,5c

Note. Values in the same row followed by different letters indicate significant differences ($p < 0.05$) between the means.

The results obtained in this research are in line with the results of other researchers. The positive effect of the application of microorganisms on the length of roots and stems of germinated seeds and on the initial growth was determined in the work of Stamenov et al. (2021). In this study, a positive effect of the application of *Azotobacter*, *Pseudomonas* and *Bacillus* isolates on the length of roots and stems of thyme was recorded. Also, Wu et al. (2005) reported that the use of PGP bacteria (*Azotobacter chroococcum, Bacillus megaterium, Bacillus mucilaginous*) resulted in higher biomass and seedling length, as well as higher assimilation of nutrients (N, P and K) in corn plants.

CONCLUSION

The optimal growth of the majority of *Azotobacter* isolates was recorded at 28 and 37°C and at pH 7 and 9. There was a complete absence of colony growth in the condition of high NaCl concentration.

Inoculation of wheat, corn and oilseed rape seeds with selected isolates had a positive effect on seed germination. The application of Azb 3, Azb5 and Azb10 isolates had the best effect on the germination of all three seeds.

All tested isolates had a positive effect on the stem and root length. On average, the application of Azb3 and Azb10 isolates had the best effect on the stem and root length of all three plants.

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THE INFLUENCE OF A PARACHUTE ON UNMANNED AERIAL VEHICLES FLIGHT PERFORMANCE

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Unmanned Aerial Vehicles (UAVs), commonly known as drones, have become significant in various research areas. However, the question of their safety is of paramount importance. The aim of this research is to analyze the characteristics of UAVs, specifically their battery and flight speed, to determine whether the use of a parachute affects UAVs functionality. The results have shown that the parachute does not significantly impact UAV flight speed or battery consumption. This study emphasizes the importance of further research on the use of parachutes with UAVs for various applications in different conditions.

***Key words**: Unmanned Aerial Vehicles (UAVs), parachute, flight performance*

INTRODUCTION

Unmanned Aerial Vehicles (UAVs), commonly referred to as drones, are aircraft capable of autonomous flight or remote operation from the ground, without the need for an onboard human pilot (Barmpounakis et al., 2016). Today, UAVs have become significant in various industries, but they face various challenges, particularly in terms of the safety of their flights and landings. Therefore, the importance of employing parachutes alongside UAVs is on the rise.

Currently, UAVs serve various purposes, including soil erosion monitoring (D'Oleire-Oltmanns et al., 2012), remote sensing (Whitehead and Hugenholtz, 2014), surveying and photogrammetry (Lopez-Rodriguez and Esteban, 2013), smart agriculture (Honkavaara et al., 2013), etc. Accident statistics during UAV operations indicate a significantly higher number of accidents compared to piloted aircraft (Wild et al., 2016). This imposes significant limitations on UAV applications, as they can encounter various issues during flight, both internal (e.g., electrical circuit failure, disrupted connection, or mechanical damage) and external (disturbances caused by external obstacles such as birds or hostile forces). Due to these problems, UAVs may crash and land on hard surfaces. In such cases, both the UAV and its equipment can be damaged, as well as objects (e.g., people or valuable property), leading to substantial financial losses, including equipment and data loss. Consequently, the use of parachutes in combination with UAVs becomes a crucial strategy for addressing safety issues. Parachutes enable controlled and precise UAVs landings, even when carrying a

certain payload. Using parachutes minimizes the risk of damage due to falls or unpredictable weather conditions.

The aim of this research is to conduct experimental tests to determine whether the use of a parachute affects the flight performance of the UAV, as well as the battery consumption during the planned flight.

MATERIAL AND METHODS

In the context of this research, a UAV equipped with a parachute, was employed as a crucial component of the study (Fig. 1). The UAV was chosen for its specific design and ability to effectively respond to research objectives. The research was carried out using a UAV, a DJI Phantom 4, which was equipped with a standard integrated RGB sensor in the first flight and an added ParaZero parachute in the second flight, with a weight of 160 g. The specifications of the UAV used, including its dimensions, weight and other relevant characteristics are shown in Table 1.

Fig. 1. UAV with a parachute
(Source, authors, 2023)

Table 1. Summary of UAV specifications
(Source, authors, 2023)

Type	Specifications
Weight (battery and propeller included)	1388g
Diagonal size (propellers excluded)	350 mm
Max ascent speed	5 m/s
Max speed	13.9 km/h
Battery	6000 mAh LiPo 25
Max flight time	Approx. 30 minutes

Study area is situated within Novi Sad, encompassing a portion of the campus, a section of the 'Djačko igralište' area, and is located at coordinates 45°14'37.6"N 19°51'11.6"E, covering approximately 1.3 hectares, which represents the football pitch (Fig. 2).

Fig. 2. Area of Experimental Research
(Source, authors, 2023)

The flight plan is defined using the DroneDeploy software package. The flight altitude was 60 m with 6 longitudinal flight strips, and the expected flight time is 5 min and 10 sec. Both flights were made according to the identical flight plan. To exclude the influence of weather conditions on flight performance, both flights were conducted in the same weather conditions over a short time span. Additionally, the DroneDeploy software automatically generated a flight report, capturing various flight parameters, including flight duration, current speed, maximum speed, aircraft condition, battery consumption, current battery condition, etc. Subsequently, flight reports from both flights were retrieved and an analysis of the parachute's influence on UAVs flight performance was conducted.The detailed procedure is shown in Figure 3.

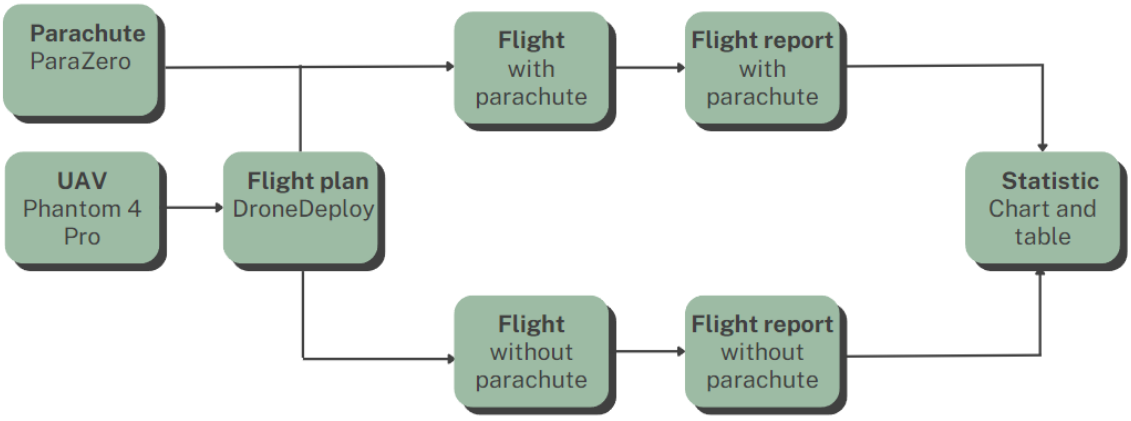

Fig. 3. Flowchart for "The Impact of a Parachute on UAVs Flight Performance"
(Source, authors, 2023)

RESULTS AND DISCUSSION

The experimental research included the analysis of the speed of the UAV in two cases: a UAV with a parachute and without a parachute. Speeds are given in km hour (km) and flight times are given in minutes and seconds. The results obtained from the experimental analysis are shown in Fig.4.

