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MICROCLIMATE CONDITIONS AS AN INDICATOR OF CALF WELFARE QUALITY

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Abstract: Microclimatic conditions in facilities for housing and rearing young category of breeding dairy cattle at the first 30 days after birth, have a significant impact on the quality of welfare, especially in intensive production. The parameters most often taken into account when evaluating microclimate conditions are: temperature and air humidity, the mutual relationship of which represents the THI (temperature-humid index) index; speed of air flow; air quality (presence of dust and ammonia) and level of light in the facility.

The quality of the microclimate in the facilities is directly influenced by the climatic conditions in the external environment, therefore study period on 2 farms (A and B) with an intensive production system was divided into 4 seasons (autumn, winter, spring and summer). Holstein Friesian calves were observed in the period from birth to 30 days of age.

The worst microclimatic conditions were recorded during the summer season on both farms (1129 on farm A and 1114 calves on farm B suffered), while the situation was more favorable during the colder period. Also, the best conditions, on both farms, were provided for calves in the first 7 days of life. The most unfavorable impact was the high air temperature, while the air flow, paradoxically, improved the air quality, especially during that period.

The overall welfare quality score was similar on the observed farms, 2.25 on farm A and 2.12 on farm B, which can be considered acceptable. At the same time, it indicates the presence of serious problems, the solution of which must be approached most seriously.

Key words: microclimatic factors, calves, welfare

Introduction

The concept of microclimate can be defined as a series of parameters forming the ambient conditions for living in a certain space. Therefore, they represent one of the indirect indicators of the quality of welfare, and belong to the so-called resource indicators.

The most important microclimate parameters are temperature and air humidity, as well as their mutual relationship (TH index), the speed of air flow and the appearance of drafts in the facility, the presence of dust and ammonia (NH_3) and level of light in the facility.

Air temperature and humidity, ventilation, concentration of harmful gases and dust in the air, and noise intensity in facilities where animals stay must be within limits that are not harmful to animals, taking into account the type and category of animals. Animals must be protected from adverse weather conditions and other dangers to their health, which is also regulated by the Rulebook on Animal Welfare, (2010). Special attention must be paid to the conditions in which the youngest categories (calves) stay in the first month of life.

In the report of EFSA (2006), it was precisely stated that the welfare of calves can be endangered by various factors, including the housing conditions. Recommendations are given related to the design of the farm, where thermal comfort in facilities, air quality, lighting, presence of noise, quality of accommodation, equipment in facilities, adoption of a plan of emergency measures and procedures in case of emergency must be foreseen.

Material and Methods

In order to determine the influence of microclimatic conditions on the quality of welfare of calves in the period from birth to the 30 days of life, research was carried out on 2 farms with Holstein-Friesian cows. The farms are marked with A and B. The period of one year, which was the duration of the study, was divided into four seasons, I (autumn - October, November, December), II (winter - January, February, March), III (spring - April, May, June) I IV (summer - July, August, September).

Both farms had a similar capacity for housing cows and a similar technological process of milk production, nutrition and work organization related to the housing system for dairy cows. Also, on both farms, animals were kept in a tied system on short beds.

On the first farm - A, the facilities for housing of animals were walled up, without the possibility of opening the side walls. Ventilation in buildings was natural, horizontal. Cows in the maternity ward were tied on one side of the feeding corridor. Calves were separated from their mothers 2-4 h after birth and tied to

beds on the other side of the feeding corridor. After 2-3 days, the calves were moved to pens for group housing, 10 calves were placed in a pen with a range. In the maternity ward, removal of manure was automatic, while the manure from boxes for housing of calves was manually removed.

Farm B differed from the previous farm, first of all, in the construction of the facilities. Namely, the buildings were of an open type, and if necessary, in the cold period of the year, the side walls could be closed with straw bales. Also, the calves in the maternity ward were not tied to beds but placed in individual boxes. In the nursery, the groups were in boxes with a capacity of 5 heads, without ranges.

The following microclimate parameters were monitored in the nursery facilities and the boxes with the calves in the first month of life: air temperature, air humidity, air flow and the presence of dust and ammonia. Measurements were made in 5 places at the height of the animals' heads, in the boxes for calves and along the beds in the case of tied animals. Temperature, air humidity and air flow speed were measured with the "TESTO 410-2" instrument. Depending on the deviation from the standard, the measured values of the mentioned parameters were graded from 5 to 0, where: 5 - excellent, 4 - very good, 3 - good, 2 - satisfactory, 1 - unsatisfactory, but with a possibility to improve, and 0 – unsatisfactory and without the possibility to improve (EFSA, 2006). Air quality was subjectively assessed based on the concentration of ammonia and the presence of dust particles in the air that could be registered by the sense of smell.

Only cases that deviate from the optimal level were analysed in the study (grades excellent and very good, 5 and 4), i.e. parameters that were evaluated with lower grades and that had caused the reduced quality of calf welfare, grades from 0 to 3. When it comes to temperature, these are values below 8^0C and above 32^0C, and for air humidity above 80%.

Calves were grouped based on age into 5 groups: 0-7 days (while housed in the nursery), 8 days (transfer to rearing facility), 15, 22 and 30 days.

Quality of welfare was assessed at the end of the one-year trial period.

Results and Discussion

The obtained research results are presented in the following tables. Table 1 shows the frequency of exposure of calves in the first month of life to adverse microclimatic conditions on farm A, depending on the calving season.

Table 1. Number of calves exposed to adverse effects of microclimatic factors in the first month of life, observed by age and season of birth on farm A

	A					
Age, day	**0-7**	**8**	**15**	**22**	**30**	**Σ**
I Season - Autumn						
Temperature						
Air humidity						
Air flow		75	75	34	15	**199**
Dust and ammonia						
Σ		**75**	**75**	**34**	**15**	**199**
II Season - Winter						
Temperature		24	30	11		**65**
Air humidity						
Air flow			9	28	30	**67**
Dust and ammonia						
Σ		**24**	**39**	**39**	**30**	**132**
III Season - Spring						
Temperature	21	32	41	42	20	**156**
Air humidity						
Air flow	25	20		11	28	**84**
Dust and ammonia			8	21	11	**40**
Σ	**46**	**52**	**49**	**74**	**59**	**280**
IV Season – Summer						
Temperature	67	72	63	42	48	**292**
Air humidity	20					**20**
Air flow		26	50	34	30	**140**
Dust and ammonia	20	21		25		**66**
Σ	**107**	**119**	**113**	**101**	**78**	**518**
ΣΣ, Disturbed microclimate	153	270	276	248	182	1129

Observed by the seasons, on farm A, it can be stated that in the first season - autumn (October, November, December), the situation was the most favourable in respect to the parameters - air temperature and humidity. Only increased air flow was recorded, which affected a total of 199 heads at the age of 8 to 30 days. In the second season - winter (January, February, March), 65 calves were exposed to low temperatures, below 8^0C, and 67 were kept in a facility with increased air flow, which in low temperature conditions had an additional adverse effect on the calves' comfort. The third season, spring, included months of April, May and June. In this

season, 156 calves of all ages were exposed to high temperatures, above 25^0C, 84 were exposed to increased air flow, and 40 of them, older than 15 days, were found in facilities with an increased amount of dust and ammonia in the air. The fourth season, summer, was the most unfavourable for the welfare of the calves in the first month of life, because it included three warm months, July, August and September, with very high temperatures, above 30^0C, (292 calves exposed) and the presence of dust and ammonia was recorded (66 heads exposed). In these circumstances, increased air flow (140 calves exposed) had a positive impact on the quality of the microclimate in the calf facilities.

Observed by age, calves at the earliest age, immediately after birth (2-3 days) and at the age of 30 days, were exposed to adverse microclimate factors in a slightly smaller number (153 and 182, respectively) than calves at other ages (270, 276 and 248 head at the age of 8, 15 and 22 days, respectively).

The values of the observed parameters at farm B are given in Table 2. At the farm B, in the first season, the situation was less favourable than at farm A, because 122 calves of all age categories were exposed to the effects of inadequate temperature, and 100 of them to the effects of increased air flow speed. Moreover, in October the temperatures were above 25^0C (42 calves), and in December lower than 8^0C (80 calves exposed). In the second season, 105 calves stayed in facilities with a temperature below the bottom limit, 7 with increased air humidity and 11 with increased air flow. In the third season, in June, 150 calves were exposed to a temperature above 25^0C. Also, 32 calves stayed in a space with an increased % of air humidity, and 78 breathed air with a high concentration of dust and ammonia. The fourth season, as in farm A, was the most unfavourable in terms of microclimatic conditions. Total of 172 calves were exposed to high temperature in the facilities, 95 of them were exposed to increased air humidity, and 73 were exposed to poor air quality with increased dust and ammonia content. In this case too, the increased air flow (169 heads) had a positive effect on the conditions in the facilities.

Immediately after birth (2-3 days) the calves were kept in the best microclimatic conditions, only 130 cows were exposed to the adverse influence of microclimatic factors. The needs of the 30-day-old calves were the most difficult to meet because 331 heads were under the influence of adverse microclimatic conditions.

Table 2. Number of calves exposed to adverse effects of microclimatic factors in the first month of life, observed by age and season of birth on farm B

	B					
Age, day	**0-7**	**8**	**15**	**22**	**30**	**Σ**
I Season - Autumn						
Temperature	26	17	34	26	19	**122**
Air humidity						
Air flow		53	26		21	**100**
Dust and ammonia						
Σ	**26**	**70**	**60**	**26**	**40**	**222**
II Season - Winter						
Temperature		17	25	38	25	**105**
Air humidity				2	5	**7**
Air flow	11					**11**
Dust and ammonia						
Σ	**11**	**17**	**25**	**40**	**30**	**123**
III Season - Spring						
Temperature	33	31	26	21	39	**150**
Air humidity		19	9	4		**32**
Air flow						
Dust and ammonia		18	20	18	22	**78**
Σ	**33**	**68**	**55**	**43**	**61**	**260**
IV Season - Summer						
Temperature	40	46	31		55	**172**
Air humidity		19		22	54	**95**
Air flow	7	22	50	26	64	**169**
Dust and ammonia	13	19		14	27	**73**
Σ	**60**	**106**	**81**	**62**	**200**	**509**
ΣΣ, Disturbed microclimate	130	261	221	171	331	1114

Figure 1 shows the share of the number of calves that were exposed to the negative influence of microclimatic factors in relation to the total number of calves by farms, seasons and days of age.

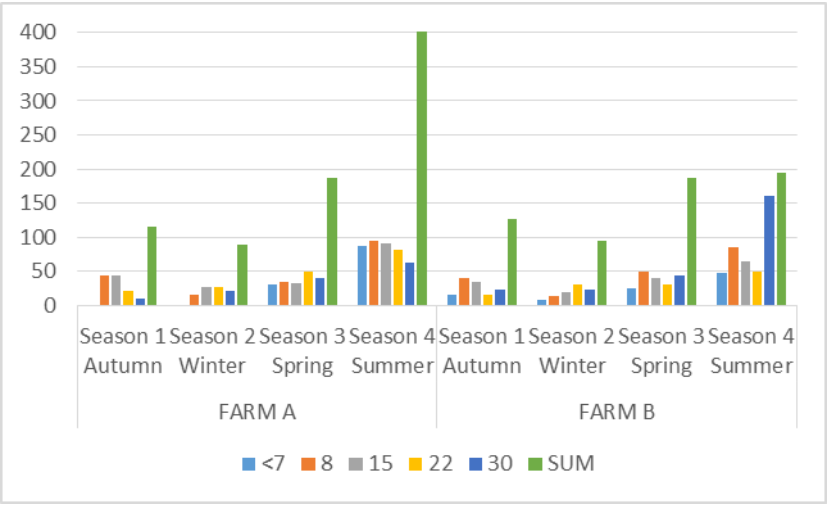

Figure 1. Number of calves exposed to the negative influence of microclimatic factors in relation to the total number of calves by farms, seasons and age

On both farms, within the overall assessment of the quality of calf welfare, the microclimate factors were rated "acceptable", 2.25 on farm A and 2.12 on farm B, which indicates major problems, but also major opportunities for improving conditions in facilities for calves in the first month of life.

Air temperature and humidity can be considered key factors of microclimate for several reasons. In case of high temperature, food intake decreases, metabolism slows down, food conversion decreases. High temperature with increased air humidity leads to an increase of the TH index. A value of the TH index above 72 leads to the appearance of heat stress, which occurs already at a temperature of 25^0C if the air humidity is over 50%, and heat stress is considered one of the most common factors that threatens the quality of animal welfare. Numerous authors have dealt with the problems caused by increased temperature and air humidity in farm facilities buildings and their impact on the quality of animal welfare.

The results obtained in the research are largely in accordance with numerous published research results, especially with those conducted in the same or similar climate zone. Also, the recorded conditions slightly deviated from the standard recommended by the Rulebook on Animal Welfare (2010), which states that the optimal air temperature in the facility for raising young cattle is 15 to 20°C with a relative humidity of 70 to 75% and an air flow speed of 0.1 to 0.3 m/s.

According to Beskorovajni et al. (2012), in research conducted in very similar conditions, the values of microclimatic parameters were in the interval from -16.3 to 38°C, temperature, and from 53 to 100%, air humidity, while the THI

index was between 53.4 and 94.0. According to numerous studies such as Bernabucci et al. (2010), Nemečkova et al. (2013), Kamal et al. (2014), Mijić et al. (2014), Dado-Senn et al. (2020), Samolovac et al. (2023), heat stress can be prevented by improving ambient conditions, primarily by good ventilation and providing natural or artificial shade although there are some contrary statements like Montevecchio et al. (2023).

On the other hand, lower-than-optimal temperatures also have multiple effects on the general condition of animals, on the rate of morbidity and mortality in the herd, on growth, etc. In the case when the temperature is lower than optimal, animals are forced to spend metabolic energy on preserving body temperature and maintaining basal metabolism as stated by Hepola et al. (2006). According to Borderas et al. (2009) and Bonizzi et al. (2020) in some cases there is also an increase in the rate of morbidity and mortality in the herd. Similar results are reported by Samolovac et al. (2019), when it comes to the effect of the season on the incidence of diseases of the digestive and respiratory system. Also, there are contrary statements made by Bickert et al. (2011) which indicate that low temperature has no negative impact if there is no draft in the buildings and if the animals are placed on a dry and clean litter.

Both temperature extremes, high and low temperature, especially if they are accompanied by high air humidity, give the same results from the point of view of economic production. Feed conversion decreases, gain and growth decrease, the degree of morbidity and mortality in the herd increases, which leads to an increase in the cost price of the product and a decrease in the quality of the final product as stated by Roland et al. (2016). Therefore, there is a constant aspiration to improve the conditions of keeping/housing in order to maximize the use of the genetic potential for high quality production of farmed animals, especially from the aspect of health status and efficiency of food utilization as reported by Hristov et al. (2011), De Vries et al. (2013), Petrovska et al. (2018).

The comfort zone for lactating cows is between 10 and 20^0C, even up to -5^0C according to Samolovac, (2016) while some authors like Majkić et al. (2017) state -16 to -37°C as the critical lower temperature limit. Younger categories (calves up to the 30 days of life) should be kept in conditions where the optimal temperature is in the range of 15-20°C, the optimal relative humidity is in the 70-75% range and the optimal speed of air flow is from 0.1 to 0.3 m /s as reported by Samolovac (2016), while Wang et al. (2020) state the temperature optimum for calves in the first month of life is from 13 to 25°C. When it comes to the top temperature limit above which the conditions for the occurrence of hyperthermia are created, it is around 25°C as reported by Kadzere et al. (2002). According to the data reported in the literature in the middle of the last century as of Beakley and Findlay (1955), Aishir calves reacted to an increase in temperature over 20^0C with rapid breathing, an increase in pulse frequency and an increase in rectal temperature.

When it comes to the presence of dust and ammonia in the air, it is inevitable considering the type of production. Every activity of people and animals in the facility leads to the creation of dust of organic origin (food, litter/bedding, animal body surface - skin and hair, dried faeces, etc.), while ammonia, along with some other gases (most often CO_2), occurs as a product of metabolism of ruminants and chemical processes in manure.

The quality of the air in the building directly depends on the ventilation and air flow, which ensure the exchange of polluted air from the building with clean air. Proper ventilation also reduces the concentration of pathogenic microorganisms in the air, which, according to various authors such as Bickert (2002), Lundborg et al. (2005), Lago et al. (2006), and Nordlund (2008), is also a measure of air quality, However, if it happens that the air flow is directed directly at the animals, if it is too strong (speed greater than 0.3 m/s) or the air temperature is very low, the air flow can have a negative impact on the health and welfare of the animals, especially younger categories.

If optimal microclimate conditions are provided in facilities for housing calves, the first prerequisite for raising healthy animals is met, whose genetic production potential will be maximal due to the positive effect on health, metabolism, feed conversion and animal welfare. Despite modern knowledge and production technology, it is still difficult to ensure optimal conditions for high quality welfare of calves according to Hristov et al. (2011), De Vries et al. (2013), Petrovska et al. (2018). Although cattle, as a species, have a pronounced ability to adapt to different microclimatic conditions, as stated by a number of authors in their studies like Beakley and Findlay (1955), Kadzere et al. (2002), Samolovac (2016), Majkić et al. (2017), Angel et al. (2018), Wang et al. (2020) this does not mean that they are insensitive and that they should not be provided with the best conditions in facilities, especially when it comes to the youngest categories, i.e. calves in the first month of life. As reported by Roland et al. (2016) any impact on the health, production capacity and welfare of animals, positive or negative, has an indirect impact on the economics of production.

Conclusion

Observing the microclimate parameter that had the most influence on the quality of rearing and the level of quality of calf welfare in the first 30 days of life on farms with an intensive method of production in closed facilities, it can be concluded that:

- Air temperature is one of the most important factors. It exceeded 30^0C on observed farms and dropped below 10^0C in facilities, depending on the season.
- The relative humidity sometimes exceeded 80%, which indicates that there is a risk of heat stress in the calves

- The appearance of increased air flow was recorded, which was expected considering that the ventilation in the facilities was natural, horizontal. In the cold period of the year, this phenomenon had a negative effect on the state of the microclimate, but in the warm summer months, it improved the overall air quality in the facilities.
- As a consequence of the lack of a ventilation system (except for natural ventilation), an increased concentration of ammonia dust in the air was observed, especially in the summer period.
- On both farms, in all four seasons, the most favorable microclimate conditions were provided to the youngest category, calves in the first 2-3 days after birth.
- On both farms, in facilities for rearing calves, more favorable microclimatic conditions were recorded during the colder period of the year.

