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SADRŽAJ / CONTENT

MULCHING EFFECTS ON YIELD AND QUALITY OF LAVANDIN
(*Lavandula intermedia*)*

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Original scientific paper

Summary

Lavandin (*Lavandula intermedia*) is a perenal aromatic plant from Lamiaceae family. Aromatic plants' qualitative and quantitative properties, including lavandin, depend on variety, environmental factors, and cultivation practices. This paper aimed to examine the impact of different mulch types on yield and bioactive compounds of lavandin. The treatments included different types of mulches (straw, alfalfa, agritela and control/without mulch). The field experiment had a split-plot design. Each plot contained ten replications. The tested traits were yield of inflorescences, contents of essential oil, contents of total phenols and total flavonoids, and antioxidant activity. The lowest amount of essential oil was recorded in the ″agritela″ treatment (5.0 mL/100g), and the highest in the ″straw″ treatment (6.4 mL/100g). The treatments with ″straw″ had the highest level of flavonoids contents (17.13 mg g^{-1} CAE), followed by the treatments with ″alfalfa″ (14.03 mg g^{-1} CAE), ″agritela″ (12.25 mg g^{-1} CAE), and ″control″ (10.32 mg g^{-1} CAE).

Key words: *lavandin, mulch, quality, essential oil, antioxidant capacity*

INTRODUCTION

Lavandin (*Lavandula intermedia*) is a perenal aromatic plant from Lamiaceae family. It is mostly cultivated for its essential oil, which is valued in the toiletry industry, agro-industry, agrifood sector, and herbal medicine (Wells *et al*., 2018; Komnenić *et al*., 2020). The quality of lavandin depends on the presence of various bioactive components and their content in the plant (Gavrić *et al*., 2024). Inherited genes regulate the type of bioactive compounds in aromatic plants, while environmental conditions and cultivation practices affect their content (Gavrić *et al*., 2023). The concentration of total phenols, flavonoids and essential oils in lavandin often serves as a reliable indicator of its quality (Zareen *et al*., 2014). During cultivation, various ecological factors such as fertility of the soil, soil and ait temperature, moisture, light and microorganisms can affect plants and their synthesis of phenolic compounds and essential oil (Sharma, 2019; Šamec *et*

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al., 2021). On the other hand, different agricultural techniques, such as tillage, fertilization, watering and mulching change the ecological conditions, directly affecting plant growth and bioactive compound synthesis (Gavrić *et al*., 2023). Mulching is an agricultural practice that is most commonly used in vegetable crops. Mulch primarily retains moisture in the soil and prevents the development of weeds (Šakonjić *et al*., 2023). Therefore, its application leads to changes in ecological conditions that can affect aromatic plants' qualitative and quantitative properties.

Considering all the above, the aim of this work was to determine the effects of mulching on yield, essential oil contents and antioxidant capacity of lavandin.

MATERIALS AND METHODS

Field Experiment. A field experimental site is located at Butmir, Bosnia and Herzegovina (B&H). The experiment was set up during the growing season of 2024. A lavandin cultivar "grosso" was used. The treatments included different mulches (straw, alfalfa, agritela and control/without mulch). The field experiment had a split-plot design. Each plot contained ten replications. The lavandin crop was one year old, that is, it was in its first year of cultivation.

Extraction and determination of essential oil content. The extraction of oils from lavandin inflorescence was carried out according to the previously described protocol by Clevenger (1928). In brief, 20 grams of dry materials were measured and transferred into a 250 mL flask containing 125 mL of distilled water. The samples underwent distillation for 120 minutes, after which the volume of essential oil was quantified.

Preparation of ethanol extracts for determination of bioactive compounds. To make the extract, 0.5 g of each pulverised sample was put into a 50 mL volumetric flask. The flask was then filled with 60% ethanol and mixed thoroughly. The extracts were later filtered and refrigerated until analysis.

Determination of bioactive compounds. The total phenolic content, standards and each ethanolic extract of lavandin were determined using the method by Gavrić *et al.* (2024). Results were expressed as mg GAE g^{-1}. Each ethanolic extract's total flavonoid content was assessed using a method previously documented by Gavrić et al. (2023). The results were expressed as mg CAE g^{-1}. The total antioxidant activity was determined using the FRAP method previously reported by Benzie and Strain (1996). The results were expressed as μM Fe^{2+} g^{-1} of dry matter.

Statistical analysis. All measures were performed ten times, and the findings were presented as the mean. Statistical analysis was conducted using SPSS software (IBM, Armonk, USA). An analysis of variance (ANOVA) accompanied by Tukey's multiple comparison tests was used or performed to compare the mean values of the results.

RESULTS AND DISCUSSION

Table 1 presents the impact of different types of mulch on fresh inflorescence yield, dry inflorescence yield, and essential oil content. The research results suggest two things. First, low yield was observed in all treatments. That is, the yield in this research is lower

compared to other research (Minev *et al*., 2022; Caccialupi *et al*., 2022). The reason for such a low yield is that the lavandin plantation was only one year old, in fact in its first year of cultivation. Second, mulch treatment significantly affected the investigated traits. The use "Alfalfa" mulch resulted in the highest inflorescence yields, measuring 27.7 g for fresh yield and 9.0 g for dry yield. This was followed by the "Straw" treatments at 19.3 g and 2.8 g, respectively, and the "Agritela" treatments at 19.2 g and 2.8 g. The control group exhibited the lowest yields, with 6.7 g fresh and 2.4 g dry yields. Observing results for the essential oil content, it can be said that there were some differences here as well. The lowest amount of essential oil was recorded in the ″agritela″ treatment (5.0 mL 100 g^{-1}), and the highest in the ″straw″ treatment (6.4 mL 100 g^{-1}). Our research indicated that mulch can influence the yield of inflorescences and essential oil content. Variations in these traits were likely due to changes in soil temperature resulting from mulch application. Namely, earlier research by Ning et al. (2021) found that soil with black mulch film is warmer than soil without mulch (the control). In contrast, Palada *et al*. (2000) noted that soil under straw mulch is cooler than soil without mulch (the control).

Table 1. Effect of mulch type on fresh inflorescence yield, dry inflorescence yield and essential oil content

Mulch type	Fresh inflorescence yield, g plant^{-1}	Dry inflorescence yield, g plant^{-1}	Essential oil contents, mL 100 g^{-1}
Agritela	19.2^{ab}	2.8^{ab}	5.0
Straw	19.3^{ab}	2.8^{ab}	6.4
Alfalfa	27.7^{a}	9.0^{a}	6.0
Control	6.7^{b}	2.4^{b}	6.3
Average	**18.2**	**4.2**	**5.9**

Different small letters (a and b) indicate significant differences between treatments at the 0.05 level

Different mulch types did not significantly affect phenolic content (Table 2), which ranged from 39.03 GAE g^{-1} (straw) to 46.50 GAE g^{-1}(control). However, mulch treatment significantly affected flavonoid content and antioxidant capacity. The treatments with ″straw″ had the highest level of flavonoids contents (17.13 mg g^{-1} CAE), followed by the treatments with ″alfalfa″ (14.03 mg g^{-1} CAE), ″agritela″ (12.25 mg g^{-1} CAE), and the ″control″ (10.32 mg g^{-1} CAE). A similar influence of mulch with differences was recorded with antioxidant capacity. The antioxidant capacity varied from 31.27 mM Fe^{2+} g^{-1} (alfalfa) to 37.71 mM Fe^{2+} g^{-1} (control). The findings were similar to the research of Šakonjić *et al*. (2023). They noted that mulching changes the content of bioactive compounds in medicinal plants. The same researchers state that different types of mulch change the growth conditions, which causes plants to react

differently to synthesize secondary metabolites. The content of flavonoids in our research is in line with Bajalan *et al.* (2016). The authors studied 30 different lavender cultivars and found that the flavonoid content was between 28.19 and 71.62 mg CAE g^{-1}. The authors highlight the importance of identifying lavender cultivars with high flavonoid content because of their beneficial therapeutic impact on human form and health.

Tabele 2. Effect of mulch type on total phenolic, flavonoid, and antioxidant capacity

Mulch type	Total phenols contents, mg GAE g^{-1}	Flavonoid contents, mg CAE g^{-1}	Antioxidant capacity, mM Fe^{2+} g^{-1}
Agritela	41.85[ns]	12.25[ab]	35.61[ab]
Straw	39.03[ns]	17.13[a]	35.36[ab]
Alfalfa	40.30[ns]	14.03[ab]	31.27[b]
Control	46.50[ns]	10.32[b]	37.71[a]
Average	**41.92**	**13.43**	**34.98**

Different small letters (a, b and c) indicate significant differences between treatments at the 0.05 level; GAE is the equivalent of gallic acid, and CAE is the equivalent of cathetin acid.

CONCLUSIONS

The experiments' results have demonstrated that mulching can affect lavandin's inflorescence yield and bioactive compounds. Alfalfa mulch had a significantly higher yield of fresh and dry inflorescence than other mulch types (straw, agritela, and control). Furthermore, the mulch used significantly affected the quality of lavandin. Given that the relatively low inflorescence yield was recorded, it is recommended to continue the research in the following years after the plant's development, that is, in the years when the plant fully matures.

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UTJECAJ MALČIRANJA NA PRINOS I KVALITET LAVANDINA (*Lavandula intermedia*)

Rezime

Lavandin (*Lavandula intermedia*) je aromatična biljka iz familije Lamiaceae. Prinos i kvalitet aromatičnog bilja, pa tako i lavandina, ovise o kultivaru, ekološkim faktorima i tehnologiji uzgoja. Cilj ovog rada bio je ispitati utjecaj različitih vrsta malča na prinos cvasti, sadržaj eteričnog ulja i antioksidativni kapacitet lavandina. Istraživanje je sadržavalo tri vrste malča i to: pšeničnu slamu, lucerku i agritelu, te kontrolnu varijantu (bez malča). Eksperiment je imao split-plot dizajn, a unutar svakog plota obavljeno je po deset mjerenja. U istraživanju su evidentirani prinos cvasti, sadržaj eteričnog ulja, ukupnih fenola, flavonoida i antioksidativni kapacitet. Najmanji sadržaj eteričnog ulja zabilježen je sa malčom od agritele (5,0 mL/100 g), a najveći sa slamom (6,4 mL/100 g). U tretmanu sa slamom evidentiran je najveći sadržaja flavonoida (17,13 mg g^{-1} CAE), a zatim su slijedili tretmani sa lucerkom (14,03 mg g^{-1} CAE), agritelom (12,25 mg g^{-1} CAE), te kontrola (10,32 mg g^{-1} CAE).

Ključne riječi: *lavandin, mulč, kvalitet, eterično ulje, antiokidativni kapacitet*

MORPHOLOGICAL AND PHYSIOLOGICAL RESPONSE OF SPINACH PLANTS TO WATER STRESS*

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Original scientific paper

Abstract

The effects of different water stress (control, low, moderate and severe) on some morphological and physiological characteristics of spinach plants were evaluated. The results showed that water stress strongly affects spinach plant growth and metabolism. The increasing water stress decreased the leaf area, petiole length and biomass accumulation, resulting in dramatically lower spinach yields. Photosynthetic pigment contents increased in response to low and moderate level water stress; however, under severe stress conditions their content started to decrease. The increase in water stress also resulted in a higher proline accumulation as well as total phenolic and flavonoid contents. These results lead to the conclusion that spinach plants could produce protective macromolecules and antioxidants in large quantities even under severe drought conditions, suggesting that spinach can be considered a drought-tolerant plant species from a survival point of view.

Keywords: *antioxidants, drought, plant growth, stress adaption*

INTRODUCTION

Drought is one of the major constraints to crop production at global scale. Stress caused by drought induces various morphology, biochemistry and physiology changes in plant, leading to considerable reductions in plant growth and productivity. This problem has been specially pronounced in last decades because of global climate changes and intensifying competition for water resources by industrial, agricultural and other users (Luo *et al.*, 2022).

To overcome the hazard effects of drought, plants have evolved different strategies through evolution, including drought avoidance and tolerance (Adel and Carels, 2023). Drought avoidance is based on constitutive and acquired traits that reduce water loss

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from plants, such as stomatal closure, reduction in the plant leaf area and increase the root-to-shoot mass ratio. On the other hand, drought tolerance refers to the ability of the plant to maintain cell turgor and scavenge harmful substances through cell osmotic regulation and synthesis of protective substances and antioxidants. The traits vary among plant species depending mainly on their genotype, age, growth stage as well as drought severity and duration (Seleiman *et al.*, 2021; Bandurska, 2022).

Spinach (*Spinacia oleracea* L.) is one of the important leafy vegetable crops worldwide. It is a rich source of iron, calcium, magnesium, potassium, carotenoids, folic acid and vitamins K, C, A, E and B-6. As part of a healthy diet, it strengthens the immune system, improves bone health and can help to protect against cardiovascular disease and obesity (El-Sayed, 2020).

Interestingly, very few studies have thus far been carried out on the drought impacts on spinach plants, although drought is the main factor restricting its growth (Kabay, 2023). Since the spinach production and consumption has rapidly increased in recent times (FAOSTAT, 2022), it is important to pay attention to this issue. Without a good understanding of the plant physiological and biochemical response to drought, farmers and scientists are unable to improve water use efficiency in plant production, especially for plants that need a lot of water to grow such as spinach (Nasarullah *et al*., 2022).

The aim of this study was to evaluate the morphological and physiological response of spinach plants exposed to different levels of drought stress. The following morphological and physiological parameters were measured: fresh mass of aerial parts, leaf area, petiole length, yield, photosynthetic pigment contents including chlorophyll *a* content, chlorophyll *b* content and total carotenoid content, proline content, osmotic potential, total phenolic and flavonoid contents and total antioxidant capacity. We hypothesized that spinach plants exposed to drought would exhibit decreases in yield and photosynthetic pigment contents. We also hypothesized that the exposure of spinach plants to drought promotes the accumulation of the protective macromolecules and antioxidants in spinach leaves.

MATERIALS AND METHODS

Plant material
Spinach (*Spinacia oleracea* L. Rembrandt F1), a popular spinach variety, was used as the plant material in this study. It has green to dark green, smooth and oblong in shape leaves, and is especially intended for winter production. Its vegetation period ranges from 45 to 60 days from sowing.

Experimental site
The present study was carried out from December 2023 to March 2024 in a double-span polyethylene-covered greenhouse with natural ventilation at the agricultural experimental station of the Faculty of Agriculture and Food Sciences in Butmir near Sarajevo. The greenhouse was equipped with two side roll-up vents due to humidity regulation. The site is located at 43°49′34.42″ N and 18°19′18.48″ E, at an altitude of 505 m above sea level. The experiment was established on sandy loam soil comprising

32.9% clay, 33.5% silt and 33.6% sand. According to World Reference Base for Soil Resources (IUSS, 2015), the studied soil can be classified as Alluvium, indicating its formation from sediments deposited by flowing water. Soil chemical analysis was performed a few weeks before sowing. The following parameters of soil chemical properties were subject of analysis: soil reaction (soil pH in H_2O and 1 M KCl), organic matter content and content of available forms of phosphorus (P_2O_5) and potassium (K_2O). The examined soil chemical parameters are presented in Tab. 1.

Table 1. Chemical properties of the studied soil

Parameter	Unit	Value	Characteristics
pH KCl	pH unit	7.5	neutral
pH H_2O	pH unit	6.7	reaction
organic matter	%	2.5	medium level
P_2O_5	mg/100 g	35.05	high level
K_2O	mg/100 g	30.04	high level

In accordance with these results the following fertilizer recommendations were given and carried out identical to all plots: the mineral NPK (10:20:30) fertilizer was added in amount of 5 kg/100 m^2 (amounts of fertilizers were recalculated based on examined soil plot area).

Experimental design and treatments
Spinach was seeded on 27 December 2023 in 1 m wide and 20 m long seedbeds at 5 cm between the plants and 20 cm between the rows. The distance between the seedbeds was 1.5 m. Four drought (water stress) levels were tested: (T1) optimum watering - 90% field capacity; (T2) low water deficit stress - 75% field capacity; (T3) moderate water deficit stress - 50% field capacity; and (T4) severe water deficit stress - 25% field capacity. Tensiometers (Soil Moisture Equipment Corp., Santa Barbara, USA) were used to monitor field capacity.
Plants were exposed to different irrigation regimes for 40 days, starting at 10 days after sowing and then re-watered until harvest. The drip irrigation system was used, supported with the water pump. In the winter period, from the end of January until the middle of February 2024, due to very low temperatures, the water was given by manual watering.

Plant sampling and analysis
At the time of technological maturity (sixty days from sowing), the spinach plants were harvested manually (above-ground spinach parts). Morphological parameters and yield were analyzed immediately after harvest. The fresh mass of spinach plants was determined using a digital electronic balance (RADWAG WPX 4500 with 0.01 g accuracy) and the total yield was calculated by summing the single plant mass from each experimental unit. The measurement of the petiole length was performed using an appropriate ruler, while the leaf area was measured using the Millimeter Graph Paper

Method (Pandey and Singh, 2011). A total of 30 spinach plants from each experimental unit were sampled to study the physiological traits: proline content, osmotic potential, photosynthetic pigment contents, total phenolic and flavonoids contents and total antioxidant capacity.

Proline estimation

Proline content was measured according to the method of Bates *et al.* (1973). Fresh leaf samples (0.5 g) were homogenized in 10 mL of aqueous sulfosalicylic acid 3% (w/v), and then filtered through a glass-fiber filter to a plastic test tube. Afterwards, 2 mL of filtrate was mixed with 2 mL of ninhydrin reagent (2.5 g of ninhydrin in 40 mL orthophosphoric acid 6 M and 60 mL of glacial acetic acid) and 2 mL of glacial acetic acid in a test tube and incubated for 1 h at 100 °C. Following incubation, 4 mL of toluene was added to the solution and vigorously mixed by vortex for 30 sec. The reddish layer of mixture was transferred to cuvette and absorbance was read at 520 nm in an Ultrospec 2100 Pro UV–Vis spectrophotometer (Amersham Pharmacia Biotech Biochrom Ltd., Holliston, MA) using toluene as blank. A proline standard curve ranging from 0 to 5 µg/mL proline was used to determine the proline levels of each sample, and then the obtained values were recalculated on fresh mass (µg/g FM).

Leaf osmotic potential estimation

Leaf osmotic potential of spinach leaves was measured according to the method of Ball and Oosterhuis (2005). Ten fully expanded spinach leaves from each experimental unit were collected by hand. Within 10 min of detachment, twenty leaf discs (6 mm diameter) were punched out of spinach leaves and then frozen at -100 °C in a refrigerator freezer. After 24 h, the filter paper disc was inserted between thawed leaf discs and pressed in a vice until a filter paper disk was saturated with the expressed sap. The osmotic potential of this liquid was then measured with a vapor pressure osmometer (Wescor, Logan, UT, USA).

Estimation of photosynthetic pigments

Photosynthetic pigments content including chlorophyll *a*, chlorophyll *b* and total carotenoids content was determined according to the method described by Lichtenthaler and Welburn (1983). 0.2 g of fresh leaves was extracted with 10 mL of pure acetone using a mortar. The pigment extracts were filtered through a coarse filter paper into a 25 mL volumetric flask and diluted to the mark with extract solution (pure acetone). The resulting extracts were assayed spectrophotometrically at 662 nm, 645 nm, and 470 nm. Concentrations of chlorophyll *a*, chlorophyll *b* and total carotenoids (mg/mL) were determined using the following equations:

$$c \text{ (chlorophyll } a) = 9.784 \text{ x } A_{662} - 0.990 \text{ x } A_{644}$$

$$c \text{ (chlorophyll } b) = 21.426 \text{ x } A_{644} - 4.650 \text{ x } A_{662}$$

$$c \text{ (total carotenoids)} = 4.695 \text{ x } A_{440} - 0.268 \text{ x } (c \text{ chlorophyll } a + c \text{ chlorophyll } b)$$

The obtained values were then recalculated to fresh mass of leaves (mg/g FM).

Extraction of phenolic compounds

At maturity stage (60 days after sowing), the leaves were picked and dried in air and then the leaves were grinded and stored in paper bags until extraction. The extraction was performed in Erlenmeyer flasks (100 mL) using a 30% aqueous solution of ethanol (1 g of air-dried leaves in a 40 mL of extract solution). The flasks were capped and incubated in a water bath at 40 °C for 2 h. Thereafter, the extracts were filtered through a coarse filter paper into a 25 mL volumetric flask and then diluted to the mark with extract solution. The extracts thus obtained were used for the estimation of the total phenolic and flavonoid content, and for the total antioxidant capacity.

Total phenolic content estimation

The total phenolic content was determined by the Folin-Ciocalteu method (Ough and Amerine, 1998). 0.25 mL of extract, 15 mL of distilled water, and 1.25 mL of Folin-Ciocalteu's reagent (previously diluted 1:2, reagent: distilled water) were placed and mixed thoroughly into a 25 mL flask for 5 min. The flask was incubated at room temperature for 15 min in the dark and then 3.75 mL of saturated sodium carbonate solution (Na_2CO_3) was added. Afterwards, the flask was filled to the mark with 30% ethanol and heated in a water bath at 50 ºC for 30 min. After cooling to room temperature, the resulting mixtures were assayed spectrophotometrically at 765 nm. The gallic acid standard curve ranging from 0 to 500 mg/L was used to determine the total phenolic content of each sample, and then the obtained values were recalculated on fresh mass (mg eq. GA/100 g FM).

Total flavonoid content estimation

The total flavonoid content was determined by the Aluminium chloride colorimetric assay (Zhishen *et al.*, 1999). 1 mL of extract, 4 mL of distilled water and 0.3 mL 5% $NaNO_2$ were placed and mixed thoroughly in a 10 mL flask. After 5 min. 0.3 mL 10% $AlCl_3$ was added, and the mixture was incubated at room temperature for 5 min. Then 2 mL of 1 M NaOH was added, and the flask was filled to the mark with distilled water. After 15 min, the resulting mixture was assayed spectrophotometrically at 510 nm. The catechin standard curve ranging from 0 to 100 mg/L was used to determine the total flavonoid content of each sample, and then the obtained values were recalculated on fresh mass (mg eq. C/100 g FM).

Total antioxidant capacity estimation

The ferric reducing antioxidant power (FRAP) assay was used to estimate the total antioxidant capacity (Benzie and Strain, 1996). 240 µL of distilled water, 80 µL of extract, and 2080 µL of FRAP reagent (0.3 mol/L acetate buffer (pH = 3.6), 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mmol/L $FeCl_3$ x 6 H_2O in a ratio 10:1:1) were added into a 10 mL Erlenmeyer flask and then heated in a water bath at 37 ºC for 5 min. After cooling to room temperature, the resulting mixtures were assayed spectrophotometrically at 595 nm. The $FeSO_4$ x $7H_2O$ standard curve ranging from 0 to 2000 µmol/L was used to determine the total antioxidant capacity of each sample and then the obtained values were recalculated on fresh mass (µmol Fe^{2+}/100 g FM).

Statistical analysis

All assays were performed in triplicates and the results were expressed as means ± standard deviation. The significance of differences was determined via one-way analysis of variance and least-significant-difference test (LSD test) using SPSS 22.0 software package program (IBM, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Growth and morphological parameters

As expected, the fresh mass of aerial parts of spinach plants were adversely affected by the drought. The highest fresh mass was obtained in the control (no-stress) treatment, whereas the lowest was recorded in spinach plants exposed to severe drought stress conditions. Reduction in fresh mass in spinach plants due to drought has also been reported earlier in numerous studies (Ors and Suarez, 2017; Yavuz *et al*., 2022).

In this study, drought also caused a reduction in leaf area and this reduction was more pronounced with increasing drought duration (Tab. II). Yang *et al*. (2021) have reported that the decrease in leaf area is mainly due to reduced cell division and expansion under drought conditions, which results in decreased spinach yield. These findings strongly support the hypothesis that spinach plants require regular watering to keep spinach growing fast. Contrastingly, if drought conditions persist, the lack of water in leaves will adversely affect cell growth, resulting in dramatically lower spinach yields (Sun *et al*., 2023). This study's results agree with such observations.

Table 2. Effects of water stress on plant morphological characteristics

Treatment[*]	Fresh mass of aerial parts (g per plant)	Leaf area (cm^2)	Petiole length (cm)	Yield (kg/m^2)
T1 (90% FC)	$93.3 \pm 10.2^{a[**]}$	80.9 ± 11.5^a	13.5 ± 0.3^a	8.9 ± 1.3^a
T2 (75% FC)	50.7 ± 7.1^b	49.4 ± 10.1^b	8.6 ± 0.1^b	4.6 ± 0.7^b
T3 (50% FC)	34.2 ± 8.0^c	32.2 ± 8.4^c	6.4 ± 0.2^c	3.0 ± 0.8^c
T4 (25% FC)	18.5 ± 9.9^d	11.2 ± 7.3^d	2.7 ± 0.2^d	1.5 ± 0.9^d
LSD $_{0.05}$	12.71	9.28	1.42	1.44

[*]Treatment: (T1) optimum watering - 90% field capacity; (T2) low stress - 75% field capacity; (T3) moderate stress - 50% field capacity; and (T4) severe stress - 25 % field capacity
[**]The mean followed by different letters in the same column indicate a significant difference at $P < 0.05$.

Drought also decreased the petiole length as compared to non-water stress treatment. Predictably, petiole length significantly decreased with increasing drought duration. Reduction in petiole length due to drought has also been reported in other studies for a number of plant species (Durigon *et al*., 2019; Enkhbat *et al*., 2022). In essence, reduced total biomass, leaf area, and petiole length are all plant mechanisms for improving water use efficiency and reducing damage under drought stress conditions (Farooq *et al*., 2009; Lovelli *et al*., 2017).

Physiological parameters

Proline, an amino acid, plays a highly important role in plants (Chahine *et al*., 2021). It stabilizes the osmotic differences between the cell's surroundings and cytosol, thus enhancing the cell's potential to maintain water without hampering the normal metabolism (Hayat *et al*., 2012). Besides acting as an osmoprotectant, proline acts as an effective quencher of reactive oxygen species. Due to its unique properties, proline greatly improve plants' ability to overcome drought stress conditions, thus the rapid increase in proline accumulation is one of the most significant responses in plants to water stress (Elmasry and Al-Maracy, 2023). In this study, the lowest values of proline content were observed in the control untreated spinach plants (FC 90%), and highest in the spinach plants under severe stress (FC 25%) (Tab. 3).

Table 3. Effects of water stress on plant physiological characteristics

Treatment[*]	Proline (µg/g)		Water potential (Mpa)		Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Carotenoids (mg/g)
T1 (90% FC)	1.36 1.2^{c**}	±	-0.56 0.1^a	±	0.97 ± 0.3^c	0.43 ± 0.2^c	0.36 ± 0.1^b
T2 (75% FC)	19.01 7.4^b	±	-0.72 0.1^b	±	1.24 ± 0.1^{bc}	0.53 ± 0.2^c	0.45 ± 0.1^b
T3 (50% FC)	24.24 9.0^b	±	-0.78 0.1^b	±	1.96 ± 0.1^a	1.05 ± 0.2^a	0.69 ± 0.2^a
T4 (25% FC)	230.54 9.9^a	±	-1.19 0.2^c	±	1.29 ± 0.2^b	0.81 ± 0.1^b	0.61 ± 0.1^a
LSD $_{0.05}$	12.01		0.11		0.28	0.22	0.14

[*]Treatment: (T1) optimum watering - 90% field capacity; (T2) low stress - 75% field capacity; (T3) moderate stress - 50% field capacity; and (T4) severe stress - 25 % field capacity
[**]The mean followed by different letters in the same column indicate a significant difference at $P < 0.05$.

The results of this study also showed that the proline content in spinach leaves increased significantly with increasing drought stress duration. Accordingly, the proline content in spinach leaves in treatments T2, T3, and T4 was 13.9, 17.8 and 169.5, times respectively higher than the control group (T1). These results indicate that cellular water uptake in prolonged drought stress conditions became more complex, and therefore, plants accumulate a large amount of osmotic active substance such as proline, which helps in maintaining cell water potential under water-deficit conditions (Chun *et al*., 2018). Numerous studies have also found evidence for a positive relationship between proline accumulation and drought treatment duration (Fu *et al*., 2018; Hosseinifard *et al*., 2022).On the other hand, an inverse relationship was observed between the level of water stress and the water potential of the leaves, indicating that drought restricts water supply and thus decreases leaf water potential (Ding *et al*., 2021). The control treatment i.e. no water stress treatment (T1) registered the highest leaf water potential value, whereas the severe stress treatment (T4) recorded the lowest water potential value (Tab.

III). These results are in line with those reported by Reyes *et al*. (2018). Hence, the decline of leaf water potential can be used as an important indicator of plant drought stress.

In this study, the photosynthetic pigment contents (chlorophyll *a*, chlorophyll *b* and total carotenoids) in plants showed considerable variations in response to the drought. The content of chlorophyll *a* and chlorophyll *b* in spinach leaves first rise in response to low-to-moderate level water stress (T2, T3) and then start to decrease with increasing drought stress duration (T4). A similar pattern was also found in total carotenoid contents; however, the changes in total carotenoid contents between spinach plants exposed to moderate and severe water stress were not statistically significant (Table 3). These results were inconsistent with the previously reported that drought reduces leaf photosynthetic pigment contents regardless of the stress level (Baccari *et al*., 2020; Juzoń *et al*., 2020). Anjum *et al*. (2011) reported that the water deficit due to drought induces the degradation of the thylakoid membrane within chloroplasts, resulting in chlorophyll breakdown.

Contrastingly, several studies have shown results where some plants including spinach exhibit increased chlorophyll content under low-level drought stress conditions (Xu and Leskovar, 2015; Rustioni and Bianchi, 2021). Interestingly, Zhang *et al*. (2014) did not observed any changes in chlorophyll content in spinach as affected by drought. In summary, these findings indicate that photosynthetic pigment contents in plants can vary significantly under water stress conditions, which primarily depends on water stress duration and intensity as well as plant tolerance to drought. Plants that maintain a relatively higher chlorophyll content under drought are generally have higher photosynthetic efficiency, and thus stronger tolerance to drought (Li *et al*., 2006). From this point of view, spinach could be considered as a drought-tolerant plant and this finding is in line with the previous studies of Yousif *et al*. (2010) and Kovár and Olšovská (2020). It is, nevertheless, important to note that the intense or prolonged drought period will undoubtedly negatively affect the chlorophyll synthesis in plants, resulting in decreased photosynthetic efficiency.

Interestingly in this study, the carotenoid content begins to decline at the same time as chlorophyll *a* and chlorophyll *b* content, but at a much slower rate, indicating that the chlorophyll content decreases faster than carotenoid content (Lichtenthaler and Babani, 2022). The ability to maintain carotenoid content can enhance plant tolerance to drought considering the fact that carotenoids protect chlorophyll pigments from photo-oxidation (Crupi *et al*., 2023). Numerous studies have also demonstrated that carotenoids play an important role in the mechanisms protecting the photosynthetic apparatus against reactive oxygen species initiated by various harmful environmental factors (Latowski *et al*., 2011; Dumanović *et al*., 2021). Moreover, this is of special significance, because the increase of reactive oxygen species in the plant cells causes oxidative damage and ultimately cell death (Huang *et al.,* 2019).

In the present study, drought stress remarkably increased the amounts of total phenolic and flavonoid contents in spinach leaves (Tab. 4). Increased synthesis of phenolic compounds under drought has also been reported in a number of plant species,

indicating that accumulation of phenolic compounds is one of the strategies adopted by plants to counteract drought stress (Cramer *et al*., 2011; Nicolas-Espinosa *et al*., 2023).

Table 4. Effects of water stress on antioxidant properties of spinach leaves

Treatment[1]	Total phenolics (mg/100 g)	Total flavonoids (mg 100/g)	Antioxidant capacity (μmol Fe^{2+}/100 g)
T1 (100% FC)	$46.5 \pm 4.6^{d**}$	12.7 ± 0.9^c	337.1 ± 11.1^d
T2 (75% FC)	67.9 ± 5.8^c	14.4 ± 1.8^c	456.6 ± 49.2^c
T3 (50% FC)	78.4 ± 4.5^b	17.4 ± 3.5^b	481.9 ± 56.1^b
T4 (25% FC)	132.4 ± 15.1^a	26.3 ± 3.3^a	769.7 ± 75.9^a
$LSD_{0.05}$	6.81	1.73	22.64

[*]Treatment: (T1) optimum watering - 90% field capacity; (T2) low stress - 75% field capacity; (T3) moderate stress - 50% field capacity; and (T4) severe stress - 25 % field capacity
[**]The mean followed by different letters in the same column indicate a significant difference at $P < 0.05$.

Study results also showed that the longer drought stress duration caused a higher increase in total phenolic and flavonoid contents. These results are expected since it is generally known that phenolic compounds play an important role in plant protection against reactive oxygen species (Andabaka *et al*., 2022), and plants therefore tend to increase their accumulation under stress conditions (Sarker and Oba, 2018; Misra *et al*., 2023). On the other hand, an opposite observation was found in several studies where exposure of plants to drought decreased the total phenolic and flavonoid contents in leaves, especially under intense drought periods (Król *et al*., 2014; Seleiman *et al*., 2021). Namely, the exposure of plant to long-term drought conditions rapidly triggers the state of stress in plants, leading to physiological destabilization, oxidative damage and disrupted metabolism. Therefore, the ability of the plants to synthesize phenolic compounds and other antioxidant substances in such conditions is significantly reduced. However, the results of this study revealed that the spinach plants could produce phenolic compounds in high amounts even in long-term drought conditions.

In this study, drought caused an increase in total antioxidant capacity (FRAP values) in comparison with control (non-stress) treatment. The study findings also showed that the FRAP values in spinach leaves increased with increasing drought stress levels. A similar pattern was observed in the relationship between drought stress levels and total phenolic and flavonoid contents, indicating that phenolic compounds greatly contribute to the antioxidant activity of plants. These results are consistent with the findings of previous studies (Lyu *et al*., 2023; Zeng *et al*., 2023).

CONCLUSIONS

The water stress resulted in reduction in spinach growth parameters and biomass accumulation and this reduction was more pronounced with increasing drought duration. The content of chlorophyll *a*, chlorophyll *b* and total carotenoid content

increased in response to low and moderate level water stress; however, under severe drought stress conditions their content started to decrease. The increase in water stress also resulted in a higher proline accumulation as well as phenolic and flavonoid contents, regardless of the stress levels. These points lead to the conclusion that spinach plants have the ability to produce protective macromolecules and antioxidants in high amounts even in severe drought conditions, suggesting that spinach could be considered as a drought-tolerant plant species from a survival point of view.

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MORFOLOŠKI I FIZIOLOŠKI ODGOVOR BILJAKA ŠPINATA NA VODNI STRES

Sažetak

U ovom istraživanju ispitivan je uticaj različitih nivoa vodnog stresa (nizak, umjeren i visok) na određene morfološke i fiziološke karakteristike biljaka špinata. Rezultati su pokazali da izlaganje biljaka špinata vodnom stresu snažno utiče na njihov rast i metabolizam. S povećanjem intenziteta vodnog stresa smanjivala se površina lista, dužina peteljke i biomasa biljke, što je rezultiralo znatno nižim prinosima špinata. Sadržaj fotosintetskih pigmenata u listovima špinata se povećao u uslovima niskog i umjerenog vodnog stresa. Međutim, u uslovima visokog nivoa vodnog stresa njihov sadržaj je počeo padati. Povećanje vodnog stresa takođe je rezultiralo većom akumulacijom prolina, kao i ukupnim sadržajem fenola i flavonoida. Ovi rezultati dovode do zaključka da biljke špinata imaju sposobnost sintetisati veliku količinu zaštitnih makromolekula i antioksidanasa čak i u uslovima kada su duže vremena izložene suši, iz čega proizilazi da se špinat s gledišta egzistencije može smatrati biljnom vrstom s visokim stepenom tolerancije na vodni stres.

Ključne riječi: *antioksidansi, suša, biljni rast, adaptacija na stres*

FREEZING DEGREE AND FERTILITY OF WINTER BUDS OF THE GRAPE VARIETIES MALVASIA ISTRIANA AND GARGANEGA IN THE ŽEPČE AREA*

Mersija Delić[1], Nejra Čoloman[1]

Original scientific paper

Abstract

The aim of this research was to examine the degree of freezing and the fertility of winter buds along the shoots of the Malvasia Istriana and Garganega vine varieties grown in the Žepče area. The vine is sensitive to low winter temperatures, and the degree of freezing of the winter buds and other organs depends on the biological characteristics of the variety and the ecological conditions of cultivation. The degree of freezing of winter buds was determined by the method of provocation of buds, while the fertility of shoots was determined by counting inflorescences per bud, shoot and reproductive shoot. Samples were taken at the beginning of March, 10 shoots with 10 buds for each variety. Fertility and the degree of freezing of winter buds along the length of the shoots were monitored. Based on the obtained results, the percentages of buds, fertility coefficients of buds and shoots, and the fertility of varieties, as well as the fertility of developed buds from the first to the tenth nodus on the shoot, were calculated. By analyzing the measured parameters, we can conclude that the tested varieties can be successfully grown in the ecological conditions of Žepče, and the values of all tested parameters were higher in the Malvasia Istriana variety compared to the Garganega variety.

Keywords: *Malvasia Istriana, Garganega, buds, fertility, freezing*

INTRODUCTION

The grapevine (*Vitis vinifera* L.) is a deciduous, shrubby, and woody climbing plant with tendrils (Hulina, 2011). It thrives in areas where the average annual air temperature is between 9 – 12ºC. Temperature is a limiting factor in grapevine cultivation, making the selection of the appropriate variety crucial depending on the climatic conditions of the specific area. Special attention should be paid to the length of the growing season and the resistance of varieties to low winter temperatures (Kojić *et al*., 2013). The resistance of grapevines to low temperatures depends on external conditions, the physiological state of the plants, and the direction and nature of metabolism in their

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tissues (Gusta and Wisniewski, 2013; Nenko *et al*., 2019). Resistance to winter temperatures primarily depends on the degree of tissue maturation and water content in them before the onset of winter dormancy (Nenko *et al.,* 2020). A key question in studying adaptation is assessing the potential genetically determined capabilities of organisms as responses to adverse environmental factors (Nenko *et al*., 2017; Yegorov *et al.,* 2017).

In the Žepče area, the average annual air temperature is 10.7ºC, so only varieties resistant to low winter temperatures and with shorter growing seasons can be cultivated there. These are primarily white wine varieties with earlier maturation epochs that require less thermal sum for their ripening (Kojić and Delić, 2009). Achieving stable high yields is limited by the impact of unfavorable environmental factors such as winter frost, especially after dry and hot summers, and spring frost following thawing (Nenko *et al*., 2017).

To achieve appropriate yield and grape quality, it is essential to know the biological characteristics of the variety and, based on that, perform the appropriate mature pruning. If the first buds are fertile in a variety, short pruning is essential, as leaving a larger number of buds on the shoot results in a higher yield but poorer grape quality. Subsequently, the shoots will not mature sufficiently, reducing their resistance to low winter temperatures, which are a regular occurrence in continental conditions. To avoid bud freezing and ensure adequate yield and grape quality, it is crucial to perform the appropriate mature pruning (Delić, 2015). Other factors are directly related to weather conditions such as temperature, light, water scarcity, or mineral nutrition. Little is known about the quantitative effects of these factors, and understanding the variation in yield from year to year and managing it remains a challenge for perennial crops (Hanke *et al*., 2007), especially for grapevine (*Vitis vinifera* L.), as recent studies have shown (Clingeleffer, 2010; Keller, 2021).

In addition to genetic potential, the position of the bud on the shoot is the main factor affecting bud fertility: it increases from the base to the middle section and decreases again towards the tip of the shoot (Huglin and Schneider, 1998). Quantitative relationships between temperature and bud fertility have been established, and an optimal temperature range for the formation of flower cluster primordia has been defined ($20°C < T < 35°C$) (Vasconcelos *et al*., 2009). Particularly valuable data about the variety are those that discuss the fertility of buds along the shoot (from the base to the top). They are significant due to the load distribution, the number of individual fruitful elements, etc. (Žunić and Matijašević, 2004).

The aim of this study was to examine the degree of freezing of winter buds in two white wine grape varieties, Malvasia Istriana and Garganega, in the Žepče area, as well as the fertility of the buds along the shoots of the examined varieties.

MATERIALS AND METHODS

Material: In early March 2022, samples of the grape varieties Malvasia Istriana and Garganega were taken from a test vineyard. Mature shoots of the examined varieties were collected from a production vineyard located in Goliješnica (44.47°N, 18.07°E,

H= 230 m), in the municipality of Žepče, using a random selection method. The training form of the vines in the vineyard is the Veronese pergola, and the planting distance is 3.5 x 0.7 m. The vineyard is in full fruit production and regular agricultural practices are applied. The climate is continental, with a regular occurrence of low winter temperatures during the dormancy period. From each variety, 10 mature shoots were collected, with each shoot having 10 buds.

Method of Bud Provocation: This method is used to determine the degree of freezing of buds in winter shoots and to quickly obtain data on bud fertility for planning pruning. During the dormancy period, representative samples of 10 shoots with 10 buds each are taken. The shoots selected are those that would be left as fruiting elements during mature pruning. The shoots are cut into cuttings with one bud each, leaving an internode length of about 5 cm below the bud, and then placed in openings on a Styrofoam plate that has 10 x 10 holes (Picture 1). The plate with the cuttings is placed in a water bath to float, in a well-lit room where the temperature is around 25ºC. After 10–15 days, the buds are activated, and young shoots appear. When these reach a length of about 15–20 cm, the flower clusters are observed, and their count is conducted according to the bud's position on the shoot (Picture 2,3). This way, the degree of freezing of the buds and the fertility of the buds along the shoots are determined. Based on these data, recommendations for pruning and yield planning are provided (Cindrić *et al*., 2000; Žunić and Garić, 2010, cited by Briza and Milosavljević, 1955, 1958; Mijatović *et al*., 2016).

Analysis of Results: A two-factor analysis of variance (ANOVA) was performed. The obtained data were processed using the SPSS software (IBM SPSS Statistics). The standard error was determined using the LSD test at a significance level of 0.05.

Ecological Conditions
For the analysis of meteorological conditions at the research site, Goliješnica in the municipality of Žepče, data from the meteorological station JUKIĆ – IEPE13 located in the test vineyard were used. The parameters were monitored for the year 2021, as the buds used in this study were formed during that growing season (Table 1).
Temperature extremes were also in line with the characteristics of a continental climate. The coldest month was January (1.8°C), while the warmest was July (22.6°C).

Table 1. Air temperature for 2021 years in Goliješnica, Žepče

Air temperature (ºC) 2021 year			
Month	Maximum	Minimum	Average
I	15.2	-11.7	1.8
II	22.7	-12.1	4.6
III	20.7	-8.2	4.6
IV	26.3	-4.7	8.0
V	30.2	0	14.7
VI	38.0	4.2	20.0
VII	38.2	10.7	22.6
VIII	37.8	6.2	20.0

IX	32.7	2.2	15.6
X	27.7	-2.3	8.7
XI	24.6	-2.2	6.7
XII	16.2	-7.5	3.2
Mean annual average temperature			10.9
Mean annual vegetative temperature			15.7
Absolute maximum air temperature	38.2		
Absolute minimum air temperature		-12.1	

Based on the presented data (Table 2), we see that the relative humidity in the Žepče area during 2021 was lowest in May (78%), while the highest was recorded in November (95%), leading us to conclude that relative humidity was high throughout the year studied. The average annual relative humidity during 2021 in the test vineyard was measured at 84%.

Table 2. Relative air humidity during 2021 year

Relative air humidity												
Month	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Annual average
%	90	82	79	79	78	79	80	82	85	93	95	91	84

RESULTS AND DISCUSSION

The results of the analysis of the freezing degree and bud fertility along the shoots of the Malvasia Istriana and Garganega varieties at the Goliješnica site in the municipality of Žepče are presented in the tables.

Freezing degree of winter buds

Picture 1. Cuttings of the Malvasia Istriana and Garganega varieties in the water bath (orig.)

From the data in Table 3, we can see that the Malvasia Istriana variety showed no freezing of the buds, and all buds were activated. Therefore, the planned mature pruning can be applied without any adjustments.

Table 3. Register activated winter buds in the Malvasia Istriana variety

Shoots	Ordinal number winter buds along the shoots									
	1	2	3	4	5	6	7	8	9	10
I	+	+	+	+	+	+	+	+	+	+
II	+	+	+	+	+	+	+	+	+	+
III	+	+	+	+	+	+	+	+	+	+
IV	+	+	+	+	+	+	+	+	+	+
V	+	+	+	+	+	+	+	+	+	+
VI	+	+	+	+	+	+	+	+	+	+
VII	+	+	+	+	+	+	+	+	+	+
VIII	+	+	+	+	+	+	+	+	+	+
IX	+	+	+	+	+	+	+	+	+	+
X	+	+	+	+	+	+	+	+	+	+

In the case of the Garganega variety (Table 4), there was freezing in 10 out of a total of 100 buds, but this is a small percentage, so no adjustments to the mature pruning need to be made. Analyzing the obtained results, we can conclude that the ecological conditions in the Goliješnica area, municipality of Žepče, are more favorable for the Malvasia Istriana variety compared to the Garganega variety when considering the indicator of the degree of freezing of winter buds.

Table 4. Register activated winter buds in the Garganega variety

Shoots	Ordinal number winter buds along the shoots									
	1	2	3	4	5	6	7	8	9	10
I	-	+	+	+	+	+	+	+	+	+
II	+	+	+	+	+	+	+	+	+	+
III	+	+	+	+	+	+	+	-	-	+
IV	+	+	+	+	+	+	+	+	+	+
V	+	-	+	+	+	+	+	+	+	+
VI	+	+	+	+	+	+	+	+	+	+
VII	+	+	+	+	+	+	+	+	+	+
VIII	-	+	+	+	-	+	+	+	+	+
IX	+	+	+	+	+	+	+	+	+	+
X	-	+	+	-	+	+	+	+	-	-

The resistance of winter buds to low temperatures depends, to a large degree, on their position on the shoot. Many studies have shown that basal buds have a higher tolerance to low winter temperatures than middle and apical buds (Köse and Kaya, 2017). In grape, the tolerance of winter buds to low temperatures is directly related with maturation cane (Fennell, 2004).

Pictures 2 and 3. Grown shoots with flowering in the Malvasia Istriana and Garganega vaieties (orig.)

Fertility of the Examined Grape Varieties

Fertility, as a biological property of grape varieties, is expressed through the following indicators:
- Percentage of activated (germinated) buds,
- Number of developed shoots,
- Number of fruiting shoots,
- Number of flower clusters,
- Fertility coefficients (potential, relative, and absolute).

The fertility indicators are influenced by the biological characteristics of the variety, the position of the bud on the shoot, ecological conditions, and applied ampelotechnics. The division into varieties of low, medium, and high fertility is primarily the result of their fruiting potential, but also of their ability to adapt to different cultivation systems, technological interventions (load, fertilization, irrigation), and adaptability to climatic and pedological conditions in various areas (Maletić *et al.,* 2008).

Percentage of Activated Buds is calculated using the formula: $Pko = B \times 100/A$, where B is the number of total developed shoots, and A is the number of retained buds.

$Pko = 114 \times 100/100$ (MI); $Pko = 92 \times 100/100$ (G)

From the obtained data, we can conclude that the percentage of activated buds for the variety Malvasia Istriana in the Žepče area was 114%. The percentage of activated buds for the variety Garganega was 92%, as not all retained buds developed into shoots. This is a high percentage of activated buds, so there is no need to adjust the pruning.

Total number of developed and fruiting shoots and number of flower clusters
During the growing season, green shoots, known as shoots, form from the buds on the vine. The number of developed shoots on the vine from the retained buds during pruning has a direct impact on the fertility of the variety (Delić, 2010).

For the variety Malvasia Istriana (114), a statistically significantly larger number of total shoots developed compared to the variety Garganega (92). From a certain number of buds, shoots developed, including from latent buds, resulting in a greater number of developed shoots than the number of activated buds. The difference in the number of fruiting shoots between the varieties Malvasia Istriana (103) and Garganega (90) was not statistically significant. The number of flower clusters for the variety Malvasia Istriana (126) was statistically significantly higher compared to the variety Garganega (95) under the same ecological conditions (Table 5).

Table 5. Total number of developed and fruiting shoots and number of flower clusters developed from 10 winter buds of Malvasia Istriana and Garganega varieties

Shoots	Total number developed shoots		Number fruiting shoots		Number of flowers	
	MI	G	MI	G	MI	G
I	10	9	10	9	11	10
II	10	10	9	10	13	12
III	10	8	10	8	12	8
IV	12	11	9	10	10	10
V	11	9	11	9	14	9
VI	12	10	11	10	15	9
VII	10	10	8	10	11	12
VIII	14	8	13	8	13	9
IX	13	11	13	10	16	10
X	12	6	9	6	11	6
Total	114[a]	92[b]	103	90	126[a]	95[b]
	LSD test $_{0.05}$= 1.252		ns		LSD test $_{0.05}$=1.754	

MI – Malvasia Istriana, G – Garganega; ns – no significant

The formation of yield in grapevines begins with the development of flower clusters in latent buds during the season 1. Unlike other perennial crops, there is no evidence of competition in grapevines between, on one hand, the initiation and differentiation of flower clusters for the following season and, on the other hand, the development of flowers and fruit set for the current season (Vasconcelos *et al.*, 2009).

According to the data in Table 6, we can see that a greater number of flower clusters developed in buds from the 5th to the 10th node on the shoots of the Malvasia Istriana variety, while for the Garganega variety, the highest number of flower clusters was formed in buds developed at the 6th and 7th nodes on the shoot. Statistically significant differences in the number of formed flower clusters based on the position of the bud on the shoot for the Malvasia Istriana and Garganega varieties were recorded only for buds

developed at the 7th, 9th, and 10th nodes. Differences in the number of flower clusters developed in buds at all other nodes for the examined varieties were not statistically significant.

Table 6. Fertility of winter buds depending on the position on the shoot

Ordinal number winter buds on the shoot	Number of flowers		Significance of the differences
	MI	G	
1	9	9	Ns
2	8	9	Ns
3	11	10	Ns
4	10	9	Ns
5	12	9	Ns
6	14	12	Ns
7	16	11	LSD test $_{0.05}$= 0.401
8	15	9	Ns
9	17	9	LSD test $_{0.05}$= 0.49
10	14	8	LSD test $_{0.05}$= 0.44
Ukupno	129	95	

MI – Malvasia Istriana, G – Garganega; ns – no significant

Malvasia Istriana has high economic value as it combines good fertility with high-quality grapes and wine, and in addition, it has a pronounced typicality and recognizability related to the areas of Istria and its surroundings. Its fertility is generally regular; however, it can sometimes be irregular due to pollination issues, which can occur when rain and cold weather happen during flowering (Maletić et al., 2015). The fertility of the Garganega variety is regular and average. It suits high cultivation systems and mixed pruning. It is sensitive to low winter temperatures (Mirošević and Turković, 2003).

Fertility coefficients

Table 7. Fertility coefficients of the tested varieties

Variety	Fertility coefficients of winter buds	Relative fertility coefficients	Absolute fertility coefficients
Malvasia Istriana	1.26	1.1	1.2
Garganega	0.95	1.03	1.05

The Malvasia Istriana variety had higher values for all three fertility coefficients compared to the Garganega variety (Table 7) in this study. According to the value of the fertility coefficient, both varieties fall into the group of varieties with a high relative fertility coefficient (0.9 – 1.1) and into the group with a very low absolute fertility

coefficient (<1.2) (Žunić and Garić, 2010; Matijašević, 2021). In studies conducted in Radmilovac, the Malvasia Istriana variety had the following coefficients: bud fertility 1.23, fertility 1.36, and productivity 1.62, while those grown in Niš had these coefficients: bud fertility 0.93, fertility 1.06, and productivity 1.2 (Sivčev *et al.*, 2003).

CONCLUSIONS

Investigation of the degree of winter bud freezing and fertility of the Malvasia Istriana and Garganega varieties in the Žepče area during 2022 showed that both varieties can be successfully cultivated under the given agroecological conditions. Greater resistance and fertility were evident in the Malvasia Istriana variety compared to the Garganega variety. Further research should be conducted to confirm the obtained results.

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STEPEN IZMRZAVANJA I RODNOST ZIMSKIH OKACA SORTI VINOVE LOZE MALVAZIJA ISTARSKA I GARGANEGA NA PODRUČJU ŽEPČA

Rezime

Cilj ovog istraživanja je bio ispitati stepen izmrzavanja i rodnost zimskih okaca duž lastara vinskih sorti vinove loze Malvazija istarska i Garganega gajenih na području Žepča. Vinova loza je osjetljiva na niske zimske temperature, a stepen izmrzavanja zimskih okaca i drugih organa zavisi od bioloških osobina sorte i ekoloških uslova gajenja. Stepen izmrzavanja zimskih okaca utvrđen je metodom provokacije okaca, dok je rodnost okaca utvrđena brojanjem cvasti po okcu, lastaru i rodnom lastaru. Uzorci su uzeti početkom marta, po 10 lastara sa po 10 okaca za svaku sortu. Praćeni su rodnost i stepen izmrzavanja okaca po dužini lastara. Na osnovu dobijenih rezultata izračunati su procenti krenulih okaca, koeficijenti rodnosti okaca i lastara, te plodnosti sorti, kao i rodnost razvijenih okaca od prvog do desetog koljenca na lastaru. Analizom izmjerenih parametara možemo zaključiti da se ispitivane sorte mogu uspješno gajiti u ekološkim uslovima Žepča, a vrijednosti svih ispitivanih parametara su bile veće kod sorte Malvazija istarska u odnosu na sortu Garganega.

Ključne riječi: *Malvazija istarska, Garganega, okca, rodnost, izmrzavanje*

EFFICACY OF NEW INSECTICIDES GENERATION IN THE CONTROL OF TOMATO LEAF MINER (*Tuta absoluta* Meyrick)*

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Original scientific paper

Abstract

The tomato leaf miner (*Tuta absoluta*) is a pest that causes significant damage to tomato production in Bosnia and Herzegovina. According to previous research, the inadequate application of older generation insecticides in the control of tomato leaf miner has led to a series of harmful consequences, such as increased resistance of the pest, reduction of the natural enemies population and an increase in pesticide residues in the agricultural products and in the environment. The aim of this work was to examine the efficacy of the newer insecticides generation in controlling of tomato leaf miner in greenhouse production in southern Bosnia and Herzegovina. Testing the efficacy of insecticides was carried out on tomato plants (Matias hybrid) in greenhouses in the localities of Višići, Gabela and Klepci. The effectiveness was tested on the following variants of insecticides: Indoxacarb, Emamectin benzoate, Methoxyfenozide, Lufenuron, Chlorantraniliprole, Azadirachtin, Spinosad and the combination of Abamectin + Lufenuron. Based on the number of live larvae in the mines, according to Abbott, the efficiency values of the applied insecticide variants were calculated. The combination of insecticides based on the active substances Abamectin and Lufenuron showed the highest percentage of efficiency (53.52%). The insecticide based on the active substance Spinosad showed the lowest percentage of efficiency. Lower efficiency values of the majority tested insecticides are probably a consequence of their specific mechanism of action, physical-chemical properties and environmental conditions in which they were applied.

Keywords: *Tuta absoluta, larvae, insecticides, Abamectin, Lufenuron*

INTRODUCTION

The tomato moth (leaf miner) (*Tuta absoluta* Meyrick, Lepidoptera: Gelechiidae) is a pest that is widespread in many parts of the world. In the territory of Bosnia and

Herzegovina, it was first noted in 2010 in the southern parts (Ostojić *et al*., 2010) and then in the northern parts of the country (Đurić & Hrnčić, 2010). After that, it gradually spread towards the central parts of the country. Since its introduction, tomato moth has become one of the most significant pests in tomato production in Bosnia and Herzegovina. Many studies have confirmed that tomato moth is a thermophilic species that prefers climatically warmer areas for the development of its life cycle (Barrientos *et al*., 1998; Borgorni *et al*., 2003; Pereyra and Sanchez, 2006). If adequate control measures are not applied, it is believed that this pest can reduce the quality and yield of tomatoes (80-100%) in both field and greenhouse production conditions. Suppression of tomato moth is very complex and limited by the specific lifestyle (behavior) and bioecological characteristics of the pest (Haseljić *et al*., 2022).

The chemical control measure of this pest was already used at the beginning of the eighties of the last century (Souza & Reis, 1986). In that period, protective agents based on organophosphates and pyrethroids were mostly used in control of tomato moth. Pyrethroids are neurotoxic insecticides, they act on sodium channels in the cell membranes of the nervous system, causing disruption of the nerve impulses flow, wich resulting in paralysis and death of the pest. Inadequate application of these preparations and other insecticides of the older generation has over time led to increased resistance of tomato moth (Siqueira *et al*., 2001; Lietti *et al*., 2005; Silva *et al*., 2011; Reyes *et al*., 2012), which is ultimately affected the reduction of efficiency. Inadequate application of the older generation of insecticides leads to a decrease in the number and activity of natural enemies, as well as to an increase in residues on agricultural products and in the environment. With insecticides of the newer generation tried to overcome the mentioned implications. The active substances of newer insecticides are Spinosad, Indoxacarb, Abamectin, Tebufenozide and Chlorfenapyr (Insecticide Resistance Pest Management - IRAC), and they were introduced into production at the end of the last century (Lietti *et al*., 2005). Insecticides based on Indoxacarb, Lufenuron, Spinosad, Thiacloprid and Imidacloprid showed a positive effect in controlling tomato moth in Malta (Mallia, 2009), and in Italy, agents based on Chlorpyrifos and Pyrethrin showed also good results (Tropea Garzia *et al*., 2009).

The aim of this research was to examine the effectiveness of the newer insecticides generation in controlling of tomato moth in Bosnia and Herzegovina.

MATERIALS AND METHODS

Testing the efficacy of new insecticides generation was carried out on tomato plants (hybrid Matias) in greenhouse production in the localities of Višići, Gabela and Klepci in the southern part of Bosnia and Herzegovina. Insecticides were applied during the growing season in the period 2012-2014.

The effectiveness was tested on the following variants of insecticides: Indoxacarb, Emamectin benzoate, Methoxyfenozide, Lufenuron, Chlorantraniliprole, Azadirachtin, Spinosad and the combination of Abamectin + Lufenuron. The mode of action and the applied concentrations of insecticides are shown in table 1. In each experimental

plantation there was a control variant with planted tomato that were not treated with insecticides.

The effectiveness of the applied insecticides was evaluated based on the number of live larvae in the mine (Braham & Hajji, 2012). Efficiency was calculated according to Abbott (1925) using the formula:

$$E = 1 - \frac{\text{number of larvae on treated plants}}{\text{number of larvae in the control}} \; x \; 100 \; (\%)$$

The obtained results were statistically processed using descriptive and inferential statistics (Kruskall Walis and post hoc Mann-Whitney U test) in the IBM SPSS program (v 27).

Table 1. Insecticide, concentration (C) and mode of action (MoA, IRAC)

Insecticide Trade name	Active Substance (A.s.)	C (%)	MoA
Avaunt 15 EC	Indoksakarb	0.1	Nerve action
Affirm	Emamectin benzoate	0.2	Nerve and muscle action
Runner 240 SC	Methoxyfenozide	0.04	Growth regulation
Match EC	Lufenuron	0.03	Inhibitors of chitin biosynthesis
Coragen 20 SC	Chlorantraniliprole	0.02	Nerve and muscle action
Neemazal- T/S	Azadirachtin	0.3	Uunknown or uncertain MoA
Laser	Spinosad	0.05	Nerve action
Vertimec 018 EC + Match EC	Abamectin + Lufenuron	0.075 + 0.03	Nerve and muscle action; Inhibitors of chitin biosynthesis

RESULTS AND DISCUSSION

Based on a three-year study, the results of the analysis (descriptive statistics) of the newer generation insecticides effectiveness in the control of the tomato miner in the area of Višići, Gabela and Klepci are shown in table 2.

The percentage of tested insecticides effectiveness ranged from 12.34 to 53.52%. The highest percentage of efficiency was achieved in the combination of active substances Abamectin + Lufenuron (trade name Vertimec + Match). Insecticides based on the active substances Indoxacarb (Avaunt) and Emamectin benzoate (Affirm) showed a slightly higher percentage of efficiency (41.88 and 41.65%). The active substances Spinosad (Laser), Chlorantraniliprole (Coragen) and Azadirachtin (Neemazal) showed the least effectiveness in controlling of tomato moth.

Considering the existence of differences in the effectiveness percentage of tested insecticides, the Kruskall Walis (K-W) test was performed. This test is a non-parametric analogue of one-factor analysis of variance. In this part of the results, the K-W test was used due to the fact that the assumption of normality data distribution and homogeneity of variances was not fulfilled.

Table 2. Efficacy of tested insecticides (descriptive statistics)

A.s.	Min	Max	Average	Std. error	Variance	Std. dev.	Coff. var.
Indoksakarb	35.04	50.63	41.88	1.81	29.59	5.44	12.99
Emamectin benzoate	31.47	51.14	41.65	2.09	39.27	6.27	15.05
Methoxyfenozide	21.5	44.71	36.97	3.03	82.74	9.1	24.6
Lufenuron	18.18	32.57	26.76	1.78	28.52	5.34	19.96
Chlorantraniliprole	11.21	22.45	16.85	1.07	10.32	3.21	19.07
Azadirachtin	10.49	22.73	17.17	1.42	18.17	4.26	24.83
Spinosad	5.38	16.26	12.34	1.53	21.03	4.59	37.16
Abamectin + Lufenuron	36.36	65.15	53.52	3.56	114.15	10.68	19.96

Table 3. Kruskall Walis test

Hc (tie corrected):	58.96
Degree Of Freedom	7
p (same):	.000

Statistical processing of the data established a very significant influence of the analyzed insecticides in the control of tomato moth. Accordingly, to test the significance of the differences between the tested insecticides in the further analysis of the results, the M-W post hoc test was used with the Bonferroni's correction of the alpha value. Statistically highly significant (p 0.001) differences were found between the combination of active substances Abamectin + Lufenuron on the one hand and Spinosad, Azadirachtin and Chlorantraniliprole on the other. There were no statistically significant differences between Methoxyfenozide, Emamectin benzoate and Indoxacarb in terms of percentage efficiency. Also, no statistically significant differences were found between Azadirachtin on the one hand, and Spinosad and Chlorantraniliprole on the other.

The satisfactory efficiency of the Abamectin + Lufenuron insecticide combination with 53.52%, is the result of the synergism of these active substances. The synergistic effect of combining insecticides can be highly effective in controlling *Tuta absoluta*. In the research carried out by Illakwahhi & Srivastava (2019) in controlled conditions, a higher efficiency with combination of the active substances Abamectin and Neem oil was determined compared to the treatments when the mentioned active substances were

applied individually. Altowayyan *et al*. (2022) determined a significant improvement in the efficiency of Abamectin in combination with two entomopathogenic fungi (EPFs) *Beauveria bassiana* and *Metarhizium anisopliae* 5 days after the treatment of *Tuta absoluta* in greenhouse conditions. In this combination, the EPFs worked gradually after the infection of the larvae, while the chemical insecticide caused a more rapid knockdown effect.