The research results indicate that in both cases, the UAV maintained relatively similar speeds throughout the flight. These results suggest that the presence or absence of a parachute does not significantly affect the speed of the UAV after it has completed its planned flight, which may be useful to consider in practical applications with the aim of protecting the UAV from adverse effects.

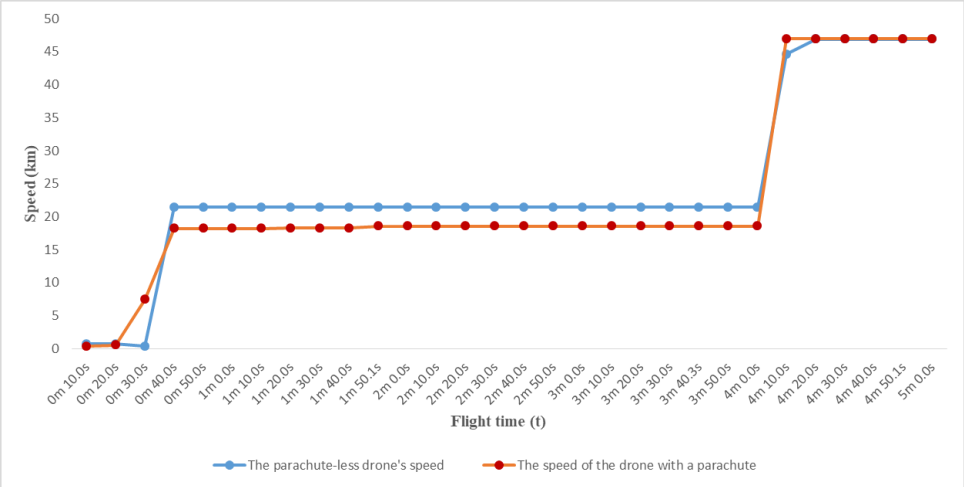

Fig.4. Graphical representation of UAV speed during flight with and without a parachute
(Source, authors, 2023)

In addition, experimental research was conducted with the aim of analyzing the battery condition of a UAV with a parachute and without a parachute (Fig. 5). The presence of a parachute did not appear to be a factor that would significantly affect the increase or decrease of the battery level during the flight. These results indicate that regardless of the presence or absence of a parachute, battery life is similar in both situations, which is beneficial in terms of UAV durability and performance.

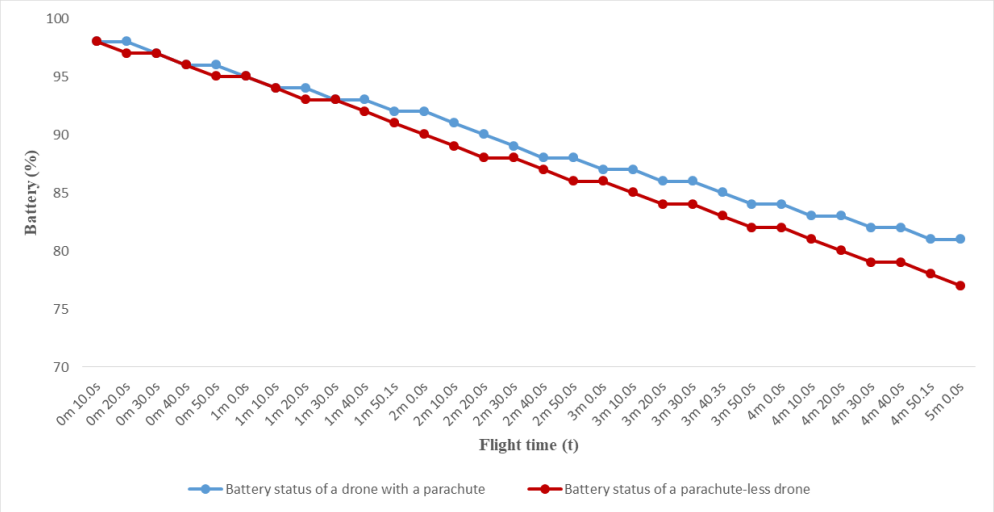

Fig. 5. Graphical representation of UAV battery status during flight with and without parachute

(Source, authors, 2023)

CONCLUSION

Concluding from the research conducted, it is evident that the presence of a parachute does not negatively affect the performance of the UAV, including specifications such as battery consumption and flight speed. The study confirmed that a UAV equipped with a parachute maintains its functionality and performance during flight, just like a UAV without a parachute.

This research has significant importance because it indicates the importance of using parachutes as an additional safety mechanism for UAVs. Parachutes can serve as a means of safely returning the UAV to the ground in case of unexpected problems during flight. This research further highlights the importance of further research and development in UAV protection to improve the safety of these devices. In the future, the application of parachutes on UAVs may become even more significant, which will contribute to the wide application of UAVs in many sectors and different weather conditions.

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INFLUENCE OF NUTRITION ON PRODUCTION QUALITY AND HYGIENIC CORRECTNESS OF COWS MILK ON HOLSTEIN-FRIESIAN DAIRY COWS IN MALESHEVO REGION NORTH MACEDONIA

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SUMMARY

Investigation was carried out in order to establish the influence of nutrition to expose of genetic potency of the Holstein-Friesian dairy cows on production, quality and hygienic correctness of cow's milk in Maleshevo region of North Macedonia. In 2022 year on the dairy cattle farm AD MALESHEVO from Berovo with capacity of 200 cows controlled 30 cows with average body weight 552.3 kg in different phases of lactation. The nutrition was compound with ration from corn silage, lucerne hay, meadow hay, brewery by-product (dry), sugar beet by-product (dry) and concentrate like a mixture. Production of milk in lactation period from 305 days is 8997.5 kg standard milk with average daily content of milk 29.50 kg with the following chemical composition of milk: average percent of milk fat is 3.99%; protein - 3.11%; lactose - 4.04% non fat dry matter - 8.39% and total dry matter - 12.38%.Total number of somatic cells in milk was 185.000/ml and the total number of microorganisms in milk 236.000/ml. Executed research of this work shown that the right or normed nutrition across creating and testing models of compartive rations who used in feeding of high milk yielding cows representing the current acknowledgments for feeding the ruminants are the key to expose of genetic potency of milk production on these cows, better chemical composition of milk (quality) and hygienic correctness. This fact looking for permanent following of cows nutrition, especially on high milk production, in all phases of production reprocycle, respective in practice necessary applying of programme of nutrition to prepare of specific of nutrition of each production periods.

Key words: *Holstein-Friesian dairy cows, nutrition, production of milk, chemical composition of milk, total number of somatic cells in milk, total number of microorganisms in milk.*

INTRODUCTION

Cattle breeding is important branch of the animal husbandry. In some European countries, like Denmark, Netherlands, Finland it contributes more than 50-60% of the agricultural production. It is same in our country where cattle products provide good results giving the best part of the milk (80%) and meat (50%) products, both as fresh or secondary

products. This, gathered with the fact that there are 296.634 cattle, 133.838 (45.12%) belonging to the Holstein-Friesian breed *(Official data from the Unit for Animal identification at the Ministry of agriculture, forestry and water management of the Republic of North Macedonia)*, makes it the major branch in the meat and milk production.

The milk is a basic cattle product consumed by the general population. It gives the highest financials to the cattle industry. Because it is a valuable product, with the add of selection, genetics and nutrition, there is an effort to create such breeds and types of dairy cattle that will give not just big amounts of milk but will improve the standard and health of the human population.