Considering the importance of quality rearing of young animals on the overall efficiency of cattle production, as well as the importance of microclimatic factors on the conditions of rearing young animals, it is necessary to improve the quality of microclimatic factors. Along with the regulation of temperature and air humidity, it is important to introduce additional methods of ventilation, and in the summer months, cooling of animals. The use of good quality litter/bedding and adequate equipment are baseline prerequisites.

Mikroklimatski uslovi kao indikator kvaliteta dobrobiti teladi

Ljiljana Samolovac, Slavča Hristov, Dragan Nikšić, Dušica Ostojić-Andrić, Marina Lazarević, Nenad Mićić, Vlada Pantelić

Rezime

Mikroklimatski uslovi u objektima za smeštaj i odgoj priplodnog podmladka mlečnih goveda u najranijem uzrastu (prvih 30 dana nakon rođenja) imaju značajan uticaj na kvalitet dobrobiti, a samim tim i na kvalitet života životinja, posebno u intenzivnom načinu proizvodnje.. Parametri koji se najčešće uzimaju u obzir kod ocene mikroklimatskih uslova su: temperatura i vlažnost vazduha, čiji međusobni odnos predstavlja THI (temperaturno humidni indeks) indeks; brzina strujanja vazduha; kvalitet vazduha (prisustvo prašine i amonijaka) i osvetljenost. Kvalitet mikroklime u objektima je pod direktnim uticajem klimatskih uslovu u spoljnoj sredini, tako da je period istraživanja na 2 farme (A i B) sa intenzivnim sistemom proizvodnje podeljen na 4 sezone (jesen, zima, proleće i leto). Posmatrana su telad Holštajn frizijske rase u periodu od rođenja do 30 dana života. Najlošiji

mikroklimatski uslovi su zabeleženi tokom letnje sezone na obe farme, dok je situacija bila povoljnija tokom hladnijeg perioda. Takođe, najbolji uslovi, na obe farme, su obezbeđeni za telad u prvih 7 dana života. Najnepovoljniji uticaj je imala visoka tempertura vazduha, dok je strujanje vazduha, paradoksalno, popravljalo kvalitet vazduha, naročito tokom tolijeg perioda. Ukupna ocena kvaliteta dobrobiti bila je slična na posmatranim farmama, 2,25 na farmi A i 2,12 na farmi B, što se može smatrati prihvatljivim. Istovremeno ukazuje na postojanje ozbiljnih problema čijem se rešavanju mora najozbiljnije pristupiti. Obzirom na značaj odgoja najmlađih kategorija životinja neophodno je unaprediti kvalitet mikroklimatskih faktora, kako bi se dobile što kvalietnije i zdravije jedinke koje će biti kasnije uključene u proizvodnju mleka.

Ključne reči: mikroklimatski faktori, telad, dobrobit

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Conflict of interest
The authors declare that they have no conflict of interest.

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IMPACT OF CLIMATE FACTORS, BREED, AND BOAR UTILIZATION FREQUENCY ON SEMEN QUALITY AND SPERM MORPHOLOGY

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Abstract: The primary objective of this research was to evaluate boar ejaculate variability and the occurrence of anomalies in spermatozoa, considering climatic factors during spermatogenesis breed, and utilization frequency. This study involved 17 boars (n=129 ejaculates) and fertility testing was conducted during the most critical period of the year, from August to October. The observed sperm characteristics included: ejaculate volume (VOL), sperm concentration (CON, spermatozoa/ml), total number and number of functional spermatozoa (NT, NF), percentage of sperm motility in the native ejaculate and after dilution (MOTN, MOTD), number of produced doses (NPD), percentage of dead and live spermatozoa (PM, PZ), and sperm anomalies. The assessment of the effect was performed using a General Linear Model procedure. The breed did not influence sperm variability, while the frequency of boar utilization impacted on the occurrence of secondary anomalies. The determined regression coefficient indicated that extending the interval by one day increased PPPK by 0.340-0.348%. The maximum daily temperature during semen collection (model 1) and the value of the TH index during semen collection (model 3) influenced ejaculate volume. An increase of one ℃ in temperature, or one unit in THI value, led to a ($p<0.05$) increase in VOL by 3.540 ml and 2.798 ml, respectively. Furthermore, the maximum daily temperature (model 2) and the TH index value (model 4) at the beginning of the epididymal phase of spermatogenesis had an impact on semen motility, as well as the percentage of live and dead spermatozoa.

Key words: boar, spermatogenesis, sperm anomalies, temperature, THI

Introduction

Modern pig farming is based on highly productive breeds with the application of modern technical and technological solutions and biotechnological methods in rearing and reproduction (Savić et al., 2015). The most common biotechnological method used in reproduction is artificial insemination (AI), which is the primary method of reproduction in intensive pig production (Lopez Rodriguez et al., 2017).

Based on the fact that the application of AI enables obtaining of a large number of offspring from one boar, the aim is to include boars with the best possible reproductive and production performance in this process (Apić et al., 2016; Gadea et al., 2005). This can be achieved by permanent monitoring of the fertility of boars and timely culling of animals that are below the average of the studied population (Savić, 2014). Therefore, it is necessary to know the characteristics of the most important ejaculate properties, as well as the factors that influence their variability. The quality and quantity of semen used for artificial insemination are determined by its main parameters: volume, concentration of spermatozoa, live spermatozoa count and number of doses for insemination obtained from one ejaculate after dilution (Smital et al., 2004). In addition, the most important qualitative and quantitative properties of the ejaculate include: sperm motility, percentage of pathological spermatozoa, percentage of dead spermatozoa, total number of spermatozoa in the ejaculate and number of functional spermatozoa (Savić and Petrović, 2019).

There are various spermatozoa defects: spermatozoa with a proximal droplet, with a distal droplet, with an incomplete abnormal head, pear-shaped, short and wide, with a large head, with an acrosomal defect, etc. (Savić, 2014). According to their origin, spermatozoa malformations are divided into two groups: primary malformations, if they arise in the testis during spermatogenesis, and secondary if their genesis takes place in the epididymis during the sperm maturation process (Briz et al., 1996).

A number of genetic and non-genetic factors influence the variability of ejaculate properties. The breed of the boar is certainly one of the most important genetic factors that affect the variability of sperm properties (Cierezko et al., 2000; Stančić et al., 2003; Okere et al., 2005; Wolf and Smital, 2009; Wolf, 2009; Savić et al., 2013; Caisin and Snitco, 2016). In addition to the breed of the boar, the intensity of the boar use has a significant influence on the variation of the properties of the ejaculate. Frequent use of the boar semen negatively affects the quality of the sperm (Lopez Rodriguez et al., 2017). High temperatures also have a negative effect on the development of the spermatogenesis process (Kunavongkrit et al., 2005; Gogić, 2020). As a result of the elevated temperature of the environment, morphological defects occur more frequently, sperm motility is reduced, and overall fertility is lower (Flowers, 2015; Shahat et al., 2020).

However, some studies indicate that not all ejaculate properties vary under the influence of high temperature, such as ejaculate volume and total sperm count (Watteman et al., 1976; Larsson and Einarsson, 1984; Wettemann et al., 1985). The differences in the results obtained in the mentioned researches indicate that it may be necessary to conduct additional research of the influence of temperature and air humidity on the variability of the most important sperm properties.

The aim of this research was to determine whether climatic factors, boar breed and intensity of exploitation influence the variability of ejaculate properties and the occurrence of spermatozoa anomalies.

Materials and Methods

The research was conducted on a pig farm consisting of a reproductive and commercial farm. The boars were placed in a special facility with microclimatic conditions under the control of a semi-automatic ventilation system, using vertical and horizontal ventilation. The animals were fed balanced feed mixtures, and had unlimited access to fresh water.

The research included 17 boars of two different breeds (4 Landrace and 13 Large White). A total of 129 ejaculates were analyzed, but the number of examined ejaculates varied based on observed traits. The fertility of those boars was examined in the time interval from August to October, which represents the most critical period of the year. Each boar had to have at least three successful jumps during the trial period to be included in the analysis. The interval between two sperm collections was average 9 days. The examination included the following parameters: ejaculate volume (VOL, ml), sperm concentration (CON, $x10^6$ spermatozoa/ml), total spermatozoa count in the ejaculate (NT, $x10^9$ spermatozoa; NT=VOL x CON), total functional spermatozoa count in the ejaculate (NF, $x10^9$ spermatozoa; NF=NT x MOTN), percentage of motility of spermatozoa mass in native state (MOTN, %), percentage of motility of spermatozoa mass after dilution (MOTD, %), number of doses produced (NPD), percentage of dead spermatozoa (PM, %), percentage of live spermatozoa (PZ, %), percentage of spermatozoa with protoplasmic droplets (PPK, %), percentage of spermatozoa with a pathological shape on the tail (PPLJR, %), percentage of spermatozoa with pathological changes on the head (POTKG, %) and percentage spermatozoa with pathological changes on the acrosomal part (PAKR, %). The volume of the ejaculate was measured with a graduated cylinder, and was expressed in milliliters with an accuracy of ±2 ml. The concentration of native sperm was assessed using a photo-colorimeter (Magacell, Magapor, Spain). The native sperm was diluted using a commercial diluent Vitasem (Magapor, Spain). The assessment of the motility of the mass of spermatozoa in the native ejaculate and after dilution was performed by subjective assessment, and observed under a microscope (BA410, Motic®, America, under a

×100 objective). The total number of sperm in the ejaculate was obtained by multiplying the volume of the ejaculate with the concentration. The number of functional spermatozoa was calculated by multiplying the total number of spermatozoa by the percentage of motility of the mass of spermatozoa in the native state. The percentage of dead and live spermatozoa in the semen was determined by observing a permanent preparation under a microscope (BA410, Motic®, America, under a ×400 objective) following staining with eosin-nigrosin (Savić and Petrović, 2019). During staining, dead spermatozoa are partially or completely stained, while live spermatozoa are visible on a dark background without staining.

Evaluation of the presence of pathological forms was performed by microscopic examination on the same permanent preparation, under an adequate immersion lens, and the total number of examined spermatozoa was 100. The number of spermatozoa with a certain anomaly was presented as a relative share of the total number.

For the entire process of spermatogenesis, i.e., the formation of mature spermatozoa from spermatogonia, according to Parrish et al. (2017), a 45 day value was taken, whereby the epididymal phase represented the last ten days of spermatogenesis (36-45 days).

Climatic data (temperature and humidity) were obtained from the weather station in Veliko Gradište near the farm and were available online (https://www.infoclimat.fr/climatologie-mensuelle/13285/juillet/2013/veliko-gradiste.html). TH index was calculated according to the formula given by Lallo et al. (2018):

$$THI = T_{max} - (0.55-(0.0055\ RH)(T_{max} - 14.5))$$

Where:

T_{max} = max temperature (℃), RH = relative air humidity (%).

The assessment of the influence of the factors on the variation of the examined properties was performed using the General Linear Model in the statistical package SAS 9.3 (SAS Inst. Inc., 2002-2010), where the following models were used:

1. $y_{ij} = \mu + B_i + b_1(x_{ij}-\bar{x}) + b_2(x_{ij}-\bar{x}) + e_{ij}$
2. $y_{ij} = \mu + B_i + b_1(x_{ij}-\bar{x}) + b_3(x_{ij}-\bar{x}) + e_{ij}$
3. $y_{ij} = \mu + B_i + b_1(x_{ij}-\bar{x}) + b_4(x_{ij}-\bar{x}) + e_{ij}$
4. $y_{ij} = \mu + B_i + b_1(x_{ij}-\bar{x}) + b_5(x_{ij}-\bar{x}) + e_{ij}$

Where:

μ = average value; B_i = fixed effect of boar breed; $b_1(x_{ij}-\bar{x})$ = linear regression effect of the interval between two jumps; $b_2(x_{ij}-\bar{x})$ = linear regression effect of maximum daily air temperature during sperm collection; $b_3(x_{ij}-\bar{x})$ = linear regression effect of the maximum daily air temperature at the beginning of the

epididymal phase of spermatogenesis (36th day); $b_4(x_{ij}-\bar{x})$ = linear regression effect of THI values when collecting sperm; $b_5(x_{ij}-\bar{x})$ = linear regression influence of THI values at the beginning of the epididymal phase of spermatogenesis (36th day); e_{ij} = random error.

Results and Discussion

The differences in all observed properties between the ejaculates included in the research were recorded, as indicated by the absolute and relative indicators of variability (Table 1).

Table 1. Basic statistical parameters of examined sperm properties

Properties	$\bar{x}$	SD	CV (%)	Minimum	Maximum
VOL (ml)	292.18	95.45	32.67	50.00	520.00
CON ($x10^6$/ml)	366.65	98.60	26.89	190.00	707.00
NT ($x10^9$)	107.49	43.72	46,68	17.50	225.00
NF ($x10^9$)	90.75	36.47	40.18	13.12	191.83
MOTN (%)	83.96	7.16	8.52	65.00	100.00
MOTD (%)	78.18	9.57	12.24	20.00	95.00
NPD	15.26	4.67	30.62	4.00	30.00
PM (%)	23.80	12.38	52.01	2.00	79.09
PZ (%)	76.19	12.38	16.24	20.91	98.00
PPK (%)	13.57	10.20	75.15	0	40.00
PPLJR (%)	2.47	3.06	123.79	0	17.00
POTKG (%)	1.32	1.84	138.99	0	8.00
PAKR (%)	0.19	0.71	368.58	0	6.00

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - native sperm motility, MOTD - sperm motility after dilution, NPD - number of produced doses, PM - percentage of dead spermatozoa, PZ - percentage of live spermatozoa, PPK - percentage of spermatozoa with a protoplasmic drop, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part; $\bar{x}$ - average value, SD - standard deviation; CV - coefficient of variation

Properties representing the occurrence of anomalies on spermatozoa (PPK, PPLJR, POTKG, PAKR) had very high coefficients of variation. The majority of all the examined anomalies were secondary (PPK; $\bar{x}$=13.57%), which occur during the maturation of spermatozoa in the epididymal phase of spermatogenesis.

In this research, PPK, unites spermatozoa with proximal and distal protoplasmic droplets, as a secondary form of anomaly. The position of the protoplasmic droplet may indicate the level of "maturity" of the spermatozoa. Namely, spermatozoa with a proximal droplet originate from the testis, with the movement of the spermatozoa toward the epididymis the drop also moves toward the distal parts of the spermatozoa tail. With the arrival of such spermatozoa into the tail of the epididymis, the distal protoplasmic droplet disappears and they take on the appearance of mature spermatozoa. Based on this, it can be concluded that the ratio of spermatozoa to proximal and distal protoplasmic drops can indicate the extent to which the epididymal sperm has matured.

The incidence of a higher proportion of abnormal forms of the secondary type and immature spermatozoa is related to the unfavorable influence of stressogenic factors. According to Savić and Petrović (2019), exposure of the animal to temperatures above 27℃ for several days can cause thermal stress that leads to an increase in abnormal forms of spermatozoa as reported by Wetteman et al. (1979), Larsson and Einarsson (1984), Flowers (1997), Flowers (2015), Gogić (2020), Shahat et al. (2020).

Table 2. Statistical significance of the effects included in models 1 and 3

Sperm property	MODEL 1			MODEL 3		
	Linear regression effect IUS	Linear regression effect Max T Uz	R^2	Linear regression effect IUS	Linear regression effect THI Uz	R^2
VOL (ml)	0.176^{NS}	3.540^{*}	0.042	0.164^{NS}	2.798^{*}	0.044
CON (x10^6/ml)	1.905^{NS}	-1.258^{NS}	0.039	1.964^{NS}	-0.736^{NS}	0.038
NT (x10^9)	0.613^{NS}	0.589^{NS}	0.024	0.625^{NS}	0.537^{NS}	0.026
NF (x10^9)	0.408^{NS}	0.101^{NS}	0.025	0.430^{NS}	0.187^{NS}	0.026
MOTN (%)	-0.061^{NS}	-0.206^{NS}	0.045	-0.053^{NS}	-0.126^{NS}	0.038
MOTD (%)	-0.079^{NS}	-0.444*	0.056	-0.064^{NS}	-0.282^{NS}	0.040
NPD	-0.013^{NS}	0.067^{NS}	0.020	-0.011^{NS}	0.062^{NS}	0.023
PM (%)	-0.214^{NS}	0.296^{NS}	0.036	-0.220^{NS}	0.198^{NS}	0.033
PZ (%)	0.214^{NS}	-0.296^{NS}	0.036	0.220^{NS}	-0.198^{NS}	0.154
PPK (%)	0.348^{*}	0.197^{NS}	0.052	0.340^{*}	-0.013^{NS}	0.052
PPLJR (%)	0.044^{NS}	-0.019^{NS}	0.023	0.046^{NS}	-0.008^{NS}	0.022
POTKG (%)	0.026^{NS}	0.036^{NS}	0.016	0.027^{NS}	0.029^{NS}	0.018
PAKR (%)	-0.013^{NS}	0.009^{NS}	0.038	-0.001^{NS}	0.007^{NS}	0.038

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - native sperm motility, MOTD - sperm motility after dilution, NPD - number of produced doses, PM - percentage of dead spermatozoa, PZ - percentage of live spermatozoa, PPK - percentage of spermatozoa with a protoplasmic drop, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part; IUS - the interval between two semen collection, Max T Uz - maximum daily air

temperature during sperm collection, THI Uz - THI value during sperm collection; R^2 - coefficient of determination, Statistical significance: ^{NS} - not significant, * - $p<0.05$

The interval between two sperm collections in both models influenced only the incidence of secondary anomalies - PPK (Table 2). The value of the determined regression coefficient indicates that by extending the interval by one day, the percentage of spermatozoa with a protoplasmic drop increases by 0.340-0.348%. This implies that non-rational boar exploitation can lead to the appearance of a greater proportion of spermatozoa with pathological changes. This is exactly what Savić and Petrović (2019) point out, who state that stressful situations or non-rational use of boars can lead to a decrease in the flow of testosterone at the level of the testes, which leads to the appearance of abnormal forms of spermatozoa or their insufficient maturation. According to Henning (2021) the incomplete maturation of epididymal sperm impairs the ability of *in vitro* capacitation and promotes the destabilization of sperm in stored boar semen.