The efficacy of Indoxacarb, Emamectin benzoate and Spinosad in this study was lower compared to the studies conducted by Moustafa *et al*. (2023) and Haseljić *et al*. (2023). It is important to single out the study conducted by Sapkal *et al*. (2018). In the conducted study, the effectiveness of chemical and biological insecticides in the control of the tomato moth was investigated. In the mentioned study, the effectiveness of the following insecticides was recorded: Chlorantraniliprole - 18.5%, Emamectin benzoate - 5%, Spinosad - 45% and Indoxacarb - 14.5%.
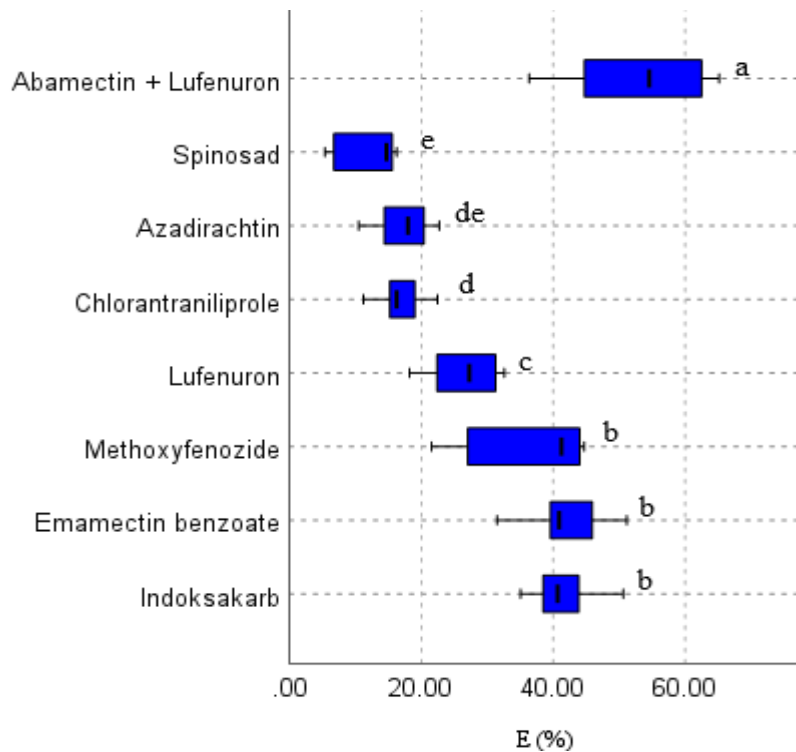
Graph 1. Efficacy of tested insecticides (Box plot, M-W test)

CONCLUSIONS

The combination of insecticides based on the active substances Abamectin and Lufenuron had a significant effect on increasing the percentage of effectiveness in controlling of tomato moth compared to all other analyzed insecticides. The efficacy of insecticides based on the active substances Indoxacarb, Emamectin benzoate and

Methoxyfenozide was satisfactory. The active substances Spinosad, Chlorantraniliprole and Azadirachtin exhibited the lowest efficiency. Lower efficiency values of the remaining insecticides are probably a consequence of their specific mechanism of action, physico-chemical properties and environmental conditions in which they are applied.

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EFIKASNOST INSEKTICIDA NOVE GENERACIJE U SUZBIJANJU LISNOG MINERA PARADAJZA (*Tuta absoluta*)

Sažetak

Miner paradajza (*Tuta absoluta*) je štetnik koji nanosi značajne štete u proizvodnji paradajza u Bosni i Hercegovini. Prema dosadašnjim istraživanjima, neadekvatna primjena insekticida starije generacije u suzbijanju minera paradajza dovela je do niza štetnih posljedica, kao što su povećana otpornost štetnika, smanjenje populacija prirodnih neprijatelja te povećanje rezidua pesticida u poljoprivrednim proizvodima i okolišu. Cilj ovog rada bio je ispitati efikasnost insekticida novije generacije u kontroli minera paradajza na području Bosne i Hercegovine. Ispitivanje efikasnosti insekticida provedeno je na paradajzu (hibrid Matias) u plastenicima na lokalitetima Višići, Gabela i Klepci. Efikasnost je ispitana na sljedećim varijantama aktivnih materija insekticida: Indoksakarb, Emamektin benzoat, Metoksifenozid, Lufenuron, Hlorantraniliprol, Azadiraktin, Spinosad i kombinacija Abamectin + Lufenuron. Na temelju broja živih gusjenica u minama, prema Abbottu, izračunat je procenat efikasnosti primijenjenih varijanti insekticida. Kombinacija insekticida na bazi aktivnih materija Abamektin i Lufenuron je ispoljila najveći postotak efikasnosti. Najniži postotak učinkovitosti pokazao je insekticid na bazi djelatne tvari Spinosad. Manje vrijednosti efikasnosti većeg broja ispitivanih insekticida su vjerovatno posljedica njihovog specifičnog mehanizma djelovanja, fizičko-hemijskih osobina i uslova sredine u kojim su primijenjeni.

Ključne riječi: *Tuta absoluta, larve, insekticidi, Abamektin, Lufenuron*

Pseudomonas SPECIES CAUSING DISEASES IN ALFALFA*

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Review paper

Abstract

The *Pseudomonas* genus includes species that are significant pathogens, causing considerable losses in plant production. Many of these pathogens lead to diseases that have global economic and environmental consequences for the trade of plants, seeds, and food. Alfalfa (*Medicago sativa*) is one of the most widely cultivated and important forage crops in the world. Its exceptional qualities, such as high biomass yield, excellent forage quality, and preference among ruminants, have earned it the nickname "Forage Queen." However, certain phytopathogenic species of *Pseudomonas* can cause substantial yield losses in alfalfa production. This review discusses the diseases caused by *Pseudomonas* species in alfalfa, their symptoms, and the mechanisms through which these pathogens cause disease. It also covers techniques for detecting and isolating these species from plants and soil, as well as methods for controlling these diseases. This review aims to provide researchers with comprehensive scientific information on managing *Pseudomonas*-related diseases in alfalfa production.

Keywords: *Pseudomonas, Alfalfa, Bacterial diseases, Medicago sativa*

INTRODUCTION

Alfalfa (*Medicago sativa*), often referred to as the "Queen of Forages," is a perennial leguminous crop that has been cultivated for over 2,000 years (Chen *et al*., 2020). It is a vital forage crop worldwide, especially for livestock production, and holds significant importance for dairy farmers. Furthermore, alfalfa plays a critical role in sustainable agricultural systems. Its benefits include high biomass yield, contributions to soil and water conservation, enhancement of soil fertility through biological nitrogen fixation, suppression of pests and pathogens in crop rotations, and provision of habitat for wildlife (Putnam *et al*., 2001).

Plant pathogens and nematodes that infect alfalfa can significantly reduce forage yield and quality, alongside shortening the plant's lifespan (Nemchinov *et al*., 2017). Most research on alfalfa diseases has traditionally focused on fungal infections (Zhibiao, 1985), while studies on bacterial diseases affecting alfalfa remain relatively limited. To

date, nine bacterial diseases have been reported in alfalfa. The bacteria responsible for these diseases belong to several genera, including *Erwinia*, *Pectobacterium*, *Agrobacterium*, *Pseudomonas*, *Xanthomonas*, *Clavibacter*, and *Xylella*. Within the genus *Pseudomonas*, two species have been documented as pathogens of alfalfa: *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* (Gray & Hollingsworth, 2015).

This review focuses on bacterial diseases affecting alfalfa globally, specifically addressing the distribution and host range of diseases caused by pathogens from the *Pseudomonas* genus. It also offers a comprehensive overview of the symptoms and identifying characteristics of these pathogens, summarizes common diagnostic methods, and discusses effective strategies for disease control.

Bacterial stem blight (*Pseudomonas syringae* pv. *syringae*)

Alfalfa bacterial stem blight (BSB) was first identified in 1904 in Colorado, where frost damage was noted as a potential entry point for the pathogen (Sackett, 1910). This disease is widespread in the central and western regions of the United States and has also been reported in Australia, the United Kingdom, the former Yugoslavia, Russia, and Iran. Although it usually results in minimal losses, it can cause 40-50% loss of forage during the first harvest in some high valleys in the western United States (Nyvall, 2013).

Symptoms of the Disease: Alfalfa bacterial stem blight typically begins in early spring and persists until the first harvest. The symptoms include necrotic lesions on the stems, chlorosis on the leaves, bending of the stems in a shepherd's crook shape, and water-soaked spots (Gray & Hollingsworth, 2015; Samac *et al*., 2014). These spots transition from light brown to black and spread toward the roots (Nyvall, 2013). Infected stems become thin, brittle, and shorter than healthy plants. While vascular tissues remain unaffected, the leaves turn yellow and eventually wither (Zhenfan & Zhibiao, 2014).

Causal Agent: The causal agent is *Pseudomonas syringae* pv. *syringae*. This bacterium is a gram-negative, motile rod with rounded ends, measuring 0.5–0.8 × 1.2–2.4 μm. Each end has one to four flagella, and the bacteria may form long chains. Colonies grown on nutrient agar appear smooth, circular, slightly convex, grayish-white, glistening, and translucent. They also produce a greenish fluorescent pigment that diffuses into the medium. The optimal temperature for growth is between 27 and 30°C. The host range for *P. syringae* strains found in alfalfa is not well-defined, but it's common for isolates within this group to have overlapping host ranges (Nyvall, 2013). *P. syringae* has two main phases: epiphytic and endophytic, and environmental factors influence its pathogenic potential in both phases (Xin *et al*., 2018).

Disease Cycle and Epidemiology: In spring, cold and humid conditions create an environment that is conducive to disease development in alfalfa. *Pseudomonas syringa*e pv. *syringae* contributes to frost damage through ice nucleation activity and can survive in plant residues in the soil. It typically enters the plant through areas that have been affected by frost. Under favorable environmental conditions, the disease can spread

within the field, and it is generally observed during the first harvest in the United States (Nyvall, 2013).

Infection Mechanism of the Pathogen: *Pseudomonas syringae* employs several virulence factors to establish infection, including the Type III secretion system (T3SS), ice nucleation activity, toxins, cell wall-degrading enzymes, and exopolysaccharides (Morris *et al*., 2013). This pathogen, specifically *P. syringae* pv. *syringae*, infects its host primarily through frost damage, with ice nucleation proteins (INPs) located in its outer membrane contributing to this process (Li *et al*., 2012). The disease progression occurs in two stages: localized leaf necrosis (or blight) and systemic vascular wilting. In the first stage, water-soaked lesions, resulting from frost injuries, spread along the stem, leading to dried bacterial exudates. The ice nucleation capability of *P. syringae* exacerbates frost damage, causing significant economic losses, particularly in plants that are vulnerable to late spring frosts (Lindow *et al*., 1982).

Management of Bacterial Stem Blight (BSB) Disease: Currently, there is no reliable method to predict BSB disease in advance, nor is there an effective chemical control available. Given the lack of commercially resistant alfalfa varieties, the importance of integrated management strategies is increasing (Baltrus *et al*., 2017; Yu & Kole, 2021). Cultural control methods, such as selecting frost-resistant alfalfa varieties and harvesting early after frost, can help reduce disease spread by eliminating infected plant material (Nemchinov *et al*., 2017). While copper-based bactericides have shown limited success in managing the disease (Scheck *et al.,* 1998), biological control options are emerging. Antagonistic bacteria like *Pantoea agglomerans* and *Bacillus* species show promising results, and natural antibacterial compounds, along with thermal seed treatments, also present alternative solutions (Balestra & Bovo, 2003). Genetic research is ongoing to develop BSB-resistant varieties. Although the ZG9830 variety has been identified as a potential candidate for resistance, its low yield poses a significant challenge (Nemchinov *et al*., 2017). Therefore, employing resistant varieties in conjunction with integrated management strategies is the most sustainable approach to mitigating the impacts of BSB.

Bacterial crown and root rot (*Pseudomonas viridiflava*)

This disease, commonly observed in many alfalfa production areas worldwide, was first described by Burkholder in 1930 and was recognized as a primary limiting factor in alfalfa production by Dowson in 1939 (Dowson, 1939).

Symptoms of the Disease: In the field, symptoms of the disease manifest as light brown, dry rot on the crown and main taproot. Light brown streaks extend beyond the rot, spreading through the vascular system along approximately one-third of the root (Nyvall, 2013). In affected plants, symptoms include yellowing, loss of green color, and wilting. This is often followed by further wilting, necrosis, stunted growth, and leaf deformation (Zhenfan & Zhibiao, 2014).

Causal Agent: The causative agent of the alfalfa bacterial crown and root rot complex is *Pseudomonas viridiflava*. This rod-shaped bacterium measures 0.5–1.0 µm in width and 1.5–5.0 µm in length. As a Gram-negative organism, it moves using a polar

flagellum. On media containing 5% sucrose, its colonies typically appear yellow, whereas on media with yeast extract and glycerol, the colonies can range from olive green to golden yellow. Additionally, some strains are capable of producing a blue pigment (Heydari *et al*., 2012).

Disease Cycle and Epidemiology: The initial infection of the disease occurs when bacteria enter the roots through wounds, infection sites, nematode feeding marks, winter injuries, or mechanical damage. Additionally, newly cut stems during harvest can serve as entry points for bacteria. The disease spreads most rapidly under moderate temperatures and moist soil conditions. Bacteria can survive in plant material, soil, and straw, and they can be transmitted from plant to plant through sprinkler irrigation or contaminated agricultural tools (Heydari *et al*., 2014).

Infection Mechanism of the Pathogen: This pathogen spreads through the xylem and obstructs water transport, leading to symptoms such as stunted growth, chlorosis, and wilting in plants. It produces exopolysaccharides that block water flow, resulting in root rot, yellowing of leaves, and large lesions on the stems and roots. The pathogen also breaks down plant tissues using various enzymes, including cutinase, pectinase, cellulase, protease, and hemicellulase (Murillo & Sesma, 2001). Infected plants exhibit color changes in their roots, shifting from yellow to brown, along with clustering of small leaves and upward curling of leaves. In severe cases, this can ultimately lead to plant death (Heydari *et al*., 2014).

Management of the Disease (Bacterial Crown and Root Rot): Although there is currently no effective management method for diseases caused by *Pseudomonas viridiflava*, the application of *Bacillus* species and *Pseudomonas fluorescens* for biological control and the use of copper compounds (such as Bordeaux mixture, copper hydroxide, and copper oxide) have shown promising results in managing epiphytic populations (Balestra & Bovo, 2003; Fascella *et al*., 2015; Al-Karablieh *et al.,* 2017). Genetic studies are focused on understanding the pathogenic characteristics of *P. viridiflava* and on developing resistance genes (Bartoli *et al*., 2015). Early diagnostic techniques, such as PCR, can help limit the spread of the disease, although no specific early detection method currently exists (Bull & Koike, 2015).

Taxonomy of *Pseudomonas* species causing disease in alfalfa

The *Pseudomonas* genus is a highly diverse group of Gram-negative bacteria that can be phylogenetically divided into two main lineages: the *P. fluorescens* lineage and the *P. aeruginosa* lineage. The *P. fluorescens* lineage includes most plant-associated species, including significant phytopathogens like *P. syringae*. *P. syringae* is recognized as a complex species group that consists of nine genomospecies, 13 phylogroups, and over 60 pathovars, many of which are responsible for diseases in various plant hosts (Bull *et al.,* 2010; Morris *et al*., 2008). Another important member of this lineage is *P. viridiflava*, which falls within phylogroups PG7 and PG8 and is characterized by distinct pathogenic traits and adaptability to different environments (Parkinson *et al*., 2011).

Pseudomonas syringae is a significant phytopathogen that has been extensively studied for its genomic and phylogenetic classifications. This bacterium causes various plant

diseases and is classified using techniques such as single-gene sequencing, multilocus sequence analysis (MLSA), multilocus sequence typing (MLST), repetitive extragenic palindromic PCR (rep-PCR), and whole-genome sequencing (Berge *et al.,* 2014). *P. syringae* belongs to the *Pseudomonas fluorescens* lineage, which contains most plant pathogens (Bull *et al*., 2010). Although *P. syringae* is often treated as a single species, it represents a complex of genetically diverse species comprising nine genomospecies, 13 phylogroups, and 64 pathovars infecting a wide range of hosts (Morris *et al*., 2008). Among these, *P. syringae* pv. *syringae*, which causes bacterial stem blight in alfalfa, is classified in phylogroup 2b (PG2b) within PG2, and is found widely in agricultural and natural environments (Morris *et al*., 2008). PG2 strains are recognized for their pathogenicity and virulence factors, including toxin production, type III secretion systems (T3SS), and quorum sensing; they often shift from epiphytic to endophytic phases (Xin *et al*., 2018). In contrast, *Pseudomonas viridiflava*, which causes bacterial crown and root rot, falls under phylogroups PG7 and PG8. Research has shown that these phylogenetic groups differ in pathogenicity and environmental adaptability, with PG2 strains generally being more aggressive in agricultural settings. The taxonomic complexity of *P. syringae* and *P. viridiflava* has led to the utilization of various genes, such as *gyrB*, *rpoD*, and *16S rRNA*, for species identification (Yamamoto *et al*., 2000). Additional housekeeping genes, including *gapA*, *cts*, and *purA*, further enhance the classification of *P. viridiflava* isolates using MLSA and single-gene analyses (Parkinson *et al*., 2011). Recent molecular methods, such as rep-PCR and MLSA, have proven effective in the precise identification of these pathogens (Bull & Koike, 2015).

Future research on the virulence and genetic diversity of *P. syringae* and *P. viridiflava* will be essential for disease management and crop loss reduction, particularly in alfalfa and similar agricultural crops.

Common diagnostic methods for identifying *Pseudomonas* species that cause diseases in alfalfa

The most important factor in fighting diseases caused by *Pseudomonas* species and in developing resistance is the precise identification of the pathogen (Guilbaud *et al*., 2016). Accurate diagnosis of *Pseudomonas* species that affect alfalfa is achieved through various methods, including culture-based techniques, PCR methods, molecular fingerprinting, and biochemical tests (Parisi *et al*., 2019).

Culture-Based and Biochemical Diagnostic Methods for *Pseudomonas* Species: Traditional culture-based methods are commonly used to diagnose *Pseudomonas* species. King's B medium is effective for isolating species that exhibit green fluorescence under ultraviolet light (Schaad *et al*., 2001).

The LOPAT test—comprising a series of determinative tests: L for levan production, O for oxidase production, P for pectinolitic activity, A for arginine dihydrolase production, and T for tobacco hypersensitivity - provides an accurate diagnosis by distinguishing between pathogenic and non-pathogenic fluorescent *Pseudomonas* species. However, it

has limitations when it comes to differentiating between strains and pathovars (Goudarzi & Mortazavi, 2020).

Biochemical tests are conducted to assess the biological characteristics of *Pseudomonas* strains. Notably, ice nucleation activity allows *P. syringae* strains to damage plant tissue at low temperatures, which can increase disease severity (Bull & Koike, 2015). Furthermore, biochemical analysis of compounds associated with virulence factors, such as lipoproteins, mono-oxygenase, and various polysaccharides, is a valuable tool in diagnosis (Bartoli *et al.,* 2015).

PCR-Based Diagnostic Methods: DNA-based techniques, especially the amplification of 16S and 23S rRNA genes via PCR, are commonly utilized to identify various *Pseudomonas* species and assess their genetic diversity (Olczak-Woltman *et al.,* 2007). In the case of *P. syringae*, the *syrB* gene serves as a marker for identifying syringomycin production. Additionally, species-specific primers targeting lipoprotein and monooxygenase genes are employed for diagnostic purposes in multiplex PCR for *P. viridiflava* (Lipps *et al*., 2019).

DNA Sequencing and Phylogenetic Analysis of *Pseudomonas* Species: Multilocus sequence analysis (MLSA) and other gene sequencing methods allow for the rapid and accurate identification of *Pseudomonas* species that cause diseases in alfalfa (Berge *et al*., 2014). By using conserved genes such as 16S rRNA, *gyrB*, and *rpoD*, researchers can reliably analyze interspecies relationships (Yamamoto *et al*., 2000). MLSA serves as an effective tool for identifying pathovars within the *Pseudomonas syringae* complex and for examining strain diversity (Parisi *et al*., 2019). The genetic data obtained from these analyses are utilized to construct phylogenetic trees, which help us understand the evolutionary relationships and the diversity of pathogenicity factors in complex species like *P. syringae* and *P. viridiflava* (Bull & Koike, 2015).

Genomic and Molecular Analyses in *Pseudomonas* Species: With the recent widespread adoption of whole-genome sequencing methods, researchers are closely examining the genetic differences among various pathovars of *Pseudomonas syringae* and the evolutionary conservation of factors related to pathogenicity. These analyses provide valuable insights into horizontal gene transfer, the diversity of effector proteins, and adaptations to different environments, significantly enhancing our ability to detect and classify pathogens (Parisi *et al*., 2019). Molecular fingerprinting techniques, such as rep-PCR, are particularly effective for the intraspecific identification of pathogenic species like *P. syringae* and *P. viridiflava*, and they play a crucial role in monitoring disease outbreaks (Bull & Koike, 2015).

CONCLUSİONS

Two species of the genus *Pseudomonas* cause alfalfa (*Medicago sativa*) diseases, resulting in serious issues such as yield losses and reduced plant lifespan. *Pseudomonas syringae* and *Pseudomonas viridiflava* play a pivotal role in the spread of these diseases. This study provides critical insights into the disease mechanisms, infection processes, and impacts of these *Pseudomonas* species on plants, contributing valuable information for developing effective management strategies for these pathogens.

In conclusion, developing resistant alfalfa cultivars and advancing biological control methods could offer long-term, sustainable solutions for combating these bacterial pathogens. Additionally, the broader adoption of modern diagnostic techniques for detecting and monitoring the spread of bacterial diseases will be an essential step in disease management. In this context, the presented findings hold significant importance for scientific research and agricultural applications.

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BOTRYTIS BLIGHT OF BLUEBERRY IN BOSNIA AND HERZEGOVINA*

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Original scientific paper

Abstract

In June 2022, blight symptoms of blueberry plants (cv. Bluecrop) were observed in a blueberry plantation in Bihać, Bosnia and Herzegovina. Blackish-brown young shoots, leaves, flower parts and fruits, and brownish mycelia and conidia were observed. Approximately 5% of the plants were affected. The causal fungus was identified as *Botrytis cinerea* based on cultural, morphological, and molecular characteristics. Pathogenicity studies were performed in a greenhouse with 2-year-old blueberry plants (cv. Bluecrop). This is the first report of *Botrytis cinerea* causing the blight disease of blueberries in Bosnia and Herzegovina.

Key words: *Botrytis cinerea, Botrytis blight, Blueberry, Bosnia and Herzegovina*

INTRODUCTION

Blueberry species, which belong to the *Vaccinium* genus of the *Ericaceae* family, are a type of fruit adapted to the temperate climate zone. There are three different types of blueberries that are economically cultivated: highbush form (*Vaccinium corymbosum*), lowbush form (*Vaccinium angustifolium*) and rabbit eye (*Vaccinium ashei*). Blueberries like organic matter-rich and acidic soils. Blueberries, which have a high antioxidant content, are used for many different purposes. These are: as fresh fruit, in the fruit juice industry, in the pharmaceutical industry, in milk and dairy products technology, in dried fruit technology, in fruit bread, buns, cakes, puddings and pastries, in the spice industry, in fruit salads, in jam, marmalade and canning industry, in tea, diet menus, in wine making and in the production of plant handles (stalks) (Çelik, 2005).

The commercial production of highbush blueberry in Bosnia and Herzegovina has been expanded in recent years. However, the limiting factor in producing plantation highbush blueberry is the soil, since this plant can only be grown on acidic soils. As an alternative, growing in bags or pot containers can be possible. Despite certain shortcomings, blueberry cultivation in containers, compared to the current cultivation method, is still much more efficient, both from a technological and an economic point of view.

The area of the city of Bihać has a moderately continental climate. It is characterized by warm summers and cold winters. Absolute summer temperatures can reach 40 °C, while winter temperatures can drop to -35 °C (Kurtović *et al*., 2013). The annual average precipitation is 1327/m², and the average monthly temperature is 10.8 ˚C.

In June 2022, blight symptoms of highbush blueberry (*Vaccinium corymbosum*) plants (cv. Bluecrop) grown in container bags were observed in a blueberry plantation in Bihać, Bosnia and Herzegovina. Blackish-brown young shoots, leaves, flower parts, and fruits and brownish mycelia and conidia were observed. Approximately 5% of the plants were affected. This study was undertaken to identify the causal agent of the disease.

MATERIALS AND METHODS

The highbush blueberry plantation surveyed was established in 2018, in Bihać (Ružica locality), Bosnia and Herzegovina, with an area of 0.65 ha. One-year-old seedlings in containers were planted in geotextile bags with a volume of 40 liters. The substrate used for planting is especially intended for blueberry, which included a mixture of white and brown peat with a pH value of 3.5 to 4.8, coconut fibers, perlite, NPK, and microelements. Natural spring water was used for irrigation with a drip system.

In the surveyed highbush blueberry plantation, disease symptoms were observed on plants. Diseased shoots, leaves, flower parts and fruits were brought to the laboratory and small pieces (0.5-1 cm) were surface sterilized with 1% NaOCl for 1 min and they were placed on Potato Dextrose Agar (PDA) plates. After fungal development in PDA, mycelia, conidiophore, conidia and sclerotia were examined under a light microscope and a stereomicroscope.

For molecular characterization of *Botrytis cinerea* isolate, mycelium grown in Potato dextrose agar (PDA) medium for 10 days at 23 ± 1 °C was scraped with a sterile spatula and disrupted with extraction buffer, and DNA was extracted with a DNA extraction kit (QIAGEN). Polymerase chain reaction (PCR) analysis, was carried out according to Rigotti et al., (2006). PCR amplification with primers BC108+(5'-ACCCGCACCTAATTCGTCAAC-3') and BC563–(5' GGGTCTTCGATACGGGAGAA-3') was performed. One cycle at 95 °C for 3 min, followed by 34 cycles at 94 °C for 20 s, 59 °C for 20 s and 72 °C for 30 s and final extension at 72 °C for 3 min were carried out. Additionally, PCR analysis was conducted on the internal transcribed spacer (ITS) region (ITS1-ITS4) in accordance with the methodology proposed by Behr et al., (2013). The initial denaturation was conducted at 98 °C for a period of two minutes. Subsequently, 30 cycles were performed, each comprising denaturation at 98 °C for 15 seconds, annealing at 64 °C for 20 seconds, and elongation at 72 °C for 20 seconds. Finally, a final elongation step was carried out at 72 °C for 10 minutes. The PCR product was electrophoresed on a 1% agarose gel containing ethidium bromide at a concentration of 0.1 µg ml⁻¹ in 0.5 x Tris-borate-EDTA (TBE) at 100 V for 3 hours. The gel was photographed under UV light (Quantum ST4, Montreal Biotech, Canada) and the DNA fragment size in the gel was

compared with the GeneRuler 100 bp DNA ladder Plus (MBI Fermentas, USA). For fulfilling Koch's postulates, pathogenicity studies were performed in a greenhouse with 2-year-old blueberry plants (cv Bluecrop). 1 x 10^5 conidia ml^{-1} were sprayed onto shoots and leaves and covered with plastic bags for 3 days. The greenhouse temperature ranged between 17±2/24±2 °C night/day. Control plants were sprayed with sterile water only.

RESULTS AND DISCUSSION

Blackish-brown symptoms on young blueberry shoots, leaves, flower parts, and fruits, and brownish mycelia and conidia were observed (Figure 1).

Figure 1. Disease symptoms observed in a highbush blueberry plantation in Bihać, Bosnia and Herzegovina

On Potato Dextrose Agar medium, brownish gray mycelia, black sclerotia (2-4 mm in diameter, n=30), and long and branched conidiophores were observed. Hyaline and ovate conidia were one-celled. Conidial measurements were (7.5) 7.95 (10) x (8.75) 13.06 (16.25) μm (n=30). The causal fungus was identified as *Botrytis cinerea* based on morphological characteristics (Ellis, 1971; Caruso & Ramsdell, 1995). PCR amplification with primers BC108+ (5'-ACCCGCACCTAATTCGTCAAC-3') and BC563–(5' GGGTCTTCGATACGGGAGAA-3') was performed and a 480 bp DNA

fragment was observed (Rigotti *et al.,* 2006). After amplification of the complete ITS rDNA of the *B. cinerea* isolate using ITS1-ITS4 primers a band was observed at 576 kb (Behr et al., 2013). ITS1-ITS4 sequence results showed 99.18% sequence similarity with other ITS sequences of *B. cinerea* in GenBank (Accession numbers: KX061437.1, KY828219.1, KY114879.1). The sequence results were submitted to NCBI (Accession No: PQ686329). These morphological and molecular studies confirmed the fungus as *Botrytis cinerea* Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel.

Rigotti *et al.* (2002) used the species-specific primers C729+/– (5'-AGCTCGAGAGAGATCTCTGA-3'; 5'-CTGCAATGTTCTGCGTGGAA-3') to detect *Botrytis cinerea* isolates. However, these primers were not sufficient to molecularly identify some of the *Botrytis cinerea* isolates obtained from different host plants. Therefore, they designed two new primers, BC108+ and BC563–. These new primers amplified a DNA fragment of 480 bp for the main group of 26 *Botrytis cinerea* strains and 360 bp for another group of 13 strains of *Botrytis cinerea*. Other closely related species such as *B. allii* and *B. fabae* from the genus *Botrytis* could not be amplified with these primers, confirming the specificity of the BC108+ and BC563– primer for *B. cinerea* and identifying it as a precision molecular tool for detecting this fungus in host plants (Rigotti *et al*., 2006). In our study, a DNA fragment was observed at 480 bp in accordance with Rigotti *et al*. (2006).

Fourteen days after inoculation of 2-year old blueberry plants (cv Bluecrop) in the greenhouse, small blackenings were observed in shoots (2 mm) and leaves (5 mm). The disease progressed with time (Figure 2). *Botrytis cinerea* was reisolated from these shoots and leaves. Control plants showed no symptoms.

These morphological, molecular and pathogenicity studies confirmed the *Botrytis cinerea* as the causal agent of blueberry blight disease observed in Bihać. This is the first report of *Botrytis cinerea* causing the blight disease of blueberries in Bosnia and Herzegovina.

Figure 2. Under greenhouse conditions, *Botrytis cinerea* showed pathogenicity to highbush blueberry leaves and shoots

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) is an ascomycetous polyphagous pathogen and affects a number important fiber, protein, oil, and horticultural crops in temperate and subtropical regions. It is also an important pathogen under storage conditions (Williamson *et al*., 2007). Foster *et al*. (2024) reported *Botrytis cinerea* causing disease in blueberries in South Africa and Kwon *et al*. (2011) reported *B. cinerea* from Korea as a postharvest pathogen. Dil *et al*. (2013) reported a *Botrytis* sp. affecting blueberries grown in Rize province of Türkiye. They reported that Botrytis leaf spot was the most common disease of blueberries in Rize, Türkiye.

CONCLUSIONS

The commercial production of highbush blueberry in Bosnia and Herzegovina has been expanded in recent years. However, diseases can hamper the blueberry production. *Botrytis cinerea* can cause disease in over 200 mainly dicotyledonous plant species and is also a significant pathogen under storage conditions (Williamson *et al.*, 2007). This study is the first to demonstrate that *Botrytis cinerea* caused disease in blueberries grown in Bihać, Bosnia and Herzegovina. It is important to implement control measures for this significant pathogen.

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SIVA TRULEŽ BOROVNICE U BOSNI I HERCEGOVINI

Rezime

U junu 2022. godine uočeni su simptomi prisustva gljivice kod mladih biljaka borovnice (cv. Bluecrop) na plantaži borovnice u Bihaću, Bosna i Hercegovina. Uočeni su crnosmeđe obojeni mladi izdanci, listovi, dijelovi cvjetova i plodovi, te smeđkaste micelije i konidije. Približno 5% biljaka je bilo zaraženo. Uzročnik je identificiran kao *Botrytis cinerea* na osnovu kulturnih, morfoloških i molekularnih karakteristika. Istraživanja patogenosti vršena su u stakleniku na dvogodišnjim biljkama borovnice (cv. Bluecrop). Ovo je prvi izvještaj o uzrokovanju sive truleži (*Botrytis cinerea*) borovnice u Bosni i Hercegovini.

Ključne riječi: *Botrytis cinerea, siva trulež, borovnica, Bosna i Hercegovina*

DISTRIBUTION OF OAT STEM RUST IN SOME PROVINCES OF V©TM [G*

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Original scientific paper

Abstract

Oat stem rust, caused by the fungus *Puccinia graminis* f. sp. *avenae*, is one of the most damaging diseases affecting oats. It is particularly problematic in regions with relatively low temperatures and high humidity, where oats are cultivated. This disease significantly reduces both the yield and quality of oat crops by diminishing the photosynthetic area of the plants. In 2023, surveys were conducted in various oat-growing areas across several provinces in Türkiye, including Bolu, Afyonkarahisar, Kastamonu, ¥ cpm̤t,."Gf ktpg."Quo cpk{ g."Cf cpc."O gtuŋp."Mcj tam cpo ctc ."I c| kcpvgr. and Hatay during May, June, and July. The findings indicated that the prevalence of oat stem rust was 25% in Bolu and Afyonkarahisar, 50% in Edirne, 48% in Kastamonu, and 39' "kp"¥ cpm̤t,0'No cases of oat stem rust were found in the surveys conducted in Quo cpk{ g." Cf cpc." O gtuŋp." Mcj tam cpo ctc ."I c| kcpvgr ."cpf " J cvc{ "r tqxkpegu0' Vq" combat this pathogen, effective control measures should be implemented.

Keywords: *Oat, Oat stem rust, Puccinia graminis f. sp. avenae, Türkiye*

INTRODUCTION

Oat (*Avena sativa* L.) is a cereal plant that serves multiple purposes in human nutrition, animal feed, and industrial applications worldwide. Compared to wheat and barley, which were cultivated long ago, oats were brought into cultivation later and were long considered just a wild grass. Despite this, oats have a history spanning two thousand years and rank third among cool-season cereals globally in terms of planting and production, and fourth in Türkiye after wheat, barley, and rye. Recently, the rising significance of oats in human nutrition and their industrial uses has led to an expansion in production areas. Oats are typically grown in marginal environments, such as cool,

rainy climates and low-fertility soils, compared to other cereals (Hoffmann, 1995; Geçit, 2016). The most common use of oats is for animal feed. Oat stalks are softer, and oat leaves are more abundant, which makes them richer in organic and mineral substances compared to wheat and barley straw. Animals enjoy eating oats, which are provided in various forms such as green grass, hay, grain, and straw. Oats are also included as a support plant in mixtures with legume forage plants (e.g., forage pea, vetch). Animals tend to prefer oats over other cereals due to their soft stalks and lush leaves. In recent years, oats have gained popularity in human nutrition, particularly in the form of oat flour and oatmeal, especially for breakfast. In addition to being rich in protein, oats contain significant amounts of oil, vitamin B1, phosphorus, iron, and various minerals, enhancing their nutritional value (Anderson, 2000; Geçit, 2016). In Türkiye, oat farming occurs in both dry and irrigated regions, with significantly more activity in dry areas compared to irrigated ones. While growing oats is generally more suitable in rainy or irrigated conditions, the actual cultivation of oats in irrigated areas remains relatively low. Oat farming takes place to varying degrees in eigj v'qh'yj g''VÃ˜tmk{ gøu''ci tkewnwtcn regions, excluding the Southeastern agricultural region. The regions where oat farming is practiced include Mid-south, Mid-north, Marmara, Aegean, Black Sea, Mediterranean, Mid-east, and Northeast (Geçit, 2016). The significance of oats has increased in Türkiye due to enhanced producer support, resulting in a rise in cultivation areas over the years. In 1991, the area allocated for oat cultivation was 132,000 hectares. This figure rose to 158,500 hectares by 2008. However, after 2008, the cultivation area dropped to 85,863 hectares in 2011, before increasing again to 109,820 hectares in 2019. Additionally, the area in Türkiye planted with oats for green grass expanded from 825,512 decares in 2012 to 2,560,078 decares in 2019 (Anonymous, 2020). Oats thrive in relatively cool temperatures and high humidity, which makes them more susceptible to fungal diseases due to their significant water requirements compared to other cool-climate cereals. One major concern for oat cultivation is rust diseases, particularly oat stem rust, which is caused by the basidiomycetous pathogen *Puccinia graminis* Pers. f. sp. *avenae* Erick. & Henn. This disease is especially problematic in regions with the cool and humid conditions that favor oat growth. Oat stem rust can affect the leaves, stems, and ears of the plant. The pustules produced by the pathogen break through the plant's epidermis, reducing the available photosynthetic area and resulting in significant decreases in both yield and quality. In severe cases, the stems and leaf sheaths may become heavily covered with rust pustules, potentially causing the stems to break. Furthermore, when oats are used as green grass, silage, grain, or hay for animal nutrition, the presence of stem rust can diminish forage quality. The fungus responsible for oat stem rust can produce new races during its sexual reproduction phase and through mutations (Martens, 1985; Rodriguez-Algaba *et al*., 2022). This adaptability allows the fungus to infect previously resistant oat varieties, potentially leading to new epidemics. Therefore, it is crucial to evaluate the impact of this disease on oat production in Türkiye and implement appropriate control measures. This study aimed to examine the distribution of oat stem rust disease across different regions of Türkiye.

MATERIALS AND METHODS

In May, June, and July 2023, surveys were conducted in the oat cultivation areas of the r tqxkpegu" qh" Mcuvco qpw." ¥ cpm,." Dqnw." Ch{qpmctcj ucr." Gf krpg." Mcj tco cpo ctc ." Adana, Mersin, Hatay, Osmaniye, and Gaziantep in Türkiye (Figure 1).
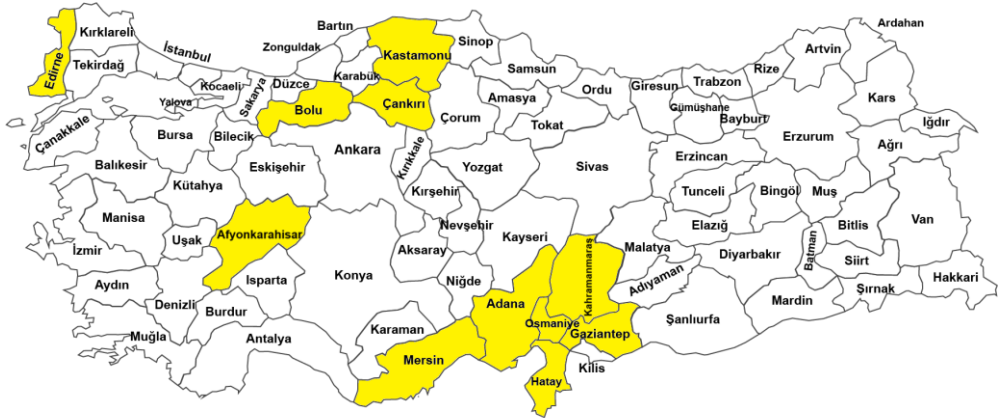
Figure 1. Turkish provinces surveyed for oat stem rust in 2023

U{uvgo cvke"uco r npi "a gyj qf "y cu"wugf "kp"yj g"uwtxg{u"*Cmc , 2021). Samples were taken from the fields by stopping approximately every 5-30 km. In these fields, sampling points were made randomly within the field according to the size of the field. The surveys took into account the grain production and grass production periods of oat plants. While the grain production periods vary by region, they generally occur in June and July, and the grass production periods typically occur in May and June. The surveys were conducted with these time frames in mind. In the surveys, prevalence of the stem rust disease in oat cultivation areas was determined. In the surveys, the presence/absence of the disease in each field was recorded. One hundred forty oat fields were surveyed in the Turkish provinces of Bolu (24 fields), Afyonkarahisar (8 fields), Mcuvco qpw"*57 hkgnf u+.'¥ cpmt,"*67 hkgnf u+.'Gf krpg"*6 hkgnf u+.'Quo cpk{ g"*6 hkgnf u+.'Cf cpc" (10 fields), Mersin (3 fields+.'Mcj tco cpo ctc "*6 hkgnf u+.'I c| kcpvgr "*4 hkgnf u+.'cpf 'J cvc{ (1 field). As the field size increased, more samples were taken (Table 1). At each location, at least 100 plants were inspected.

Vcdng"30'Hkgnf "uk| g"cpf "pwo dgt "qh'uco r npi "nqecvkqpu"*Cmc , 2001).

Field size	Number of sampling locations
Up to 10 decares	At least 5 different locations
11-100 decares	At least 10 different locations
101-500 decares	At least 15 different locations
Over 501 decares	At least 20 different locations

RESULTS AND DISCUSSION

Oat stem rust was detected in 6, 2, 2, 17, and 8 oat fields in Bolu, Afyonkarahisar, Edirne, Kastamonu, cpf "¥cpmᵣ,"rtqxⱡpegᵤ, respectively (Figure 2). The percentages of oat stem rust disease was found to be 25% in Bolu and Afyonkarahisar provinces, 50% ⱡp"Gfⱡᵣpg"rtqxⱡpeg."6:'"ⱡp"Mcuⱱcoqwⱼ"rtqxⱡpeg."cpf"39'"ⱡp"¥cpmᵣ,"rtqxⱡpeg"fwtⱡpi" field inspections (Table 2). Oat stem rust disease was not encountered in the surveys eqpfwⱱeⱱf"ⱡp"Quoⱥpkⱡⱥg."Cfⱥpc."Ogtuⱡp."Mcjtcoⱥpⱥtⱥ."Icⱬⱡcpⱱgr."cpf"Jⱥvⱥⱥ{" provinces. However, the number of investigated fields were limited in some provinces including Hatay and Gaziantep. The lack of oat rust disease in these regions may be attributed to the climatic conditions and the specific oat cultivars grown. It is possible that the disease was not detected in surveys in these regions due to insufficient spring rainfall and high temperatures.

Figure 2. Oat stem rust symptoms observed in Kastamonu province of Türkiye

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces qh''Dqnw.''Ch{qpnctcj kuct.''Mcuvco qpw.''¥cpnµt,.''Gf ktpg.''Quo cpk{g.''Cf cpc.''O gtukp.'' Mcj tco cpo ctc .''I c| kcpvgr .''cpf''J cvc{''vq''cuuguu''vj g''status of oat stem rust disease

Province	District	Village	GPS Coordinates	Altitude	Disease situatio
Bolu	Merkez	Karamanlar	40.792432 31.806046	724 m	Absent
	Merkez	Mcpf co ,	40.782618 31.838197	776 m	Absent
	Merkez	[c{ncf ,nct	40.789156 31.846775	769 m	Absent
	Merkez	Ulumescit	40.791873 31.868373	892 m	Absent
	Merkez	Uc| cnnp,m	40.768776 31.824105	810 m	Absent
	Merkez	Yuva	40.763587 31.788537	747 m	Absent
	Merkez	¥c{i ¾nr ,nct	40.719842 31.737113	859 m	Absent
	Merkez	Vakifgeçitveren	40.724709 31.693848	757 m	Absent
	Gerede	Sofular	40.762166 32.199602	1213 m	Absent
	Gerede	Sofular-C wtnwo gxnkk	40.770856 32.189385	1245 m	Absent
	Gerede	Sofular-Two cj mt, mevkii	40.764579 32.199440	1240 m	Absent
	Gerede	[c| ,nctc	40.754919 32.210221	1206 m	Absent
	Gerede	[c| ,nctc-O g| ctn,m mevkii	40.757737 32.200750	1206 m	Absent
	Gerede	¥q wnw	40.726320 32.200560	1162 m	Present
	Gerede	¥q wnw- Gübüzler mevkii	40.736601 32.184480	1171 m	Absent
	Gerede	Nuhören	40.771136 32.166371	1265 m	Absent
	Gerede	Nuhören-Mcf ,mt, mevkii	40.762729 32.169082	1261 m	Present
	[gpk±c c	Hamzabey	40.783159 31.998186	998 m	Present

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces of Dqnw."Ch{qpnɪtcjkuct."Mcuvcoqpw."¥cpn,t,."Gfktpg."Qoocpk{g."Cfcpc."Ogtukp. Mcjtcocpoctc ."Ic|kcpvgr."cpf"Jcvc{vq"cuuguu"vjge"uɪvcvwu"qf"qcv"uvgo"twuv"fkugcug (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
Bolu	[gpkɪtc c	Dereköy	40.814708 31.961013	1121 m	Present
	[gpkɪtc c	Kemaller	40.779020 31.958742	1185 m	Present
	[gpkɪtc c	Örenköy	40.766063 32.001083	1022 m	Absent
	[gpkɪtc c	M,pf ,tc	40.822883 31.981536	1168 m	Absent
	[gpkɪtc c	M,pf ,tc-Büyükçam mevkii	40.812813 31.981651	1121 m	Present
	[gpkɪtc c	Gunkɪtc c	40.818581 32.034908	1008 m	Absent
Afyonkarahisar	Bolvadin	F k ɪk˙Mcucdcu,	38.780853 31.053032	1075 m	Present
	Bolvadin	F gtgmɪtcdc	38.712369 31.162270	969 m	Absent
	Bolvadin	DÃ{Ãmɪtcdc	38.766096 31.251273	1101 m	Absent
	Bolvadin	Yörük Karacaören	38.826965 31.079002	1206 m	Present
	Go ktf c	Güneysaray	38.990078 31.070070	1132 m	Absent
	Go ktf c	Güneysaray- Belen mevkii	39.004113 31.037627	1127 m	Absent
	Go ktf c	Tabaklar	39.031513 31.090677	1021 m	Absent
	Go ktf c	Tabaklar-Köyönü mevkii	39.048908 31.087348	960 m	Absent
Kastamonu	Merkez	Gömeç	41.438023 33.827262	713 m	Absent
	Merkez	Gölköy	41.456969 33.746371	732 m	Absent
	Merkez	Ömerli	41.437114 33.866896	797 m	Absent

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces qh¨Dqnw."Ch{qpmctcj kuct."Mcuvco qpw."¥¨cpmt,."Gf ktpg."Qom cpk{ g."Cf cpc."O gtukp." Mcj tco cpo ctc ."I c| kcpvgr ."cpf "J cvc{"vq"cuuguu"vj g"utcvwu"qh"qcv"uwgo "twuv"f kugcug (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
	Merkez	Dq|q rcm	41.461317 33.895264	736 m	Present
	Merkez	Q wm¾{	41.461279 33.909330	747 m	Present
	Merkez	Emirler	41.463075 33.765818	741 m	Present
	Merkez	C c ,dcvcm	41.478971 33.951202	688 m	Absent
	Merkez	Alatarla	41.495506 34.028122	643 m	Present
	Merkez	J crc±n,	41.461743 33.960728	685 m	Absent
	Merkez	Duruçay	41.489096 33.769956	813 m	Absent
	Merkez	Emirli	41.453496 33.875009	743 m	Absent
Kastamonu	Merkez	Vc n,m	41.421244 33.712529	857 m	Present
	Merkez	Çavundur	41.472517 33.984066	645 m	Present
	Merkez	Teke	41.316831 34.067255	907 m	Present
	Merkez	Kasaba	41.479390 33.681155	832 m	Present
	Merkez	Küçüksu	41.515614 33.717935	967 m	Present
	Merkez	J ce, cdcp	41.504560 33.694899	946 m	Present
	Devrekani	Cucte,m	41.706252 34.046871	1214 m	Present
	Vc n¾r tÃ	Merkez	41.494221 34.210672	582 m	Present
	Vc n¾r tÃ	M,tj c	41.484328 34.192886	621 m	Present
	Vc n¾r tÃ	Cnf g kto gp	41.469180 34.008389	648 m	Absent

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces qh˝Dqnw.˝Ch{qpnıtcj kuct.˝Mcuvco qpw.˝¥cpnıt,.˝Gf ktpg.˝Quo cpk{g.˝Cf cpc.˝O gtukp.˝ Mcj tco cpn ctc .˝I c| kcpvgr.˝cpf ˝Hatay to assess the status of oat stem rust disease (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
Kastamonu	j ucpi c\ k	Çay	41.220979 33.549267	864 m	Present
	j ucpi c\ k	Sevindik	41.220043 33.575209	882 m	Present
	j ucpi c\ k	Mw ±wnct	41.222985 33.535342	834 m	Present
	j ucpi c\ k	pek g\	41.254972 33.494885	1099 m	Absent
	j ucpi c\ k	Enbiya	41.216431 33.513004	824 m	Present
	Seydiler	Merkez	41.617861 33.707887	1039 m	Absent
	Seydiler	[wnct,˝Cuncpn,	41.636943 33.708615	1030 m	Absent
	Seydiler	C c ,˝Cuncpn,	41.638740 33.682305	1015 m	Absent
	Seydiler	[c nct	41.611671 33.743468	1054 m	Absent
	Seydiler	cni co	41.653166 33.668125	1018 m	Absent
	Seydiler	Demirciözü	41.611946 33.645084	1080 m	Absent
	Seydiler	Qf cdc ,	41.638487 33.655707	1014 m	Absent
	Seydiler	Sahbat	41.628096 33.711774	1027 m	Absent
	Seydiler	O cpe,nık	41.646982 33.627923	1021 m	Absent
¥cpnıt,	[cr tcnk,	Merkez	40.758731 33.782780	1173 m	Present
	[cr tcnk,	[wnct,˝o cj cnkg	40.753591 33.771949	1184 m	Absent
	[cr tcnk,	Cnk(c\ ,	40.743919 33.832042	1024 m	Present
	[cr tcnk,	f kt	40.770089 33.814044	1176 m	Present

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces of Bolu, Ch{qpmctcj kuct."Mcuvco qpw."¥ cpmt,."Gf ktpg."Quo cpk{ g."Cf cpc." O gtukp. Mcj tco cpo ctc ."I c| kcpvgr ."cpf "J cvc{ "vq "cuuguu "vj g "uvcvwu "qh "qcv "uvgo "twuv'f kugcug (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
¥ cpmt,	[cr tcm,	Yüklü	40.681434 33.792520	867 m	Present
	[cr tcm,	Bugay	40.720040 33.76322	1028 m	Absent
	[cr tcm,	Buluca	40.739735 33.794060	1116 m	Absent
	[cr tcm,	Uc| ec ,|	40.706255 33.801085	937 m	Absent
	[cr tcm,	nk| ¾gp	40.682676 33.87468	1176 m	Absent
	Merkez	pc±	40.627833 33.700085	827 m	Absent
	Merkez	Tuzlu	40.597248 33.678758	904 m	Absent
	Merkez	Dc{,pf ,t	40.621879 33.819211	1107 m	Absent
	Merkez	Küçüklü	40.589638 33.807994	1126 m	Absent
	cdcp¾ Ã	I Ãtr ,pct	40.503781 33.307665	1136 m	Absent
	cdcp¾ Ã	Mart	40.424840 33.393881	995 m	Absent
	cdcp¾ Ã	I Ão gtf k kp	40.457119 33.250321	1077 m	Absent
	Korgun	Büyük mahalle-Ova mevkii	40.715897 33.523893	848 m	Absent
	Korgun	Wnwf c	40.747414 33.498544	1011 m	Absent
	Eldivan	Merkez-Höyük	40.516843 33.522116	1202 m	Absent
	Eldivan	Sarayköy	40.535606 33.480773	946 m	Absent
	Eldivan	Merkez-Çelebi	40.511324 33.518367	1182 m	Absent

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces of Dqnw." Ch{qpnctcj kuct." Mcuvco qpw." ¥cpm̦t,." Gfktpg." Qom cpk{g." Cfcpc." M gtukp. Mcj tco cpm cta ." I c| kcpvgr." cpf "J cva{ vq "cuuguu" vj g "uvcvwu" qh" qcv" uvgm "twuv" f kugcug (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
¥cpm̦t,	Eldivan	Seyidi	40.570706 33.473780	912 m	Absent
	Eldivan	Çiftlik	40.581513 33.493342	887 m	Absent
	Eldivan	I ¾g| mc{,	40.515514 33.545795	1000 m	Absent
	M,| ,n̦to cm	Uctc{e,m	40.290132 33.928717	648 m	Absent
	M,| ,n̦to cm	Mctcm,	40.289432 33.863436	678 m	Absent
	M,| ,n̦to cm	Mctcm,-[c{,ri cp mevkii	40.289764 33.871309	674 m	Absent
	M,| ,n̦to cm	Merkez-Hüseyinli	40.348376 34.02119	569 m	Absent
	M,| ,n̦to cm	[wmct, "Crci ¾	40.393057 33.891104	636 m	Absent
	M,| ,n̦to cm	Tepe Alagöz	40.381526 33.966605	600 m	Absent
	M,| ,n̦to cm	J cmc±n̦	40.286649 33.943914	635 m	Absent
	Orta	M,uc±"	40.648418 33.034105	1346 m	Present
	Orta	Mcpnec	40.640342 33.088706	1287 m	Present
	Orta	Kalfat	40.687073 33.082792	1292 m	Absent
	Orta	Dw w¾gp	40.639973 33.005142	1367 m	Absent
	Orta	Dodurga	40.603362 32.99397	1370 m	Absent
	Orta	DÃ f Ã̦	40.595664 33.067013	1284 m	Absent
	Orta	J cucp "J ce,	40.593198 33.060053	1285 m	Absent

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces qh''Dqnw.''Ch{qpnctcj kuct.''Mcuvco qpw.''¥cpnt,.''Gf ktpg.''Quo cpk{g.''Cf cpc.''O gtukp.'' Mcj tco cpn ctc `.''I c| kcpvgr.''cpf ''J cvc{''vq''cuuguu''vj g''utcvwu''qh''qcv''uvgo ''twuv''f kugcug (continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
¥cpnt,	Orta	Yaylakent	40.599518 33.076971	1276 m	Present
	¥gtng	Dc{,pf ,t	40.826971 32.780433	1098 m	Absent
	¥gtng	Kuzdere	40.819080 32.708355	1202 m	Absent
	¥gtng	Göynükçukuru	40.828919 32.659420	1144 m	Present
	¥gtng	Çördük	40.849609 32.718056	1308 m	Absent
	Bayramören	Dalkoz	40.947670 33.225827	904 m	Absent
	Bayramören	Erenler	40.940311 33.134386	1000 m	Absent
Edirne	Enez	Karpuzlu	40.804066 26.280752	5 m	Absent
	Enez	Yeniceköy	40.686800 26.138559	38 m	Present
	Enez	Sultanice	40.612551 26.145290	9 m	Absent
	Enez	Küçükevren	40.640566 26.171860	25 m	Present
Osmaniye	Toprakkale		37,076015 36,121265	55 m	Absent
	Toprakkale	Sayhüyük	37,077370 36,121281	46 m	Absent
	Toprakkale	Atatürk cad.	37,072376 36,113327	47 m	Absent
	Merkez		37,346863 36,078526	76 m	Absent
Adana	Ceyhan		37,0088987 35,706173	24 m	Absent
	Ceyhan		37,008377 35,701595	28 m	Absent

Table 2. In 2023, surveys were conducted in oat cultivation regions across the provinces qh "Dqnw." Ch{qpnctcj iuct." Mcuvco qpw." ¥ cpnt,." Gf ktpg." Quo cpk{g." Cf cpc." O gtukp." Mcj tco cpo ctc ." I c| icpvgr ." cpf "J cvc{ vq "cuuguu"vjg "uvcvwu"qh"qcv"uvgo "ruwv"f ikugcug"
(continued)

Province	District	Village	GPS Coordinates	Altitude	Disease situation
Adana	Ceyhan		36,997749 35,679161	48 m	Absent
	[Ãtg kt	Cnr ,pct	36,820152 35,595577	128 m	Absent
	[wo wtvcn,m		36,785324 35,642174	24 m	Absent
	Mctckucn,		37,065678 35,171108	274 m	Absent
	Kozan		37,427738 36,707478	107 m	Absent
	Kozan		37,465374 35,669548	156 m	Absent
	Kozan	[cj {cn,	37,457054 35,682716	135 m	Absent
	Kozan	[cj {cn,	37,447369 35,693237	121 m	Absent
Mersin	Silifke	Sömek	36,591892 34,062874	855 m	Absent
	Silifke		36,591831 34,058262	5 m	Absent
	Silifke		36,628815 33,999493	6 m	Absent
Mcj tco cpo ctc	Rc| cte,m		37,447224 37,211380	901 m	Absent
	Rc| cte,m		37,355000 37,018230	749 m	Absent
	Rc| cte,m		37,310482 36,793736	763 m	Absent
	VÃtnq nw		37,244095 36,776432	485 m	Absent
Gaziantep	Araban		37,205166 37,474949	540 m	Absent
	Q w| gnk		37,183266 37,450790	746 m	Absent
Hatay	M,t ,nj cp		36,249306 36,4176933	84 m	Absent

Oat stem rust has been detected in several provinces of Türkiye. The significant presence and prevalence of this disease in some areas highlight the urgent need for prompt and effective action. The oat stem rust fungus is capable of producing new races, and currently cultivated varieties may be vulnerable to these emerging races (Boshoff *et al.,* 2019; Fetch *et al*., 2021; Sowa *et al*., 2021; Li *et al*., 2022). Comprehensive studies need to be conducted to assess the prevalence of oat stem rust and to identify the different races of the fungus. Additionally, it is essential to develop resistant oat varieties to combat these races. In a study conducted by Kahraman & Akan (2017a) in Türkiye, the responses of 48 oat genotypes to oat stem rust were evaluated at both the seedling and adult plant stages. Among these genotypes, 38 (79%) exhibited resistance during both the seedling and adult plant tests. Furthermore, the genotypes that demonstrated resistance in field trials also showed resistance in the seedling tests. In another study by Kahraman & Akan (2017b) conducted in Türkiye, the reactions of 40 oat genotypes to oat stem rust were evaluated. The results showed that 83% of the lines tested were resistant to oat stem rust at both the seedling and adult stages. Akan *et al*. (2016) examined the reactions of seven oat varietiesô Checota, Faikbey, Fetih, Mcj tco cp." Uct,." Ug{f k gj k.." cpf " [gpk±gtkô cv" y q" mecvkqps:" mk eg" *Cpmctc+" cpf " Seydiler (Kastamonu) using isolates collected from those regions.Greenhouse tests kpf kecvgf " vj cv" vj g" Ej geqvc." Hckmdg{." Hgvkj ." Mcj tco cp." Uct,." cpf " [gpk±gtk" xctkgvkgs" displayed resistance ratings (0-4+" ci ckpuv" vj g" mk eg" kuqmcvg0" Cf f kkqp cm{." vj g" Uct," cpf Yeniçeri varieties also demonstrated resistance (0-2) to the Seydiler isolate. Field tests tgxgcmgf " vj cv" kp" vj g" mk eg" mecvkqp." vj g" Ej geqvc." Hckmdg{." Hgvkj ." Mcj tco cp." Uct,." cpf " Yeniçeri varieties were classified as resistant (42Ö+0" Kp" vj g" Ug{f kmt" mecvkqp." vj g" Uct, and Yeniçeri varieties were found to be resistant (42Ö) as well. In South Africa, oat stem rust races RSJ, RJS and RJJ were identified (Boshoff *et al*., 2019) and moderate levels of adult plant resistance of seven cultivars against oat stem rust races RSJ, and RJS in 2017 and 2018 were reported. In Poland, 57 pathotypes of oat stem rust were detected, and the most dominant race was SSK (Sowa *et al*., 2021). Eight oat stem races were identified in China and using oat stem rust races TJD, TJN, and TKN, the resistance of 30 Chinese oat cultivars was evaluated at the seedling and adult plant stages. Oat cultivars Bayan 1, Baiyan 2, Baiyan 3, Baiyan 5, and Baiyan 9 were found resistant to three races in seedling and adult plant tests (Li *et al.,* 2022). In a study by Huerta-Espino *et al*. (2022), the resistance of 13 oat varieties and 37 lines to stem rust caused by *Puccinia graminis* f. sp. *avenae* was evaluated in both seedling and adult plant stages in Mexico. The study used TNQ and TFQ races, as well as isolates AMEX18.21.1.1 and AMEX18.18.1.1 for seedling evaluation, and AMEX18.18.1.1 isolate for adult plants. The Diamante, Menonita, Karma, and Turquesa varieties were found to be resistant to the TFQ race in the seedling stage but susceptible to the TNQ race and isolates AMEX18.21.1.1 and AMEX18.18.1.1. The other varieties were susceptible to both races and isolates. All varieties were susceptible to isolate AMEX18.18.1.1 in seedlings, but in adult plants, they were classified as susceptible, and intermediate, and only Teporaca was resistant. The 37 lines were categorized into 17 groups based on final severity and sister lines. Genotypes 25, 28, 34, 39, and 48 were seedling-resistant to AMEX18.18.1.1, while the rest were susceptible. In the field, all the lines were resistant,

and only genotype 19 of group 14 reached a 20% infection. Based on the response in seedlings and adult plants of the advanced lines, it is inferred that resistance is conditioned by adult plant genes. In Canada, the most common race of *Puccinia graminis* f. sp. *avenae* found in Manitoba and Saskatchewan was TGN, followed by SGB and TJS. The most frequent races identified in Ontario were TDJ, TDD, and TGN (Menzies *et al*., 2024).

CONCLUSIONS

Oat stem rust, caused by *Puccinia graminis* f. sp. *avenae*, has been found in the Turkish r tqxkpcegu"qh"Dqpw."Ch{qpmctcj ksct."Gf ktpg."Mcuvmo qpw"cpf "¥ kcpmt,0"Vj g"f kugcug"j cu"
pqv"dggp"f gvgevgf "kp"Qmo cpki{g."Cf cpc."O gtukp."Mcj tmc cpo ctc.,"I cz kcpvgr ."cpf "J cvc{
provinces. However, in some provinces disease was found in half of the fields inspected. Control measures should be implemented for this pathogen. Races of the pathogen should be determined and resistant oat varieties to the disease should be developed.

ACKNOWLEDGEMENT

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AMMONIUM OXALATE EXTRACTION OF IRON TRIAD FROM NATURAL CLAY SAMPLES*

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Original scientific paper

Summary

Acidic ammonium oxalate extraction is used to estimate the amount of amorphous or poorly crystalline iron in soils and sediments. Iron is the one element from the "iron triad" which have similar chemical behaviour. Other two elements are nickel and cobalt. The aim of this study was to evaluate acid ammonium oxalate extraction of all elements from iron triad. The samples of natural clay were used in the investigation. Samples for total amounts of metals were prepared with microwave digestion with HNO_3 and HF, and measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Ammonium oxalate extraction was done by earlier established procedure in acid medium and with absence of light. Measurements were conducted with atomic absorption spectroscopy with flame atomization (FAAS). For statistical evaluation, maximum, minimum, average, median and correlations were used. The investigated iron triad elements lie in the next sequence: Fe>Ni>Co. The poorly crystalline iron and ammonium oxalate extractable Ni showed higher correlation (r = 0.874) than ammonium oxalate extractable cobalt (r = 0.446), suggesting that iron and nickel are more similar regarding ammonium oxalate extractability.

Key words: *iron, nickel, cobalt, clay, ammonium oxalate extraction*

INTRODUCTION

It is common practice to employ the acid ammonium oxalate extraction method (McKeague and Day, 1966) to determine the quantity of amorphous or poorly crystalline Fe in soils and sediments. Actually, oxalate-extractable Fe is a measure of the free (x-ray) amorphous iron oxides, which are basically ferrihydrite and small amounts of organically-bound Fe (Cornell and Schwertmann, 2003). Ammonium oxalate is also used for sequential extraction procedures (Benitez and Dubois, 1999). Clay minerals belong to the family of phyllosilicate or sheet silicate minerals, which

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have layered structures (Edi *et al*., 2015). Clay minerals, which are primarily negatively charged, are highly effective at adsorbing metal ions. The charge on clay is mostly depending on the pH level. The adsorption of cations on the surface or between layers of clay is dependent on the pH level. The mineral surface of clay also attracts heavy metals. Heavy metal attraction is increased by smaller particle size (greater active surface)(Chen *et al*., 2016). Heavy metals directly connect to the clay surface by a number of intricate processes, including complexation, ion exchange, and direct bonding of metal cations (Yuan *et al*., 2013). The elements that belong to iron triad are all heavy metals. The Iron Triad is composed of three elements: iron (Fe), cobalt (Co), and nickel (Ni), which share similar chemical and physical characteristics (Muhammad e*t al*., 2022). They are found adjacent to each other in period 4 of the periodic table. Similarities of iron triad metals are presented in the table 1.