Milk production in R. North Macedonia is generally achieved by breeding Holstein-Friesian dairy cattle that have genes for high production that measure 8-10 tones of standard milk and one calf in a 365-day reproduction cycle. But, the big production can be achieved only by controlling the paragenetic factors (nutrition, management, animal welfare, animal health) that are specific and can differ quite from the ones used in our general practice.

One of the major paragenetic factors in milk production is the nutrition. Without adequate nutrition considering all of the ingredients needed by the cows, the high milk production is not possible. Although a lot has been done on this issues through the years, the science can only give the numbers in part on what a successful ration is about *(Grubic and Adamovic, 2003)*. We consider that the key to economical production of milk is creating and testing different models of rations for dairy cattle, with the help of the modern science of ruminants nutrition.

The purpose of this paper is to stress the importance of the nutrition in expressing the genetic potential in Holstein-Friesian dairy cattle in conditions found in Maleshevo region of North Macedonia.

MATERIAL AND METHODS

The research has been done on a commercial dairy cattle farm AD „MALESHEVO" from Berovo, Maleshevo region, where cows have been fed with rations made of corn silage, lucerne hay, meadow hay, brewery by-product (dry), sugar beet by-product (dry) and concentrate like a mixture. All the forage and mixture samples were analysed according to *AOAC (1980)* analysis by the method of Weende.

The results of the chemical composition of milk (% of milk fat, % of proteins, % lactose, % of defatted dry matter and % of total dry matter), the total number of somatic cells and the total number of microorganisms in milk were analyzed *(Regulations for the storage of raw milk - Article 9 and Article 13, Official Gazette of the RM No. 151/2011)*.

Milk samples were taken in sterile plastic bottles, in the amount of 50 ml per sample. The samples were taken from the collected morning milk.

The chemical composition of milk was determined using a Foss Milkoscan 4000 apparatus (according to the Instructions for the Operation of Infrared Spectrophotometry Instruments), manufactured by Foss Electric from Denmark. When determining the chemical composition of cows milk, the content of: milk fat, proteins, lactose, defatted dry matter and total dry matter in milk was determined.

The total number of somatic cells was determined using a Foss Fossomatic 5000 apparatus, somatic cell enumeration (According to the Operating Instructions with Fluoro-Opto-Electronic Counters), manufactured by Foss Electric of Denmark.

The microbiological composition of the milk was determined using an apparatus, Bentley Bactocount IBC 50 – (Quantitative determination of the bacteriological quality of raw milk.

All analyzes were performed in the PROANALYZ Laboratory - Strumica, North Macedonia. The purpose of these tests was to determine the quality, that is, the hygienic correctness of the collected milk obtained from the dairy cattle farm AD MALESHEVO from Berovo, Maleshevo region, based on that, to propose measures for its improvement.

RESULTS AND DISCUSSION

The ration composition for nutrition of high-productive dairy cattle that were object in this research is given in Table 1.

Tab.1. Composition of ration for nutrition of high productive cows on the cattle farm AD MALESHEVO-Berovo

	Daily	***DM***	***NEL-total***	***Total protein***	***Digestable protein***	***Undigestablep rotein***	***Crude fibre***	**ADF**	**NDF**
	kg.	**kg.**	**MJ**	**g.**	**%/g.**	**%/g.**	**g.**	**%/g.**	**%/g.**
Rouhage feedstuffs									
Corn silage	13	4.16	23.40	130.21	65.7/85.55	25.3/32.94	467.58	10/46.76	17.2/80.42
Brewery by-product (dry)	1.5	1.32	10.04	331.06	5.2/17.22	57.5/190.3	193.38	4.9/9.48	11/21.26
Lucerne hay	4	3.60	17.32	354.96	48.8/173.2	33.3/118.2	1405.1	30.1/422.9	40.4/567.6
Meadow hay	4	3.56	18.80	239.94	20/47.99	56/134.36	1428	38.3/546.9	62.7/895.3
Sugar beet pulp by-product (dry)	1.5	1.27	10.14	119.60	5.2/6.22	61.6/73.7	319.38	32/102.2	41.1/131
Total rouhage feedstuffs	**24**	**13.91**	**79.70**	**1175.77**	**330.18**	**549.56**	**3813.44**	**1128.24**	**1695.58**
Concentrate	**9**	**7.83**	**68.31**	**1202.4**	**155.7**	**616.5**	**454.5**	**61.11**	**120.51**
Total (rouhage feedstuffs+ concentrate)	**33**	**21.74**	**148.01**	**2378.17**	**485.88**	**1166.06**	**4267.94**	**1189.4**	**1816.1**

The results of the average content of milk produced on the cattle farm AD MALESHEVO - Berovo is given in Table 2.

Tab. 2 Average content of milk produced on the cattle farm AD MALESHEVO - Berovo, kg

Groups	**n**	Measures of variation				
		x	**Sx**	**Sd**	**Cv**	**Iv**
		Milk, kg				
I	15	28.00	0.43	2.33	8.33	24-34
II	15	31.00	0.93	5.09	16.43	22-40
Both groups-Maleshevo region	***30***	***29.50***	***0.54***	***4.21***	***14.27***	***22-40***

The results presented in Table 2 shows that the average milk production was 28.00 kg in group I, 31.00 kg in group 2, and the average milk production for both groups was 29.50 kg while the standard milk production for 305 days of lactation was 8997,5 kg.

Our results on the total lactation in the period of 305 days are in the limits of 7420-8894 kg given by *Sretenovic Ljiljana et al., (2007)*, but are above 5795-7190 kg and 7290 kg given by *Palasevski et al., (1995)* and *Shokarovski et al., (2001)* respectevelly, and much better than 5849 kg in the first lactation of Holstein-Friesian cows in the Pelagonia region given by *Kitanovski et al., (1998)* and *Trajkovski and Bunevski (1999)*.

It can be noted that the average milk production of the cows that were tested in our conditions related to that of some European countries *(Arend, 1999)* like Netherlands, Sweden, Italy where for the lactation period of 305 days of the tested black and white cows it is 8000 kg (8003, 8504, 8134 kg respectively) and Germany, Finland and UK where it measures more than 7000 kg (7438, 7496, 7109 respectively), is in the limits of the genetic potential of the breed of the cows.

In table 3 presents the results of the chemical composition of the milk in the farm tested.

Tab. 3 Chemical composition of milk on farm AD MALESHEVO - Berovo, %

Groups	**n**	Measures of variation				
		x	**Sx**	**Sd**	**Cv**	**Iv**
Milk fat, %						
I	15	3.91	0.10	0.53	13.47	2.32-4.74
II	15	4.07	0.09	0.47	11.59	2.81-5.20
Both groups-Maleshevo region	**30**	***3.99***	*0.06*	*0.50*	*12.59*	*2.32-5.20*
Proteins, %						
I	15	3.09	0.03	0.19	6.04	2.34-3.33
II	15	3.14	0.02	0.11	3.41	2.84-3.31
Both groups-Maleshevo region	**30**	***3.11***	*0.02*	*0.15*	*4.91*	*2.34-3.33*
Lactose, %						
I	15	4.01	0.04	0.23	5.84	3.07-4.31
II	15	4.07	0.02	0.14	3.42	3.71-4.31
Both groups-Maleshevo region	**30**	***4.04***	*0.02*	*0.19*	*4.78*	*3.07-4.31*
Non fat dry matter, %						
I	15	8.32	0.09	0.49	5.92	6.35-8.99
II	15	8.46	0.05	0.28	3.34	7.58-8.88
Both groups-Maleshevo region	**30**	***8.39***	*0.05*	*0.40*	*4.82*	*6.35-8.99*

Total dry matter, %						
I	15	12.23	0.18	0.96	7.88	8.67-13.73
II	15	12.53	0.12	0.63	5.06	10.39-13.52
Both groups-Maleshevo region	**30**	***12.38***	*0.11*	*0.82*	*6.64*	*8.67-13.73*

According to the results shown in the Table 3, the average chemical content of the milk is 3.99% fats, 3.11% protein, 4.04% lactose, 8.39% non-fat dry matter and 12.38% total dry matter.