The maximum daily temperature during semen collection (Model 1) and the TH index value during semen collection (Model 3) influenced the ejaculate volume. Increasing the temperature by 1℃, i.e. THI value by one unit, increased ($p<0.05$) VOL by 3.540 ml, and by 2.798 ml, respectively. On the other hand, an increase in temperature by one unit leads to a decrease in the mass of sperm motility (MOTD) by 0.444%. The low values of the coefficients of determination (<5.2 – Model 1; <15.4 – Model 3), determined by the applied models, indicate that the examined influences explain the variability of the examined sperm properties to a lesser extent. These results are partially similar to those reported by Larsson and Einarsson (1984). Namely, in their research, the ejaculate volume did not vary under the influence of elevated ambient temperature, which is the case in our research, however, there is agreement between the results related to reduced mobility due to elevated temperatures. Wettemann et al. (1985) also report no effect of breed on ejaculate variability. According to Suriyasomboon et al. (2004) the difference in the obtained results may be due to different duration of exposure to elevated temperature, or individual differences in the susceptibility of boars to stress caused by high temperatures.

The maximum daily temperature (Model 2) and the value of the TH index (Model 4) at the beginning of the epididymal phase of spermatogenesis had an impact on the motility properties of native ejaculat and semen after dilution, as well as on the percentage of live and dead spermatozoa (Table 3).

Table 3. Statistical significance of the effects included in models 2 and 4

Sperm property	MODEL 2			MODEL 4		
	Linear regression effect IUS	Linear regression effect Max T Ep	R^2	Linear regression effect IUS	Linear regression effect THI Ep	R^2
VOL (ml)	0.109^{NS}	1.641^{NS}	0.016	0.022^{NS}	1.135^{NS}	0.012
CON (x10^6/ml)	1.700^{NS}	-1.407^{NS}	0.045	1.713^{NS}	-1.152^{NS}	0.045
NT (x10^9)	0.597^{NS}	0.283^{NS}	0.022	0.578^{NS}	0.184^{NS}	0.021
NF (x10^9)	0.349^{NS}	-0.144^{NS}	0.026	0.345^{NS}	-0.135^{NS}	0.026
MOTN (%)	-0.012^{NS}	-0.336^{**}	0.112	-0.115^{NS}	-0.256^{NS}	0.103
MOTD (%)	-0.166^{NS}	-0.538^{***}	0.135	-0.153^{NS}	-0.418^{**}	0.127
NPD	-0.029^{NS}	-0.025^{NS}	0.015	-0.033^{NS}	0.030^{NS}	0.017
PM (%)	-0.049^{NS}	0.693^{***}	0.154	-0.059^{NS}	0.540^{**}	0.149
PZ (%)	0.049^{NS}	-0.693^{***}	0.154	0.059^{NS}	-0.540^{**}	0.149
PPK (%)	0.340^{NS}	-0.011^{NS}	0.052	0.337^{NS}	-0.017^{NS}	0.052
PPLJR (%)	0.066^{NS}	0.063^{NS}	0.040	0.065^{NS}	0.057^{NS}	0.038
POTKG (%)	0.019^{NS}	-0.007^{NS}	0.007	0.019^{NS}	0.005^{NS}	0.007
PAKR (%)	-0.013^{NS}	0.002^{NS}	0.034	-0.001^{NS}	0.006^{NS}	0.035

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - native sperm motility, MOTD - sperm motility after dilution, NPD - number of produced doses, PM - percentage of dead spermatozoa, PZ - percentage of live spermatozoa, PPK - percentage of spermatozoa with a protoplasmic drop, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part; IUS - the interval between two semen collection, Max T Ep - maximum daily air temperature during the epididymal phase, THI Ep - THI value during the epididymal phase; R^2 - coefficient of determination, Statistical significance: ^{NS} - not significant, **- p<0.01, ***-p<0.001

In model 2, based on the estimated regression coefficient, increasing the maximum daily temperature by 1℃, decreases (p<0.01; p<0.001) motility (MOTN by 0.336%, MOTD by 0.538%) as well as PZ by 0.693%, which indicates the negative influence of high temperatures during the epididymal phase of spermatogenesis on spermatozoa. A similar regularity is observed when examining the influence of the TH index at the beginning of the epididymal phase of spermatogenesis (reduction in the motility of the mass of spermatozoa after dilution and reduction in the percentage of live spermatozoa). The low values of the determination coefficients obtained by these applied models also indicate that the studied influences explain to a lesser extent the variability of the studied sperm properties.

The interval between two consecutive sperm collections did not affect the variation of the examined sperm properties, which is in contrast to numerous previous studies, showing that the intensity of boar exploitation had an effect on: volume as reported by Smital (2010), Savić and Petrović (2015), Savić et al.

(2015), concentration according to Wolf and Smital (2009), the total spermatozoa count in the ejaculate as reported by Wolf and Smital (2009), Savić et al. (2015), the functional spermatozoa count in the ejaculate according by Smital (2010).

Regardless of which model was applied, the breed of the boar did not affect the average expression and variability of the tested traits (Table 4). The results obtained are contrary to numerous studies such as Stančić et al. (2003), Okere et al. (2005), Smital (2010), Savić and Petrović (2015) which indicate the variation of ejaculate volume under the influence of boar breed. It is similar to sperm concentration (p=0.354), which is contrary to research by Chinchilla-Vargas et al. (2018) and Savić et al. (2020).

This research is in agreement with the research of Savić (2014), who concluded that the mobility of native semen and semen after dilution did not vary under the influence of boar breed. In contrast, in the research of Okera et al. (2005),breed has a significant effect on the variation of native semen motility. Also, Savić et al. (2020) state that the breed exerted an influence on the variability of semen mobility after dilution. Knecht et al. (2014*)* find in their research that the percentage of dead and live spermatozoa varies under the influence of the boar breed, so the results of this research differ from the aforementioned.

Table 4. The average expression of sperm properties under the influence of the boar breed evaluated by the applied models

Sperm property	MODEL 1			MODEL 2			MODEL 3			MODEL 4		
	Boar breed LSMean		p	Boar breed LSMean		p	Boar breed LSMean		p	Boar breed LSMean		p
	VJ	L		VJ	L		VJ	L		VJ	L	
VOL (ml)	304.76	305.19	0.980	304.66	305.40	0.967	304.74	305.23	0.978	304.69	305.33	0.971
CON ($x10^6$/ml)	372.72	350.80	0.354	372.68	350.87	0.354	372.78	350.67	0.354	372.65	350.95	0.357
NT ($x10^9$)	116.71	107.11	0.343	116.67	107.18	0.349	116.70	107.11	0.343	116.67	107.18	0.349
NF ($x10^9$)	98.36	88.12	0.241	98.32	88.18	0.245	98.37	88.09	0.239	98.32	88.20	0.246
MOTN (%)	84.18	81.68	0.106	84.19	81.66	0.090	84.18	81.68	0.107	84.19	81.68	0.095
MOTD (%)	78.55	76.74	0.416	78.59	76.63	0.357	78.55	76.74	0.423	78.58	76.65	0.368
NPD	15.13	16.00	0.335	15.13	15.99	0.339	15.13	16.00	0.334	15.13	16.00	0.339
PM (%)	25.44	22.86	0.401	25.51	22.70	0.327	25.42	22.90	0.411	25.51	22.71	0.331
PZ (%)	74.56	77.13	0.401	74.48	77.29	0.327	74.58	77.09	0.411	74.49	77.28	0.331
PPK (%)	13.24	13.38	0.951	13.23	13.40	0.939	13.24	13.38	0.946	13.22	13.40	0.936
PPLJR (%)	2.29	3.12	0.273	2.31	3.08	0.304	2.29	3.12	0.277	2.31	3.08	0.303
POTKG (%)	1.33	1.52	0.688	1.32	1.54	0.636	1.33	1.52	0.688	1.32	1.54	0.636
PAKR (%)	0.28	0.02	0.155	0.28	0.03	0.165	0.28	0.02	0.157	0.29	0.03	0.164

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - motility of native semen, MOTD - motility of semen after dilution, NPD - number of produced doses, PM - percentage of dead spermatozoa, PZ - percentage of live spermatozoa, PPK - percentage of spermatozoa with a protoplasmic droplet, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part, VJ –Large white, L-Landrace , p – statistical significance

Conclusions

Of all the investigated factors, only the boar breed showed no significant effect on the properties of the ejaculate in any of the analyzed models. However, it was established that there is a correlation between the interval between two sperm collections and the incidence of secondary anomalies, especially in relation to the percentage of spermatozoa showing certain pathological changes. These results suggest that improper collection of sperm samples can increase the presence of abnormal spermatozoa. Also, the maximum daily temperature and the value of the TH index during the collection of sperm samples affect the ejaculate volume, where higher values of these parameters lead to a larger ejaculate volume. On the other hand, the temperature and the value of the TH index at the beginning of the epididymal phase of spermatogenesis affect the decrease in the mobility of spermatozoa in native and semen after dilution, as well as the decrease in the percentage of live spermatozoa. This indicates that high temperatures during the epididymal phase of spermatogenesis have a negative effect on ejaculate characteristics and sperm vitality.

It is important to note that the analyzed factors explained to a lesser extent the variability of the examined sperm properties, which is shown by the low values of the coefficients of determination in the applied models.

Uticaj klimatskih faktora, rase i intenziteta korišćenja nerasta na kvalitet semena i morfologiju spermatozoida

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Rezime

Osnovni cilj ovog istraživanja bio je da se oceni varijabilnost osobina ejakulata nerasta i pojava anomalija na spermatozoidima, uzimajući u obzir klimatske faktore tokom spermatogeneze, rasu i učestalost korišćenja nerasta. U istraživanje je uključeno 17 nerasta (n=129 ejakulata), a testiranje plodnosti sprovedeno je tokom najkritičnijeg perioda godine, od avgusta do oktobra. Posmatrane karakteristike sperme obuhvatale su: zapreminu ejakulata (VOL), koncentraciju spermatozoida (CON, spermatozoida/ml), ukupan broj i broj funkcionalnih spermatozoida (NT, NF), procenat pokretljivosti spermatozoida u nativnom ejakulatu i nakon razređenja (MOTN, MOTD), broj proizvedenih doza (NPD), procenat mrtvih i živih spermatozoida (PM, PZ) i anomalije spermatozoida. Procena uticaja izvršena je primenom General Linear Modela. Osobine ejakulata nisu varirale pod uticajem rase nerasta, dok je učestalost korišćenja nerasta uticala na pojavu sekundarnih anomalija. Utvrđeni regresioni koeficijent pokazao je da produženje intervala za jedan dan povećava PPK za 0,340-0,348%. Maksimalna dnevna temperatura tokom prikupljanja semena (model 1) i vrednost TH indeksa tokom prikupljanja semena (model 3) uticali su na zapreminu ejakulata. Povećanje temperature za jedan ℃ ili vrednosti THI za jednu jedinicu, dovelo je ($p<0,05$) do povećanja VOL za 3,540 ml, odnosno 2,798 ml. Takođe, maksimalna dnevna temperatura (model 2) i vrednost TH indeksa (model 4) na početku epididimalne faze spermatogeneze uticali su na pokretljivost sperme, kao i na procenat živih i mrtvih spermatozoida.

Ključne reči: nerast, spermatogeneza, anomalije spermatozoida, temperatura, THI

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Conflict of interest
The authors declare that they have no conflict of interest.

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SITUATION ON THE MARKET OF EGGS FROM NON-CAGE PRODUCTION SYSTEMS

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Abstract: The objective of the study is to determine the share of eggs from non-cage production systems compared to eggs from cage systems, in the year when the extended transitional period expires (December 2023) for Serbian producers to comply with the welfare legislation, mandating the transition from the conventional cage breeding system to permitted systems (enriched cage system and non-cage systems (floor, aviary, free breeding – free ranges, organic production). Also, the objective of the study is to determine the structure of consumers within the group valueing the breeding system, which declares that it prefers free range eggs and organic eggs. The examination of the Belgrade market supply of eggs, including supermarkets (11), green markets (5) and specialized stores (5), and consumer attitudes was conducted through a survey (247 respondents in the Belgrade region). According to the results of the survey, it can be concluded that the supply of eggs from non-cage systems is minor, compared to eggs from the cage system, which have a share of 100%, it is 81.82% for eggs from the floor system, 54.54% for eggs from free range and 9.10% for organic eggs. The supply of organic eggs on the market is insufficient and mainly associated with specialized stores. For about 30% of consumers, the egg production system is very important, whereby the majority of consumers (53.45%) would prefer the eggs from free ranges, and among them the largest share are women (76.03%), consumers with higher education (67.10%), employed (78.38%), aged 36-55 and with higher monthly incomes. Based on the research results, it can be concluded that the process of harmonizing egg production with welfare regulations has an impact on the egg market, i.e. resulting in changes in the sense of greater share of eggs from non-cage systems compared to similar research in the earlier period. At the same time, although there is a growing awareness among consumers about the

importance of the production/rearing system, it is still insufficient compared to certain European countries. The development of non-cage systems, as more favourable from the point of view of welfare and consumer expectations, should be given greater attention in the coming period, by acting through regulatory measures, education of producers and consumers, research and support in terms of incentives, subsidies and loans to producers who switch to alternative systems of raising laying hens.

Key words: layer hens, non-cage rearing systems, market, consumers, survey

Introduction

The ban of conventional cage systems in the EU countries followed different dynamics, from the slow process in case of certain member countries, to those that completed the process quickly and even introduced stricter national laws. Looking at egg production from the perspective of the representation of individual systems, it can be concluded that of the total of 376 million laying hens in the European Union in 2022, the smallest share is in organic production (7.1%) and in the in the so-called "Free range" or free keeping system (15.5%), while the largest share of hens is still in the cage system, i.e. in "enriched" cages around 39.7%, with similar representation of hens in the floor system (37.8%) (European Commission, 2023). Enriched cages are dominant in EU member states from Eastern, Central and Southern Europe, while in Northern and Western European countries they are represented in low percentage (Urios et al., 2022). The percentage of individual birds that are grown in one of the so-called "uncaged methods" is constantly increasing, so that in Great Britain the percentage is already over 60% (United Kingdom egg statistics, 2022), in Ireland, Austria and France 46.1, 31.5 and 29.8%, respectively, while some European countries, such as Austria, the Czech Republic and Germany, are gradually excluding enriched cages from use (Rodenburg et al., 2022). Further developments in the egg production sector may be affected by the citizens' initiative launched in Europe, called "End the Cage Age", to which the European Commission responded positively on June 30, 2021 and presented plans for a legislative proposal to ban cages for numerous domestic animals (European Commission, 2021).

In Serbia, harmonization with European standards (Directive 1999/74/EC) is related to the Law on Animal Welfare (2009) and the Rulebook on Breeding Conditions (2010). The process of abonding the conventional cage system and transition to permitted systems is slow. Tolimir et al. (2020) reported that by the end of 2020, only around 16% of surveyed producers have switched to permitted systems. The egg producers in our country, in addition to the challenges related to

the large investments that require the transition from conventional cages, are also faced with the decision to choose a housing system. According to the above mentioned research, all surveyed producers (100%) declared that their choice would be an "enriched" cage system. However, the fact should also be taken into account that at the time of the survey, within the mentioned research, only 61.7% of producers were aware of the initiative on the complete abolition of cages, which could have an impact on the decision on the choice of the system. This statement of the producers was probably influenced by the perception of the negative sides of non-cage systems, in terms of high costs for setting up production, reduction of production capacity, higher costs in production and a more complex management system compared to the cage system. However, although non-cage rearing systems have certain disadvantages, for making the final decision on the choice of the system, it would be important to look at the advantages, which are primarily related to respect for welfare, product quality, environmental protection and competitive advantages in the market, as well as a better status with consumers.

In the world, consumers have a differently developed awareness in individual countries about animal welfare, which often depends on gender, education, occupation, eating habits, understanding of ethical value and welfare, economic opportunities and personal preferences (Cornish et al., 2016). Research on laying hens housing systems in Serbia (Tolimir et al., 2019; 2020) indicate that there is a tendency of increasing welfare awareness, but there is still a need for education on the benefits of cage-free systems and transparency regarding production, which could play a key role in promoting positive attitudes towards these systems.

In anticipation of future changes in the egg production sector in Serbia, the objective of the paper is to determine how the changes so far in the process of compliance with regulations have been reflected in the market regarding the supply of eggs from non-cage production systems compared to eggs from cage systems. Also, the paper aims to indicate the structure of consumers who prefer eggs from cage-free systems, which can be important for producer decisions regarding the choice of rearing system.

Materials and Methods

The examination of the supply of eggs from non-cage farming systems on the Belgrade market was conducted in the period from April to June 2023, by monitoring the supply in 11 of the most represented super markets, 5 green markets and 5 specialized stores. The supply and price of eggs were monitored on a monthly basis, in the first week of the month, and recorded within each of the sales facilities, including: the number of producers, the representation of eggs according to the production system - floor system, free range eggs, eggs from organic

production, as eggs from the cage system for the purpose of comparison. The average price per egg for grade M was calculated for each of the rearing systems, including the price of eggs at all test sales points. The survey of consumers of edible eggs from the Belgrade region included 247 respondents who filled out the survey questionnaire. The structured questionnaire consisted of: a) data on the respondent, obtained by rounding the offered answers, for the following categories: gender (male, female), education (secondary school, higher education), status (student, employed, unemployed, retired), age (below 18, 26-35, 36-55, over 55); income (less than 40,000 dinars, 40-70,000 dinars and more than 70,000 dinars) and b) questions with suggested answers: 1) "How important is the rearing system when buying eggs" - the answers were marked from 1 - not important at all to 9 - very important (Likert scale was used) and 2) "Which eggs would you most often buy" - answers offered: eggs from a cage system, free range eggs, organically produced eggs and others. Within the group declaring that the farming system is important (the group that scored 7 to 9 for the importance of the farming system) and the group of consumers who declared that they would prefer to buy free range eggs, the structure of the respondents was made. Standard methods of analysis in the Microsoft Excel program were used for data processing.

Results and Discussion

Table 1 shows the results related to the monitoring of the situation on the Belgrade consumption egg market, in terms of the supply and prices of eggs from different rearing systems and at different points of sale, i.e. in supermarkets, markets and specialized organic food stores.