Table 1. Physic chemical characteristics of iron triad metals

	Iron	Cobalt	Nickel
Atomic Number	26	27	28
Molar mass, g mol^{-1}	55.85	58.93	58.69
Electron Configuration	$3d^64s^2$	$3d^74s^2$	$3d^84s^1$
Metallic radius, pm	124	125	125
Ionization Energy, kJ mol^{-1}	762.5	760.4	737.1
First	759	758	737
Second	1561	1646	1753
Third	2957	3232	3393
Common (+) Oxidation States	2, 3, 6	2, 3	2, 3
Melting Point (°C)	1530	1495	1455
Boiling Point (°C)	2862	2927	2732
Density, g cm^{-3}	7.87	8.90	8.91

From all the facts stated above arise the aim of this study: to evaluate possibilities to extract all of the elements of iron triad from natural clay with ammonium oxalate, and to get insight into extent of their similar behaviour.

MATERIALS AND METHODS

Samples
The "Rapailo" clay deposit in Central Bosnia and Herzegovina (43° 51′ 54″ N, 18° 13′ 36″ E) was used to provide seventeen natural clay samples with similar colour and texture. The site is situated in the Sarajevo Canton, near to Sarajevo, the country's capital (Figure 1). This region's clay material is dug up for brick manufacture. The area of the clay pit is 300 m wide and 1300 m long. The region with the clay deposits has a temperate continental climate. The average yearly temperature in this climate zone is 12 °C, with average highs of 1 °C in January and average lows of 22 °C in July. Precipitation reaches up to 1000 mm on average every year.
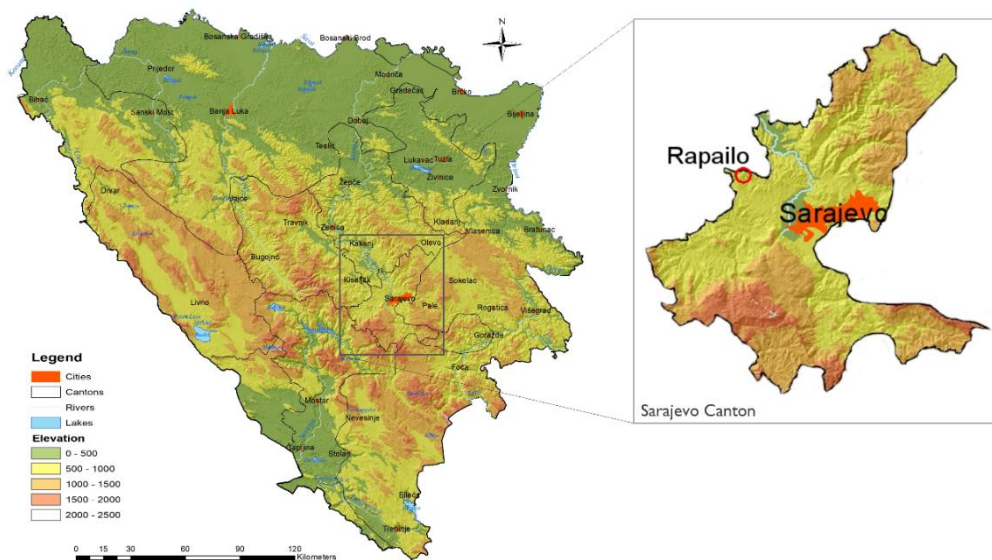
Figure 1. Map of Bosnia and Herzegovina with sampling position (Jurkovic *et al.*, 2021)

Methods

Several analytical techniques were applied in this study. The following parameters were examined for the fundamental physicochemical analysis: electrical conductivity (EC), oxidation-reduction potential (ORP), and pH values in water and calcium chloride. Measurements were also made of the total and ammonium oxalate amounts of iron, nickel and cobalt. Samples were dried, homogenized, and sieved.

The pH value was measured with a Mettler Toledo MP 220 instrument. The instrument was calibrated with 4.21 and 7.00 buffers. Extraction solution ratio was 1:5, according to (ISO 10390, 1994). Two different extraction solutions were used: $CaCl_2$ (0.01 mol/L) and distilled water. EC was evaluated using a Mettler Toledo MC 126 instrument in the clay sample/ultrapure water (1:5) ratio. The approach outlined in the ("US EPA 9050 A - Specific conductance," 2005) method was used to calibrate the instrument and measure electrical conductivity using the conductivity standards. Potential for Oxidation and Reduction was determined using a HANNA HI98201 instrument. The calibration standards with a known ORP potential were used to set the instrument. For the purposes of the study, the quantities of iron, nickel, and cobalt were quantified as "total." In addition, measurements of ammonium oxalate extractable amounts of iron, nickel and cobalt were conducted.

Sample preparation and measurement for metals analyses

For determination of total amounts of metals (M_{total}), around 0.2 g (0.001 g accuracy) of sample was placed in tubes made of Teflon. Concentrated nitric acid and concentrated hydrofluoric acid were added as a next step. The samples were digested in microwave oven. The digestion lasted for 3 h, with a pressure of 55 bar and temperature gradually increasing to 230 °C.

Total element content was determined using ICP-OES (Agilent 7000) instrument. The total concentrations of iron, nickel and cobalt were examined in this manner. In order to prevent excitation interference, 1% CsCl was added to all of the samples. The analytical findings were in good agreement with the utilized (BAM 112 and sandy loam) reference materials. Every sample was diluted either by ten or one hundred. Ammonium oxalate that has been acidified was employed in the extraction process to extract amounts of iron, nickel and cobalt. McKeague and Day were the ones who first presented this technique for amorphous iron extraction (McKeague and Day, 1966).

Dried, homogenized and sieved through a 150 m sieve were all the samples used for measurement. Sample mass of 1.0 ± 0.0001 g was placed in 100 mL polyethylene bottles. An extraction solution of 40 mL has been added. The extraction solution has been prepared according to the following procedure: 28.4 g of $(NH_4)_2C_2O_4$ was dissolved in distilled water in a 1000 mL volumetric bottle. For. A brown coloured glass bottle was used to transfer the solution. Immediately after mixing the sample and extraction solution the bottles were kept in dark (aluminium foil), to ensure that there was no light at them. The plastic bottles containing the sample and extraction solution were placed in an shaker for a period of 4 h. After shaking, the samples (still in dark) were centrifuged at 2400 rpm for 10 min. 5 mL of the supernatant was placed in a 100 mL volumetric flask. Each aliquot was added 4 drops of H_2SO_4 and 0.5 mL each of concentrate HNO_3. The flasks were placed on a heater, which had to be digested for 30 minutes. Three drops of concentrate HNO_3 were added at about 15 minutes into the digestion procedure. The residual solution was quantitatively transferred to a 50 mL flask by means of a plastic tube and washed with distilled water after cooling the samples. 10,000 ppm K solution (5 mL). Measurements with an atomic absorption spectrophotometer (Shimadzu AA7000) were made after the sample was prepared (Singer and Janitzky, 1986).

Statistical evaluation

Statistical Evaluation. The results of this study are as follows: statistically evaluated using the SPSS 20.0 (SPSS Inc., Chicago, IL, USA) statistical program through the analyses as follows:

Descriptive statistics - was presented by maximum, minimum, mean, average, and median value.

Correlations - Description of correlation coefficients which describe the strength of connection between two variables is as follows: 0.0−0.19 very weak connection, 0.2−0.39 weak connection, 0.40−0.59 moderate connection, 0.60−0.79 strong connection, and 0.80−1.0 very strong connection.

RESULTS AND DISSCUSION

Basic quality parameters

The results descriptive statistics of basic quality parameters are shown in the table 2.

Table 2. Basic quality parameters

	pH (H$_2$O)	pH (CaCl$_2$)	EC (mS/cm)	ORP (mV)
Maximum	8.49	7.69	15.99	297
Minimum	3.72	3.63	0.59	141
Average	6.56	6.14	4.92	236
Median	7.03	6.63	3.55	240

The pH value of samples variated greatly. Minimal pH was very acidic, while maximal was basic. pH value in deionized water was greater than value in CaCl$_2$. Lower pH value when CaCl$_2$ is applied is because some of H$^+$ ions from clay particle is replaced by Ca^{2+}, thus resulting in higher amounts of H$^+$ ions in solutions and lower pH. The electrical conductivity was also very different among samples, ranging from very low to very high amounts. Higher electrical conductivity means higher amounts of water-soluble ions. ORP was quite uniform trough the samples, and positive in every case, meaning oxidative medium.

Iron triad content

The results descriptive statistics of iron, nickel and cobalt content gained by two extraction procedures are shown in the table 3.

Table 3. Average contents of measured iron triad metals

	Fe$_{total}$	Ni$_{total}$	Co$_{total}$	Fe$_{AmOx}$	Ni$_{AmOx}$	Co$_{AmOx}$
Maximum (mg/kg)	127725.9	898.24	105.40	47123.68	421.34	2.99
Minimum (mg/kg)	26324.32	46.68	14.53	1289.82	3.41	0.15
Average (mg/kg)	57599.22	290.01	38.37	11367.54	96.40	1.26
Median (mg/kg)	58881.74	141.84	32.17	4884.93	57.93	1.18

The metal with highest concentrations was iron. Maximal concentration was 12.77%. Minimal concentration was 2.6%. Similar finding was reported in research of (Mohamed *et al*., 2022). Maximum value for nickel was 898.24 mg/kg, and minimal 46.68 mg/kg. The metal with lowest concentrations was cobalt. Maximal concentration was 105.40 mg/kg and minimal 14.53 mg/kg. Results presented in (Williams *et al*., 2008) are in the similar interval to ours. Certain amount of every metal from iron triad could be extracted with acid ammonium oxalate. The average difference between total

and ammonium oxalate extraction lies in the next sequence: Co>Fe>Ni, meaning that cobalt has the lowest extractability (highest percentage difference) with ammonium oxalate.
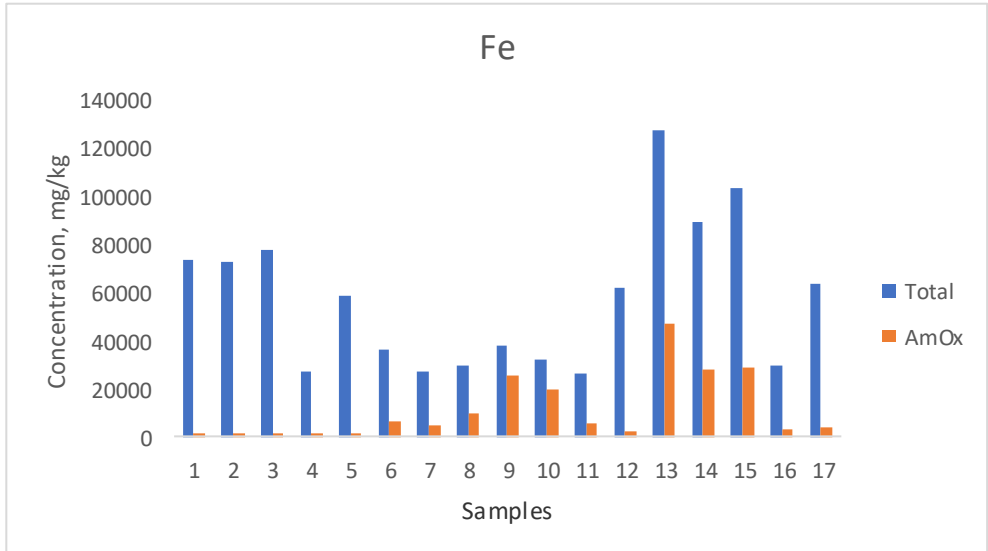
Graph 1. Total and ammonium oxalate extractable amounts of iron
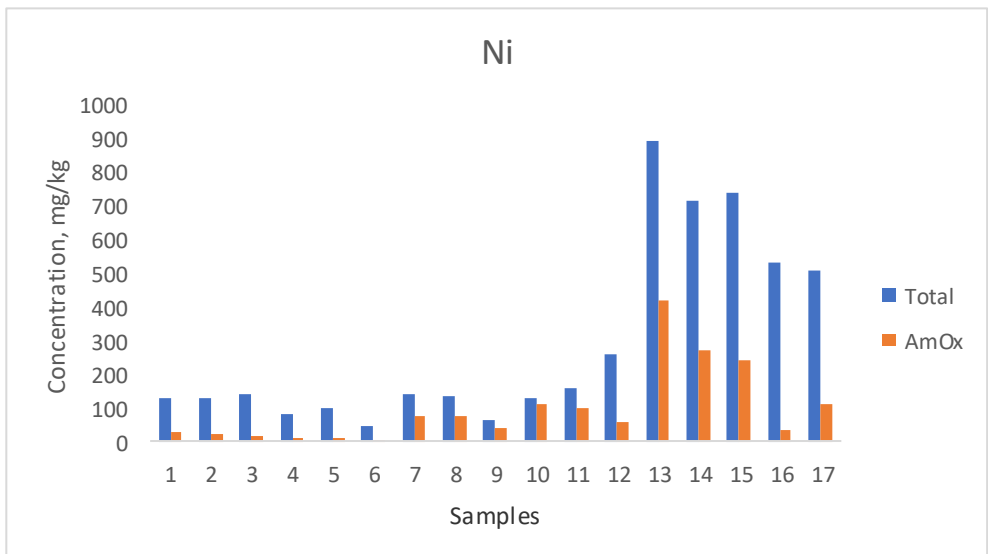
Graph 2. Total and ammonium oxalate extractable amounts of nickel
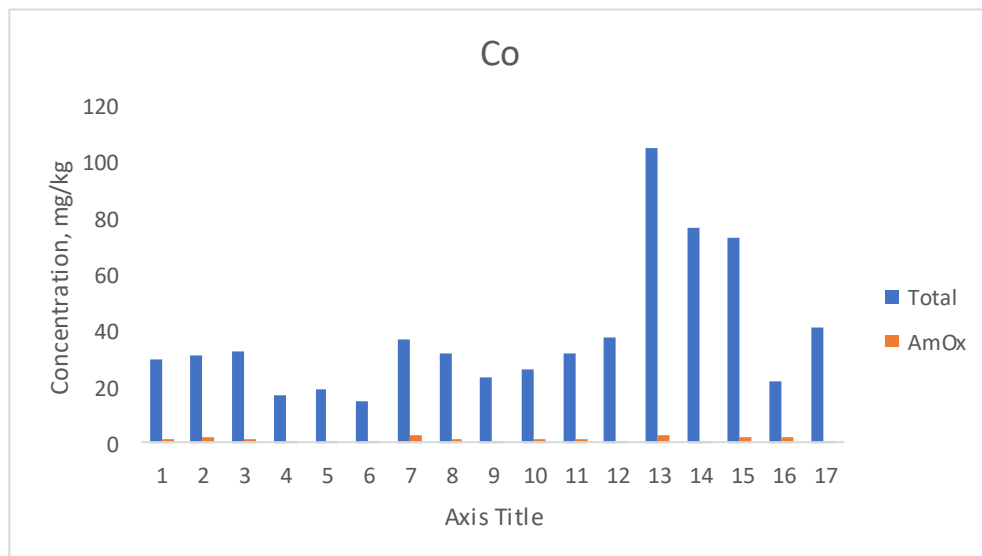
Graph 3. Total and ammonium oxalate extractable amounts of cobalt

In cases of iron and nickel, when there is a higher amount of total form of metal, ammonium extractable amount is also higher.

Correlations

Correlations between measured metals concentrations after two different extractions are shown in the Table 4.

Table 4. Correlations among metals

	Fe_{total}	Ni_{total}	Co_{total}	Fe_{AmOx}	Ni_{AmOx}	Co_{AmOx}
Fe_{total}	1					
Ni_{total}	**0.713**	1				
Co_{total}	**0.824**	**0.876**	1			
Fe_{AmOx}	**0.569**	**0.678**	**0.801**	1		
Ni_{AmOx}	**0.709**	**0.876**	**0.766**	**0.874**	1	
Co_{AmOx}	0.304	0.379	0.472	0.446	0.478	1

High and positive correlations were found in all cases dealing with total amounts and ammonium oxalate extractable amounts. The lowest correlations were found in case of total and ammonium oxalate extractable cobalt.

CONCLUSIONS

High similarity was found in case of iron and nickel. Cobalt did not show high similarity to other two elements of iron triad, meaning that although those metals are very similar, cobalt shows different ammonium oxalate extraction characteristics in natural clays.

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EKSTRAKCIJA TRIJADE ŽELJEZA AMONIJ OKSALATOM IZ UZORAKA PRIRODNE GLINE

Sažetak

Ekstrakcija kiselim amonijevim oksalatom koristi se za procjenu količine amorfnog ili slabo kristalnog željeza u tlu i sedimentima. Željezo je jedini element iz "trijade željeza" koji ima slično kemijsko ponašanje. Druga dva elementa su nikal i kobalt. Cilj ovog rada bio je ispitati ekstrakciju kiselim amonijevim oksalatom svih elemenata iz trijade željeza. U istraživanju su korišteni uzorci prirodne gline. Uzorci za ukupne količine metala pripremljeni su mikrovalnom digestijom s HNO_3 i HF, te izmjereni induktivno spregnutom plazma-optičkom emisijskom spektroskopijom (ICP-OES). Ekstrakcija amonijevim oksalatom obavljena je ranije utvrđenim postupkom u kiselom mediju i bez svjetla. Mjerenja su provedena atomskom apsorpcijskom spektroskopijom s plamenom atomizacijom (FAAS). Za statističku procjenu korišteni su maksimum, minimum, prosjek, medijan i korelacije. Ispitivani elementi trijade željeza nalaze se u sljedećem nizu: Fe>Ni>Co. Nikal koji se može ekstrahirati slabo kristalnim željezom i amonijevim oksalatom pokazao je veću korelaciju (r = 0,874) od kobalta koji se može ekstrahirati amonijevim oksalatom (r = 0,446), što sugerira da su željezo i nikal sličniji u pogledu ekstrakcijske sposobnosti amonijevog oksalata.

Ključne riječi: *željezo, nikal, kobalt, glina, ekstrakcija amonij oksalatom*

MONITORING OF THE DRINA RIVER QUALITY IN THE CITY OF GORAŽDE*

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Original scientific paper

Abstract

Surface water quality is a variable value that depends on the impact of many factors. The Drina River Quality Testing was one of the main motives for writing this paper. In order to get as complete and accurate insight into the water quality as possible, the monitoring lasted for two months (April-May). Sampling was carried out in five locations on the territory of the municipality of Gorazde over the given time intervals, at the hydrological station, 500 and 1000 meters downstream and 500 and 1000 meters upstream of it. The monitoring was carried out to determine the content of heavy metals: Pb, Cd, Fe, Zn, Cu, Ni, and selected alkali and alkaline earth metals: K, Na, Mg, and Ca. In order to complement the analysis, basic physical and chemical parameters of the water quality were also monitored: temperature, conductivity, redox potential, pH value and dry residue. The experiment was carried out in the Chemistry Laboratory at the Faculty of Agriculture and Food Sciences in Sarajevo.

Despite numerous attempts to disturb the Drina River's recognizable quality, the measured parameters did not show significant chemical contamination. The measured parameters should be broaden in the future in order to include organic pollutants too.

Keywords: *chemical contamination, monitoring, evaluation, water quality.*

INTRODUCTION

Water is of crucial importance for the survival of all living beings. The fact that water occupies about 75% of the Earth's total surface is not something to be taken for granted. As such, water has an unquestionable influence on the living world.

Human existence depends on the use of a large amount of healthy water (microbiologically, physically and chemically), which unfortunately is becoming increasingly scarce today because not enough attention is paid to this extremely valuable resource. Today, due to human civilization practices, water contains many toxic

products. More than 800 different impurities in water have been identified, and the number is still growing. All toxicants present in water have a direct or indirect effect on humans since they are at the top of the food pyramid (Mačkić et al. 2010). In order to raise awareness of the preservation of this resource, an ambitious water management concept is being introduced, addressing the importance of water quality preservation systems, including monitoring. Monitoring, together with evaluation, are the elements of water quality control, which aims to prevent or suppress the spread of infectious diseases caused by water. There is no ideally pure water in nature, because it dissolves numerous compounds from the place of origin to the place of capture or the way of use. Dissolution of various compounds leads to mineralization or contamination (depending on the type of dissolved particles). The state border between Bosnia and Herzegovina and Serbia spreads across the middle and lower water channel of the Drina River, and spans through four countries: Bosnia and Herzegovina, Serbia and Montenegro. It is a part of the Danube Basin, which empties into the Black Sea, and is the biggest tributary of the Sava River. A million or so people live in its watershed, and their activities (mining, industry, agriculture, and developments in rural and urban populations) significantly impact the aquatic ecology (Ivanovic *et al*., 2011). River water sources could be influenced by natural factors and human activities (Who *et al*., 2016). Increased urbanization and population growth have increased the strain on water environments and ecosystems. In order to accommodate the population increase, industrial activities have been encouraged, which may result in the release of pollutants and wastewater into the environment (Chen *et al*., 2018; Estrada-Rivera *et al.*, 2022). The quality of river water may decline as a result of pollutants from emissions or wastewater. Seasonal and geographic climate differences result in seasonal and spatiotemporal variations in the quality of river water (Cruz *et al*., 2019; Mena-Rivera *et al*., 2017). The city of Goražde is situated on the banks of the Drina River in southeastern Bosnia. The city lies at the foot of the eastern slope of the Jahorina mountain at a height of 345 m (1,132 ft) above sea level. The settlement is situated on the alluvial terrace in a broad valley, formed by the erosion of the Drina River. With around 30 000 inhabitants it is one of the biggest settlements on the Drina River bank ("Goražde - Wikipedia" n.d.).

The purpose of this paper is to monitor and evaluate the content of selected metals and physicochemical parameters of water quality, and to compare the results with the current legal regulations and to define measures for improvement if the chemical contamination is found.

MATERIALS AND METHODS

Samples and sampling

The first step of the experiment was the river water sampling. The entire sampling process lasted for seven weeks (labeled as W_1, W_2, W_3, W_4, W_5, W_6 and W_7) and took place according to the pre-defined procedures (every Sunday around 12:00 p.m., always

from the same locations). Sampling was carried out in the municipality of Goražde in five locations within 2 km of the river course.

In the very center of the town of Goražde, there is a hydrological station that measures basic parameters (water and air temperature, flow and water level of the river) on a daily basis. Below the hydrological station, on the left bank of the Drina River, is the mouth of the Podhranski stream. As the stream seemed to be contaminated, the sampling was targeted at this location and the locations around it. The water samples were taken facing the opposite direction of the river flow.

The locations from which the samples were collected (Figure 1) are numbered so that the location numbered 1 (the location from which the first sample was taken) was the lowest and is located 1000 meters below the hydrological station. Location number 2 was 500 meters below the hydrological station and location 3 was right next to the hydrological station. Location 3 is also close to the Hranjen stream, which is close to the source of contamination and industrial discharge pipe. Locations 4 and 5 were 500 and 1000 meters upstream from location 3. Locations of sampling are labeled as: L_1, L_2, L_3, L_4 and L_5.
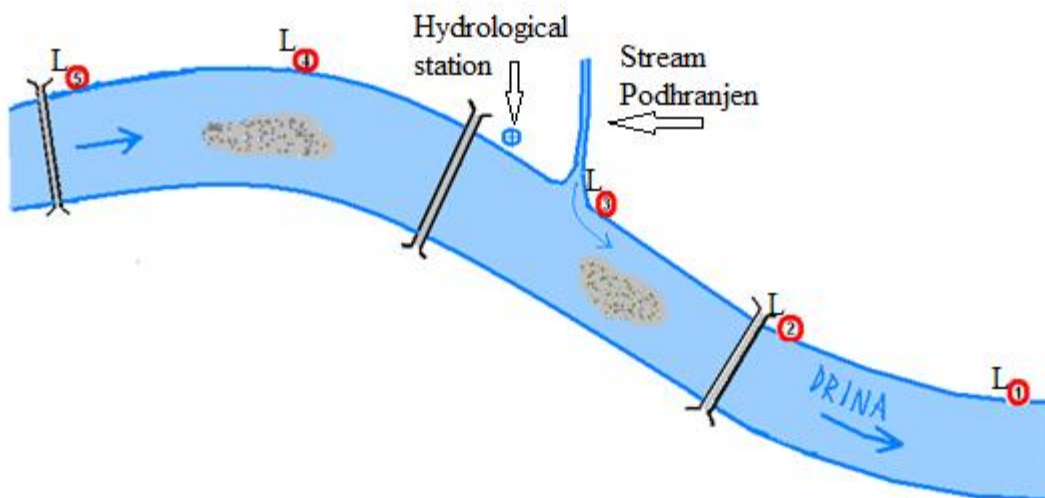
Figure 1. Drina River sampling locations

Methods of analysis

Dry residue
15 250-ml laboratory glasses were oven-heated for one hour at 105 °C. After cooling in desiccator, the glasses were weighed using an analytical balance. The volume of 100 ml water samples were then added into the each of the marked glasses. The oven-drying method was performed at a temperature of around 105 °C until the constant mass was reached. The obtained results were expressed as mg/L (ppm).

pH value
The pH value was measured using a laboratory pH meter (Mettler Toledo MP 230) with a combined ionselective electrode. Prior to the measurement, the device was standardized with buffer solutions (4.00 and 7 .00 of pH).

Electrical conductivity
Electrical conductivity was measured using a conductometer (Mettler Toledo MC 126). The instrument was standardized with solutions of a known electrical conductivity.

Oxidation reduction (redox) potential (ORP)
The redox potential was measured in a similar way as electrical conductivity. The difference was in the instrument (HANNA ORP) and the standards which were used.

Metals content determination
In order to test the content of heavy, alkali and alkaline earth metals, it was necessary to conserve or acidify each batch of the samples. Preservation of the samples was carried out in special containers to which two to three drops of concentrated nitric acid (63% HNO_3) were added. After adding the acid, the samples were archived in chronological order (by dates and the markings of sampling locations) in a refrigerator.
Different metals, due to their different physicochemical properties, required different test methods. Lead, cadmium, iron, zinc, magnesium, calcium, copper and nickel were tested using the atomic absorption spectrophotometry with flame atomization (FAAS), while potassium and sodium were measured by flame photometry (FP). The first step in the analysis was the preparation of working standards in order of increasing concentration. An unknown concentration of the sample can be determined by way of a calibration curve created by entering the values of increasing concentrations of standards and absorbance. After standardization, the analysis of elements in a series of prepared samples was performed.

RESULTS AND DISCUSSION

The next chapter shows, in tables and figures, and discusses the measurement results.

Determination of basic quality parameters

Table 1. Results of the basic quality parameters

Residue mg/kg	W_1	W_2	W_3	W_4	W_5	W_6	W_7
L_1	244.33	154.00	108.66	156.66	197.00	127.33	176.66
L_2	274.00	201.00	138.33	148.66	**228.00**	149.33	203.66
L_3	**319.00**	**277.00**	239.66	220.00	155.33	**236.66**	**241.00**
L_4	299.00	107.00	177.00	**298.33**	170.00	184.66	202.66
L_5	254.00	104.00	**240.00**	318.66	141.66	163.66	107.33
Average	278.06	168.66	180.73	228.46	178.39	172.32	186.26

pH value	W_1	W_2	W_3	W_4	W_5	W_6	W_7
L_1	6.87	7.33	6.69	7.13	7.40	7.21	7.11
L_2	7.69	7.75	8.11	7.58	7.55	7.61	7.52
L_3	7.75	7.67	8.08	7.71	7.64	7.65	7.62
L_4	**7.94**	**7.95**	8.18	**7.83**	7.78	7.74	**7.78**
L_5	7.91	7.91	**8.22**	7.79	**7.80**	**7.78**	7.71
Average	7.63	7.72	7.85	7.60	7.63	7.59	7.54

EC (µS/cm)	W_1	W_2	W_3	W_4	W_5	W_6	W_7
L_1	267.5	267.3	376.7	389.3	361.3	253.3	284.3
L_2	266.3	284.3	409.3	380.7	393.0	398.0	310.7
L_3	**401.0**	**375.7**	**448.3**	**467.3**	**495.3**	**509.7**	**364.0**
L_4	305.0	253.0	354.7	334.0	344.3	346.0	269.0
L_5	280.7	252.7	349.3	335.7	340.7	342.7	269.0
Average	304.1	286.6	387.66	381.4	386.92	369.94	299.4

ORP (mV)	W_1	W_2	W_3	W_4	W_5	W_6	W_7
L_1	**197.7**	46.3	**167.7**	77.7	73.0	**53.7**	**43.0**
L_2	153.0	46.0	137.3	82.7	70.0	52.0	39.0
L_3	183.0	77.7	111.3	82.3	77.0	51.3	31.0
L_4	122.3	87.7	120.0	**87.7**	**79.0**	53.0	35.0
L_5	74.66	**92.3**	143.7	86.3	78.0	53.7	38.0
Average	146.1	70.0	136.0	83.3	75.4	52.7	37.2

Table 1 clearly shows that sampling location 3 leads in the content of dry residue in relation to other sampling locations due to the vicinity of the extended discharge pipe and this is the case in almost all of the sampling series. The lowest values in all of the sampling series were found in the samples from location 1 and location 2. The results for dry residue correspond to the ones reported recently (Rajković *et al*., 2012). Going upstream towards the location 5, the pH value increases. Going downstream from location 3 lower pH value was recorded, probably as a result of the presence of acidic substances in the wastewater. It is important to mention that all the measured pH values

were within the permitted limits defined by the applicable legislation. The case study of the Sutla River in Croatia (Dragun *et al*., 2011) showed comparable pH ranges. Sample 3 had the highest value of EC. Wastewater, which enriches the mentioned location with its present ions, contributed to this increased value. As a result of ion dilution in the water, a rapid drop in values is noticeable from location 3 downstream. Samples 4 and 5 have the lowest values because they were collected upstream of the critical zone and do not have the potential for contamination like the samples from other locations. According to the Regulation on dangerous and harmful substances in water ("Pravilnik o opasnim materijama u vodama" 2007), the conductivity value must not exceed 500 µS/cm. In the present case, the sixth series of sampling, namely in the sample 3, slightly exceeded the upper limit, which is in line with the expectations. Leščešen *et al*. (2016) presented almost the same results for electrical conductivity of the Drina River samples. By observing Table 1., it is clearly seen that the total redox potential per sampling series was highest in the first sampling series, which may be a consequence of the low water level and temperature of the river, and the lowest in the sixth and seventh series when the water level was increased. In general, the redox potential results were irregularly distributed within the different sampled locations, making their interpretation difficult. It can be seen that the highest value was found in sample number 1 in a larger number of series, and the lowest in sample number 3, but in each case the obtained values were positive (oxidative medium). All positive and similar values of ORP were found by Ting and Rizaludin Mahmud (2020) and Mehmedinović *et al.* (2021).

The greatest attention during the entire process of preparing the paper was paid on the monitoring of heavy and selected metals, i.e. their quantitative analysis. In this section, the analysis of alkali and alkaline earth metals (Ca, Mg, K and Na) are presented and discussed. The measured metal content was compared with the limits defined in the applicable regulations, the Regulation on dangerous and harmful substances in water ("Official Gazette of the Federation of Bosnia and Herzegovina", number 286/07). In order to obtain more complete information, the mean, minimum and maximum values and their maximum allowable limits are presented in Table 2 below.

Table 2. Maximal, minimal values and maximum allowable limits

	Ca	Mg	K	Na
Maximum (mg/kg)	49.06	18.56	5.40	86.00
Minimum (mg/kg)	18.50	3.18	3.72	14.40
Maximum allowable limit (mg/kg)	200	30	12	150
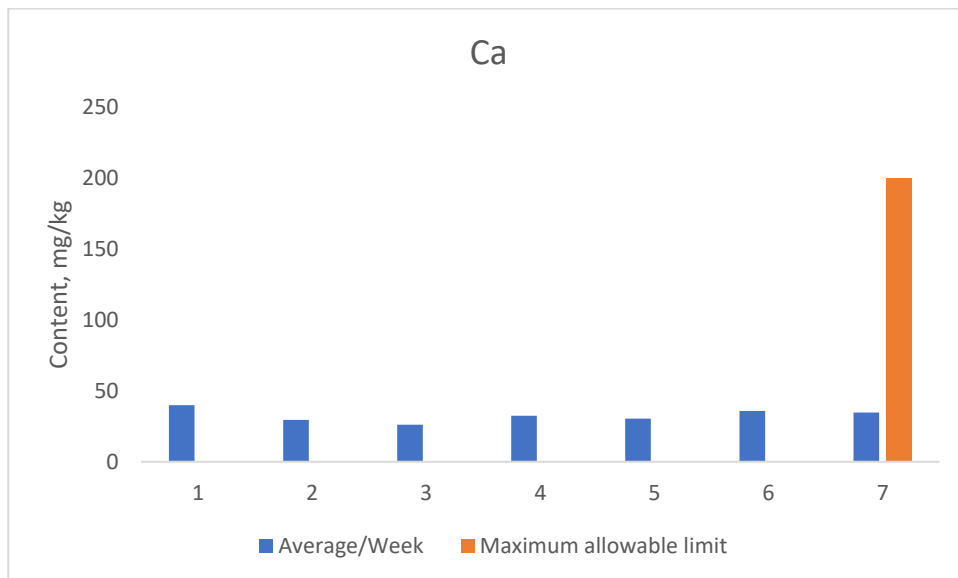
Figure 2. Results for calcium content compared with a maximum allowable limit

The analysis established that the examined calcium content was unevenly distributed within the sampling series, and for this reason, no single sampling series or sample can be singled out as the richest in that element. The Figure 2 shows that the element is present in all of the samples, with sample 1 of the second and sixth series having the highest results. Sample 3, which proved to have highest values in earlier tests carried out in this analysis, did not continue the same tendency for calcium, that is, the metal concentration in the sample was relatively stable and ranged from 30 to 40 mg/L. Despite the fact that the Drina River belongs to the type of surface waters that are abundant with this metal, this was not recorded in this case. It is clearly seen that the calcium content was far within the permissible limits.
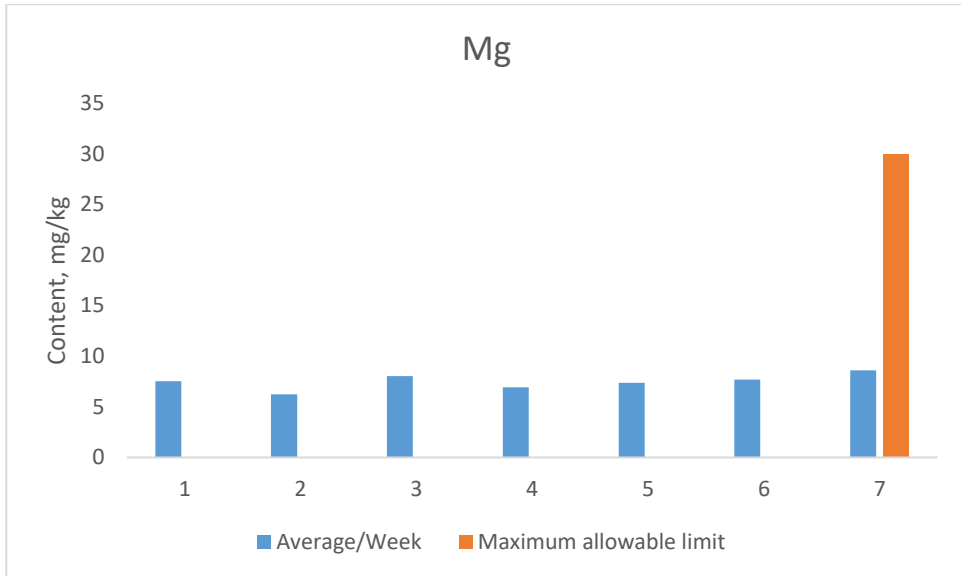
Figure 3. Results for magnesium content compared with a maximum allowable limit

The Figure 3 above clearly indicates that sample number 3 in all of the sampling series was the richest in magnesium content which was relatively equally distributed within different sampling locations. Samples 2 and 1 followed, while samples 4 and 5 were characterized by the lowest and almost equal content of the tested metal. Analyzing the overall picture, it becomes clear that the industry wastewater that reaches the watercourse of the river near location 3 (sample 3) is rich in chemical substances that contain this metal, enriching the river flow downstream from it. A regressive drop in the content of the element is noted downstream from location 3, with uniform content upstream from it. This means that location 3 is a point with the highest content, making it a "demarcation line" between the two concentration intervals. It is important to note that all the measured values of the metal content were within the maximum permissible limit defined by the applicable legal regulations.
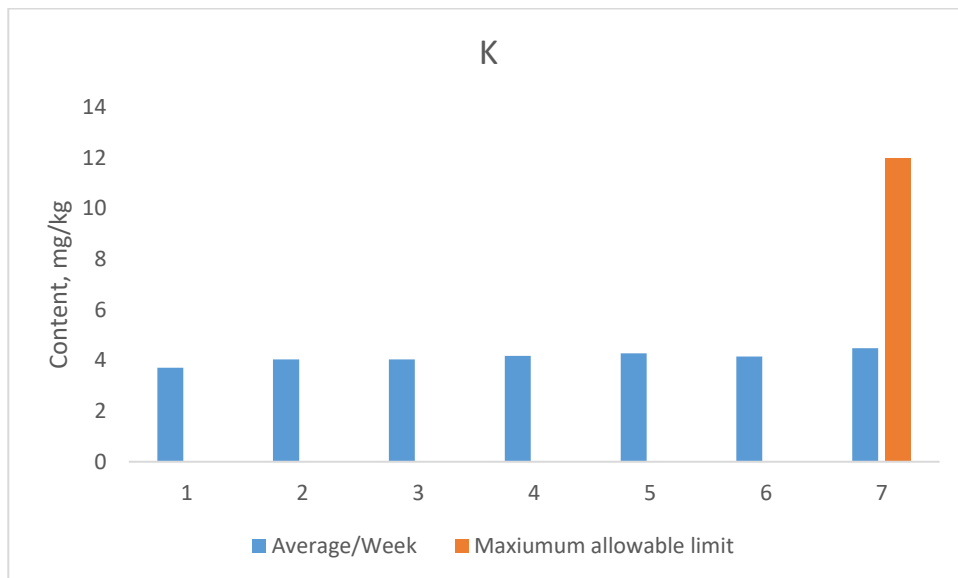
Figure 4. Results for potassium content compared with a maximum allowable limit

Observing the Figure 4, it can be seen that sample 3 leads in terms of potassium content compared to the other tested samples. The reason for this increase is the wastewater that reaches the river at this point. It is likely that the wastewater contains a chemical substance rich in potassium that enriches the water mass from the point of inflow and downstream from it. Going upstream from the point where the stream enters the river, the location 3, the content of the element decreases. Samples 4 and 5 contain smaller amounts of the tested element. It is worth noting that the content of the element in all of the tested samples was below the maximum allowed limit defined by the applicable legal regulation.
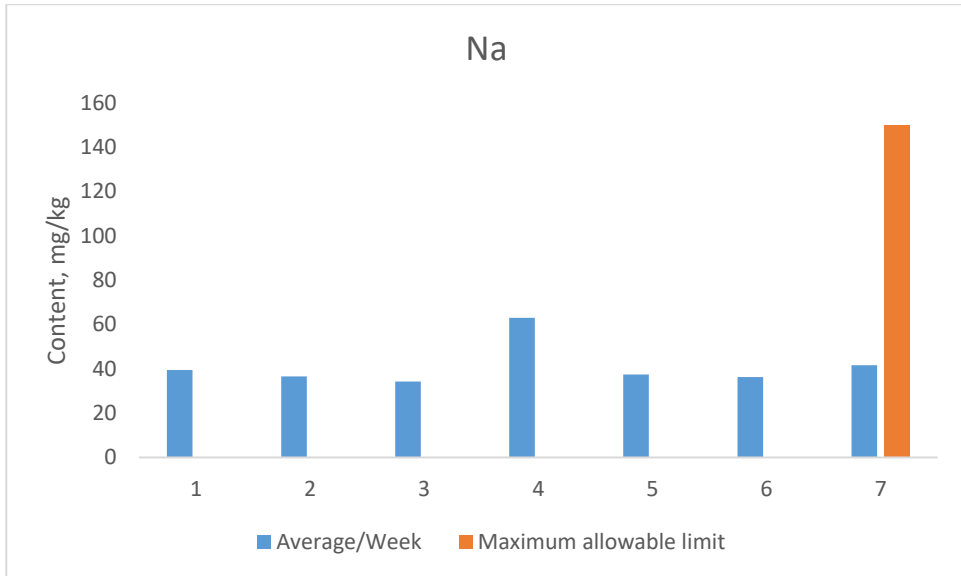
Figure 5. Results for sodium content compared with a maximum allowable limit

As the above figure shows, sample 3 was the richest in sodium content, while sample 5 was the poorest. The reason for this increase in sample number 3 is the same as in the previous cases. Going up or down towards the initial or final location, the metal content decreases, making this location again the line of demarcation between the two value intervals. The obtained values were below the margin of the maximum allowed content determined by the relevant regulation.

Sodium was the last surface water quality element to be monitored. All the obtained results were at a satisfactory level, which will be generally discussed in the next chapter. It is noted that the content of the measured metals (average values) was highest in the period of reduced river water level and vice versa. The reason for the increase in their concentration when the water level was low is due to the reduction of the amount of water to the same amount of metal in a given volume unit. Similar concentrations of calcium and magnesium, but lower content of sodium and potassium, were reported by (Grsic *et al.*, 2018).

Determination of heavy metals content
The content of heavy metals (lead, cadmium, iron, zinc, nickel and copper) was too low to be determined by means of atomic absorption spectrophotometry. The detection limit for many of the heavy metals was above 1 mg/kg and the content of heavy metals in the present case were much lower.

CONCLUSIONS

The results of the analysis of the basic indicators or physicochemical parameters: pH value, conductivity, turbidity, redox potential and dry residue of the Drina River (for the implementation of which appropriate test methods are specified by law) were within the permitted limits. Therefore, the water quality of the Drina River, according to the mentioned parameters, during the mentioned monitoring period (April/May), was at a satisfactory level.

The obtained values of the analysis of heavy and selected metals, in accordance with the Regulation on hazardous and harmful substances ("Official Gazette of the Federation of Bosnia and Herzegovina", number 286/07), indicate that the quality of the Drina River meets the criteria of the first category of surface waters as defined by this Regulation.

The results reported in this paper should be considered in the light of some limitations. Namely, the instrumentation used herein was not sensitive enough which is why we suggest sampling in the larger area of the Drina River and usage of more sensitive equipment in future research investigations (GFAAS, ICP-MS, ICP-OES).

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MONITORING KVALITETE RIJEKE DRINE NA PODRUČJU GORAŽDA

Sažetak

Kada se govori o kvalitetu kao jednoj cjelini, bitno je naglasiti da u njegovoj izgradnji učestvuje veći broj elemenata. Kvalitet površinskih voda je varijabilna vrijednost koja zavisi od uticaja mnogobrojnih faktora. Ispitivanje kvaliteta rijeke Drine je bio jedan od osnovnih motiva izrade ovog rada.

Da bi se dobila što potpunija i tačnija slika kvaliteta vode vršen je njen monitoring. Monitoring je trajao dva mjeseca (april-maj), kada je primjetan povećan vodostaj rijeke. Uzorkovanje je vršeno sa pet lokacija na teritioriji opštine Goražde u zadanim vremenskim intervalima i to kod hidrološke stanice, zatim 500 i 1000 metara nizvodno i uzvodno od nje. Praćenje je ustremljeno na elemente hemijske kontaminacije, odnosno

na kvantitativni sadržaj teških metala (olovo i kadmij) i odabranih elemenata: željezo (Fe), cink (Zn), magnezij (Mg), kalcij (Ca), bakar (Cu), nikl (Ni), kalij (K) i natrij (Na). U cilju kompletnosti analize praćeni su i osnovni fizičko-hemijski parametri kvaliteta vode. Mjerena je temperatura, provodljivost, redoks potencijal, pH vrijednost i suhi ostatak. Ispitivanje je vršeno u hemijskoj laboratoriji Poljoprivredno-prehrambenog fakulteta u Sarajevu, a dobiveni rezultati su hronološkim redom pohranjivani radi evaluacije kvaliteta vode. Evaluacijom dobivenih rezultata, odnosno usporedbom sa važećom regulativom zaključuje se da je kvalitet rijeke Drine ispitivanog područja u vremenskom periodu mjesec april-maj bio na zavidnom nivou.

Uprkos mnogobrojnim pokušajima narušavanja prepoznatljivog kvaliteta, rijeka Drina se uspjela odbraniti od nemilih destruktivnih pokušaja.

Ključne riječi: *hemijska kontaminacija, monitoring, evaluacija, kvalitet vode.*

HISTOPATOLOŠKA I PARAZITOLOŠKA ANALIZA TKIVA VRSTE *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002 KAO BIOINDIKATORA DEGRADACIJE RIJEKE MILJACKE

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Rezime

Zagađenje vodenih ekosistema uzrokuje stres kod ribljih vrsta i dovodi do bolesti kao posljedice interakcije sa uvjetnim patogenom. Posebnu opasnost za zdravlje riba predstavljaju parazitske vrste koje se prenose s jedne ribe na drugu neposrednim i/ili posrednim putem. U ovom istraživanju je izvršena parazitološka analiza bioindikatorske, riblje vrste *Barbus balcanicus.* Histološka analiza vršena je u svrhu detekcije veze s parazitskim infekcijama, kao i povezanosti između bakterioloških parametara u vodi i upalnih procesa u tkivima riba. Uzorkovanje je izvršeno u julu 2021. godine, na dva lokaliteta rijeke Miljacke. Analizom tkiva jedinki detektovano je prisustvo parazita iz grupa Protozoa i Trematoda, koje su do nivoa roda ili vrste identificirane korištenjem relevantnih ključeva i radova. Uporedo sa ihtioparazitološkim istraživanjima provedena je i analiza fizičko - hemijskih parametara vode te mikrobiološka analiza, čiji rezultati ukazuju na zagađenost rijeke Miljacke i povoljne uslove za razvoj parazita. Histološkom analizom utvrđeno je prisustvo patoloških lezija u tkivu škrga i jetre jedinki, koje su dovedene u vezu s parazitskim infekcijama i prisustvom patogenih mikroorganizama. Različiti polutanti vodenih ekosistema uslovljavaju prisustvo parazita i histopatoloških promjena u ribljim vrstama, ali i obrnuto – parazitske infekcije i promjene u tkivima ukazuju na loš ekološki status i kontaminiranost površinskih voda.

Ključne riječi: *potočna mrena, parazitologija, patohistologija, polutanti, biološki elementi kvaliteta vode*

UVOD

Kontaminiranost vodenih ekosistema uzrokuje stres i potencijalni razvoj bolesti kod ribljih vrsta. Stepen opterećenosti vodenog ekosistema procjenjuje se, između ostalog, mikrobiološkom analizom vode. Mikrobna populacija u površinskim vodama potiče iz zraka (kiša), ispiranjem tla, iz otpadnih industrijskih voda i/ili iz postrojenja za obradu otpadnih voda. Među glavnim skupinama mikroorganizama, kontaminanata

[1]W˛ ã¸^¦: ã⹀o⌐ ⌐U˛ã⹀a⹀ǫ˘ ⹀U¦ã[å} [¦ᵗ æᵉ^{ æ⹀ã\ã⌐äᵗ ¦c^o⌐
[2]W˛ ã¸^¦: ã⹀o⌐ ⌐U˛ã⹀a⹀ǫ˘ ⹀X^c˟¦ä] aᵗ•\ã⌐äᵗ ¦c^o⌐
S[¦^•][}å^]&ã⹀o⹀⌐ä˛ã⌐o⹀}ä¦{ O]{ ⹀È}•a⹀ä˛aᵒ⌐

površinskih slatkih voda su: koliformne bakterije, anaerobne vrste bakterija, alge i cijanobakterije te protozoe (F r e c e & M a r k , 2015).

Uticaj parazita na organizme je varijabilan, a u konačnici može dovesti i do smrti domaćina/domadara (L e v s e n *et al*., 2008). Potencijalni domaćini različitih obligatnih ili fakultativnih parazita su i mnogobrojne vrste slatkovodnih riba (R i c k l e f s & M i l l e , 2000; P e e k , 2012). Egzoparazitske vrste se kod riba nalaze na koži ili krljuštima, perajima te na škrgama i u usnoj šupljini. Endoparaziti se lociraju u mišićima, unutrašnjim organima (najčešće crijevima) i membranama koje obavijaju unutarnje šupljine organizma (P e e k , 2012).

Razvoj specifičnih životnih ciklusa ribljih parazita je najvećim dijelom posljedica načina ishrane riba (B e l l a y *et al*., 2015). Tako za određene vrste riba koje se hrane organskim detritusom na dnu voda postoji mogućnost unosa jaja parazita koja su odložena od strane drugih inficiranih jedinki (I y a j i & E y o , 2008). Predatorske vrste riba se također mogu inficirati ako u ishrani koriste drugu riblju vrstu zaraženu parazitima. Pojedine riblje vrste imaju tendenciju migracije iz određene riblje vrste u povoljne dijelove organskih sistema (najčešće probavnog) druge vrste (Y a n o n g , 2017). Zbog svojih biološko-ekoloških karakteristika ribe se koriste kao bioindikatori dalekosežnijih i dugoročnijih posljedica promjena u akvatičnim ekosistemima te detekciju specifičnih kontaminanata (R i đ a n o v i ć & R i đ a n o v i ć , 2016). Kod riba postoje dva jednako važna načina unosa polutanata: preko škrga – direktno iz vode i oralnim putem – asimilacijom zagađene hrane (K l e i n o w *et al*., 1987).

Histopatološka analiza ribljih tkiva također omogućava detekciju izlaganja nizu antropogenih zagađivača. Simptomi kod inficiranih jedinki se mogu uočiti makroskopskim pregledom jedinki, a također i kasnijim seciranjem ribe i analizom histoloških preparata pojedinih tkiva. Najčešće korišteni organi riba za ove analize su škrge i jetra (F a t i m a *et al*., 2014). Uspješnost odbrane od patogena zavisi od stanja površine tijela, imunog sistema i cijelog organizma riblje vrste te o broju i osobinama patogena (F i j a n , 2006).

Fizičko – hemijski parametri vodenih ekosistema ukazuju na trenutno stanje kvaliteta vode određenog akvatičnog ekosistema. Odabir parametara zavisi od tipa istraživanja i drugih okolnosti, a neki od njih su: gustoća i boja vode, površinska napetost, temperatura, svjetlost, kisik (koncentracija i zasićenost), minerali, tip supstrata (dna, korita) te koncentracija ugljika i azotnih jedinjenja, boja vode, pH vrijednost, elektroprovodljivost itd. (S m i t h & S m i t h , 2001; B o g u t *et al*., 2006).

Istraživanja u ovoj studiji su vršena na ribljoj vrsti *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002 (potočna mrena, balkanska/velika mrena, balkanska sapača, engl. *Danube Barbel*, *Mediterranean Barbel*). Kao sinonimi za ovu vrstu u literaturi se mogu naći *Barbus meridionalis* Risso, 1826 i *Barbus caninus* (Bonaparte, 1839) (M r a k o v č i ć *et al*., 2004). Njen IUCN status je osjetljiva (VU) te nije na Crvenoj listi. Međunarodno je zaštićena Bernskom konvencijom (Dodatak III) i Europskom direktivom o zaštiti staništa. Potočnu mrenu ugrožava onečićenje vodotoka, nestajanje prirodnih i mrijesnih staništa, pregradnja rijeka i regulacija gornjih tokova rijeka (M r a k o v č i ć *et al*., 2004; M i l o š , 2009). Predstavlja pogodnu bioindikatorsku,

riblju vrstu (objekat/model) za ispitivanje zagađenosti vodenih ekosistema zbog njene osjetljivosti na različite promjene u okolišu (B a š i ć, 1980) te odsustvo s crvene liste.

Miljacku, rijeku sliva rijeke Bosne, najvećim dijelom sačinjavaju Paljanska i Mokranjska Miljacka. Cjelokupni riječni sistem Miljacke uzvodno od proširenja aluvijalne ravni (kod naselja Babića Bašta) ima obilježja planinskog toka, s vrlo strmim i neusaglašenim uzdužnim profilom. Nizvodno od navedenog lokaliteta rijeka Miljacka teče kroz aluvijalnu ravan sa vrlo malim padom. Nalazi se između 487 m i 1699 m nadmorske visine (Ž u n i ć, 2010). Rijeka Miljacka je izložena različitim izvorima zagađenja, naročito u donjem dijelu toka koji je pod velikim antropogenim pritiskom.

Cilj ove studije je bio parazitološka i histopatološka analiza tkiva vrste *Barbus balcanicus* iz rijeke Miljacke, kao vodenog ekosistema izloženog negativnim antropogenim uticajem. Svrha je bila detektiranje povezanosti i komplementarrnosti između prisustva parazita i histopatoloških promjena na tkivima odabrane vrste s kontaminiranošću vode rijeke Miljacke. Eventualno prisustvo parazita je značajno zbog toga što nivo parazitarnosti može značajno uticati na strukturu zajednice te se može koristiti kao biološki parametar ekološkog statusa kompletnog akvatičnog ekosistema. Potencijalna detekcija parazitskih vrsta opasnih za riblje vrste na primjeru ove bioindikatorske vrste podrazumijeva i detekciju „biološke opasnosti“, kako po ostale vrste ihtiofaune, tako i po cjelokupan živi svijet ovog akvatičnog ekosistema. Isto tako, u drugom smjeru posmatrano – nivo zagađenosti vode može odrediti uslove za razvoj parazita (pogodovati ili ne). Histopatološka analiza je vršena s ciljem detekcije veze s parazitskim infekcijama te povezanosti između bakterioloških parametara u vodi i upalnih procesa u tkivima riba, te ona na neki način korelira i objedinjuje sve aspekte.

MATERIJAL I METODE RADA

Uzorkovanje vrste *Barbus balcanicus* iz rijeke Miljacke je izvršeno u mjesecu julu, 2021. godine na dva lokaliteta rijeke Miljacke, Dariva (L1) i Otoka (L2) (slika 1). Korištena je metoda „elektroribolova" uz korištenje agregata "Honda" EZ 2.200 jačine 2 kW i "ELT 61 II" jačine 2 kW. Ukupno je izlovljena 21 jedinka potočne mrene od čega 12 jedinki sa lokaliteta Dariva i 9 sa lokaliteta Otoka. Identifikacija istraživane vrste rađena je na terenu i u laboratoriji Centra za ihtiologiju i ribarstvo (CIR) Prirodno-matematičkog fakulteta Univerziteta u Sarajevu prema referentnom ključu za ovu vrstu (Kottelat & Freyhof, 2007).
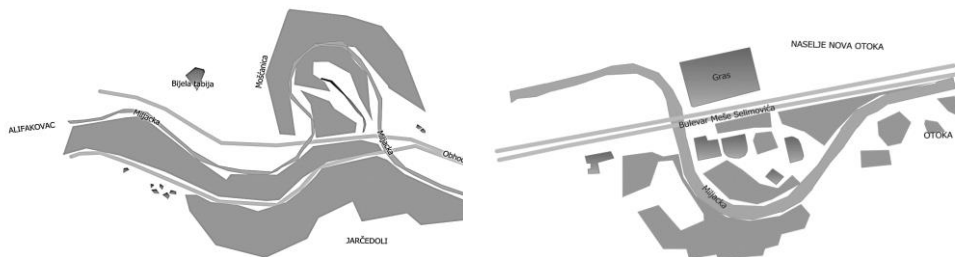
Slika 1. Mjesto uzorkovanja na lokalitetima: Dariva (lijevo), Otoka (desno)
Figure 1. Sampling sites: Dariva (left), Otoka (right)

Pomoću digitalnog ihtiometra na terenu su izmjerene osnovne morfometrijske mjere svih prikupljenih jedinki riba: ukupna dužina tijela (longitudo totalis, TL) i standardna dužina (longitudo corporis, SL). Masa uzoraka određena je pomoću analitičke vage sa tačnošću od 0,1 g. Dob jedinki je određena na osnovu uzetih krljušti, a spol na osnovu gonada tokom kasnije disekcije jedinki. Osim što je ovo standardna procedura, određeni parametri su bitni u tumačenju rezultata.

Provedena je i standardna analiza odabranih fizičko – hemijskih parametara vode. Ispitivani fizičko-hemijski parametri prilikom ovog istraživanja bili su: temperatura vode na mjestu uzorkovanja (°C), rastvoreni O_2 (mg/l), zasićenje O_2 (%), pH vrijednost vode i elektro-provodljivost vode (µS/cm). Navedeni parametri su mjereni na terenu pomoću multi-set aparata za mjerenje (WTW Oxi 3310 IDS Set 1), a odabrani prema svrsishodnosti ciljeva studije. Na odabranim lokalitetima rijeke Miljacke određena je nadmorska visina i geografska širina i dužina. Mjerenja su izvršena pomoću GPS-a, Garmin, oregon 600.

Uzorci vode za mikrobiološku analizu uzorkovani su u skladu sa BAS EN ISO 19458. Nakon uzorkovanja vode na lokaliteteima, uzorci su u ručnom frižideru transportovani do laboratorije za Mikrobiologiju voda na Prirodno-matematičkom fakultetu u Sarajevu. Sve analize su urađene u okviru 24h, standardnim postupcima i metodama. Za određivanje ukupnog broja aerobnih/fakultativno anaerobnih heterotrofnih bakterija (cfu/ml pri 22ºC/68h) i mezofilnih bakterija (cfu/ml pri 35 ±1 ^{0}C/48h) korištena je metoda zasijavanja u agar ploču u skladu sa BAS EN ISO 6222. Za određivanje broja ukupnih koliformnih bakterija i fekalnih (termotolerantnih) koliformnih bakterija i detekciju *Escherichia coli* primijenjena je metoda membranske filtracije u skladu sa ISO 9308-1:2014. Za određivanje ukupnog broja koliformnih bakterija i termotolerantnih koliformnih bakterija koristio se hromogeni koliformni agar i nM-TEC agar. Za potvrdu prisustva *Escherichia coli* na suspektne kolonije apliciran je Kovačev reagens, a pojava crvene boje bila je potvrda stvaranja indola. Za potvrdu *E. coli* određivana je i reakcija aktivnosti oksidaze tako da se jedna pojedinačna suspektna kolonija bakterije, prenijela sterilnom ezom na trakicu za određivanje reakcije. Nakon pet sekundi izostanak

tamnoplave boje na trakici ukazivao je na negativnu reakciju koja je karakteristična za bakteriju *E. coli*. Ukupan broj i detekcija fekalnih enterokoka u 100 ml izvršena je membranskom filtracijom prema metodi ISO 7899-2:2000. Za određivanje fekalnih enterokoka koristio se eskulin azidni žučni agar i temperatura inkubacije od 44,0±0,5ºC. Nakon izlova i identifikacije jedinki, uzeti su uzorci škrga za daljnje laboratorijske analize. Važno je napomenuti da nisu svi uzorci podvrgnuti i parazitološkim i histopatološkim analizama, budući da to nije bilo izvodivo. Dio uzoraka, sedam jedinki sa L1 i četiri sa L2, protokolarno su pohranjeni u plastične vreće i potom transportovani u prenosivom frižideru (s ledom) do Odjeljenja za akvakulturu Veterinarskog fakulteta u Sarajevu, u svrhu parazitološke analize.

Drugi dio uzorka, pet jedinki sa L1 i pet sa L2, transportovano je u prenosivom frižideru do Laboratorije za Histologiju i embriologiju životinja i čovjeka Prirodno-matematičkog fakulteta Univerziteta u Sarajevu za potrebe dalje histološke analize.

Vanjske i unutrašnje parazitološke pretrage ribe

Parazitološka istraživanja su bila usmjerena na pregled peraja, škržnih listića, kože i organa probavnog sistema. Najprije je izvršen spoljni, adspekcijski pregled jedinki vrste *Barbus balcanicus*, utvrđivanjem eventualnih povreda na tijelu, crvenila na različitim dijelovima tijela, kao i drugih promjena na tijelu, te prisustva cisti i ektoparazita.

Nakon adspekcijskog pregleda izvršena je eutanazija ulovljenih jedinki potočne mrene, radi daljne analize.

Za parazitološku analizu napravljeni su nativni preparati uzoraka uzetih sa kože i škrga. Uzimane su strugotine blagim struganjem površine kože i peraja skalpelom u kranialno-kaudalnim smjeru po tijelu ribe te napravljeni trajni preparati. Posmatranje je vršeno pod mikroskopom Leica DM 500, pod uvećanjem 150x i 600x, te je vršena detekcija i determinisanje parazita.

Potom se pristupilo disekciji jedinki pri čemu je dobijen jasan uvid u organe trbušne šupljine i škrge. Svi unutrašnji organi pregledani se u svrhu analize boje, konzistencije i patoloških promjena (krvarenja, edemi, ciste, neoplazije, paraziti). Nakon toga su analizirane izvađene škrge, a potom odvajani škržni listići, te pravljene strugotine kao u prethodnom slučaju. Materijal za mikroskopski pregled škrga, listić škrga, odrezan je na drugom luku. Strugotine su pretraživane na eventualno prisustvo parazita (u Petrijevim posudama) korištenjem binokularne lupe.

Digestivni trakt je seciran iz svježe ribe standardnim priborom. Pravljenjem strugotina crijeva se mogu detektirati paraziti. Crijevni sadržaj je bio bez parazita i razvojnih oblika parazita, te se nije pristupalo daljnjoj analizi.

Determinacija svih parazitskih vrsta vršena je prema relevantnim ključevima za identifikaciju (Byhovskaja – Pavlovskaja *et al*., 1962; Bruno *et al*., 2006), a u nedostatku većeg broja adekvatnih i referentnih ključeva konsultirane su knjige, priručnici i časopisi/radovi sljedećih autora: Stefanović (1948), Čanković *et al*. (1968), Antal *et al*. (1974), Kiškarolj (1977), Brajković (2001, 2004), Fijan (1974, 2006) te djelo *Opredelitel parazitov presnovodnih rib fauni SSSR* (1962., 1984., 1985. i 1987.).

Histološka analiza tkiva

Nakon disekcije, uzeti su uzorci jetre i škrga (drugi desni škržni luk) koji su fiksirani u 10% puferiranom neutralnom formalinu. Dehidracija tkiva rađena je sa uzlaznom serijom alkohola (30, 50, 70, 90 i 100%) tokom 2h, a zatim očišćena u ksilenu i uklapana u parafinske blokove. Mikrotomom (LEICA RM 2145) su izrezani presjeci debljine 5-6 mikrona. Tkiva su zatim hidratizirana opadajućom serijom alkohola. Presjeci su obojeni hematoksilinom i eozinom za opštu strukturu, a zatim je izliven kanada balzam i prekriven pokrovnim staklom (Presnell & Schreibman, 1997). Preparati su pregledani pomoću svjetlosnog mikroskopa BestScope BS-2035DA1 pri uvećanju od 400x i 1000x, u programu ScopeImage 9.0.

REZULTATI RADA I DISKUSIJA

U julu 2021. godine, iz rijeke Miljacke uzorkovano je i analizirano ukupno 21 jedinka vrste *Barbus balcanicus* (potočne mrene) sa dva odabrana lokaliteta (Dariva i Otoka).

Rezultati hidromorfoloških i fizičko-hemijskih analiza

Lokalitet Dariva (L1) je smješten u kanjonu Miljacke na istočnom ulazu u grad. Tokom uzorkovanja pH vrijednost je bila blago alkalna sa vrijednostima od 8,4 što ukazuje na III - IV klasu kvaliteta vode. Pri temperaturi vode od 13,1 °C, koncentracija rastvorenog O_2 iznosila je 10,75 mg/l, a zasićenje O_2 94,9% (tabela 1).

Lokalitet Otoka (L2) pripada gradskom dijelu i smatra se zagađenim područjem, jer je izložen uticaju otpadnih voda okolnih naselja. Ovdje je pH bila blago alkalna sa vrijednostima od 8,1 što je također ukazivalo na III - IV klasu kvaliteta. Pri temperaturi vode od 16,2 °C, koncentracija rastvorenog O_2 iznosila je 10,28 mg/l, a zasićenje O_2 88% (tabela 1).