Our results for the average content of fat, protein and lactose in the milk are in the limits of those reported by *Djordevic et al., (2005)* where the fat content in the milk is 3.34-3.81% depending on the diet and those of *Gutic et al., (2001)* where the milk protein is 3.56%. Our results are measurably better than those of *Bobos et al., (2001)* where the control group of cows gave milk with average milk fat of 3.47%, 3.20% of protein, 8.27% of non-fat dry matter and 11.47% of total dry matter, while the tested group that were given the preparation Sel-Plex TM in the ration yielded better results of 3.59%, 3.38%, 9.04% and 12.61%, respectively. Our results were also better than those of *Adamovic et al., (2004)*, where the control group gave milk with average fat content of 3.29%, 2.90% of protein, 11.62% of dry matter, while the tested group that were given buffer (mineral mixture of magnesium oxide, sodium bicarbonate, bentonite and organic zeolite) in the ration yielded better results of 3.58%, 3.03% and 11.99% respectively.

Sretenovic Liljana et al., (2007) in their research on the use of yeasts in combination with probiotics and enzymes in the diet of the dairy cow and its effect on the milk production gave results of 3.91% fat, 3.05% protein, 4.91% lactose and 11.65% of dry matter in the control group of cows, and 4.19%, 3.11%, 5.16% and 11.72% respectively in the tested group. Those results are similar to ours.

In Table 4 shows the results of the average total number of somatic cells in the milk obtained at the examined farm AD MALESHEVO-Berovo, Maleshevo region.

Tab. 4 Average amount of total number of somatic cells (x1000/ml) in the milk obtained from the tested farm AD MALESHEVO-Berovo, Maleshevo region

Groups	**n**	Measures of variation				
		x	**Sx**	**Sd**	**Cv**	**Iv**
		Total number of somatic cells (x1000/ml) in milk				
I	15	180.00	15.26	59.08	32.82	104-328
II	15	190.00	16.03	62.06	32.66	137-337
Both groups-Maleshevo region	**30**	***185.00***	***15.64***	***60.57***	***32.73***	***104-337***

From the presented table 4, it can be seen that the average content of total number of somatic cells in the milk ranges from 180.000/ml in the I group to 190.000/ml in the II group

or the average for the both groups - Maleshevo region average content of total number of somatic cells in cow's milk is 185.000/ml.

Our found results in relation to the total number of somatic cells in the cow's milk at the examined farm AD MALESHEVO-Berovo, as well as the average for the Malesevo region (185.000/ml) compared to those in the *Regulation on the storage of raw milk - article 13 (Official Gazette of RM No. 151/2011)* belongs to the EXTRA CLASS ≤ 400.000/ml, which means that the cow's milk in the Maleshevo region is of excellent quality.

In Table 5 shows the results of the average content of total number of microorganisms in the milk obtained at the examined farm AD MALESHEVO-Berovo, Maleshevo region.

Tab. 5 Average amount of total number of microorganisms (x1000/ml) in the milk obtained from the tested farm AD MALESHEVO-Berovo, Maleshevo region

Groups	**n**	**Measures of variations**				
		x	**Sx**	**Sd**	**Cv**	**Iv**
		Total number of microorganisms (x1000/ml) in milk				
I	15	202.00	15.07	58.31	28.86	101-275
II	15	270.00	22.24	86.08	31.88	126-380
Both groups-Maleshevo region	**30**	***236.00***	***18.65***	***72.19***	***30.37***	***101-380***

From the presented table 5, it can be seen that the average content of total number of microorganisms in the milk ranges from 202.000/ml in the I group to 270.000/ml in the II group or the average for the both groups - Maleshevo region average content of total number of microorganisms in cow's milk is 236.000/ml.

According to the results of microbiological analyzes in relation to the total number of microorganisms in the milk at the investigated farm AD MALESHEVO-Berovo, as well as the average for the Maleshevo region (236.000/ml), compared with those in the *Regulation for the storage of raw milk - article 13 (Official Gazette of RM No. 151/2011)* belongs to CLASS I from 100.001/ml – 700.000/ml, which means that for cow's milk from the Maleshevo region, greater hygiene and hygiene measures should be proposed before, during and after milking as well as proper storage and cooling the milk, which would achieve the extra class and the milk would be of excellent quality.

CONCLUSION

The results from the research done on Holstein-Friesian dairy cows in conditions found in the Maleshevo region, North Macedonia focused on the effect of the nutrition as a factor for expressing their genetic potential led us to the conclusion that:

- ✓ The milk production in the lactation period of 305 days with the use of a ration (corn silage, lucerne hay, meadow hay, brewery by-product (dry), sugar beet by-product (dry) and concentrate mixture) in the cattle dairy farm AD MALESHEVO-Berovo, Maleshevo region was 8997.5 kg standard milk and average daily milk production of 29.50 kg;
- ✓ The milk quality had the following chemical content: average percentage of milk fat of 3.99%, 3.11% protein, 4.04% lactose, 8.39% non-fat dry matter and 12.38% total dry matter.
- ✓ The hygienic correctness of milk had the following content: total number of somatic cells is 185.000/ml and total number of microorganisms is 236.000/ml.
- ✓ As a general conclusion from these tests, we can highlight the fact that the cow's milk obtained in the Maleshevo region in terms of the two investigated parameters (chemical composition and content of somatic cells) fulfills the stipulated criteria for EXTRA class and is of excellent quality, while in terms of the total number of microorganisms belongs to the I CLASS of quality and it is necessary to take a set of hygiene measures in order to improve its microbiological composition.

Our research done on the Holstein-Friesian breed of dairy cows in the North Macedonia establish the genetic potential of milk production in this breed, and the fact that the breed has been totally adapted, acclimatized and accommodated in the conditions found in Maleshevo region, North Macedonia, so the farmers should learn how to use its genetic and production potential solely through correct nutrition.

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REGULATION ON THE STORAGE OF RAW MILK (OFFICIAL GAZETE OF RM No. 151/2011).