Based on the data in Table 1, it can be concluded that the number of producers within the single market ranged from 1 to 4, within green markets from 2 to 5, and within specialized stores from 1 to 2. All markets (11) offered eggs from the cage system, while the supply of eggs from non-cage systems was minor, that is, eggs from the floor system were represented in 9 markets, eggs from free ranges in 6 markets and eggs from organic production in only one market. If the supply of eggs from the cage system is taken as a basis for comparison, with a representation of 100% in markets, the supply of eggs from the floor system was 81.82%, from free range for 54.54% and from organic production 9.10%. Analysing the supply of eggs from different systems, it can also be concluded that eggs from the cage system were represented in all markets (100%), and the supply also included eggs from free ranges, with 60% share in relation to the cage system, while eggs from the floor system and organic production were not on offer. The supply of eggs from organic production is mainly related to specialized stores, in which one to two producers are represented, offering eggs from non-cage systems (free range, free range eggs and organic eggs).

Table 1. Supply of eggs from cage-free systems on the Belgrade market (market, green market, specialized store)

Sale point	Total No. of egg producers	No. of present production systems	No. of egg producers			
			Cage systems	Floor system	Free range	Organic eggs
Supermarkets						
SM1	2	2	2	-	1	-
SM2	2	2	2	2	-	-
SM3	3	3	1	1	1	-
SM4	3	2	3	1	-	-
SM5	1	3	1	1	1	-
SM6	1	3	1	1	1	-
SM7	3	2	2	1	-	-
SM8	3	2	2	1	-	-
SM9	3	1	3	-	-	-
SM10	2	3	2	1	2	-
SM11	4	4	3	2	2	1
Green markets						
GM1	5	2	5	-	2	-
GM2	2	1	2	-	-	-
GM3	5	2	3	-	2	-
GM4	3	1	3	-	-	-
GM5	3	2	2	-	1	-
Specialized stores of organic products						
SS1	2	2	-	1	-	1
SS2	2	2	-	-	1	1
SS3	1	1	-	-	-	1
SS4	2	2	-	1	-	1
SS5	2	1	-	-	-	-

The results of the research, compared to a similar research by Tolimir et al. (2017), when only eggs from cage systems were offered, indicate that the process of harmonizing the egg production sector with welfare regulations in Serbia resulted in an increase in the supply of eggs from non-cage systems. The supply of eggs from cage-free systems in the market is in line with the representation of these systems in Serbia (about 15%) (Krnjaić, 2019; Tolimir et al., 2020). In the coming period, given that the process of harmonization of egg production sector with welfare regulations will continue in Serbia, a growing trend in the supply of eggs from non-cage systems can be expected. Such expectations are also based on tendencies in EU countries, where an increase in the number of producers, as well

as buyers, is observed, who commit to supply only eggs from non-cage systems (cage free) (Egg Track Report, 2021). Also, the strengthening of the "End of the cage age" movement should be taken into account, which could also affect further changes in the egg production sector in EU countries, given that the share of hens (39.7%) is still in enriched cages. Allowed, cage-free systems, which could be the choice of the producers, are the following: 1) Free-Range rearing system, in which layers have access to an open area, usually a grassy area; 2) A free-range system of rearing with portable poultry facilities, whereby layer hens are allowed to consume grass in one place before moving to another; 3) Organic production - implies free access to open space and eating organically produced food; 4) Floor system, as free rearing in a closed space (Cage-Free); 5) Aviary system - when several levels or floors are used for rearing layer hens and implies optimal use of space. It should be noted that for small farms, the traditional system (Backyard Farming), in which hens are reared in their backyards for their own use of eggs, can be important on a smaller scale, and can be useful for local food production.

Based on the results related to the share of production systems by producers, it was found that out of nine producers present in markets, 7 of them have the cage system, 5 producers have the floor system, 3 produce free range eggs and one producer has organic production. The number of systems per one producer ranges from one system (4 producers only produce eggs from the cage system, 1 producer only eggs from the floor system and 1 producer only eggs from organic production) to a maximum of 3 systems per one producer (3 producers simultaneously produce eggs in cage, floor system and on free range). The producers present on the market mostly have cage system, and from non-cage systems, free range eggs. Producers in specialized stores are committed to non-cage systems, primarily organic production.

Analysing the data, it can be concluded that certain producers decide for simultaneous production in several systems, which provides them with greater market security in the transition period. Although the producers in the research by Tolimir et al. (2020) state the enriched cages as their choice when switching to other rearing systems (100%), the situation in the market indicates that a number of producers are opting for non-cage systems. Bearing in mind the tendencies in the EU, it would be important in Serbia in the coming period to engage in the development of non-cage systems, through a systemic approach, which implies a combination of: regulatory measures, producer education, research and development of new technologies and approaches that improve the efficiency and sustainability of alternative cultivation systems, support for this sector in terms of incentives, subsidies and loans to producers who switch to alternative systems of raising laying hens and strengthening consumer awareness. This approach is in agreement with Đoković et al. (2018), who point out that a series of measures are important during the transition period, which include the joint work of the

administration, scientific and professional institutions and the creation of economic and infrastructural conditions.

The research included the price of eggs from different farming systems, grade M, which is shown in the Figure 1.

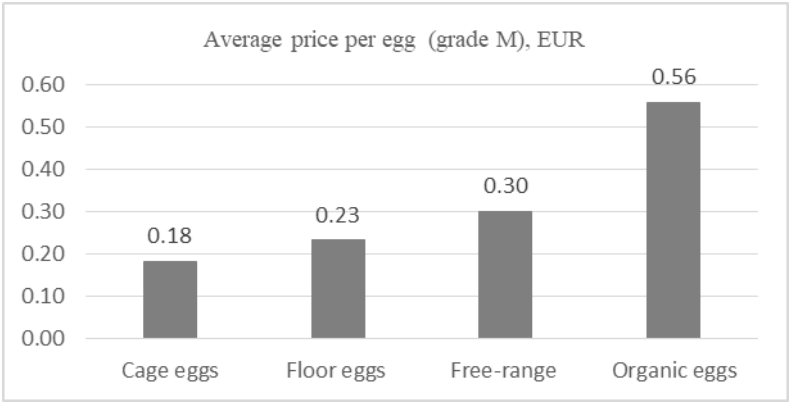

Figure 1. Price of eggs from different rearing systems

According to the obtained results, the average price of eggs from different rearing systems, on the Belgrade market, for grade M, was the lowest for eggs from the cage system (0.18 EUR), while the average price of eggs from non-cage systems was higher, by 26.70% for eggs from the floor system, 63.40% for eggs from free range and 204% for organic eggs. By comparing the results obtained from the EU, a greater difference in the price of cage and non-cage systems can be noted. According to data of the European Commission (2023), the price of eggs from the free range system was higher by 45.60%, and organic by 108%, compared to the cage system.

According to the results of the survey of consumer attitudes, it is determined that for 29.96% of consumers the production system is very important, and within the group of these respondents, the majority are female (76.03%), consumers with higher education (67.10%), age structure from 36 to 55 years (59.46%), employed (78.38%), with high income (55.41%) (Table 2). Also, on the basis of the survey research, it is determined that more egg consumers (53.45%) declared that they would prefer to buy free range eggs, whose structure is very similar to the group of consumers for whom farming systems are important, i.e. this farming system is mostly preferred by women (76.52%), consumers of higher education (59.85%), age structure from 36 to 55 years (40.90%), employed (71.21%), with high income (50.00%) (Table 2).

Table 2. Structure of egg consumers within the group that finds the hen farming system very important and the group that would buy free range eggs

Parameters	Structure of egg consumers who find the rearing system important, %	Structure of egg consumers who would buy free range eggs, %
Sex		
Male	23.97	23.48
Female	76.03	76.52
Education		
Highschool	31,51	40.15
Faculty degree	67,10	59.85
Status		
Student	9.46	15.91
Employed	78.38	71.21
Unemployed	4.05	3.03
Retiree/Pensioner	8.11	9.85
Age		
18-25	12.16	19.70
26-35	14.86	22.00
36-55	59.46	40.90
Over 55	13.52	17.40
Monthly income (RSD)		
30.000-40.000	10.81	15.15
40.000-70.000	33.78	34.85
>70.000-100.000	55.41	50.00

Knowing the attitudes of consumers is important for the process of transitioning to permitted systems and can be one of the factors in making decisions for manufacturers when choosing a system. By looking at earlier research in this area, it can be concluded that in Serbia, consumer preferences regarding free range eggs have not changed significantly for more than a decade, for which, according to research by Pavlovski et al. (2010), 51.2% declared as their choice, which can be explained by the traditional understanding that "real" eggs are those laid by chickens that walk around.

By comparing the obtained data on the structure of consumers for whom the rearing system is important, with the results of research on the structure of consumers for which the welfare of hens is very important (Tolimir et al. 2019), differences can be noted that indicate that consumers do not see a more complete connection between the rearing system and welfare. The mentioned differences point to the need for consumer education in the area of rearing systems and the welfare of laying hens, with special reference to the advantages of non-cage rearing systems. In addition to the question of understanding and connecting the system of rearing and welfare, there is also an open question of whether the statements of consumers about commitment to a certain system coincide with the actual

purchase, and this problem was pointed out by the European Commission in 2007 and 2016 (European Commission, 2007; European Commission, 2016).

Conclusion

Based on the results of the research, which aimed to determine the situation on the egg market, i.e. the supply of eggs from non-cage systems, it can be concluded that the process of compliance with welfare regulations resulted in changes in the Belgrade market, in terms of a greater share of eggs from non-cage systems compared to similar researches in the earlier period. In the following period, in accordance with the developments in the EU countries, where consumers increasingly appreciate products that reflect ethical values, animal care and sustainability, as well as considering that the "Cage Free" movement has an increasingly noticeable effect, for the further development of the egg production sector in Serbia, it is important to focus attention on the establishment of production from non-cage systems on a larger scale. When choosing a system, the attitudes of consumers in Serbia should also be taken into account, who recognize free range eggs as their first choice (53.45%). For the development of the egg production sector in Serbia, systemic action aimed at producers and consumers, through a combination of regulatory measures, education, research and financial support, is of key importance.

Stanje na tržištu jaja iz nekaveznih sistema gajenja

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Rezime

Cilj rada je da utvrdi zastupljenost jaja iz nekaveznih sistema proizvodnje u poređenju sa jajima iz kaveznog sistema, u godini kada za proizvođače u Srbiji, ističe produženi prelazni rok (decembar 2023. godine) za usaglašavanje sa zakonskom regulativom o dobrobiti, koja nalaže prelazak sa konvencionalnog kaveznog sistema gajenja na dozvoljene sisteme (obogaćeni kavezni sistem i nekavezni sistemi (podni, avijarni, sloboda uzgoj – ispusti, organska proizvodnja). Takođe, cilj rada je bio da se utvrdi struktura potrošača unutar grupe kojoj je važan sistem gajenja, koja se deklariše da najviše preferira jaja sa pašnjaka i organska jaja. Ispitivanjem tržišne ponude jaja obuhvaćeni su supermarketi (11), pijace (5) i specijalizovane prodavnice (5), a stavovi potrošača utvrđeni su anketnim

istraživanjem (247 ispitanika u Beogradskom regionu). Prema rezultatima ispitivanja može se konstatovati da je na tržištu Beograda ponuda jaja iz vankaveznih sistema manja, u poređenju sa jajima iz kaveznog sistema, koji imaju zastupljenost od 100%, iznosi 81.82% za jaja iz podnog sistema, 54.54% za jaja sa pašnjaka i 9.10% za organska jaja. Ponuda organskih jaja na tržištu je nedovoljna i vezana za specijalizovane prodavnice. Za oko 30% potrošača, sistem proizvodnje jaja je veoma važan, pri čemu bi se najveći broj potrošača (53,45%) najradije opredelio za jaja sa pašnjaka, a među njima je najveći udeo žena (76.03%), potrošača sa visokim obrazovanjem (67,10%), zaposlenih (78,38%), starosti od 36-55 godina i sa najvišim mesečnim primanjima. Na osnovu rezultata istraživanja može se zaključiti da proces usaglašavanja proizvodnje jaja sa regulativama o dobrobiti ima uticaja na tržište jaja, odnosno da je rezultirao promenama u smislu veće zastupljenosti jaja iz nekaveznih sistema u poređenju sa sličnim istraživanjima u ranijem periodu. Istovremeno, iako je prisutna rastuća svest kod potrošača o značaju sistema gajenja, ona je i dalje nedovoljna u odnosu na pojedine evropske zemlje. Razvoju nekaveznih sistema, kao povoljnijim sa aspekta dobrobiti i očekivanja potrošača, u narednom periodu treba posvetiti veću pažnju, delovanjem kroz regulatorne mere, edukacij proizvođača i potrošača, istraživanja i podrške u smislu podsticaja, subvencija i kredita proizvođačima koji prelaze na alternativne sisteme gajenja kokoši nosilja.

Ključne reči: kokoš, nekavezni sistemi gajenja, tržište, potrošači, anketa

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Conflict of interest
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DETERMINATION OF THE EFFECT OF A LACTIC ACID BACTERIA+ENZYME MIXTURE ON THE SILAGE QUALITY AND DIGESTIBILITY OF VETCH-OAT MIXTURE SILAGES

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Abstract: This study was carried out to determine the effects of the addition of lactic acid bacteria+enzyme (LBE) mixture on the fermentation characteristics and digestibility of Hungarian vetch-oat silages. An inoculant was used as additive which contains *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, alpha-amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger* in its biological composition. While additive was not used for control, LBE1 and LBE2 groups were inoculated with LBE as 300 000 and 500 000 cfu/g of silage material, respectively. After 60-d of incubation, no significant difference was observed in pH values among silages, but the highest lactic acid value was detected in the LBE2 group silages ($P<0.01$). *In vitro* neutral detergent fiber digestibility (IVNDFD) values of the silages were 41.65, 44.14 and 47.38% for the control, LBE1 and LBE2 groups, respectively, and it was determined that there was a linear correlation ($r=0.945$) between the inoculant doses and the IVNDFD values. As a result, LBE improved fermentation characteristics and IVNDFD values of the Hungarian vetch-oat mixture.

Key words: digestibility, enzyme, lactic acid bacteria, vetch-oat silage

Introduction

Ensiling allows large amounts of forage to be conserved rapidly. The harvesting and storing process of ensiling is less dependent on the weather compared to haymaking. Corn, alfalfa, and other legumes, grasses, sorghum, and other alternative crops are commonly ensiled crops (Grant and Adesogan, 2018).

Lactic acid bacteria (LAB) play a crucial role in promoting a rapid and efficient fermentation process in silage. They convert plant sugars into lactic acid (LA) leading to a decrease in pH, which helps in preserving the forage and inhibiting the growth of undesirable microorganisms (Dunière et al., 2013). Futhermore, enzyme inoculants also play a crucial role in the breakdown of complex carbohydrates present in forage crops into simpler sugars. Both cellulolytic and other hydrolytic enzymes facilitate the degradation of plant cell walls, thereby increasing the availability of fermentable substrates for microbial fermentation (Irawan et al., 2021). This synergistic action not only improves the overall fermentation process and results in better silage quality (Dunière et al., 2013; Irawan et al., 2021), but also results in improved nutrient release and availability for animal digestion and utilization (McDonald et al., 1991; Ogunade et al., 2019). The application of enzymes at ensiling might have a beneficial effect on forages with low sugar contents (Nadeau et al., 2000) like as forage legumes which have higher crude protein but lower easily fermentable carbohydrate content than those of cereals. To our knowledge, the addition of lactic acid bacteria+enzyme (LBE) mixture for Hungarian vetch-oat silage has not been extensively explored. Therefore, the present study was conducted to assess the effect of lactic acid bacteria+enzyme mixture on the fermentation quality, nutrient composition, and *in vitro* digestibility values of Hungarian vetch-oat silage.

Material and Method

Silage Material and Experimental Design

The experiment was carried out at the field of Asarcık, Samsun, in the middle Black Sea (41.0346 N, 36.2451 E) on Hungarian vetch-oat mixture, grown for ensiling. The mixture with 75% Hungarian vetch seeds and 25% oat seeds was sown, and during sowing a composite fertilizer was applied. The Kansur variety of Hungarian vetch *(Vicia pannocia)* and a local variety of oats commonly grown in the region was used in the mixture. The Hungarian vetch-oat mixture was harvested at the early bloom stage of Hungarian vetch. Before ensiling, the silage material was wilted and chopped into approximately 1.5-2.0 cm size pieces. The commercial LBE mixture consisted of *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, and also it contained alpha-amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger*. The inoculant was dissolved in a small amount of distilled water prior to ensiling, and it was sprayed evenly onto the silage material of LBE groups, and

thoroughly mixed for homogenization. Hungarian vetch-oat mixture was inoculated following as Control (without LBE), LBE1 (300 000 cfu/g of silage material), and LBE2 (500 000 cfu/g of silage material). The silage material was tightly packed into laboratory-scale silos with a volume of 1 L, ensuring no air pockets. A total of 15 silos, five silos for each group, were prepared and incubated at room temperature (21±2°C) for 60 days.

Determination of Nutrients and Fermentation Characteristics of Silages

The dry matter (DM) content of the silages was determined by drying the samples in a circulating air oven at 60°C for 48 hours. The dried samples were ground to achieve homogeneity for analysis. Obtained in this way was held in a circulating air oven at 105°C until a constant weight was reached. All results related to chemical composition and content of nutrients are expressed in absolute dry matter. The ash content was calculated after the organic matter portion of the samples was incinerated in a muffle furnace at 560°C for 4 hours. The crude protein (CP) content was determined using the Kjeldahl method. The values for acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) were analyzed using the Ankom 200/220 Fiber Analyzer following the method described by Van Soest et al. (1991). After 60-d of incubation, a total of 25 g sample of silage was taken from each silo and homogenized in approximately 100 ml of distilled water for about 10 minutes (Polan et al., 1998). The pH value of the silage liquid was measured using a pH meter. Silage samples were prepared following the method reported by Tjardes et al. (2000) and the detection of silage acids was performed using an HPLC following the procedure described by Canale et al. (1984).