Tabela 1. Hidromorfološki, fizički i hemijski parametri lokaliteta istraživanja
Table 1. Hydromorphological, physical, and chemical parameters of research sites

Parametar	L1	L2
Dužina lokaliteta (m)	100	60
Širina lokaliteta (m)	12	15
Površina lokaliteta (m^2)	1200	900
Prosječna dubina vodenog stuba (m)	0,50	0,60
Nadmorska visina (m)	510	558
Geografska dužina	N 43º50'47"	N 43º51'14"
Geografska širina	E 18º21'39"	E 18º22'27"
Vrijeme uzimanja uzorka	13.00	9.00
Temperatura vode na mjestu uzorkovanja (°C)	16,2	13,1

Rastvoreni O$_2$ (mg/l)	10,28	10,75
Zasićenje O$_2$ (%)	88,0	94,9
pH vrijednost vode	8,10	8,40
Električna provodljivost vode µS/cm	419	412

Temperatura vode, kao jedan od glavnih faktora kada su u pitanju paraziti, ovisila je uglavnom o temperaturi okolnog zraka. Zabilježene vrijednosti su se kretale između 13,1ºC (Dariva) i 16,2ºC (Otoka). Količina rastvorenog kisika i zasićenost vode kisikom su kompatibilni na oba lokaliteta. Vrijednosti zasićenja vode kisikom niže od 40% nepovoljno djeluju na prehranu riba, a time i na njihov prirast (Debeljak *et al.*, 1993). Posljedice toga su slaba tjelesna kondicija, te pojava i širenje bolesti kod riba (Teskeredžić & Račić, 1985).

Vrijednosti fizičko-hemijskih parametara generalno pokazuju trenutno, a ne dugoročno stanje kvaliteta vode i opterećenosti vodenog ekosistema. U istraživanjima ovog tipa mogu poslužiti kao zanačajna dopuna procjene stanja, naročito kada je u pitanju parazitološka i histopatološka analiza ribljih vrsta. No, bitno je detektirati da je pH vrijednost vode na oba lokaliteta ulazivala na kontaminiranu vodu (III-IV klasu kvaliteta).

Rezultati mikrobiološke analize vode

Rezultati mikrobiološke analize vode na dva istraživana lokaliteta rijeke Miljacke potvrđuju prisutnost koliformnih bakterija, *Escherichia coli* i *Enterococcus* sp. koji su značajni pokazatelji fekalne kontaminacije vode (tabela 2). Prema Kohlu (1975, izmijenjeno), Kavka i Poetsch (2002, modificirano) i prema EPCEU direktivama 2006/7 EEC voda na lokalitetu Dariva (L1) pripada II klasi kvaliteta površinske vode na osnovu broja intestinalnih enterokoka, dok klasi III na osnovu broja fekalnih koliformnih bakterija. Analizirana voda na lokalitetu Otoka (L2) pripada IV klasi kvaliteta vode na osnovu svih analiziranih parametara što ukazuje na prekomjerno fekalno zagađenje.

Tabela 2. Rezultati mikrobiološke analize vode rijeke Miljacke
Table 2. Results of the microbiological analysis of the Miljacka River water

Parameter	L1	L2
Ukupne koliformne bakterije cfu/100ml	13023	980 300
Fekalne koliformne bakterije cfu/100ml	4738	61 829
E. coli cfu/100ml	3789	49 456
Enterococcus cfu/100ml	3834	198 300

Rezultati analize starosne dobi, spola i osnovnih morfoloških mjera uzorkovanih jedinki potočne mrene

Rezultati mjerenih morfometrijskih parametara jedinki uzrokovanih za histološku analizu dati su u tabeli 3. Vrijednosti parametara predstavljene su kao srednje vrijednosti, standardna devijacija, minimalne i maksimalne vrijednosti uzoraka. Za potrebe ove analize analizirano je 10 uzoraka od čega (4 mužjaka i 6 ženki). Na lokalitetu Dariva analizirane jedinke dominantno su pripadale starosnoj dobi od jedne godine, a na lokalitetu Otoka podjednako su bile zastupljene jedinke starosti dobi od 2-3 godine.

Tabela 3. Morfometrijski parametri jedinki uzorkovanih za histološku analizu organa od interesa
Table 3. Morphometric parameters of specimens sampled for histological analysis of organs of interest

Lokalitet	Dariva			Otoka		
Morfometrijski parametri	TL (mm)	SD (mm)	Masa (g)	TL (mm)	ST (mm)	Masa (g)
Srednja vrijednost	126	108	21,93	211	182,9	104,09
Stand. devijacija	5,3	5,1	4,77	17,7	17,4	24,68
Min. vrijednost	118,8	99,9	18,2	183	155	73,6
Maks. vrijednost	135,7	115,7	32,1	236	209	136,3

Rezultati mjerenih morfometrijskih parametara (7+4 jedinke), starosti i spola jedinki uzrokovanih za parazitološku analizu dati su u tabeli 3 uz podatke o prisustvu parazita. Prosječna masa uzorkovanih riba je iznosila 61,29 g. Prosječan SL za uzorkovane ribe je iznosio 76,85 mm, s tim da je najmanji SL iznosio 18,7 mm (lokalitet Otoka), a najveći 115,77 mm (lokalitet Dariva). Prosječan TL je 89,83 mm (najmanji je evidentiran na L1 - 22,3 mm, a najveći na L2 - 135,75). Dobna struktura jedinki se kretala od 1-3 godine, s tim da su bile dominantne jedinke starosne dobi 1+. Mlade jedinke ribljih vrsta su prijemčivije za razvoj parazita, jer je veća mogućnost invazije i pojave znakova bolesti (Yanong, 2017). Ovi navodi su išli u prilog parazitološkoj analizi istraživane vrste.

Rezultati parazitološke analize istraživane riblje vrste

Na osnovu adspekcijskog i sekcijskog pregleda svih uzoraka riblje vrste *Barbus balcanicus*, na oba lokaliteta, nisu ustanovljeni klinički manifestni simptomi koji bi ukazivali na prisustvo bolesti.
Mikroskopskim pregledom strugotina ustanovljene su ukupno četiri vrste parazita kod svih 11 uzoraka/jedinki vrste *Barbus balcanicus*. U tabeli 4 su pored toga prikazani

rezultati svih ostalih već spomenutih mjerenih parametara (spol, starost, morfometrijski parametri i masa).

Tabela 4. Spol, starost, morfometrijski parametri (TL i SL), masa i prisustvo parazita (*Barbus balcanicus* iz rijeke Miljacke na oba lokaliteta uzorkovanja)
Table 4. Sex, age, morphometric parameters (TL and SL), weight, and presence of parasites (Barbus balcanicus from the Miljacka River at both sampling sites)

Broj uzorka (lokalietet)	Masa (g)	TL (mm)	SL (mm)	Spol	Starost	Prisustvo parazita
1 (L1)	22,6	130	112,9	M	2+	+
2 (L1)	26,7	135,75	115,77	M	2+	+
3 (L1)	22,65	127,93	110,72	M	1+	+
4 (L1)	18,26	123,14	99,94	Ž	1+	+
5 (L1)	18,62	125,72	107,6	M	1+	+
6 (L1)	32,08	129,44	109,38	M	1+	+
7 (L1)	20,19	124,57	109,04	M	1+	+
8 (L2)	136,3	22,7	20,9	Ž	3+	+
9 (L2)	136,1	23	20,6	Ž	3+	+
10 (L2)	135,2	23,6	19,9	Ž	3+	+
11 (L2)	105,5	22,3	18,7	Ž	3+	+

Kod sedam analiziranih jedinki potočne mrene iz rijeke Miljacke sa lokaliteta Dariva (L1) ustanovljena je jedna vrsta protozoa i dvije vrste metilja (Trematoda). Na lokalitetu Otoka (L2) analizirane su četiri jedinke istraživane vrste, kod kojih je potvrđeno prisustvo dvije vrste protozoa i jedna vrsta trematoda (tabela 5). Prisustvo vrste *Ichthyophthirius multifiliis* utvrđeno je kod svih analiziranih jedinki, na oba lokaliteta. Do nivoa vrste identifikovana je i vrsta *Chilodonella cyprini.* Ostali paraziti su determinisani do nivoa roda, konkretno rodovi *Dactylogirus* i *Diplozoon.*
Rod *Dactylogirus* (Monogenea; Trematoda), evidentiran na oba lokaliteta istraživanja, je metilj koji je ustanovljen i na koži i na perajima ispitivanih uzoraka (tabela 4). Daktilogiroza je vrlo česta bolest slatkovodnih riba koju uzrokuju paraziti iz grupe homogenih („jednodomaćinskih") metilja (Yamaguti, 1968). Na perajima i koži ciprinidnih riba, parazitira veći broj vrsta metilja roda *Dactylogyrus*, što može uzrokovati ugibanje mlađi posebno u prvom mjesecu života (Ćirković *et al.*, 2002).
Diplozoon je jedinstven rod unutar monogenetskih trematoda u kojoj odrasle jedinke ostaju ujedinjene u parovima u stalnoj kopulaciji u obliku križa (Yamaguti, 1963). Prema podacima od strane Kawatsu (1978) *Diplozoon* može da izazove hipohromnu anemiju. Lokalizacija parazitiranja vrste roda *Diplozoon* (tabela 4) kod svih inficiranih jedinki je koža.
Praživotinje (Protozoa; Protista) parazitiraju na koži, škrgama, u unutrašnjim organima i krvi riba. Parazitski oblici Protozoa u malom broju su bezopasni, međutim kada se namnože mogu izazvati ozbiljne posljedice u razvoju riba, pa čak i smrt. Na istoj ribi može se javiti više vrsta parazitskih Protozoa što predstavlja dodatni problem.

Protozojska vrsta trepljara, *Ichthyophthirius multifiliis*, je ustanovljena kod svih ispitivanih jedinki analizirane riblje vrste. Lokalizacija ove vrste je koža, peraja i škrge (tabela 5). U ukupnom ihtiouzorku najvišu prevalenciju infestacije ostvarila je, dakle, ova vrsta. Izgleda da *I. multifiliis* parazitira na svim slatkovodnim ribama i nema podataka o vrstama koje su prirodno otporne na nju, iako postoje razlike u osjetljivosti među vrstama (Molnar, 1995). Pogodni faktori za širenje bolesti su gustoća populacije u uzgoju, stres, loši parametri okoliša i temperatura. Više temperature ubrzavaju razvojni ciklus parazita. Prema svemu navedenom, a s obzirom na prethodno navedene rezultate, ova parazitska vrsta nije mogla doseći svoj „maksimum" u vrijeme uzorkovanja. Morbiditet i mortalitet je izražen u visokom postotku, naročito kod mlađi, dok starije ribe ugibaju sporadično, ali su istovremeno parazitonoše (Jažić *et al*., 2009). Protozojska, cilijatna vrsta *Chilodonella cyprini*, evidentirana je samo na lokalitetu Otoka na koži istraživane vrste (tabela 4). Ove parazitske vrste parazitiraju na tijelu i perajima riba. Izazivaju iritaciju kože i pojačavaju mukoznu sekreciju. Utvrđeno je da trajanje aktivnog stanja parazita izvan tijela domaćina/ribe ovisi o temperaturi. Najaktivnije se dijeli na 5 - 10°C. Na 20°C podjela praktično prestaje (Bauer & Nikol'skaya, 1957). Izmjerene tmperature na mjestu uzorkovanja naše vrste nisu pogodovale ni jednom ni drugom. Odlučujući uticaj ima svakako fiziološko i kondiciono stanje ribe.

Vrsta *Barbus balcanicus* u našoj studiji ima evidentirane parazite čiji je intenzitet infestacije generalno slabog stepena (+) (tabela 5). *Dactylogirus sp.* i *Chilodonella cyprini* parazitiraju na koži i perajima, *Ichthyophthirius multifiliis* i *Diplozoon sp.* na škrgama.

Tabela 5. Mikroskopski pregled strugotina istraživane riblje vrste (Ø/O/+/++/+++ - simboli uzlazne gradacije promjena izazvanih parazitskim vrstama)
Table 5. Microscopic examination of scrapes from the investigated fish species (Ø/O/+/++/+++ - symbols indicating the ascending severity of changes caused by parasitic species)

Lokalitet Dariva		
Organ	**Nalaz**	**Stepen promjena (Ø/O/+/++/+++)**
Koža, peraje	*Dactylogirus sp., Ichthyophthirius multifiliis*	O/+
Škrge	*Diplozoon sp., Ichthyophthirius multifiliis*	O/+
Crijeva	Bez parazita i razvojnih oblika parazita	Ø
Lokalitet Otoka		
Organ	**Nalaz**	**Stepen promjena (+/++/+++)**
Koža, peraje	*Chilodonella cyprini, Dactylogirus sp.,*	=/+
Škrge	*Ichthyophthirius multifiliis*	O/+
Crijeva	Bez parazita i razvojnih oblika parazita	Ø

Rezultati histološke analize tkiva

Histološkom analizom utvrđeno je prisustvo patoloških lezija u tkivu škrga i jetre jedinki sa oba lokaliteta (slika 2). Veći stepen promjena zabilježen je u uzorcima jedinki koje su prikupljene na lokalitetu Otoka u odnosu na lokalitet Dariva.

Analizom škrga uočene su lezije u lamelarnom epitelu ili dublje u tkivu lamela (slika 2a, b, c). Zabilježene lezije okarakterisane su kao nodule uglavnom sfernog do jajolikog oblika i potpuno zatvorene unutar zida epitela. Naši rezultati su u korespondenciji sa brojnim studijama koje ove formacije pripisuju različitim parazitskim infekcijama, ali također navode i značajnu vezu između bakterioloških parametara u vodi i upalnih procesa u tkivima riba (Azevedo *et al.*, 2009; Kilian and Avenant-Oldewage, 2013; Kaur and Katoch, 2014; Dar *et al.*, 2020).

Histopatološkom analizom hepatocelularnog parenhima uzoraka sa oba lokaliteta zabilježene su ozbiljne patološke lezije (slika 2d,2e,2f). Veći stepen ovih promjena također je konstatovan kod uzoraka sa lokaliteta Otoka, što povezujemo sa većim antropogenim pritiskom na ovom dijelu vodotoka rijeke Miljcke. Zabilježene su proliferativne i multifokalne nodularne lezije različitih veličina i sastava (slika 2d). Okarakterizirane su nodularne lezije sa nekrotičnim centralnim dijelom (slika 2e) i nodule ograničene fibroznim tkivom (slika 3d). Nodularne lezije zabilježene su u nekoliko studija koje su sugerisale njihovu povezanost sa različitim vrstama parazitskih infekcija (Mahmoud *et al.*, 2016; Alexandre Sebastião *et al.*, 2019). Promjene hepatocelularnog parenhima mogu biti i rezultat odgovora organizma na prisustvo patogenih mikroorganizama u vodenim ekosistemima (Novotny *et al.,* 2010; Rajme-Manzur *et al.*, 2021).

Slika 2**.** Histopatološke lezije u škrgama (a, b, c) i jetri (d, e, f) *Barbus balcanicus*
Figure 2. Histopathological lesions in gills (a, b, c) and liver (d, e, f) of Barbus balcanicus

Rezultati parazitoloških i histoloških analiza tkiva vrste *Barbus balcanicus* u odnosu na stepen opterećenosti/kontaminiranosti vode rijeke Miljacke

Poulin (2014) navodi kako raznolikost parazitskih vrsta ovisi o više faktora. Jedan od faktora se odnosi prvenstveno na specifičnost parazitskih vrsta. Manje specifične vrste inficiraju pripadnike različitih taksonomskih skupina, dok su one visoko specijalizirane vrste najčešće ograničene na samo jednu vrstu ili rod.

Ekološki faktori u kojima domaćin obitava također mogu imati veliki uticaj na brojnost parazitskih vrsta, a utiču i na opće stanje domaćina koje predstavlja još jedan od značajnih elemenata pri čemu Poulin i Morand (2000) spominju i važnost raspoloživog prostora za parazite, tačnije samu veličinu domaćina. Bagge *et al.* (2004) ističu veću važnost u brojnosti jedinki u populaciji naspram gustoće populacije, što

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znači da prema navedenom autoru za daljnje širenje parazita veću ulogu igra broj raspoloživih jedinki od bliskog kontakta između domaćina.

Riblji paraziti se obično nalaze u hrskavici škržnih lukova, slušnog i statičkog organa, kao i u kičmi. Spore se mogu naći u svim dijelovima tijela. Ovom činjenicom možemo tumačiti da i žive, prividno zdrave ribe koje su preboljele invaziju mogu lučiti spore, te na taj način predstavljaju direktnu opasnost za širenje oboljenja. Parazitarne bolesti mogu izazvati ogromne štete, naročito ukoliko nastanu takvi ekološki uslovi koji djeluju negativno na otpornost riba, kao što su nepravilna ishrana, promjene fizičko-hemijskog sastava vode i prenaseljenost pojedinih rijeka (Skenderović, 2015).

Vrijednosti temperature i vodostaj u vrijeme uzorkovanja tokom našeg istraživanja nisu bili izuzetno povoljni za razvoj kožnih, ektoparazitskih protozoa. S tim smo povezali izostanak invazije i samim tim ispoljavanje simptoma bolesti. Sprovedena ihtioparazitološka istraživanja su time potvrdila da je pojava ektoparazita usko povezana sa mikroklimatskim faktorima sredine, gustinom ihtiopopulacija u akvatičnom ekosistemu, stepenom kvaliteta vode i ekoloških uslova datog ekosistema. Na temelju detektovanih parazita, kao svojevrsnih ekoloških pokazatelja, evidentno je da je voda oba lokaliteta rijeke Miljacke zagađena. To je svakako potvrdila mikrobiološka analiza vode na osnovu čijih je parametara određena kategorizacija vode (umjereno do jako zagađena) te djelimično i fizičko-hemijska analiza vode.

Histološke analize su također pokazale promjene u tkivima (škrgama i jetri) sa većim stepenom upravo na onom lokalitetu gdje voda ima veći stepen zagađenija na osnovu mikrobiološke analize, ali i sa dominacijom opasnih protozojskih parazita. Osim što se pregledom literature ove promjene povezuju s bakteriološkim patogenima, kako i jeste potvrđeno u ovoj studiji, također indiciraju prisustvo parazita. Jednokratno uzorkovanje i tada prisutni parametri su svakako nedostatak za donošenje definitivnih zaključaka, ali prediktivni su potpuno osnovani. Već sama detekcija ovih parazita je utemeljenje za navedeno, ali i potvrda da se i u limitiranoj frekvenciji uzorkovanja, pa i broju lokaliteta i jedinki, ovi aspekti analiza mogu dovesti u vezu i dati značajnu kompenzaciju cjelokupnog ekološkog stanja istraživanih lokaliteta. Odabrana riblja vrsta sigurno može biti dobar model upravo za ovakav tip istraživanja, naročito što je relativno kvantitativno dobro zastupljena u istraživanom području.

Rezultati mikrobiološke analize kvaliteta vode rijeke Miljacke i analize fizičko-hemijskih parametara vode, kao i ostalih ekoloških uslova istraživanog područja, svakako ukazuju da je rijeka Miljacka veoma povoljna sredina za razvoj parazita. Samo prisustvo parazita i histološka analiza tkiva određene vrste i njihovo frekventnije praćenje bi nam sigurno ukazalo na kvalitet vode istraživane rijeke, u slučaju da nismo u mogućnosti izvršiti mikrobiološku analizu ili nekog drugog biološkog elementa kvaliteta vode. Iako je multidisciplinarnost analiza jednog akvatičnog ekosistema i bioindikatorskih vrsta koji tu obitavaju najsigurniji način za dobivanje uvida u realno i kompletno stanje i ekološki status površinskih voda, uspostava i značaj ovih korelacija je veliki.

ZAKLJUČAK

Vrsta na kojoj je vršena parazitološka i patohistološka analiza, *Barbus balcanicus* Tsigenopoulos, Ráb & Berrebi, 2002, predstavlja adekvatan model i bioindikator za ispitivanje zagađenosti vodenih ekosistema zbog senzitivnosti na parazitske vrste i manifestno djelovanje različitih mikrobioloških patogena na njena tkiva. U ovom radu je tako njena bioindikativnost podignuta na veći nivo, zbog njene osjetljivosti na parazite i uzročno-posljedično pratećih histopatoloških promjena.

Ihtioparazitološka istraživanja u rijeci Miljacka, na odabranoj vrsti, su pokazala prisustvo četiri vrste parazita: *Dactylogyrus sp., Ichtyophtirius multifiliis, Diplozoon sp.* i *Chilodonella cyprini,* s tim da najveću infestiranost pokazuje vrsta *Ichtyophtirius multifiliis.* Adspekcijskim i sekcijskim pregledom jedinki nisu ustanovljeni klinički manifestni simptomi koji bi ukazivali na prisustvo bolesti. Sprovedena ihtioparazitološka istraživanja su pokazala da je pojava ektoparazita usko povezana sa mikroklimatskim faktorima sredine, gustinom ihtiopopulacija u akvalnom ekosistemu, stepenom kvaliteta vode i ekološkim uslovima datog ekosistema. Uvidom u rezultate mikrobiološke analize te analize fizičko-hemijskih parametara vode utvrđen je stepen zagađenosti, pri čemu je očekivano veći na lokalitetu Otoka usljed veće naseljenosti i izraženijeg antropogenog uticaja. Histopatološkom analizom utvrđeno je prisustvo patoloških lezija u tkivu škrga i jetre jedinki sa nešto većim stepenom promjena u uzorcima jedinki uzorkovanih na navedenom lokalitetu. Povezanost ovih promjena sa parazitskim infekcijama i prisustvom patogenih mikroorganizama u našem istraživanju je potvrđena primarna hipoteza rada.

Uzimajući u obzir rezultate svih aspekata istraživanja, rijeka Miljacka pogoduje razvoju parazita. Da bismo u potpunosti potvrdili ovaj zaključak, te da bismo ispitali prisustvo, brojnost i diverzitet parazitskih vrsta u perspektivi, neophodno je sprovesti istraživanje u svim sezonama na istim ili pak na više lokaliteta. Preporuka za sveobuhvatnost procjene stanja površinskih voda i jasnije procjene korelacije između ovih značajnih segmenata istraživanja ribljih vrsta i ekološkog statusa njihovog biotopa bi bila da se uključi i više ribljih vrsta i jedinki uz neophodne dodatne analize. Bez obzira na neophodne dopune, iz ovog istraživanja je jasno da kontaminirani akvatični ekosistem uslovljava prisustvo parazita i histopatološke promjene određenih tkiva ribljih vrsta, ali i obrnuto – parazitske infekcije i promjene u tkivu ukazuju na kontaminiranost određenog vodenog ekosistema. Navedeno predstavlja više „alata" kao i mogućnost izbora za ostvarivanje ovakvih ili sličnih ciljeva istraživanja.

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HISTOPATHOLOGICAL AND PARASITOLOGICAL ANALYSIS OF *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002 TISSUES AS A BIOINDICATOR OF MILJACKA RIVER

Summary

Water ecosystem pollution causes stress in fish species and leads to diseases as a consequence of interaction with opportunistic pathogens. Parasitic species pose a particular threat to fish health, being transmitted from one fish to another through direct and/or indirect means. This research conducted a parasitological analysis of the bioindicator species Barbus balcanicus. Histological analysis was performed to detect the relationship with parasitic infections, as well as the correlation between water bacteriological parameters and inflammatory processes in fish tissues. Sampling was carried out in July 2021 at two locations in the Miljacka River. Analysis of the tissue samples from the individuals detected the presence of parasites from the groups Protozoa and Trematoda, which were identified up to the level of genus or species using relevant keys and literature. Alongside ichthyoparasitological research, an analysis of physicochemical water icrobiological analysis was conducted, with results indicating pollution of the Miljacka River and favorable conditions for parasite development. Histological analysis confirmed the presence of pathological lesions in the gill and liver tissues of individuals, linked to parasitic infections and the presence of pathogenic microorganisms. Various pollutants in water ecosystems contribute to the presence of parasites and histopathological changes in fish species, while parasitic infections and tissue changes indicate poor ecological status and contamination of surface waters.

Key words: *barbel, parasitology, pathohistology, pollutants, biological elements of water quality*

AVAILABILITY AND TECHNICAL ASSESSMENT OF TRACTOR PURCHASING POWER AMONG FARMERS IN CEFTA COUNTRIES*

Nermin Rakita[1], Zuhdija Omerović[1], Zoran Maličević[2], Muamer Bezdrob[1], Enver Karahmet[1] and Bešćo Alibegović[3]

Abstract

The tractor is a key driving unit in modern agricultural production. This study explores the possibilities and capabilities of farmers in Bosnia and Herzegovina (BiH) compared to other CEFTA members in purchasing an adequate, quality, and suitable new tractor. The research analyzes the process of trade liberalization in Southeast Europe, including the Agreement on Amendment and Accession to CEFTA. The study covers an analysis of ten global tractor brands across 110 different models that are available in CEFTA countries market. The average tractor prices and prices per kW of power were calculated by brand, and a brendization (according to the Analytical Hierarchy Process method (AHP)) into three groups: A, B, and C was performed. The research results showed that New Holland brand tractors are the most expensive, while Foton brand tractors are the cheapest. In terms of price per kW, Ferrari brand tractors are the most expensive, while ArmaTrac brand tractors are the cheapest. Differences in the purchasing power of farmers in BiH compared to CEFTA members were also analyzed, with a focus on differences in average salary in countries across three years (2018, 2019, and 2020). The purchasing power of farmers was determined by calculating the purchasing power coefficient (PPC), which showed that farmers from Serbia and farmers from Montenegro have the highest purchasing power for all examined tractor brands, while Moldova is the weakest by this indicator, and theoretically, farmers do not have the ability to purchase tractors of any examined brand. Farmers from BiH do not have the purchasing power to buy Kubota and Ferrari brand tractors but have the purchasing power to purchase Foton, ArmaTrac, and Case brand tractors.

Keywords: *tractor, brand, CEFTA, purchasing power, farmer.*

INTRODUCTION

Agricultural tractors play a crucial role in modern farming by delivering the power and efficiency needed to carry out essential tasks like tillage, sowing, feeding, harvesting, and transportation. Their importance makes selecting the right tractors, along with

favorable purchasing terms, vital for boosting productivity and optimizing the economics of agricultural operations. Today, the focus is on reducing production time, lowering costs, and automating human labor through the use of various tools and machines. Modern tractors enable faster, better, and more efficient execution of agro-technical operations. In BiH, farmers must remain competitive with producers from the European Union and CEFTA countries, making the choice and availability of agricultural machinery even more critical to their success.

CEFTA (*the Central European Free Trade Agreement*) is an economic organization comprising Albania, Bosnia and Herzegovina, Montenegro, North Macedonia, Moldova, Serbia, and Kosovo. Initially, the organization included countries that later became members of the European Union, such as the Czech Republic, Hungary, Poland, Slovakia, Slovenia, Bulgaria, Romania, and Croatia. The 2006 CEFTA agreement marked a commitment by its members to enhance trade and investment policies across Southeast Europe, with the goal of establishing a free trade zone aligned with the regulations of both the World Trade Organization and the European Union.

The objective of this paper is to analyze the availability of agricultural tractors in CEFTA countries and examine purchasing options in relation to the average salaries of their residents. Research into the tractor market in this region provides valuable insights for agricultural producers, traders, and other stakeholders regarding tractor availability, technical specifications, and the financial aspects of acquisition. Previous studies and research (Škaljić *et al.*, 2014; Škaljić *et al.*, 2017) have shown that the technical equipment and characteristics of tractors have a significant impact on the efficiency of agricultural operations, highlighting the importance of a comprehensive market analysis. In BiH, farmers have long been affected by outdated and poorly maintained agricultural machinery, resulting in increased fuel consumption and environmental pollution due to the declining operational efficiency of tractors (Rakita and Škaljić, 2016).

Machinery costs represent a significant portion of expenses for farms operating in highly mechanized systems. In the last 10 years, the costs have risen due to machines with high engine power, advanced technology, expensive spare parts, frequent repairs, and increased fuel consumption (Sopegno *et al*., 2016; Bochtis *et al*., 2014; Najafi *et al*., 2015; USDA, 2014). Škaljić *et al*. (2017) point out that the utilization of agricultural machinery in BiH is notably low, primarily due to outdated equipment, declining reliability, and high decommissioning costs. A study conducted in the Tuzla Canton (Škaljić *et al.*, 2017) administrative area revealed that 23% of tractors are operated for less than 100 hours annually, 43% between 100-200 hours, and 34% over 200 hours. The average age of these tractors is 25 years. On average, each two-axle tractor covers 3.2 hectares, with an output power of 10.9 kW per hectare of arable land.

This analysis aims to provide farmers in CEFTA countries, particularly in BiH, with valuable insights to make informed decisions when purchasing tractors, ultimately enhancing the productivity and competitiveness of their agricultural operations.

MATERIALS AND METHODS

Data on tractor prices per kilowatt (kW) of engine power and their sales were collected over three years (2018, 2019, and 2020) at the International Agricultural Fair in Novi Sad, Serbia. The research covered 10 tractor brands, with prices defined for specific models, resulting in the analysis of 110 tractor models. For each model, the engine power was measured in kilowatts (kW), and the price per kilowatt of installed power was recorded, with all prices expressed in euros (€). The purchasing power of farmers was estimated based on the average salaries in all CEFTA member countries during this period.

The methodology involved collecting and processing data from the International Agricultural Fair, online sources, and relevant literature. Basic mathematical operations such as addition, subtraction, and multiplication were used to calculate tractor prices per kW. Additionally, statistical methods were applied, including the calculation of average, maximum, and minimum prices. Statistical analysis was conducted using IBM SPSS Statistics software, employing one-way analysis of variance (ANOVA), the Tukey test, and descriptive statistics.

It has been challenging to find a way to determine the purchasing power of farmers due to the lack of available data on farmers. For this purpose, a purchasing power coefficient was created, which is based on simple and easily available data, and was used as such for this research. The purchasing power coefficient (PPC) was calculated by comparing the average salary in each country to the average price of a tractor per kW of engine power. This approach provided insights into the affordability of tractors across different CEFTA nations.

$$PPC = \frac{AS}{AP}$$

Where: PPC represents the purchasing power coefficient, AS is the average salary in the country, and AP is the average tractor price per kilowatt (kW) of engine power. Intervals of coefficient values were determined and the values of 1 or higher indicate that farmers have sufficient purchasing power to afford tractors from that brand. A coefficient between 0.7 and 1 suggests that farmers can afford some of the lower-priced models, while a coefficient below 0.7 indicates insufficient purchasing power to afford any models from that brand.

To further refine the classification of tractors and incorporate multiple relevant factors into the decision-making process, the Analytical Hierarchy Process (AHP) method was employed (Saaty, 1980). The AHP method structures the problem by establishing a hierarchy of criteria, such as price, engine power, quality, reliability, and other technical specifications. A comparative evaluation of tractors based on each criterion was conducted, and the results were used to calculate weighting coefficients for each factor. This enabled the ranking of tractors and their classification into three groups (A, B, and C) based on a comprehensive set of parameters.

This research examined 10 tractor brands: Ferrari, Kubota, Case, Steyr, Claas, New Holland, Same, Ursus, ArmaTrac, and Foton. The brands were classified into three

groups based on a combination of average power output and technological advancement:

- **Group A** (high power and advanced technology): Steyr, Claas, and New Holland
- **Group B** (moderate power and technology): Kubota, Same, and Ursus
- **Group C** (lower power and technological development): Ferrari, Case, ArmaTrac, and Foton.

This classification helps distinguish the brands by their performance and innovation levels.

Group A comprises tractors from Steyr, Claas, and New Holland, all recognized for their long-standing traditions, innovative technologies, and diverse range of high-quality models.

- **Steyr** is an Austrian brand with a rich history dating back to the 1940s. Renowned for producing high-performance tractors, Steyr stands out for its cutting-edge technologies and commitment to quality.
- **Claas**, a German brand, offers not only tractors but a comprehensive range of agricultural machinery, including combines, balers, and lawn mowers. The diversity of its models and equipment options make Claas highly competitive in the agricultural sector.
- **New Holland**, an American brand, is a global leader in the production of specialized tractors, celebrated for its continuous innovation and extensive range of models.

Group B comprises tractors from Kubota, Same, and Ursus, brands recognized for offering moderate power and technological advancement.

- **Kubota**, a Japanese brand, has been gaining popularity in the Balkans due to its competitive pricing and strong price-performance ratio, making it an attractive option for many farmers.
- **Same**, an Italian brand under the Same Deutz-Fahr Group, is known for its high-quality tractors and diverse model range, providing reliable solutions across various farming needs.
- **Ursus**, a Polish brand with a long-standing tradition in tractor manufacturing, offers a variety of models with different engine types, catering to a range of agricultural applications.

Group C encompasses tractors produced by Ferrari, Case, ArmaTrac, and Foton.

- **Ferrari**, an Italian brand, offers tractors designed to meet the latest European standards, ensuring compliance and quality.
- **Case IH**, an American brand, is renowned for its innovative agricultural solutions and a diverse range of equipment, including tractors, harvesters, and various other agricultural machinery.

- Á **ArmaTrac**, a Turkish brand, produces tractors through Erkunt Traktor Sanayii A.S., known for its extensive selection of tractor models catering to various farming needs.
- Á **Foton**, a Chinese brand, has made its mark in the European market by providing tractors that adhere to high-quality and safety standards.

RESULTS AND DISCUSSION

Group A tractors

The research examined 12 Steyr models, 8 Claas models, and 14 New Holland models. Table 1 presents an overview of key performance indicators for tractors in this group, based on the average values of various models observed over a three-year period.

Table 1. Key performance indicators for tractors in group A

Brand	Engine power (kW)	Price (€)	Price per kW (€/kW)
Steyr	101.3±50.2	68000±42012	647.8±102.9
Claas	82.6±22.7	52125±19482	621.0±92.1
New Holland	123.2±75.5	68214±52450	531.7±85.6
Average	**102.4±49.5**	**62779±37981**	**600.2±93.5**

The data in Table 1 highlights several key findings. New Holland tractors have the highest average engine power, at 123.2 ± 75.5 kW, while Claas tractors offer the lowest average power, at 82.6 ± 22.7 kW. In terms of pricing, New Holland also leads with the highest average price of €68,214 ± 52,450, whereas Claas is the most affordable in this group, with an average price of €52,125 ± 19,482.

Interestingly, despite New Holland's higher average price, Steyr tractors have the highest price per kW of engine power, at €647.8 ± 102.9/kW. This suggests Steyr tractors emphasize premium components and advanced technology. In contrast, New Holland, with the lowest price per kW (€531.7 ± 85.6/kW), offers excellent value for those seeking high power at a relatively lower cost.

The overall averages for Group A tractors show a power output of 102.4 kW, an average price of €62,779, and a price per kW of €600.2/kW. These figures demonstrate that tractors in this group offer a strong combination of high power and advanced technological features.

In summary, Group A tractors not only deliver superior power but also incorporate cutting-edge technologies, making them ideal for farmers focused on maximizing productivity and efficiency.

Group B tractors

The analysis covered 13 Kubota models, 19 Same models, and 3 Ursus models. Table 2 presents an overview of key performance indicators for tractors in this group, based on the average values of various models observed over a three-year period.

Table 2. Key performance indicators for tractors in group B

Brand	**Engine power (kW)**	**Price (€)**	**Price per kW (€/kW)**
Kubota	48.7±27.9	35092±18880	762.5±158.8
Same	76.2±36.6	41731±19939	555.1±77.3
Ursus	65.4±13.6	35473±4722	547.3±37.9
Average	**63.4±26.0**	**37432±14513**	**621.6±91.3**

Table 2 reveals that Same tractors boast the highest average engine power at 76.2 ± 36.6 kW, while Kubota presents the lowest average power at 48.7 ± 27.9 kW. When it comes to pricing, Same models are the most expensive, with an average price of €41,731 ± 19,939, whereas Kubota tractors are the most affordable, averaging €35,092 ± 18,880. An analysis of the price per kW of engine power indicates that Kubota has the highest cost at €762.5 ± 158.8 per kW, while Ursus offers the most competitive price at €547.3 ± 37.9 per kW. The overall average engine power for this group is 63.4 kW, with an average tractor price of €37,432 and an average price per kW of €621.6.

Tractors in Group B present a balanced relationship between power, price, and technological advancement. This group features various advantages: Same provides higher power at a premium price, while Kubota offers more affordability but with less power. Ursus stands out for its lowest price per kW, making it an appealing choice for farmers seeking a cost-effective option without compromising too much on power.

Group C tractors

The analysis included 14 models from Ferrari, 7 models from Case, 4 models from ArmaTrac, and 16 models from Foton. Table 3 presents an overview of key performance indicators for tractors in this group, based on the average values of various models observed over a three-year period.

Table 3. Key performance indicators for tractors in group C

Brand	**Engine power (kW)**	**Price (€)**	**Price per kW (€/kW)**
Ferrari	40.3±13.4	28107±8322	786.7±446.2
Case	57.8±16.5	28085±10842	474.5±69.3
ArmaTrac	67.7±12.5	26297±3894	392.7±42,4
Foton	44.9±16.6	18850±7041	433.3±32.9
Average	**52.7±14.7**	**25334±7524**	**521.8±147.7**

Table 3 reveals that among group C tractors, ArmaTrac has the highest average engine power at 67.7 ± 12.5 kW, while Ferrari provides the lowest average power at 40.3 ± 13.4 kW. In terms of pricing, Ferrari models are the most expensive, with an average price of €28,107 ± 8,322, whereas Foton offers the most affordable models, averaging €18,850 ± 7,041.

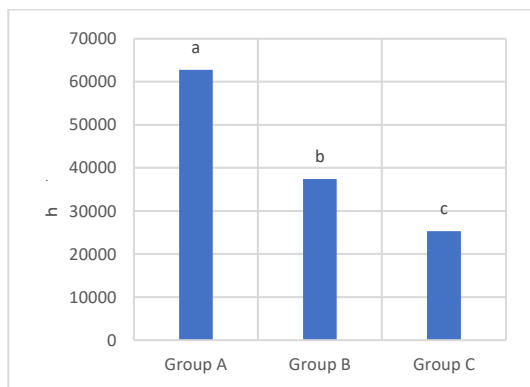An analysis of price per kW of engine power shows that Ferrari has the highest cost at €786.7 ± 446.2 per kW, suggesting a focus on premium quality and specialized features. In contrast, ArmaTrac offers the most economical price per kW at €392.7 ± 42.4, making it a value-driven option for those seeking more power at a lower cost.

The average engine power across group C is 52.7 kW, with an average tractor price of €25,334 and a price per kW of €521.8. Tractors in this group cater to lower power and technological development, emphasizing affordability. ArmaTrac stands out for delivering the highest power at a competitive price, while Ferrari commands the highest price per kW, reflecting its premium positioning. Foton emerges as the most budget-friendly option, appealing to cost-conscious buyers.

Price differences between tractor brands

The brand is a significant factor influencing tractor prices, similar to its impact on other consumer products. Well-established and widely recognized brands typically command higher prices per kW of engine power. Buyers with greater purchasing power often choose these premium brands due to their reputation for quality, reliability, and advanced features.

This analysis highlights the average tractor prices and price per kW of engine power across the three tractor groups, as illustrated in Graphs 1 and 2. These comparisons provide valuable insights into how brand reputation and performance affect overall pricing in the tractor market.
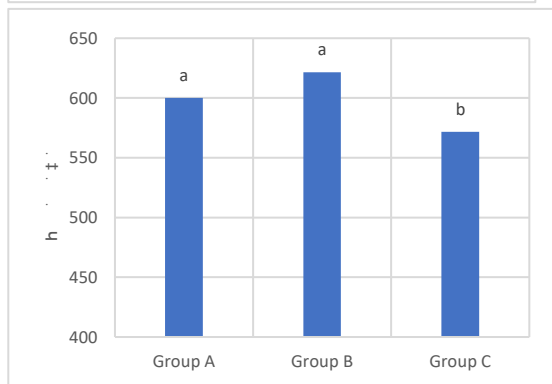
Figures 1 and 2. Average tractor prices and prices per kW of engine power of different brands offered to farmers from CEFTA member countries

A one-factor analysis of variance (ANOVA) reveals that tractor brand significantly affects average tractor prices (F=5.09, p=0.000 < 0.05). To further explore the price differences between brands, the Tukey test was employed. The results indicate that Group A tractor brands have significantly higher average prices, while Group C brands offer the lowest prices. Specifically, New Holland (€68,214.3), Steyr (€68,000), and Claas (€52,125) have the highest average prices, followed by Same (€41,731), Ursus (€35,473.3), and Kubota (€35,092.3). On the lower end, Ferrari (€28,107.1) and Case (€28,085.7) are priced modestly, with ArmaTrac (€26,297.5) and Foton (€18,850) being the most affordable options.

However, these average prices don't fully capture the complexity of pricing, as some brands offer tractors with higher engine power and more advanced features, while others provide lower-powered, basic models. Analyzing prices per kW of engine power provides a more accurate comparison among brands. The ANOVA also shows that brand significantly impacts the price per kW of engine power (F=5.06, p=0.000 < 0.05). According to the Tukey test and supporting graphs, Group B (€621.6/kW) and Group A

(€600.2/kW) brands have significantly higher prices per kW, while Group C brands have the lowest price per kW (€521.8/kW).

These findings suggest that although Kubota is part of Group B, it has a relatively high cost per kW, and Ferrari, from Group C, also has a higher price per kW due to the lower engine power offered in the market.

For farmers seeking high engine power, it is advisable to consider brands with a lower cost per kW, such as Case, Foton, and ArmaTrac. Conversely, farmers who don't require high engine power may opt for technologically advanced brands, accepting a higher price per kW in exchange for superior performance and additional features.

Average salaries in CEFTA member countries

The member countries of the Central European Free Trade Agreement (CEFTA) include BiH, Serbia, Montenegro, Albania, Moldova, North Macedonia, and Kosovo. The figure below presents a comparison of the average monthly salaries across these CEFTA nations, providing insight into the economic disparities and income levels within the region.
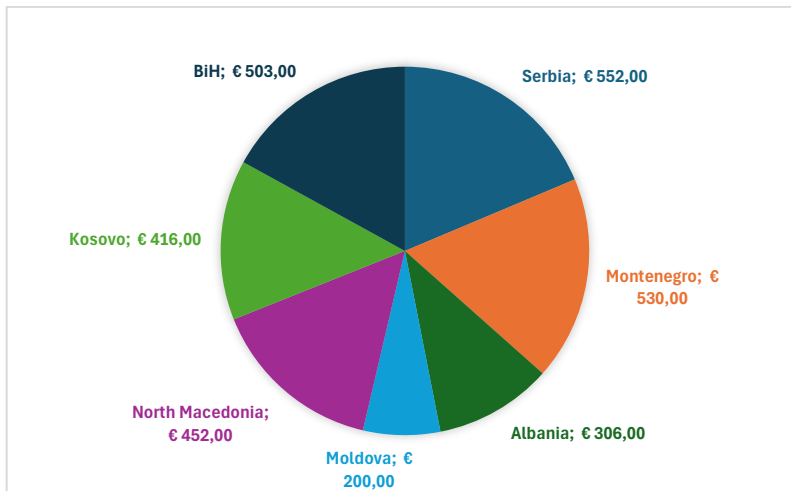
Figure 3. Average monthly salaries in CEFTA member countries

The average monthly salaries of residents in CEFTA member countries were calculated based on a three-year average (2018-2020). Among the seven CEFTA countries, Serbia[1] recorded the highest average salary at €552, while Moldova[2] had the lowest, with an average of €200. Other member countries showed the following averages: BiH[3] at

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[1] www.stat.gov.rs

[2] www.statistica.gov.md

[3] www.bhas.gov.ba

€503, Montenegro[1] at €530, Albania[2] at €306, North Macedonia[3] at €452, and Kosovo[4] at €416.

These figures offer valuable insights into the economic landscape of CEFTA nations, highlighting regional income disparities and living standards when compared to more developed economies. Understanding these differences can help in assessing the broader economic conditions within the region.

Purchasing power of farmers in CEFTA member countries

To achieve the established goals, it is essential to analyze the purchasing power of farmers in CEFTA member countries concerning various tractor brands. This analysis will be based on the average salary in these countries and the average prices of tractors per kW of engine power.

Purchasing power is assessed by comparing the average salary to the price of a tractor per kW of power, using the PPC coefficient. If the average salary exceeds the average tractor price per kW, the coefficient will be greater than 1, indicating that farmers in that country have sufficient funds to purchase such tractors. Conversely, a coefficient of less than 1 suggests insufficient purchasing power.

Table 4 illustrates the purchasing power of farmers from CEFTA member countries in relation to different groups of tractor brands.

Table 4. Purchasing power of CEFTA farmers by tractor brand groups

CEFTA member countries	Group A			Group B			Group C			
	Class	Steyr	New Holland	Kubota	Ursus	Same	Foton	ArmaTrac	Case	Ferrari
BiH	+	+	+	-	+	+	++	++	++	-
Serbia	+	+	++	+	++	++	++	++	++	+
Montenegro	+	+	++	+	+	+	++	++	++	-
Albania	-	-	-	-	-	-	+	+	-	-
Kosovo	+	+	+	-	+	+	+	++	+	-
North Macedonia	+	+	+	-	+	+	++	++	+	-
Moldova	-	-	-	-	-	-	-	-	-	-

In Table 4, the presence of two plus signs (++) indicates an PPC coefficient of 1 or higher, signifying that farmers in those countries possess sufficient purchasing power

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[1] www.monstat.org

[2] www.instat.gov.al

[3] www.stat.gov.mk

[4] www.ask.rks-gov.net

to buy tractors from the specified brand. A single plus sign (+) indicates an PPC coefficient ranging from 0.7 to 1, meaning that farmers can afford some of the more affordable and basic versions of tractors from that brand. Conversely, a minus sign (-) denotes an PPC coefficient below 0.7, indicating that farmers in those countries cannot afford tractors from that brand.

Based on the presented results, it can be concluded that only farmers in Serbia possess the purchasing power to acquire tractors from all brands, particularly those in group C, as well as some more affordable models from groups A and B. Farmers in Montenegro demonstrate similar purchasing power, enabling them to purchase tractors from group C, with the exception of the Ferrari brand, along with select lower-priced models from groups A and B. Farmers in BiH can also afford tractors from group C, excluding Ferrari, and some basic models from groups A and B, except for Kubota. Kosovo's farmers similarly have the purchasing power for group C tractors (excluding Ferrari) and certain models from groups A and B. The situation is comparable for farmers in North Macedonia. However, farmers in Moldova exhibit the lowest purchasing power, as they cannot afford any models from the offered brands and groups. Only those with above-average incomes in Moldova can purchase select models of tractors from the available brands.

To more accurately assess the purchasing power of agricultural producers when purchasing a tractor, it would be beneficial to consider factors such as yield and profit per hectare of agricultural land. Key indicators include the age of machinery, available power (kW/ha), useful lifespan, and maintenance costs. The average age of agricultural machinery is notably high. According to Zimmer *et al*. (2018), the total average age of tractors examined in their study is 20.71 years, which aligns closely with the average ages of tractors in many EU countries. In the United States, the average age of tractors exceeds 25 years, as reported by Murphy *et al*. (2010). In the research involving OPGs, the total average available power of tractors per unit of agricultural area is 5.59 kW/ha. In comparison, Zimmer *et al*. (2018) notes that the average available power of tractors in the USA is about 1 kW/ha of utilized land, while in Germany it is approximately 2 kW/ha, and in France around 6 kW/ha. In BiH, Škaljić *et al*. (2017) state that the average age of tractors is 25 years. The load of two-axle tractors per unit area is 3.2 hectares per tractor, with an output power of 10.9 kW/ha for arable land.

CONCLUSIONS

This paper explores the purchasing capabilities and potential of farmers in BiH compared to other CEFTA member countries in acquiring suitable, high-quality new tractors. The analysis covered ten global tractor brands and examined 110 different models, calculating their average prices and prices per kW of power. These brands were categorized into three groups (A, B, and C) using the Analytic Hierarchy Process (AHP) method. The research revealed that tractors in group A (Steyr, Claas, New Holland) offer superior technology and power, but they come with the highest price tags, making them more suitable for farmers with larger budgets. Group B tractors (Kubota, Same, Ursus) strike a balance between price and performance, providing solid options for those

looking for value. In contrast, tractors in group C (Ferrari, Case, ArmaTrac, Foton) are more affordable but typically lack advanced technologies. Additionally, the findings indicate that tractor brand significantly influences the price per kW of power. Well-known and technologically advanced brands tend to be the most expensive per kW. The purchasing power of farmers across CEFTA countries varies, impacting the availability of tractor brands and their technological features in these regions. Specifically, the results show that farmers in BiH often lack the purchasing power to acquire tractors from more renowned and technologically advanced brands. However, they can afford tractors from less technologically developed brands such as Foton, ArmaTrac, and Case. Farmers are advised to choose tractors based on their specific needs and budget constraints. In some instances, it may be more beneficial to invest in a technologically advanced tractor with lower engine power, while in other situations, purchasing a less technologically developed tractor with higher engine power might be more profitable, depending on the farmer's operational requirements. Further research is needed to develop models for incentive policies, as factors such as farm size, tractor operating hours, and yield potential significantly influence decisions to invest in more expensive machinery.

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PONUDA POLJOPRIVREDNIH TRAKTORA I TEHNIČKA PROCJENA KUPOVNE MOĆI POLJOPRIVREDNIKA U BOSNI I HERCEGOVINI I CEFTA REGIJI

Sažetak

Traktor je ključna pogonska jedinica u savremenoj poljoprivrednoj proizvodnji. Ovaj rad istražuje mogućnosti i sposobnosti farmera u BiH u odnosu na ostale članice CEFTA-e za kupovinu adekvatnog, kvalitetnog i odgovarajućeg novog traktora. U okviru istraživanja analiziran je proces liberalizacije trgovine u jugoistočnoj Europi, uključujući Sporazum o izmjeni i pristupanju CEFTA-i. Rad obuhvata analizu deset svjetskih brendova traktora kroz 110 različitih modela koji su dostupni na tržištu zemalja CEFTA-e. Izračunate su prosječne cijene traktora i cijene po kW snage po brendovima i izvršena kategorizacija (prema AHP metodi) u tri grupe: A, B i C. Rezultati istraživanja pokazali su da su traktori brenda New Holland najskuplji, dok su traktori brenda Foton najjeftiniji. Po cijeni po kW, traktori brenda Ferrari su najskuplji, dok su traktori brenda ArmaTrac najjeftiniji. Analizirane su i razlike u kupovnoj moći farmera u BiH u poređenju s članicama CEFTA-e, s fokusom na razlike u prosječnim platama kroz tri godine (2018, 2019 i 2020). Kupovna moć farmera određivana je računanjem koeficijenta mogućnosti kupovine (MK) koji je pokazao da farmeri Srbije i Crne Gore imaju najveću kupovnu moć za kupovinu traktora svih ispitivanih brendova, dok je Moldavija najslabija po tom pokazatelju i teoretski farmeri nemaju mogućnost kupovine traktora bilo kojeg ispitivanog brenda. Farmeri iz BiH nemaju kupovnu moć za kupovinu traktora bredova Kubota i Ferrari, a posjeduju kupovnu moć za kupovinu traktora brendova Foton, ArmaTrac i Case.

Ključne riječi: *traktor, brend, CEFTA, kupovna moć, farmer.*

KNOWLEDGE AND APPLICATION OF HORSE LEARNING THEORY*

Maja Gregić[1], Tea Zaharov[1], Vesna Gantner[1] and Tina Bobić[1]

Review paper

Abstract

The aim was to present an overview of the application of learning theory in practical horse training. Learning theory helps humans understand the learning process, information retention, and creates better communication during work and performances with horses. In the mid-20th century, new insights into the learning processes of animals emerged, leading to the development of a modern approach to horse training. The horse's brain acquires knowledge through non-associative learning (habituation and sensitization) and associative learning (classical conditioning and operant learning). The learning process in horses is influenced by ethology, emotions, mental state, and the environment. Training is subject to the simultaneous use of multiple learning processes, which are sometimes ambiguous, and for successful training, these processes need to complement each other. More research is needed in these areas for the field of equine science to advance and improve the clarity and application of learning processes. Learning theory is an excellent tool for trainers, enabling them to work with horses in a way that facilitates and accelerates learning while maintaining well-being. Training based on learning theory aims to individualize the process, making it essential to give horses time to familiarize themselves with their environment and maintain constant interaction to improve relationships and communication. Optimal use of learning theory should be established as a fundamental principle in working with horses, making it crucial to educate all those involved in horse training. In the 21st century, horses are partners and companions to humans, and their work experience must be mutually enjoyable and beneficial.

Keywords: *learning theory; horse; training*

INTRODUCTION

In the mid-20th century, new insights into the learning processes of animals emerged, sparking significant interest in the study of learning and cognition across various species. This research was largely driven by a fascination with animal intelligence, often

intensified by reports of sophisticated intellectual skills exhibited by animals. Thorpe (1963) defined learning as a process of adaptive changes in an individual's behavior resulting from experience. In-depth studies on the learning abilities and brain mechanisms of mammals and birds have contributed to the development of learning theory. This theory describes an approach that explains behavioral changes induced by mental and physical practice, as opposed to factors like physiological development.

Learning theory is divided into two main categories: non-associative learning, which includes habituation and sensitization, and associative learning, which consists of classical and operant conditioning (McLean and Christensen, 2017). It is widely regarded as the most appropriate scientific foundation for horse training methods. However, research indicates that a rider's knowledge of learning theory was not significantly related to improvements in horse welfare or rider safety (Luke *et al.,* 2023). The aim of this paper was to present an overview of the application of learning theory in practical horse training.

EQUINE LEARNING THEORY IN PRACTICE

The application of learning theory has transformed training methods for a variety of animal species. At the dawn of the 21st century, literature on equine training began to enlighten trainers and riders about the practical implementation of learning theory in horse training (McLean and Christensen, 2017; McGreevy, 2012; McGreevy and McLean, 2010; McLean, 2005a, 2005b, 2008). Learning theory has proven to be a valuable tool for horse trainers, enabling them to foster faster learning while ensuring the well-being of the horse. Foals naturally learn important skills from their mothers and the herd, which highlights the horse's inherent way of learning. In the rehabilitation of traumatized horses, the presence of a herd can have a stabilizing effect, aiding in recovery by providing a familiar social environment (Hurst, 2015). However, when interacting with humans, horses encounter an interspecies barrier that presents unique challenges. If a horse becomes agitated, its survival instincts kick in, releasing adrenaline that prepares it for a "fight or flight" response. In such heightened states, the horse instinctively looks for safety, which can hinder its ability to think rationally. Horses learn based on the outcomes of their actions. For instance, if a behavior leads to a positive result - like receiving a treat or alleviating discomfort - they're more likely to repeat it. On the flip side, if an action ends in a negative consequence - such as pain from an electric fence or losing access to food - they will likely avoid that behavior in the future. Learning theory enables trainers to structure training in clear, logical steps, rewarding desired behaviors and reinforcing learning in the process (Skipper, 2007).

When selecting a training method, it's crucial for trainers to consider the individual experiences of each horse, steering clear of methods that could inflict physical or emotional harm. Such approaches can jeopardize the horse-human relationship since horses may connect negative experiences with people. Techniques that induce fear or stress can obstruct learning and diminish training effectiveness. Horses typically respond to potential threats with flight, fight, or freezing. In these heightened emotional states, effective learning is not possible (Huntington *et al.,* 2004). Jones (2020)

challenges the notion that horses can plan their actions, arguing that it's easy to project human traits onto them, as their behavior may seem intentional. However, since horses lack a prefrontal cortex, they cannot deliberate or act with foresight.

NON-ASSOCIATIVE LEARNING IN HORSES

Non-associative learning refers to a lasting change in an animal's response to a single stimulus due to repeated exposure. This type of learning can be divided into two categories: habituation and sensitization (Blumstein *et al.,* 2017). Habituation, in particular, is a process where an animal gradually decreases its behavioral response to a repeated stimulus that doesn't provide any reward or threat. At first, animals may react strongly, but if the stimulus is deemed neutral, they will slowly lessen their response over time. This process is crucial for other forms of learning, as it helps animals filter out non-threatening stimuli and focus on what truly matters. Given that animals are constantly bombarded by environmental stimuli, habituation allows them to distinguish between important signals and those they can ignore. This foundational learning process is essential for adapting to ever-changing surroundings (Rankin *et al.,* 2009; Schmid *et al.,* 2015).

Habituation involves a slow decline in the intensity or frequency of responses to repeated sensory stimulation. It differs from sensory receptor adaptation or physical fatigue (Rankin *et al.,* 2009; Schmid *et al.,* 2015; McLean and Christensen, 2017). In horses, an individual's temperament can play a significant role in how they habituate, largely due to its genetic underpinnings. For instance, naturally cautious horses may continue to flee from new objects, even after undergoing training, while others may be more curious and approach unfamiliar stimuli with ease. Training techniques that use desensitization can leverage this natural curiosity, encouraging the horse to maintain a safe distance from something feared until it shows interest and a willingness to investigate (McLean and Christensen, 2017). Habituation has been observed in nearly all animal species, including some larger protozoans like *Stentor coeruleus*. Dishabituation occurs when an animal encounters a new stimulus, prompting an increased response to the original one. Typically, a strong stimulus is employed to bring about this dishabituation. In the context of horse training, horses become accustomed to various aspects of their surroundings, including both dynamic environmental factors and training gear, such as saddles and bridles. For younger horses, adapting to the consistent pressure of training can be particularly challenging, as some may find the intensity of training overwhelming (McGreevy and McLean, 2010).

Sensitization, a type of non-associative learning, involves the gradual intensification of a response following repeated exposure to a stimulus. Unlike habituation, which results in diminished responses, sensitization actually heightens the intensity of reactions. For example, when an animal is faced with a series of unpleasant stimuli, sensitization can increase the likelihood of a stronger response to similar or even unrelated stimuli presented shortly after. This phenomenon is evident when continuous stimulation of peripheral nerves, like persistent scratching, ultimately leads to pain. Sensitization acts

as an adaptive warning for harmful stimuli, but during training, it can lead to adverse reactions to stimuli that would usually be considered neutral (McLean and Christensen, 2017).

In training contexts, painful or distressing experiences can cause horses to become sensitized not only to the initial frightening stimulus but also to other triggers. For instance, a horse that has bolted from a sudden encounter with a dog may become more reactive to even minor similar stimuli. While this response mechanism ensures quick reactions to potential dangers, it can complicate training by causing the horse to overreact to neutral or minor stimuli. To counteract this, desensitization techniques focus on reducing these exaggerated responses through controlled exposure (McLean and Christensen, 2017). Horses are naturally neophobic and often perceive certain stimuli as threatening. Systematic desensitization, borrowed from human psychotherapy (Wolpe and Lazarus, 1969), involves gradually exposing the horse to low levels of a stimulating trigger and then slowly increasing the intensity. Rewards are given when the horse stays calm or displays appropriate behavior, and the intensity is only ramped up when the horse reliably responds to the previous level. For example, police horses undergo systematic desensitization to various stimuli, including sounds, smoke, flags, people, and rapidly moving objects.

Counterconditioning, also known as response substitution, is a technique often used in conjunction with systematic desensitization. This method aims to teach the horse a behavior that is the opposite of an unwanted reaction. By making the desired behavior more rewarding, the horse learns to adopt this new response in the presence of a problematic stimulus. For instance, when a challenging stimulus is paired with something rewarding, like food, the horse begins to associate the problematic stimulus with a positive experience, thus promoting an alternative response (Taylor, 2010; McLean and Christensen, 2017).

The concept of overshadowing comes into play when multiple stimuli vie for the same reaction, leading an individual to become desensitized to the less prominent ones. In horse training, this technique can be particularly effective in helping horses become accustomed to aversive stimuli, such as clippers and needles, which typically provoke withdrawal responses. Once a horse learns to respond to subtle rein cues for forward or backward movement, its initial instinct to withdraw lessens, allowing it to become more attuned to gentler signals. For this approach to succeed, the horse must first be well-trained to respond to rein cues through operant conditioning (McLean and Christensen, 2017).

Flooding is another method that involves restraining the animal and exposing it to a feared situation in such a way that it cannot avoid the stimulus until its resistance diminishes. In contrast to gradual habituation, flooding exposes the animal to the full intensity of the aversive stimulus for an extended duration. To effectively extinguish the fearful response, the stimulus must remain present until the response completely fades away (McLean and Christensen, 2017).

Approach conditioning taps into a horse's natural curiosity by encouraging it to move towards a feared object, which then retreats as the horse approaches, positively reinforcing this behavior. Riders can signal their horse to halt before it reaches its fear

threshold, allowing the object to pull back. This method has proven effective for helping horses overcome their fear of tractors, machinery, and moving vehicles (McLean and Christensen, 2017).

Stimulus blending involves gradually desensitizing the horse by beginning with a familiar, non-threatening stimulus and then slowly introducing the fearful one with increasing intensity. For example, a horse that is afraid of aerosol sprays but not of water might be exposed to both at the same time, reducing its awareness of the fear-inducing spray. Once the familiar stimulus is removed, the horse becomes more comfortable with the new one. This technique can be particularly useful for addressing aversions to stimuli such as reins and leg pressure in training, which can cause confusion if overdone (McLean and Christensen, 2017).

Without proper control over an aversive stimulus, horses might develop learned helplessness, which negatively affects their well-being (McGreevy and McLean, 2010). A "hard mouth" can occur when a horse associates certain signals with pain or aversion. If the pain threshold is crossed, habituation might escalate to learned helplessness, where chronic variability in pressure creates confusion. In equestrian settings, horses often encounter pressures that obscure the line between habituation and response; hence, clarity in the application of pressure is vital in order to prevent maladaptive reactions (McGreevy and McLean, 2010; McLean and Christensen, 2017).

ASSOCIATIVE LEARNING IN HORSES

Associative learning refers to the process of making connections between two stimuli or between a behavior and a stimulus, primarily through two main forms: classical conditioning and operant conditioning. In classical conditioning, a previously neutral stimulus is paired repeatedly with a stimulus that elicits a reflexive response. Over time, this neutral stimulus alone can trigger the response. On the other hand, operant conditioning involves modifying behavior based on reinforcement or punishment, which ultimately affects the likelihood of that behavior occurring again (McLean and Christensen, 2017).

Classical conditioning, one of the foundational concepts of learning, is observable across various species (McLean and Christensen, 2017). It essentially establishes an association between two stimuli - one neutral, and the other biologically significant, whether it's something aversive like pain or something pleasant like food. The classical conditioning process includes three basic components. In practical applications, such as training horses, this type of conditioning helps the animal link neutral stimuli with specific events or reactions (McLean and Christensen, 2017).

For instance, consider a horse being trained with food. The unconditioned stimulus - food - naturally prompts the unconditioned response of approaching or wanting to eat. The neutral stimulus could be a sound, like a whistle, which on its own doesn't provoke a response. However, if the whistle is consistently sounded just before the horse receives food, it eventually becomes associated with the arrival of food. This connection is akin to clicker training (Gregić *et al.,* 2023a). As a result, the whistle transforms into a

conditioned stimulus, leading the horse to respond to it as if food is forthcoming, even in its absence.

The significance of classical conditioning in psychology lies in its ability to explain how organisms learn to anticipate environmental events. In horse training, the stimuli typically employed are mainly tactile. Trainers might use pressure from a halter or reins to encourage or refine responses through negative reinforcement. Voice cues, body balance control, and certain visual cues also become part of the learned responses through classical conditioning (McGreevy and McLean, 2010; McLean and Christensen, 2017).

In contrast, operant associative learning - also known as operant or instrumental conditioning - focuses on how the consequences of an organism's behavior can lead to changes in that behavior (McLean and Christensen, 2017). According to McLean and Christensen (2017), Skinner's work (1938) showed that behaviors can be encouraged or discouraged based on the outcomes that follow them. The core components of operant conditioning include the behavior itself, which is any action by the organism that can be subject to rewards or punishments.

In operant conditioning, positive reinforcement occurs when a horse receives a reward, like a carrot, after successfully completing a task. This increases the chances that the horse will repeat the behavior to earn the reward. On the other hand, negative reinforcement happens when the rider applies pressure with their legs to prompt the horse to move, then releases the pressure once the horse starts to respond. The horse learns that moving will alleviate the uncomfortable pressure, making it more likely to repeat the behavior in the future.