EDIBLE DIPLOTAXIS FLOWERS AS SOURCE OF BIOACTIVE COMPOUNDS- HOW SOLVENT SYSTEM INFLUENCE ON PHYTOCHEMICALS YIELD AND ANTIOXIDANT ACTIVITY

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Among decorative petals of some plant species, lies a hidden wide array of bioactive components, representing a potential source of health-promoting compounds. Predominantly known as a group of vegetables with great economic importance, the Brassicaceae family stands out with an abundant number of species whose flowers are edible. One such species is Diplotaxis tenuifolia (L.) DC., a versatile plant, commonly grown for its deeply lobed leaves which are widely used in gastronomy. However, there is limited available data regarding the phytochemical profile of its edible flowers. The aim of this study was to compare two different extraction solvents (80% acetone and 80% methanol) with regard to the phytochemical yield and antioxidant activity of D. tenuifolia flowers. Furthermore, using spectrophotometric methods, the analyses of total phenolic content (TPC), total flavonoid content (TFC), and total hydroxycinnamic acid derivatives content (HCAs) were performed, in addition to determining the total content of pigments (chlorophyll a, chlorophyll b, and carotenoids) from acetone extract. In terms of the total content of pigments, chlorophyll b was the most abundant (27.83 µg/g of fresh weight (FW)), while chlorophyll a exhibited the lowest concentration (9.34 µg/g FW). The content of TPC was higher in the acetone extract (8.29 mg GAE /g FW), whereas the yield of TFC was greater in the methanolic extract (2.00 mg QE /g). As for the total hydroxycinnamic acid derivative content (HCAs) the applied solvents did not statistically significantly influence the yield. Nevertheless, the antioxidant properties of both extracts were assessed through four distinct assays: DPPH$^{\bullet}$ quenching assay, ferric reducing power (FRP), in vitro phosphomolybdenum total antioxidant capacity (TAC), and cupric reducing antioxidant capacity (CUPRAC). Phytochemical analyses revealed differences in the quencher assay results when the two solvents were used. The methanol extract exhibited higher DPPH$^{\bullet}$ activity at 10.17 µmol Trolox/g FW, as confirmed by statistical analysis, which demonstrated significant variations among the solvents used. Similarly, regarding the TAC results, methanol extract yielded higher values (6.61 mg AAE /g FW) as opposed to the acetone extract (4.85 mg AAE /g FW), although the statistical analysis indicated a lack of statistically significant differences among the solvents. Moreover, the results of FRP and CUPRAC assays were contrasted. In the FRP analysis, acetone extracts exhibited superior antioxidant activity (5.07 mg AAE/g FW) compared to the methanol extract (4.24 mg AAE/g), whereas in the CUPRAC assay, methanol extracts displayed a

higher level at 29.19 mg AAE/g FW indicating possible differences in extracted bioactive compounds. The values exhibited statistically significant differences among the solvents used in each assay. The obtained findings reveal that wild rocket flowers can serve as a valuable source of bioactive compounds with health-promoting properties, and the choice of solvent influences their yield, which is significant for possible pharmaceutical and/or food applications.

***Keywords**: antioxidant activity; bioactive compounds; Diplotaxis tenuifolia; edible flowers; extraction solvents*

INTRODUCTION

Flowers have long been cherished for their beauty and symbolic meaning. Their ornamental value enriches our everyday lives, enhancing the ambiance of gardens, green spaces, and balconies. Among the decorative petals of certain plant species lies a true treasure trove of diverse bioactive compounds with well-documented health-promoting properties. The consumption of edible flowers has ancient origins spanning centuries and various cultures worldwide (Fernandes et al., 2020). Whether fresh or dried, these plant parts served myriad purposes, from garnishing dishes to being combined with ingredients such as honey, sugar, and butter to create unique culinary delights. They also imparted their aromas and vibrant colors to drinks and alcoholic beverages, while also finding use as remedies (Rop et al., 2012; Takahashi et al., 2020). Nowadays, the market for edible flowers is expanding, driven by a growing number of consumers seeking healthier dietary approaches. Still, it is important to note that not all flowers are edible as some may be toxic to humans.

Predominantly known as a group of vegetables with great economic importance, the Brassicaceae family is distinguished by a plethora of species whose flowers are edible. These include cabbage (*Brassica oleracea* L. *var. capitata)*, broccoli (*B. oleracea* L. *var. italica* Plenck), cauliflower (*B. oleracea* L. *var. botrytis*), mustard (*Sinapis alba* L.), artichoke (*Cynara scolymus* L.), arugula (*Eruca sativa* Mill.), *Diplotaxis* spp., etc. (Falleh et al., 2013; Raza et al., 2020; Shantamma et al., 2021; Takahashi et al., 2020). Nonetheless, the flower composition is a distinctive trait shared by all members of this family (Nikolov, 2019). Four sepals encircle an equal number of petals, arranged in a cross-like shape when viewed from above, which explains the previously used family name- the Cruciferae. The varying lengths of filaments divide the six stamens into two groups – two shorter ones and four longer ones, positioned closer to the pistil. In addition, the gynoecium is syncarpous, composed of two fused carpels leading to a divided ovary.

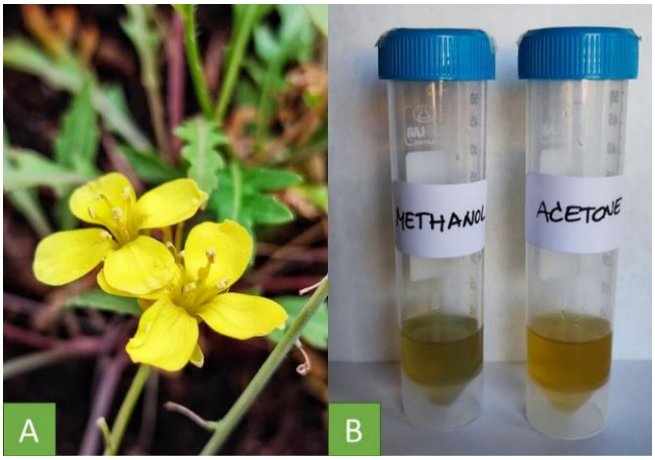

Figure 1-Diplotaxis tenuifolia *(L.) DC. flowers (A); Different solvent extracts of flowers (B);*

Diplotaxis tenuifolia (L.) DC. is a versatile plant, commonly grown for its deeply lobed leaves with peppery taste (Figure 1). In recent years, wild rocket has gained attention due to its application in gastronomy, usually as a salad mixture or garnish. However, despite the growing interest in this perennial plant, there is limited available data regarding the phytochemical constituents of its edible flowers. Moreover, a comprehensive analysis of wall rocket flower antioxidant properties and bioactive compound content, including pigments (chlorophylls and carotenoids), and phenolics (both simple acids and flavonoids), could provide insight into its potential impact on enhancing overall human well-being due to its cardioprotective, antioxidant, anti-inflammatory, antitumor, and antibacterial properties (Ballard and Maróstica, 2019; Kolašinac et al., 2021).

The aim of this study was to compare two different extraction solvents (80% acetone and 80% methanol) regarding the phytochemical yield and antioxidant activity of *D. tenuifolia* flowers. Furthermore, using spectrophotometric methods, the analyses of total phenolic content (TPC), total flavonoid content (TFC), and total hydroxycinnamic acid derivative content (HCAs) were performed, in addition to the total content of pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) from acetone extract. Nevertheless, the antioxidant properties of both extracts were assessed through four distinct assays: $DPPH^{\bullet}$ quenching capacity, ferric reducing power (FRP), *in vitro* phosphomolybdenum total antioxidant capacity (TAC), and cupric reducing antioxidant capacity (CUPRAC). Finally, the impact of the solvent on the obtained results was subjected to statistical evaluation. The findings indicate that wild rocket flowers are a valuable source of bioactive compounds with health-promoting properties.

MATERIAL AND METHODS

D. tenuifolia plants were cultivated in polystyrene containers within the Faculty of Agriculture's greenhouse. The flowers were collected at full bloom in July 2023.

Upon harvest, the plant material was ground, and extraction was carried out in 80% methanol and 80% acetone as solvents (with plant material to solvent ratio of 1:10). The samples were placed in two plastic cuvettes (Figure 1), each saturated with the respective

solvent. They were then vigorously shaken and kept protected from the light for 3 hours at room temperature. After extraction, the resulting extracts were filtered through filter papers, and the cuvettes containing the supernatant were stored in a refrigerator at a temperature of 4°C until further analyses were conducted.