Determination of Digestibility Values

The Ankom Daisy incubator was used to determine the *in vitro* digestibility values in the study. For the fermentation system, rumen contents were obtained from adult cattle slaughtered at a local abattoir in Samsun province. Immediately after slaughter, rumen contents were collected and filtered through four layers of cheesecloth into a thermos preheated to 39°C and filled with CO_2. The rumen fluid's pH was measured to be 6.25. The preparation of the buffers, 48-h of incubation procedure and the calculations of *in vitro* true digestibility (IVTD), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true organic matter digestibility (IVTOMD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) values were done according to the operation manual of the Ankom Daisy incubator.

Statistical Analysis

The Shapiro-Wilk normality test was applied to all data in the study. The Levene's test for homogeneity was conducted for data that showed a normal distribution. One-way analysis of variance (ANOVA) was applied to data that exhibited homogenous distribution, and post-hoc multiple comparisons using the Tukey test were conducted to determine group differences. For data that did not show homogenous distribution, group differences were determined using the Tamhane test. The Kruskal-Wallis non-parametric test was applied to data that did not follow a normal distribution, and group differences were determined using the Mann-Whitney test. The SPSS software package was used for statistical analysis (SPSS 2012).

Results

The different amounts of LBE inoculant dramatically affected DM, CP, ash, LA and propionic acid (PA) values of 75% Hungarian vetch-25% oat mixture silages (Table 1). *In vitro* digestibilty values of the silages were presented in Table 2. There was an increase ($P<0.01$) in IVNDFD in the LBE-supplemented silages. There was a linear correlation ($r=0.945$) for IVNDFD value among the control, LBE1, and LBE2 groups were given in Figure 1.

Table 1. Effects of lactic acid bacteria+enzyme (LBE) additive on conservation characteristics (% on dry matter, unless otherwise stated) of 75% Hungarian vetch-25% oat mixture silages

Characteristics	Control x ± Sx	LBE 1 x ± Sx	LBE 2 x ± Sx	P value
Fresh DM %	23.17 ± 0.13^{a}	22.02 ± 0.61^{b}	22.33 ± 0.10^{b}	0.01
Ash %	9.77 ± 0.07^{b}	10.11 ± 0.12^{ab}	10.28 ± 0.11^{a}	0.01
CP %	14.43 ± 0.18^{b}	14.96 ± 0.09^{a}	13.82 ± 0.11^{c}	0.00
ADF_{OM} %	32.36 ± 0.53	31.33 ± 0.13	32.38 ± 0.20	0.07
NDF_{OM} %	44.09 ± 1.11	43.99 ± 0.76	45.31 ± 0.60	0.54
ADL_{OM} %	3.43 ± 0.18	3.09 ± 0.28	2.78 ± 0.07	0.10
pH	4.14 ± 0.03	4.19 ± 0.01	4.13 ± 0.00	0.16
LA %	17.31 ± 0.32^{b}	18.95 ± 0.29^{ab}	20.54 ± 0.85^{a}	0.00
AA %	1.60 ± 0.09	1.64 ± 0.05	1.59 ± 0.06	0.83
PA %	4.80 ± 0.07^{c}	6.40 ± 0.05^{b}	12.80 ± 0.01^{a}	0.01

DM: Dry matter; CP: Crude protein; ADF_{OM}: Acid detergent fibre exclusive of residual ash; NDF_{OM}: Neutral detergent fibre exclusive of residual ash; ADL_{OM}: Acid detergent lignin exclusive of residual ash; LA: Lactic acid; AA: Acetic acid; PA: Propionic acid

Table 2. Effects of lactic acid bacteria+enzyme (LBE) additive on digestibility values of 75% Hungarian vetch-25% oat mixture silages

Digestibility%	Control x ± Sx	LBE 1 x ± Sx	LBE 2 x ± Sx	P value
IVTD	76.58 ± 0.67	77.35 ± 0.51	77.95 ± 0.25	0.21
IVTDMD	74.26 ± 0.69	75.41 ± 0.56	76.16 ± 0.28	0.08
IVTOMD	71.94 ±0.80	73.07 ± 0.66	73.77 ± 0.27	0.15
IVNDFD	41.65 ± 0.29^{c}	44.14 ± 0.66^{b}	47.38 ± 0.22^{a}	0.00

IVTD: *In vitro* true digestibility; IVTDMD: *In vitro* true dry matter digestibility; IVTOMD: *In vitro* true organic matter digestibility; IVNDFD: *In vitro* neutral detergent fiber digestibility

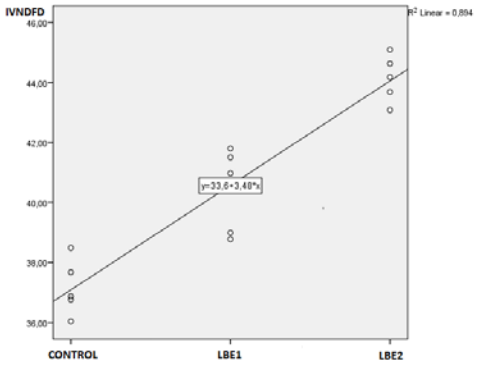

Figure 1. A linear correlation for IVNDFD value among groups
IVNDFD: *In vitro* neutral detergent digestibility; LBE: Lactic acid bacteria+enzyme

Discussion

To get good-quality silage, forages having high carbohydrate and low protein contents are generally preferred but this results in silage with high-energy but low-protein contents. However, a good feed material has to have a balanced energy and protein content. Therefore, energy-rich forages may be mixed with protein-rich forages or mixed planting (Şen et al., 2022). Kılıçalp et al. (2022) found that the LAB+Enzyme inoculant added to triticale and Hungarian vetch silages with different seed ratios resulted in lower crude protein contents in the groups with LAB+Enzyme compared to the control group. In the current study, the lower crude protein content of the LBE2 group compared to the control and LBE1 groups was similar to the findings of Kılıçalp et al. (2022). Some previous studies (Coskuntuna and Gül, 2020; Şen et al., 2022; Li et al., 2022; Marbun et al., 2020) reported that the addition of inoculant to silage material did not affect the ADF and

NDF contents of the silage. The results of the current study for ADF, NDF, and ADL were compatible with previous studies. Marković et al. (2018) stated that the pH values of silages made from 75:25 common vetch-oat mixture with or without inoculant were between 4.22-4.32. According to Weissbach (1996), to obtain well-fermented and stable silage with a dry matter content of 20%, pH values below 4.2 were necessary. The results of the current study were comply with this requirement.

In the silages, lactic acid produced by LAB is the primary acid and it contributes significantly to the decrease in pH during silage fermentation. Silages with low dry matter content (<30%) have higher LA concentrations (Kung Jr et al., 2018). In the current study, the lactic acidification in LBE2 silage was enhanced, which might be due to the dose of the inoculant that improved fermentation, resulting in higher LA and lower pH values compared to the other groups. The second silage acid is acetic acid (AA) and it can increase the stability of silage because of its negative effects on the growth of yeasts when silage is exposed to air. High concentrations of AA in silage (>4-6%) generally occur in undesirable silage fermentations dominated by bacteria such as enterobacteria and clostridia. The concentration of AA in grass silage is typically between 1% and 3% (on a DM basis) (McDonald et al., 1991). In the current study, the AA concentration in silages untreated or treated with LBE inoculant was similar and consistent with that of McDonald et al. (1991). In a previous study (Flores-Galarza et al., 1985) it was reported that propionic acid bacteria increased PA concentration and reduced the proliferation of yeasts and moulds of high-moisture maize silages. Kung Jr et al. (2018) mentioned that Propionibacteria that convert glucose and LA to PA and AA have been found in silages. In the current study, PA concentration was increased in the silages inoculated with the different doses of LBE, which might be because the LBE inoculant used in the study contained *P*ropionibacterium acidipropionici which produced PA and caused an increase in PA concentration. Butyric acid (BA) is an acid that should not be detected in well-fermented silage. The presence of BA in silage is associated with the metabolic activity of Clostridium species (Pahlow et al., 2003). It is stated that the amount of BA in high-quality silage should be less than 0.1% (Seglar, 2003). In this study, the presence of BA could not be determined, which is consistent with the findings of Pahlow et al. (2003) and Seglar (2003).

Roughage digestibility is one of the most important parameters that directly reflects the quality of silage (Li et al., 2022; Liu et al., 2019). No significant difference was observed between the control and the silages inoculated with LBE in terms of the IVTD, IVTDMD, and IVTOMD values, which was consistent with Li et al. (2022) who reported the lack of effects of a commercial lactic acid bacteria-based inoculant as a silage additive on IVDMD. Another study conducted by Başkavak et al. (2008) indicated that LAB and enzyme inoculation did not affect the IVDMD and IVOMD values of wheat silage, but the addition of

inoculants tended to increase these values like the findings of the present study. However, in this study, there was an increase in IVNDFD value of the silages inoculated with LBE was consistent with the results of Guo et al. (2020), who reported that L. plantarum and E. faecalis increased IVNDFD. Similarly, in this study, it was found that LBE additions at different levels increased the IVNDFD at 48 h. The positive response to LBE addition in this study may be attributed to the disruption of plant cell wall structures. This disruption may effectively release intracellular contents, providing more substrate for binding and fermentation by rumen microorganisms. Oba and Allen (1999) stated that a 1-unit improvement in IVNDFD is positively associated with 0.17 kg of DM and 0.25 kg of corrected milk yield per 4% fat. In this study, increases of 5.97% and 13.75% were observed in NDFD values for silages in the LBE1 and LBE2 groups, respectively. According to Oba and Allen (1999), the increased IVNDFD values of the silages in the LBE1 and LBE2 groups compared to the silages in the control group potentially can resulted in an increase of 0.42 kg and 0.97 kg in DM intake and 0.62 kg and 1.43 kg in corrected milk yield per 4% fat, respectively.

Conclusion

Based on the obtained data, the mixture of Hungarian vetch-oat silage could be preserved without additive. However, the use of LBE inoculant up to 500 000 cfu/g had a positive response in the fermentation quality and IVNDF digestibility.

Određivanje uticaja mešavine mlečne kiseline i enzima na kvalitet silaže i svarljivost silaže mešavine grahorice i ovsa

Zeki Kılın, Zehra Selçuk

Rezime

Ovo istraživanje je sprovedeno da bi se utvrdili efekti dodavanja mešavine bakterija mlečne kiseline i enzima (LBE) na karakteristike fermentacije i svarljivost mađarske silaže od grahorice i ovsa. Kao aditiv je korišćen inokulant koji sadrži *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilacti* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4U, alfa amilaze iz *Bacillus amyloliquefaciens*, celulaza iz *Trichoderma reesei*, ksilanaza iz *Trichoderma longibrachiatum*, beta-glukanaza iz *Aspergillus niger* u svom biološkom sastavu. Dok aditiv nije korišćen za kontrolu, LBE1 i LBE2 grupe su inokulisane sa LBE kao 300 000 i 500 000 cfu/g silažnog materijala, respektivno. Posle 60 dana inkubacije, nije primećena

značajna razlika u pH vrednostima među silažama, ali je najveća vrednost mlečne kiseline otkrivena u silažama grupe LBE2 (P<0,01). *In vitro* vrednosti svarljivosti vlakana neutralnog deterdženta (IVNDFD) silaža su bile 41,65, 44,14 i 47,38% za kontrolnu, LBE1 i LBE2 grupu, respektivno, i utvrđeno je da postoji linearna korelacija (r=0,945) između doza inokulanta i IVNDFD vrednosti. Kao rezultat toga, LBE je poboljšao karakteristike fermentacije i IVNDFD vrednosti mađarske mešavine grahorice i ovsa.

Ključne reči: svarljivost, enzim. bakterije mlečne kiseline, silaža grahorica-ovas

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CHEMICAL COMPOSITION AND SILAGE QUALITY OF UREA, MOLASSES, AND UREA AND MOLASSES ENSILED SOYBEAN HUSK

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Abstract: The study evaluated the effect of additives treatment on the chemical composition and silage quality of soybean husk in a 28-day experiment, using completely randomised design. Soybean husk was chopped into 1-3 cm length and ensiled in a laboratory silo. There were four treatments consisting of the control (no additive treatment; T1), urea treatment (T2), molasses treatment (T3) and urea and molasses treatment (T4). The colour of the silages was goldenrod, dark goldenrod, peru and burly wood for T1, T2, T3 and T4 respectively. Except for T2 which had a pungent smell, silage smell was generally pleasant. All the silages had firm texture. Silage temperature, ether extract and propionic acid were ($P>0.05$) not affected by treatments. Silage dry matter and ammonia-nitrogen were higher ($P<0.05$) in T2 than in other treatments. Silage organic matter, non-fibre carbohydrates, neutral detergent fibre, acid detergent fibre, acid detergent lignin, hemicellulose, cellulose and butyric acid were higher $P<0.05$) in the control relative to the additive treatments. Crude protein of the silages was lowest and highest ($P<0.05$) in T1 and T4 respectively. Silage pH was lower in T3 than in other treatments. Acetic and lactic acids of the silages were affected by additives treatment, and increased in the order: T1 < T2 < T3 < T4 ($P<0.05$). In conclusion, additives treatment enhanced the nutritive and fermentation qualities of soybean husk. However, urea and molasses treatment produced the best results.

Key words: additives, ensiling, fermentation quality, nutritive value, soybean husk

Introduction

Insufficient availability and fluctuating quantity and quality feed is one of the major prevalent constraints to livestock production (Olafadehan and Adewumi,

2009). This problem becomes exacerbated during the dry season when native pastures are senesced, fibrous and lignified, and have little or no nutritional value (Olafadehan et al., 2009). The scarcity of green fodder and escalating demand for conventional ingredients consumed by humans have led to the utilisation of non-competitive and non-conventional agricultural wastes in livestock feeding. Similarly, occasional scarcity and high cost of conventional feed ingredients have prompted researchers to employ different processing methods and technologies such as urea ammoniation (Olafadehan and Adebayo, 2016; Olafadehan and Okoye 2017; Olafadehan et al., 2017), urea and molasses treatment (Lunsin et al., 2018) and solid state fermentation (Anaso and Olafadehan, 2021; Olafadehan et al., 2021, 2023) to improve the feeding value of poor quality agricultural wastes. However, additive treatment of lignocellulosic materials to improve the nutritive value is an easier and cheaper technology for farmers' adoption than the solid state fermentation, which requires some level of proficiency and expertise to practice.

Presently, in Nigeria, agro-industrial by-products and crop residues are copiously available due to increasing expansion of agro-industrial activities and diversion of open grazing land to crop production in an attempt to feed the ever teeming human population (Olafadehan and Adebayo, 2016). However, they constitute nuisance and environmental hazards due to the problem of poor disposal. Most often than not, they are either heaped and left to decay or burnt. If well harnessed and processed, they could guarantee all-year round feed availability for ruminant stock. One of such agricultural wastes is soybean husk (SBH) obtained from processing of soybean (*Glycine max L.*) after harvesting and threshing. Soybean is one of the commonly consumed legumes worldwide, with 200 million metric tons produced per year (Lim et al., 2011). However, the inedible SBH (the pod that covers the seeds) which is removed during threshing and processing, represents a major disposal problem for soybean industries.

Unlike cowpea husk, which is commonly fed to ruminant stock in Nigeria, SBH is not presently used as a feed ingredient. Therefore, the husk is mostly heaped and sometimes burnt or left on the field, thereby constituting environmental hazards. The fibrous, poor quality SBH can be harnessed and enhanced by ensiling with additives, such as urea and molasses, to improve its nutritive value, ameliorate the problem of environmental pollution due to its disposal and circumvent the problem of dry season feeding of ruminant animals. Silage, anaerobically fermented, preserved substrates or plant materials, has now become an increasingly important source of animal feed in the tropics in both dry and rainy seasons (Pholsen et al., 2016). Its production is an efficient conservation technology to improve the feeding value and ensure adequate feed supply when required. Urea and molasses have been used as additives for nutritional fortification or enhancement of lignocellulosic materials in a process involving ensiling. Previous studies involving use of urea and/or molasses (Olafadehan and Adebayo, 2016;

Abera et al., 2108; Lunsin et al., 2018) to ensile crop residues showed improvement in the nutritive value.

It was hypothesized that additive treatment of ensiled SBH would enhance its silage quality and nutritive potential. This study was conducted to investigate the effect of urea, molasses, and urea and molasses treatment and ensiling of SBH on its silage quality and nutritive value.

Materials and Methods

Experimental site

The experiment was conducted at the University of Abuja Teaching and Research Farm, Federal Capital Territory, Nigeria. The site is at 456 m altitude and lies between latitude 8º 55' N and 9º 00' E and longitude 7º 00' N and 7º 05' E. It has a tropical climate with temperature and annual rainfall ranging from 25.8 to 42°C and 1100 to 1650 mm respectively.

Silage preparation

Soybean husk, obtained from farmers, was chopped into 2-3 cm length for ease of compaction during ensiling. There were four treatments with four replicates per treatment. In treatment 1 (no additive; T1), 100 L of water was sprayed and mixed on 100 kg dry matter (DM) basis of SBH. In treatment 2 (T2), 4 kg of urea was dissolved in 100 L water, and the solution was carefully sprinkled and mixed with 100 kg (DM basis) of SBH. In treatment 3 (T3), 10 L of molasses was dissolved in 100 L of water, and the solution was thoroughly mixed with 100 kg (DM basis) of SBH. In treatment 4 (T4), 4 kg of urea and 10 L of molasses were carefully dissolved in 100 L of water, and the solution was mixed with 100 kg DM of SBH. The treated SBH was placed in individual labelled polyethylene bags which were compressed to eliminate air and the mouths tightly sealed. The polyethylene bags were then placed inside a large drum (experimental silo), compacted to make it air tight, sealed tightly with polyethylene sheets and then covered with a heavy object placed on the lid to prevent aeration and allow anaerobic fermentation for 28 days.

Physical evaluation of silage quality

After the 28 days ensiling, silage colour, smell, texture and temperature were determined immediately. Silage temperature was determined by inserting laboratory thermometer. Colour was assessed by visual appraisal with the aid of a colour chart. The aroma or smell and texture assessment were by five trained panellists.