Positive punishment involves correcting undesirable behavior, such as biting, through immediate action (like a sharp sound), which decreases the likelihood of that behavior happening again. Conversely, negative punishment occurs when a horse avoids a task and thereby misses out on a reward, like scratching or food, which can help curb unwanted actions. According to McLean and Christensen (2017), Skinner (1938) pointed out that if a specific action is followed by positive outcomes, it will be remembered and more likely to recur, while negative consequences will tend to eliminate the behavior.

Reinforcers can be categorized as primary (unconditioned), which means their reinforcing nature doesn't need to be learned (for example, food, water, or discomfort), and secondary (conditioned), which acquire their value through association with primary reinforcers, such as praise or treats. Generalized conditioned reinforcers, through higher-order conditioning, become linked to multiple other reinforcers (e.g., attention). Essentially, both positive and negative reinforcement aim to enhance behavior, though positive reinforcement adds something pleasing, while negative reinforcement removes something unpleasant.

In the context of training horses, positive reinforcement means providing something the horse enjoys immediately after it exhibits a desired behavior. The reward doesn't have to be food - it could also be a scratch, a break, a word of praise, or relaxation—anything the horse views as positive. It's crucial for the reward to follow promptly after the desired action to effectively motivate the horse. Research has shown that horses trained

with positive reinforcement exhibit fewer stress-related behaviors (Gregić *et al.,* 2023b). Elements such as cues during training, the environment, the equipment used, and the presence of the trainer all play a role in how the horse associates these reinforcers (Sankey *et al.,* 2010).

For instance, if the goal is for the horse to stop, a cue is given, the horse understands and halts, then is rewarded with scratching, praise, or another form of positive reinforcement. Studies have shown that petting a horse's neck can reduce its heart rate (Normando *et al.,* 2003), making this area ideal for primary reinforcement and often paired with secondary reinforcement, like a verbal cue or clicker sound. Thorbergson *et al.* (2015) found that scratching along the neck vein is more effective than patting, whereas Hancock *et al.* (2014) demonstrated that patting doesn't speed up learning in riding.

The underlying principle of positive reinforcement is that rewarded behavior is more likely to be repeated. At the start of training, behaviors are rewarded more frequently, and small steps toward the goal are acknowledged to help the horse grasp each stage of the learning process. Maintaining low stress levels during training allows the horse to mentally and emotionally process everything, building trust in the trainer or rider (McLean and Christensen, 2017). It's important to reward the right behavior, as horses respond emotionally to the same cues, environments, and equipment they dislike (Innes and McBride, 2008). A horse trained with positive reinforcement will eagerly look for cues from the rider and respond more readily, confident that its actions will ultimately lead to a reward.

Positive reinforcement is a powerful tool in training, but it's important to recognize that it can also lead to frustration. This frustration not only affects the horse but can also pose a risk to humans, particularly if it escalates into aggression (McLean and Christensen, 2017). As the use of positive reinforcement becomes more prevalent in horse training, it's essential to carefully identify elements of negative reinforcement in these interactions and avoid attributing results solely to positive methods (Stacey *et al.,* 1999). Another potential issue arises when a cue consistently precedes food reinforcement. This can result in the task becoming reliant on the cue or the person delivering it, rather than on the desired behavior. This phenomenon, known as "sign tracking," leads the horse to fixate on the reward-giver, striving to stay too close to them (Hearst and Jenkins, 1974). If the horse does not respond to the cue that once led to a reward, it offers immediate insight into their mental state.

Additionally, it's interesting to compare the motivations of horses trained with negative reinforcement versus those trained with positive reinforcement. A horse's fundamental drive is often to escape or avoid, so if we're not careful with our reinforcement methods, the horse might try various behaviors just to prevent us from issuing a cue. According to Gregić *et al.* (2023b), while training with positive reinforcement is gaining traction, there's still limited research on its long-term benefits concerning stress, emotional well-being, and the horse-human relationship. Ultimately, the outcomes we observe are influenced more by the testing methods than by the actual approach, the horse, or its level of training.

Negative reinforcement in learning, as described by McLean and Christensen (2017), involves a process of pressure and release, forming a core aspect of operant conditioning. In a herd, for instance, when one horse wants to move another, it might turn its ear, position its legs, direct its head, lower its ears and neck, or even bite or kick. However, as soon as the second horse responds by moving, the first horse ceases applying pressure. This form of negative reinforcement rests on the principle that the subject - in this case, the horse - aims to avoid negative stimuli. It's crucial, however, to apply this pressure in a correct and ethical manner (Hockenhull and Creighton, 2013).

When pressure is applied, the horse's response is influenced by the level of pressure it has associated with the handler. Thus, it's vital to start with the least possible pressure and gradually increase it if the horse doesn't respond. Importantly, pressure should be released the moment the horse starts to engage in the desired behavior, not just when it completes the task (Innes and McBride, 2008). McLean and Christensen (2017) outline what behavioral scientists refer to as the four quadrants, illustrating the interactions of these variables and how they define learned responses. Even when employing the mildest forms of negative reinforcement, it's essential that the horse is encouraged to respond to cues; otherwise, it may need to be desensitized gradually. Resensitizing is crucial, meaning the cue must become sufficiently distinct and unpleasant to elicit a response (Hockenhull and Creighton, 2013).

Riders, regardless of experience, often overlook how they might unintentionally reinforce fearful or reactive behaviors, such as raising the front legs or abruptly changing direction, which can unintentionally reward the horse for such actions (McLean and Christensen, 2017). The use of aversive stimuli in horse training is only effective when situated within the framework of negative reinforcement, where aversive pressure is applied before a conditioned signal (like light pressure or a voice cue) and is removed following the correct response. Releasing pressure from the reins, especially, demands a high level of skill across various gaits. Egenvall *et al.* (2012) noted that the release of rein pressure should occur precisely as the desired behavior is demonstrated; this can be challenging to identify. Conversely, any delay in releasing pressure can heighten signs of conflict or stress in the horse.

Key elements of riding involve maintaining a steady connection between the rider's hands and the horse's nose, as well as upholding stable leg and seat positions that adapt to the horse's body movements at every gait, speed, and direction. Mistakes in managing negative reinforcement can pose significant training obstacles, especially when the pressure is released incorrectly, reinforcing undesirable responses and leading to conflict and extended stress (McLean, 2005a). The communication between horse and rider relies on the physical presence of both partners, necessitating sensitivity, emotional awareness, and kinetic intelligence (Maurstad *et al.,* 2013). Research indicates that combining reinforcement strategies can reduce the negative impact of negative reinforcement (McKinley, 2004; Warren-Smith *et al.,* 2007). To foster the development of training methods that enhance horse welfare, experts in riding science advocate for techniques grounded in a more comprehensive understanding and ethical application of learning theories, including operant and classical conditioning (ISES, 2018; McLean and Christensen, 2017).

CONCLUSIONS

The theory of learning provides a rich toolkit for working with horses when well understood and appropriately applied. The training process is subject to the simultaneous use of multiple learning processes, which can sometimes be ambiguous; for successful training, these processes must complement one another. More research is needed in these areas to advance the field of equine science and improve the clarity and application of learning processes. Optimal utilization of learning theory should be established as a fundamental principle in horse training, making it essential to educate all individuals involved in the training of horses. By fostering a comprehensive understanding of learning mechanisms and their practical implications, practitioners can enhance training efficacy, promote better welfare for the horses, and ultimately contribute to the advancement of equestrian practices.

Note: The paper is the result of collaboration with a student Tea Zaharov in preparing her graduation thesis "Theory of Learning in Horses" at the Faculty of Agrobiotechnical Sciences Osijek.

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SPOZNAJE I PRIMJENA TEORIJE UČENJA KONJA

Sažetak

Cilj ovoga rada bio je prikazati pregled primjene teorije učenja u praktičnoj obuci konja. Teorija učenja konja pomaže čovjeku u shvaćanju procesa učenja, zadržavanju informacija i stvaranju bolje komunikacije u radu te izvedbama s konjem. Sredinom dvadesetog stoljeća čovjek dolazi do novih spoznaja o procesu učenja životinja te se od tada počinje raditi na modernom pristupu obuci konja. Konjski mozak usvaja znanje neasocijativnim učenjem (navikavanjem i senzibilizacijom) te asocijativnim učenjem (klasično uvjetovanje i operantno učenje dicioniranje). Proces učenja kod konja pod utjecajem je etologije, emocija, mentalnog sklopa i okoline. Proces obuke je podložan istodobnoj upotrebi višestrukih procesa učenja koji su ponekad dvosmisleni, za uspješnu obuku oni se trebaju nadopunjavati. Potrebno je više istraživanja u tim područjima, kako bi polje znanosti o radu s konjima napredovalo i poboljšalo jasnoću i korištenje procesa učenja. Teorija učenja izvrstan je alat trenerima kako bi radili s konjima na način koji omogućuje i ubrzava učenje, ali zadržava dobrobit. Obuka s pomoću teorije učenja teži individualizaciji treninga stoga je važno dati konju vremena za upoznavanje okoline i imati konstantnu interakciju s konjem sve u svrhu poboljšanja odnosa i komunikacije. Optimalna uporaba teorije učenja treba biti uspostavljena kao temeljno načelo rada s konjima. Stoga je važno educirati sve koji se bave treningom konja. Konji su u 21. stoljeću čovjeku partneri i suputnici, njihovo iskustvo rada mora biti obostrano ugodno i korisno.

Ključne riječi: teorija učenja, konj, obuka

DETECTION OF LAMENESS IN DAIRY COWS USING INFRARED THERMOGRAPHY*

Tina Bobić[1], Pero Mijić[1], Maja Gregić[1] and Vesna Gantner[1]

Review paper

Abstract

Lameness in dairy cows is one of the bigest problems in dairy farming. Lameness compling with pain and discomfor of the animals witcht disrupt the welfare. Besides that, requires medical care and additional costs resulting in large economic losses. Early detection of hoof inflammation in animals is important to prevent lameness. Infrared thermography (IRT) is a non-contact method for recording the temperature on the body surface of animals or other objects. IRT has great potential as an automated diagnostic method for lameness in dairy cows. Each method has its advantages and disadvantages, including IRT, so all positive and negative influences should be taken into account before making a final conclusion. Due to the great influence of the environment and the animal current biological status, it is crusual to ensure adequate conditions at the location of the recording and to consider the influence of the animal itself. It is necessary to have additional support from other lameness detection techniques and data sets that will complement the application of IRT, such as visual hoof observation and behavioural observation methods for dairy cows.

Keywords: *lameness, detection, infrared thermography, dairy cows, risk factors*

INTRODUCTION

The lameness in lactating cows is one of the major problems in intensive milk production (Whay *et al*., 2003). Lameness caused by various hooves diseases affects animal welfare, leads to reduced milk production, increases feeding costs and causes huge economic losses (Green *et al*., 2002; Ózsvári, 2017). According to different authors, the average lameness rate of cows ranged between 18 to 37% in Europa (Griffiths *et al*., 2018; Sjöström *et al*., 2018). Cha *et al.* (2010) have established the average cost per cow from 120.70 to 216.07 US dollars depending on the type of the hoof's diseases (sole ulcer, digital dermatitis, foot rot) and according to various costs of lameness (milk loss, decreased fertility, treatment costs). Because of that is crucial to prevent the incidence of lameness and detect those animals which are in the initial phases of illnesses. Those cows are not yet have walking problem, not yet experienced

the pain or discomfort. Detection of cows in that stage is possible using newer technologies such as infrared thermography. Automated lameness detection systems are very useful, as farmers do not have to spend time watching cows walk. For that purpose can be used a pressure platforms that can measure the cows pressure (separately for each hoof) when the cow walks or stands (Pastell *et al*., 2010; Maertens *et al*., 2011). In addition, there are systems that allow automatic tracking the changes in feeding behavior and activity associated with lameness (Blackie *et al*., 2011; Beer *et al*., 2016). According to previous research, it was observed the necessity of using multiple monitoring systems, i.e. different sensors, in order to increase accuracy of problem detection (Borchers *et al*., 2017). Many automated lameness detection technologies have been proposed to assist dairy farmers in managing their herds. However, reasons such as cost and skepticism among farmers themselves, who do not see the usefulness of these new technologies for application on their farms, are limiting factors for the greater application of such innovative systems on farms (Dutton-Regester *et al.*, 2020). Dutton-Regester *et al.* (2020) also points out that is a need to more thoroughly evaluate the effectiveness of these technologies under on-farm conditions in order to generate the necessary data required to show dairy farmers that these technologies are reliable and are economically rational for their dairy business.

ADVANTAGES OF INFRARED THERMOGRAPHY

Infrared thermography (IRT) is a contactless method that measures emitted infrared radiation of the surface temperature of an object (Alsaod *et al.*, 2015). Lin *et al.* (2018) states that IRT is such tip diagnostic tool which can detect mininor changes in skin temperature without tachung the animals. Which can indirectly measures blood flow changes associated with inflammation from foot lesions. This method has been widely used in industry, veterinary medicine and in livestock production (Hurnik *et al.*, 1985; Harper, 2000; Eddy *et al*., 2001). Furthermore, according to Alsaaod and Büscher (2012) and Bobić *et al.* (2017) this method could be used as tool for the prevention of lameness in dairy production. That support also Schaefer and Cook (2013), who emphasizes that there are compelling thermal signatures that can be identified in the image when lameness occurs due to inflammation. It is known that during inflammation, vascular circulation increases, which consequently leads to increased blood flow to the inflamed area, thereby changing the temperature of the inflamed part of the body. The temperature of extremities and skin is largely dependent on the underlying circulation and tissue metabolism rate. Inflammation or metabolic activity can be associated with changes in blood flow which lead to changes in the amount of heat radiated and consequently with surface thermal patterns, which can be easily identified using infrared thermography (Bobić *et al.,* 2024). Nikkhah *et al.* (2005) concluded that IRT could detect inflammation due to laminitis in cows during early and midl lactation. In the research of the Alsaood *et al.* (2014) was condacted a threshold value of 0.99 °C in maximum coronary band temperature difference between hind and front feet for detection of dermatitis digitalis. According to Harris-Bridge *et al.* (2018) the maximum temperature measured at the heels had the highest accuracy in detecting and predicting

of lameness despite of the risk of contamination through dirt and faeces in housed dairy cattle. Furthermore, the opposite opinion was held by Marti *et al.* (2015) who consider that infrared thermography was not a useful tool for differentiating of the claw area temperatures between beef feedlot cattle diagnosed with foot rot and digital dermatitis. Another study that also gave a positive opinion about thermal imaging is a study from LokeshBabu *et al.* (2018) whitch emphasises that the infrared thermography is a method with high rate of success methods for early detection of lesions before the occurrence of visibly symptoms. And they also add that infrared thermography have good results especially in combination with other diagnostic tools.

DISADVANTAGES OF INFRARED THERMOGRAPHY

Each diagnostic method has its advantages but unfortunately also certain disadvantages. One of the main disadvantages of infrared thermography is the susceptibility to environmental conditions. The accuracy and the interpretation of thermograms of thermal imaging is determined by many things, such as: ambient temperature, current environmental conditions, solar radiation, airflow, the size of the scanned object and background radiation, angle and distance (Alsaaod and Büscher, 2012; Westermann *et al.,* 2013; McManus *et al.,* 2022). Besides that, the influence of the animal itself is great, because of the different blood circulation and body status of ich animal can influence the thermal radiation thus complicating the interpretation of thermograms (Alsaaod *et al.,* 2015). Body temperature varies throughout the day and depends on different activities of the animal (physical activity, food intake, time of day etc. (Novotna *et al.*, 2019; Moreira *et al.,* 2021). In addition to the influence of animals, environmental factors also affect the appearance of the thermogram, for exemple by dirt that covers the animal, which impairs the normal emissions of infrared radiation from the body surface. Montanholi *et al.* (2015) state that image quality can be affected by the distance from which it is captured. The choice and quality of thermocamera is very important, because the infrared thermography sometimes gives false positive results and can lead to wrong conclusions (Novotna *et al.,* 2019). High-quality thermocameras are expensive, but cheaper models are available on market have lower image quality and resolution (Lokesh-Babu *et al.,* 2018). Therefore, it is necessary to select suitable equipment for thermographic measurements (Montanholi *et al.,* 2015; Werema *et al.*, 2023), and take into account all the possible negative influences that can lead to an incorrect reading of the thermogram.

CONCLUSIONS

Infrared thermography has great potential as an automated diagnostic method for lameness in dairy cows. Due to the great influence of the environment and the animal itself, it is crucial to ensure adequate conditions at the location of the recording and to consider the influence of the animal itself and its current biological status. It is necessary to have additional support from other lameness detection techniques and data sets that will complement the application of IRT.

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OTKRIVANJE ŠEPAVOSTI KOD MUZNIH KRAVA PRIMJENOM INFRACRVENE TERMOGRAFIJE

Sažetak

Šepavost mliječnih krava jedan je od najvećih problema na farmama za proizvodnju mlijeka. Šepavost uz bol i nelagodu životinja narušava dobrobit. Osim toga, zahtijeva medicinsku skrb i dodatne troškove koji rezultiraju velikim ekonomskim gubicima. Rano otkrivanje upale papaka kod životinja važno je za sprječavanje šepavosti. Infracrvena termografija (IRT) je beskontaktna metoda za bilježenje temperature na površini tijela životinja ili drugih predmeta. IRT ima veliki potencijal kao automatizirana dijagnostička metoda za šepavost kod mliječnih krava. Svaka metoda ima svoje prednosti i nedostatke, pa tako i IRT, pa prije donošenja konačnog zaključka treba uzeti u obzir sve pozitivne i negativne utjecaje. Zbog velikog utjecaja okoliša i trenutnog biološkog statusa životinje, ključno je osigurati odgovarajuće uvjete na mjestu snimanja te uzeti u obzir utjecaj same životinje. Neophodno je imati dodatnu podršku drugih tehnika za otkrivanje šepavosti i skupova podataka koji će nadopuniti primjenu IRT-a, kao što su vizualno promatranje papaka i metode promatranja ponašanja mliječnih krava.

Ključne riječi: *šepavost, otkrivanje, infracrvena termografija, mliječne krave, čimbenici rizika*

PRODUCTION POTENTIAL AND NUMBER OF IMPORTED DAIRY SHEEP BREEDS IN THE REPUBLIC OF CROATIA*

Zvonko Antunović[1], Josip Novoselec[1], Krunoslav Zmaić[1], Željka Klir Šalavardić[1]

Review paper

Summary

This paper aims to show the production potential and the number of imported herdbook dairy sheep breeds that are reared in the Republic of Croatia (CRO), as well as the prospects for the development of this production sector. The research included data from research conducted with imported sheep breeds, East Friesian and Lacaune sheep, which are dairy sheep breeds, and Travnik sheep, a combined breed that is also used for milk production in the Republic of Croatia. In the Republic of Croatia, 2521 breeding valued Lacaune sheep, 75 East Friesian sheep, and 263 bred Travnik sheep are reared. An analysis of the number of these breeds revealed an increase in Lacaune sheep and a decrease in East Friesian sheep, while the number of Travnik sheep is standard. The reasons for the above are numerous and include the weaker resistance and more prominent commitment required by the East Frisian sheep, also the increasing depopulation of rural areas, and the lack of labor, where the majority of sheep farming is located in the Republic of Croatia. This gives the Lacaune sheep a considerable advantage, especially in the continental regions of the Republic of Croatia, rich in fodder. The development of the dairy sector in sheep farming has good prospects in the Republic of Croatia, considering that the demand for sheep's milk has increased, which is accompanied by an increased interest in the purchase of Lacaune sheep, but also that the results of research conducted so far in the Republic of Croatia with Lacaune sheep indicate a high milk yield and good milk quality and adaptability.

Keywords: *dairy imported breeds, sheep, production potential, population size, Republic of Croatia*

INTRODUCTION

Dairy sheep farming is developing more and more in Europe and the dairy industry's demand for sheep's milk is increasing. Pulina *et al.* (2018) point out that considerable improvements are expected in the dairy sector for small ruminants and that a significant increase in production of 30-50% is to be expected by 2030. In the last five years, the

production and purchase of sheep's milk in EU countries has increased significantly, amounting to 2 116 000 tons in 2021. In the EU countries, the majority of sheep's milk is processed into various types of cheese, and 223 000 tons of cheese were produced in 2021 (Croatian Agency for Agriculture and Food - CAAF, 2023). In 2022, 2 231 355 kg of sheep's milk was purchased in the Republic of Croatia (CRO). According to the CAAF, 553 673 sheep were bred on 18 663 farms in the Republic of Croatia on 31.12.2022, while 425 breeders had 41 768 sheep in the selection. The interest in sheep milk and the import of highly selected dairy sheep breeds has also increased in the Republic of Croatia (Antunović *et al*., 2022a). The rearing of Lacaune sheep in the Republic of Croatia has increased significantly in recent years (Antunović *et al*., 2022a). This is also supported by data from the CAAF (2023), according to which the Lacaune sheep is the second most common rearing sheep in the Republic of Croatia (2521 sheep), just behind the Romanov sheep. In the Republic of Croatia, 21 sheep breeds are reared (9 domestic and 12 imported sheep breeds). Larger imports of East Friesian sheep took place in the 1980s at the instigation of the dairy industry in the areas of northwestern Croatia (Antunović *et al.,* 2012). The Travnik sheep (Dubska, Vlašićka) belongs to the breeds with combined production traits, which have significant milk and meat production. It is assumed that the Travnik sheep are about 10,000 animals that were kept on the territory of the Republic of Croatia after the war in Central Bosnia and Herzegovina, when the shepherds went into exile, in Western Slavonia (Voćin, Daruvar, Pakrac) and Lika (Otočac, Udbina), Korenica, Vrhovine), where their breeding was continued (Pavić *et al.,* 1999). Looking at the percentages of all breeds bred in the Republic of Croatia, imported dairy sheep breeds, namely Lacaune sheep, are represented with 6.04%, Travnik sheep with 0.63%, and East Friesian sheep with only 0.18%. Comparing only the imported sheep breeds reared in the Republic of Croatia, 26% are Lacaune, 2.7% are Travnik sheep and 0.8% are East Friesian sheep (CAAF, 2023). This article aimed to show the production potential and number of imported dairy sheep breeds reared in the Republic of Croatia, as well as the prospects for the development of this production sector.

MATERIAL AND METHODS

Data from the Sheep Breeding Program (Mioč *et al.,* 2011) and the annual reports of the Croatian Agency for Agriculture and Food (CAAF, 2020, 2022, and 2023) were used to investigate the number and production potential of imported dairy sheep breeds reared in the Republic of Croatia. Two imported sheep breeds with high milk production (Lacaune and East Friesian sheep) and the Travnik Pramenka, a combined sheep breed that is also reared for milk production in the Republic of Croatia, were included in the research. Data from previous studies published in several publications (Antunović *et al.,* 2013, 2020, 2022a,b,c and 2024a,b; Mioč *et al.,* 2004; Pavić *et al.,* 2002 and 1999; Antunac *et al.,* 2002) were used to assess the production potential of imported dairy sheep breeds reared in the Republic of Croatia.

RESULTS AND DISCUSSION

Table 1 shows the production potential and the population size of herdbook imported dairy breeds of sheep reared in the Republic of Croatia.

Table 1. Production potential and size of the population of herdbook imported dairy sheep breeds reared in the Republic of Croatia
Tablica 1. Proizvodni potencijal i veličina populacije uzgojno valjanih inozemnih mliječnih pasmina ovaca koje se uzgajaju u Republici Hrvatskoj

Indicator- Pokazatelj	Breed of sheep - Pasmina ovaca		
	East Friesian sheep Istočnofrizijska ovca	Lacaune sheep Lakon ovca	Travnik sheep Travnička pramenka
Production indicators[1] - Proizvodni pokazatelji:			
Body weight of adult sheep, kg Tjelesna masa odraslih ovaca, kg	60-110	60-100	70-90
Withers height, cm Visina grebena, cm	60-80	55-75	55-75
Index of lambing[2*] Indeks janjenja	1.01	1.02	1.04
Litter size[2**] Veličina legla	1.24	1.03	1.09
Milk yield, l Količina mlijeka, l	250-400	200-250	130-180
Wool yield, kg Prinos vune, kg	4.0-6.0	2.0-3.0	2.5-4.5
Indicators of male lamb in field conditions (duration of 105 days)[2]- Pokazatelji muške janjadi u terenskim uvjetima (105 dana trajanje):			
Lambing weight, kg Porodna masa, kg	-	4,1	4.1
Daily gain, g Dnevni prirast, g	-	320	290
Final weight gain, kg Završna tjelesna masa, kg	-	37.65	34.10[3]
Population size[2] - Veličina populacije:			

Number of heardbook sheep Broj uzgojno-valjanih ovaca	75	2521	263
Average flock size Prosječna veličina stada	25	180	88

[1]Sheep breeding program in the Republic of Croatia - Program uzgoja ovaca u Repu lici Hrvatskoj; [2]CAAF (2023); [3]CAAF-a (2021); *Lambing index = number of lambs/number of ewes – Index janjenja = broj janjadi/broj ovaca; **Litter size = number of lambs/numbers of lambing – Veličina legal = broj janjadi/broj janjenja

The analysis of the results shown in Table 1 shows that in the Republic of Croatia, Lacaune sheep are reared from imported dairy breeds (a total of 2.521 herdbook head), while the breeding of East Friesian sheep (only 75 herdbook sheep) is much lower, and the rearing's of the combined breed Travnik sheep, which is grown and intended for milk production, comprises 263 herdbook head. In terms of production potential, it should be noted that all three breeds have a similar body performance, with the milk yield of the East Friesian sheep being more pronounced, considering the data presented in the Sheep Breeding Program of the Republic of Croatia (Mioč *et al.,* 2011), but also a larger amount of wool. However, the ever-increasing genetic profiling of Lacaune sheep towards milk production has led to a significant increase in the number of Lacaune dairy sheep in the Republic of Croatia, amounting to almost 62% from 2018 to 2023. The significantly higher milk yield of Lacaune sheep in the Republic of Croatia is also confirmed by CAAF data (2023), which highlights the average milk yield in lactating Lacaune sheep according to the control of a total of 457.5 kg, which was achieved by monitoring 464 lactations. However, there has been a significant decline in the number of East Friesian sheep, as well as a stagnation in the number of Travnik sheep in the selection area. The reasons for this are manifold, but among others, it is certain that the weaker resistance and greater dedication required by the East Friesian sheep, but also the increasing depopulation of rural areas and the lack of labor, where most of the sheep breeding is stationed in the Republic of Croatia, including the breeding of Travnik sheep. This gives the Lacaune sheep a considerable advantage over the other two breeds mentioned, especially in the forage-rich continental regions of the Republic of Croatia. Graph 1 shows the distribution of herdbook sheep of the Lacaune, East Friesian, and Travnik sheep breeds by county in the Republic of Croatia.
It can be seen that the largest number of Lacaune sheep are reared in Osiječko-Baranjska (230) and Vukovarsko-Srijemska (181) counties, East Friesian sheep in Krapinsko-Zagorska and Travnik sheep in Bjelovarsko-Bilogorska county (175).

Table 2 shows the external characteristics of imported dairy breeds of sheep reared in the Republic of Croatia.
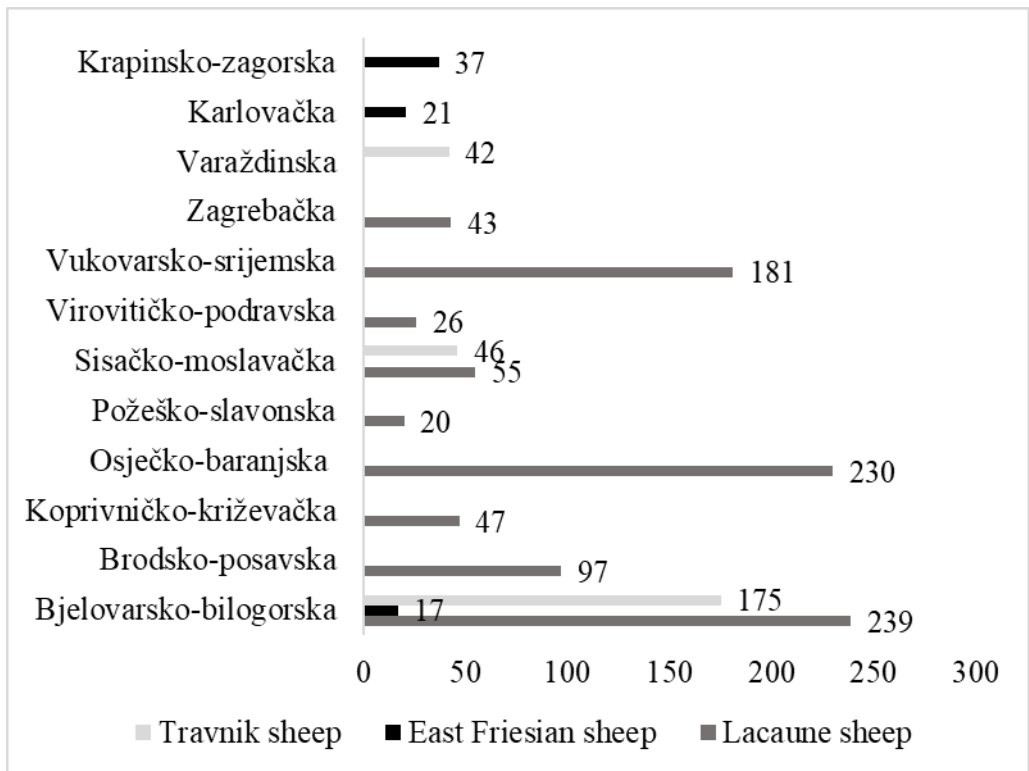
Graph 1. Distribution of herdbook sheep of the Lacaune, East Frisian and Travnik sheep breeds by county in the Republic of Croatia (CAAF, 2023).

Grafikon 1. Raspored uzgojno valjanih ovaca pasmine lakon, istočnofrizijska i travnička pramenka po županijama u Republici Hrvatskoj (HAPIH, 2023).

The analysis of the results of body mass and body measurements of Lacaune sheep and Travnik sheep carried out in the Republic of Croatia shows that they are different. Namely, the Lacaune sheep has a lower body weight, height at the withers, body length, chest girth, and width than the Travnik sheep, which is also a characteristic of the breed, considering that the Travnik sheep is a combined breed with a stronger body frame. In the available literature, there is no research related to the external characteristics of East Friesian sheep, but we have provided data from the Sheep Breeding Program in the Republic of Croatia (Mioč *et al.,* 2011). Comparing the external characteristics of Dubska pramenka sheep (Travnik sheep) in research conducted in the area of western Herzegovina, Bosnia and Herzegovina, Dokso *et al.* (2011) determined a higher withers height (74.57 cm) and shorter body length and chest girth (79.37 and 110.11 cm). This mostly indicates somewhat weaker physical performance. The authors associate this with the failure to carry out systematic selection in the selected flocks of Dubska sheep.

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Table 2. External characteristics of imported sheep dairy breeds in the Republic of Croatia

Tablica 2. Odlike vanjštine inozemnih mliječnih pasmina ovaca koje se uzgajaj u Republici Hrvatskoj

Characteristic - Odlika	Breed of sheep - Pasmina ovaca		
	East Friesian sheep[3] Istočnofrizijska ovca	Lacaune sheep[1] Lakon ovca	Travnik sheep[2] Travnička pramenka
Body weight, kg Tjelesna masa, kg	60-110	60.94	66.20
Withers height, cm Visina grebena, cm	60-80	68.05	69.80
Body length, cm Dužina trupa, cm	-	78.49	85.70
Chest girth, cm Opseg prsa, cm	-	94.82	117.00
Širina prsa, cm Chest width, cm	-	20.86	27.40
Circumference of shin bone, cm Opseg cjevanice, cm	-	8.68	9.00
Head length, cm Dužina glave, cm	-	20.04	24.20
Head width, cm Širina glave, cm	-	12.97	13.10

[1]Antunović *et al.* (2022b); [2]Antunović *et al.* (2013); [3]Sheep Breeding Program in the Republic of Croatia- Program uzgoja ovaca u Republici Hrvatskoj

Table 3. Lactation characteristics of imported dairy breeds of sheep reared in the Republic of Croatia

Tablica 3. Laktacijska svojstva inozemnih mliječnih pasmina ovaca koje se uzgajaj u Republici Hrvatskoj

Indicator - Pokazatelj	Breed of sheep - Pasmina ovaca		
	East Friesian sheep[1] Istočnofrizijska ovca	Lacaune sheep[1] Lakon ovca	Travnik sheep Travnička pramenka
Number of recorded lactations Broj zaključenih laktacija	40	464	-
Lactation length, days Dužina laktacije, dani	237	183.4	240[2]; 235[3]

Milking period, days / Razdoblje mužnje, dani	181	139.8	-
Milk yield in lactation, kg / Mlijeko u laktaciji, kg	389.9	457.5	137[2]
Milk yield in suckling period, kg / Posisano mlijeko, kg	124.3	128.4	-
Milk yield in milking period, kg / Količina mlijeka u razdoblju mužnje, kg	265.6	329	-

[1]CAAF (2023); [2]Pavić *et al.* (2002); [3]Antunac *et al.* (2002)

Analysis of the results from Table 3 shows that Lacaune sheep produced a higher average total milk yield (457.5 kg) in lactation of shorter duration (183.4 days) compared to East Friesian sheep (389.9 kg and 237 days) and Travnik sheep (137 kg and 240 days). In East Friesian sheep in Macedonia, Paćinovski *et al.* (2007) determined that 346 L of milk was produced in a lactation of 232 days, with an average milk fat content of 5.01% and protein content of 4.68%.

Table 4. Milk quality of imported dairy breeds of sheep reared in the Republic of Croatia in available research
Tablica 4. Kvaliteta mlijeka inozemnih mliječnih pasmina ovaca koje se uzgajaj u Republici Hrvatskoj u dostupnim istraživanjima

Indicator - Pokazatelj	Breed of sheep - Pasmina ovaca		
	East Friesian sheep[1] Istočnofrizijska ovca	Lacaune sheep[2] Lakon ovca	Travnik sheep[3] Travnička pramenka
Basic milk chemical composition, % - Osnovni kemijski sastav mlijeka, %			
Milk fat / Mliječna mast	5.68	5.3	7.43
Milk protein / Bjelančevine	4.92	5.9	5.70
Lactose / Laktoza	4.59	4.5	4.33
Dry matter without fat / Suha tvar bez masti	-	1.,29	11.10
Total dry matter / Ukupna suha tvar	-	17.40	18.45
Mineral composition mg/kg DM - Sadržaj minerala, mg/kg ST:			

Calcium	-	2373	1838-1993
Kalcij			
Phosphorus	-	1634	1485-1851
Fosfor			
Potassium	-	1330	1224-1435
Kalij			
Sodium	-	662	567-517
Natrij			
Magnesium	-	183	179-200
Magnezij			

[1]Mioč *et al.* (2004); [2]Antunović *et al.* (2024b); [3]Antunović *et al.* (2020); ST-suha tvar - DM-dry matter

Analyzing the quality of milk of imported dairy breeds of sheep reared in the Republic of Croatia from the available research, it is evident that Travnik sheep, in particular, had a significantly higher content of fat and dry matter in their milk compared to Lacaune sheep and East Friesian sheep. The milk of Lacaune sheep had a higher calcium and sodium content, while the content of other macroelements (phosphorus, potassium, and magnesium) was similar compared to the milk of Travnik sheep. We did not find any research on the mineral composition of milk East Friesian sheep in the available literature.

CONCLUSION

Increased demand for sheep milk on the market and an increased interest in the purchase of the high-milk Lacaune breed indicate that the development of the dairy sector in sheep production has good prospects in the Republic of Croatia. In addition, previous research conducted in the Republic of Croatia with Lacaune sheep indicates high milk yield, good milk quality, and adaptability. It is to be expected that the above will contribute to the further development of the dairy sheep sector.

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PROIZVODNI POTENCIJAL I ZASTUPLJENOST INOZEMNIH MLIJEČNIH PASMINA OVACA U REPUBLICI HRVATSKOJ

Sažetak

Cilj ovoga rada bio je prikazati proizvodni potencijal i zastupljenost inozemnih uzgojno valjanih mliječnih pasmina ovaca koje se uzgajaju u Republici Hrvatskoj (RH) te perspektive razvoja ovoga proizvodnog sektora. U istraživanje su uključeni podaci iz istraživanja provedenih s inozemnim pasminama, istočnofrizijskom i lakon ovcom, koje su mliječne pasmine ovaca te travničkom pramenkom, kombiniranom pasminom, koja se u RH koristi i za proizvodnju mlijeka. Analizom brojnosti ovih pasmina utvrđeno je povećanje broja lakon pasmine, a smanjenje broja istočnofrizijske ovce, dok je broj travničke pramenke standardan. U RH se uzgaja 2521 uzgojno valjana lakon ovca, 75 istočnofrizijskih ovaca te 263 uzgojno valjane travničke pramenke. Razlozi su višestruki, ali između ostalih, slabija otpornost i veća posvećenost koju zahtijevaju istočnofrizijske ovce, ali i sve veća depopulacija ruralnih prostora i nedostatak radne snage gdje je stacionirana većina uzgoja ovaca u RH. Navedeno daje značajnu prednost lakon ovci, osobito u kontinentalnim krajevima RH bogate krmnom bazom. Razvoj mliječnog sektora u ovčarskoj proizvodnji ima dobre perspektive u RH s obzirom na pojačanu potražnju za ovčjim mlijekom što prati i povećan interes za nabavkom lakon ovaca, ali i da dosadašnji rezultati istraživanja s lakon ovcom provedenim u RH, ukazuju na visoku mliječnost te dobru kvalitetu mlijeka.

Ključne riječi: *mliječne inozemne pasmine, ovca, proizvodni potencijal, veličina populacije, Republika Hrvatska*

THE EFFECT OF GREEN LENTIL FLOUR ADDITION ON THE SNACK QUALITY*

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Original scientific paper

Abstract

Enhancing the nutritional quality of plant-based food has become necessary since the research about plant-based nutrition is a trending topic. Legumes such as chickpeas, beans, and lentils are the main components for the fortifying these products. Green lentils are considered a rich source of dietary fibre and protein as well as low in fat. Therefore, it is highly preferred as a meat substitute for consumers. This study determined the influence of green lentil (*Lens culinaris* Medik.) flours (GLF) in snack formulation in terms of nutritional, physical, and sensory analysis. The lentil flour was added to the formula as a replacement of wheat flour with the ratio of 5%, 10% and 20%. According to the total phenolic content analysis results, the nutritional qualities of snacks were improved by adding green lentil flour to the formula. Physical assessment pointed out that the brightness of snacks was slightly affected by enhancement, and the yellowness of samples increased by the addition rate. Furthermore, the sensory analysis pointed out that a 10% addition of GLF into the snack formula had the highest score in terms of overall acceptability. This study showed that GLF addition can improve the quality and acceptability of the snack.

Keywords: *Green lentil flour, snack, sensory, nutrition, food*

INTRODUCTION

The increasing rates of obesity and chronic diseases, including cardiovascular diseases, diabetes, and cancers, have raised awareness of balanced diets and lifestyle changes. As a result, some modern diets have become prominent among others (Fehér *et al.*, 2020). Plant-based diets and vegan/vegetarian diets have become more popular in recent years due to their positive effects on health. Furthermore, these diets are mostly chosen for their environmentally friendly characteristics since the crops need less land and water, resulting in less air pollution (Ewy *et al*., 2022). However, the main drawbacks of these diets are their nutritional quality, especially the containment of lower amounts of protein. Proteins are the primary macronutrients crucial for the human body and growth;

their essential amino acid contents measure their quality and digestibility in the human body (Lopez and Mohiuddin, 2024).

Legumes are the second most important food group after cereals, which contain high amounts of protein, carbohydrates (starch and dietary fibre), minerals and vitamins (Tiwari *et al*., 2011). Because of those characteristics, they are highly preferred in plant-based diets for developing healthy formulas as an alternative flour substance. Lentils (*Lens culinaris)* are ancient pulses widely consumed worldwide (Khazaei *et al*., 2019). Research on lentils showed their lowering effect on cholesterol-total lipids, colon cancer, and diabetes in humans owing to dietary fibre and bioactive contents (Roy *et al*., 2010). Lentils are not only rich in soluble dietary fibre, but they also comprise a high amount of protein (including globulins, albumins, glutelins and prolamins), between 20.6% and 31.4%. They include low amount of sulphur-containing amino acids (Jarpa-Parra, 2018). Besides this, the digestibility is higher in lentils than other plant protein sources and the PDCASS (protein digestibility-corrected amino acid score) and DIASS (digestible indispensable amino acid score) were found to be 50.8 and 0.49, respectively, for green lentils. Due to this nutritional quality, it can be used as a functional ingredient in different formulas such as pasta (Levent *et al*., 2023), gluten-free biscuits (Di Cairano *et al*., 2021), gluten-free cookies (Hajas *et al*., 2022b, Silva-Paz *et al*., 2024), muffins (Gülhan and Karaça, 2023) and bread (Marchini et al., 2021). Snacks are food, which are consumed small portions, inexpensive and ready-to eat packaged foods. These foods generally assummed as unhealthy because of including multiple processing and food additives (Hess *et al*., 2016). Therefore, this study assessed the influence of green lentil (*Lens culinaris* Medik.) flour addition in the snack formula by developing the formulation using only plant-based substances. Furthermore, the impact of green lentil flour's replacement ratio on protein ratio also was evaluated.

MATERIALS AND METHODS

Materials: Margarine, wheat flour, baking powder and salt were purchased from a local market in Bayburt Türkiye. Green lentil flour (GLF) was obtained from KB Gurme Gıda (Isparta, Türkiye) and soya lecithin was obtained from Smart Kimya (İzmir, Türkiye).

Snack Production: Snacks were produced according to the method Çetin-Babaoğlu *et al*. (2021). The snacks were prepared using wheat flour, green lentil flour, margarine, baking powder, soya lechitin, salt and water. The formulations of snacks are given in Table 1. Green lentil flour was added into the formula as flour replacement with a ratio of 5,10, and 20%. For production, all the ingredients were mixed by using a tilt-head stand mixer (Bosh, Türkiye) until it became a homogenous dough. After this, the dough was sheeted with using a rolling pin. Then the dough shaped with a cookie cutter (35 mm) and prepared doughs were baked in an electric baking oven (Arçelik, Türkiye) at 175°C for 20 min. Then, they were cooled down at room temperature and stored in polyethylene bags in dark conditions at room temperature (Çetin-Babaoğlu *et al*., 2021). The proximate anlaysis of raw materials were given by the brand in Table 2. Therefore, no more analysis were applied to determine their nutirional value.

Table 1. Formulation of snacks

Formulations	Control	GLS1 (5%)	GLS2 (10%)	GLS3 (20%)
Wheat flour (%)	100	95	90	80
Green Lentil Flour (GLF) (%)	0	5	10	20
Lecithin (%)	0.3	0.3	0.3	0.3
Salt (%)	2.3	2.3	2.3	2.3
Water (%)	53.3	53.3	53.3	53.3
Margarine (%)	8.67	8.67	8.67	8.67
Baking Powder (%)	2	2	2	2

Table 2. Nutritional value of the wheat flour, green lentil flour and margarin.

Product	Carbohydrate (%)	Protein (%)	Lipid (%)
Wheat Flour	73.8	11.5	1.5
Green Lentil Flour (GLF)	62	24	1
Margarine	0.40	0.40	75

Proximate analysis: The moisture, total ash and fat contents of snacks were analysed according to AOAC (1995). The Bradford method was applied for the protein analysis (Moraşan et al. 2022).

Physical Evaluation of Snacks: Just after cooling down of the snack for 30 min, the physical properties of samples were evaluated by measuring the diameter, height, and spread ratio with digital calliper. The analysis was performed at room temperature and to determine the spread ratio, the following equation was used.

Spread ratio= Diameter of snacks / Height of snacks (Ho and Pulsawat, 2020).

The colour of snack samples was determined using Hunter Lab Chroma Meter (Minolta CR-400, Osaka, Japan) in terms of the L* (white; black), a* (red; green) and b* (yellow; blue) values of the samples.

Total Phenolic Content Determination: Total Phenolic Content Determination: Total phenolic contents (TPC) of snacks were measured according to Zlatanovic *et al*. (2019) by using the Folin Ciocalteu method. For analysis; 100 mg grounded samples were extracted with ethanol-water (1:1, 1400 µL) at ambient temperature for 60 min. After this, mixture was centrifugated at 5000 rpm for 20 min and the supernatants were used

for analysis. Then, 0.25 mL extracts were mixed with of 1.25 mL Folin-Ciocalteu's phenol reagent and left for 6 min for reaction. Then, 1 mL of Na2CO3 solution (75 g/L) was added and left for 2 h at ambient temperature in the dark. After this, the absorbance was measured at 765 nm and the results are determined as mg gallic acid equivalent/g (Zlatanović *et al*., 2019).

Sensory Analysis: The sensory analysis was conducted according to Rajagukguk *et al*. (2022) and the analysis was carried out with 20 untrained panelists (staff and students of the Faculty of Health Sciences, Bayburt University). The sensory characterstics of snacks were evaluated using a 7-point hedonic scale. The fresh snacks samples were presented to pannellist and they assessed the snacks in terms of their appearance, flavour, taste, texture, after taste, and overall acceptability (Rajagukguk *et al*., 2022).

Statistical Analysis: Obtained results were compared by ANOVA including post hoc comparison Tukey's test, at the probability level $p = 0.05$ using IBM SPSS Version 22 for Windows.

RESULTS AND DISCUSSION

Results of the proximate analysis are given in Table 3. The differences between samples were not significant statistically in terms of ash, which indicates the importance of the addition ratio. Mosture content ranged found between 4.94 ± 0.03 and $10.90 \pm 0.11\%$. Addition of GLF increased the moisture content of samples since dried lentil flour absorbs water more quickly than wheat flour (Nonogaki *et al*., 2010). As the snack samples were evaluated regarding lipid content, there was a significant difference between samples ($p<0.05$). The control sample has the highest ratio since green lentil flour includes less lipid than wheat flour. However, the lipid ratio was the smallest for the samples with 5% GLF added, and the lipid proportion slightly increased as the GLF replacement ratio rose in the formulation. Polat *et al*. (2020) reported the similar results even though they found that the ash amount is higher with an increasing ratio of green lentil. The protein ratio increased with the higher in snack formulation ratio of GLF. Although there were no significant differences between GLS2 and GLS3, the protein amount in the control sample was significantly lower than in the fortified samples. Similar results were obtained by Levent *et al*. (2023) for pasta samples and Yaver (2022) for snacks, including GLF and carob flours.

Table 3. Results of proximate analysis of the snacks

Samples	Ash (%)	Moisture (%)	Protein (%)	Lipid (%)
Control	3.41 ± 0.05^a	4.94 ± 0.03^c	11.04 ± 0.83^b	7.30 ± 0.35^a
GLS1	3.67 ± 0.08^a	10.90 ± 0.11^a	11.58 ± 0.10^{ab}	5.50 ± 0.15^c
GLS2	3.44 ± 0.04^a	10.40 ± 0.38^{ab}	13.80 ± 0.88^a	6.67 ± 0.03^b
GLS 3	3.16 ± 0.50^a	10.26 ± 0.16^b	13.86 ± 1.52^a	6.94 ± 0.01^{ab}

* Different superscript letters (a,b,c) in the same columns indicate significant differences ($p<0.05$), (mean±standard deviation of triplicate analysis).

The total phenolic content (TPC) of the samples is given in Table 4. The values varied between 0.521 to 0.340 mg GAE/ g, and the differences were obtained as statistically significant. Although the TPC ratio increased with the increasing ratio of GLF in snack formulation, the control sample was still higher than fortified snacks. Previous studies were found to be quite the opposite of the results of this study. Di Stefano *et al*. (2020), Levent *et al*. (2023), Yaver *et al*. (2022), Polat *et al*. (2020) found that the TPC ratio increased with the addition of GLF in the formula (Di Stefano *et al*., 2020; Levent *et al*., 2023; Yaver, 2022; Polat *et al*., 2020).

Table 4. Total phenolic content (TPC) of snacks

Samples	TPC (mg GAE /g snack)
Control	0.521 ± 0.01^a
GLS1	0.340 ± 0.01^c
GLS2	0.378 ± 0.03^{bc}
GLS 3	0.422 ± 0.00^b

* Different superscript letters (a,b,c) in the same columns indicate significant differences ($p < 0.05$), (mean±standard deviation of triplicate analysis).

The thickness, weight and spread ratio of fortified samples were found to be between 1.26 and 1.66 cm, 2.76 and 2.93g, and 1.68 and 2.32, respectively. Despite not observing the significant differences between the control and GLS1 in terms of thickness, weight and spread ratio, a significant difference was found between samples GLS2 and GLS3 ($p < 0.05$). Yaver (2022) also stated that both carob flour and green lentil flour did not show any different effect on the thickness, diameter and spread ratio of the enhanced snacks (Yaver, 2022), it can be seen that the spread ratio increased with the increased GLF ratio. A similar situation was observed by Portman *et al*. (2020) and Hajas *et al*. (2022a) as they added lentil flour to the cookie formula (Portman *et al*., 2020; Hajas *et al*., 2022a).

Table 5. Physical properties of snacks

Samples	Thickness (cm)	Weight (g)	Spread Ratio
Control	1.66 ± 0.05^a	2.80 ± 0.00^b	1.68 ± 0.05^c
GLS1	1.57 ± 0.05^a	2.87 ± 0.05^{ab}	1.83 ± 0.09^{bc}
GLS2	1.26 ± 0.05^c	2.93 ± 0.05^a	2.32 ± 0.15^a
GLS 3	1.41 ± 0.02^b	2.76 ± 0.05^b	1.95 ± 0.04^b

* Different superscript letters (a,b,c) in the same columns indicate significant differences ($p < 0.05$), (mean±standard deviation of triplicate analysis).

The colour of the snack samples and the GLF were analysed with a Hunter colourimeter, and the results are shown in Table 6, the image of the samples was given in Figure 1. As it was seen from the figure 1, the surface colours were very similar for all analysed

samples. However, the measurements were performed by grounding the samples. Thus, the colour of the inner part was also taken into account.

Figure 1. Image of the snack samples

The brightness of samples indicated that the addition of GLF into the formula increased the brightness, and the differences between samples were significant (p<0.05). Although the wheat flour used for snack production was refined, the brightness of GLF was also higher, which was reflected in the L* value. The a* values ranged between 0.21 and 5.24, with significant differences between all samples. The most yellowness was found for the GLF, and the highest was recorded as a control sample. Hajas *et al*. (2022a) and Oskaybaş-Emlek *et al.* (2021) found that the brightness was decreased by increasing of GLF replacement ratio (Hajas *et al*., 2022a; Oskaybaş-Emlek *et al*., 2021). However, both studies examined the effect of GLF on cookies; but the lower brightness was obtained probably because of the high sugar content in the formulation, which might lead to more caramelisation. Therefore, the brightness of the cookies was observed to be darker than in this study.

Table 6. Colour properties of the snacks

Sample	L*	a*	b*
(GLF)	87.05 ± 0.02^a	0.21 ± 0.01^e	23.76 ± 0.04^d
Control	77.98 ± 0.09^e	5.24 ± 0.02^a	27.67 ± 0.14^b
GLS1	81.14 ± 0.01^b	2.99 ± 0.01^d	25.29 ± 0.61^c
GLS2	79.51 ± 0.09^c	3.98 ± 0.05^c	27.37 ± 0.34^b
GLS 3	78.82 ± 0.02^d	4.51 ± 0.01^b	29.20 ± 0.13^a

* Different superscript letters (a,b,c) in the same columns indicate significant differences (p<0.05), (mean±standard deviation of triplicate analysis).

The sensory analysis was performed to determine the potential consumer acceptability of the snacks on a small scale, and the data are given in Figure 2. The data did not vary considerably from each other (p<0.05). Except for texture scores, other sensory parameters such as appearance, flavour, taste, after taste and overall acceptability showed that these fortified snacks had potential positive responses from the consumer. Since the average hedonic scale was found to be around 5, it can be considered that the snacks were liked slightly. According to overall acceptibility, GLS2 had the highest

score among samples as well as taste and after taste. This sample had also the highest spread ratio. The lowest overal acceptability was noticed in the GLS1, which had the lowest scores for taste and texture, the darkest colour, the lowest fat and phenolic compounds content an dthe highest moisture content. Because of that the GLS2 and the GLS1 could be considered as the most preferable and the least preferable samples. On the hand, Yaver (2022) stated that overall acceptibility had been found for the least added GLF in the snack formulation with carob flour.
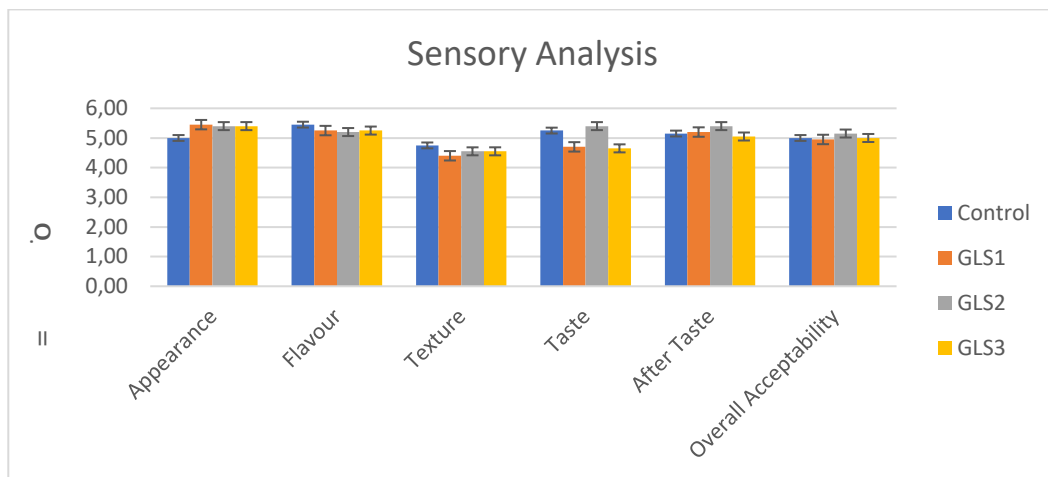
Figure 2. Sensory analysis results of snacks

CONCLUSION

In conclusion, this study showed that GLF can be used as a fortification and functional agent for plant-based snack products. However, to improve the protein ratio, the replacement ratio needs to be increased, but an increased ratio of GLF might lead to unwanted lentil flavour and might negatively affect the dough systems. Therefore, the formulations can be improved for future studies to enhance the formulation and sensory scores.

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UTJECAJ ADITIVA SERVOMYCES® NA KVASCE RAZLIČITIH GENERACIJA TIJEKOM VRENJA PIVA

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Rezime

Pivo se procesom alkoholnog vrenja proizvodi iz slada, hmelja, vode i pivskog kvasca. U proizvodnji piva koriste se odabrani sojevi pivskog kvasca vrste *Saccharomyces uvarum* za tzv. lager piva (piva donjeg vrenja), dok se za proizvodnju ale piva (piva gornjeg vrenja) koristi vrsta *Saccharomyces cerevisiae*, a mogu se koristiti i različiti hibridi tih kvasaca. U toku vrenja kvasac razlaže šećere iz sladovine na alkohol i CO_2. Proizvodi njegova metabolizma su i nusproizvodi kao što su vicinalni diketoni koji značajno utječu na aromu i kvalitetu piva. Posebna se pažnja posvećuje procesu propagacije kvasca. Servomyces® jedan je od aditiva koji je proizveden kao dodatak za potrebe pivarske industrije. Cilj ovoga rada bio je utvrditi učinak na kvasce 1., 2. i 3. generacije, te utvrditi eventualne razlike između piva kojemu je pri kraju procesa kuhanja dodan aditiv Servomyces®, u odnosu na pivo koje je proizvedeno standardnim postupkom. U tu svrhu, tijekom procesa vrenja praćeni su prividni ekstrakt i koncentracija vicinalnih diketona, a na kraju vrenja izmjeren je stupanj prevrelosti i postotak mrtvih stanica kvasca. Redukcija vicinalnih diketona se pratila 6 - 9 dana nakon završetka vrenja. Utvrđeno je da je redukcija vicinalnih diketona brža uz dodatak Servomycesa®, te da je udio prividnog ekstrakta ustaljen nakon šestog dana. Broj mrtvih stanica kvasca bio je manji u kvascu s dodatkom Servomycesa®. Stupanj prevrelosti bio je znatno niži u uzorcima kojima je dodan Servomyces®.

Ključne riječi: *pivo, kvasac, aditivi, vicinalni diketoni, vrenje*

UVOD

Pivo je osvježavajuće gazirano piće s malim udjelom alkohola. U proizvodnji piva velika se pažnja posvećuje kvascu koji se koristi za nacjepljivanje pivske sladovine. Koriste se odabrani sojevi vrste *Saccharomyces uvarum* i *Saccharomyces cerevisiae* (Marić i Navodnik, 1995). U toku vrenja kvasac razlaže šećere iz sladovine na alkohol i CO_2. Kvasac tijekom uzgoja proizvodi i druge spojeve osim CO_2 i etanola, primjerice

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glicerol, više alkohole, ostale arome okusa i mirisa te malu količinu biomase (Overkamp i sur., 2000).

Gotovo 90% aminokiselina koje su prisutne u sirovinama za proizvodnju piva se u procesu proizvodnje u anaerobnim uvjetima, tj. tijekom vrenja konvertiraju u više alkohole piva (Vuralhan i sur., 2005).

Anaerobnim vrenjem dio nikotinamid adenin dinukleotid hidrida (NADH) se oksidira u reakcijama sinteze glicerola, no to je za stanicu energetski nepovoljno jer se troši adenozin trifosfat (ATP) (Overkamp i sur., 2000). Vieira i sur. (2018) navode da kvasac može biti značajan izvor biogenih elemenata: željeza (1,76 mg/ 100 g otopljene vode), cinka (119 mg/100 g otopljene vode), mangana (0,564 mg/100 g otopljene vode) i bakra (0,364 mg/100 g otopljene vode). Proizvodi njegova metabolizma su i nusproizvodi kao što su vicinalni diketoni (VDK), koji značajno utječu na aromu i kvalitetu piva. Posebna se pažnja posvećuje procesu propagacije kvasca (razmnožavanje čiste kulture kvasca na veće količine) (Bohunicki i sur., 1972). Kvasci iz roda *Saccharomyces* vrlo dobro rastu na jednostavnoj podlozi sa šećerima kao izvorom energije i ugljika, amonijevim solima kao izvorom dušika, mineralnim tvarima (izvori Fe, K, Mg, Zn, Mn, Cu) potrebnih za funkcioniranje aktivnog metabolizma i tvarima rasta (Stewart i Russell, 1993). Kvasci su jednostanične mikroskopske gljivice kugličasta, eliptična ili izdužena oblika, promjera 5 - 8 µm. Osnovni tip stanice je blastospora, koja se razmnožava nespolno, pupanjem. Na blastospori izrasta pup koji se razvija te kada dosegne veličinu matične stanice otkida se od nje. Roditeljska stanica stvara pup na vanjskoj površini, kako pup raste dijeli se jezgra roditeljske stanice i potom jedna odlazi u pup. Na mjestu gdje se otkinuo pup ostaje ožiljak na roditeljskoj stanici i kada je velika površina prekrivena ožiljcima, stanica odumire (Duraković i Duraković, 2003). Inokulum mora imati više od 95 % živih stanica, ako nema potrebna je veća količina inokuluma. Veliki broj mrtvih stanica loše utječe na pivo koje poprima okus i miris po kvascu. Loša manipulacija kvascem i pretjerano kiselinsko pranje smanjuju životnost kvasca (Wackerbauer i sur., 1997). Optimalna temperatura vrenja za ale pivo (pivo gornjeg vrenja) je 16 – 20 ºC, a za lager pivo (pivo donjeg vrenja) 9 - 13 ºC. Općenito, vrijedi pravilo da je vrenje brže što je temperatura viša, ali ona ne smije biti previsoka jer utječe na sintezu metabolita koji mijenjaju okus i miris piva (Marić, 1996). Alkoholno vrenje obuhvaća niz enzimatskih procesa, čiji je konačni rezultat metaboliziranje heksoza na etanol i ugljični dioksid CO_2, te se u tim procesima oslobađa energija koju stanice kvasca koriste u sintezi sirovina potrebnih za njihove životne aktivnosti, rast i razmnožavanje (Chul i sur., 2007). Ukoliko nema dovoljno pojedinih hranjivih tvari u odabranim količinama ekstrakta kvasca, mogu se koristiti specifični sojevi. Što je endogena hidrolitička aktivnost stanica kvasca veća tijekom autolize, više aminokiselina se može osloboditi iz proteina u ekstrakt kvasca (Sommer, 1998). Znatnije promjene uočene su tijekom ulaska u stacionarnu fazu (Hans i sur., 2001). Vieira i sur. (2018) su otkrili da je proteolitička aktivnost za autolizu bila najveća nakon druge uporabe pivskog kvasca. *S. cerevisiae* (uzgaja se na melasi trske) i *K. marxianus* (uzgaja se na permeatu sirutke) imali su utjecaj na opseg autolize (stanično otapanje komponenata, oslobađanje amino i nukleinskog dušika), te su utjecali na aktivnosti lipolitičkih enzima (Amrane i Prigent, 1998, Yamamura i sur. 1991). Ponovno korištenje kvasca u procesu proizvodnje piva

rezultiralo je povećanjem sadržaja proteina u ekstraktu kvasca (Vieiri i sur., 2018). Visokoproteinski sojevi *S. cerevisiae* mogu poslužiti kao početni materijal za proizvodnju ekstrakta kvasca (Jacob i sur., 2019). Upotreba kvasaca koji imaju intrizični katalitički mehanizam za razgradnju glutaminske kiseline tijekom autolize mogu utjecati na promjenu niza slobodnih aminokiselina (Masuda i sur., 2008). Danas se u proizvodnji piva mogu koristiti različiti aditivi. Cvengroschová i Šmogrovičová (2005) u svom radu su proučavale utjecaj upotrebe sredstva protiv pjenjenja i hrane za kvasce na tijek vrenja, redukciju diacetila i kvalitetu gotovog piva u sladovini gravitacije 13 °P. Prema njihovom istaživanju, dodatak Antifoam Foamsol u koncentraciji od 4,2 ml/hl značajno je smanjio pjenjenje u kotlu za sladovinu tijekom vrenja, tako da je omogućio smanjenje doze hmelja. Jedinice gorčine smanjene su za oko 9,7 BU tijekom vrenja s upotrebom Foamsola, ali bez sredstva protiv pjenjenja smanjene su za oko 12,6 BU. Stabilnost pjene je bila konstantna. Hranjivi dodatak Yeastlife Extra u koncentraciji od 4 g/hl pomogao je bržem smanjenju sadržaja diacetila i VDK.

Servomyces® jedan je od aditiva koji su proizvedeni kao dodatak za potrebe pivarske industrije. To je osušeni pivarski kvasac u koji je ugrađen cink. Patentiran je u Americi (Lallemend) kao dodatak pivskoj sladovini (Lallemandbrewing, URL). Odobren je kao dodatak za korištenje u njemačkoj pivarskoj industriji u skladu s njemačkim zakonom o čistoći piva. Svrha njegovog dodatka je ubrzavanje procesa vrenja i životnosti kvasca. Kada je kvasac pohranjen, on crpi rezervne hranjive tvari koje kasnije moraju biti kompenzirane da bi kvasac mogao uspješno obaviti vrenje sladovine (Schuster i sur., 1988). Sastav ekstrakta kvasca može biti obogaćen s različitim ugljikohidratima koji će se moći koristiti pri određenim uvjetima okoline kao što su povišene temperature i nedostatak pojedinih nutrijenata (Bokulich, 2017). Na tržištu patentiran "Servomyces", tvrtka Lallemand Inc., trgovački naziv aktivnog i osušenog kvasca (taksonomski naziv: *S. cerevisiae*), je zadržao visoke koncentracije cinka u svom metabolizmu. Dodavanjem ovog kvasca obogaćenog cinkom u sladovinu tijekom kuhanja dolazi do otapanja ekstrakta u sladovini (Fischborn i sur., 2004). Prema navodima proizvođača na 100 litara sladovine za vrenje koristi se 1 g Servomycesaa. Praktična ispitivanja su pokazala da Servomyces treba dodati desetak minuta prije kraja kuhanja.