The determination of TPC, TFC, and HCAs, as well as the assessment of antioxidant properties ($DPPH^{\bullet}$, FRP, TAC, CUPRAC) for both extracts, were conducted in triplicate, following the procedures outlined by Kilibarda et al. (2022). The quantification of pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids) in the 80% acetone solvent was performed following the procedure documented in the work of Gordanić et al. (2022). All results were expressed as fresh weight (FW) of the plant material.

Statistical analysis was performed using *R Studio 4.3.1* software, employing analysis of variance (ANOVA) and Tukey's post-hoc test to determine significant differences (considered statistically significant at $p < 0.05$.).

RESULTS AND DISCUSSION

Natural pigments serve as sustainable and healthier alternatives to synthetic colorants and find applications in both the food and textile industries (Mohammad Azmin et al., 2022). Furthermore, the literature highlights their bioactive properties such as antioxidant, anticancer, antimutagenic, anti-inflammatory, neuroprotective, eye-protective, etc. (Eggersdorfer and Wyss, 2018; Martins et al., 2023) Table 1 presents the content of selected phytochemical compounds found in the flowers of *D. tenuifolia*, including the overall pigment content obtained using 80% acetone as the solvent. Regarding the latter, chlorophyll *b* was the most abundant (27.83 μg/g of fresh weight (FW)), while chlorophyll *a* exhibited the lowest amount (9.34 μg/g FW). Notably, the color of the wild rocket flower petals reflects the dominant presence of chlorophyll *b*, attributed to the pigment's natural yellow color and carotenoids (Pareek et al., 2018). Moreover, Petrova et al. (2016) reported the content of chlorophyll *b* in 95% ethanol extracts of widely consumed *Tagetes erecta* L. flowers (7.6 μg/g FW), indicating lower values compared to wild rocket flowers. Notably, the comparison might have been influenced by the solvent used. The total carotenoid content in *D.tenuifolia* was 11,98 μg/g FW.

The selection of appropriate extraction solvents is crucial as they have a significant impact on the total phenolic content (including flavonoids), ultimately influencing the desired biological activity of the extracts (Dirar et al., 2019). Therefore, Table 1 displays the values of TPC and TFC in the examined *D. tenuifolia* flower extracts using 80% acetone and 80% methanol. The results of these two analyses are in contrast to each other, with the content of TPC being higher in acetone (8.29 mg GAE /g FW), while the yield of TFC was greater in the methanol extract (2.00 mg QE /g FW). This suggests differences in the phytochemical composition of the obtained extracts, likely due to varying polarity of applied systems. It is noteworthy that the values showed statistically significant differences between the solvents used in each assay. Li et al. (2014) reported the TPC of edible flowers from some Brassicaceae species using a methanol–acetic acid–water extraction system. The results for

Brassica campestris L. were 3.32 mg/g GAE (FW), whereas *Matthiola incana* (L.) W.T. Aiton displayed 1.70 mg/g GAE (FW), both lower than *D. tenuifolia* in both solvents. However, the differences might be attributed to the choice of solvents. Falleh et al. (2013) investigated the total phenolic and total flavonoid content of stem, leaves, and flowers of two *Diplotaxis* species (*D. harra* and *D. simplex),* with the flower organs demonstrating the highest values. The results indicate that the flowers of this genus species hold promising potential for both dietary and medicinal applications.

Table 2. General phytochemical composition of Diplotaxis tenuifolia flowers in different solvents

Extraction solvent	Chlorophyll *a* (µg/g FW*)	Chlorophyll *b* (µg/g FW)	**Carotenoids** (µg/g FW)	TPC (mg/g GAE FW)	TFC (mg/g QE FW)	HCAs (mg/g CGAE FW)
80% Acetone	9.34 ± 0.23	27.83 ± 0.61	11.98 ± 0.23	8.29 ± 0.17^{a,**}	1.14 ± 0.03^{b}	1.18 ± 0.01^{a}
80% Methanol	/	/	/	7.24 ± 0.15^{b}	2.00 ± 0.05^{a}	1.27 ± 0.13^{a}

*FW-fresh weight; TPC—total phenolic content; TFC—total flavonoid content; HCA—total dyhydroxicinnamic acid derivative content; GAE—gallic acid equivalents; QE—quercetin equivalents; CGAE—chlorogenic acid equivalents; / -not analysed ** Different superscript letters (a and b) in a same column indicate significant differences at $p < 0.05$.

In addition to flavonoids, phenolic acids (including hydroxycinnamic acids) are the main group of phenolics (Kandylis, 2022). In terms of total hydroxycinnamic acid derivative content (HCAs) in *D. tenuifolia* flower extracts (Table 1), the choice of solvents did not statistically significantly influence the yield.

Table 3. Antioxidant properties of D. tenuifolia extracts

Extraction solvent	DPPH• * (µmol Trolox/g FW)	TAC (mg/g AAE FW)	FRP (mg/g AAE FW)	CUPRAC (mg/g AAE FW)
80% Acetone	8.59 ± 0.29^{b**}	4.85 ± 0.15^{a}	5.07 ± 0.08^{a}	22.58 ± 1.17^{b}
80% Methanol	10.17 ± 0.14^{a}	6.61 ± 1.14^{a}	4.24 ± 0.31^{b}	29.19 ± 2.70^{a}

*DPPH—2,2-diphenylpicrylhydrazyl radical; TAC—total antioxidant capacity determined via in vitro phosphomolybdenum assay; FRP—Ferric Reducing Power; CUPRAC—Cupric Reducing Antioxidant Capacity; Trolox- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; FW- fresh weight; AAE—ascorbic acid equivalents. ** Different superscript letters (a–f) in the same column indicate significant differences at $p < 0.05$.

Moreover, the antioxidant properties of wall rocket edible flowers using different solvents were also investigated (Table 2). This provides valuable data for pharmaceutical and

functional food applications of *D. tenuifolia* flowers. Conducted analyses revealed differences in the quencher assay results when the two solvents were used. The methanolic extract exhibited the highest antioxidant activity (10.17 μmol Trolox/g), confirmed by statistical analysis. It demonstrated significant variations among the different solvents used. Similarly, regarding the TAC results, methanol extract yielded higher values (6.61 mg /g AAE) compared to the acetone extract (4.85 mg /g AAE), However, the obtained results did not show statistically significant differences. Furthermore, the results of FRP and CUPRAC assays were conflicting. In the FRP analysis, acetone extracts exhibited superior antioxidant activity (5.07 mg/g AAE) compared to the methanol extract (4.24 mg/g AAE), whereas in the CUPRAC assay, methanol extracts displayed a higher level at 29.19 mg/g AAE. The values exhibited statistically significant differences among the solvents used in each assay. The observed differences in FRP/CUPRAC assays resulted from the fact that CUPRAC assay includes both hydrophilic and lipophilic redox substances while during FRP assay redox activity is expressed only by hydrophilic compounds (Kostić et al., 2023).

CONCLUSION

Natural pigments can provide sustainable alternatives to synthetic colorants along with exhibiting bioactive potential including antioxidative, anticancer, antimutagenic, anti-inflammatory, neuroprotective, and eye-protective properties. In this context, the presented results demonstrate the potential of wild rocket flowers. Moreover, this study highlights the influence of solvent choice during the extraction process on the quantity of obtained bioactive compounds. The flower organs of *D. tenuifolia* show promising levels of total phenolic content, flavonoids, and hydroxycinnamic acid derivatives, while their antioxidant activity varies depending on the solvent used. In summary, the results underscore the importance of solvent selection for both experimental and pharmaceutical applications. Furthermore, they provide insights into the potential use of wild rocket flowers as functional food ingredients.