Chemical analyses and calculation

The DM of the silages was determined by drying the samples at 60°C in a forced air oven until a constant weight was achieved (AOAC, 1995). After drying, the samples were ground through a 1 mm screen (Wiley mill, Standard Model 3, Arthur H. Thomas Co., Philadelphia, PA) for chemical analyses. Samples of the silages were analysed for their proximate constituents in accordance with the procedures of AOAC (1995). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed using the procedures of Van Soest et al. (1991). NDF was analysed using sodium sulphite and amylase, and expressed with residual ash. Acid detergent lignin (ADL) was determined by solubilisation of cellulose with sulphuric acid. Concentrations of hemicellulose and cellulose were calculated as the differences between NDF and ADF, and ADF and ADL respectively. Non-fibre carbohydrate (NFC) was calculated using the following formula, NFC = 100 – (CP + EE + ash + NDF) %. Silage fermentation parameters were determined by mixing another sample (20 g) with 180 mL sterile water which was suspended at 4°C overnight and then filtered through four layers of cheesecloth. The filtrate was used to measure pH, ammonia-nitrogen (NH_3-N) and organic acids. NH_3-N content was determined according to the method of Broderick and Kang (1980). Organic acids (acetic, propionic, butyric and lactic acids) were analysed using high-performance liquid chromatography according to the methods of Wang et al. (2019).

Statistical analysis

Data were subjected to analysis of variance in a completely randomized design using SPSS Base 23.0 (SPSS software products, USA). Duncan multiple range test of the same software was used to test the significance of the differences among the means at $P \leq 0.05$. The statistical model is:

$Yij = \mu + ti + eij$

where Yij = the general response to the specific parameter under investigation, μ = the general mean peculiar to each observation, *ti* = the fixed effect of the additive treatments on the observed parameters and *eij* = the random error term for each estimate.

Results and Discussion

Chemical composition of the additives enhanced soybean husk silage

Silage DM was lower ($P<0.05$) in T3 and T4 compared to T1 and T2, though T2 had the highest ($P<0.05$) value. The lower DM of T3 and T4 relative to the other treatments (Table 1) suggests higher extent of fermentation by providing soluble substrates from molasses and the effect of urea on cell wall content of the

substrates (McDonald et al., 1991). The result, therefore, implies loss of soluble carbohydrates during fermentation for T3 and T4. This decreased in DM of T3 and T4 concurs with the findings of Pour et al. (2018), who observed lower DM for silages ensiled with molasses and urea-molasses additives relative to untreated and urea-treated silages. The highest DM content of T2 indicates reduced DM loss during ensiling. Organic matter and NFC were lower ($P<0.05$) in the additives-treated silages compared to the control. The decrease in OM and NFC contents of the additives-treated groups suggests the availability and utilisation of the two chemical components by certain microbes for their growth and enhanced fermentability of the substrates (Olafadehan et al., 2012), resulting in increased lactic acid production. Non-fibre carbohydrate supplies readily fermentable carbohydrates which are fermented into organic acids, particularly lactic acid, under conducive anaerobic condition. However, the lower OM and NFC of T2 relative to T3 and T4 indicates less fermentability of the OM and NFC contents of urea ensiled SBH compared to molasses, and urea and molasses treated SBH. Crude protein was highest in T4, followed by T2, then T3, and lowest in T1 ($P<0.05$). The increased CP content of the additives-treated SBH indicates that addition of urea (a non-protein nitrogen source) and molasses (an energy source) improved microbial proliferation during fermentation to produce microbial protein (Kung et al., 2000). Moreover, both urea and molasses have been used to improve the CP contents of silages, in consistence with previous reports (Wanapat et al., 2013; Norrapoke et al., 2018, 2022). The increase in the CP content of T2, T3 and T4 also suggests efficient fermentation, preservation and stability of the silages, which possibly arrested and hindered the activities of different types of bacteria from performing their activity, thus making them available and becoming part of the silage. However, the higher CP content of T2 than T3 indicates that urea, might have been utilised to produce protein by certain organism, in corroboration previous findings (Norrapoke et al., 2022). The greater CP of T4 suggests that urea and molasses combination reduced proteolysis during ensiling and thus enhanced CP level better than urea only. The crude protein content of the additives-treated silages met the critical threshold requirement of 7% recommended for small ruminants (NRC, 1981).

Fibre fractions, hemicellulose and cellulose were lower ($P<0.05$) in the treatment groups compared to the control group, implying enhanced structural carbohydrates degradation with additives treatment. The lower fibre fractions of the T2 than the control may be due the enhancement of the nitrogen content of the urea-ensiled SBH which perhaps resulted in release of ammonia that reacted with cell wall components and thus reduced the fibre fractions. Similar observations were made by Olafadehan et al. (2017) and Lunsin et al. (2018). The reduction in structural carbohydrates of T3 relative to the control suggests increased activity of certain fibre-degrading microbes during the ensiling (Olafadehan et al., 2012; Olafadehan and Adebayo, 2016). Molasses, a good source of readily fermentable,

is likely to have furnished certain fibre-degrading microbes with energy to enhance the fibre fractions, hemicellulose and cellulose degradation and thus reduced their concentrations in the silage. However, the lowest structural carbohydrates of T3 indicates that urea and molasses additive enhanced fibre degradation more effectively to produce silage of better quality and nutritive value than either urea only or molasses only, in tandem with earlier findings (Kang et al., 2018; Lusin et al., 2018).

Table 1. Effect of additives treatment on chemical composition (% DM unless otherwise stated) of soybean husk silage

Item	Treatment				SEM
	T1	T2	T3	T4	
Dry matter (% fresh matter)	31.5^{b}	32.7^{a}	28.3^{c}	27.7^{c}	3.89
Organic matter	96.9^{a}	94.8^{b}	92.9^{b}	91.3^{c}	1.66
Crude protein	5.80^{d}	8.84^{b}	6.70^{c}	9.55^{a}	0.32
Ether Extract	2.20	2.40	2.50	2.30	0.19
Non-fibre carbohydrate	29.72^{a}	29.28^{b}	28.54^{c}	28.30^{c}	0.29
Neutral detergent fibre	59.08^{a}	54.28^{b}	54.96^{b}	51.15^{c}	1.09
Acid detergent fibre	45.61^{a}	42.23^{b}	42.19^{b}	40.51^{c}	1.23
Acid detergent lignin	10.77^{a}	10.59^{b}	10.34^{b}	10.49^{c}	0.09
Hemicellulose	10.08^{a}	9.05^{b}	8.72^{b}	6.94^{c}	0.12
Cellulose	33.84^{a}	29.64^{b}	26.9^{b}	24.02^{c}	3.02

Means in the same row without a common superscript letter are significantly different ($P<0.05$)
T1: untreated soybean husk silage; T2: urea-treated soybean husk silage; T3: molasses-treated soybean husk silage; T4: urea and molasses treated-soybean husk silage

Physical characteristics of additives enhanced soybean husk silage

The silage colour was golden rod, dark golden rod, peru and burly wood for T1, T2, T3 and T4 respectively (Table 2). The peru colour of T3 is similar to the burly wood colour of T4 but the dark goldenrod colour of T2 may be due to the addition of urea, which perhaps affected the smell also. The colour of the silages was in order because good silage usually preserves well the original colour of the ensiled substrates or materials. All the silages had pleasant smell except T2 silage that had pungent smell due to addition of urea, which usually generates ammonia, a choking and pungent gas, during fermentation. Therefore, the pungent smell of T2 silage cannot be attributed to spoilage. All the silages had a firm texture which was desirable and previously reported as the best texture for a good silage (Kung and Shaver, 2001). The temperature of the silages was not affected ($P>0.05$) by additives treatment. It is worthy to say that the temperature of the silages was below 28°C, indicating well preserved silages. Bolsen et al. (1996) reported that excessive heat production during ensiling can result in maillard or browning

reaction which compromises silage quality and nutritive value due to reduced protein and fibre digestibility when fed.

Table 2. Physical characteristics of additives treated soybean husk silage

Parameter	Treatment				SEM
	T1	T2	T3	T4	
Colour	Goldenrod	Dark goldenrod	Peru	Burly wood	-
Smell	Pleasant	Pungent	Pleasant	Pleasant fruity	-
Texture	Firm	Firm	Firm	Firm	-
Temperature (℃)	26	25.8	27.5	27.0	0.91

Means in the same row without a common superscript letter are significantly different ($P<0.05$)
T1: untreated soybean husk silage; T2: urea-treated soybean husk silage; T3: molasses-treated soybean husk silage; T4: urea and molasses treated-soybean husk silage

Silage fermentation quality

The preservation of ensiled substrates/materials depends on the adequate acid production to arrest activity of undesirable microorganisms under anaerobic conditions. pH is a critical index of the extent of silage fermentation and quality, and a low pH ensures better aerobic stability, and inhibits further fermentation and development of undesirable aerobic fungi, particularly yeast and mould, which cause aerobic deterioration of silage. In the present study, all the silages (Table 3) had either below or the benchmark pH 4.20 for well-fermented high moisture silage (McDonald et al., 1991). Urea treatment increased ($P<0.05$) silage pH compared to control and other additive treatments. However, molasses-treated and urea-treated silages had the lowest and the highest pH respectively ($P<0.05$). Whereas urea buffers the decrease in silage pH, molasses enhances the pH. The highest pH of T2 is obviously the result of hydrolysis of urea by enzyme urease to ammonia, which possibly improved the buffering capacity of the silage and bacterial activity. The lowest pH of T3 can be attributed to the treatment with molasses, a sugar-rich ingredient, which is commonly used to upgrade the water-soluble carbohydrates content of poor quality, fibrous substrates. Molasses has been used to enhance fermentation quality and nutritive value of silage by enhancing the supply of fermentable carbohydrates for improved growth of lactic acid bacteria (LAB) (Li et al., 2010). Though the silage microbes were not monitored in the current study, it, however, appears plausible to infer that the decreased pH values due to the increased acidity of both T3 and T4 inhibited the less acid-tolerant bacteria, like Clostridium and Enterobacter, which perhaps consequently reduced undesirable fermentation and proteolysis of the silages.

Though NH_3-N (as g/kg of total N) was highest ($P<0.05$) in T2 compared to other treatments, the values of the silages were below the threshold value of 100 g/kg of total N that indicates extensive proteolysis during ensiling (McDonald et

al., 1991). Generally, high concentration of NH_3-N (12–15% of total N) in silages indicates highly degraded, ensiled substrate proteins due to increased number and activities of Enterobacter or Clostridia (Kumar and Singh, 1984). Clostridia usually produce NH_3-N from decomposed protein in silage materials. Since silage with NH_3-N level of less than 70 g/kg total N has been reported as excellent (Lima et al., 2010), the silages in the current study can be said to be excellent and of good quality. The pronounced NH_3-N (g/kg total N) of T2 silage was due to the presence of urea, which on hydrolysis releases NH_3.

Similarly, the organic acid values of all the silages in this study were within or similar to the ranges for good-quality silages in which the values for lactic acid, acetic acid, propionic acid and butyric acids are 4–7%, 1–3%, <0.1% and 0% respectively (Kung, 2008). Acetic acid was higher ($P<0.05$) in the additive treatments relative to the control. However, among the additive treatments, it was highest in T4, followed by T3 and lowest in T2 ($P<0.05$). The higher acetic acid in the additives-treated silages, particularly molasses, and urea and molasses treatments, suggests the activity of heterofermentative LAB which perhaps increased aerobic stability and anti-fungal activity, thus decreasing proliferation and growth of undesirable spoilage microbes, and improving silage fermentation quality. Though not determined in the current study, the large amounts of acetic acid in the molasses, and urea and molasses based silages probably reduced the yeast count, and resulted in greater aerobic stability compared to the control and urea-treated silages. Propionic acid concentration is usually almost negligible (especially in drier silages) or in very low concentrations (<0.1%) in good, well-fermented silages (Kung et al., 2018). Propionic acid was not ($P>0.05$) affected by additives treatment. The generally low and unaffected propionic acid indicates well-fermented and preserved silages. Propionic acid, in addition with other organic acids such as sorbic, benzoic and acetic acids, has reported to improve aerobic stability of silage at feed out through direct inhibition of yeasts and moulds (Auerbach et al., 2012).

Butyric acid was lower ($P<0.05$) in the additive treatments than the control. However, the concentration was far below the critical threshold level at which it depresses feed intake by animals. The presence of butyric acid, an acid with strong, foul rancid-butter smell, in silage is undesirable because its production is an energy-waste metabolism, and concentration > 5 g/kg DM indicates substantial clostridial metabolic activity, large DM losses and poor energy recovery, which compromise feed intake and health of animals (McDonald et al., 1991; Muck, 2010). Clostridial silages indicate excessive proteolysis producing a putrid, fishy or ammonia-like odour (Kung et al., 2018), and also have a low level of energy and high soluble protein, which reduce feed intake when fed to animals (Muck, 2011). From the foregoing, the generally low butyric acid content is thus an indicator of reduced clostridial fermentation during ensiling.

Lactic acid was affected (P<0.05) in the order: T4 > T3 > T2 > T1. As previously explained, the higher lactic acid in the molasses-based silages is desirable as it suggests stoppage of bacterial activity and thus nutrient losses during the ensiling. The molasses in both T3 and T4 must have furnished LAB with soluble carbohydrates, which perhaps resulted in increased accumulation of lactic acid and subsequent reduction of the pH of the silages. The result suggests relative abundance of Lactobacillus and decreased Enterobacter because Lactobacillus, a common bacterium in silages, plays an important role in lactic acid accumulation and pH decline (Ni et al., 2018; Yan et al., 2019). However, the presence of Enterobacter is undesirable in silage because they may compete with LAB for nutrients and produce NH_3-N. The reduced lactic acid concentration of the molasses-based silages has some implications in ruminant nutrition. This is because addition of molasses contributed to high lactic acid contents and low pH during silage fermentation to produce high quality silages which when fed to ruminants may mitigate methane production. Generally, lactic acid is secondarily fermented in the rumen by lactate-utilising bacteria, such as *Megasphaera elsdenii*, *Selenomonas ruminantium*, *Fusobacterium necrophorum* and *Veillonella parvula*, which use hydrogen to convert lactic acid to propionate (Dawson et al., 1997; Russell and Wallace, 1997), thus providing alternative pathway for utilising hydrogen that could have otherwise been used for methanogenesis.

Table 3. Fermentation quality of additives treated soybean husk (g/kg DM)

Parameter	Treatment				SEM
	T1	T2	T3	T4	
pH	4.11^{a}	4.24^{a}	3.89^{c}	4.02^{b}	0.24
NH_3-N (g	42.80^{d}	55.14^{a}	44.94^{c}	46.86^{b}	0.69
Acetic acid	16.26^{d}	19.23^{c}	22.29^{b}	25.28^{a}	0.39
Propionic acid	0.004	0.004	0.003	0.003	0.00
Butyric acid	1.93^{a}	1.80^{b}	1.52^{c}	1.21^{d}	0.09
Lactic acid	52.22^{d}	59.14^{c}	67.24^{b}	69.80^{a}	1.46

Means in the same row without a common superscript letter are significantly different (P<0.05)
T1: untreated soybean husk silage; T2: urea-treated soybean husk silage; T3: molasses-treated soybean husk silage; T4: urea and molasses treated-soybean husk silage

Conclusion

Additives (urea, molasses, and urea and molasses) treatment of soybean husk improved the quality and nutritive value of emanating silages. However, molasses only, and urea and molasses treatments produced silages of superior quality relative to urea treatment only. It is, therefore, concluded that although soybean husk can be ensiled without any additives, additives treatment enhanced

the silage quality with urea and molasses treatment producing silage of superior quality. Further research to evaluate the feeding value of urea and molasses treated soybean husk silage *in vitro* and *in vivo* should be conducted.

Hemijski sastav i kvalitet silaže sojine ljuske tretirane ureom, melasom, i ureom i melasom

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Rezime

U ovoj studiji je ispitivan efekat tretmana aditiva na hemijski sastav i kvalitet silaže sojine ljuske u 28-dnevnom eksperimentu, koristeći potpuno randomizovan dizajn. Sojina ljuska je iseckana na 1-3 cm dužine i silirana u laboratorijskom silosu. Ispitivanje se sastojalo od četiri tretmana - kontrola (bez aditivnog tretmana; T1), tretmana ureom (T2), tretmana melasom (T3) i tretmana ureom i melasom (T4). Boja silaže je bila zlatna, tamno zlatna, tamno narandžasta i narandžasta za T1, T2 T3 i T4 respektivno. Osim T2 koji je imao oštar miris, miris silaže je uglavnom bio prijatan. Sve silaže su imale čvrstu teksturu. Temperatura silaže, etarski ekstrakt i propionska kiselina (P>0,05) nisu bili pod uticajem tretmana. Suva materija silaže i amonijak-azot su bili veći (P<0,05) u T2 nego u drugim tretmanima. Organska materija silaže, nevlaknasti ugljeni hidrati, NDF, ADF, kiseli deterdžent lignin, hemiceluloza, celuloza i buterna kiselina bili su viši P<0,05) u kontroli u odnosu na tretmane aditiva. Sirovi protein silaže je bio najniži i najviši (P<0,05) u T1 i T4 respektivno. Vrednost pH silaže je bio niži u T3 nego u drugim tretmanima. Sirćetna i mlečna kiselina silaže su bile pod uticajem tretmana aditiva i povećavale su se po redosledu: T1 < T2 < T3 < T4 (P<0,05). U zaključku, tretman aditiva je poboljšao hranljive i fermentacione kvalitete sojine ljuske. Međutim, tretman ureom i melasom dao je najbolje rezultate.

Ključne reči: aditivi, siliranje, kvalitet fermentacije, hranljiva vrednost, sojina ljuska

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Conflict of interest
The authors declare no conflict of interest.

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ASSESSMENT OF RESIDUAL NITRITE LEVELS IN COOKED SAUSAGES: COMPLIANCE, THERMAL PROCESSING EFFECTS, AND CONSUMER SAFETY

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Abstract: The increasing global consumption of processed meat, which often contains nitrite as a preservative, raises health concerns due to potential adverse effects from its metabolites, such as nitric oxide and N-nitroso compounds. The study sought to evaluate the food safety of processed meat products within the Serbian market, specifically in the Belgrade region. Nitrite levels were analysed in cooked sausages, both in their raw state and after undergoing the manufacturer's recommended thermal processing prior to consumption, if applicable. Additionally, thus far, there has been a lack of research exploring the potential influence of residual nitrite levels in the meat products prepared as per manufacturer recommendations prior to the consumption of meat products, as well as their contribution to acceptable daily intake (ADI), which provides crucial insights into the overall dietary safety of processed meats. During a three-year period, the study performed analysis on a total of 77 cooked sausages, following the standard ISO methodology. Boiling the cooked sausages led to a major reduction in this meat-product additive, whereas frying led to a comparatively smaller decrease in nitrite concentration. Additionally, the greatest exposure to nitrite compounds occurs when consuming meat products without prior preparation, i.e., without thermal treatment by consumers before ingestion. In summary, the assessment of the ADI for nitrites revealed a high level of food safety, with all values noticeably below the maximum permitted levels specified by national legislation (150 mg/kg).