Za uspješnost vrenja presudna su slijedeća svojstva kvasaca: flokulacija; potrošnja šećera, aminokiselina, malih peptida i amonijevih iona iz ekstrakta; tolerancija kvasca na visoke osmotske tlakove; tolerancija na etanol i potreba za kisikom. Navedena svojstva su vezana uz temperaturu vrenja koja je različita za kvasce donjeg i gornjeg vrenja. Kvasci donjeg vrenja počinju vrenje na 6 °C, završavaju na 18 °C, maksimalno na 34 °C. Kvasci gornjeg vrenja započinju vrenje sladovine pri temperaturi od 18 °C, završavaju na oko 25 °C, maksimalno na 37 °C (Stewart, 2009). Kvasac u hmeljnoj sladovini apsorbira otopljene šećere, vitamine, aminokiseline i amonijeve ione (Stewart, 2016). Dodatkom Servomycesa® tj. mineralnih tvari ugrađenih u živo tkivo (stanice pivskog kvasca) postiže se efekt boljeg iskorištenja tih hranjivih tvari, tj. veći stupanj prevrenja (Sp). Servomyces® može značajno smanjiti vrijeme vrenja, poboljšati taloženje kvasca, povećati prinos alkohola, stimulirati sintezu proteina i rast kvasaca i time doprinijeti porastu biomase u procesu propagacije kvasca, reducirati sumporne

komponente, poboljšati zdravlje i životnost kvasca, reducirati koncentraciju vicinalnih diketona u glavnom vrenju. Thomas i sur. (1994) utvrdili su u svojoj studiji da ekstrakt kvasca i drugi složeni aditivi kao što su tripton i pepton pokazuju dvojak mehanizam kod vrenja piva. Posljedično, oni ne samo da opskrbljuju faktore rasta za nedovoljno opskrbljene stanice, nego potiču rast i vrenje sladovine visoke gravitacije, te održavaju vitalnost stanica na 80% i djeluju kao osmoprotektori. Učinak glicin betaina, koji je sastavni dio ekstrakata kvasca, bio je umjeren i povezan s poteškoćama u staničnom metabolizmu, što je najvjerojatnije uzrokovano njegovim pozitivnim nabojem. Osim toga, utvrđeno je da prolin proizveden katabolizmom arginina djeluje kao osmoprotektor prilikom vrenja pšenice (Thomas i sur., 1993). Korištenje nukleinskih kiselina povećalo je rast kvasca i učinak vrenja, iako se vitalnost stanica nije mogla održati Thomas i sur. (1994).

MATERIJAL I METODE RADA

Uzorci piva su priređeni u industrijskoj proizvodnji piva. Nakon što je slad priređen mljevenjem, izvršeno je ukomljavanje (oko 1 h; tem. 45 – 72 °C), odvajanje sladovine (1- 3 h; 73 – 78 °C). Dobivena sladovina se kuhala 2,5 h pri tem 100 °C i bistrila 60 min. pri temp 90 – 100 °C. Potom je slijedila inokulacija sladovine (dodatak kvasca *Saccharomyces uvarum*). Vrenje i zrenje trajali su 9 dana, početak vrenja je bio pri 6 °C, a kraj pri 25 °C. Praćeni su procesi glavnog vrenja sladovine, te su stanice kvasca izdvojene po završetku glavnog vrenja sladovine. Otvaranjem ispusta pri dnu kotla za vrenje izuzeti su uzorci za provođenje analitičkog dijela eksperimenta. Na početku vrenja izmjeren je sadržaj ekstrakta u sladovini, te je iznosio 14,1% u uzorcima nacijepljenim prvom generacijom kvasaca i 13,8% u uzorcima nacijepljenim drugom i trećom generacijom. Napravljena su tri pokusa, u svakome su korištena po dva cilindrično konusna kotla za vrenje (CKF). U jednom CKF – se radilo po standardnoj proceduri vrenja, a drugom uz dodatak aditiva Servomyces®. U prvom pokusu je inokulum bio kvasac prve generacije, u drugom je korišten kvasac druge generacije, a u trećemu kvasac treće generacije. Praćeno je ukupno šest CKF-a koji su nacijepljeni s tri različite generacije kvasca. Tri uvarka sladovine su nacijepljena i proizvedena standardnim postupkom (uz dodatak $ZnCl_2$), ostala tri uz dodatak aditiva Servomyces®, 10 minuta prije završetka procesa kuhanja u količini od 310 g po jednom uvarku sladovine. U prvom pokusu korišten je kvasac *Saccharomyces uvarum* prve generacije proizveden aerobnom propagacijom, u drugom pokusu kvasac *Saccharomyces uvarum* druge generacije, a u trećem kvasac *Saccharomyces uvarum* treće generacije. Od šestog do devetog dana vrenja određivana je koncentracija VDK mjerenjem na spektrofotometru na valnoj duljini 335 nm. Za mjerenje VDK priređen je uzorak piva temperiran na sobnoj temperaturi. Dodatkom filtracijskog sredstva dobiven je bistri uzorak. U 10 ml filtrata dodano je 5 ml 1% -tne otopine o-fenilendiamina, te mu je nakon stajanja 20 – 30 minuta na mračnom mjestu, dodano 2 ml 4M HCl. Potom je dodano tri žličice filtracijskog sredstva i profiltrirano preko lijevka prekrivenog satnim staklom. Koncentracija uzorka mjeri se na spektrofotometru, na valnoj duljini 335 nm. Aparat mjeri apsorpciju.

$$VDK = 2.7 * A \qquad (1)$$

2.7 - koeficijent

A – apsorbancija, nm

VDK - vicinalni diketoni, ppm

Ekstrakt u mladom pivu izmjeren je pomoću DMA 35N aparata direktnim očitavanjem vrijednosti (Anton-paar, URL). Prije očitavanja uzorak je degaziran postupkom prelijevanja iz jedne posude u drugu. Za mjerenje stupnja prevrelosti, uzorak iz CKF - a temperiran je u vodenoj kupelji pri temperaturi od 20 °C, a zatim je stavljen u tikvicu od 1000 ml, te mu je dodano 2 žličice filtracijskog sredstva Potom je tikvica začepljena i dobro promućkana osam puta, uz povremeno otvaranje čepa (sve dok se više ne čuje zvuk izlaženja CO_2). Nakon toga je izvršena filtracija. Bistri i degazirani uzorak uliven je u pripadajuće posudice i postavljen u bubanj „Alcolyser Plus Beer" (Anton – paar, URL).

Određivanje broja mrtvih stanica kvasca u uzorku kvasca vršen je brojanjem stanica kvasca na Thomaovoj komorici mikroskopiranjem (Evropska pivarska konvencija, 1985). Pri tom se koristilo metilensko plavilo koje je obezbojilo žive stanice, te ih je pod mikroskopom lako razlikovati. Potom se izvršilo brojanje stanica kvasca na Thomaovoj komorici mikroskopiranjem. Najprije su se izbroje sve stanice kvasca (obojene i neobojane) u svakom pojedinom kvadratu, a potom su brojane samo mrtve stanice kvasca (obojene u plavo).

REZULTATI I DISKUSIJA

Parametri koji su praćeni tijekom procesa vrenja bili su prividni ekstrakt i koncentracija vicinalnih diketona, a na kraju vrenja izmjeren je stupanj prevrelosti i postotak mrtvih stanica kvasca. Navedeni parametri su izuzetno važni za kvalitetu piva (Kunze, 1988), jer tijekom vrenja nastaju karbonilni spojevi, kao što su aldehidi i vicinalni diketoni koji negativno utječu na aromu i okus piva (Briggs i sur., 2004). Za potrebe provedbe eksperimenta napravljena su tri pokusa, svaki s po dva CKF-a. U svakom pokusu pratila su se istovremeno po dva CKF-a. U prvom pokusu, sladovina u dva kotla za vrenje (CKF 5 i CKF 7), nacijepljena je recikliranim kvascem prve generacije, u drugom pokusu u iduća dva kotla za vrenje (CKF 10 i CKF 15) sladovina je nacijepljena recikliranim kvascem druge generacije, te u trećem pokusu (CKF 12 i CKF 11) nacijepljeni su kvascem treće generacije. Pokusima se uspoređivao i kraj vrenja tj. dan kada se prividni ekstrakt ustalio na konačnoj vrijednosti (ista vrijednost ekstrakta izmjerena u dva uzastopna dana).
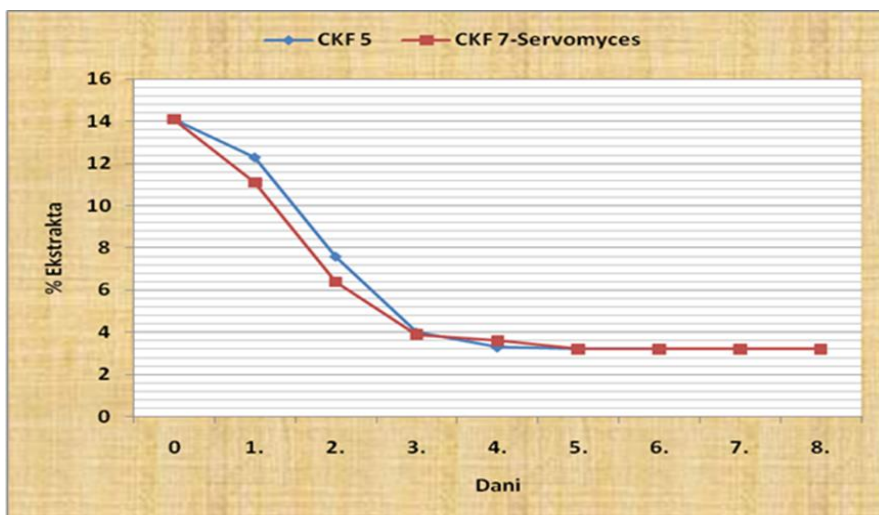
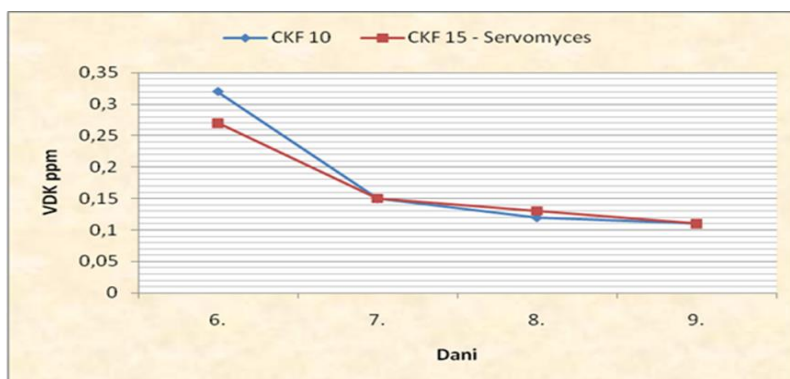Grafikon 1. Promjena udjela ekstrakta po danima vrenja piva iz dva usporedna
CKF-a, nacijepljenog kvascem prve generacije
Graph 1. Change in the proportion of extract by days of beer fermentation from two
comparative CKFs, inoculated with first generation yeast

U prvom pokusu (korišten je reciklirani kvasac prve generacije). Izmjerena vrijednost
početnog ekstrakta u obadva tanka bila ista (14,1%). Prva tri dana ekstrakt je brže padao
u CKF 7, tj. u tanku gdje je bio dodan Servomyces® (Grafikon 1). Razlog tome je
najvjerojatnije veća aktivnost kvasca što je dovelo do brže razgradnje ekstrakta. Četvrti
dan, ekstrakt u CKF 7 padao je sporije od ekstrakta u CKF 5, a peti su se izjednačili.
Glavno vrenje je u obadva tanka završilo šesti dan (Grafikon 1), tj. kada se vrijednost
prividnog ekstrakta ustalila na 3,2%. Prema tome u prvom pokusu utvrđeno je da je
proces vrenja u prvim danima tekao brže u tanku s dodatkom Servomyces®-a (CKF 7),
no nakon toga je usporio i u konačnici je glavno vrenje završilo isti (šesti) dan u obadva
tanka. Redukcija VDK se pratila 6 – 9 dana, nakon završetka glavnog vrenja. U prvom
pokusu početna je koncentracija VDK (mjerena 6 - ti dan) u CKF 7 (Servomyces®) bila
nešto niža nego u CKF 5. Diacetili, odnosno VDK daju okus i miris po maslacu.
Redukcija diacetila se odvija pomoću kvasaca, na način da kvasac konvertira diacetil u
acetoin, a potom u butandiol. Udio ukupnog diacetila (vicinalnih diketona i prekursora)
u pivu bi trebala iznositi 0,1 ppm (Palmer, 2006). Navedeno je u skladu s dobivenim
rezultatima za kraj vrenja u 2 i 3. pokusu u slučaju korištenja Servomyces®, te je za
očekivati da će tako proizvedeno pivo imati dobru aromu. Deveti dan izmjeren je stupanj
prevrelosti u tankovima i utvrđeno da je pivo u CKF 7 (dodan Servomyces®) imalo
znatno niži stupanj prevrelosti od piva u CKF 5. Na kraju procesa vrenja ispušten je
kvasac i napravljena analiza kojom se utvrdio postotak mrtvih stanica kvasca. Analizom
je utvrđeno da je u kvascu iz CKF 7 (dodan Servomyces®) bio prisutan manji postotak
mrtvih stanica kvasca nego u CKF 5 (Grafikon 2).
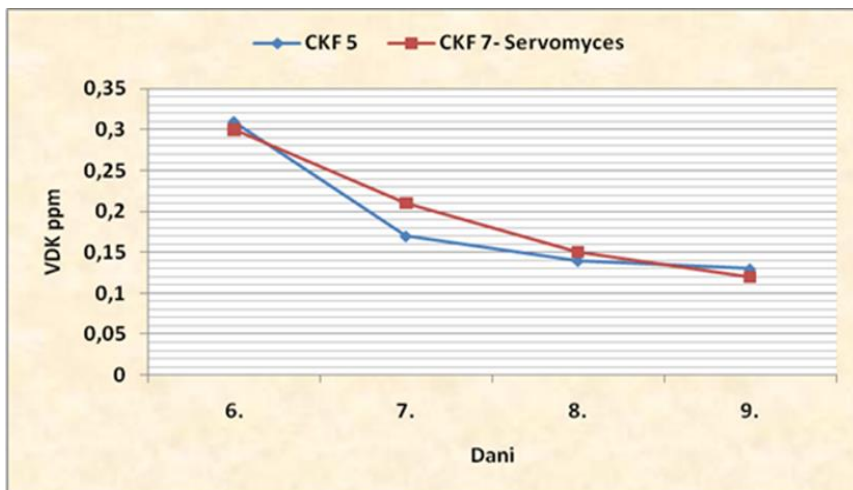
Grafikon 2. Redukcije VDK po danima vrenja piva iz dva usporedna CKF-a,
nacijepljenog kvascem prve generacije
*Graph 2. VDK reductions by days of beer fermentation from two comparative CKFs,
inoculated with first generation yeast*

U drugom pokusu CKF 10 je napunjen sladovinom po standardnom postupku vrenja, a
CKF 15 je napunjen sladovinom kojoj je dodan aditiv Servomyces®. Za nacjepljivanje
je korišten kvasac druge generacije. Početni ekstrakt bio je u obadva tanka isti (13,8%)
(Grafikon 3). Prva dva dana ekstrakt je brže padao u CKF 15. Razlog tome je očita veća
aktivnost kvasca što je dovelo do brže razgradnje ekstrakta, što je u skladu s
istraživanjima Fischborna i suradnika (2004). Naime, oni navode da se bolja
ekstakcijska razgradnja postiže dodatkom Servomycesa® u odnosu na dodatak čistog
ZnCl$_2$., te smatraju da je bioraspoloživost Zn veća u stanicama Servomycesa®. Van
Zandycke i Fischborn (2008) smatraju da deaktivirani kvasac obogaćen cinkom kao
hrana za kvasce, daje slične razultate kao i Servomyces® i slični aditivi, te da je važno
uskladiti sastav hranjivih tvari sa specifičnim potrebama kvasaca.
Treći dan, ekstrakt u CKF 15 padao je sporije od ekstrakta u CKF 10, a peti su se gotovo
izjednačili. Vrijednost prividnog ekstrakta ustalila se na 3,1% u obadva tanka. Vrenje je
u CKF 10 završilo šesti dan, a u CKF 15 (dodan Servomyces®) sedmi dan. Prema tome,
u drugom pokusu utvrđeno je da je proces vrenja u prva dva dana tekao brže u tanku sa
dodatkom Servomyces® - a (CKF 15), no nakon toga je usporio i u konačnici je glavno
vrenje završilo ranije u CKF 10, rađenom po standardnoj proceduri.
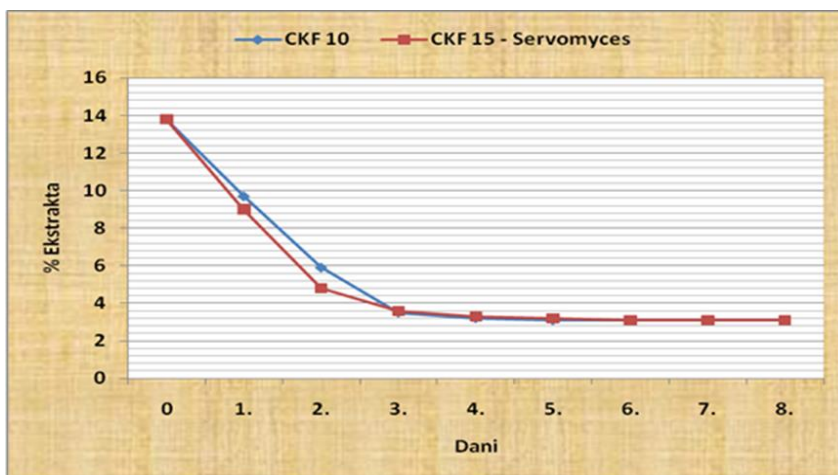
Grafikon 3. Promjena udjela ekstrakta po danima vrenja piva iz dva usporedna CKF-a nacijepljena kvascem druge generacije
Graph. 3. Change in the proportion of extract by days of beer fermentation from two comparative CKFs inoculated with second generation yeast

U drugom pokusu početna je koncentracija VDK (mjerena 6 - ti dan) u CKF 15 (dodan Servomyces[®]) bila niža nego u CKF 10, zatim je iduća dva dana padala sporije, da bi konačan rezultat izmjeren 9 – ti dan bio isti u obadva tanka (slika 4). Smanjenje njihove koncentracije tijekom glavnog i naknadnog vrenja, omogućuje da esteri i viši alkoholi budu presudni za aromu i okus gotovog piva (Kunzle, 2010.) Kits i Garshol (2021) navode kako pri uobičajenim temperaturama vrenja, odnosno pri temperaturama unutar optimalnog raspona, kvasac SafAleTM US-05 daje pivo dobro uravnoteženog aromatskog profila, kojim dominiraju voćni esteri. Povećanjem temperature vrenja uočen je snažan trend porasta nepoželjnih tvari arome.

Deveti dan izmjeren je stupanj prevrelosti u tankovima i utvrđeno da je pivo u CKF 15 imalo niži stupanj prevrelosti od piva u CKF 10. Analizom je utvrđeno da je u kvascu iz CKF 15 (dodan Servomyces[®]) bio prisutan manji postotak mrtvih stanica kvasca nego u CKF 10 (Grafikon 8). U trećem pokusu CKF 12 je napunjen sladovinom po standardnom postupku, a CKF 11 je napunjen sladovinom kojoj je dodan Servomyces[®]. Za necijepljivanje je korišten kvasac treće generacije. Početni ekstrakt u CKF 12 bio je nešto viši (13,8 %) od ekstrakta u CKF 11 (13,5%). Prva dva dana ekstrakt je brže padao u CKF 11. Treći dan, ekstrakt je bio isti u obadva tanka (3,5%), dalje je padao približno istom brzinom (Grafikon 5). Prividni ekstrakt se ustalio na 3,1% u CKF 12 i 2,8% u CKF 11. Glavno vrenje je u CKF 12 završilo šesti dan, a u CKF 11 (Servomyces[®]) sedmi dan (Grafikon 3). Prema tome u trećem pokusu utvrđeno je da je proces vrenja u prva dva dana tekao brže u tanku sa dodatkom Servomyces-a (CKF 11), no nakon toga je usporio i u konačnici je glavno vrenje završilo ranije u CKF 12 (rađenom po standardnoj proceduri). Prema istraživanjima Cvengroschove i Šmogrovičove (2005) dodatak Servomyces[®] u količini 1g/hl skratio je vrijeme fermentacije za 12 sati, a poželjna vrijednost diacetila postignuta je šesti dan. U trećem pokusu koncentracija

VDK (mjerena 6. dan) u CKF 11 (Servomyces®), bila je sva četiri dana niža nego u CKF 12 (Grafikon 6). Deveti dan izmjeren je stupanj prevrelosti u tankovima i utvrđeno da je pivo u CKF 11 (dodan Servomyces®) imalo niži stupanj prevrelosti od piva u CKF 12, te je utvrđeno da je u kvasacu iz CKF 11 (Servomyces®) bio prisutan manji postotak mrtvih stanica kvasca nego u CKF 12 (Grafikon 3). Deveti dan izmjeren je stupanj prevrelosti u tankovima i utvrđeno da je pivo koje je sadržvalo Servomyces imalo znatno niži stupanj prevrelosti od piva koje je proizvedeno standardnim procesom proizvodnje. Najniži stupanj prevrelosti je imalo pivo proizvedeo u CKF 15, u kom su nacjepljeni kvasci druge generacije uz dodatak Servomyces® – a (Grafikon 7).

Grafikon 4. Redukcije VDK po danima vrenja piva iz dva usporedna CKF-a nacijepljena kvascem druge generacije
Graph 4. VDK reductions by days of beer fermentation from two comparative CKFs inoculated with second generation yeast
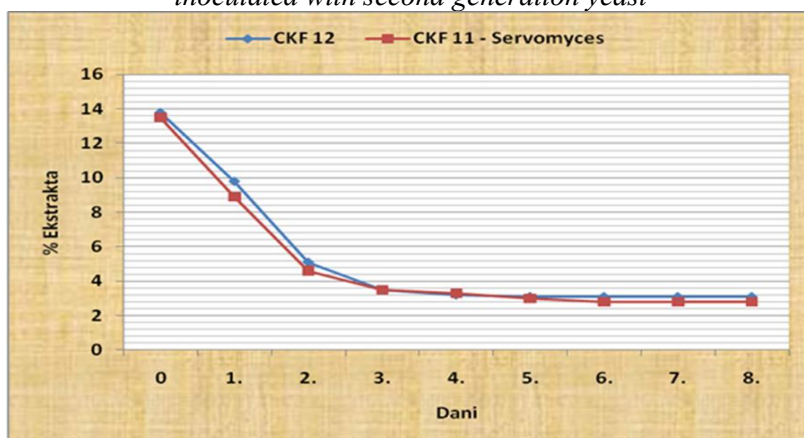
Grafikon 5. Promjena udjela ekstrakta po danima vrenja piva iz dva usporedna CKF-a nacijepljena kvascem treće generacije

Graph 5. Change in the proportion of extract by days of beer fermentation from two comparative CKFs inoculated with third generation yeast
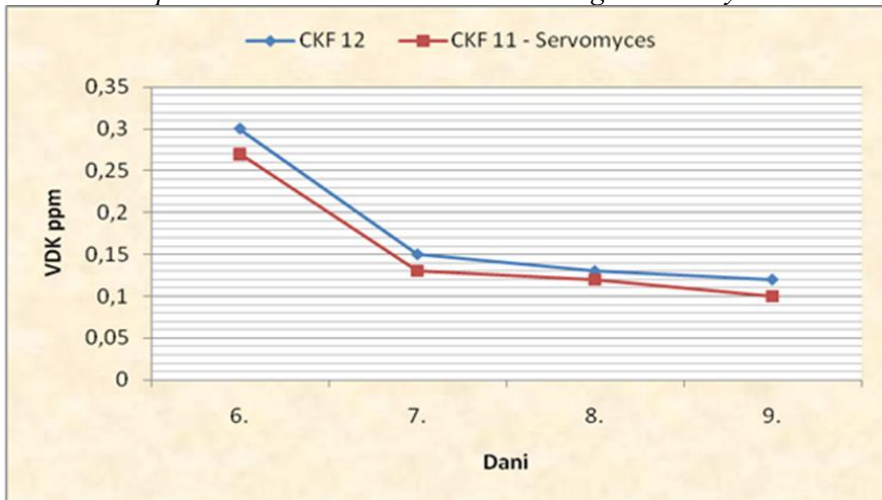
Grafikon 6. Redukcije VDK po danima vrenja piva iz dva usporedna CKF-a nacijepljena kvascem treće generacije
Graph 6. VDK reductions by days of beer fermentation from two comparative CKFs inoculated with third generation yeast
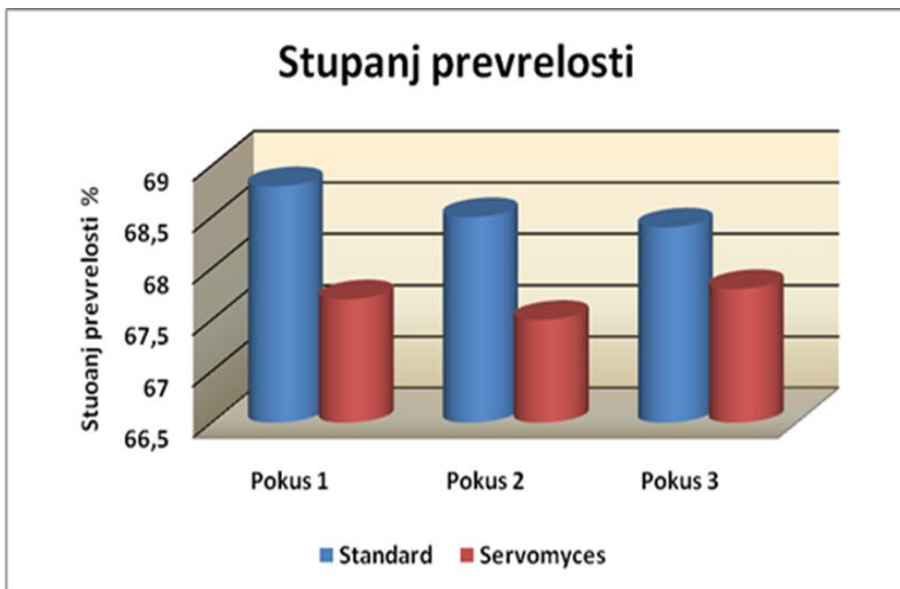
Grafikon 7. Usporedba stupnja prevrelosti za sva tri pokusa
Graph 7. Comparison of the degree of prevalence for all three experiments
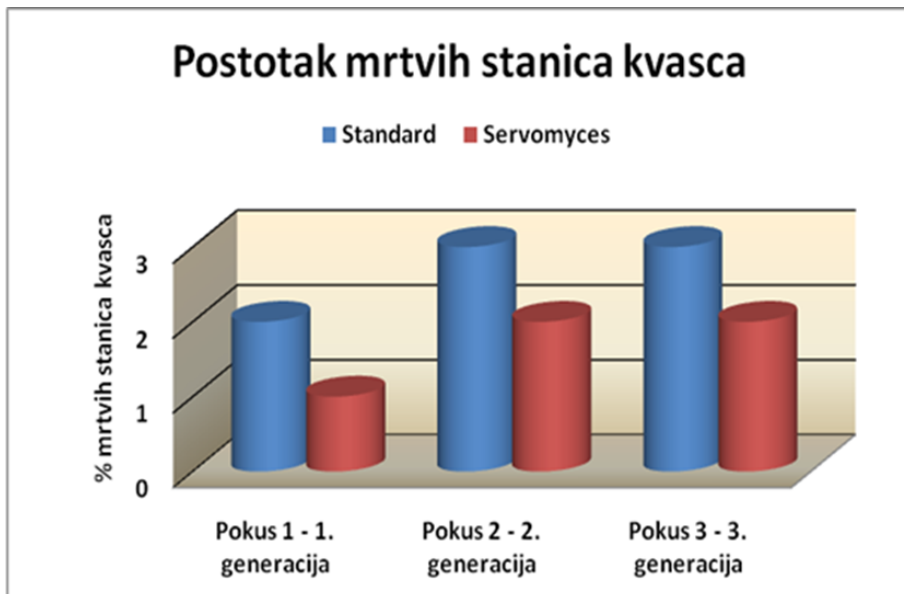
Grafikon 8. Usporedba postotka mrtvih stanica kvasca u sva tri pokusa
Graph 8. Comparison of percentage of dead yeast cells in all three experiments

ZAKLJUČAK

Dodatak Servomycesa® povećava brzinu vrenja u prvim danima vrenja, te zamjetno utječe na smanjene broja vicinalnih diketona pri korištenju kvasaca prve generacije. Učinak na redukciju vicinalnih diketona je uz dodatak Servomycesa® najočitiji u uzorcima nacjepljenim kvascima treće generacije, te je njihov udio na kraju vrenja niži u odnosu na pivo proizvedeno bez Servomycesa®. Stupanj prevrelosti manji je u tankovima s pivom kojem je u procesu kuhanja dodan Servomyces®. Broj mrtvih stanica kvasca manji je u kvascu iz tankova sa Servomycesom®, što ukazuje na veću životnost toga kvasca. S obzirom da vicinalni diketoni negativno utječu na aromu i okus piva, a veći broj mrtvih stanica kvasca daje pivu okus i miris po kvascu, dodatak Servomycesa® u konačnici će se odraziti na poboljšan okus i miris piva. Udio ekstakta se uz dodatak Servomycesa® je brže padao i ustalio se nakon šestog dana vrenja, te bi bilo dobro razmotriti mogućnost smanjenja vremena vrenja u industrijskoj proizvodnji piva.

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EFFECT OF SERVOMYCES® ADDITIVES ON DIFFERENT GENERATIONS OF YEAST DURING BEER FERMENTATION

Summary

Beer is produced from malt, hops, water and brewer's yeast through the process of alcoholic fermentation. In the production of beer, selected strains of brewer's yeast of the Saccharomyces uvarum species are used for the so-called lager beer (bottom-fermented beer), while to produce ale beer (top-fermented beer) the species Saccharomyces cerevisiae is used, and different hybrids of these yeasts can also be used. During fermentation, the yeast ferments the sugars from the wort into alcohol and CO_2. The products of its metabolism are also by-products such as vicinal diketones, which significantly affect the aroma and quality of beer. Special attention is paid to the yeast propagation process. Servomyces® is one of the additives produced as a supplement for the needs of the brewing industry. The aim of this work was to determine the effect on 1st, 2nd and 3rd generation yeasts, and to determine possible differences between fermented beer to which the additive Servomyces® was added at the end of the brewing process, compared to fermented beer that was produced by the standard process. For this purpose, the apparent extract and concentration of vicinal diketones were monitored during the fermentation process, and at the end of fermentation, the degree of overcooking and the percentage of dead yeast cells were measured. The reduction of vicinal diketones was monitored 6-9 days after the end of fermentation. It was found that the reduction of vicinal diketones is the same or slightly faster with the addition of Servomyces®, with a slightly lower concentration at the end of fermentation, and that the proportion of the apparent extract remained unchanged. The number of dead yeast cells was lower in yeast supplemented with Servomyces®.

Keywords: *beer, yeast, additives, vicinal diketones, fermentation*

NATURAL ENZYMES AND ALLERGIES*

Lejla Avdić[1], Ziha Ašimović[1]

Review paper

Abstract

Enzymes play a crucial role in speeding up chemical reactions in living organisms. Discovered in the late 19th century, these complex proteins are essential for various biological processes. Food allergies are immune responses to specific proteins in food. Common allergens include proteins from eggs, milk, peanuts, tree nuts, and seafood. Allergic reactions can vary from mild to severe and affect different body systems. The prevalence of food allergies is rising, with some estimates suggesting that up to 10% of the population may be affected.

The aim of this paper is to investigate the influence of some natural enzymes on allergies.

Enzymes used in food processing, like lysozyme in wine or alpha amylase in baked goods, can pose risks for those with sensitivities. Lysozyme, an enzyme that breaks down bacterial cell walls, can cause allergic reactions in some people, particularly those allergic to eggs. Similarly, enzymes like enolases and aldolases, which are involved in energy production, can trigger allergies, especially among those sensitive to fish proteins. Alpha-amylase, an enzyme that breaks down starches, is a well-known allergen, particularly in occupational settings like baking. Arginine kinase, found in seafood and insects, can also cause allergic reactions, which is a concern for people handling these products. Chitinase, which breaks down chitin in insects, and papain from papaya, used in meat tenderizers, can lead to allergies as well.

Conclusion: Clearly specifying the natural or synthetic origin of potentially allergenic substances enables the avoidance of allergic reactions and directly contributes to maintaining the health of individuals sensitive to these ingredients.

Keywords: *lysozyme allergy, α-amylase, chitinase, aldolase, papain*

INTRODUCTION

The concept of enzymes in nature has been recognized for over a century. The German physiologist Wilhelm Kühne introduced the term enzyme in 1878 to describe yeast's ability to ferment sugar into alcohol. The term originates from the Greek words "en" (within) and "zyme" (yeast) (Robinson, 2015). In 1926, James B. Sumner achieved a

milestone by isolating the first enzyme, urease, in crystalline form from beans (Sumner, 1926). Enzymes, often referred to as biocatalysts, are specialized biological molecules that speed up biochemical reactions without being consumed. While they are synthesized within living cells, enzymes remain functional outside of their natural environment, enabling their use in industrial applications. Most enzymes are proteins and serve as essential biocatalysts in sustaining life by facilitating vital biochemical processes (Kuddus, 2018).

Allergies are defined in various ways depending on the context. According to the World Health Organization (2003), they involve hypersensitivity reactions initiated by immune mechanisms. Another perspective describes allergies as an overreaction of the immune system to typically harmless environmental substances (Dougherty *et al*., 2023). The term "allergy" was first introduced in 1906 by Clemens von Pirquet, a Viennese pediatrician, who used it to broadly describe alterations in an organism's reactivity, considering factors such as timing, quality, and intensity of the response (Huber, 2006). Substances that provoke allergic reactions are referred to as allergens. These can be biological or chemical agents and often consist of foreign proteins or glycoproteins that stimulate IgE antibody responses in humans (Judith *et al*., 2015; Mekori, 1996). The International Union of Immunological Societies stipulates that a substance must induce IgE-mediated allergic reactions in at least 5% of the population to qualify as an allergen under their nomenclature (Løwenstein, 1996).

Food allergies, a specific category, are defined as adverse health effects stemming from a reproducible immune response triggered by exposure to foods (Boyce *et al*., 2010). These reactions are usually linked to specific proteins within the food, though occasionally chemical haptens may also act as allergens. While some food allergens, particularly those in fruits and vegetables, provoke reactions only when consumed raw, most can still elicit allergic responses even after being cooked or digested (Boyce *et al*., 2010).

Protein-rich foods are common sources of allergens, with most food-induced allergic reactions attributed to specific food proteins (James *et al*., 2012). The distribution of proteins, fats, and carbohydrates varies across food types, but allergens are predominantly associated with the protein fraction.

Food allergies exhibit varying patterns of persistence and resolution. While allergies to eggs, milk, and wheat often diminish during early childhood, allergies to peanuts, tree nuts, sesame seeds, fish, shellfish, and buckwheat are more likely to persist throughout life. However, the likelihood of resolution can differ significantly across studies for a given food allergen (James *et al*., 2012).

According to the World Allergy Organization (WAO), adverse reactions to food are collectively referred to as food hypersensitivity. This term is further divided into immune-mediated responses, known as food allergies, and non-immune-mediated responses, termed food intolerances. Food allergies themselves can be classified into two main categories based on the immune mechanism involved: immunoglobulin E (IgE)-mediated reactions, which occur rapidly, and non-IgE-mediated reactions, which have a delayed onset (James *et al*., 2012).

MATERIALS AND METHODS

This study examines the interrelationships between natural enzymes and allergies. Key terms such as "enzyme" and "allergy" are defined to establish their fundamental meanings. The research focuses on natural enzymes that can stimulate the immune response and lead to adverse reactions in individuals. To conduct this research through literature review, methods classification, description and comparison were used. The most well-known natural enzymes associated with unwanted allergic reactions were identified and described, highlighting their potential to threaten human life through inhalation or consumption.

RESULTS AND DISCUSSION

Over recent decades, researchers have identified over 2,000 allergens, with detailed information available in online databases. Among these, the allergen nomenclature database maintained by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) is one of the most rigorous and extensively peer-reviewed resources, accessible at www.allergen.org. Another key resource, the Allergen Online database, provided by the Food Allergy Research and Resource Program (FARRP) (www.allergenonline.org), offers valuable data on allergenic proteins (Karnaneedi *et al*., 2021).

Food allergies have become a growing public health issue worldwide. Approximately 2.5% of the global population is estimated to be affected, though prevalence rates vary widely between 1% and 10% depending on the region and study. Applying this percentage to the global population of 8.1 billion suggests that around 202.5 million people could have food allergies (Fiocchi & Fierro, 2017). This issue not only affects individual health but also imposes substantial socioeconomic challenges on patients, families, and society. Although fatalities are rare, the fear of severe outcomes, including death, remains a significant concern for those with food allergies.

Proteins, including some enzymes, are key triggers of allergic sensitization. Despite the vast number of proteins classified in databases, allergenic proteins belong to a relatively small number of protein families. The Pfam protein family database (http://pfam.xfam.org/) identifies 19,632 families, while the Structural Database of Allergenic Proteins (SDAP; http://fermi.utmb.edu/) lists 1,908 allergens and isoallergens, along with 233 novel Pfam classes. SDAP combines an allergenic protein database with bioinformatics tools to study structural features and epitope characterization. The most impactful plant- and animal-derived food allergens are concentrated in eight protein superfamilies (James *et al*., 2012).

Symptoms of allergic reactions can affect the skin, gastrointestinal tract, respiratory system, and cardiovascular system. Common signs include abdominal cramps, vomiting, hives, difficulty breathing, persistent coughing, hoarseness, trouble swallowing, tongue swelling that interferes with speaking or breathing, weak pulse,

pallor or bluish skin, dizziness, and fainting. Severe reactions, such as anaphylaxis, can be life-threatening, causing breathing difficulties and potentially leading to shock (American College of Allergy, Asthma and Immunology).

Lysozyme

Lysozyme is a versatile enzyme found across living organisms and viruses, showing considerable diversity in its origin, abundance, and biochemical characteristics (Ferraboschi *et al*., 2021). First identified by Alexander Fleming, lysozyme is recognized for its bacteriolytic properties and classified as a hydrolase under EC 3.2.1.17 (Figure 1). The enzyme's name reflects its function: breaking down bacterial cell walls through hydrolysis of the β-1-4-glycosidic bond between N-acetylglucosamine and N-acetylmuramic acid, a process earning it the alternative name "muramidase" (Daniel *et al*., 2015).
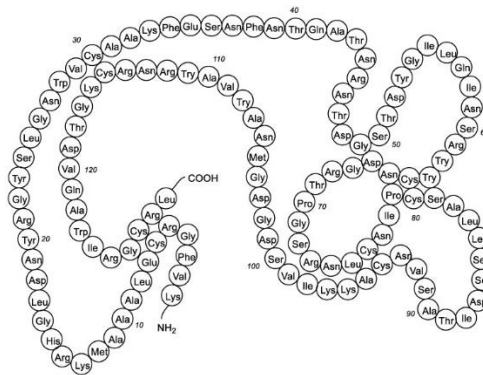
Figure 1. Representation of the lysozyme structure
https://doi.org/10.1016/j.foodchem.2018.09.017

Lysozymes are categorized into three primary families: chicken type (c-type), goose type (g-type), and invertebrate type (i-type) with additional varieties, such as phage, bacterial, and plant lysozymes, also identified (Table 1). C-type lysozyme, including the chicken egg white lysozyme (HEWL), plays a significant role in the pharmaceutical industry due to its efficacy against bacterial, viral, and inflammatory conditions (Wu *et al*., 2019).

Egg white is composed of approximately 90% water and 10% protein, with around 40 proteins identified. Among these, lysozyme (Gal d 4) is considered a minor allergen but has been associated with sensitization in roughly one-third of egg-allergic individuals. The allergenicity of Gal d 4 diminishes with heat or enzymatic treatment, making reactions more likely when raw egg is consumed (Lopata, 2013).

Table 1. Overview of some characteristics of lysozyme

Biological function	Glycosyl hydrolase
Allergen code	362
Source	Egg extract
Latin name	*Gallus domesticus*
Other names	Gal d IV, lysozyme,E1105
Molecular weight	14 kDa
Categories	Eggs, food animal-derived food

Source:https://www.thermofisher.com/diagnostic-education/hcp/wo/en/resource-center/allergen-encyclopedia/allergen-component.html?key=k208

The EU Food Safety Authority has found that wines treated with egg-derived lysozyme (used as a microbiological stabilizer or additive) to control lactic acid bacteria can cause undesirable allergic reactions in some individuals. At the request of the European Commission, the Panel on Dietetic Products, Nutrition, and Allergies (NDA) was asked to provide a scientific opinion regarding the labeling of egg lysozyme, where the panel concluded that wines treated with lysozyme may cause undesirable allergic reactions in sensitive individuals (EFSA Journal 2011).

Egg-derived lysozyme is increasingly used as an antibacterial additive to prevent spoilage in cheese, wine, or other foods, as well as in medications for respiratory tract infections and congestion, which usually do not specify the source, posing a risk to consumers allergic to chicken eggs (Benede *et al.*, 2014).

Lysozyme can be used as a preservative in cheese to prevent the late release of gas caused by the genus *Clostridium* (anaerobic Gram-positive bacteria) and represents an alternative to nitrate (Schneider & Pischetsrieder, 2013).

Enolase and Aldolase

Enolases and aldolases, which are part of the lyase enzyme class, play a vital role in glycolysis, a fundamental metabolic process found across all tissues. Glycolysis is a highly conserved pathway comprising ten steps that break down glucose into pyruvate, generating adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) as high-energy molecules (Pirovich *et al*., 2021).

Fish allergies can trigger adverse reactions in sensitive individuals through ingestion, inhalation of cooking vapors, or direct contact. Symptoms range from mild, such as oral itching and hives, to severe manifestations like asthma and systemic anaphylaxis. Around half of individuals with fish allergies may experience cross-reactivity with other fish types (Kuehn *et al*., 2014).

Epidemiological studies highlight the prevalence of fish allergies. In Norway, a survey of 3,623 children revealed nearly 3% of reported food-related reactions by age two were linked to fish. In Spain, a cohort of 355 children with IgE-mediated food allergies showed that fish allergies often began before two years of age. In the United States, approximately 5.9% of 14,948 respondents reported seafood allergies (Sharp & Lopata,

2013). Similarly, in Australia, research at a specialized clinic involving 2,999 children indicated a 5.6% prevalence of fish allergies, with white fish, tuna, and salmon as the most common culprits. In Asian populations, fish allergies are also significant, as shown by a Singaporean study where 13% of 227 children with food hypersensitivity were sensitized to fish, often introduced into diets by seven months of age (Sharp & Lopata, 2013).

While proteins such as enolases and aldolases were initially linked to fish-specific allergies, it has been observed that many individuals allergic to fish with IgE targeting enolase and aldolase also react to parvalbumin. However, there are documented cases where patients with significant sensitization to enolase or aldolase lack IgE specific to parvalbumin. These findings suggest that such sensitizations can lead to species-specific fish allergies. Despite this, cross-reactivity among enolase and aldolase allergens from fish species like cod, salmon, and tuna remains possible (Dijkema *et al*., 2020).

α-Amylase

Amylase, a hydrolase-class enzyme, was first identified in the early 19th century and is among the earliest enzymes to be studied scientifically (Akinfemiwa *et al*., 2023). Its primary role is to break down glycosidic bonds in starch, transforming complex carbohydrates into simpler sugars. There are three primary types of amylase enzymes: alpha-amylase, beta-amylase, and gamma-amylase, each targeting specific parts of carbohydrate structures. Alpha-amylase is found in humans, animals, plants, and microorganisms, while beta-amylase occurs in plants and microbes (Table 2). Gamma-amylase is present in both animals and plants (Akinfemiwa *et al*., 2023).

In the baking industry, amylase is a key enzyme used to enhance and optimize various processes, playing a critical role in improving the quality of baked goods.

Table 2. Overview of some characteristics of fungal α-amylase

Properties	Fungal α-amylase
Physical Appearance	White to gray powder
EC Code	3.2.1.1
Ph	4,5-7.0
Temperature	30 °C -60°C

Source:https://www.foodnetworksolution.com/company/angel-yeast-co-ltd/products/1019/Enzymes-for-baking

In humans, α-amylase is synthesized by the exocrine pancreas and also secreted in saliva. It is a recognized occupational allergen when inhaled. In cases of bakery asthma associated with the flour processing industry, the allergenic α-amylase typically originates from fungal contamination (Pali-Schöll *et al*., 2018).

The enzyme α-amylase (4-α-D-glucan glucanohydrolase; EC 3.2.1.1) is produced using a non-genetically modified strain of *Aspergillus oryzae* (Lambre *et al*., 2023). It is utilized across seven food production processes: starch processing to produce glucose, maltose syrups, and other hydrolysates; brewing, baking, production of distilled alcohol, grain-based processes, manufacturing of milk analog products and processing fruits and

vegetables for juice production. During the production of glucose syrups and distillation, residual total organic solids (TOS) from the enzyme are removed, so dietary exposure is not calculated for these processes. For the other five processes, the estimated dietary exposure in European populations is up to 0.134 mg TOS/kg body weight per day.

Genotoxicity assessments raised no safety concerns, and systemic toxicity was evaluated in a 90-day repeated oral toxicity study in rats. Based on the proposed uses (excluding distilled alcohol production), the risk of allergic reactions due to food exposure cannot be entirely ruled out, although the likelihood is considered low (Lambre *et al.*, 2023).

Arginin kinase

Arginine kinase (EC 2.7.3.3) is a transferase enzyme that facilitates the transfer of phosphate groups from high-energy molecules like ATP (adenosine triphosphate) to specific substrate molecules. Specifically, it catalyzes the transfer of a phosphate group from ATP to the amino acid arginine, resulting in the formation of phosphoarginine. Arginine kinases have been identified as allergens in various invertebrates, including food sources such as shrimp, and in cross-reactive species like the Indian meal moth, royal shrimp, lobster, and mussel (Binder *et al.*, 2001). This enzyme has a molecular weight of 40-42 kDa and is considered unstable in acidic or basic conditions (Laly & Sankar, 2020).

Inhaled bioaerosols containing seafood allergens can provoke allergic responses, posing a risk to individuals working in seafood-related industries. This includes those involved in seafood processing, food preparation (e.g., chefs and restaurant staff), and collection activities like fishing and aquaculture. While the percentage of shrimp-sensitized individuals recognizing arginine kinase is not definitively established, estimates suggest it ranges between 10 and 51% (Giovannini *et al.*, 2023). Additional studies indicate that IgE sensitization to arginine kinase is observed in 21-50% of adults and as much as 67% of children (Kleine-Tebbe & Jakob, 2017).

Chitinase

Chitinases (EC 3.2.1.14), classified under hydrolases, are enzymes that break down β-1,4-N-acetyl-D-glucosamine bonds in chitin polymers. They are produced by various organisms, including bacteria, fungi, insects, plants, and vertebrates, and play roles in nutrition, morphogenesis, and defense against pathogens containing chitin (Rathore *et al.*, 2015; Leoni *et al.*, 2019).

An allergenic form of chitinase has been identified in the silkworm pupa (*Bombyx mori L.*), which is consumed as food (Zhao *et al.*, 2015). This is notable as it is the first allergenic chitinase linked to food that is not plant derived. This finding is significant, particularly given that silkworm pupae are a traditional food in East Asia and may become part of emerging dietary trends in other regions (Leoni *et al.*, 2019). Table 3 outlines various food sources of chitinases that could potentially induce allergic reactions.

Table 3. Chitinases identified as food allergens

Source	Molecule	Assay
Class I (GH19, with chitin binding module)		
Kiwi fruit *(Actidinia chinensis)*	I	IR
Papaya *(Carica papaya)*	I	
Chestnut *(Castanea sativa)*	I	
Tomato *(Lycopersicum esculentum)*	I	IR
Banana fruits *(Musa sp.)*	P	IR, SPT
Avocado (*Persea americana*)	P, R	IR
Green bean (*Phaseolus vulgaris*)	I	SPT
Wheat (*Triticum aestivum*)	I	IR
Class II (GH19, without chitin binding module)		
Tomato *(L.esculentum)*		
Wheat (*T. aestivum)*	I	
Rice (*Oryza sativa)*	R	IR
Class III (GH18, without chitin binding module)		
Coffee green beans (*Coffea arabica*)	I, R	IR
Raspberry berries (*Rubus ideaeu*)	I	IR
Indian jujube fruit (*Zizyphus mauritiana*)	P, R	IR
Pomegranate (*Punica granatum*)	I	IR, SPT
Class IV (GH19, with chitin binding module)		
Grape (*Vitis vinifera*)	I, R	IR
Maize (*Zea mays)*	R	IR
Insect chitinases (GH18)		
Silkworm (*Bombyx mori)*	I	IR

Source:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6600546/#:~:text=Chitinases%20are% 20a%20group%20of,been%20only%20partially%20carried%20out.

For plant and insect chitinases, it is considered that the allergic reaction may be a result of the so-called cross-reactivity syndrome, meaning the occurrence of a food allergy in individuals who have already been exposed and sensitized to structurally similar non-food allergens. Specifically, for many plant chitinases, structural similarity with hevein (a protein identified as a significant allergen for patients allergic to latex extracted from the rubber tree) was noted in the first descriptions of allergenic chitinases in plants. Furthermore, for the recently identified insect allergenic chitinase, structural similarity with the allergenic chitinase from house dust mites was reported (*Leoni et al.*, 2019).

Papain

Papain (EC 3.4.22.2) is a proteolytic enzyme in the hydrolase class, derived from the latex of the papaya plant (*Carica papaya*). It is extensively utilized in various industries, including as a meat tenderizer, beer clarifier, and contact lens cleaner, and plays a significant role in the pharmaceutical and cosmetic sectors. The first IgE-mediated allergic reaction to papain in workers was documented in 1975 (Milne & Brand, 1975). The initial report of papain allergy dates to 1928. Since then, numerous cases of occupational asthma linked to papain exposure have emerged, primarily among workers in the pharmaceutical and cosmetic industries (Soto-Mera *et al*., 2000). Papain adversely affects the skin by impairing its protective barrier. It can quickly increase vascular permeability and attract inflammatory cells to the skin (Medical University of Vienna, 2015).

Structurally, papain shares significant similarities with a major allergen found in house dust mites. Due to these properties, individuals with sensitive skin and young children are advised to avoid papain-containing products and carefully review ingredient labels for consumer items (Medical University of Vienna). Tests are now available to identify allergic reactions to this enzyme (https://www.healthlabs.com/papain-allergy-testing). Papain allergy can arise in both occupational and non-occupational settings, with most cases involving individuals employed in factories where papain is processed, biochemical laboratories, or cosmetic facilities.

Triosephosphate Isomerase

Triosephosphate isomerase (TIM or TPI) is a key enzyme in glycolysis and has been identified as an allergen in saltwater products, belonging to the class of isomerases (Yang *et al.* 2017). TIM (EC 5.3.1.1.) has been described as a new allergen, mainly in marine organisms and certain invertebrates. Some of these species include *Octopus fangsiao* (webfoot octopus), *Blattella germanica* (German cockroach), *Penaeus monodon* (black tiger shrimp), *Procambarus clarkii* (red swamp crayfish), and *Citrullus lanatus* (watermelon) (Yang *et al.,* 2017). Its clinical and immunological relevance and cross-reactivity are still not well researched (Lopata, 2017).

CONCLUSIONS

Food allergies are an important public health issue that affect both children and adults and may be on the rise. An allergy manifests as a hypersensitivity reaction to environmental factors known as antigens or allergens. Despite the significant risk of severe allergic reactions, and even death, there is currently no treatment for food allergies. The symptoms of an allergic reaction vary greatly in terms of onset speed and intensity. The condition can only be managed by avoiding allergens or treating symptoms. Every food or allergen derivative must be clearly labeled and indicated on food, medication, and other product labels that people consume to improve the safety of individuals prone to allergic reactions. Reviews of the literature often focus on egg

and milk derivatives. It is essential to clearly state the natural or synthetic origin of potentially allergenic substances, such as lysozyme, casein, lactose, albumin, papain, arginine kinase, and aromatic essences. Providing such information enables the avoidance of allergic reactions and directly contributes to maintaining the health of people who may be at risk when consuming these ingredients.

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PRIRODNI ENZIMI I ALERGIJE

Sažetak

Enzimi igraju ključnu ulogu u ubrzavanju hemijskih reakcija u živim organizmima. Otkriveni krajem 19. stoljeća, ovi složeni proteini su neophodni za različite biološke procese. Alergije na hranu predstavljaju imunološke reakcije na specifične proteine u hrani. Uobičajeni alergeni uključuju proteine iz jaja, mlijeka, kikirikija, orašastih plodova i morskih plodova. Alergijske reakcije mogu varirati od blage do teške i utjecati na različite sisteme tijela. Učestalost alergija na hranu raste, a neka istraživanja sugeriraju da čak do 10% populacije može biti pogođeno.

Cilj ovog rada je istražiti utjecaj nekih prirodnih enzima na alergije. Enzimi korišteni u preradi hrane, poput lizozima u vinu ili alfa-amilaze u pečenim proizvodima, mogu predstavljati rizik za osobe sa senzibilitetom. Lizozim, enzim koji razgrađuje ćelijske zidove bakterija, može izazvati alergijske reakcije kod nekih ljudi, posebno onih alergičnih na jaja. Slično tome, enzimi poput enolaza i aldolaza, koji su uključeni u proizvodnju energije, mogu izazvati alergije, posebno kod osoba osjetljivih na proteine ribe. Alfa-amilaza, enzim koji razgrađuje škrob, poznati je alergen, posebno u radnim okruženjima kao što je pekarstvo. Argininska kinaza, koja se nalazi u morskim plodovima i insektima, također može izazvati alergijske reakcije, što predstavlja zabrinutost za osobe koje se bave ovim proizvodima. Hitinaza, koja razgrađuje hitin u insektima i papain iz papaje, koji se koristi za omekšavanje mesa, također mogu dovesti do alergija.

Zaključak: Jasno specificiranje prirodnog ili sintetičkog porijekla potencijalno alergenih supstanci omogućava izbjegavanje alergijskih reakcija i izravno doprinosi održavanju zdravlja osoba osjetljivih na ove sastojke.

Ključne riječi: *alergija na lizozim, alfa-amilaza, hitinaza, aldolaza, papain*

THE INFLUENCE OF MICROWAVE HEATING AND ANTIOXIDANTS ON THE OXIDATIVE STABILITY OF COLD-PRESSED VEGETABLE OILS*

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Original scientific paper

Abstract

Lipid oxidation is a significant problem in the production, processing and use of edible oils, which causes significant changes in chemical, sensory and nutritional properties. Therefore, the aim of this research was to examine the influence of microwave heating and antioxidants, natural and synthetic, on the oxidative stability of cold-pressed vegetable oils (rapeseed oil, sesame oil and milk thistle seed oil). Cumin and fennel essential oils were used as natural antioxidants, and propyl gallate (PG) and butylhydroxytoluene (BHT) as synthetic antioxidants. The test was carried out by determining the value of the peroxide value in the tested samples, first through the impact of microwave heating of different intensity at the same sampling time, as well as microwave heating of the same intensity sampled every 5 minutes up to a maximum of 25 minutes. By extending the time of microwave heating at constant power (140 W), the values of peroxide value increased in all tested samples with and without the addition of antioxidants. The biggest changes in the value of the peroxide value were recorded in milk thistle seed and rapeseed oil, and the least in sesame oil. The best protection against oxidative deterioration was seen in BHT in all tested samples, as well as in fennel essential oil in rapeseed oil. With the increase of the power intensity of the device during microwave heating for the duration of 5 minutes, the values of the peroxide value of all tested samples increased, both with and without the addition of antioxidants. Using different powers of microwave heating (140 W, 280 W and 420 W), the best protection against oxidative deterioration of sesame oil was shown by PG, milk thistle seed oil by BHT and PG, and rapeseed oil by fennel essential oil and BHT.

Keywords: *oxidative stability, cold-pressed oils, antioxidants, microwave heating.*

INTRODUCTION

Cold-pressed vegetable oils are obtained exclusively by mechanical pressing and are consumed directly, without any subsequent refining. They are a group of important food ingredients. Novel specialty seed oils rich in health-promoting factors are in high demand due to consumer interest in disease prevention and health promotion through improved nutrition. These beneficial factors include special fatty acid (FA) compositions such as high content of monounsaturated FA (MUFA) or n-3 FA, tocopherols, carotenoids, and antioxidative phenolic compounds (Parry *et al.,* 2006). Cold pressing of oilseeds enables maximum retention of bioactive compounds, such as essential fatty acids, phenolic and flavonoid substances, tocopherols, tocotrienols, phytosterols, etc. (Radočaj and Dimić, 2013), as well as of the characteristic sensory properties of the oils (Moslavac *et al.,* 2023). Rapeseed oil is a rich source of natural components with an antioxidant effect. It is well-known for its high content of unsaturated fatty acids, especially polyunsaturated fatty acids, which make it of great nutritional value (Shen *et al.,* 2023). Rapeseed oil contains about 65% oleic, 20% linoleic, 9% linolenic and 2% stearic acid (Beyzi *et al.,* 2019). The low content of saturated fatty acids in rapeseed oil has led to it being considered one of the most health-promoting edible oils (Coughlan *et al.,* 2022). Sesame oil, also known as gingelly oil or til oil, is a highly unsaturated edible oil rich in essential fatty acids such as linoleic acid. It is also rich in various bioactive compounds including phytosterols, tocopherols and lignans such as sesamin, sesamolin and sesaminol, which are known to play an important role in providing stability against oxidation of oil and contribute to antioxidative activity (Bopitiya and Madhujith, 2013). Oxidative stability of sesame oil is superior to that of other vegetable oils although it contains nearly 85% unsaturated fatty acids (Abou-Gharbia et *al.,* 2000; Lončar *et al.,* 2012; Moslavac and Vuković, 2013; Zahran *et al.,* 2020). It may be added to other oils to enhance oxidative stability such as in the preparation of frying oils (Gunstone, 2004). Milk thistle seed oil is an important byproduct of industrial production of silymarin. It oil has a high concentration of unsaturated fatty acids, especially linoleic and oleic acid, which are beneficial to human health in preventing arteriosclerosis, diabetes, and cancer (Tarasevičiene *et al.,* 2023; Zhang *et al.,* 2020). Milk thistle seed oil has also been documented as a potential natural source of vitamin E, and has often been recommended as a favorable edible oil (Meddeb *et al.,* 2017). Oil oxidation is problematic, reducing the oil quality over the storage period due to the breakdown of essential fatty acids, resulting in increased free fatty acids, resulting in oil rancidity, reducing the nutritional benefits significantly (Coughlan *et al.,* 2022). Oxidative stability is an important indicator of the quality and shelf life of products. Oxidative stability of edible oils is defined as resistance to oxidation during processing, storage, and cooking (Loganathan *et al.,* 2022). Today, many methods are used to determine the oxidative stability of vegetable oils based on accelerated oil oxidation: Schaal Oven test, AOM test and Rancimat test (Shahidi, 2005). Antioxidants are used to prevent oxidation of edible oils and fats. Synthetic antioxidants are cheaper than natural ones, while natural antioxidants are safer than

synthetic ones. Extracts of various plants are used to stabilize oils and fats, as well as essential oils obtained by extracting aromatic, spice and medicinal plants. Microwave heating is a modern and widely used method for food preparation (Dostálová *et al.,* 2005). It represents the conversion of electromagnetic field energy to thermal energy, in materials that possess dielectric properties (Moslavac *et al.,* 2012). Microwave heating of different vegetable oils and fats causes the formation of free radicals that rapidly react with atmospheric oxygen to produce hydroperoxides and secondary oxidation products (Kishimoto, 2019). It was found that the rate of quality deterioration, such as oxidation, depends on the polyunsaturated fatty acid (PUFA) content (Lukešová *et al.,* 2009). In vegetable oils exposed to microwave energy, the higher the amount of PUFA in the oils, the greater was the rate of quality deterioration of the oils. The levels of free fatty acids also increased in vegetable oil heated in microwave oven (Hassanein *et al.,* 2003). In this study, the influence of microwave heating and antioxidants on the oxidative stability of cold-pressed rapeseed, sesame and milk thistle oil was investigated.

MATERIALS AND METHODS

As material for the experimental part of the work, three samples of cold-pressed oils were used, i.e. rapeseed oil, sesame oil and milk thistle seed oil from the Sarajevo Canton market. For the distillation of essential oils, samples of dried fennel and cumin herbs were taken. Butylhydroxytoluene (BHT) and propyl gallate (PG) were used as synthetic antioxidants. The extraction of essential oils was performed by hydrodistillation using an adapted Neo Clevenger apparatus (ISO 8571, 1988, E). To evaluate the sustainability, i.e. the oxidative stability, a sustainability test with microwave heating was carried out. A microwave oven was used for research purposes (Tristar, output intensity 700 W). Natural antioxidants (fennel and cumin essential oils) in a concentration of 0.1% and synthetic antioxidants (BHT-butylhydroxytoluene and PG-propyl gallate) in a concentration of 0.01% have been added to the cold-pressed oils (rapeseed oil, sesame oil and milk thistle seed oil). For each base oil sample, a control sample without added antioxidants was prepared, which was further treated like all samples. The tested samples were treated with microwave heating under the following conditions:

-Á power 140 W for 5, 10, 15, 20 and 25 minutes and
-Á powers of 140 W, 280 W and 420 W for 5 minutes.

During each sampling, the value of the peroxide value (PV) was determined using the method according to ISO 3960 (2017). The Past 3.15 program (Hammer *et al.,* 2001) was used for statistical data processing. To determine a statistically significant difference in the PVs under the influence of the type of added antioxidants, sampling time and different intensity of microwave heating a two-factor analysis of variance was applied. In case of statistically significant differences, Tukey's post-hoc test was used (significance level $\alpha=0.05$).