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RP-HPLC METHOD FOR DETERMINATION OF CAFFEINE IN BEVERAGES

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SUMMARY

Caffeine is often added as a stimulant to various beverages in order to provide consumers with extra energy and mental alertness. Today, over 90 % of the population regularly uses caffeine through coffee, tea, soft drinks, energy drinks and other beverages. Due to the possible adverse effects on the consumers' health, it is important to determine and control the concentration of caffeine in beverages.

This study presents a developed and validated reversed-phase high-performance liquid chromatography (RP-HPLC) method with ultraviolet-diode array detection (UV-DAD) for determination of caffeine in beverages, such as refreshing carbonated and non-carbonated drinks, sports and energy drinks. Separation and quantitative determination ofthe analyte were carried out on the reversed-phase octyldecylsilane column as stationary phase, and methanol and diluted phosphoric acid as mobile phase, applying isocratic elution with the flow rate of 1 mL/min. The chromatographic process was followed at 210 nm, and under constant column temperature at 25 ºC. The developed method was validated by testing linearity, precision, accuracy, the limit of detection (LOD) and quantification (LOQ). All the validation parameters were within the acceptance range. The method was successfully applied to determine of caffeine in various beverages that were taken randomly from local markets.

Key words: *beverages, caffeine, RP-HPLC method.*

INTRODUCTION

Caffeine (Figure 1) is a white crystalline substance, with the molecular formula $C_8H_{10}N_4O_2$. It is a natural substance, an alkaloid of the methylxanthine family, found in the leaves, seeds or fruits of many plant species worldwide (Wanyika et al., 2010; Nour et al., 2010).

It is most common in beverages such as coffee (71 %), soft drinks (16 %) and tea (12 %). The range of caffeinated beverages has grown over the past decade with the introduction of functional beverages, including the energy drink category, as well as other beverages such as sports drinks, juices and caffeinated water. In addition to these beverages, caffeine is also

found in cocoa, chocolate, and various medications such as some pain relief formulations and dietary supplements (Heckman et al., 2010).

Numerous research reports show that high caffeine intake is associated with various clinical diseases such as coronary heart disease, myocardial infarction, cancer (urinary tract, kidney and pancreas), anxiety and fibrocystic breast disease, nervous system disorders, digestive system and the respiratory system. Also, caffeine increases heart rate, dilates blood vessels, and raises plasma free fatty acids and glucose levels (Heckman et al., 2010).

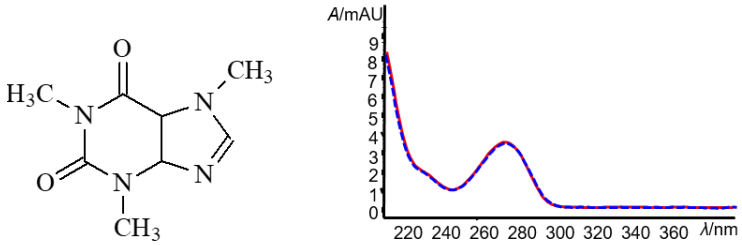

Figure 1. Chemical structure of caffeine (a) and its UV spectrum in phosphoric acid aqueous solution (pH = 3.8) and methanol (80/20, *V/V*) (b)

Considering the negative impact of caffeine on human health, their determination is not only important for product quality assurance, but also for consumer's health protection (Shaikh et al., 2018). Various analytical methods can be found in the literature for the determination of caffeine in food such as UV-Vis spectroscopy, electroanalytical methods, electrophoresis and chromatographic methods (Aşçı et al., 2016; Nour et al., 2010; de la Calle et al., 2018). Among these analytical methods, high performance liquid chromatography (HPLC - High Performance Liquid Chromatography) combined with UV - DAD detector is the most commonly used method due to its sensitivity, selectivity and high resolution (Martins et al., 2019; Aşçı et al., 2016).

Therefore, the aim of this paper was to develop a simple reversed-phase high-performance liquid chromatography (RP-HPLC) method for determination of caffeine in beverages. The developed method was successfully applied for the determination of caffeine in nine different beverages.

MATERIAL AND METHODS

The pure analytical standard of caffeine (99.9 ± 0.1 %) was purchased from CPAchem Ltd. (Bulgaria). The HPLC-grade chemicals acetonitrile, methanol and water, and phosphoric acid (85.35 %) employed for the preparation of the mobile phase were produced by Fisher Chemical, United Kingdom.

The chromatographic analyses were carried out using Agilent 1260 Infinity Rapid Resolution Liquid Chromatography system equipped with vacuum degasser, binary pump, autosampler, a thermostatted column compartment, ultraviolet-visible (UV-Vis) diode array detector (DAD), and ChemStation software. For optimization of the method for qualitative and quantitative determination of caffeine, the following analytical columns with same

stationary phase were used: Poroshell 120 EC - C18 (3.0 × 50 mm, 2.7 μm) and Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 μm).

Preparation of Standard and Sample Solutions. Stock solution was prepared by dissolving 0.01 g (with an accuracy of 0.0001 g) of the pure analytical standard of caffeine in water in a 10 mL volumetric flask. The solution was stored in a refrigerator at 4 °C. The stock solution was used to prepare a series of standard solutions with different concentrations of caffeine (10.00 - 100.00 mg/L), by taking an appropriate volume of stock solution (10, 25, 50, 75, and 100 μL) and transferring to 10 mL volumetric flasks, filled up to the mark with water.

Nine different beverages (ice tea, cola drink, energy drinks, sport drink, carbonated drinks, and fruit nectars) were randomly taken from local markets in N. Macedonia. The carbonated drinks were degassed in an ultrasonic bath (Elma Schmidbauer GmbH, Germany). Before HPLC analysis, all beverages were filtered with 0.22 μm nylon syringe filters (produced by ALWSCI Group, China). All samples were analysed in triplicate with injection volume of 2.5 μL.

The solution for recovery experiment was prepared by adding a known amount of caffeine (10 mg/L) in a volumetric flask of 10 mL filled to the mark with cola drink which contain the analyte. The spiked solution was analysed in triplicate (2.5 μL injections).

RESULTS AND DISCUSSION

Reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for determination of caffeine in beverages. The specificity, selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were evaluated for the method validation (Meyer, 2013).

The choice of wavelength on which the chromatographic analyses were performed was based on the UV spectrum of caffeine recorded in a mixture of phosphoric acid aqueous solution (pH = 3.8) and methanol (80/20, *V/V*). The UV spectrum of caffeine is presented in the Figure 1b. As can be seen from the UV spectrum of caffeine the absorption maximum band can be noticed at around 270 nm, but it is observed that the absorption increases with decreasing wavelength. Hence, the chromatographic analyses for determination of caffeine in the beverages were carried out at 210 nm. In addition, to confirm the specificity of the developed method, UV-diode array detection was used to check the peak purity and analyte peak identity. The purity index for caffeine was greater than 999 (the maximum value for the peak purity index (PPI) should be 1000), which means that the chromatographic peak was not affected by any other compound.

In order to determine the caffeine, two analytical columns: Poroshell 120 EC - C18 (3.0 × 50 mm, 2.7 μm) and Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7) were tested.

The first column was tested with mobile phase consisting of phosphate buffer (KH_2PO_4/H_3PO_4) with pH = 3.8 and acetonitrile in different volume ratio, and the best results were obtained using volume ratio of 15:85 (*V/V*) (Fig. 2a, c).