Key words: residual nitrite, thermal processing, cooked sausages, ADI

Introduction

Early human consumption of meat, beginning over 2 million years ago, likely contributed to cerebral evolution due to its rich energy and nutrients. Initially, humans consumed freshly hunted meat due to the lack of long-term storage methods, later developing techniques for prolonged preservation. Today, meat processing not only extends shelf life but also facilitates the creation of diverse products, enhancing the value of less preferred meat types or its by-products commonly used in products like sausages (Rodrigues et al., 2022). Meat curing, an age-old technique for food preservation, continues to be widely practiced even today. It entails adding salt and spices to fresh meat, typically at different levels of comminution and different stages of processing (Shakil et al., 2021). Synthetic nitrite, typically in the form of nitrite salt combined with common salt (NaCl), is commonly used in the meat processing industry due to its cost-effectiveness and ease of application. Nitrite serves as a multifunctional agent in meat products, such as colour preservation, flavour enhancement, antioxidant properties, and antimicrobial function, especially *Clostridium botulinum*, whose toxins could lead to food poisoning (Dragoev et al., 2014; Hospital et al., 2016).

The colour of meat products exhibits considerable variation and is influenced by numerous factors. Upon the introduction of nitrite to meat, it undergoes several chemical reactions, ultimately converting into nitric oxide (NO). NO subsequently binds with the iron found in both myoglobin (Fe^{2+}) and metmyoglobin (Fe^{3+}), resulting in the development of a cured pink hue in the meat products (Alahakoon et al., 2015). Myoglobin, a sarcoplasmic protein responsible for the occurrence of the red colour in meat, experiences oxidation to form metmyoglobin, which gives the products a brownish hue. The interaction of nitric oxide with myoglobin results to the formation of a vibrant red nitrosyl-myoglobin complex, which forms the basis for the characteristic colour of cured meat. However, this complex is highly unstable and transforms into a stable, visually appealing reddish-pink pigment known as nitroso-hemochrome during the heat treatment process (Shakil et al., 2021). As the hematin and heme proteins, such as myoglobin, hemoglobin, cytochrome oxidase, and peroxidases, possess the ability to trigger lipid peroxidation processes (Chabi et al., 2008), added nitrites, and consequentially, formed nitric oxide, exerts antioxidant effects through various mechanisms: (1) It reduces the formation of Fe^{2+} ions, preforming as a radical acceptors, thereby minimizing oxidative stress by inhibiting Fenton's reaction (d'Ischia et al., 2011); (2) NO suppresses the activities of enzymes which play roles in lipid oxidation processes (Dragoev, 2023); (3) During the smoking of meat products, nitrogen oxides formed outside the membranes stabilize unsaturated lipids, contributing to lipid stability (Zhang et al., 2023). In general, the antioxidant

effect of nitric oxide is diverse, enhancing the oxidative stability of meat products not just during processing but also during storage.

Nevertheless, despite its benefits, the use of sodium nitrite in meat and its link to N-nitrosamine (NAs) formation raised health concerns among consumers as early as the 1970s (Hur et al., 2015; Lee et al., 2021). NAs can form in meat products during production processes, as previously described, during at-home preparation, and even in the digestive tract after consumption (Shakil et al., 2021). Currently, it is understood that there is a positive relationship between the amount of nitrite added and the production of NAs, although this relationship does not follow a straightforward pattern. The phenomenon of "endogenous nitrosation" primarily occurs within the digestive system, particularly in organs such as the stomach (at lower pH), rectum, colon, and urinary bladder, and is typically considered organ-specific (Said Abasse et al., 2021). Overconsumption of nitrite can cause respiratory center paralysis and hypoxia symptoms by reducing hemoglobin's oxygen-carrying capacity (Chen et al., 2022). Methemoglobinemia, or "blue baby syndrome," is a life-threatening condition resulting from high nitrite intake, causing reduction of oxygen supply to tissues, leading to cyanosis. This condition is most severe in infants under six months, although it has been reported in children and adults (Sorour et al., 2023).

In the Republic of Serbia, nitrites in processed meats are listed among permitted additives in meat products (in form of potassium and sodium salts, E 249 and E 250, respectively), and the maximum permitted level (MPL) is defined by the Rulebook on Food Additives (2018), which is harmonized with European Directive (EFSA, 2011), setting the maximum permissible amount that can be added during the production process expressed as $NaNO_2$ or $NaNO_3$ to 150 mg/kg of the product, whereas the residual amount of nitrites in meat products is not set, leaving it with quite unclear boundaries. However, according to the latest European Food Safety Authority Panel on Contaminants in the Food Chain opinion (EFSA, 2023), meat and meat products are identified as the primary source of public exposure to carcinogenic nitrosamines, prompting recent legislative changes by the EU Commission to establish lower limits for nitrites as food additives. This aims to reduce consumer exposure to carcinogenic NOs while ensuring food safety. Specifically, heat-treated meat products like cooked sausages now have a maximum permissible nitrite concentration during production of 80 mg/kg under the latest EU Commission legislation (EFSA, 2023). Additionally, there's a prescribed maximum residual nitrite amount for products ready for market and throughout their shelf life, capped at 45 mg/kg as NO_2^-. Moreover, there is a set value for the maximum acceptable daily intake (ADI) of synthetic nitrite at 70 μg/kg body weight/day, established by both The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA, 2002) and the European Food Safety Authority (EFSA, 2003).

Cooked sausages are meat products processed before consumption - cured, often lightly smoked, and precooked. The residual nitrite content in sausages decreases over time during storage and thermal treatment during preparation before consumption (Paudel et al., 2021). This research aims to investigate the residual nitrite levels in selected categories of cooked sausages available in the market of the Republic of Serbia. It seeks to provide insights from the consumers' perspective, considering how the products are consumed as marketed by the manufacturers—whether raw, boiled, or fried. Our research aimed to address several key questions: What is the amount of nitrite ingested by consumers, allowing us to assess the estimated daily intake (EDI)? How much of these nitrites originate from meat products in comparison to the allowable daily intake (ADI)? Do all products comply with national legislative standards regarding the maximum permitted levels (MPLs) of nitrites, as well as the proposed new regulations by the EU Commission (EFSA, 2023) on MPLs and residual nitrite levels? Furthermore, we explored the impact of frying and boiling processes on residual nitrite levels in sausages.

Materials and Methods

Over a three-year period (2021-2024), nitrite analysis was conducted on a total of 77 products sourced from various market-chains in the Belgrade region. The analysis followed random selection method and aimed to determine the presence of residual nitrite in cooked sausage samples. Samples were sent to the laboratory while maintaining the cold chain, vacuumed if needed, and stored at 4°C in a refrigerator prior to analysis. The selected samples are categorized into three groups of products: finely ground cooked sausages (small- and large-diameter) with 41 samples, coarsely ground cooked sausages with 33 samples, and cooked sausages with meat chunks (Šunkarica sausage) with 3 samples. The nitrite concentration was determined following the spectrofotometric reference ISO method (SRPS ISO 2918:1999). It is noteworthy to mention that all of the analysed products exclusively listed E 250 (sodium nitrite) additive on their food labels. Each sample underwent triplicate analysis to determine its residual nitrite content both before and after thermal processing, following the manufacturer's guidelines specified on the packaging. Approximately 250 g of each sample was selected for analysis. Samples intended for consumption in their raw state, such as Šunkarica sausage, Extra sausage, Tirolska sausage, Toast sausage, and Novosadska sausage, were solely analysed in their raw form, with the entire 250 g homogenized. For small- and large-diameter finely ground cooked sausages, including Hot dogs, Hot dog-type sausages, and Debreciner sausage, a portion of approximately 120 g was analysed in its raw state, while the remaining portion was analysed after boiling for

5 minutes in water maintained at 100 ± 5°C, as recommended by the manufacturer. The same division of the sample was applied to coarsely ground sausages, such as Grill sausage, Srpska sausage, Domaća sausage, and Beef sausage, with a portion analysed in its raw state and another portion analysed after frying at 149 ± 2°C for 5 minutes (2.5 minutes on each side) in a frying pan. Following the heat processing, the samples were cooled to room temperature and thoroughly homogenized in a food processor before analysis for residual nitrite levels.

The average daily consumption of processed meat products was calculated using the latest data available from the Statistical Office of the Republic of Serbia in 2022 (SZS, 2023). Estimated daily intake (EDI) was determined following the method outlined by Temme et al., 2011. The EU Commission Authority (EFSA, 2023) established an acceptable daily intake (ADI) for nitrites at 0.07 mg/kg body weight per day. Thus, the contribution of nitrite (expressed as $NaNO_2$) to ADI was evaluated. The EFSA Scientific Committee (EFSA, 2012) recommended using an estimated average body weight of 70 kg as the default for the European adult population (aged 18 years and above).

For data analysis, the MS Excel was used.

Results and Discussion

According to the latest data from the Statistical Office of the Republic of Serbia in 2022 (SZS, 2023), the average number of household members is 2.67, and the average consumption of processed meat items per household per year is 36.1 kg. Therefore, the estimated average consumption of processed meat, including cooked sausages, is approximately 37.04 g/day/person. This allows for further calculation of the average EDI (mg/kg bw/day) and its contribution to ADI (%) of nitrites for individuals above 18 years of age. The nitrite levels considered for this calculation are residual nitrite levels, which vary based on the preparation method recommended by the manufacturer (whether the product is intended to be consumed raw, boiled, or fried). The results for the finely ground cooked sausages and cooked sausages with meat chunks are presented in Table 1, while the results for the coarsely ground cooked sausages are shown in Table 2.

Table 1. Nitrite concentrations in finely ground cooked sausages (small- and large-diameter) and cooked sausages with meat chunks samples analysed raw and after boiling (where needed, according to manufacturers' label), percentage loss from thermal processing, and contribution to final residual nitrite levels in relation to EDI and ADI

Product type (sausage type)	Average nitrite levels (mg/kg) (min – max)		Average percentage decrease of nitrites after thermal process (%) (min – max)	EDI (mg/kg bw/day) (min – max)	Average contribution to ADI (%) (min – max)
	Raw	Boiled			
Small-diameter finely ground cooked sausages, n=33					
Hot dog sausage, n=6	33.89 (4.40 - 47.29)	13.53 (0.70 - 22.67)	50.70 (40.76 - 58.49)	0.007 (0.000 - 0.012)	10.23 (0.53 - 17.14)
Hot dog-style sausage, n=27	31.86 (5.57 - 68.22)	14.24 (1.40 - 35.29)	48.59 (30.03 - 69.30)	0.008 (0.001 - 0.035)	10.75 (1.06 - 26.68)
Large-diameter finely ground cooked sausages, n=8					
Debreciner sausage, n=3	44.59 (33.78 - 60.64)	23.27 (18.42 - 29.95)	47.33 (45.48 - 50.61)	0.012 (0.010 - 0.016)	17.59 (13.93 - 22.64)
Extra sausage, n=5	42.10 (29.30 - 58.82)	-	-	0.022 (0.016 - 0.031)	31.83 (22.15 – 44.47)
Finely ground cooked sausages with meat chunks, n=3					
Šunkarica sausage, n=3	33.56 (14.42 - 51.22)	-	-	0.018 (0.008 - 0.027)	25.37 (10.90 - 38.72)

EDI - estimated daily intake; ADI - acceptable daily intake; n – number of samples;

Table 2. Nitrite concentrations in coarsely ground cooked sausages samples analysed raw and after frying (where needed, according to manufacturers' label), percentage loss from thermal processing, and contribution to final residual nitrite levels in relation to EDI and ADI

Product type (sausage type)	Average nitrite levels (mg/kg) (min – max)		Average percentage decrease of nitrites after thermal process (%) (min – max)	EDI (mg/kg bw/day) (min – max)	Average contribution to ADI (%) (min – max)
	Raw	Fried			
Srpska sausage, n=4	40.81 (34.18 - 52.68)	26.21 (20.29 - 31.91)	35.54 (23.86 – 43.31)	0.014 (0.011 - 0.017)	19.81 (15.34 - 24.12)
Grill sausage, n=6	40.69 (18.99 - 54.92)	33.32 (17.67 - 43.17)	16.32 (6.95 - 24.11)	0.018 (0.009 - 0.023)	25.19 (13.36 - 32.64)
Domaća sausage, n=4	39.52 (36.37 - 42.58)	33.42 (25.24 - 40.11)	16.37 (7.90 - 32.22)	0.018 (0.013 - 0.021)	25.26 (19.08 – 30.32)
Beef sausage, n=4	48.94 (24.34 - 59.30)	42.57 (20.81 - 54.38)	14.16 (8.31 - 17.58)	0.023 (0.011 - 0.029)	32.18 (15.73 - 41.11)

Product type (sausage type)	Average nitrite levels (mg/kg) (min – max)		Average percentage decrease of nitrites after thermal process (%) (min – max)	EDI (mg/kg bw/day) (min – max)	Average contribution to ADI (%) (min – max)
	Raw	Fried			
Tirolska sausage, n=7	41.53 (18.91 - 67.49)	-	-	0.022 (0.010 - 0.036)	31.40 (14.30 – 51.02)
Toast sausage, n=4	29.74 (17.53 - 49.21)	-	-	0.016 (0.009 - 0.026)	22.48 (13.25 – 37.20)
Novosadska sausage, n=4	46.81 (34.91 - 52.60)	-	-	0.025 (0.018 - 0.028)	35.39 (26.39 – 39.76)

EDI - estimated daily intake; ADI - acceptable daily intake; n – number of samples;

For heat-treated processed meat like cooked sausages, Denmark reported a consumption of 5.6 mg/kg, while the mean reported value for other EU countries (excluding Denmark) was 11.6 mg/kg (EFSA, 2017), indicating significantly lower exposure to nitrites from meat products compared to the population of the Republic of Serbia. Moreover, the EFSA's research (EFSA, 2017) reveals that the population across European countries is exposed to levels of nitrites in meat products, ranging from 0.01 to 0.04 mg/kg body weight/day, or 14.3% to 57.1% of the ADI. Similarly, our study found that exposure to residual nitrites from cooked sausages varied from 0.00 (from hot dog sausages) to 0.036 mg/kg body weight/day (Tirolska sausage), representing a wide range of 0.56% to 51.02% of the ADI, with an average of 22.91%.

All products, both raw and post-heat treatment, complied with the national legislation, with a set value of 150 mg/kg $NaNO_2$ (Rulebook on Food Additives, 2018), and remained below the maximum permissible nitrite concentration of 80 mg/kg under the latest EU Commission legislation (EFSA, 2023).

The results of nitrite concentrations in raw sausages varied widely, with the small-diameter finely ground cooked sausage exhibiting the highest range, ranging from 4.40 mg/kg for hot dog samples to 68.22 mg/kg for chicken hot dog-style sausage samples. The variation of nitrite values in the samples may result from nitrite depletion during product storage, from manufacturing to analysis. It is typical to use 50-100 mg/kg $NaNO_2$ during the manufacturing process in cooked sausages products to inhibit *Clostridium botulinum* growth (Paudel et al., 2021). Nitrite loss over time depends on factors such as heat processing, product pH, storage temperature, and the presence of reducing agents (Paudel et al., 2021). Approximately one-fourth (20.78%) of all the raw analysed samples had nitrite content less than 20 mg/kg, while the majority of the samples (74.04%) fell within the range of 20-60 mg/kg. Only 6.50% of the samples had nitrite contents in the range of 60 mg/kg and higher, but remained well below 80 mg/kg. However, according to the latest EU Commission legislation (EFSA, 2023), which sets the

maximum residual nitrite amount for meat products at 45 mg/kg, 39% of the cooked sausage samples intended for consumption without prior preparation exceed this threshold. Among these, the majority (6 out of 23) are coarsely ground cooked sausages, with three out of four product samples from Novosadska sausages surpassing this limit.

All cooked sausage samples exhibited a reduction in nitrite levels following both boiling and frying treatments, with wide variations in the percentage decrease, as depicted in Tables 1 and 2. Thermal processing significantly decreased residual nitrite levels, particularly in products subjected to boiling, with reductions ranging from 30.03% to 69.30%, and an average reduction of approximately 50%. These findings are consistent with previous studies. Nurlailah et al. (2020) observed a 45% reduction in nitrite levels in sausages boiled for 5 minutes at 90°C. Paudel et al. (2021) reported reductions ranging from 7.83% to 75.23%, indicating varying effects not attributed to common parameters such as emulsion type, product size, or initial nitrite concentration. Additionally, Iammarino et al. (2023) found that boiling treatment significantly decreased residual nitrite levels ($p < 0.05$) in sausage samples, with reductions ranging from 25.8% to 43.5%. On the other hand, Merino et al. (2016) argue that the results of their pilot study indicated that the boiling process did not alter the residual nitrite level at all, whereas frying resulted in a decrease of around 50% from the initial level. Our results differed from these findings. While frying of the sausages has led to decrease in residual nitrite levels as well, this reduction was not as substantial as that observed with the boiling process. Abdel-Atty et al. (2022) reported a decrease of approximately 26% in sausages marketed in Egypt following the frying process, consistent with our findings, where nitrite levels were reduced by 20.21%. The next question is: how did thermal processing affect residual nitrite levels, and do they comply with the newest requirements from the EU Commission (EFSA, 2023)? Out of a total of 36 cooked sausages subjected to boiling, all of them had residual nitrite levels well below 45 mg/kg. Regarding sausages subjected to frying, all of which were coarsely ground cooked sausages and initially had higher nitrite levels when analysed raw, only 2 out of 18 exceeded the newly set values by the EU Commission (EFSA, 2023).

Conclusion

The use of synthetic nitrites in meat processing, particularly in the production of cooked sausages, serves various functions such as colour preservation, flavour enhancement, and antimicrobial action. However, concerns regarding the formation of carcinogenic N-nitrosamines and potential health risks associated with nitrite consumption have prompted regulatory measures to ensure food safety. Our investigation into residual nitrite levels in cooked sausages

available in the Serbian market revealed several key findings. While there were variations in nitrite content among different sausage types, our findings indicate that all products met national legislative standards. However, there are areas for improvement, particularly concerning products intended for consumption without prior thermal preparation, as some exceeded the newest legislative threshold for residual nitrite set by the EU Commission. Thermal processing, especially boiling but also frying, resulted in substantial reductions in residual nitrite levels, thereby enhancing food safety.