RESULTS AND DISCUSSION

The results of testing the influence of microwave heating time at a power of 140 W of natural and synthetic antioxidants on the oxidative stability of rapeseed, sesame and milk thistle seed oil are shown in Table 1. A two-factor analysis of variance determined that there is a statistically significant influence of experimental factors (microwave heating time and type of antioxidants) on the PV of the examined oil samples with the addition of different antioxidants ($p < 0.05$). From the results shown in Table 1, it can be seen that with the increase in the time of treatment with microwave heating at a constant power of the device, the PV of the tested oils with and without the addition of antioxidants increase, but with a significantly lower intensity in the case of sesame oil compared to the other two test samples in this work. All applied antioxidants, natural and synthetic, as can be seen in Table 1, stabilized rapeseed and milk thistle seed oil, i.e. protected them from oxidative deterioration, while the PV in all samples with the addition of antioxidants were lower than the values of the control sample. On the other hand, the addition of antioxidants to sesame oil did not lead to a stabilization of the oil, except when BHT was added. Low peroxide values of the control sample of sesame oil during treatment with microwave heating without any additives indicate good stability. The PV of the sesame oil sample with the addition of BHT increased with a lower intensity compared to the control sample up to a value of 0.50 mmol O_2/kg, i.e., the same value as the control sample at the end of the treatment of 25 minutes. BHT and fennel essential oil exhibited the best stabilization of rapeseed oil, i.e. protection against oxidative deterioration. Also, the synthetic antioxidant BHT showed the best protection in milk thistle seed oil. The most intense changes were recorded in the sesame oil sample with the addition of PG. The results of the study by Labrović (2011) are consistent with the results of this work. Namely, the authoress states in her research that increasing the time of treatment of rapeseed and sesame oil with microwave heating decreases the sustainability of the oil, and that the addition of natural antioxidants to the oil leads to a change in stability towards oxidative deterioration. The sample with the addition of pomegranate extract showed the best stability of rapeseed oil, while the mentioned antioxidant was the only one that did not protect sesame oil from spoilage. Pavlović (2014) noted in her research that the viability of rapeseed oil reduced by applying microwave heating at constant power (450 W) with increasing treatment time, as well as stabilization with the addition of natural and synthetic antioxidants. The best protection of rapeseed oil against oxidative deterioration was achieved by the addition of rosemary extract, as well as the addition of the synthetic antioxidant octyl gallate. Examining the oxidative stability of rapeseed oil through microwave heating with increasing treatment duration in the research of Dostálová *et al.* (2005) and Lukešová *et al.* (2009), the PV increased up to a certain point in treatment, after which the value started to decrease. In the research of Nikolov (2018), using the Rancimat method, better oxidative stability of sesame oil was determined, compared to hemp, flax and sunflower oil. Of the applied extracts, the winter savory extract had the least influence on the oxidative stability of the examined vegetable oils. Pelko (2019), in his research shows similar results to the ones in Table 1, i.e. an increase in the PV of cold-pressed milk

thistle seed oil with an extension of the microwave heating time (300 W). Stabilization of milk thistle seed oil was achieved by the addition of rosemary extract, while no further stabilization of the oil was achieved with the combination of the mentioned extract and synergist, citric acid. The synthetic antioxidant tert-butylhydroquinone (TBHQ) showed a significant protection of milk thistle seed oil from oxidative spoilage using the Schaal Oven test at a constant temperature of 63°C, was recorded in the research of Furundžija (2020).

Table 1. The influence of microwave heating time at 140 W power and antioxidants on the oxidative stability of rapeseed, sesame and milk thistle seed oils (expressed as peroxide value mmol O_2/kg)

Sample	Antioxidant	%	Microwave heating time (minutes)					
			0	5	10	15	20	25
Rapeseed oil	-		$2.46^{Aa}\pm0.02$	$2.72^{Aa}\pm0.04$	$3.17^{Aa}\pm0.07$	$4.19^{Ab}\pm0.32$	$4.46^{Ab}\pm0.00$	$5.17^{Ac}\pm0.38$
	PG	0.01	$2.46^{Aa}\pm0.02$	$2.55^{Aa}\pm0.24$	$2.69^{Aa}\pm0.31$	$2.94^{Ba}\pm0.04$	$3.43^{Bb}\pm0.06$	$4.06^{Bb}\pm0.49$
	BHT	0.01	$2.46^{Aa}\pm0.02$	$2.46^{Aa}\pm0.02$	$2.96^{Aa}\pm0.06$	$3.06^{Ba}\pm0.13$	$3.09^{Ba}\pm0.26$	$3.47^{Bb}\pm0.05$
	Cumin essential oil	0.10	$2.46^{Aa}\pm0.02$	$2.50^{Aa}\pm0.00$	$2.70^{Aa}\pm0.35$	$3.21^{Ba}\pm0.34$	$3.96^{Ab}\pm0.00$	$4.19^{Bb}\pm0.44$
	Fennel essential oil	0.10	$2.46^{Aa}\pm0.02$	$2.72^{Aa}\pm0.35$	$3.00^{Aa}\pm0.00$	$3.00^{Ba}\pm0.00$	$3.00^{Ba}\pm0.00$	$3.47^{Ba}\pm0.05$
Sesame oil	-		$0.00^{Aa}\pm0.00$	$0.00^{Aa}\pm0.00$	$0.25^{Aa}\pm0.35$	$0.25^{Aa}\pm0.35$	$0.50^{Aa}\pm0.00$	$0.50^{Aa}\pm0.00$
	PG	0.01	$0.00^{Aa}\pm0.00$	$0.25^{Aa}\pm0.35$	$0.50^{Aab}\pm0.00$	$0.98^{Bb}\pm0.02$	$0.98^{Bb}\pm0.02$	$0.98^{Ab}\pm0.02$
	BHT	0.01	$0.00^{Aa}\pm0.00$	$0.00^{Aa}\pm0.00$	$0.00^{Aa}\pm0.00$	$0.17^{Aa}\pm0.35$	$0.25^{Aa}\pm0.00$	$0.50^{Aa}\pm0.00$
	Cumin essential oil	0.10	$0.00^{Aa}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.61^{Aab}\pm0.15$	$0.66^{Aab}\pm0.14$	$0.74^{Ab}\pm0.34$
	Fennel essential oil	0.10	$0.00^{Aa}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.50^{Aab}\pm0.00$	$0.86^{Ab}\pm0.17$
Milk thistle seed oil	-		$3.98^{Aa}\pm0.03$	$4.46^{Aa}\pm0.00$	$4.75^{Ab}\pm0.35$	$4.95^{Ab}\pm0.00$	$5.17^{Acb}\pm0.29$	$5.47^{Acb}\pm0.04$
	PG	0.01	$3.98^{Aa}\pm0.03$	$4.04^{BAa}\pm0.12$	$4.41^{Aa}\pm0.06$	$4.51^{BAa}\pm0.14$	$4.87^{Ab}\pm0.03$	$5.34^{Ab}\pm0.07$
	BHT	0.01	$3.98^{Aa}\pm0.03$	$4.23^{Aa}\pm0.32$	$4.35^{Aa}\pm0.03$	$4.47^{BAa}\pm0.03$	$4.62^{Bba}\pm0.29$	$4.80^{Bba}\pm0.14$
	Cumin essential oil	0.10	$3.98^{Aa}\pm0.03$	$4.29^{Aa}\pm0.05$	$4.56^{Aa}\pm0.27$	$4.78^{Ab}\pm0.09$	$5.03^{Acb}\pm0.14$	$5.08^{Acb}\pm0.25$
	Fennel essential oil	0.10	$3.98^{Aa}\pm0.03$	$4.17^{Aa}\pm0.06$	$4.43^{Aa}\pm0.03$	$4.90^{Ab}\pm0.11$	$4.95^{Ab}\pm0.07$	$5.23^{Acb}\pm0.39$

*A-B Different capital letters in columns indicate statistically significant differences in peroxide values for antioxidants added to oils

a-c Different lowercase letters in rows indicate statistically significant differences in peroxide values at different microwave heating times

The results of determining the oxidative stability of rapeseed, sesame and milk thistle oils by the effect of different powers of microwave heating at the same time are shown in Table 2.

Table 2. The influence of microwave heating power for 5 minutes and antioxidants on the oxidative stability of rapeseed, sesame and milk thistle seed oils (expressed as peroxide value mmol O_2/kg)

Sample	Antioxidant	%	Microwave heating power		
			140 W	280 W	420 W
Rapeseed oil	-		$2.72^{Aa}\pm0.04$	$2.94^{Aa}\pm0.00$	$3.94^{Ab}\pm0.03$
	PG	0.01	$2.55^{Aa}\pm0.24$	$3.11^{Aa}\pm0.20$	$5.42^{Bb}\pm0.04$
	BHT	0.01	$2.46^{Aa}\pm0.02$	$3.00^{Aa}\pm0.00$	$4.73^{Cb}\pm0.39$
	Cumin essential oil	0.10	$2.50^{Aa}\pm0.00$	$4.25^{Bb}\pm0.00$	$5.50^{Bc}\pm0.00$
	Fennel essential oil	0.10	$2.72^{Aa}\pm0.50$	$2.85^{Aa}\pm0.53$	$4.29^{ACb}\pm0.11$
Sesame oil	-		$0.00^{Aa}\pm0.00$	$0.74^{Aa}\pm0.35$	$1.59^{Ab}\pm0.17$
	PG	0.01	$0.25^{ABa}\pm0.35$	$0.50^{Aa}\pm0.00$	$0.50^{Ba}\pm0.00$
	BHT	0.01	$0.50^{ABa}\pm0.00$	$0.75^{Aa}\pm0.01$	$0.75^{Ba}\pm0.01$
	Cumin essential oil	0.10	$0.50^{ABa}\pm0.00$	$0.87^{Aa}\pm0.18$	$3.19^{Cb}\pm0.35$
	Fennel essential oil	0.10	$1.00^{Ba}\pm0.01$	$1.00^{Aa}\pm0.01$	$2.35^{Ab}\pm0.18$
Milk thistle seed oil	-		$4.46^{Aa}\pm0.00$	$4.73^{Aab}\pm0.32$	$5.45^{Ab}\pm0.00$
	PG	0.01	$4.04^{Aa}\pm0.12$	$5.20^{Ab}\pm0.35$	$6.09^{BAc}\pm0.13$
	BHT	0.01	$4.23^{Aa}\pm0.32$	$4.93^{Aab}\pm0.10$	$5.20^{Ab}\pm0.28$
	Cumin essential oil	0.10	$4.29^{Aa}\pm0.05$	$5.47^{Abc}\pm0.04$	$6.00^{BAc}\pm0.00$
	Fennel essential oil	0.10	$4.17^{Aa}\pm0.06$	$5.23^{Ab}\pm0.39$	$7.30^{Cc}\pm0.18$

*A-C Different capital letters in columns indicate statistically significant differences in peroxide values for antioxidants added to oils

a-c Different lowercase letters in rows indicate statistically significant differences in peroxide values at different microwave heating powers

A two-factor analysis of variance determined that there is a statistically significant influence of experimental factors (microwave heating power and type of antioxidants) on the PV of the examined oil samples with the addition of different antioxidants ($p<0.05$). The investigation of the influence of the power of microwave heating and the

addition of antioxidants on the oxidative stability of the tested oils resulted in different changes depending on the applied power. By increasing the power of microwave heating, there was an increase in the PV of the tested oils with and without the addition of antioxidants. By applying a microwave heating power of 140 W, the antioxidants used stabilized rapeseed and milk thistle seed oil, while the addition of fennel essential oil to rapeseed oil resulted in the same PV as the control sample (2.72 mmol O_2/kg). The best protection against oxidative deterioration was shown by the synthetic antioxidant BHT in the case of rapeseed oil, or PG in the case of milk thistle oil. Contrary to the above, accelerated oxidative deterioration was recorded with the addition of antioxidants in sesame oil. Microwave heating with a power of 280 W for 5 minutes did not stabilize the tested oils with the addition of antioxidants, except for the addition of fennel essential oil in the case of rapeseed oil and PG in the case of sesame oil. The most intense changes were recorded in the samples of rapeseed and milk thistle seed oil with the addition of caraway essential oil and in the case of sesame oil with the addition of fennel essential oil. By treating the tested oils with microwave heating at 420 W, no stabilization was recorded with the addition of antioxidants, except for sesame oil with the addition of synthetic antioxidants and milk thistle seed oil with the addition of BHT. The most intensive changes were recorded in the samples of rapeseed and sesame oil with the addition of caraway essential oil, and milk thistle seed oil with the addition of fennel essential oil. According to the above, the stabilization of oil with the addition of antioxidants is more difficult to achieve with an increase in the power of microwave heating. Also, in the research of Labrović (2011), it was determined that the application of higher power of microwave heating leads to greater oxidative deterioration of rapeseed and sesame oil. By microwave heating of the mentioned oils with a power of 600 W, a decrease in the PV was observed in relation to the value recorded when the oil was treated at 450 W. The authoress states that the reason for the phenomenon mentioned is most probably due to the high temperatures measured in the treated oils, at which the decomposition of the peroxide and hydroperoxide took place. The stabilization of cold-pressed rapeseed oil through the addition of antioxidants was determined in studies by Moslavac *et al.* (2023) and Bigić (2014). According to the Schaal Oven test of accelerated oil oxidation at 63°C, the PV of rapeseed oil with and without the addition of antioxidants increased with the extension of the treatment time. The best protection of rapeseed oil was achieved with the addition of green tea extract (Moslavac *et al.,* 2023; Bigić, 2014). The conducted research found that increasing the content of natural antioxidants resulted in greater stability of the oil. In the research of Moslavac and Vuković (2013), it was determined that by adding sesame oil (50%) to other types of tested edible vegetable oils, there was an increase in the stability of the oil mixture against oxidative deterioration. The research carried out by Ghosh *et al.,* (2014) recorded an increase in the peroxide value of sesame oil with an increase in the power and length of microwave heating. During microwave heating of the highest power after 10 minutes of treatment, the PV decreased. In a study by Pelko (2019), when the power of microwave heating was increased from 180 W to 300 W, an increase in the PV of samples of cold-pressed milk thistle seed oil with and without the addition of

antioxidants and synergists was recorded. However, by further increasing the power of the device to 450 W and 600 W, there was a decrease in the PV of the mentioned samples. In the research of Tomljenović (2021), an increase in the PV of cold-pressed milk thistle oil with and without antioxidants was recorded using the Schaal Oven test at 63℃ with an extension of the heating time. The best protection of milk thistle seed oil from oxidative deterioration is achieved by a combination of the addition of a natural antioxidant and a synergist, i.e. rosemary extract and citric acid.

CONCLUSIONS

The tests carried out showed that it is possible to effectively protect cold-pressed rapeseed and milk thistle seed oil from oxidative spoilage by adding antioxidants during microwave heating, while sesame oil behaved differently. In a comparison of the three tested samples, the highest stability was found in sesame oil, while rapeseed and milk thistle seed oils are more susceptible to oxidative deterioration. By increasing the time of microwave heating at a constant power of the device, an increase in the peroxide value was recorded, and stabilization of the tested oils was achieved with the addition of antioxidants, except for sesame oil. By increasing the power of the microwave heating device, an increase in the peroxide value was recorded at a constant heating time. The stabilization of rapeseed and milk thistle seed oil with the addition of antioxidants was achieved at a power of 140 W, and as the power of the device was increased (280 W and 420 W) it was found that oxidative deterioration was accelerated by the addition of antioxidants.

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UTICAJ MIKROVALNOG ZAGRIJAVANJA I ANTIOKSIDANASA NA OKSIDATIVNU STABILNOST HLADNO PREŠANIH BILJNIH ULJA

Sažetak

Oksidacija lipida je značajan problem u proizvodnji, preradi i korištenju jestivih ulja, što uzrokuje značajne promjene u hemijskim, senzornim i nutritivnim svojstvima. Stoga, cilj ovog istraživanja bio je ispitati uticaj mikrovalnog zagrijavanja i antioksidanasa, prirodnih i sintetskih, na oksidativnu stabilnost i kvalitet hladno prešanih biljnih ulja (ulje repice, susamovo ulje i ulje sikavice). Od prirodnih antioksidanasa korišteni su eterična ulja kima i komorača, a od sintetskih propil galat (PG) i butilhidroksitoluol (BHT). Ispitivanje je provedeno određivanjem vrijednosti peroskidnog broja kod ispitivanih uzoraka djelovanjem različitih snaga mikrovalnog zagrijavanja pri istom vremenu uzorkovanja, kao i kod jedne snage mikrovalnog zagrijavanja pri uzorkovanju svakih 5 minuta do maksimalne od 25 minuta. Produžavanjem vremena mikrovalnog zagrijavanjem pri konstantnog snazi (140 W) vrijednosti peroksidnog broja su rasle kod svih ispitivanih uzoraka bez i sa dodatkom antioksidanasa. Najveće promjene vrijednosti peroksidnog broja zabilježene su kod ulja sikavice i repice, a najmanje kod susamovog ulja. Najbolju zaštitu od oksidativnog kvarenja pokazao je BHT kod svih ispitivanih uzoraka, kao i eterično ulje komorača kod repičinog ulja. Porastom snage uređaja tokom mikrovalnog zagrijavanja pri jednakom trajanju od 5 minuta, rasle su vrijednosti peroksidnog broja svih ispitivanih uzoraka bez i sa dodatkom antioksidanasa. Djelovanjem različitih snaga mikrovalnog zagrijavanja (140 W, 280 W i 420 W) najbolju zaštitu od oksidativnog kvarenja susamovog ulja pokazao je PG, ulja sikavice BHT i PG, te repičinog ulja eterično ulje komorača i BHT.

Ključne riječi: *oksidativna stabilnost, hladno prešana ulja, antioksidansi, mikrovalno zagrijavanje.*

MINERAL COMPOSITION OF MEAT FROM DIFFERENT SPECIES OF ANIMALS FROM SERBIA*

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Original scientific paper

Abstract

This study aimed to provide information on levels of magnesium (Mg), potassium (K), calcium (Ca), copper (Cu) and zinc (Zn) in 154 meat samples from six species of animals. Samples (chicken, turkey, pork, lamb, equine and beef meat) were gathered from different meat processing facilities in Serbia during 2023. The levels of macro- (Mg, K, Ca) and micro- (Cu, Zn) elements were determined by inductively coupled plasma mass spectrometry (ICP-MS). The highest significant mean content ($p < 0.05$) of Mg was measured in chicken meat. The highest values of K and Ca were found in pork and equine meat, respectively, and there were no significant differences between other meat species. Equine meat had the highest significant mean levels of Cu while the significantly highest Zn levels were determined in beef meat. The distribution of the elements in meat samples was examined by applying principal component analysis (PCA).

Keywords: *macro- elements; micro-elements; meat; species of animals*

INTRODUCTION

Being known as rich and suitable sources of nutrients, which are vital for the normal functioning of almost all biochemical and enzymatic processes in the body (Geiker *et al.,* 2021), meat and meat products represent a valuable part of human nutrition. For this reason, meat takes leading position among the food of animal origin (Nikolic, 2018). On the other hand, meat is probably controversial food today and is subject to the greatest number of ethical and moral, health, environmental and economic dilemmas (Font-i-Furnols & Guerrero, 2022).

Global meat production has been rising. According to Food and Agriculture Organization of the United Nations (FAO, 2023) 357 million tonnes of meat were produced in 2021, 53% more than in 2000, with chicken meat representing more than

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half the increase. According to the EFSA (European Food Safety Authority) (https://www.efsa.europa.eu/en/data-report/food-consumption-data) Comprehensive European Food Consumption Database, the average amount (kg) of fresh and frozen meat, consumed per capita per year, for Serbian population is following: beef – 8.78, pork - 14.98, poultry - 18.61, turkey – 0.18 and ovine – 0.82. With respect to the significant meat consumption pattern in Serbia, modern consumers have shown an increasing interest in meat quality and safety, especially in relation to their health.

Nowadays, authentic scientific information on the content of macro- and micro-elements in meat from different animal species from Serbia are limited (Nikolic *et al.,* 2015; Djinovic-Stojanovic *et al.*, 2017; Nikolic *et al.,* 2017). Levels of these elements in meat vary according to geographical origin, sex, animal category, their breeding and production system etc. (Nikolic, 2018; Dehelean *et al.*, 2023; Hoffman *et al.*, 2024). Therefore, this study aimed to comparatively examine meats in terms of mineral composition originating from turkey, chicken, pork, beef, lamb, and equine consumed in Serbia. Moreover, such data could be useful for future studies on the total dietary intake of these elements by the Serbian population. Principal component analysis (PCA) was used to assess the effect of species of animals on mineral composition of muscle.

MATERIALS AND METHODS

Sample collection
In total, 154 red meat samples (thigh muscles) from different species of animals (19 chicken, 8 turkey, 73 pork, 10 lamb, 10 equine and 34 beef meat) were collected in different meat processing facilities in Serbia during 2023. After the collection had been carried out, the samples were homogenized, labeled and stored in polyethylene bags and frozen at -18 °C prior to analysis.

Sample preparation and reagents
Frozen meat samples were thawed at 4 °C and homogenized, then approximately 0.5 g (wet weight) of sample was mineralized by adding 5 mL of nitric acid (67-70%, TraceMetal grade, Fisher Chemical, Belgium) and 5 mL deionized water, purity of 0.067 µS/cm, produced by a Purelab DV35 water purification system (ELGA, Buckinghamshire, UK). Microwave assisted digestion was performed in a MARS 6 iWawe Microwave Digestion System (CEM Technology, USA). After cooling at room temperature, the digests were quantitatively transferred into polypropylene volumetric flasks and diluted to 100 mL with deionized water.

Analysis of the following five elements, Mg, K, Ca, Cu and Zn, was performed by inductively coupled plasma mass spectrometry (ICP-MS) (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). The most abundant isotopes were used for quantification. Operating conditions of the ICP-MS system were: RF power (1550 W); cooling gas flow (14 L min^{-1}); nebulizer flow (1 L min^{-1}); collision gas flow (1 mL min^{-1}); operating mode (Kinetic Energy Discrimination); dwell time (10 ms).

Standards

Standard stock solutions of each element (Mg, K, Ca, Cu and Zn) were obtained from CPA Chem (Stara Zagora, Bulgaria). The purity of the starting material in standards was 99.999% for each element. For quantitative analysis of the samples, a five-point calibration curve (including zero) was constructed for the ^{24}Mg, ^{39}K, ^{44}Ca, ^{63}Cu and ^{66}Zn isotopes.

Statistical analysis

Statistical analysis of experimental data was performed using software Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA). For testing the differences in elements levels between different meat and between total group results one-way analysis of variance (ANOVA) and Tukey's HSD were applied. Statistically significant differences were expressed at the probability level of 0.05. PCA was used to group the observed samples and to discover any possible correlations among the element levels.

RESULTS AND DISCUSSION

The contents of five elements (Mg, K, Ca, Cu and Zn) in chicken, turkey, pork, lamb, equine and beef meat, expressed in terms of mean and standard deviation (SD) are presented in Table 1.

Table 1. Levels (mg/kg) of five selected elements (Mg, K, Ca, Cu and Zn) in meat from six animal species

Animal species	Element levels (mg/kg)				
	Mg	K	Ca	Cu	Zn
Beef, n=34					
Mean	218.76 [a]	3913.62 [a]	44.00 [a]	0.70 [b]	47.43 [d]
SD	34.32	506.69	18.89	0.30	16.20
Equine, n=10					
Mean	230.78 [a, b]	3605.53 [a]	62.51 [a]	1.37 [c]	33.12 [c]
SD	43.06	498.54	19.19	0.53	14.47
Lamb, n=10					
Mean	235.52 [a, b]	3720.47 [a]	38.53 [a]	0.77 [b]	29.95 [b, c]
SD	41.23	413.53	25.00	0.23	6.99
Pork, n=73					
Mean	255.55 [a, b]	4120.41 [a]	51.56 [a]	0.55 [a, b]	20.60 [a, b]
SD	46.12	471.33	27.98	0.27	9.34
Turkey, n=8					
Mean	269.59 [b, c]	3955.08 [a]	56.76 [a]	0.49 [a, b]	24.01 [b, c]
SD	39.15	428.40	19.86	0.26	14.75
Chicken, n=19					
Mean	307.15 [c]	3820.04 [a]	51.67 [a]	0.30 [a]	11.16 [a]

SD	49.05	495.24	13.65	0.13	4.47

[a–d] Values within the same column with different superscripts are significantly different (p < 0.05).

The mean levels (mg/kg) of elements were in the range of 218.76-307.15 (Mg), 3605.53-4120.41 (K), 38.53-62.51 (Ca), 0.30-1.37 (Cu) and 11.16-47.43 (Zn). Some significant differences in the levels of Mg, Cu and Zn (p<0.05) were found between meat from different animal species, while K and Ca levels were not significantly different.

The highest mean level of Mg was determined in chicken meat (307.15 mg/kg). It was significantly higher than Mg mean levels measured in all other analysed meat while the mean Mg level in turkey meat was only significantly higher than the Mg level in beef meat. The mean Mg levels in the current study were in line with the data reported by the Frida Food Data (2024).

The highest mean levels of K (4120.41 mg/kg) and Ca (62.51 mg/kg) were measured in pork and equine meat, respectively, but there were no significant differences between meat species. Lower levels of K were found by Bilandzic et al. (2021) in beef and pork than in this study (Table 1). The mean K level in chicken meat was close to the data reported by Bilandzic *et al*. (2021) while the data from USDA Food database Central (2024) showed lower concentration of K in beef, turkey and chicken meat than in this study. The mean Ca levels in all analysed meat species (Table 1.) were lower while the mean K levels were higher than those published by Frida Food Data (2024).

The mean level of Cu in equine meat (1.37 mg/kg) was significantly higher than Cu mean level measured in all other analysed meat. Also, the mean levels of Cu in lamb and beef meat were significantly higher than measured Cu mean level in chicken meat. The mean Cu levels in meat from six animal species were similar to concentrations reported by Lombardi-Boccia *et al*. (2005), ((mg/kg): Beef: 0.3-0.9; lamb: 1.0; horse: 1.2; pork: 0.4-0.7; chicken: 0.4-1.1; turkey: 0.3-1.2). Considerable higher levels of Cu in sheep and horse (8.53 and 8.45 mg/kg) were found by Bilandzic et al. (2010).

The mean level of Zn in beef meat (47.43 mg/kg) was significantly higher than Zn mean level measured in all other analysed meat and was in line with the data for raw beef meat (39.4-47.5 mg/kg) from Italian markets (Lombardi-Boccia *et al*., 2005). The obtained mean levels of Zn in equine, lamb and turkey meats were significantly higher than Zn mean level measured in chicken meat (11.16 mg/kg), which was similar with those found in our previously investigation (chicken cuts – drumstick: 14.1 mg/kg) (Djinovic-Stojanovic *et al*., 2017).

Principal component analysis (PCA)

PCA was applied to the correlation matrix, which included the five parameters for the meat from six animal species (Hammer *et al*., 2001). PCA was applied to group the observed the possible correlations among the measured Mg, K, Ca, Cu and Zn levels and the animal species (chicken, turkey, pork, lamp, equine and beef meat) (Figure 1). The first two components (PC1 and PC2) resulting from the examination of the levels

of micro- and macro-elements in meat from different animal species accounted for 82.48% of the total variance (PC1 53.37%, PC2 29.11%). In the case of PC1, the levels of Cu and Zn (significant positive correlations) as well as the Mg level (significant negative correlation) contributed the most to the variability of the meat samples. In the case of PC2, significant positive correlation was established for Ca level, while a significant negative correlation was established for the K level. For the third principal component (PC3), the level of K achieved a strong positive correlation, while the Mg level produced a strong negative correlation. For the fourth principal component (PC4), the level of Zn achieved strong positive correlations, while the Cu level produced a strong negative correlation. Figure 1 shows the Cu and Zn levels, as well as the Mg and K levels were highly positively correlated. However, the Mg and Zn levels, as well as the Cu and K levels were highly negatively correlated.
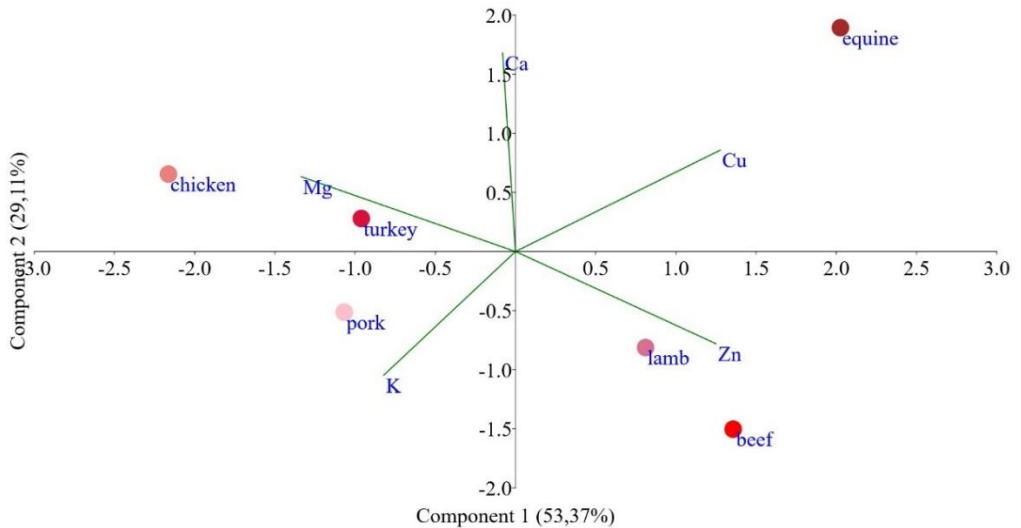
Figure 1. Bi-plot graphic of PCA of Mg, K, Ca, Cu and Zn levels in meat from different animal species.

The influence of different parameters, that described the examined meat samples, can be evaluated from Figure 1. Examined meat from different animal species are located on different sides of the graphic. Equine meat, in which the highest Ca and Cu levels were observed, was located on the upper right side of the graphic. The chicken and turkey meat were on the opposite side of the graphic (upper left), in which the highest Mg levels were observed in comparison with other meat. Chicken meat was located the furthest on that side, since chicken meat contained higher Mg levels than turkey meat. The pork meat samples, in which the highest K levels were observed, were located on the lower left side of the graphic. The lamb and beef meat samples were on the opposite side of the graphic (lower right) with regard to their high Zn levels. Beef meat was

located the furthest on that side, since beef meat contained highest Zn levels than other analysed meats.

CONCLUSIONS

Levels of Mg, K, Ca, Cu and Zn in chicken, turkey, pork, lamp, equine and beef meat were determined and estimated by comparing with levels found in similar meat samples from literature data. There is a variability in elements content in meat among studies. Our findings suggest some significant differences in Mg, Cu and Zn levels among meat from different animal species. The significantly highest Mg, Cu and Zn levels were determined in chicken, equine and beef meat, respectively. The highest K and Ca levels were determined in pork and equine meat, respectively. Considering the importance of meat as a source of needed minerals, our results can be useful for future studies on total dietary intake of human population. Having in mind the permanent breeding improvement of animals, further control of meat mineral composition is recommended.

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MINERALNI SASTAV MESA OD RAZLIČITIH VRSTA ŽIVOTINJA U SRBIJI

U ovom radu određen je sadržaj magnezijuma (Mg), kalijuma (K), kalcijuma (Ca), bakra (Cu) i cinka (Zn) u 154 uzoraka mesa koje potiču od šest vrsta životinja. Uzorci mesa (piletina, ćuretina, svinjetina, jagnjetina, junetina i konjsko meso) su uzeti iz različitih postrojenja za preradu mesa u Srbiji tokom 2023. godine. Sadržaji makro- (Mg, K, Ca) i mikro- (Cu, Zn) elemenata određeni su primenom induktivno kuplovane plazme sa masenom spektrometrijom (inductively coupled plasma mass spectrometry, ICP-MS). Najveći, statistički značajan ($p < 0.05$), sadržaj Mg utvrđen je u uzorcima pilećeg mesa. Svinjsko meso imalo je najveći sadržaj K, a konjsko meso najveći sadržaj Ca, ali se ti sadržaji nisu statistički razlikovali u odnosu na sadržaje tih elemenata u drugim analiziranim vrstama mesa. Statistički značajne razlike utvrđene su između sadržaja Cu, kao i između sadržaja Zn u analiziranim vrstama mesa. Konjsko meso je imalo najveći sadržaj Cu dok je u uzorcima junetine utvrđen najveći sadržaj Zn. Distribucija elemenata u uzorcima mesa analizirana je primenom PCA analize (principal component analysis, PCA).

Ključne riječi: *makroelementi, mikroelementi, meso, vrsta životinje*

IMPACT OF LOCALITY ON THE SENSORY PROFILE AND PHYSICOCHEMICAL CHARACTERISTICS OF KEFIR FROM BOSNIA AND HERZEGOVINA*

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Original scientific paper

Abstract

Kefir is a fermented milk beverage originating from the Caucasus region. When compared to other fermented dairy products, kefir is unique due to the complex microbial flora in kefir grains, resulting in a combined fermentation process of the milk. Kefir grains are primarily composed of lactic acid bacteria, yeasts, and acetic acid bacteria. The microbial composition of kefir grains largely determines the formation of kefir's qualitative and technological properties, such as the physicochemical, biochemical, microbiological, and sensory characteristics of the ferment, as well as the growth rate of kefir grains (biomass).

The production area can influence the qualitative parameters. The aim of this study was to determine the influence of kefir grain origin on kefir quality. Kefir grains were collected from different regions of Bosnia and Herzegovina.

The results of the analysis of kefir produced from kefir grains from different locations showed a biomass increase above 5%, with pH values ranging from 4.1 to 4.6. The average sensory score of the analyzed kefirs (18.28) classified the kefir as extra class. The highest-rated samples were characterized by the following attributes: milky-sour, sharp, pleasant, with the typical flavor of kefir.

Keywords: *kefir, kefir grain, microbial composition, sensory analysis*

INTRODUCTION

Kefir is a carbonated fermented milk beverage similar to yogurt, with its earliest production linked to the nomadic Ossetian and Karbadinian tribes in the Caucasus region (Samaržija, 2015). The word 'kefir' is of Turkish origin, coming from 'keyif,' which means joy or satisfaction. However, kefir grains are often also called 'Grains of the Prophet,' as it is believed that the Prophet Muhammad entrusted kefir grains to the people in the mountains of the northern Caucasus (Lopitz-Otsoa *et al*., 2006; Gaware *et al*., 2011; Seifi 2016).

Traditionally, kefir was made using kefir grains to ferment the milk, but today industrial starter cultures are primarily used. Kefir grains, which act as a natural starter culture, and can range from 0.2 to 3.0 cm in size. It is white to yellow in color, with an irregular round or elongated shape similar to cauliflower. It consists of proteins, a mixture of lactic acid bacteria, acetic acid bacteria, and yeasts bound together in a biofilm formed by a polysaccharide matrix (Loretan *et al.*, 2003; Shah, 2014; Dong *et al*., 2018).

In kefir production, cow's milk is most commonly used as the medium, but sheep's, goat's, or even buffalo's milk can also be used (Cais-Sokolińska *et al*., 2015). The symbiotic interaction is seen in the exchange of bioproducts, which act as energy sources or growth and survival factors for microbial species within the kefir grain. This results in the creation of a product with specific sensory, nutritional, and functional properties. The ratio and number of individual microbial species within the grain largely depend on its origin and storage conditions.

The first written records of kefir production and consumption in Bosnia and Herzegovina are linked to the Milkos dairy in Sarajevo, which operated as part of the UPI conglomerate. The technologists from this dairy brought kefir grains to Bosnia and Herzegovina during professional training in the USSR in 1960. For several years, kefir technology was refined under industrial conditions, and this method is still used in production today (Parijez, 1973). Over 65 years of kefir production, kefir grains have become popularized and widely adopted for home production.

Figure 1. Macroscopic view of kefir grain BH 22
(Source: Sakić-Dizdarević, 2021)

It has been found that the characteristics of kefir grains from Bosnia and Herzegovina are not sufficiently researched. The aim of this study was to examine the qualitative characteristics of kefir produced using kefir grains from different locations in Bosnia and Herzegovina.

MATERIALS AND METHODS

To achieve the set objective, a three-month activation of kefir grains was conducted, during which their technological properties (titratable acidity, pH, and biomass) were examined, and the fermentation products were sensory evaluated.

For the experimental production of kefir, 26 different kefir grains from the collection of the Faculty of Agriculture and Food Sciences, University of Sarajevo, were used. The kefir grains were grouped into two categories: the SA group consisted of 13 kefir grains from the Sarajevo area, and the BH group consisted of 13 kefir grains from various regions of Bosnia and Herzegovina. During the 90-day reactivation period, kefir was produced from each individual grain (26 samples), with six pH measurements taken over this period, while the biomass (mass of kefir grains) was measured weekly. The sensory evaluation was conducted after 90 days.

The kefir grain, in a 1:10 ratio, was inoculated into sterile milk (ZZ with 2.8% milk fat) and incubated for 24 hours. The resulting mother culture (5%) was used for inoculation, and inoculated into fresh milk with gentle stirring and fermented for 20 hours. After fermentation, the ripening process occurred at a temperature of 8°C for 24 hours.

Determination of kefir pH value

The pH value was measured using a pH meter (Methrom 632, Switzerland) with an electrode for liquid media (WTW SenTix 41, Germany), which was previously calibrated with standard buffers at pH=4 and pH=7.

Determination of kefir grain biomass

The increase in kefir grain biomass was measured gravimetrically. After 24 hours of fermenting the grains in milk at a ratio of 1:10, the grains were separated from the fermented medium using a strainer, rinsed with water, and weighed on an analytical scale. The mass was recorded daily, and biomass increase was calculated according to the formula (Guzel-Seydim *et al*., 2021).

Biomass increase (%) = $\frac{W2-W1}{W1}$ X 100

In the formula, W1 represents the initial mass of the kefir grain, and W2 is the mass measured 24 hours after fermentation.

Sensory analysis of kefir

The sensory analysis of individual kefir samples produced using 26 kefir grains was conducted by a panel of 5 trained evaluators. A scoring system with a maximum of 20 points was used, assessing the sensory characteristics of appearance (maximum 1 point), color (maximum 1 point), consistency (maximum 4 points), odour (maximum 2 points), and taste (maximum 12 points). In addition, the kefir was evaluated qualitatively, with descriptions of its individual characteristics.

RESULTS AND DISUSSION

Determination of kefir pH value

Figure 2. shows the average pH values of kefir samples from the SA group over six measurements during the 90-day kefir grain reactivation period.
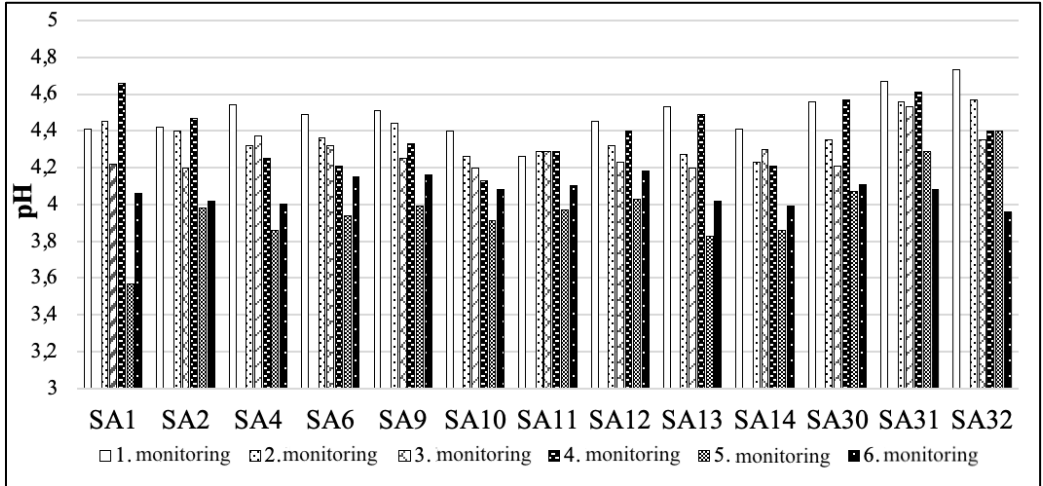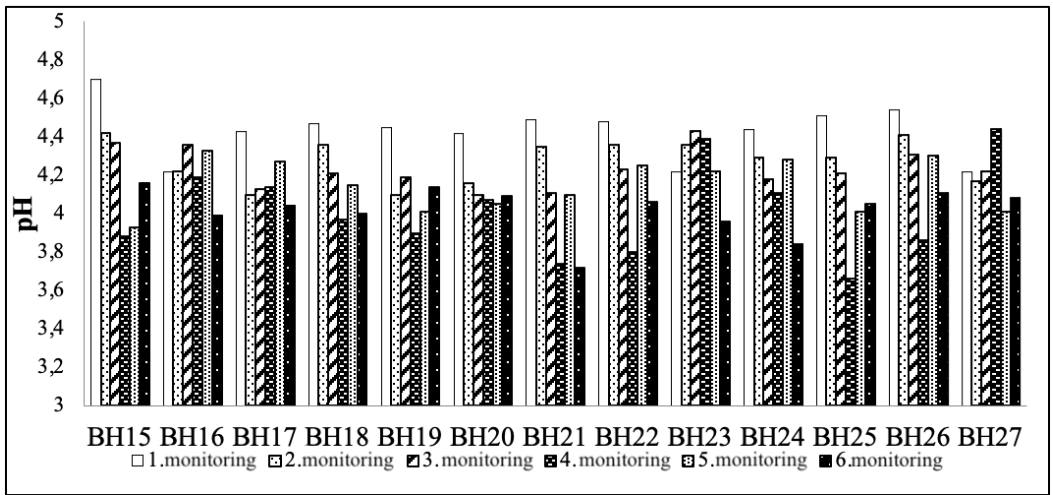

Figure 2. Average pH values of kefir samples from the SA group

The pH values of kefir samples produced from SA group grains ranged from 4.10 to 4.50, with the lowest average value found in sample SA10 and the highest in SA31. A lower pH value in kefir grains indicates an increased presence of lactic acid bacteria. Sulmiyati *et al.* (2019) state that lactic acid bacteria convert lactose into lactic acid through metabolic activity, resulting in a reduction of the medium's pH value. Figure 3 shows the average pH values of kefir samples from the BH group over six measurements during the 90-day kefir grain reactivation period.

Figure 3. Average pH values of kefir samples from the BH group

The pH values of kefir samples produced from BH group grains ranged from 4.00 to 4.30. Most kefir samples had an average pH value around 4.20, with sample BH21 having the lowest (4.09) and samples BH23 and BH26 the highest (4.26). A lower pH value in kefir is desirable for certain micronutrients. For example, a lower pH in fermented milk enhances calcium absorption, as this mineral is present in ionic form in such a medium (Hui *et al*., 2007).

Based on the analysis results, it can be concluded that a three-month reactivation period was necessary for the grains to fully activate, thereby enriching the grain microbiota, primarily with lactic acid bacteria.

Determination of kefir grain biomass
The average biomass growth values of the 26 kefir grains (SA and BH) monitored over the three-month period are shown in figure 4. The highest average biomass increase was observed in sample BH16 (13.87%), while the lowest was in SA4 (1.40%). According to Libudzisz & Piatkiewicz (1990), the average daily biomass increase for kefir grains ranges from 5 to 7%.
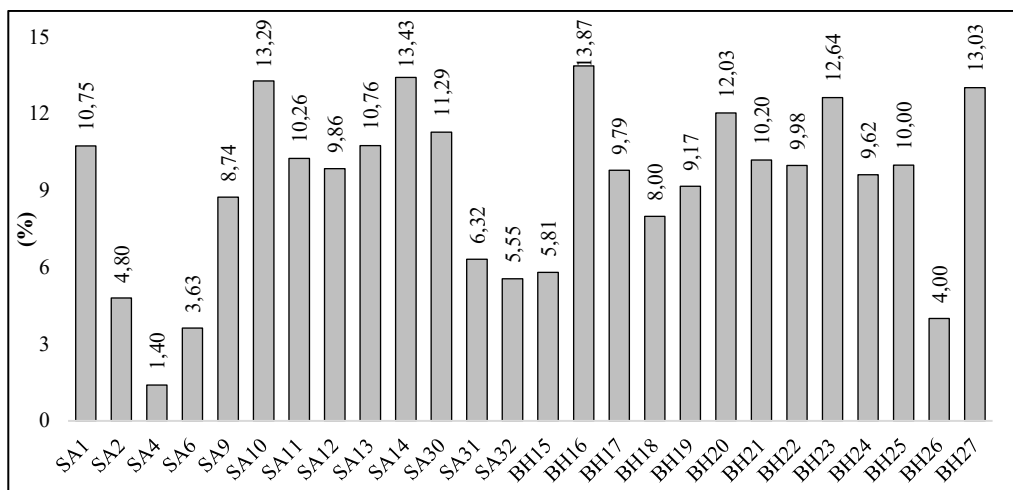
Figure 4. Average biomass growth values of tested kefir grains from groups SA and BH

The biomass increase is driven by the growth of microorganisms that use milk or other media as a food source. Additionally, frequent inoculation leads to the synthesis of kefir grain components. In contrast to these findings, the kefir grains SA2, SA4, and SA6 showed an average biomass increase below 5% (figure 4). This result may be due to damage to the grains before the reactivation process, which can impoverish or completely degrade the microbiota.

The biomass of kefir grains, as an essential technological parameter, showed a consistent increase depending on the intensity of the fermentation process repetitions, while simultaneously establishing a stable ratio of the main microorganisms in the grain, namely lactic acid bacteria and yeasts (Gorek & Tramsek, 2007). This characteristic is particularly significant in industrial kefir production, as it enables continuous production and accelerates the process of obtaining the final product (Rosa *et al*., 2017). Biomass increase is also an important parameter for the potential use of kefir grains for other applications, such as polysaccharide production (Gorsek & Tramsek, 2008). A certain number of analyzed kefir grains from Bosnia and Herzegovina demonstrated technological potential (biomass production).

The analysis of kefir grain growth dynamics (biomass) based on the locations BH and SA is shown in figure 5.
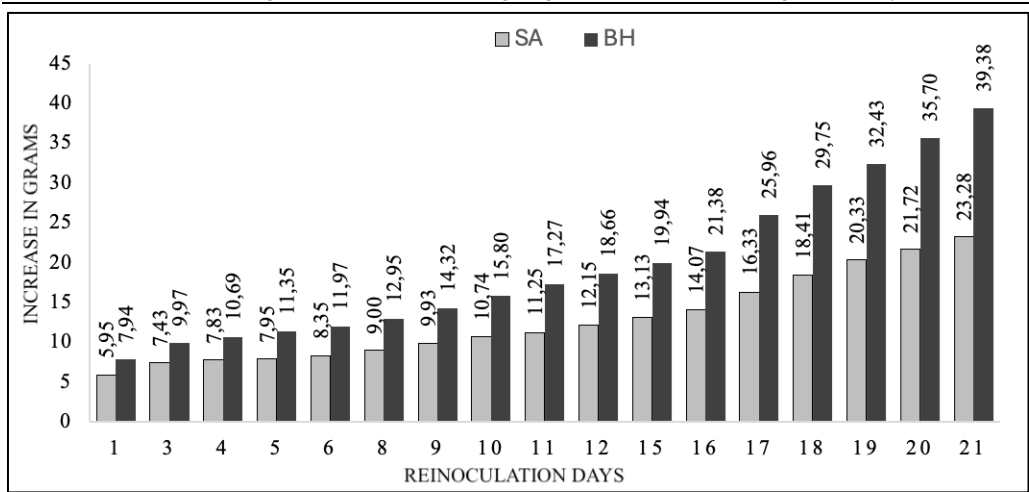
Figure 5. Biomass dynamics of kefir grains from groups SA and BH over a 90-day period

The BH kefir grains showed more efficient biomass increase compared to the SA group, except for grain BH26, which had a biomass increase below 5%.

Sensory analysis of kefir grains
The results of the sensory analysis of kefir produced from kefir grains BH and SA are shown in graph 5.
The highest overall scores were observed in samples SA10 (19.50), BH17/BH26 (19.50), with characteristics such as pleasant, clean, milky-sour, and kefir-like. The main components contributing to the pleasant aroma of kefir include alcohols (ethanol), ketones (acetoin and 2-3 butanedione), esters (ethyl acetate), aldehydes (acetaldehyde), and acids (lactic acid) (Farag *et al*., 2020). Lactic acid bacteria play a crucial role in the creation of aromatic components in kefir, while yeast activity is also significant for alcohol production. In their study, Walsh *et al*. (2016) highlight *Lactobacillus kefiranofaciens* among the notable lactic acid bacteria that impact the pleasant aroma. Additionally, other bacteria significant for aroma creation include *Lactobacillus acidophilus, Levilactobacillus brevis, Lactobacillus helveticus, Lacticaseibacillus casei, Lactococcus cremoris, Lactococcus lactis, and Leuconostoc mesenteroides* (Farag *et al*., 2020).
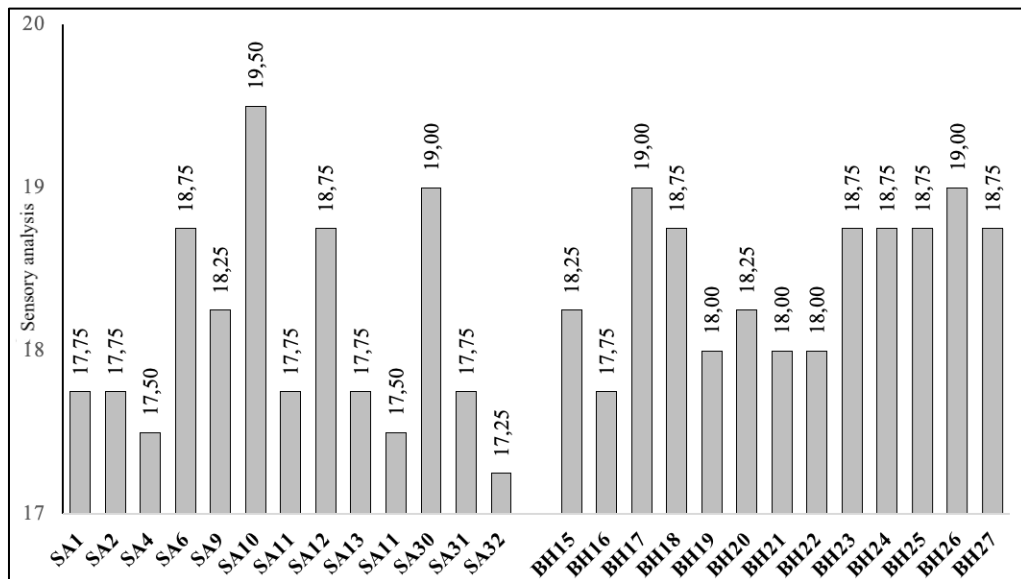
Figure 6. Average sensory score of kefir produced from 26 kefir grains

The samples with the lowest scores were SA32 (17.25) and BH16 (17.75), with the following characteristics: intensely sour, unusual yeast and mold flavor, bitter, with an atypical taste. The pronounced sour taste may be attributed to acetic acid, which is produced as a result of the metabolic activity of heterofermentative lactic acid bacteria or acetic bacteria (Magalhães *et al*., 2011; Irigoyen *et al*., 2012). Some acids that may negatively affect the overall aroma of kefir include octanoic and n-decanoic acids, which contribute to a soapy, pungent, waxy, and rancid aroma (Farag *et al*., 2020). Diacetyl is one component that, if present in excessively high concentrations, can lead to the formation of an atypical, sharp odor in kefir (Ott *et al*., 1999).

CONCLUSIONS

Kefir is considered a functional product due to its diverse microbial flora. It was found that kefir produced from SA kefir grains had a slightly higher pH compared to BH. The highest average biomass increase was recorded in samples SA14 and BH16, indicating technological potential. BH kefir samples had a higher overall sensory score (between 18 and 19) compared to SA samples. A significant number of tested kefir grains showed good technological potential, suggesting that future efforts should focus on standardizing this product and studying its functional characteristics. Most kefir grains were collected from the Sarajevo Canton area, indicating a long tradition of use and production of this product.

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UTJECAJ LOKALITETA NA SENZORNI PROFIL I FIZIČKO-HEMIJSKE OSOBINE KEFIRA SA PODRUČJA BOSNE I HERCEGOVINE

Sažetak

Kefir je fermentirani mliječni napitak koji potiče sa područja Kavkaza. U odnosu na ostale fermentirane mliječne proizvode razlikuje se po kompleksnoj mikrobnoj flori kefirnog zrna koja dovode do kombinovane fermentacije mlijeka. Sastav kefirnog zrna čine dominantno bakterija mliječne kiseline, kvasaci i sirćetne bakterija. Mikrobni sastav kefirnog zrna u znatnoj mjeri određuje formiranje kvalitativnih i tehnološki osobina kefira kao što su fizičko-hemijske, biohemijskih, mikrobiološke i senzorne osobine fermenta, kao i brzinu rasta kefirnih zrna (biomasa).

Područje proizvodnje može utjecati na kvalitativne parametre. Cilj ovog rada bio je da se odredi utjecaj lokaliteta kefirnog zrna na kvalitetu kefira. Kefirna zrna prikupljena su sa različitih područja Bosne i Hercegovine.

Rezultati analiza kefira proizvedenog od kefirnih zrna sa različitih lokaliteta pokazali su porast biomase iznad 5%, pH vrijednost varirala je u intervalu od 4,1 do 4,6. Prosječna senzorna ocjena analiziranih kefira (18,28) kategorisala je kefir u ekstra klasu. Najbolje ocijenjene uzorke karakterisale su sljedeće osobine: mliječno-kiseo, rezak, prijatan sa tipičanim okusom po kefiru.

Ključne riječi: *kefir, kefirno zrno, mikrobiološki sastav, senzorna analiza*

VGEJ PQNQI [.'RJ [UEEQ/EJ GO KECN'CPF'UGPUQT["
EJ CTCEVGTKUVKEU'QH'KPFK GPQWUI QCV'EJ GGUG'NKFGT , "

\ nvcvp'Uctk [1].'Pgvto kpc'J cucpdc–k [1].'Ucpjc'Qtu gvki fiwrlgvk [1].'Vctkm'Fk\ fctagvk [1],
O wpgvcvg'Dgi k [1], Amila Oras[1].'O kjorlwu'Dctc [2], Slobodan Rgtqvk [3].'Uxkjgnvcpc'Ucnk -
Fk\ fctagvk [1].'Clgmucpdtra'Octvkpqxk [4].'Tadkvqjg'O krilcpk [3]

Cduvtcev'

The technology and quality of indigenous Lider goat cheese are examined in this research. It is a hard, full-fat cheese produced from pasteurized goat's milk. Goat's milk is obtained from goats that are raised in the mountainous area of Pkmk .'O qpvgpgi tq0' The aim of the work is to present the Lider goat cheese technology as well as to examine the physico-chemical and sensory characteristics of this cheese. Cheese samples were tested after ripening for 21 days (three weeks). The average chemical composition of the cheese was (g/100 g): dry matter 61.85; fat 30.17; proteins 27.59; salt 3.96 and lactic acid 0.185. The average content of fat on dry basis (FDM), moisture on fat-free basis (MFFB) and salt in moisture (S/M) were 48.94%, 54.42% and 10.89%. Average pH was 5.25, water activity 0.895, while hardness and adhesiveness were 13.844N and -0.213N. The average rind thickness of 2.57 mm was established, while the average cross-sectional height and cross-sectional area of the cheese were 33.33 mm and 9.57 cm^2/100g. The average density of the cheese was 1.338 g/ml. The total sensory evaluation averaged 17.34, which places this cheese in the high first class. One-way analysis of variance (p<0.05) revealed that there is a statistically significant influence of the production day on all physico-chemical and sensory parameters of the cheese, except for the external appearance and color.

Key words: *ej ggug. 'vgej pqnqi {. 's wcnkv{.'ej go kecn'ugpuqt{'*

KPVTQFWEVKQP "

The goat has a great ability to produce milk, so during one lactation period it gives 10 to 30 times more milk than its weight. She needs more food per kilogram of her own weight, but for that weight she gives more milk than cows and sheep *Uctk .'3; ; 9=Uctk "

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[1] Uaˉ}^!Áˉ!^•^}cˆåˌaˍˌcbˍ@-Á˲H ˳ĲQˌcˆˌ}ˌæaˌ}aˌÛ&aˉ}ˌãæˌĚ˛¢]^!ˌoˆÕ[}-^¦^}&^Áˉ-ÁⱭˌ¦ˌˌˌˉ¦ˌcˉˉ^Áæ}ˌåÁſQ[ˌåÁʠⱱˈ•dˆˉĚⱭU&ʠˌàˉ^¦ÁˉĚⱤⱤÊ
G€GⱯĚⱯÚˌ˛æ˛æ˛ɓ^ç[ˌĚⱭ[•]ˉㆄㆄˉ}ˌåÁⱤ^¦:-^*[çㆀˌæ
[2] Wˌˌㆃç^!•ㆃˆÁˉ-ÁÚˌ˛æ˛æ˛ɓ^ç[ˌĚⱭⱧ&˛¦cˉÁˉ-ÁⱭ[!ˌㆀˉ¦cˉ^Áæ}ˌåÁſQ[ˌåÁⱱ&aˉ}&^•Ɑ̂Ⱡ€€€ÁÚˌ˛æ˛æ˛ɓ^ç[ˌĚⱭ[•]ˉㆄㆄˉ}ˌåÁⱤ^¦:-^*[çㆀˌæÁ
[3] Wˌˌㆃç^!•ㆃˆÁˉ-ÁⱭ^\[*!æㆄˆ‖ſ₣Ⱡ€ⱠᴢZˉ{ ˙ˇ}Ɑ̂Úˉ^!ˌàㆃæ
[4] ᵻ̂Ɑæˉㆌㆌ˝farm "Miljanić", 81400 Nikšić, Montenegro
[5] Wˌˌㆃç^!•ㆃˆÁˉ-ÁⱭ[}ㆄæᴢÕ[!ㆀ˛æㆄㆀ⓪æ˛ɓ¦cˉÁˉ-ÁⱭ[ˌåÁⱱ^&Ⱪ[ˉ[*ˆĚⱭ[ˌåÁⱱæˆĉˉÁæ}ˌåÁⱱ&[ˉ[*ˆĚⱭ[ˌ}æÕÕ[!ㆀ˛æ˛æᴀⱭ̂Ⱡ€€€ÁÚ[ˌå*[!ㆀ˛æㆀⱭ
T{}cˉ}^*¦[
Ô[!ˉ•]!}ㆃˉ^}˛&^K̂ÁĚˈæ̂ㆀZO]]-Ě̂}•æᵻⱦæ

gv'cn, 2005). Kp´'\vj g'r tqf wevkqp´'qh'ht guj '¡ qcv'u´ej ggug.'¡ qcv'o]kn\¡ ej pqrqj kecm{¡ 'f qgup¿v¡ fall behind cow's milk, except for the separation of small fat droplets in the whey, slower coagulation and softer rennet coagulated gel. However, this property contributes to the softness and characteristic properties of goat cheeses. Goat's milk casein with rennet enzyme gives a less solid curd than cow's and sheep's milk, and is better and easier to digest, which contributes to better digestibility (Dozet *gv'cn*, 2226="Uctk "*gv'cn*, 2005). Such production characteristics of the goat and favorable physico-chemical, nutritional and technological properties have influenced that goat's milk is predominantly processed into cheese today. Therefore, cheese is the basic product of goat's miln"'*Uctk " & Brenjo, 2022). About 140 types of goat cheese are produced in the world (Rubino *gv' cn*, 2004; Zervas & Tsiplakou, 2013). The processing of goat's milk into cheese has a very long tradition, and goat's cheese was, and still is, an important element in the population's diet. Goat cheeses have been known since ancient times and were an important element in the diet of the inhabitants (Dozet *gv'cn*, 2004). The origin of goat cheeses is Mesopotamia and their production spread especially in the Mediterranean countries (Greece, Turkey, Syria, Israel, Iraq and also Iran) (Kosikowski, 1986). The milk was probably originally processed into soft cheeses and then into hard mature cheeses (Park & Hanlein, 2010). In the households, the traditional method of production based on old, original, traditional recipes was preserved, from which different types of cwwqej yj qpqwu"¡ qcv'ej ggugu."ej ctcevgtkuvke"hqt"egtvckp"ctgcu."y gtg"etgcvgf "*Dqflocpk "*gv' cn*, 2002; 2018). Over time, the production of goat cheeses in a semi-industrial manner in smaller capacity plants had appeared and they became popular and sought after due to their quality. These cheeses have retained the characteristics of goat's milk with better hygienic production conditions required by modern dairying. One such cheese is the hard, full-fat Lider cheese, which is produced from pasteurized goat's milk at the family hcto "õO knlcpk ö"kp´'vj g'o qwpvckpqwu'ctgc'qh'P km-k "o wpkekr ckv{ ."O qpvgpgi tq0'Vj gtg"ku" no much information about this cheese in the literature except in the recent work r wbnkuj gf "d{"Dctc "*gv'cn* (2024). Therefore, the aim of this work is to present the technology, chemical composition, physical and sensory properties of this cheese.

O CVGTKCNU'CPF 'O GVJ QFU'

"

In the research, Lider goat cheese technology was monitored and cheese samples were taken from 3 different production days after 21 days of ripening, which represents the usual ripening period of this cheese. The chemical composition of Lider cheese (g/100g) was examined: dry matter by drying in oven at $102\pm2^{\circ}C$ (ISO 5534:2004), fat by Van Gulik method (ISO 3433:2008), proteins using Kjeldahl method (ISO 8968-1:2014), salt by the potentiometric method using the device *O M'"'KK'ej pqt\f g"Cpcn{|gt"; 48." Uj gty qqf* and lactic acid by titration (AOAC, 1995). By calculation were determined (%): fat on dry basis - FDB (Bylund, 1995), moisture on fat-free basis ó MFFB (CODEX ALLIMENTARIUS, 1978) and salt in moisture ó S/M (Scott, 1998; Guinee & Fox, 2004). Following physical properties of cheese were analyzed: pH (pH-meter *O gvtqj o " 854*), water activity - a_w (*Tqvtqpke*;´"texture (*VCŒV"Rnuu"Vgzwtg"Cpcn{ugt*;´ rind thickness (mm), cross-sectional height (mm) and cross-sectional area (cm^2/100g) as

well as density (g/ml) *Tc–gxk "gv'cr0'4239='Qtcu"("Qtw gxk "fiwdgxk ."4246+0'Vj g"
results for texture obtained by *VCO/Z"Rnuu'Vgzwtg'Cpcn{ugt* in this paper are expressed
in N (1N = 101.97g-force, Stable Micro Systems, UK). The sensory evaluation of the
cheese was carried out by an expert panel using a point scale of a maximum of 20 points.
The data were statistically processed. In order to examine influence of day-by-day
variations in milk composition and applied technology to cheese properties one-way
analysis of variance and Tukey post-hoc vguv'* ≤ 2.05) were done (Hammer gv'cr0' 2001).

TGUWNVU'CPF 'FKUEWUUKQP "

Vj g'hco kn{ 'hcto "\$M krcpk \$"y cu'hqwpfgf "kp'4234.'cpf 'vj g'r tqeguukpi 'qh'b kmlkpvq'ej ggug"
dgi cp'kp"42360'Fckt{ "ku'r ncegf "cv'rqecvkqp'I qtprjg'r qrlg."Pkm-k ."M qpvgpgi tq0'I qcv'
cheeses are the main product of this company, and among them Lider goat cheese is
certainly the most important one. Lider cheese is a hard, full-fat cheese produced from
pasteurized goat's milk obtained from goats that are raised in the mountainous area of
Pkm-k 0Nkf gt 'ej ggug'ejej pqnqi { 'ku'dcugf "qp'vj g'tuse'qh'r cuvgwtkzgf "tpumko o gf 'i qcv'o kk'
with the addition of selected bacterial cultures (Figures 1- 4). Ex-farm milk is cooled
and stored to temperatures from 3 to 4ºC. The milk is pasteurized at a temperature of
63°C for 30 minutes. After cooling to a temperature of 33°C, CaCl$_2$, lyophilized starter
culture (DELVO TEC, DX-33C DSL DSM Food Specialities consisted of mesophylic
lactic acid bacteria Ncevqeqeewu'rcevku'ssp0*rcevku'and "ssp0'etgo qtku. "Ncevqeqeewu'rcevku"
ssp0'rcevku'biovar0f kcegv{rcevku. "Ngwcqpquoqe "spp.) and rennet (šOczktgpõ."FUO "Hqqf "
Specialities, 1g to 100L of milk) are added. Upon coagulation of milk the curd is cut
and heated at a temperature of 36-38ºC. The curd is not subjected to pre-pressing, but
is immediately poured into the tub, where the molds are manually filled.

Pressing lasts for 12 hours (the cheese is turned over 4 times at the beginning, on average
every hour, and the load is 5 kg per tube). Brining is method of salting. The brine was
made from whey and consist of 16% salt in which the cheese is soaked for 24 hours.
The ripening of the cheeses takes place at a temperature of 11-12ºC and a humidity of
65-70% for 30 days but usually the cheese ripening is shorter, 21 days. The weight of
the cheese after pressing is 720-820g while after ripening it is sold with average weight
of 550g. Very often, depending of requirement of distribution, Lider cheese is vacuum
sealed (Figure 5) before ripening. In that case it has longer ripening, somewhat softer
consistency and can be clasified into category of semi-hard cheeses.