The retention time of caffeine in these conditions was short (up to 2 minutes), but the purity index was low, which means that other unknown components also appear simultaneously that interfere with the caffeine. The best results, with symmetrical peak shapes and good purity index, were achieved with the other column (Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 μm) and mobile phase consisted of phosphoric acid aqueous solution (pH = 3.8) / methanol (80/20, *V*/*V*), flow rate of 1 mL/min, and column temperature of 25 °C. The run time of analyses was 10 min. A successful separation of the analyte from other potentially present components in the samples was obtained under the stipulated chromatographic conditions. The mean retention time for caffeine was about 2.82 min (Fig. 2b, d). The chromatograms comparison of a caffeine standard solution (50 mg/L) and a caffeine in sample, obtained on the Poroshell 120 EC - C18 (3.0 × 50 mm, 2.7 μm) column with phosphate buffer (KH_2PO_4 / H_3PO_4) with pH = 3.8 and acetonitrile in volume ratio of 15:85 (*V/V*), with the other column (Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 μm) and mobile phase composed of phosphoric acid aqueous solution (pH = 3.8) and methanol (80/20, *V*/*V*) is presented in Figure 2.

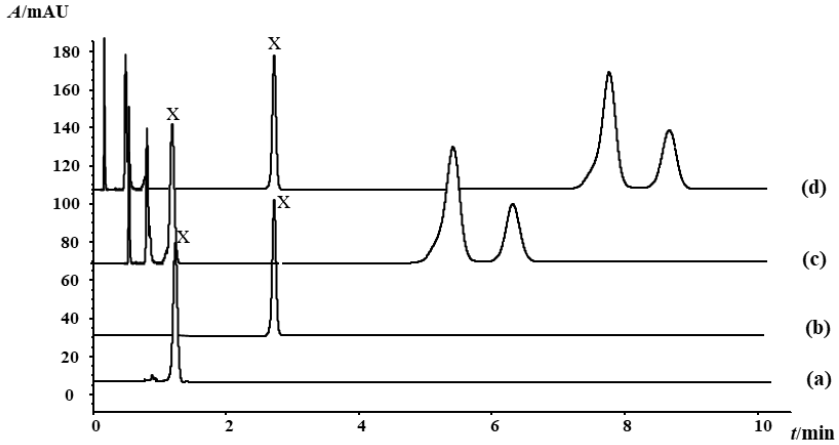

Figure 2. Chromatograms of a caffeine standard solution (a) and sample (c) obtained using Poroshell 120 EC - C18 (3.0 × 50 mm, 2.7 μm) column and a caffeine standard solution (b) and sample (d) obtained using Poroshell 120 EC-C18 (4.6 x 50 mm, 2.7 μm) column

*x – peak of caffeine

The linearity of the method was tested by constructing calibration curves that give the dependence of the peak area and the peak height on the injected amount of the analyte. For this purpose, a series of 5 working solutions of different concentration (Table 1) was prepared. As can be seen from this table, the values of the multiple correlation coefficients were $R^2 \geq 0.9998$, which means that the developed method was characterized by excellent linearity in the investigated concentration range. The obtained results for multiple correlation coefficients (R^2) indicated, preferably the use of peak area as a variable.

Table 1. Linearity range, limit of detection (LOD), limit of quantification (LOQ), regression equations, and correlation data of caffeine (210 nm)

Linearity range (mg/L)	**Regression equation**	R^2	**LOD (mg/L)**	**LOQ (mg/L)**
10 – 100.00	$y^A = 17.452x - 5.1025$	0.9999	0.03	0.1
	$y^H = 4.3416x + 14.854$	0.9998		

[A]Area. [H]Height.

LOD was determined as a signal to noise ratio 3:1, while LOQ value was calculated as 10 times the signal height to the baseline (S/N = 10). The determined values of LOD and LOQ for the investigated compound are given in Table 1.

Precision expressed as relative standard deviation (RSD, %) was obtained by 10 successive injections of analytical standard of caffeine with concentration of 50 mg/L. The intra-day (n = 10) precision was evaluated for the retention times, peak areas, and peak heights of caffeine. The computed values of RSD were the following: 0.11 % for retention time, 0.77 % for peak area and 0.93 % for peak height, indicated an excellent precision of the proposed method.

The accuracy of the method was confirmed by standard additions (Snyder et al., 2012). Accuracy of the method was expressed as recovery of the method and was calculated as the deviation between the calculated mean value obtained by examination and the true value of the spiked amounts of the analyte into a sample matrix that already contains some quantity of the analyte (Table 2). The obtained values for the recovery and RSD values less than 1% (Table 2) found suggested that the proposed method can be satisfactory used for determination of caffeine in beverages. Acceptance criteria for mean recovery and RSD are in accordance with guidelines for standard method performance requirements (Meyer, 2013).

Table 2. Results from recovery studies (n = 3)

Concentration of standard solution added (mg/L)	**Caffeine concentration determined in a blank sample (mg/L)**	**Caffeine concentration determined in a spiked sample (mg/L)**	**Recovery (%)**	**RSD (%)**
10	18.91	28.90	99.91	0.17

The developed method was applied to the analysis of investigated caffeine in 9 different beverages. The presence of caffeine was indicated on the labels of the analysed drinks. On the label of some beverages, caffeine was declared, but the amount was not given. All the samples were analysed without previous preparation, only by filtering throught syringe

filters, which contributes to develop a rapid, cheap, and extraction-free method for the determination with almost no analyte loss in the samples. The concentration of caffeine found in the analysed beverages is presented in Table 3.

Table 3. Concentration of caffeine in beverages

Beverage	**Concentration (mg/L)**	**On label**
I (Energy drink)	252.89	Declared
II (Cola drink)	94.59	Declared
III (Pear soda drink)	*NF	*ND
IV(Orange soda drink)	*NF	*ND
V (Lemon soda drink)	*NF	*ND
VI (Ice tea peach)	37.37	Declared
VII (Energy drink)	235.63	Declared
VIII (Peach juice)	*NF	*ND
IX (Sport drink)	2.34	Declared

*ND - not declared, and *NF - not found.

According to the obtained results (Table 3) five beverages contained caffeine. Four of the analysed beverages were declared without caffeine, and the obtained results confirmed that. In beverages number I and VII (Energy drink), caffeine has the highest concentration, but it is within the maximum allowed concentration (RULEBOOK ON ADDITIVES USED IN FOOD PRODUCTION, 2012). According to Directive 2002/67/EC (2002), beverages containing more than 150 mg/L of caffeine must provide a warning message on the label indicating the caffeine content, "High caffeine content (X mg/100 mL)".

CONCLUSION

Caffeine is the most commonly used stimulant in beverages. A fast, simple, low-cost, selective, and reliable RP-HPLC method for the quantitative determination of caffeine in beverages was developed and validated. Successful separation and quantification was achieved using the Poroshell EC 120-C18 (4.6 x 50 mm, 2.7 μm) analytical column and isocratic elution with mobile phase consisted of phosphoric acid aqueous solution (pH = 3.8) / methanol (80/20, *V/V*), flow rate of 1 mL/min, constant column temperature at 25 °C and UV detection at 210 nm. The results from the method validation revealed that the proposed method has an excellent linearity and precision of retention time, peak area and height. The obtained values for recovery (99.91 %) and RSD = 0.17 %, revealed that the proposed method is convenient for routine determination of caffeine in beverages. The run time of

analysis under the stipulated chromatographic conditions was about 10 min. The presented results show that HPLC is a powerful technique for the caffeine determination in beverages.

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