However, conducting a survey on current consumption habits among the population of the Republic of Serbia is necessary to obtain more precise insights into the assessed exposure to nitrites from meat products, thereby enabling the accurate determination of nitrite EDI and, consequently, ADI.

Procena nivoa rezidualnih nitrita u barenim kobasicama: usklađenost sa regulativom, uticaj termičke obrade i bezbednost potrošača

Tamara Stamenić, Maja Petričević, Tanja Keškić, Boris Pisinov, Aleksandar Stanojković, Ivica Kos, Maša Radojičić

Rezime

Rastuća globalna potrošnja proizvoda od mesa, koji često sadrže nitrite kao konzervans, dovodi do povećane zabrinutosti za javno zdravlje usled postojanja potencijalno štetnih efekata njihovih metabolita, poput azot-oksida i N-nitrozo jedinjenja. Istraživanje je imalo za cilj da izvrši evaluaciju bezbednosti proizvoda od mesa na srpskom tržištu, fokusirajući se na region grada Beograda. Izvršena je analiza određivanja koncentracije nitrita u barenim kobasicama, kako u nativnom stanju, tako i nakon podvrgavanja preporučenoj termičkoj obradi preporučenoj od strane proizvođača (ukoliko je postojala). Pretragom literature, došlo je do shvatanja da dosadašnja istraživanja nisu obuhvatila analizu potencijalnog uticaja rezidualnih nitrata u mesnim proizvodima koji su termički obrađeni u skladu sa preporukama proizvođača pre njihovog konzumiranja, odnosno da nije ispitan njihov doprinos prihvatljivom dnevnom unosu (ADI) za nitrite. Ovakva istraživanja su od suštinskog značaja za dublje razumevanje ukupne bezbednosti ishrane proizvodima od mesa. Tokom trogdišnjeg perioda, u studiji je izvršena analiza na 77 uzoraka barenih kobasica, prema ISO standardu. Termički proces barenja kobasica doveo je do bitnog smanjenja ovog aditiva, dok je proces prženja doveo do nešto manjeg smanjenja koncentracije nitrita. Pored toga, najveća izloženost nitritnim jedinjenjima se javlja pri konzumiranju proizvoda od mesa koji

ne zahtevaju prethodnu pripremu, odnosno termičku obradu od strane potrošača pre konzumacije samog proizvoda. Procenom ADI za nitrite u odnosu na dostupne proizvode na tržištu mesa, utvrdili smo visok nivo bezbednosti barenih kobasica, pri čemu su vrednosti za sve proizvode primetno ispod maksimalno dozvoljenih nivoa propisanih nacionalnim zakonodavstvom (150 mg/kg).

Ključne reči: rezidualni nitriti, termička obrada, barene kobasice, ADI

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Conflict of interest
The authors declare that they have no conflict of interest.

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IMPACT OF STORAGE CONDITIONS ON THE INSTRUMENTAL COLOUR OF BUCKWHEAT PRODUCTS FOR MEAT INDUSTRY APPLICATIONS

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Abstract: Buckwheat is one of the functional pseudocereals rich in antioxidants, nutrients, bioactive compounds, and phytochemicals. Colour represents one of the most important sensory parameters in the quality assessment of the meat products. The aim of this study was to determine the possible effect of 9-month artificial aging storage at 40 ± 2 °C on the change in instrumental colour of buckwheat products used in the meat industry. In the CIE L*a*b* system, L*, a*, and b* coordinates are used to specify the data of instrumental colour properties. The results were statistically processed by two-way ANOVA ($P<0.001$) and Tukey's Honestly Significant Difference post-hoc tests ($P<0.05$). Storage and product type (and their interaction) factors significantly influenced instrumental colour. During storage, significant differences were observed between the time points (0, 3, 6, and 9 months) in flour, with trends of decreasing lightness (L*) and increasing red (a*) and yellow (b*) colour intensity values. Regarding product type, significantly higher L* values were observed in flour, while a* values were higher in grains. Except for the 3rd month, b* values did not show significant differences. The insights gained in this study may indicate the further application of stored buckwheat flour and grains in obtaining technologically justified and colour-sensory acceptable meat end-products for consumers.

Key words: colour, buckwheat, flour, grains, storage, meat products

Introduction

In the era focused on nutritionally justifiable and health-beneficial food, the spotlight falls on functional food enriched with bioactive ingredients.

Pseudocereals, with buckwheat as a notable example, stand out in this category. Buckwheat is a good source of nutrients, bioactive components, phytochemicals, and antioxidants (Salejda et al., 2022; Sofi et al., 2023). Traditionally, buckwheat products such as grains, seeds, and flour are usually consumed as breakfast cereals and bakery products, or incorporated into enriched products such as bread, dough, pancakes, noodles, honey, sprouts, tea, and snacks (Devrajan et al., 2018; Małgorzata et al., 2018; Sofi et al., 2023).

In recent years, there has been a growing trend in utilizing buckwheat ingredients in processed meat products, marking a significant development in the modern meat industry. Numerous studies have demonstrated that incorporating buckwheat raw materials (grains, flour, husks) into meat product recipes and technologies can effectively enhance various physical and chemical, functional, technological, structural, mechanical and organoleptic parameters of combined meat products, such as semi-smoked sausages, frankfurter-type sausage, pork meatballs, horsemeat patties, chicken patties etc. (Hęś et al., 2017; Salejda et al., 2022; Atambayeva et al., 2023; Yessengaziyeva et al., 2023). Moreover, buckwheat and its products offer numerous health-related benefits, such as hypoglycemic, anticancer, hypocholesterolemic, anti-hypertensive, and anti-inflammatory properties, significantly enhancing their agricultural, industrial, and pharmaceutical utility value (Fotschki et al., 2020; Mondal et al., 2021; Salejda et al., 2022; Sofi et al., 2023).

Given its nutritional and functional importance, storing large quantities of post-harvest grains for extended periods is essential. However, prolonged storage negatively impacts both the quality and nutritional attributes of grains (Rakić et al., 2014), as elevated temperatures during storage could lead to the loss of soluble sugars (Zia-Ur-Rehman, 2006). Similarly, storage time is a factor that significantly affects flour quality, with higher temperatures accelerating deterioration reactions, while low-temperature (e.g., −20 °C) conditions have a positive effect on storage stability (Lancelot et al., 2021). During storage, grain quality inevitably deteriorates due to degradative changes in its biological, physical, and chemical properties. These changes negatively affect the quality of flour obtained from the grain, including its colour as a sensory property (Ping-Ping et al., 2019; Suzuki et al., 2020; Rakić et al., 2023).

In the meat industry, long-term storage and high temperature adversely impact the sensory and physicochemical characteristics of meat products due to accelerated proteolysis, glycolysis or hydrolytic, and oxidative processes of adipose tissue (Augustyńska-Prejsnar et al., 2023). The modulated sugar profile of buckwheat-flour-enriched meat products could also influence shelf life. For instance, the increase in reducing sugars resulting from the hydrolysis of buckwheat-flour-enriched products by either meat or microbial enzymes alters the quantity and composition of sugars available for further microbial proliferation

during storage. This can potentially result in off-odors, negatively impacting another sensory characteristic - smell (Mendiolea et al., 1995).

On the other hand, preserving of quality and nutritional traits of buckwheat products and meat products could be managed for a prolonged period with appropriate storage conditions (Fleurat-Lessard, 2002; Wagh et al., 2015; Augustyńska-Prejsnar et al., 2023), which would also keep the colour unchanged for a certain time. Many papers have examined the effects of storage conditions, such as temperature variations and prolonged storage duration, on the colour changes of flour and grains in various cereals, as well as in various meat and meat products. However, limited attention has been given to the impact of these factors on buckwheat material and meat products combined with it. The colour of meat originates from myoglobin (or its three forms), and is further influenced by lipid oxidation and the subsequent formation of oxidation products, which can further affect myoglobin. Conversely, buckwheat contains flavonoids, pigments responsible for its coloration (Wang et al., 2021; Atambayeva et al., 2023). It is well known that changes in colour provide information on the extent of browning reactions (Maillard reaction, pigment degradation, caramelization, etc.) or lipid degradation and oxidation, which are mostly but not always caused by thermal processes (Anberbir et al., 2023; Atambayeva et al., 2023). Storage conditions, particularly temperature, and duration of storage, have a significant impact on the colour of the flour (Muneer, 2015). Moreover, antioxidants present in buckwheat products, such as polyphenolic compounds, can mitigate the development and slow down the intensity of chemical transformations associated with lipid degradation, oxidation, or pigment oxidation in meat products by scavenging free radicals, thereby impeding color changes. (Wagh et al., 2015; Salejda et al., 2022; Atambayeva et al., 2023).

These findings are part of the ongoing investigation into the properties of buckwheat products and their application in meat products. Thus, in this study, the impact of storage conditions on buckwheat flour and grains was observed from the perspective of their possible further use in obtaining technologically justified and colour-sensory acceptable meat end-products for consumers.

Materials and Methods

Buckwheat (Novosadska variety) harvested in 2022 at the technological maturity stage was used for research. The obtained grains were cleaned of impurities and damaged grains. Sampling was performed according to ISO 24333 (2009), and a 2 kg sample of freshly harvested buckwheat grain was brought to the laboratory. A detailed description of sample storage has been previously reported (Ping-Ping et al., 2019). Briefly, the grain sample was equally distributed into eight

closed plastic containers of the same volume. Six containers were placed in a drying oven Digitheat-TFT (J.P Selecta, Barcelona, Spain) at a temperature of 40 ± 2 °C with thermoregulation and common relative humidity for periods of 3, 6, and 9 months. At the end of each period, two containers were taken, their contents mixed, and a sub-sample (about 0.5 kg) was formed. Half of this sub-sample was kept as whole grains, while the other half was crushed and ground at a speed of 20.000 rpm using a laboratory mill A10 (IKA Works Inc., NC, Wilmington, USA) to obtain flour with a particle size of 1 mm for analysis. Both flour and grain samples were prepared in triplicate.

The instrumental colour was measured at five opposite points around each flour and grain sample on chromameter CR-410 (Konica Minolta Sensing Inc., Osaka, Japan). Diffuse light D-65 was applied, as it represents average daylight that correlated with a colour temperature of approximately 6 500 K. The standard angle of 2 degrees of shelter and 50 mm aperture of the measuring head were used. The results were expressed in CIE L*a*b* system (CIE Colorimetry, 1986) as L* (psychometer light), a* (psychometer tone) and b* (psychometer chroma), where L* value indicates the lightness from black (0) to white (100), a* value varies from green (-) to red (+), and b* value ranges from blue (-) to yellow (+). Before measurement, chromameter was calibrated regarding the white standard (tile).

To interpret the instrumental colour results of experiment, the obtained data were presented as mean ± standard deviation (M±SD) and statistically processed. The two-factor analysis of variance (two-way ANOVA, $P<0.001$) was used to evaluate the effect of storage time, product type and their interaction, and Tukey's HSD (Honestly Significant Difference, $P<0.05$) post-hoc test to determine differences between means, applying Statistica 12.5 software (StatSoft, Inc., Tulsa, OK, USA).

Results and Discussion

Table 1 presents the values of determined instrumental parameters of buckwheat colour in relation to storage time and product type factors. Notably, both storage time and product type, as well as their interaction, significantly affected instrumental colour parameters ($P<0.001$). During storage, there were no significant changes in any instrumental colour parameters of the buckwheat grain. Moreover, progressive changes were noted with increasing storage time in buckwheat flour for all observed instrumental colour parameters. Significant alterations were revealed by product type in all instrumental colour parameters, except for yellow tone parameters (except for the third month).

Table 1. Changes on buckwheat instrumental colour parameters in relation to storage time (S), product type (P), and their interaction (S×P)

n=3 (five points each)	Product type	Months				Level of significance		
Parameters		0 month	3 months	6 months	9 months	S	P	S×P
L*	grains	32.68 ± 0.764^{A}	31.60 ± 0.694^{A}	31.67 ± 0.376^{A}	32.53 ± 0.781^{A}	***	***	***
	flour	73.84 ± 0.208^{bB}	71.95 ± 0.945^{bB}	69.17 ± 1.235^{aB}	76.17 ± 0.497^{cB}			
a*	grains	5.41 ± 0.074^{B}	5.54 ± 0.152^{B}	5.72 ± 0.240^{B}	5.77 ± 0.191^{B}	***	***	***
	flour	2.00 ± 0.049^{aA}	3.01 ± 0.064^{cA}	2.64 ± 0.040^{bA}	2.43 ± 0.021^{bA}			
b*	grains	9.21 ± 0.294	8.97 ± 0.265^{A}	8.93 ± 0.302	9.47 ± 0.329	***	***	***
	flour	8.80 ± 0.100^{a}	11.26 ± 0.071^{cB}	9.09 ± 0.100^{ab}	9.69 ± 0.121^{b}			

$^{a, b, c}$ Means within the same row with different superscripts differ significantly ($P<0.05$); $^{A, B}$ Means within the same column with different superscripts differ significantly ($P<0.05$); *** $P<0.001$

As the storage time increased, the flour in the sections exhibited a decreasing trend in lightness. However, a statistically significant difference ($P<0.05$) was observed at 6 months and beyond. Additionally, L* on the 9th month was significantly different from all others. This inconsistency in the decreasing pattern could possibly be attributed due to the mechanically easier shredding of the more dried shell and greater homogeneity of the sample thus prepared. Product type affects lightness changes. Throughout the storage period, buckwheat grains consistently demonstrated significantly lower lightness compared to the results obtained in flour ($P<0.05$). In contrast to the decrease in lightness, the a* and b* values of flour showed a progressive increase with the passage of storage time. Compared to the start of storage, redness significantly increased ($P<0.05$) until the end of storage, but without significant changes after 6 months. A similar trend was observed in the yellow tone parameter during storage.

The effect of product type on a* remains consistent throughout the storage period, with significantly higher values ($P<0.05$) in buckwheat grains compared to flour. On the other hand, there were no significant differences in b* values between buckwheat grain and obtained flour, except for the 3rd month, which exhibited a significantly increased yellowness in the instrumental colour of the flour.

Our study aligns with analysis conducted by several researchers (Muneer, 2015; Anberbir et al., 2023), highlighting the impact of various natural processes, especially of the chemical type, such as the Maillard reaction, pigment degradation, and caramelization, known to collectively contribute to browning. These processes are notably heightened by extended storage duration and thermal effects, leading to significant alterations in the instrumental colour parameters. Additionally, Muneer

(2015) reports that increasing the storage time by one more day leads to a decrease in the colour value of the stored flour, which agrees with our L* values.

For our further research, it can be assumed that combining buckwheat and meat products can lead to the instrumental colour change in the final product, mainly by decreasing L* and increasing a* and b* values, with significant control of the applied formulation. In combined meat products, Atambayeva et al. (2023) reported that the addition of buckwheat and its flour to horsemeat and chicken patties resulted in a lower L* value and slightly higher a* and b* values, while during storage, L* values decreased slowly, whereas a* and b* showed higher values. All value changes could indicate the preservation of product colour during the prolonged storage time, due to the presence of phenolic compounds that stabilizes oxymyoglobin and delays its deterioration. On the contrary, the increasing addition of buckwheat husks in frankfurter-type sausage (3% or more) resulted in a decrease in L* and b* values, which sets colour change at a lower level of consumer acceptability, but with minimal changes in colour-sensory properties during 2-weeks of storage period (Salejda et al., 2022). Similarly, Yessengaziyeva et al. (2023) stated that incorporating buckwheat flour in semi-smoked sausage (12% or more) made meat colour unsatisfactorily desirable and less attractive.

Conclusion

Based on the obtained results, which form a part of the ongoing investigation into the application of buckwheat products in the meat industry, it can be concluded that storage time, product type, and their interaction influenced the instrumental colour of buckwheat flour and grains. During storage, a trend of decreasing lightness and increasing intensity of red and yellow colours was observed in the flour over intervals of 0, 3, 6, and 9 months. According to product type, significantly higher L* and certain b* values were found in flour, while higher a* values were observed in grains. These results provide valuable guidelines for meat producers and the meat processing industry regarding the management and storage conditions of buckwheat flour and grains. The inclusion of buckwheat in meat products can impact the visual appear and overall attractiveness of meat end-products to consumers.

Uticaj uslova skladištenja na instrumentalnu boju proizvoda od heljde za primenu u industriji mesa

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Rezime

Heljda je jedna od funkcionalnih pseudožitarica bogata antioksidansima, hranljivim materijama, bioaktivnim jedinjenjima i fitohemikalijama. Boja predstavlja jedan od najvažnijih senzornih parametara u proceni kvaliteta proizvoda od mesa. Cilj ovog istraživanja je bio da se utvrdi mogući uticaj 9-mesečnog veštačkog starenja u skladištu na 40 ± 2 °C na promenu instrumentalne boje proizvoda od heljde koji se koriste u mesnoj industriji. CIE L*a*b* sistem sa L*, a* i b* koordinatama je bio korišćen za specifikaciju podataka o svojstvima instrumentalne boje. Rezultati su bili statistički obrađeni testovima dvofaktorska ANOVA ($P<0.001$) i Tukey-jev Honestly Significant Difference post-hoc ($P<0.05$). Faktori skladištenje i tip proizvoda (i njihova interakcija) su značajno uticali na instrumentalnu boju. Tokom skladištenja uočene su značajne razlike između preseka (0, 3, 6 i 9 meseci) u brašnu, sa trendovima opadanja svetloće (L*) i povećanja vrednosti intenziteta crvene (a*) i žute (b*) boje. Prema tipu proizvoda, značajno veće vrednosti L* su bile u brašnu i a* u zrnu, dok vrednosti b*, sa izuzetkom u 3. mesecu, nisu bile značajno različite. Uvidi stečeni u ovoj studiji mogu ukazati na dalju primenu uskladištenog heljdinog brašna i zrna u dobijanju tehnološki opravdanih i boja-senzorno prihvatljivih krajnjih proizvoda od mesa za potrošače.

Ključne reči: boja, heljda, brašno, zrna, skladištenje, meso

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Conflict of interest
The authors declare no conflict of interest.

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