Ceeqtfkpi "vq'Dklgrlce'("Uctk "*4227+'j ctf 'i qcv'ej ggug'ctg'uo cmgt. "y kij 'fkco gvgt'33-
15 cm and height 3-5 cm. In Balkan countries goat cheeses appear usually in cattegories
of white brined, semi-hard or hard ones. Hard and semi-hard cheeses are usually small
and their weights are between 60 g and 1.5 kg (Dozet gv'cn, 2004). This is consistent
with results obtained in this work.

Figure 1. Filling of molds with curd

Figure 2. Pressing of curd

Figures 3. and 4. Brining and ripening of Lider cheese

Figure 5. Vacuum sealed Lider cheese

In this work chemical composition, physical and sensory properties of Lider cheese are tested. They are shown in following tables.

Table 1. Chemical composition of goat cheese Lider

Component (g/100g)	Production day			Xs	Min.	Max.	St. Dev.	CV (%)
	1	2	3					
Dry matter	60.73[a]	62.20[b]	62.64[c]	61.85	60.73	62.64	1.00	1.62
Fat	29.00[a]	30.50[b]	31.00[c]	30.17	29.00	31.00	1.04	3.45
FDB* (%)	47.99[a]	49.27[b]	49.56[c]	48.94	47.99	49.56	0.83	1.69
MFFB** (%)	55.21[c]	54.22[b]	53.82[a]	54.42	53.82	55.21	0.72	1.32
Proteins	27.15[a]	27.30[b]	28.33[c]	27.59	27.15	28.33	0.64	2.32
Salt	4.53[c]	4.35[b]	3.00[a]	3.96	3.00	4.53	0.84	21.21
S/M (%)***	12.14[b]	12.10[b]	8.43[a]	10.89	8.43	12.14	2.13	19.56
Lactic acid	0.132[a]	0.199[b]	0.226[c]	0.185	0.132	0.226	0.05	27.03

*a-c Different small letters in the columns show statistically significant differences in the values of the examined chemical parameters in the goat cheese samples depending on the production day; *FDB-fat on dry basis; **MFFB ó moisture on fat-free basis; ***S/M-salt in moisture

Content of dry matter, fat, FDB and MFFB were typical for hard, full-fat cheese. These xcn\/gu"ctg"uko knct"\q"\j qug"tgr qt\/gf "d{ "Dctc "*gv'cn* (2024) for Lider cheese after 15 and 30 days of ripening. Chemical composition for hard goat cheese from Balcan countries is following (%): dry matter 67.21; fat 32.94; FDB 48.78; proteins 29.51 and salt 3.35 (Dozet *gv'cn*0"3; ; 8=Dklgrlce"*ˈˈUctkˈ.ˈ4227+0"Eqpvgpv'qh'hce\/e"cekf "y cu'uwr r tkpuki n{"'rqy "

but corresponding to relatively high pH (Table 2). One-way analysis of variance revealed that there is a statistically significant influence of the experimental factor (production day) on all examined chemical parameters of goat cheese (p<0.05).

Table 2. Physical properties of goat cheese Lider

Property	Production day			Xs	Min.	Max.	St. Dev.	CV (%)
	1	2	3					
pH	5.50[a]	5.11[b]	5.15[b]	5.25	5.11	5.50	0.21	4.00
Water activity (a_W)	0.892[ab]	0.890[a]	0.902[b]	0.895	0.890	0.902	0.01	1.12
Hardness (N)	17.230[b]	17.870[c]	6.432[a]	13.844	6.432	17.870	6.43	46.45
Adhesiveness (N)	-0.153[b]	-0.345[a]	-0.142[c]	-0.213	-0.345	-0.142	0.11	51.64
Rind thickness (mm)	2.56[b]	2.72[c]	2.42[a]	2.57	2.42	2.72	0.15	5.84
Cross-sectional height of cheese (mm)	34.24[b]	30.85[a]	34.91[c]	33.33	30.85	34.91	2.18	6.54
Cross-sectional area of cheese (cm^2/100g)	9.81[c]	9.41[a]	9.50[b]	9.57	9.41	9.81	0.21	2.19
Density (g/ml)	1.277[a]	1.317[b]	1.421[c]	1.338	1.277	1.421	0.07	5.23

*a-c Different small letters in the columns show statistically significant differences in the values of the tested physical parameters in the goat cheese samples depending on the production day

In the literature there are no a lot of results on physical properties of cheese compared to chemical composition. In this work pH was at normal level for hard cheeses as well as for watter activity. T gi i "cpf "Dncpe"*3; 99+"tgr qtvgf "xcnwgu" for the water activity of different cheeses, from 0.886 and 0.892 for Bergkäse and Parmesan, to 0.995 for Quark. Average values of hardness and adhesiveness for Lider cheese were 13.844 resp. -0.213 N (expresssed in g-force 1411.67 resp. -21.72) and were in accordance with values tgr qtvgf "hqt"f kffgtgpv'v{ r gu"qh"ej ggugu0"Vj wu."Uctk "gv'cn (2024) established hardness (g) for hard and extra hard, semi-hard, mold ripened and white brined cheeses 2143.43, 1047.56, 1673.40 and 205.17 resp. In the same research, results for adhesiveness (g) were -30.69, -94.57, -540.40, and -26.24 resp. Density was typically high for hard cheeses and in accordance with values obtained for hard and extra hard cheeses (1.276 i]o n+'tgr qtvgf "d{ "Uctk "gv'cn (2024). One-factorial analysis of variance revealed that there is a statistically significant influence of the experimental factor (production day) on all examined physical parameters of goat cheese (p<0.05).

Rind thickness, cross-sectional height of cheese and cross-sectional area of cheese are physical parameters of high important in sensory evaluation and control of cheese technology. They give valuable data on cheese ripening conditions (length, humidity and temperature). Maybe more important, all of them, especially, number, shape and size of cheese spores (results not shown in paper but can be visible in Figure 5) play an ko r qtvcpv'tqng'kp'ugpuqt{ 'lwf i kpi "qh'ej ggug0'Cm'õdki ö'ej ggugu'kp'yj g'y qtf "j cxg'uvtqng judgement and clasification according to spores on cut and therefore their clasifying depends on it.

Figure 5. Cut of goat Lider cheese after 21 days of ripening

Sensory evaluation showed high quality of Lider cheese where cheeses were clasified in first class according to total average score. All of them had odour typical for goat milk, pronounced but moderate and pleasant. As shown on the figure 5 spores are equally distributed in cheese body and appropriate shape and size.

Table 3. Sensory evaluation of goat cheese Lider

Property	Max.	Production day			Xs	Min.	Max.	St. Dev.	CV (%)
		1	2	3					
External appearance	2	1.82[a]	1.73[a]	1.80[a]	1.78	1.73	1.82	0.05	2.81
Color	1	1.00[a]	1.00[a]	1.00[a]	1.00	1.00	1.00	0.00	0.00
Consis-tency	2	1.86[c]	1.62[a]	1.74[b]	1.74	1.62	1.86	0.12	6.90
Cut	3	2.54[b]	2.49[ab]	2.35[a]	2.46	2.35	2.54	0.10	4.06
Odor	2	1.68[a]	1.83[b]	1.94[b]	1.81	1.68	1.94	0.13	7.18
Taste	10	8.63[b]	8.35[a]	8.64[b]	8.54	8.35	8.64	0.16	1.87
Total	20	17.53[b]	17.02[a]	17.48[b]	17.34	17.02	17.53	0.28	1.61

One-way analysis of variance revealed that there is a statistically significant influence of the sample factor (production day) on all examined sensory parameters of goat cheese except for the evaluation of external appearance and color of goat cheese (p>0.05). This shows the influence of day-by-day variations in applied cheese technology which should be improved.

CONCLUSIONS

Goat Lider cheese is produced in a region of full quality Nikšić Montenegro from high quality goat milk obtained from pasture-fed goats. In terms of FDB, dry matter content and MFFB Lider cheese is a full-fat, hard cheese that is very close to semi-hard cheeses. pH, a_w, hardness and adhesiveness are common for hard and semi-hard types of cheese. According to the overall sensory evaluation, it is classified as high first class. Sensory properties, convenient small dimensions and well-known positive characteristics of goat's milk give it special characteristics and quality.

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TEHNOLOGIJA, FIZIČKO/J GO KLUMG'KUGP\ QTP G'MCT CMVGT KUVKMG " CWQJ VQP QI 'MQ\ KLGI 'UKT C'NKF GT "

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THE EFFECT OF STRESS ON EATING HABITS AND BODY COMPOSITION OF THE STUDENT POPULATION*

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Original scientific paper

Abstract

Obesity is emerging as a serious problem worldwide. One of the factors contributing to obesity is stress. It appears to be particularly important, as it leads to poor diet, lack of physical activity, lack of sleep. The transition from high school to a university can increase perceived stress levels, which affect eating behavior and metabolism. This change alone leads to a difference in eating habits compared to the general population, which tends to rely on meals that are quick and easy to access.There are two main components of the stress response: the autonomic nervous system (ANS), which includes the sympathetic and parasympathetic nervous systems and the HPA axis. The goal of research was to assess the impact of stress on eating habits and body composition of the student population. The respondents were 50 students from the Faculty of Agriculture and Food Sciences UNSA. The devices used for body composition measurements are BIA-ACC and PPG Stress Flow (BioTekna S.r.l., Marcon-Venice, Italy). Statistical analyses included student's T-test to determine differences between the observed parameters regarding the gender. The biggest differences with respect to gender can be observed in the parameters of the HPA axis index ($p < 0,000$), which is one of the indicators of stress proving higher stress among women (M = 3.8±0.4 vs. F= 2.4±0.7) and between BMI in men and women (p=0.2). Men had a higher BMI than women (M=24.4±3.2 vs. F=21.6±3.7) but both were in the normal weight category.

Keywords: *students*, *stress, BIA-ACC, PPG Stress Flow, body composition.*

INTRODUCTION

The beginning of university life represents an important transition period from adolescence to young adulthood, which can often be challenging due to the interaction between individual psychological characteristics and common stressors, such as academic demands, lifestyle changes, leaving home, separation from family, inadequate living conditions, physical and emotional issues, and financial concerns (Baumann *et al*., 2011).

Weight gain is a common occurrence among students, especially those in their first year of the university (Choi, 2020). Obesity is recognized as a major global issue, impacting not just adults but also children, adolescents, and young individuals. Among the factors contributing to obesity, stress seems to play a particularly crucial role, as stressful situations result in unhealthy eating patterns, reduced physical activity, and dependencies, all of which are considered independent factors leading to obesity (Kumar *et al.*, 2007).

High levels of psychological stress have numerous detrimental effects on the academic, physical, and mental health of students (Chen *et al.*, 2020). Research has shown that psychological stress is a risk factor for overweight and obesity through various interactions between biological and behavioral mechanisms (Tomiyama, 2019). Meta-analysis of 14 longitudinal studies found that stressors, *including* general life stress (caregiver stress, major life events) and work-related stress, were positively associated with the risk of obesity with a moderate effect (Wardle *et al.*, 2011).

There are two main components of the stress response: the autonomic nervous system (ANS), which encompasses the sympathetic and parasympathetic nervous systems, and the HPA axis. These systems function centrally and peripherally to produce several responses. The 'fight or flight' response is an active reaction to either confront the stressor or flee from the confrontation. The 'defeat response' occurs when an individual engages in neither fight nor flight, ultimately 'losing' the confrontation; this is the primary stress response in modern society and is associated with changes in the HPA axis (Bose *et.al*., 2009).

The aim of this study is to assess the impact of stress on dietary habits and body composition in the student population.

RESULTS AND DISCUSSION

Men represented 26% of the participants, while women accounted for 74%. On average, 38.3% of the respondents were in the second year of the first cycle of study, while 27.7% were in the third year. The minimum BMI was 16.4, indicating underweight, while the maximum BMI was 33.2, defined as obesity. The average BMI was 22.7±3.7 kg/m², which falls within the normal weight range. Most participants (65.96%) were within the normal weight range, while only 4.3% of respondents fell within the obesity range, which is a satisfactory result. In a study conducted on 50 students in Croatia, the average BMI was 22.1 ± 2.7 kg/m² (Keser *et al*., 2020) indicating a very small difference compared to the results of this study. The differences in BMI by gender were statistically significant (p=0.23, p<0.05). Men (24.4±3.1) had a higher BMI than women (21.6±3.6) who exhibited greater variations in their BMI values (Table 1).

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Table 1. BMI results by the Year of Study

BMI	I year (%)	II year (%)	III year (%)	MA (%)
<18.5	10	17	12	0
18.5-24.9	60	63	61	100
25-29.9	30	20	24	0
≥30.0	0	0	3	0

In a study conducted in 2007 at Auburn University (Gropper *et al*., 2012) a significant three-year weight gain of 2.1±4.7 kg and a BMI increase of 0.7±1.6 kg/m² were observed. Absolute gains in weight, BMI, percentage and absolute body fat were highest during the first year, followed by the third year, while the second year saw the lowest gains. Among 70% of students who gained weight over the three years, the average weight gain was 4.3 kg. The number of female participants with over 30% body fat doubled, while the number of male participants with over 20% body fat increased fivefold. Initially, 15% of students were classified as obese, while 79% had a normal weight; by the end of the second year, 24% were classified as obese, and 70% had a normal weight.

Comparing the results from other studies with those of this research, it can be concluded that students from Bosnia and Herzegovina have significantly better outcomes. The results for participants with normal body weight are quite similar, while there is a substantial difference in obesity classification results, which is not the case in the Auburn study. Additionally, variations in body weight during different years of study were noted in both studies.

Table 2. Dietary habits of the participants

Frequency of food consumption	Yes (%)	No (%)
Vegetables	72	28
Fruits	70	30
Poultry	56	44
Red meat	26	74
Milk and dairy products	45	55
Grains and grain-based products	85	15
Coffee with sugar	44	20
Coffe without sugar	16	20

Table 2 presents the dietary habits of respondents with a frequency of at least 4 times per week. The most of participants (72%) indicated that they consume vegetables at least 4 times a week. For fruits, a similar proportion (70%) reported the same. There were greater variations in the intake of poultry and red meat. Regarding red meat, 74% of respondents stated that they do not consume it 4 times a week, with some claiming that they never consume it, while 56% of participants reported eating poultry almost daily. The results show that 85% of respondents consume grains and grain-based products at least 4 times a week, whereas 15% expressed that they do not consume grains and grain-based products that frequently.

In a study conducted in 2019 with Korean students using a dietary habits questionnaire (Choi, 2020) it was recorded that 20.9% of respondents consume meat 5-6 times a week, while 39.7% consume meat 3-4 times a week. Milk and dairy products are consumed by 43.3% of respondents 1-2 times a week, while more than two pieces of fresh fruit are consumed by 40.7% of respondents 1-2 times a week. Grains and grain-based products are consumed 3-4 times a week by 43.0% of participants.

In a study conducted in 2019 in Romania, respondents with an average age of 21 were surveyed about their dietary habits using a questionnaire. The respondents reported consuming the following foods daily: 31% consumed meat and fruit, 28.5% reported consuming milk and dairy products, while less than half of the respondents (45.7%) consumed vegetables, and more than half of the respondents (52.61%) consumed grains and grain-based products every day (Pop *et al.*, 2021). By comparing the results from other studies with those of this research, it can be said that students from Bosnia and Herzegovina consume the highest amounts of fruits, vegetables, white meat, and grains (at least 4 times a week).

In a 2018 study involving Polish students with an average age of 22.7 years, bioimpedance was used to measure average fat tissue values, which were found to be 26.0 ±7.9% for women and 15.8±5.3% for men. A statistically significant difference was identified with respect to sex (Keska *et al.*, 2018).

In a study conducted in 2016 in Italy, participants aged 18-21 with an adequate body mass index were measured using the BIA-ACC device via bioimpedance method. Men showed an average fat tissue percentage of 22.5 ± 4.8%, while women had an average of 25.3±4.4% (Stefanaki *et al.*, 2016). By comparing these results from the two studies with the findings presented in Table 3, a difference in fat mass (FM) values among men can be observed, whereas no such difference was noted among female participants. Bosnian students in this study exhibited significantly higher average FM values compared to students from Poland and Italy.

Table 3. Results of body composition parameters categorized by gender

SEX	M (%)	F (%)
BMR (kcal/day)	1577.6	1358.5
TBW	49.6	45.0
ICW	60.1	53.0
ECW	39.8	46.9
FFM	70.8	74.6
FM	29.2	25.4
ECMatrix	21.4	29.6
HPA INDEX	3.8	2.5
STM (kg)	0.5	0.4
Bm (kg)	2.4	1.6
ALST (kg)	19.5	11.8
AT (kg)	29.4	20.5
IMAT (kg)	1.4	0.8

The HPA index greater than 3.5 is considered normal or circadian, indicating good cell health and an optimal level of stress hormones (cortisol). In addition to this reference value, there are two more categories: Flat low (low cortisol level throughout the day, with no fluctuations) and Flat high (high cortisol level throughout the day, with no fluctuations) are presented in Table 4.

Table 4. Norms for HPA axis index values

Norms for HPA axis index values	HPA axis index values
Flat low	1.0 – 2.6
Flat high	2.7 – 3.4
Circadian	> 3.5

The average value for the HPA index is 2.8, which, according to Table 4, falls within the Flat high category. This means that, on average, the students in this study do not have good cell health, indicating that they have a system with poorly intact cell membranes and that cortisol levels are high throughout the day, without fluctuations.

Table 5. HPA axis index results categorized by three norms

HPA axis index values	Average (%)
1.0 – 2.6 (Flat low)	42.5
2.7 – 3.4 (Flat high)	36.2
>3.5 (Circadian)	21.3

It can be concluded from Table 5. that 42.5% of participants have a Flat low value of the HPA axis index, indicating that their cortisol levels are low, without fluctuations, and that cell health and cell membrane integrity are compromised. Only 21.3% of participants have HPA axis index values greater than 3.5, which represents the norm considered optimal, indicating excellent cell health and normal cortisol levels throughout the day.

Table 6. HPA axis index results categorized by gender

HPA index osi	M (%)	F (%)
1.0 – 2.6	0	55.6
2.7 – 3.4	27.2	38.8
>3.5	72.8	5.6

It can be seen from the Table 6 that 72.8% of men had an HPA axis index value greater than 3.5, which is considered a satisfactory norm. In contrast, 55.6% of women had HPA axis index values ranging from 1 to 2.6, which falls into the flat low norm and is not satisfactory. Furthermore, only 5.6% of women had this value of 3.5, which is a very small number compared to men. Differences in HPA axis index by gender are statistically significant (p=0.000, p<0.05). Men (3.8±0.4) had a higher HPA axis index than women (2.4±0.7), with women showing greater variability in their HPA axis index values. Men exhibited more balanced cortisol secretion throughout the day, while women experienced greater fluctuations.

In a study conducted in 2016 in Italy, participants were measured with the BIA-ACC device using the bioimpedance method and they had a suitable body mass index. The average HPA axis index for men was 4.45±1.3, while for women, it was 6.02±2.26 (Stefanaki *et al*., 2016). Comparing the results of these two studies reveals a significant difference between them. Therefore, it can be concluded that Italian students have excellent cell health compared to Bosnian students, whose results indicate possible damage to cell membrane integrity and injury.

Table 7. HPA axis index results categorized by year of study

HPA AXIS INDEX	I year (%)	II year (%)	III year (%)	MA (%)
1.0 – 2.6	60	38	31	50
2.7 – 3.4	20	34	54	33.5
>3.5	20	25	15	16.5

Table 7 shows the results for the HPA axis index categorized by year of study. It was observed that 60% of participants in their first year have a Flat low HPA axis index, while only 20% show a normal HPA axis index. In the following years, the results vary: the percentage of participants with a Flat low HPA axis index decreases to 38% in the second year and 31% in the third year, before rising again to 50% in the master's program. On the other hand, the Flat high HPA axis index increases during the first three

years of study but decreases by almost half during the master's program. The normal HPA axis index remains above 20% of participants during the first three years, while this percentage drops to 16.5% in the master's program. These results suggest that stress levels vary during the years of study and are not solely elevated in the first year.

Table 8. Spearman correlation results of body composition index (first part)

		BMI	TBW	ECW	ICW	FFM	FM	HPA INDEX OSI
BMI	Correlation Coefficient	1.000	-0.201	**-0.777****	**0.777****	-0.923**	0.924**	0.417**
	Sig. (2-tailed)	.	0.176	0.000	0.000	0.000	0.000	0.004
	N	47	47	47	47	47	47	47
TBW	Correlation Coefficient	-0.201	1.000	0.027	-0.027	0.465**	-0.464**	0.475**
	Sig. (2-tailed)	0.176	.	0.856	0.856	0.001	0.001	0.001
	N	47	47	47	47	47	47	47
ECW	Correlation Coefficient	-0.777**	0.027	1.000	-1.000**	0.774**	-0.776**	-0.642**
	Sig. (2-tailed)	0.000	0.856	.	.	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
ICW	Correlation Coefficient	0.777**	-0.027	-1.000**	1.000	-0.774**	0.776**	0.642**
	Sig. (2-tailed)	0.000	0.856	.	.	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
FFM	Correlation Coefficient	**-0.923****	0.465**	**0.774****	**-0.774****	1.000	-1.000**	-0.273
	Sig. (2-tailed)	0.000	0.001	0.000	0.000	.	0.000	0.063
	N	47	47	47	47	47	47	47
FM	Correlation Coefficient	**0.924****	-0.464**	-0.776**	0.776**	-1.000**	1.000	0.278
	Sig. (2-tailed)	0.000	0.001	0.000	0.000	0.000	.	0.058
	N	47	47	47	47	47	47	47
HPA INDEX OSI	Correlation Coefficient	0.417**	0.475**	**-0.642****	**0.642****	-0.273	0.278	1.000
	Sig. (2-tailed)	0.004	0.001	0.000	0.000	0.063	0.058	0.000
	N	47	47	47	47	47	47	47

**The correlation is significant at the 0.01 level (2-tailed)

Table 9. Spearman correlation results of body composition index (second part)

		BMI	BMR (kcal/day)	STM (kg)	Bm (kg)	ALST (kg)	AT (kg)	IMAT (kg)
BMI	Correlation Coeffic.	1.000	0.590**	0.752**	0.747**	0.767**	**0.928****	**0.907****
	Sig. (2-tailed)	.	0.000	0.000	0.000	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
BMR (kcal/day)	Correlation Coeffic.	0.590**	1.000	**0.926****	**0.927****	**0.921****	0.718**	0.757**
	Sig. (2-tailed)	0.000	.	0.000	0.000	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
STM (kg)	Correlation Coeffic.	0.752**	0.926**	1.000	0.998**	0.995**	0.766**	0.808**
	Sig. (2-tailed)	0.000	0.000	.	0.000	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
Bm (kg)	Correlation Coeffic.	0.747**	0.927**	0.998**	1.000	**0.993****	0.760**	0.802**
	Sig. (2-tailed)	0.000	0.000	0.000	.	0.000	0.000	0.000
	N	47	47	47	47	47	47	47
ALST (kg)	Correlation Coeffic.	0.767**	0.921**	0.995**	0.993**	1.000	0.790**	0.833**
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	.	0.000	0.000
	N	47	47	47	47	47	47	47
AT (kg)	Correlation Coeffic.	0.928**	0.718**	0.766**	0.760**	0.790**	1.000	0.985**
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	.	0.000
	N	47	47	47	47	47	47	47
IMAT(kg)	Correlation Coeffic.	0.907**	0.757**	0.808**	0.802**	0.833**	0.985**	1.000
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	.
	N	47	47	47	47	47	47	47

**The correlation is significant at the 0.01 level (2-tailed)

Detailed results of Spearman correlation of body composition index are divided in two tables and the first part can be found in Table 8. The correlation coefficient between BMI and Fat Mass (FM) is 0.924. This value is very close to 1, indicating a very strong relationship between these two variables. The significance level is 0.000, which is less than 0.01, making the relationship highly significant. Therefore, there is a very strong and significant correlation. The positive correlation indicates that as the value of BMI increases, the value of FM also increases.

The correlation between BMI and Fat-Free Mass (FFM) is -0.923, suggesting a very strong and significant negative relationship; as BMI decreases, the value of FFM increases. The correlation between BMI and Extracellular Water (ECW) is 0.777, while the correlation between BMI and Intracellular Water (ICW) is -0.777. The correlations among these variables are strong and highly significant.

The difference lies in the signs of the correlations. The correlation between BMI and ICW is positive, meaning that as one value increases, so does the other. Conversely, the correlation between BMI and ECW is negative, indicating that as BMI decreases, the value of ECW increases. The correlation between the HPA axis index and BMI is 0.417. This correlation is moderate but not statistically significant ($p = 0.004$).

The correlation between the HPA axis index and ECW is -0.642, while the correlation between the HPA axis index and ICW is 0.642. Both correlations are strong and highly significant, as the significance level is less than 0.01. The key difference is in the sign of the correlation. The correlation between the HPA axis index and ECW is negative, suggesting that the variables move in opposite directions, while the correlation between the HPA axis index and ICW indicates that the variables move in the same direction. This reflects on cell health and membrane integrity, indicating whether the systems are compromised or not (Weir, 2016).

The other part of correlations is presented in the following Table 9. The correlation between BMI and AT is 0.928**, indicating a very strong and significant correlation. The sign is positive, meaning that the variables move in the same direction. As a person gains weight and their BMI increases, the level of AT in the body also rises and changes. The correlation between BMI and IMAT is 0.907**. This correlation is also very strong and significant.

One of the results of the PPG stress flow is a graph that shows the strength of the balance of the ANS (autonomic nervous system). The graph shows the strength of the SNS (Sympathetic Nervous System) and PNS (Parasympathetic Nervous System) strength and whether these strengths are in a state of homeostasis or in a state of allostasis (Vodopianov *et al.*, 2022). An example of homeostasis can be normotonia of the autonomic nervous system (ANS) shown in Figure 1.
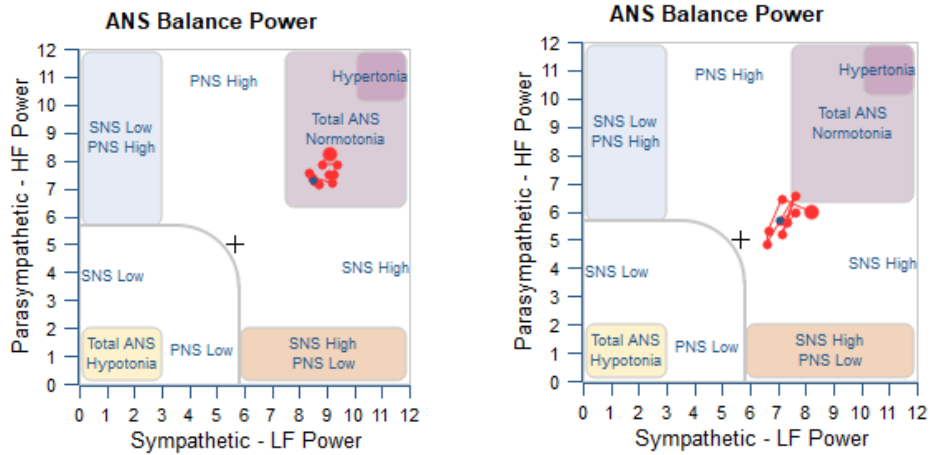
Figure 1. Homeostatic Balance of SNS and PNS Power (left) and allostatic Load of SNS and PNS Power (right) (BioTekna, 2021)

Table 10. Results of the balance of ANS strength categorized by gender

ANS Balance Strength	M (%)	F (%)
Allostasis	0	25
Homeostasis	100	75

It can be observed that 100% of male participants are in a state of homeostasis, while there is a division among women (Table 10), 25% of women are in a state of allostasis, where both PNS and SNS levels are high. The remaining 75% of women have achieved total ANS normotonia (homeostasis).

Table 11. Results of ANS balance strength divided by year of study

ANS Balance Strength	I year (%)	II year (%)	III year (%)	MA (%)
Allostasis	33.4	33.4	22.1	11.1
Homeostasis	18.5	39.5	29	13

The results of ANS balance (table 11) strength are presented according to the year of study, where the results for first and second-year students show that 33.4% of participants are in a state of allostasis. These results are significantly lower for the other years. However, during the second year, 39.5% of participants were in homeostasis, which represents excellent results. The lowest percentage of participants in a state of allostasis is found among master's students, at 11.1%.

CONCLUSIONS

Based on the research results, it can be concluded that the majority of students adhere to the recommended daily intake of certain foods that are essential for daily consumption (fruits, vegetables, meat, milk and dairy products, grains and grain-based products, and fats).

There is a statistically significant difference between BMI in men and women (p=0.23). Men had a higher BMI (24.4±3.2) compared to women (21.6±3.7). Both results indicate that the participants have a normal nutritional status. The only reason men included in this study have a higher BMI is due to their larger physique.

The average percentage of body fat in women is 25.36%, while in men, it is 29.36%, which exceeds the normative range (min 7%, max 25%) set for men. Male participants still had a higher percentage of body fat compared to female participants, whose body fat percentage fell within optimal parameters.

The values of the HPA index, which is an indicator of chronic stress, differ significantly between male and female participants ($p < 0.000$). Men exhibit statistically significantly higher values compared to women (M = 3.8 ± 0.4 vs. F = 2.4 ± 0.7), indicating that male participants predominantly have a circadian rhythm of cortisol secretion compared to females.

According to the results concerning the balance of the autonomic nervous system (ANS), it can be concluded that male participants (100%) have better outcomes than female participants (75%). This means that all male participants are in a state of homeostasis, while changes in the strength of ANS balance are observed in female participants, with 25% of women experiencing allostasis. The results for homeostasis and allostasis vary significantly depending on the year of study.

BMI had a positive correlation with fat mass (FM), adipose tissue (AT), intramuscular adipose tissue (IMAT), and intracellular water (ICW), which means that as the BMI of the participants changes, so do the other parameters. Additionally, the HPA axis index has a positive correlation with ICW and a negative correlation with extracellular water (ECW), which indicates that a low HPA index suggests compromised cellular health, while a high HPA index indicates optimal cellular health.

The overall conclusion of this research is that female participants experience higher stress levels compared to their male colleagues.

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UTICAJ STRESA NA PREHRAMBENE NAVIKE I TJELESNU KOMPOZICIJU STUDENTSKE POPULACIJE

Sažetak

Gojaznost postaje ozbiljan problem širom svijeta. Jedan od faktora koji doprinosi gojaznosti jeste stres. Čini se da je ovaj faktor posebno važan, jer dovodi do nepravilne ishrane, nedostatka fizičke aktivnosti i nedostatka sna. Prijelaz iz srednje škole na fakultet može povećati nivo percipiranog stresa, što utiče na prehrambene navike i metabolizam. Ova promjena sama po sebi utiče na razlike u prehrambenim navikama u odnosu na opću populaciju - studenti se oslanjaju na obroke koji su lako dostupni i brzi za pripremu. Postoje dvije glavne komponente stresnog odgovora: autonomni nervni sistem (ANS), koji uključuje simpatički i parasimpatički nervni sistem, te HPA os.

Cilj istraživanja bio je procijeniti uticaj stresa na prehrambene navike i tjelesnu kompoziciju studentske populacije. U istraživanju se učestvovalo 50 studenata sa Poljoprivredno-prehrambenog fakulteta Univerziteta u Sarajevu. Uređaji korišteni za mjerenje tjelesne kompozicije su BIA-ACC i PPG Stress Flow (BioTekna S.r.l., Marcon-Venice, Italija). Studentov T-test korišten je da bi se utvrdilo postoji li statistički značajna razlika između parametara u odnosu na spol. Najveće razlike s obzirom na spol mogu se primijetiti u parametrima HPA indeksa (p < 0,000), koji je jedan od pokazatelja stresa, što dokazuje viši nivo stresa među ženama (M = 3,8 ± 0,4 vs. F = 2,4 ± 0,7) i između BMI kod muškaraca i žena (p = 0,23). Muškarci su imali viši BMI od žena (M = 24,4 ± 3,2 vs. F = 21,6 ± 3,7), ali oba spola spadaju u kategoriju normalne tjelesne mase.

Ključne riječi: *studenti*, *stres, BIA-ACC, PPG Stress Flow, tjelesna kompozicija*.

TRADITIONAL DISHES OF BOSNIA AND HERZEGOVINA*

Almira Avdić[1], Amina Kozlić[1], Irzada Taljić[1]

Original scientific paper

Abstract

Traditional dishes of Bosnia and Herzegovina represent a wealth of flavors and culture shaped over centuries by the historical and social influences of various conquerors. These dishes reflect a blend of Ottoman, Mediterranean, Austro-Hungarian and Balkan cuisines, where locally sourced ingredients have been transformed into diverse and recognizable dishes. Each region in Bosnia and Herzegovina boasts its specific culinary traditions. In eastern Herzegovina, cuisine is known for dishes like *japrak* and *cicvara*, while Prijedor and Banja Luka are famous for *banjalučki ćevap* and *pita*. Sarajevo offers iconic dishes such as *sarajevski ćevap* and *begova čorba*. The Krajina region is renowned for *kljukuša* and other varieties of *pita*, Zenica and Žepče areas preserve authentic recipes that combine simplicity with rich flavors.

Materials for the study include books, journals, academic theses, video materials, websites and live communication (interviews and conversations). Descriptive and analytical methods were used to document and analyze culinary customs. The descriptive method explains traditional dishes' ingredients, preparation methods, and cultural significance. The analytical method focused on comparing and contrasting these customs across different regions. The research revealed significant dish differences regarding preparation methods, names and habits across the country. Preserving these customs is essential for maintaining cultural identity and heritage. Efforts to document and promote these culinary traditions can help and maintain the cultural heritage of Bosnia and Herzegovina for future generations.

The research has been conducted in the frame of the subject „Culture of eating in Bosnia and Herzegovina".

The aim was to collect and preserve traditional dishes of Bosnia and Herzegovina highlighting regional differences between Mediterranean-influenced Herzegovina and Central European-inspired northern and central Bosnia.

Keywords: *Bosnia and Herzegovina, culinary heritage, traditional cuisine, customs, culture preservation*.

INTRODUCTION

The culinary heritage of Bosnia and Herzegovina represents one of the most important elements of its cultural heritage, providing a deep insight into the historical, geographical and ethnic characteristics of the country.

Located at the crossroads of East and West, Bosnia and Herzegovina has been the scene of numerous historical events and meetings of different civilizations throughout the centuries (Šarić-Halilović, 2014.)

Traditional foods are given a positive image across Europe (Almli *et al*., 2011) because consumers want less industrial products with additives (Dickson – Spilman *et al*., 2011). Diverse influences in Bosnia and Herzegovina left an indelible mark on gastronomy, shaping a unique cuisine that combines elements of Ottoman, Mediterranean, Central European, and Slavic traditions (EuropaAdventure, 2024). Traditional Bosnian-Herzegovinian cuisine is the result of a long-term process of adaptation and synthesis of different culinary styles and techniques, which over time have adapted to local conditions, climate and available foods. This cuisine is not only a reflection of eating habits but also a mirror of social, cultural and religious customs that are deeply rooted in people's daily lives. Dishes such as pies, soups, roasts, and various sweets carry stories about history, customs, and ways of life that have been passed down from generation to generation (Samardžić *et al*., 2021; Alibabić *et al*., 2012). Traditional food were popularized from the aspect of tourist attractions and territorial brands for tourist development, and promoted in terms of healthy eating (Pestek and Nikolic, 2011).

Bosnia and Herzegovina is lagged because of the war (1992-1994) and the consequences but some steps are made in preserving geographical mark of products like: cheese from Livno (*Livanjski sir*), potato from Nevesinje (*Nevesinjski krompir*), sirloin steak from Visoko (*Visočka pečenica*), Original cheese from Livno (*ivory Livanjski sir*), Whipped jam from Drvar (*Drvarski mućeni pekmez*) (Brenjo, 2022).

This research has been conducted in the frame of the subject „Culture of eating in Bosnia and Herzegovina" and this paper aims to provide a comprehensive overview of the culinary heritage of Bosnia and Herzegovina with special reference to regional differences and specificities.

MATERIALS AND METHODS

The descriptive method is used to describe the ingredients, preparation techniques, and cultural significance of traditional dishes, while the analytical method will be applied to compare and contrast these customs in different parts of the country. Through this analysis, the paper will investigate how dishes differ in the way they are prepared, their names and ingredients, and how these customs reflect the wider cultural and historical picture of Bosnia and Herzegovina.

Materials used in this research were:

-Á Published literature covering culinary traditions, recipes, and cultural significance of food as scientific articles and magazines related to gastronomy, cultural aspects of food and food customs.
-Á Video materials: tv programmes, documentaries and educational videos.
-Á Websites of non-governmental organizations, restaurants…
-Á Live communication: Interviews and conversations with people who practice traditional culinary customs**.**

RESULTS AND DISCUSSION

The results are described for a few dishes in each of the aforementioned regions of Bosnia and Herzegovina. The results show variations in food preparation. Traditional culinary techniques are used. Long cooking processes (from 3 to 4 hours) are used in recipes such as agaric and fish, showing the tradition of slow cooking to produce flavor and the shared pleasure of a meal between family and community (Alibabić *et al*., 2012). Regions in Bosnia and Herzegovina have unique ways of creating dishes based on local ingredients and cultural traditions. For *pita* (Orašje) and Herzegovinian lamb skewers (Nevesinje, Blieće, Trebinje) locally available meat and vegetables, along with special spices are used (Samardžić *et al*., 2021).

Table 1. Bosnian-Herzegovinian traditional dishes across the region of the country

Region	Dish Name	Main ingredients	Recipe Source
East Herzegovina	Hercegovački jagnjeći ražnjići (Ražnjići od jagnjetine)	Lamb (leg, shoulder, or another tender cut of meat) Olive oil Garlic Rosemary (or a mix of Mediterranean herbs) Salt and pepper Lemon juice	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Bilećki sir	Fresh milk (usually sheep or cow's milk) Rennet (a natural or industrial enzyme used to coagulate milk) Salt	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf

	Trebinjska musaka	Minced meat (veal or pork) Potatoes Onion Carrot Tomato Salt, pepper, spices	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Teletina ispod sača	Veal (leg or shoulder blade) Olive oil Garlic Rosemary Salt and pepper	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Lučenica	Rennet Fresh milk Salt	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Herzegovačka kalja	Smoked meat Potatoes Onion Carrot Dried spices	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Patišpanja	Flour Eggs Sugar Vanilla	https://www.tkh.ba/wp-content/uploads/2018/02/gastro_hercegovina_eng_final.pdf
	Japrak	Grape leaves Minced meat Rice Onion Salt, pepper	https://www.tasteatlas.com/japrak
Sarajevo	Sarajevski burek	Dough Minced meat (or Onion Salt	https://www.recepti.com/kuvar/pite-i-testa/48843-sarajevski-burek

	Sarajevski ćevapi	Minced meat (veal, beef or pork) Onion Salt, pepper, spices	https://www.tasteatlas.com/sarajevski-cevapi/recipe
	Kadaif	Kadaif pastry Butter Sugar Walnuts	https://www.dishesorigins.com/kataifi-kadayif-kadaif/
	Begova čorba	Lamb or veal Okra (or substitutes like carrots or peas) Flour Onion Lemon	https://www.krstarica.com/kuvar/supe-i-corbe/corbe/begova-corba-originalni-sarajevski-recept/
Zenica, Žepče, Maglaj	Čimbur	Eggs Milk Cheese	https://www.zenicablog.com/preporuke-zenicka-jela/
	Žepačka patka-ramazanija	Duck Tomato Rice Onion Garlic	https://zepce.ba/udruzenje-spektar-objavilo-brosuru-ramazanska-prica-zepacka-patka/
	Ćetenija	Smoked meat Tomato Onion	https://www.zenicablog.com/preporuke-zenicka-jela/
	Tagarica	Dough (bread dough) Meat Onion	https://www.aa.com.tr/ba/balkan/neizostavan-dio-iftarske-sofre-te%C5%A1anjska-tagarica-je-djetinjstvo-

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vqi gyi gt'hkuj 'cpf 'ewnpcnr{'gpyj wukcuu'htqo 'cetquu'yj g'eqwpvr{.'cmyy kpi 'yj go 'vq'eqo r gvg"
kp'eqqmkpi 'cpf 'gplq{ 'c'xctkgv{ 'qh'hkuj 'f kuj gu0'"

Cv'yj g'uco g'vko g. 'yj g'hngzkdknkv{ 'qh'tgekr gu. 'uwej 'cu'ecmgu'qr 'r kgu. 'o cmgu'kv/r quukdng'vq'
cf cr v'f kuj gu'vq'f khhgrgpv'qeecukqpu'cpf 'vuuvgu.'uj qy kpi 'r tcevkecnkv{ 'cpf 'etgcvkxkv{ 'kp'hqqf "
r tgr cticvkqp'*Uco ctf fik "gv'cn0 "4243=Gwtqr cCf xgpwtg."pd+0"

EQPENWUKQPU'

Dqupkc'cpf "J gt| gi qxkpc"ewkukpg"eqo dkpgu"gmgo gpvu"qh"Qwqo cp."Cwuvtq/J wpi ctkcp."
O gf kgttcpgcp. "Egpvtcn Gwtqr gcp'cpf "Uncxke "tcf kvkqpu'etgcvkpi 'c 'ur gekhke "i cuvrqpqo ke "
kf gpvkv{0'Gcej 'f kuj 'tgfngevu'yj g'cf cr vcvkqp'qh'f khhgrgpv'ewnvwtgu'uv{ngu'vq'nqecn'eqpf kvkqpu."
enko cvg'cpf 'cxckncdng'kp tgf kgpvu0'Vj g'ewkukpg'qh'Dqupkc'cpf "J gt| gi qxkpc'cnuq 'tghngevu'
uqekcn'cpf 'tgnki kqwu'euwuqo u0'Uqo g'f kuj gu'ctg'r tgr ctgf "qp'ur gekcn'qeecukqpu."uwej "cu"
Gkf."Ej tkuvo cu'qr 'y gf f kpi u.'y j kej 'j cxg'ur gekhke 'ewnwwtcn'uki pkhkecpeg0'"

Vj ku'tgugctej "go r j cuk| gu'yj g'pggf "hqr 'cf f kvkqpcn'tgugctej "kpvq 'ewnkpctk/v tcf kvkqpu'cpf "
f kuj 'xctkcvkqpu'kp'Dqupkc'cpf "J gt| gi qxkpc0'Fkxgrukv{ 'kp'hqqf 'r tgr ctcvkqp'ecp'dg'uugf 'cu'
c'y c{ 'qh'r tgugrxkpi 'cpf 'r tqo qvkpi 'ewnwwtcn'j gtkvci g.'etgcvkpi 'c'eqo o qp'r ncvhqto "hqr "
f khhgrgpv'eqo o wpkvkgu'cpf 'vqwtkuo 0'"

TGHGTGPEGU"

Cnkdcdk . "X0 'O wlk . 'K0 'Twf k . 'F 0 'Dcltco qxk . 'O 0 'Iqmk . 'U0' ᵀ Yṙ tqxk ."G0 '*4234+0'
Vtcf kvkqpcn'F kgwu'qh'Dqupkc'cpf 'yj g'tgr tgugpvcvkqp'qh'tcf kvkqpcn'hqqf "kp'yj g'
ewkukpg'hkgnf 0'Rtqecf kc'/ 'Uqekcn'cpf "Dgj cxkqtcn'Uekgpeu'68. '3895'ᵔ '389: 0'f qk<'
320823811 0udur tq042342705057; "

Cmo k'X0 N0 'Xgrdgmg. 'Y 0 'Xcpj qpcemgr. 'H0 'P cgu. 'V0 'J gtureqj . 'O 0 '*4233+0'I gpgrcn'
ko ci g'cpf 'cwtkdwg'r gtrgr vkqpu'qh'tcf kvkqpcn'hqqf 'kp'ukz 'GGwtqr gcp'eqwpvrkgu0'
Hqqf 'S wcnkv{ 'cpf 'Rtghgtgpegu. '44. '34; '35: 0'

Dtgplq." F 0' *4244+0' Vtcf kekqpcnk' r tgj tcm dgpk' r tqk| xqf k' Dqupg' k' J gtegi qxkpg' k'
r qvgpekcnk i gqi tchuvk/ q| pcmc. 'WUCKF . 'HCQ. 'Ci gpekc' | c 'uki wtpquv'j tcpg"
Dqupg' K' J gtegi qxkpg0' Cxckncdng "cv^ 'j w r u‹1lwwtk| co dkj 0dc1r wdlkmceklc/q/
r qvgpekcnko c/i gq/q| pcm/k/tcf kekqpcnkj /r tgj tcm dgpkj /r tqk| xqf c/w/dkj 1"

F kemuqp/Ur kmo cpp. 'O 0 'Ukgi tkuv. 'O " (' 'Mgmer. 'E0'*4233+0'Cwukwf gu'vq 'ctf "ej go kecnu'
ctg 'cuuqekcvgf "y kyj 'r tghgtgpeg 'hqr 'pcwtcn'hqqf 0'Hqqf 'S wcnkv{ 'cpf 'Rtghgtgpegu."
44. '36; '3780'

Gwtqr cCf xgpwtg'*4246+'Vtcf kekqpcnc'dqucpunc'mwj kplc<'Qmwu'pcunlg co 0'Cxckncdng 'cv^ '
j w r u‹1y y y 0gwtqr ccf xgpwtg0eqo lgpldqupkc/cpf /j gt| gi qxkpc/tcf kvkqpcn/
dqupkcp/ewkukpg/c/vcuvg/qh/j gtkvci g10'"

Rguvgm'C0 (' P kmqṙk . 'C0 '*4233+0Tqng 'Qh 'Vtcf kvkqpcn'Hqqf ' Kp "Vqwtkuv' F guvkpcvkqp' Ko ci g'
Dwknf kpi <'Gzco r ng'Qh'Vj g'Ekv{ 'Qh'O quvct0WVO U'Lqwtpcn'qh'Geqpqo ku. '4. ': ; "
3220'

Uco ctf ffk .'U0' wtk .'I 0'Twfk /I twlk .'X0'Tcf qxcpqxk .'I 0'Fk| fctgxk .'V0' qt gxk /
O kq–gxk .'U0'*4243+0'Hqqf ."P wtkkqp"cpf "J gcnj "kp"Dqupkc"cpf "J gt| gi qxkpc."
7ʲ "ej cr vgt"kp<'P wtkkqpcn'cpf "J gcnj "Curgewi"qh"Hqqf "kp"vj g"Dcmcpu."Gnugxkgt"
Vtcf kkqpcn' cpf " Gvj pke" Hqqf " Ugtkgu." Cecf gm ke" Rtguu0' r r 0' 69/880'
j wr u‹1lf qk|qti 132032381D; 9: /2/34/: 429: 4/8022229/6"

ᵀᶜtrk /J cnkqxk ."G0' *4236+0' Vtcf kekqpcnc" dqucpumc" j tcnc" *Kp' Gpi rkuj <' Vtcf kkqpcn"
Dqupkn" hqqf +0' Cxcknadrg" cv<'
j wr u‹1ly y y 0dlcuvkpc0dclko ci gulurqtkgulucfr| cl14239142392538l4vtcf keklc1Gf kpc
' 42J cnkqxk' " E6' : 9/
' E7' C2ctk' E6' : 9' 42Vtcf kekqpcnc' 42dqucpumc' 42j tcnc0r f h@'

VcuvgCvncu"ʰbd+'cxaknadrg"cv‹'j wr u‹1ly y y 0cuvgcvncu0eqo 1lcr tcn0""

j wr u‹1ly y y 0 gpkecdmqi 0eqo 1r tgr qtwng/| gpkenc/lgnc10'

j wr u‹1ly y y 0 gpkecdmqi 0eqo 1r tgr qtwng/| gpkenc/lgnc10'

j wr u‹1lr wo '0dc'1hkuklcf c'/k'/eqdcpklcf c'/w'/r tqi tco w'/f cpc'/qr ekpg'/qtcusgl0'

VTCF KEKQP CNP C'LGNC'DQUP G'KJ GTEGI QXKP G''

Ucfigvcnl'

Vtcf kekqpcnc" lgnc" Dqupg" k' J gtegi qxkpg" r tgf uvcxnlclw" dqi cuvuq" qmwuc" k' mwnwtg"
qdrkkqxxcpg'htq| 'urqnlg c.'r qf 'wlgeclgqm 'tc| pkj 'quxclc c'k'l'ftw–xtgpkj 'r tqm lgpc0Xvc'lgnc"
qf tcdlcxclw'ur ql"quo cpukg."o gf kvgtcpukg."cuvtqwi utunrg'k'dcmacpuke"mwj kplie. g f lg"uw'
qmcnpki'ucuvqlekc'r tgvxqtrgpk'w'tc| pqtxupc' k'r tgr q' cnvrlkc'lgnc0'Uxcka "tgi kc"Dqupg" k'
J gtegi qxkpg"lo a "uxqje"ur ekhkk ng "mwnkpctunrg "tcf kekclg0'Vcmq 'lg. "pc"r tko lgr ."mwj kplie"
kuvq ng"J gtegi qxkpg'r q| pctc'r q"lgnk c"mcq"–vq"uw"*lartal"k'ekcxctc.'f qm'uw'Rtklgfqr "k'
Dcplc"Nwmc" wxgkn'r q"*dcpnanw mqm " gxcrw"k'rkk0'Uctalqxq'pwfk'kmqpki pc."lgnc'r qr w'
uctalgxuqi " gxcrc" qrbg0'Mtclkpa"lg'r q| pctc'r q"mlwmw–k'k'ftwi ko "xtuvam a"
rkc."f qm'r qf tuw lc'\ gnkeg'k'figr c" vuclu'cwgpvk pg'tcegp vg'mqlk'ur clalw'lgf pquvcxpqur"
u'dqi cvko "qmwuko c0'

O cvgtklcrl| a'uuwf kl'lurnlw uvlw'hnplki g."cuqr uug."cmaf gm um g'tcf qxg.'xlf gq' a'cvtklcnlg."y gd"
uvtcnice" k' ffkxw' mqm wnkmcklw" *kpvgtxlvkk" k' tc| i qxqtk+0' F gunmkr vkpko " k' cpcnkk mko "
o gvqf am a" f qmm gnvktcpki uw' k' cnpali tktcnk' mwnkpctunki' bdi clkl0' F gunmkr vkpa" m gvqf a"
mqm wixkcnc'lg'bdlc–plenlerg'ucuvqlakac.'pc kpa'r tkr gm g'k'mwnwtpqi '| pc cla'tcf kkqpcnki "
lgnc0'Cpankik na"m gvqf a'dka'lg "wtgf vq' gpc"pc"uur qtdfw'k'mqpvtau"vk "bdi cla"w'
tc| pk kko "tgi kqma c0'Knrtcf kxanig'lg'qvuknkq| pa clpg'tc| nkkg'w'lgm a'w'pa kpw'r tkr tgm g."
pa| kxka c'k'pnxknam a'f krlem " | gm r lg0'Vqmo "kurtcf kxana"cik–lk'uo q" a"qri cka| cakw"
tc| rk kkj 'f qi a cnpa'mqlc'ucnxe'j tanw0Rtko lgr 'lg'Hk–klcf a'l'Qtc–lw'mqlc'qmwrrla'ilwdkyerlg"
tkpe'k'mwnkpctuvxc'| 'elkgng"lgm rlg."pavlg w k'ug'w'mwj cnlw'k'fkkxqtkv k'w'tc| pka tkdlko "
lgmc a0'Ucuvqlekl'uw'–ctar."uo a"k'fklgm a"tkdlc'r qr w'f gxgtkmng'kdrk'r nqukeg."nwm"urctvnac"k'rdwa"
r cr tkm a."hqxqtqx'kkuv.'wo cma'qf 'r ctaf alz'k'dklgmq'xkpq0'

Očuvanje ovih običaja ključno je za očuvanje kulturnog identiteta i baštine. Napori da se dokumentiraju i promoviraju ove kulinarske tradicije mogu pomoći u očuvanju kulturnog naslijeđa Bosne i Hercegovine za buduće generacije.

Ključne riječi*: Bosna i Hercegovina, kulinarsko naslijeđe, tradicionalna kuhinja, običaji, očuvanje kulture.*

Index autora / Authors' index

UPUTSTVO ZA OBJAVLJIVANJE RADOVA

Radovi Poljoprivredno-prehrambenog fakulteta Univerziteta u Sarajevu (Radovi) su godišnjak u kojem se objavljuju naučni, izuzetno i stručni radovi, te izvodi iz doktorskih i magistarskih teza odbranjenih na Poljoprivredno-prehrambenom fakultetu Univerziteta u Sarajevu (Fakultet).

Radovi imaju karakter naučnog časopisa i kao takvi podliježu propozicijama za takve publikacije. Od broja 52 Radovi su indeksirani kod CAB Publishing - UK.

Članci za objavljivanje se klasificiraju, po preporuci UNESCO-a, u ove kategorije: naučni radovi, prethodna saopštenja, pregledni i stručni radovi. Autori predlažu kategoriju za svoje članke, recenzenti preporučuju, a konačnu odluku o kategorizaciji donosi Redakcija Radova. Naučni radovi sadrže rezultate izvornih istraživanja. Njihov sadržaj treba da bude izložen tako da se eksperiment može reprodukovati i provjeriti tačnost analiza i zaključaka. Prethodna sopštenja sadrže one značajne naučne rezultate, koji zahtijevaju hitno objavljivanje. Ova istraživanja mogu biti vremenski kraća od uobičajenih. Pregledni radovi sadrže pregled neke problematike na osnovu već publikovanih tekstova, koja se u pregledu analizira i diskutuje. Stručni radovi su korisni prilozi iz područja struke, koji ne predstavljaju izvorna istraživanja. Članci se pišu na bosanskom, srpskom, hrvatskom ili engleskom jeziku. Na početku rada treba pisati naziv rada (velikim slovima) na maternjem i na engleskom jeziku, a nakon toga ime (imena) autora. Naziv radne organizacije autora upisuje se u fusnotu (Ariel 7). Ispod imena autora obavezno se upisuje i kategorija rada.

U časopisu se publikuju radovi iz oblasti: poljoprivredna biljna proizvodnja, animalna proizvodnja, prehrambene tehnologije i održivi razvoj agrosektora i ruralnih područja.

Poželjno je da članci naučnog karaktera imaju uobičajenu strukturu naučnog rada i to: rezime (na bosanskom, srpskom i hrvatskom), uvod, pregled literature (može se dati i u uvodu), materijal i metode rada, rezultati istraživanja, diskusija (može biti objedinjeno sa rezultatima istraživanja), zaključci, literatura, summary na engleskom jeziku. Rezime i summary na našim jezicima i engleskom jeziku mogu imati maksimalno 200 riječi, uz obavezno upisivanje ključnih riječi. U spisku literature daju se samo autori i radovi koji se spominju u tekstu. Imena autora u tekstu pišu se spacionirano (sa razmakom). Latinska imena biljaka, životinja i mikroorganizama treba (osim imena autora) pisati kurzivom. Tabele, grafikoni i slike moraju imati svoj naziv, a ako ih je više i broj. Broj i naziv tabele pišu se u istom redu, iznad tabele, dok se broj i naziv grafikona, crteža i slika pišu ispod tih priloga. U tabelama, grafikonima i slikama naslove, zaglavlja i objašnjenja poželjno je dati i na stranom jeziku. Grafikone i crteže treba raditi isključivo u crnobijeloj tehnici. Tabele uokviriti linijama debljine 1/2 pt, bez sjenčenja pojedinih ćelija, ili redova i kolona. Slike i grafički prikazi treba da budu besprijekorne izrade radi kvalitetne reprodukcije u knjizi.

Radovi, po pravilu, ne treba da budu duži (sa prilozima) od 12 kucanih stranica. Izvodi iz magistarskih teza mogu biti dugi do 15, a iz doktorata do 25 kucanih stranica.

Za sadržaj članka odgovara autor. Članci se prije objavljivanja po "*double blind*" principu recenziraju od strane dva nezavisna recenzenta. Redakcija, uz konsultovanje sa autorima, zadržava pravo manjih redaktorskih i jezičkih korektura u člancima.

Autor dostavlja Redakciji rukopis putem e-maila uređen prema uputstvima za pisanje radova. Prilikom slanja radova Redakciji obavezno je naznačiti kontakt adresu i e-mail adresu u posebnom dokumentu. Svi prispjeli rukopisi će biti podvrgnuti inicijalnoj provjeri u pogledu zadovoljenja kriterija oblasti iz kojih časopis objavljuje radove i tehničke pripreme rukopisa u skladu sa uputstvima autorima.

Podneseni rukopis nakon inicijalne provjere od strane Redakcije može biti odbijen bez recenzija, ako uredništvo ocijeni da nije u skladu s pravilima časopisa. Autoru će u roku od 20 dana biti upućena informacija o inicijalnom prihvatanju rada ili razlozima za njegovo neprihvatanje.

Po završetku postupka recenziranja koji, u pravilu, ne bi trebao trajati duže od tri mjeseca Redakcija, na osnovu konačnih preporuka recenzenata, donosi odluku o objavljivanju, odnosno neobjavljivanju rada. Nakon toga rad se šalje na Univerzitet, Službi za izdavačku djelatnost, koja vrši provjeru potencijalnog plagijarizma. O svojoj odluci Redakcija informiše autora, uz informaciju o broju i terminu izlaska časopisa u kojem će rad prihvaćen za objavljivanje biti štampan.

Elektronsku verziju rada treba pripremiti u Wordu u formatu stranica 170 x 240 mm, sa slijedećim veličinama margina: gornja i donja 2,2 cm, lijeva 2,0 cm, a desna 1,5 cm, te formatirati parne i neparne stranice. Isključivo koristiti font Times New Roman, veličina 11, dok za fusnote treba koristiti font Arial, veličina 7. Tekst treba da je obostrano poravnat. Nazive poglavlja u radu treba pisati velikim slovima, boldirano i sa srednjim poravnanjem, te jednim redom razmaka od teksta.

Prilikom formatiranja članka ne treba uređivati zaglavlje i podnožje članka (Header and Footer) niti numerisati stranice. Autorima kojima engleski jezik nije maternji, strogo se preporučuje da obezbijede profesionalnu korekturu teksta koji će biti recenziran. Prilikom pisanja na engleskom jeziku treba koristiti jasne engleske izraze bez žargona i izbjegavati duge rečenice. Strogo se preporučuje da autor prije slanja rukopisa izvrši provjeru teksta na engleskom jeziku koristeći opciju „spelling and grammar". Prihvatljivi su i britanski i američki „spelling", ali on mora biti konzistentan u cijelom tekstu rada na engleskom jeziku. Prije pisanja članaka za Radove, poželjno je da autori pogledaju formu radova već objavljenih u jednom od zadnjih brojeva ili da na web stranici: www.ppf.unsa.ba (radovi.ppf.unsa.ba), pronađu uputstva sa primjerom pravilno uređenog članka.

Pridržavajući se ovih uputstava, autori ne samo da olakšavaju posao Redakciji, nego i doprinose da njihovi radovi budu pregledniji i kvalitetniji. Više informacija, autori mogu dobiti obraćanjem Redakciji na e-mail: radovi@ppf.unsa.ba.

<div align="right">Redakcija</div>

INSTRUCTION FOR WRITING PAPERS

"Radovi Poljoprivredno-prehrambenog fakulteta Univerziteta u Sarajevu" ("Works of the Faculty of Agriculture and Food Sciences of University of Sarajevo), hereinafter: "Radovi" (the "Works") is an almanac in which (original) scientific papers, exceptionally professional papers, and also some excerpts from doctoral/PhD or master theses defended at the Faculty of Agriculture and Food Sciences (the Faculty) of University of Sarajevo (Univerzitet u Sarajevu) are published.

"Radovi" (the "Works") has a character of scientific magazine and, as such, is subject to the propositions for such publications. Since its issue no. 52, "Radovi" (the "Works") has been indexed at CAB Publishing - UK.

Articles for publishing are classified, according to the recommendation by the UNESCO, into these categories: (original) scientific papers, previous statements, (scientific) review and professional papers. The authors propose the category for their articles, critics recommend it and final decision on their categorisation is made by the Editorial Board of the "Radovi" (the "Works"). (Original) Scientific papers contain results of authentic researches. Their content should be presented in such a manner that an experiment may reproduce and verify accuracy of the analyses and conclusions. Previous statements contain those significant scientific results that require urgent publishing. These researches can be shorter in time than the usual ones. (Scientific) Review papers contain an outline of certain problems on the basis of previously published texts that are analysed and discussed about in the review. Professional papers are useful articles/works from the professional domain that do not present authentic researches.

Articles are written in one of the three official languages of BiH (Bosnian/Serbian/Croatian) or English. The title of the paper should be written at the beginning of the paper (in capital letters) in one's mother tongue and in English and after that the author's name (authors' names). The author's working organisation name is written in the footnote (Ariel 7). It is mandatory to write out the category of the paper below the author's name as well.
Papers from the areas of: agricultural plant production, animal production, food technologies and sustainable development of agro-sector and rural areas are published in the journal.

It is desirable that articles of scientific character have common structure of a scientific paper, namely: summary in one of the three official languages of BiH (Bosnian/Serbian/Croatian), introduction, references (may be given in the introduction, too), material and methods, results of research, discussion (may be integrated with results of research), conclusions, bibliography and summary in English. Summary in one of the three official languages of BiH (Bosnian/Serbian/Croatian), and summary in English respectively may have maximum 200 words, with mandatory enlisting of the key words. In the list of bibliography, only authors and papers that are mentioned in the text are given. The authors' names in the text are written with expanded spacing. Latin

names of plants, animals and micro-organisms should be written in italics. Tables, graphs and pictures must have their title and also if they are numerous, their number. The number and the title of the table are written in the same row above the table while the number and the title of the graph, drawing and pictures are written below them. It is desirable to give titles, headings and explanations in the tables, graphs and pictures in the foreign language, too. Graphs and drawings should be done exclusively in black-and-white technique. Tables should be framed in lines of thickness of 1/2 pt, without shading of individual cells or rows and columns. Pictures and graphic illustrations should be done impeccably in order to be top-quality reproduced in the book.

Papers, as a rule, should not be longer than 12 typed pages (with appendices). Excerpts from master theses may be even up to 15 pages, and from doctoral/PhD theses up to 25 typed pages.

The author is responsible for the contents of the article. Prior to their publishing, articles are reviewed under "*double blind*" principle by two independent reviewers. The Editorial Board, in consultations with the authors, reserves the right to minor editorial and linguistic corrections in the articles.

The author submits one's manuscript to the Editorial Board by the means of e-mail edited according to the instructions for writing papers. On the occasion of sending papers to the Editorial Board it is obligatory to indicate the contact address and e-mail address in a separate document.

All the submitted manuscripts shall be subject to initial check in terms of meeting the criteria of the field which the magazine publishes papers from as well as technical preparation of the manuscript in accordance with the instruction to the authors.
Upon the initial check by the Editor, the submitted manuscript may be rejected without review if the Editor evaluates it is not in accordance with the journal's rules. Within the term of 20 days, the notification shall be sent to the author about either initial acceptance of the paper or reasons for its rejection.

Upon completion of the reviewing procedure which, as a rule, should not last longer than three months, the Editorial Board, on the basis of final recommendations by reviewers, makes decision on publishing the pertinent paper or not. Subsequently, the manuscript is submitted to the University's Publishing Office, where it undergoes a thorough review for potential plagiarism. The Editorial Board then informs the author about their decision, in addition to the information on the issue and term of the article publishing which the paper accepted for publishing is going to be published in.

Electronic version of the paper should be prepared in Word, in page format of 170 x 240 mm, with the following size of margins: the upper and lower ones of 2,2 cm, the left one of 2,0 cm and the right one of 1,5 cm and then the even and odd pages formatted. The font of Times New Roman, size 11, is to be exclusively used, while for footnotes the font of Arial, size 7 should be used. The text should be aligned on both sides. The title of chapters in the paper should be written in capital letters, bold and with medium alignment as well as with one row of space from the text.

While formatting the article, neither header and footer nor page numbering should be arranged.

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